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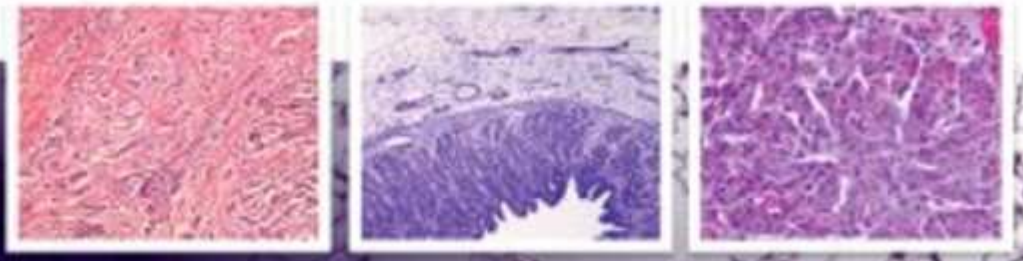

FUNDAMENTALS OF

TOXICOLOGIC PATHOLOGY

SECOND EDITION

AP

"To teach is to learn …" Japanese Proverb

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Preface

"What is toxicologic pathology?" Although the answer to this question is not simple, toxicologic pathology can be defined as a medical science focusing on the study of structural and functional changes in cells, tissues, and organs that are induced by toxicants. The list of toxicant includes drugs, industrial and agricultural chemicals, environmental contaminants, toxins (chemicals of biological origin such as mycotoxins and phycotoxins), and physical agents such as heat and radiation. Toxicologic pathology also includes the investigation of the mechanisms by which these changes are induced and the development of risk assessment and risk management policies based on this information. Therefore, toxicologic pathology relies heavily on disciplines that are included within toxicology (e.g., biochemistry, pharmacodynamics, and risk assessment), those that are prerequisites for pathology (e.g., physiology, microbiology, immunology, and molecular biology), and other associated disciplines. Since most toxicologic pathology is performed in an experimental setting, knowledge of experimental design and biostatistics is essential. In addition, toxicologic pathologists need to be cognizant of newly emerged and emerging scientific areas of study (e.g., genomics and proteomics), and relevant technologies (e.g., tissue microarray analysis and new imaging technologies). Obviously it is too much to expect individuals to have in depth expertise in all of these areas; therefore, there is a critical need for individuals in this discipline to have a broad background in related disciplines as well as strong communication skills and the ability to work in teams, in order to function effectively in solving problems and resolving issues in this field.

Emergence of toxicologic pathology as a discipline was stimulated by the advent of worldwide concerns about the adverse health effects of pollutants and food contaminants, as well as the human and ecological safety of pesticides and pharmaceuticals. The first formalized program in the United States, the cancer bioassay, was originally under the auspices of the National Cancer Institute (NCI) and later transferred to the National Toxicology Program (NTP), both within the National Institute of Health (NIH). With the advent of toxicologic pathology there have been marked advances in understanding the mechanisms of toxicity and the manifestations of toxic effects.

Most toxicologic pathologists are veterinarians who are trained in diagnostic pathology and, ideally, in toxicology and experimental methods. A smaller number of toxicologic pathologists are physicians or biologists with advanced training in pathology. Toxicologic pathologists are employed in industry, academia, government, contract research laboratories or as consultants. Diagnostic and forensic pathologists also need to be familiar with tissue responses to xenobiotics. The ultimate goal of toxicologic pathology is to protect the health of people, animals and the environment.

The purpose of this revision of the *Fundamentals of Toxicologic Pathology* is to update the information presented in the first edition and to broaden the scope of the book to appeal to a wider range of readers interested in toxicologic pathology. The current edition targets toxicology and pathology graduate students and residents as well as pathologists and toxicologists who need an overview of the integration of structure and functional changes to toxic injury. The increased scope of the revised text should appeal to diagnostic and forensic pathologists and toxicologists (both graduate students and professionals) as well as professionals in industry, contract research organizations and government.

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CHAPTER

1

Principles of Toxicology

OUTLINE

THE EFFECT OF THE BODY ON THE CHEMICAL

ABSORPTION

Passage across Membranes

Simple Diffusion

With the process of simple diffusion there is no substrate specificity and no receptor requirements. It involves the entire membrane and depends solely on the lipid–water partition. Polar, or water-soluble, compounds are in equilibrium between ionized and non-ionized forms. The ionized form has such a low lipid–water partition that it is essentially insoluble in lipid membranes, and only the non-ionized portion is available for diffusion across the membrane. Ionization is dependent upon the pK_a of the compound and the acidity of the environment. Due to the volume disparity between aqueous and lipid areas of the cell, diffusion is rate-limited by lipid solubility, increasing with increasing lipid solubility. Once through the membrane the substance re-equilibrates between ionized and non-ionized forms, depending on the pH of the aqueous intracellular environment.

Passage through Pores

Plasma membranes contain pores that allow small, ionized particles to pass through them. Typically the pore size is only 2–7 Å, and only 2% or 3% of the membrane is devoted to these pores. As the pressure rises

on one side of the membrane, particles that are almost the same size as the pores (about 100 MW) are forced through these pores. There are larger intercellular pores, or gaps, between the endothelial cells of the capillary walls in most tissues, allowing passage of larger water-soluble compounds from plasma into the extracellular space. The number and size of these interendothelial gaps varies widely: they are absent in the brain due to the tight junctions between cells, ~40 Å in most other tissues and larger (70 or 80 Å) in the renal glomerulus, allowing molecules of less than 69 000 MW to filter into the urine.

Specialized Transport Systems

Substrate-specific carrier proteins allow rapid transport of polar compounds across membranes. Some carrier proteins facilitate diffusion, transporting compounds down a concentration gradient, while others are integrated into an energy-requiring active transport system for transport of substances against a concentration gradient. Xenobiotics bearing structural or charge similarities to nutrients and endogenous substrates can interact with the specific carrier systems, competing with the endogenous substrate for uptake. For example, the active transport system for uracil and other pyrimidine bases is involved in the uptake of the chemotherapeutic agents, 5′-fluorouracil and 5′-bromouracil. Since these systems do not normally function at saturation, this additional uptake usually has little effect on transport of the true substrate.

Absorption Routes

Toxicologically significant routes of absorption are the gastrointestinal tract, the lungs, and the skin. During transport, some chemicals are modified through metabolism and/or binding. Because of this modification, correct modeling of absorption is essential in toxicological evaluations. Although weak acids and neutral compounds can be absorbed by simple diffusion from the acid stomach, the small intestine is the major site for absorption of xenobiotics from the gastrointestinal tract. Because of the large surface area, the rapid blood flow, and the thin alveolar wall, pulmonary absorption is a rapid and effective route of uptake for gases, volatile compounds, and even some small particulates. Ionizable compounds are absorbed rapidly across the alveolar wall by passive diffusion. For compounds that are relatively poorly water soluble, such as ethylene, uptake is limited by blood flow. Metals do not accumulate in the lungs, but pass directly into plasma so that metal absorption is many times greater in the lungs than in the intestine. The skin is an excellent barrier to all but highly lipid-soluble compounds, such as solvents. However, if the keratinized epidermal layer is removed by abrasion or hydrated by soaking the skin for a prolonged period of time, absorption is greatly increased.

DISTRIBUTION

Volume of Distribution

Body water may be divided into three compartments: the vascular, extracellular, and intracellular spaces. To pass from plasma to extracellular fluid, a compound must be either lipid soluble for diffusion across the endothelial membrane, or sufficiently small to pass through an interendothelial pore. To pass on from the extra- to the intracellular space, a compound must either diffuse or pass through the very much smaller pores of the plasma membrane. With no further refinements, the volume of distribution of a compound would be either 3, 12, or 41 liters in the average adult male, depending on whether distribution was limited to the vascular space alone, the vascular and extracellular spaces, or freely diffusible throughout total body water, respectively.

Two factors serve to add complexity to this distribution pattern. First, excretion is continuous, so that after an initial distribution period of a few minutes, even compounds totally confined to the plasma compartment slowly disappear as they are excreted from the body. Second, very few compounds are evenly distributed within each compartment. This simple three-compartment model must therefore be refined to contain multiple compartments depending on: (1) variation in capillary interendothelial pore size, from very large in the liver to almost absent in the brain; (2) presence of transport systems that permit organ-specific concentration of toxic compounds, such as uptake of iodine in the thyroid; and (3) presence of intracellular storage sinks which shift the equilibrium toward the storage organ, for example, the uptake of fluoride, lead, or strontium into the hydroxyapatite lattice of bone.

Barriers to Distribution

Blood–Brain Barrier

The historical concept of an impenetrable blood–brain barrier is no longer valid. Although water-soluble compounds are effectively excluded, lipid-soluble substances can pass freely across this barrier to concentrate in the fatty nervous tissue. Exclusion of polar substances depends on the fact that junctions between the capillary endothelial cells are far tighter in the brain than in the rest of the body, eliminating interendothelial pores. Astrocyte end feet tightly abut the endothelium, so that a compound must pass through,

rather than around, two extra cell layers to pass into the cerebrospinal fluid. Furthermore, active transport systems similar to those found in kidney serve to transport organic acids and bases out of the brain. The blood–brain barrier is undeveloped in immature animals, and develops incompletely in certain areas of the brain, such as the olfactory bulbs. Somewhat similar to the blood–brain barrier is the blood–testis barrier, protecting the male gamete from many xenobiotics, but there is no known corresponding barrier protecting the female gamete.

Placental Barrier

Lipid-soluble xenobiotics freely diffuse from maternal to fetal blood, and thus to the fetus. The less lipid-soluble a compound, the more effectively it is excluded by the placenta. This barrier is equally effective from either direction; therefore any fetal metabolism of a xenobiotic to a more polar metabolite would tend to trap the metabolite in the fetus. Fortunately, most drug-metabolizing systems develop after birth, so that this particular event is avoided.

BIOTRANSFORMATION

Biotransformation, or metabolism, of a xenobiotic can dramatically alter its distribution and action, leading to detoxification and excretion, or to bioactivation and toxicity. Compounds that are so physically similar to an endogenous compound that they enter the body via its active transport mechanism may also share its sites for biochemical action and its route of metabolism, leading to eventual catabolism and excretion. For xenobiotics entering by diffusion, several organs, particularly the liver, contain enzymes with very broad substrate specificity that will metabolize a wide variety of lipid-soluble compounds. Biotransformation to a more water-soluble product usually enhances excretion, decreasing the likelihood of accumulation to toxic levels. However, these same enzymes can bioactivate a number of xenobiotics to reactive intermediates, producing cytotoxicity or carcinogenicity.

Biotransformation has traditionally been divided into two phases. Phase I metabolism is degradative, involving oxidative, reductive, and hydrolytic reactions that cleave substrate molecules. Products may be more or less toxic than the parent compound. Phase II metabolism is synthetic, involving conjugation or addition of xenobiotics to endogenous molecules. While traditionally phase II metabolites have been considered as almost invariably nontoxic, exceptions are growing with our increasing knowledge base. Frequently phase I metabolism produces a suitable site on the metabolized molecule to allow phase II conjugation to occur. For example, benzene is not a substrate for any phase II reaction, but can undergo phase I oxidation to phenol. Phenol can undergo phase II glucuronidation, forming phenol-*O*-glucuronide, which is excreted.

Phase I Metabolism

Cytochrome P450

The cytochromes P450 (CYP) are a family of enzymes involved in oxidation and reduction of lipid-soluble compounds. In highest concentration in the liver, they are present in most tissues, including kidney, lung, gut, and nasal epithelium. Several CYP are dedicated to specific endogenous metabolic steps, such as kidney mitochondrial 1-a-hydroxylase, which is highly substrate specific and appears to metabolize 25-hydroxycholecalciferol and no xenobiotics. However, the CYP in the hepatic endoplasmic reticulum show far less substrate specificity, catalyzing oxidation or reduction of many chemicals. Furthermore, a single substrate may be metabolized to the same product by several CYP, each with its own kinetic characteristics. In excess of 20 isozymes are now recognized. CYP-dependent oxidation frequently leads to a decrease in the lipid–water partition, decreasing fat storage and increasing the fraction in body water, resulting in an increased rate of urinary excretion. Oxidative attack occurs at N, S, and C bonds, resulting in the insertion of one atom of oxygen. Occasionally, the oxidized products are highly toxic unstable electrophiles. Of the many mechanisms of oxidative attack, *N*-hydroxylation and aromatic C-oxidation (epoxide formation) are most frequently associated with bioactivation to toxic intermediates. Rather than aiding excretion, this change traps the electrophile inside the cell. Excretion is dependent on further metabolism by epoxide hydration or glutathione (GSH) conjugation. When this does not occur, GSH stores are depleted, covalent binding to cellular components occurs, and excretion is inhibited.

Epoxide Hydrase

Epoxide hydrase (or hydrolase) hydrates epoxide products of CYP oxidation to form the corresponding dihydrodiols. The enzyme most studied is microsomal and forms *trans*-diols. A cytosolic epoxide hydrase exists which may be more active towards lipid epoxides. Epoxide hydration is associated with detoxification of reactive epoxides, increased water solubility, and increased excretion. For example, epoxide hydrase catalyzes the hydrolysis of the toxic 3,4-epoxide of bromobenzene to an excretable product, 3,4-dihydrodiol. For a few compounds, epoxide hydration is a step

towards further metabolism to form a toxic product. Benzo[*a*]pyrene is oxidized by CYP to the 7,8-epoxide. This is hydrated by epoxide hydrase, and the dihydrodiol is further oxidized at the 9,10-position, forming the ultimate carcinogen benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide.

Other Phase I Reactions

Hydrolyzing enzymes are widespread throughout the body, including the plasma, and catalyze the hydrolysis of esters. Classification is ill-defined, due to the wide substrate distribution and overlapping substrate specificity.

A number of nitro and azo compounds undergo an NADPH-dependent reduction that can be inhibited by carbon monoxide, implicating cytochrome P450 in this reaction, although a separate flavin nitroreductase also exists. Carbon tetrachloride and halothane are metabolized, both oxidatively and reductively, by cytochrome P450. In both cases, reduction causes bioactivation to a toxic intermediate, while oxidation is a detoxification step. The oxygen tension in the liver affects the route of metabolism, and reduction is favored as the oxygen tension falls. A number of aldehyde reductases, including alcohol dehydrogenase (named for the reverse reaction), are found throughout the body, and may catalyze reduction of toxic lipid-oxidation products.

There is an FAD-containing mono-oxygenase that is a non-CYP, microsomal system able to oxidize secondary amines and several sulfur compounds, including sulfides, thiols, and thioesters. Another microsomal oxidation system, particularly prevalent in kidney, is prostaglandin synthetase, a glycoprotein with a heme center. This enzyme produces peroxide as a byproduct during synthesis of prostaglandins from arachidonic acid. A number of xenobiotics are co-oxidized during this process by the peroxide. However, the *in vivo* significance of this co-oxidation is unknown at present. There are also at least two other distinct groups of amine oxidases, both widely distributed. The monoamine oxidases are mitochondrial flavoproteins involved primarily in catabolism of monoamine neurotransmitters and several xenobiotic amines. The diamine oxidases are vitamin B6-dependent cytosolic enzymes that preferentially metabolize short chain aliphatic diamines.

Alcohol dehydrogenase is a cytosolic enzyme present in liver and eye which catalyzes the oxidation of a variety of alcohols, including methanol, ethanol, and ethylene glycol. In the reverse direction, it catalyzes the reduction of a number of xenobiotics, including chloral hydrate. There are also large numbers of aldehyde dehydrogenases and oxidases with no metabolic role yet assigned to them.

Phase II Metabolism

Most frequently, products of phase II metabolism are more water soluble, more easily excretable, and less toxic than either parent compounds or phase I metabolites. Typically, conjugation involves the addition of an endogenous compound (e.g., glucuronic acid) to a xenobiotic in a two-step reaction, each step requiring an enzyme. Step 1 is the high-energy activation of either the conjugating agent (e.g., UDP-glucuronic acid formation for glucuronidation), or the xenobiotic (e.g., benzoyl-CoA formation for benzoic acid conjugation to glycine). Step 2 is the synthesis of the conjugate.

Glucuronidation

Glucose is readily available for glucuronidation, while the pool of sulfate or glycine available for conjugation is relatively small. This availability of endogenous substrate serves to increase the importance of glucuronidation relative to sulfation in endogenous and xenobiotic metabolism. The enzymes responsible for glucuronide conjugation, the glucuronosyl transferases, are present in the microsomal fraction of liver and other tissues. N-, O-, and S-glucuronides are formed and excreted into bile and urine.

Glutathione Conjugation

Glutathione (GSH), either spontaneously or with the aid of transferase enzymes, conjugates electrophiles, allaying potential toxicity of reactive metabolites. The resultant conjugate is further metabolized to the cysteinyl conjugate by removal of glutamate and glycine. The cysteinyl conjugate is acetylated, and the product formed, a mercapturic acid, is readily excreted in the urine. The hepatic synthesis of GSH, limited by the availability of cysteine, is frequently slower than conjugation. For this reason, GSH stores can fall during conjugation, leading to a temporary inability to conjugate electrophiles, loss of redox potential, and inability to quench peroxidation via GSH peroxidase. Since GSH levels exhibit a distinct diurnal variation, xenobiotics that only exert their toxicity after GSH has been depleted exhibit greatest potency at approximately 6 pm in rodents, the nadir in their diurnal variation in hepatic GSH levels. Recently, a number of GSH conjugates, including many allylic compounds, have been found to undergo further metabolism to reactive thionocompounds, producing nephrotoxicity.

ENZYME LOCATION IN TOXICITY

When a reactive intermediate is formed during metabolism, often it will exert its toxicity in the

immediate vicinity. Many of the xenobiotics which are bioactivated by hepatic CYP cause centrilobular necrosis. The highest concentration of CYP is in the centrilobular region. The centrilobular region is also rich in conjugating enzymes. The oxygen-rich periportal region contains mostly enzymes of intermediary metabolism for plasma protein synthesis, and lipid and carbohydrate metabolism. Necrosis of the periportal region is associated with direct-acting hepatotoxic agents that enter the liver at the portal triad via the portal vein, and do not require bioactivation. Examples are hepatotoxic metals such as iron, manganese, and arsenic, and also phosphorus. Because the biliary tree drains from this region, bile salt damage and hepatic necrosis resulting from accumulation of biliary excretory products during cholestasis is first seen in the periportal region.

The kidney contains many of the same metabolizing enzymes as the liver, but at lower concentration. Also, depending on the route of exposure, the kidney receives a smaller dose of xenobiotic than the liver, because of the first-pass effect. The enzymes for bioactivation are located in the straight portion of the proximal tubule, resulting in necrosis at this site following bioactivation of a xenobiotic. The kidney also contains the enzyme cysteine β-lyase, which deconjugates cysteinyl conjugates formed in liver or kidney, producing reactive sulfur intermediates. This pathway is responsible for the nephrotoxicity of vinyl halides. A similar intestinal pathway exists whereby intestinal microflora deconjugates biliary metabolites of aromatic halides. This is followed by portal uptake of the sulfur metabolite, and transport to the liver for hepatic *S*-methylation and *S*-oxidation. The toxicologic consequences of this intestinal pathway have yet to be determined.

EXCRETION

Urinary Excretion

Excretion of xenobiotics into urine depends on filtration without reabsorption, and/or active secretion through the renal tubular epithelial cell. Components greater than 69 000 MW, such as those bound to plasma proteins, are too large to pass through the glomerular filtration apparatus, and therefore are not available for filtration. In addition, active secretory systems catalyze excretion through the proximal tubule cell into the tubular lumen, regardless of plasma protein binding. Since proximal tubule cells contain many of the drug-metabolizing enzymes found in the liver, xenobiotics often undergo metabolism during passage through these cells.

Once filtered, nutrients such as glucose are reabsorbed in the proximal tubule. This is also the site for reabsorption of a number of xenobiotics, leading to recirculation or accumulation in kidney. Both passive and active reabsorption processes exist. There also are at least two separate systems for active secretion in which xenobiotics (or metabolites) might interact, one for organic acids and the other for organic bases. The physiologic role for the acid system is clearance of uric acid and the glucuronide and sulfate conjugates of endogenous compounds, such as steroids. That this is a single carrier-mediated transport system is shown by competition between the various endogenous acids.

Biliary Excretion

Unlike urinary excretion, biliary excretion is relatively independent of hydrostatic pressure. Rather, biliary excretion depends on hepatic concentration of the xenobiotic. Substances passing into the bile have been grouped according to their plasma:bile ratios. Group A, those with a plasma:bile ratio approximating 1.0, are thought to pass into bile passively down a concentration gradient. Substances in Group A are mostly small molecules in equilibrium with total body water. Group B substances, those that are concentrated in the bile, constitute a growing number of compounds known to be actively secreted into the biliary canaliculi. Group C, those with a plasma:bile ratio greater than 1.0, tend to be bulky polar molecules, such as insulin and mannitol, that cannot readily cross membranes. Compounds of less than a certain molecular weight (325 in rats; 500–700 in humans) are thought to be excreted mainly in urine, while larger compounds are excreted into bile. However, this generalization is not accurate for highly polar substances.

As in the kidney, there appear to be two separate carrier-mediated biliary transport systems for excretion of organic acids and bases. A third transport system is responsible for excretion of steroids. Unless biliary metabolites are ionized at intestinal pH, excretion is thwarted by reabsorption, termed enterohepatic recirculation. Glucuronides are often deconjugated by intestinal microflora; this increases lipid solubility, permitting reabsorption and recirculation. This enterohepatic circulation can greatly increase the biological half-life of a xenobiotic, prolonging toxicity by producing chronic exposure.

Glutathione conjugates are excreted into the biliary canaliculi, where *gamma*-glutamyl transpeptidase and a dipeptidase on the luminal surface remove first glutamate and then glycine, leaving the xenobiotic conjugated only to cysteine. All three products are reabsorbed into the liver, from where the cysteinyl conjugate

travels to the kidney for acetylation and excretion as a mercapturic acid.

Pulmonary Excretion

The lungs are an important site for absorption and excretion of volatile substances including solvents, alcohols, anesthetic gases, pesticide fumigants, and cyanide. Excretion is passive, and hyperventilation improves excretion of these substances by maximizing the concentration gradient. The Clara cell, present within the bronchiolar epithelial lining, contains an active drug metabolizing system, and therefore can bioactivate certain xenobiotics, causing necrosis. With such a ready source of oxygen for production of free radicals, alveolar cells are particularly susceptible to compounds that produce oxidative stress, such as bleomycin and paraquat.

INTERACTION OF CHEMICAL AND THE BODY

ENZYME INDUCTION AND INHIBITION

Activation, Induction, or Synergism

Interaction of xenobiotics with enzymes at sites other than the substrate-binding site can either increase or decrease enzyme activity. An increase in maximal velocity of an enzyme, with no concomitant increase in the amount of that enzyme, is termed activation and may be caused, for example, by allosteric addition of a cofactor which might aid in bringing substrates into juxtaposition at the site of action of an enzyme. In contrast, chelation and removal of the cofactor could inhibit the enzyme.

While activation/deactivation causes no change in the absolute amount of enzyme, many xenobiotics can cause the induction of enzyme, resulting in increased flux due to increased amount of enzyme. Commonly, xenobiotics cause the increased synthesis of the enzymes specifically involved in their own metabolism. Due to lack of substrate specificity of most drug-metabolizing enzymes, this induction also increases the rate of metabolism of other xenobiotics. While xenobiotics can be grouped according to which of several different isozymes they induce, the mechanism of induction of drug-metabolizing systems remains unknown for all but a select few xenobiotics. A cytosolic carrier has been identified that binds a number of polycyclic hydrocarbons, such as TCDD and 3-methylcholanthrene, traveling with them into the nucleus, where DNA-directed synthesis of new cytochrome P450 occurs. This carrier does not bind phenobarbital, and a search for a cytosolic protein binding to phenobarbital or to any xenobiotic that induces the same CYP enzymes as phenobarbital has been unsuccessful.

Synergism is an increase in the activity or toxicity of two or more components, over and above the added effects of the individual components, regardless of mechanism. Ethanol and carbon tetrachloride, both hepatotoxins, produce unexpectedly severe hepatotoxicity when administered together. Cigarette smoking and occupational carcinogen exposure in miners and steel workers appears to be synergistic with relation to induction of lung cancers. Similar to synergism is potentiation, where the potentiator alone appears to have no adverse effect, but greatly increases the toxicity of another xenobiotic. GSH depletors potentiate the toxic effect of many xenobiotics normally removed harmlessly via GSH conjugation. Diethylmaleate removes GSH by direct binding and buthionine sulfoximine by actual inhibition of GSH synthesis. Both potentiate the toxicities of acetaminophen and bromobenzene. The area of synergism and potentiation of toxicities deserves a great deal of attention if our knowledge of toxicology is to be more than academic, since exposure to a polluted environment seldom consists of exposure to a single chemical.

INHIBITION

A number of xenobiotics can inhibit the normal action of an enzyme by competing as substrates. Often these are not very potent *in vivo* inhibitors, because neither the xenobiotic nor the natural substrate is present at a sufficiently high concentration for the enzyme to become rate-limiting. Conversely, xenobiotics that form a stable substrate–enzyme complex that dissociates only very slowly are potent *in vivo* inhibitors. For example, organophosphate pesticides and related chemical warfare agents form a lasting complex with acetylcholine esterase which, when involving greater than 50% of the enzyme, cause accumulation of the natural substrate, acetylcholine, to toxic levels.

Some xenobiotics, while still interacting at the substrate-binding site, are termed suicide substrates, because they destroy the enzyme during metabolism, effecting a long-lasting noncompetitive inhibition that requires synthesis of new enzyme to reverse the inhibition. Many xenobiotics containing allyl groups, such as allylisopropyl acetamide, are bioactivated to such highly reactive intermediates that, immediately upon formation, they covalently bind to cytochrome P450, causing breakdown of the heme center of the CYP.

Some xenobiotics, termed antimetabolites, successfully replace the normal enzyme substrate, forming an

abnormal product that then disturbs metabolism at a later point in intermediary metabolism. Fluoroacetate, galactosamine, and ethionine are examples. A number of antimetabolites have been developed as anticarcinogenic drugs, becoming incorporated into the pathways of purine and pyrimidine biosynthesis. Chemotherapeutic efficacy relies on sensitivity being greatest in cells that are most actively replicating. For example, the antifolate methotrexate, while nonspecifically inhibiting all DNA synthesis, is most toxic to those cells in the logarithmic growth phase, including cells of growing tumors.

DOSE DEPENDENCY AND SITE OF ACTION

Interaction between a toxic agent and an organism depends on the dose arriving at a particular site, and the affinity of the xenobiotic for that site. If a xenobiotic or nutrient interacts with more than one site in the organism, each site will have its own peculiar affinity for the xenobiotic, measurable as the dissociation constant K_d (or K_m if the site is an enzyme). As the dose of a xenobiotic is increased, the xenobiotic interacts with an ever-increasing number of different sites, with ever-decreasing affinities. In the test tube or cell culture, and to a lesser degree even in whole animals, it is possible to reach concentrations seldom reached in the environment. Thus, while the interaction of a biological ligand with very high concentrations of a xenobiotic may be a useful tool for mechanistic studies, it does not necessarily portray the site of toxicity of that same xenobiotic at lower concentrations.

ORGAN SPECIFICITY FOR TOXICITY

Site specificity, as described in the preceding sections, refers to a particular intracellular site, due to specificity of a chemical interaction. Often a xenobiotic interacts at that same subcellular site in several organs, although a particular organ is always the first to exhibit toxicity. This organ specificity, termed the critical organ, is due to a variety of causes. In toxicities causing generalized loss of energy metabolism, such as cyanide inhibition of cytochrome oxidase, the critical organ is the central nervous system (CNS), and death due to respiratory arrest occurs because of the greater oxygen sensitivity of the respiratory center, even though cytochrome oxidase is inhibited in all tissues. Similarly, radiation and the antimetabolites that interfere with purine and pyrimidine metabolism first affect organs undergoing most rapid DNA turnover, such as bone marrow, intestinal mucosa, and actively growing tumors.

SITE-SPECIFIC INTERACTIONS AND TOXICITY

Receptors and Enzymes

The more site-specific a toxic agent, the more likely it is that its action depends on physically mimicking the natural component that interacts at that site. Once at the site, its difference from the natural component produces the characteristic toxic action. A xenobiotic may interact with a receptor, either causing (as an agonist) or preventing (as an antagonist) the effect evoked by the endogenous substrate for this receptor. For example, atropine is a muscarinic antagonist, interacting with the acetylcholine-binding site at nerve terminals, but eliciting no signal. If a xenobiotic is only able to evoke a submaximal response, the term "partial agonist" may be applied.

Direct and Cascade Effects

The initial insult by a toxic xenobiotic may be one or two steps removed from the final physiological change that overwhelms the body. For many chemicals, the initial insult causes a sequence or cascade of effects, for example, by switching on a cAMP-dependent pathway, or causing the slow accumulation of an endogenous metabolite. Thus the normal balance of intermediary metabolism is disrupted. Triglyceride accumulation is a common response to a number of hepatotoxic agents, because of a disruption of the balance between uptake, synthesis, and release of triglycerides. However, individual xenobiotics vary in the mechanisms by which they cause this imbalance. For example, ethanol is thought to inhibit mitochondrial utilization of lipids, hydrazine to increase uptake and synthesis of lipids, and carbon tetrachloride to inhibit release of triglycerides by inhibition of lipoprotein synthesis.

NONSPECIFIC INTERATIONS AND TOXICITY

Electrophiles and Covalent Binding

Xenobiotics that are bioactivated to electrophiles bind to certain tissue macromolecules—lipid, protein, or DNA—depending on the xenobiotic in question. The nucleophile GSH is utilized by the family of GSH S-transferases, enzymes of which have a great affinity for electrophiles. Toxicity appears to ensue only after GSH levels have been depleted by conjugation to less than 20% of normal, and irreversible covalent binding to cellular components has commenced. Loss of GSH *per se* does not appear to be the toxic event, since a number

of GSH-depleting agents are relatively nontoxic; rather, covalent binding after depletion would appear to be the culprit. However, the role of covalent binding in the toxicity of electrophiles is controversial, since the extent of covalent binding does not always correlate with toxicity. This has led to the suggestion that, at least for some xenobiotics, covalent binding may play no role in toxicity, while for others toxicity correlates closely with covalent binding. One possible reason for this seeming dichotomy is that covalent binding at a specific site produces toxicity, while concomitant binding at several nonspecific sites is without toxicity. To date, no target protein binding with subsequent inhibition of any specific cellular process has been found to connect covalent binding to cell death. Determination of the site of covalent binding and the specificity or lack thereof is presently an active area of research.

These findings are somewhat in contrast to studies of covalent binding of xenobiotics to DNA, where carcinogenic electrophiles have been found bound to specific sites on DNA. Some studies report generalized binding of a carcinogen to a specific base, and a specific location on that base, followed by site-specific repair of bases at certain sites and not others. The anticarcinogenic action of selenium has been proposed to protect specific bases from accumulating covalently bound material, while other bases continue to exhibit binding, with no carcinogenic outcome. Carcinogenic electrophiles also exhibit a specificity of binding to cellular components. Benzo[*a*]pyrene incubated with GSH, DNA, and microsomes will bind GSH most avidly, then DNA, and exhibits least affinity for microsomal protein, reflecting the binding pattern that this carcinogen exerts in the whole animal. The basis for this specificity, whether due to the strength of the nucleophilic tissue site, to the lack of hindrance about that site, or to some other characteristic, is presently unknown. Discovery of the basis for this specificity may lead to a method for inhibition of carcinogenesis.

Free Radicals and Lipid Peroxidation

When a xenobiotic is bioactivated to an intermediate that breaks down to a free radical, an available source of hydrogen atoms to quench the radical is the unsaturated lipid of the cell membranes. The lipid radical thus formed by removal of the hydrogen atom readily interacts with oxygen to form a peroxy radical. Quenching of the peroxy radical by a hydrogen atom abstracted from a second unsaturated lipid leaves a lipid peroxide, and produces a new lipid radical, thus propagating lipid peroxidation. Peroxidation may also be initiated by xenobiotics, such as paraquat and menadione, that cycle between oxidized and reduced states, producing an oxygen radical each time it cycles back to the oxidized form. The oxygen radical then initiates peroxidation. Any other sources of partially-reduced oxygen, such as that produced by uncoupling of oxidative phosphorylation, can also initiate peroxidation. Unless removed by catalase or GSH peroxidase, hydrogen peroxide formed as a byproduct of metabolism, or as a result of uncoupling of mixed function oxidation, forms hydroxyl radicals which initiate peroxidation and cause single strand breaks in DNA. Inhibition of peroxidation by GSH peroxidase, catalase, or vitamins C and E can often forestall cytotoxicity and necrosis, and toxicity generally ensues only after GSH depletion.

The precise mechanisms whereby peroxidation causes cytotoxicity and necrosis are controversial. One possibility is that breakdown products of peroxidized lipids, such as 4-hydroxynonenal, are cytolytic and destroy the membrane. Alternatively, radical products of peroxidation may covalently bind to essential cellular components. Another possibility is that peroxidation of the plasma membrane may cause sufficient change in its physical properties that basic functions such as calcium homeostasis are lost. Whatever the cause, cell death appears to be directly preceded by a loss of calcium homeostasis, a massive influx of calcium, a loss of ATP, and an activation of a number of calcium-dependent phospholipases and proteases.

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CHAPTER

2

Manifestations of Toxic Cell Injury: Cell Injury/ Death and Chemical Carcinogenesis

OUTLINE

SECTION I CELL INJURY, CELL DEATH AND SEQUELAE

INTRODUCTION

Whatever the cause of injury to a cell, toxic or otherwise, whatever the biochemical events, simple or complex, that lead to the injury or death of that cell, the pathologist must rely on a visible morphologic change to detect a disruption of homeostasis. This visible manifestation of disrupted function, at the ultrastructural, microscopic or macroscopic levels, is a lesion. The lesion is still the primary means by which a toxicologic pathologist arrives at a diagnosis, one that ideally includes the etiology, as well as a description of the underlying morphologic alterations.

The basic unit of life is the cell; therefore, any morphologic alterations to a tissue as a result of injury must begin with the response of the cell itself to injury. A thorough evaluation and understanding of a lesion must logically begin at the cellular level. The development of a lesion is dependent on a variety of factors which influence how the cell responds to the disruption in homeostasis forced upon it.

Key Cellular Components in Cell Injury

Although damage to any one organelle or structure in a cell can result in injury to the cell as a whole, there are several critical cell systems that are of prime importance in cell injury. It is these structures where disruptions of structure or function will almost always result in injury to, or death of, the cell. These structures are: the plasma membrane—site of osmotic, electrolyte and water regulation, as well as signal transduction; the mitochondrion—site of aerobic respiration; the endoplasmic reticulum—site of much protein synthesis, as well as calcium storage; and the nucleus, where the genetic material of the cell is sequestered, and in which transcription of the genetic code takes place. Accordingly, it is these structures on which toxicologists and pathologists have focused in their studies of the genesis of cell injury and death.

Factors Influencing Injury

Perhaps the most obvious factor influencing lesion development is the severity of the damage itself. If damage to the cell is mild, with rapid recovery, a lesion may never manifest itself morphologically, although a temporary functional disruption may occur or a biochemical alteration may become detectable. On the other hand, the damage may be so severe that tissue structure is obliterated to the point where determining a pathogenesis or etiology is impossible. Another possibility is that the damage is so swift and so profound that not only the cell dies, but also the entire individual is affected and death of the individual occurs before the injury can manifest morphologically. Acute cyanide intoxication is a classic example.

The overall metabolic rate of a cell has a significant effect on lesion development. Cells with high metabolic activity tend to suffer injury more easily and quickly. One need only compare the response of a neuron to that of a fibroblast under conditions of hypoxia to observe the effect of high metabolic demand on a cell's ability to adjust to injury. Metabolically active cells such as neurons, myocardial cells, and renal proximal convoluted tubule epithelium, are absolutely dependent on a continuous oxygen supply for normal function. They need uninterrupted and high concentrations of O_2 for oxidative phosphorylation, to provide the necessary ATP for maintenance of membrane polarity and membrane integrity (neurons), for continual muscular contraction/relaxation and Ca^{2+} transport (myocardium), and for transport of fluids, electrolytes, and metabolites (PCT of kidney). Even small changes in O_2 tension mildly decreasing ATP production will cause serious disruptions of the essential functions of these cell types, with serious consequences for the survival of the individual. By contrast, cells with low metabolic activity, such as fibroblasts and adipocytes, are less affected by low O_2 supply, and can tolerate a very low oxygen environment—hence their prominent role in regeneration and scarring.

Related to this, the degree of specialization can be an important determinant of a response to injury. Some cells, such as the fibroblast, a cell which is quite "plastic" in its adaptability to a variety of conditions and which can assume an assortment of different roles due to its relatively undifferentiated nature, can tolerate almost totally anaerobic conditions and a variety of toxic insults. Retinal rods and cones, on the other hand, which must expend much energy in maintaining their highly specialized membrane structures for trapping photons of light, can only tolerate an absolute minimum of disruption of homeostasis before degeneration or death ensues.

The "metabolic peculiarities" of a particular cell may have a substantial impact on its response to a particular injurious stimulus. The presence of specific receptors on a cell—the *fas* receptor, for example, may induce a cell to undergo apoptotic necrosis (i.e., apoptosis) when a *fas* ligand binds to it, whereas a cell without this receptor will be totally unaffected.

In similar fashion, certain cells may accumulate toxic concentrations of xenobiotics because they have specific uptake systems which allow the cell to "mistakenly" accumulate a substance that it either cannot metabolize or that it bioactivates. Gentamicin toxicity in the proximal convoluted tubular epithelium is an example of this. Gentamicin is taken up by the organic transport system, accumulates in the lysosomes, and eventually interferes with lipid metabolism and lysosomal function, to the point where the lysosomes malfunction and kill the cell. The sensitivity of pancreatic acinar cells to excessive dietary lysine or arginine is another example. Possession of certain phase I metabolizing enzymes, especially certain members of the cytochrome P450 family, may result in bioactivation, rather than detoxification of certain xenobiotics, leading to an enhanced or selective susceptibility to damage by a particular xenobiotic. The presence in the hepatocyte of CYP 2E1 makes it uniquely susceptible to damage by acetaminophen, which is bioactivated to a highly reactive quinone imine by this P450 isozyme.

The "innate" ability of a cell to respond to the injurious stimulus—a high activity of antioxidant enzymes, for example—can have a substantial impact on its ability to handle injury. The hepatocyte, for example, which receives 60% of its blood supply directly from the gastrointestinal tract, can tolerate a high degree of "toxic" insult entering from that source. This is due to its tremendous complement of phase I and phase II detoxification enzymes, as well as its high concentrations of antioxidants such as glutathione, Vitamin E, and Vitamin C. These feature all the hepatocytes to accomplish this within in a relatively oxygen-poor environment. However, a high complement of phase I and phase II detoxification enzymes, while protective under most conditions can be liability as well as an asset in some cases, where phase I- or phase II-mediated bioactivation of a highly lipid-soluble, but otherwise innocuous, compound can result in the production of highly damaging soluble intermediates. Cells lacking high concentrations of endogenous antioxidant or antioxidant enzymes, or lacking the appropriate concentrations and/or combinations of phase I and phase II enzymes, can be especially prone to toxic injury, especially if cells like the hepatocyte or the Clara cell fail to do their job of detoxifying toxins before they reach the general circulation.

Reaction of the Body to Injury

The reaction of surrounding viable tissues to the injured or dead cell has a key role in the morphologic manifestation of toxic cell injury. In some cases, it is the exuberance of the inflammatory cells attracted into the affected area, rather than the injury itself, that leads to the development of an overt lesion. Although in most cases the inflammatory reaction is an essential component in ridding the areas of damaged cells, the inflammatory reaction can be a "two-edged sword," converting a relatively mild injury into a severe one. Much of the severe necrotizing damage in acute toxic pancreatitis, for example, arises not from the release of zymogen granules from dying acinar cells, but rather from the activated neutrophils attracted into the lesion by the released cell contents. Such a reaction is typically associated with the type of cell death that has been termed "oncotic necrosis" or "oncosis," whereas the inflammatory reaction is much more likely to be muted, or even absent, with the type of cell death described as "apoptotic necrosis" or "apoptosis." The differences in response, to be discussed in more detail below, are linked to the leakage of intracellular components out of the cell dying by oncotic necrosis, as well as pro-inflammatory substances produced by the dying cell itself. These substances elicit a response from neutrophils, the "foot soldiers" of the acute inflammatory response. On the other hand, in situations where cells have undergone apoptotic necrosis, with its orderly sequence of disintegration and preservation of membrane integrity, local macrophages, rather than neutrophils attracted from the circulation, ingest and break down the dead cells with no overt inflammatory response.

Adaptation

A cell typically exists within a very narrow range of physiochemical conditions, and it will exert much of its metabolic energy and resources towards maintaining these conditions. This process is termed homeostasis. Ion gradients, intracellular pH and cytosolic osmolarity, for example, are vigorously maintained by the cell, even at the risk of losing its own specialized functions. A cell threatened with a loss of homeostasis will often jettison its specialized structures and cut back on its specialized functions, no matter how important they may be to the rest of the organism, in order to maintain its internal environment. Substantial deviations from homeostasis may lead to the death of the cell, while less substantial deviations can lead to a new level of function or metabolic activity in an attempt to maintain internal environment. A cell dealing with disrupted homeostasis can respond in a variety of ways to maintain itself short of death. This is called adaptation.

Atrophy

One form of adaptation is atrophy, which is a reduction of mass in a cell, tissue, or organ. At the cellular level, atrophy is often a response to decreased demand for the specialized functions of a particular cell. Decreased workload on a skeletal muscle attached to a limb immobilized by a cast will lead to a decrease in content of actin, myosin, and other proteins associated with muscle contraction. This will in turn be reflected as decreased diameters and volumes of myofibers within that muscle (Figure 2.1). Loss of appropriate stimulation needed for specialized function may result in a decrease not only in the metabolic activity of the affected cell, but also a decrease in overall content as well, including loss of organelles. Loss of innervation to a muscle, or lack of hormonal stimulation to an endocrine-dependent tissue such a thyroid follicular cell, will result in a reduction in cell size, if not ultimately the death of the cell itself. A reduction in nutrient or oxygen supply due to inadequate or reduced blood flow will almost certainly result in a reduction in the mass of a cell.

At the most basic level, it can be said that an atrophied cell has undergone catabolism. Ultrastructurally, this is reflected by an overt breakdown of mitochondria, endoplasmic reticulum, microtubules and microfilaments. Typically, there are increased numbers of autophagic vacuoles within the cell. These are often fused with lysosomes. Specialized structures such as cilia, contractile apparatuses, secretory granules, and microvilli may be reduced in number or even absent.

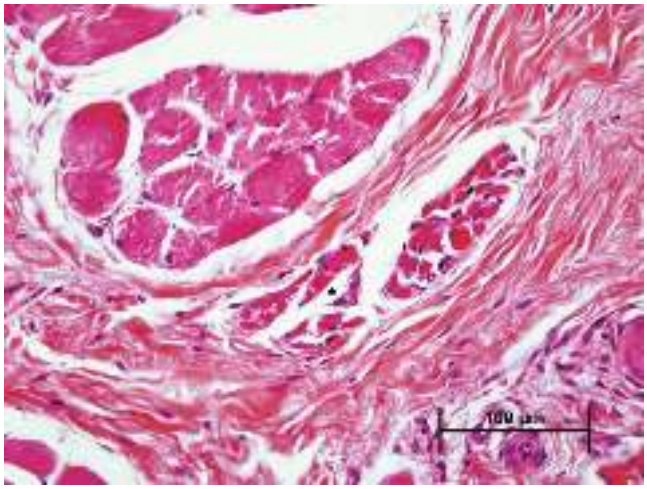

FIGURE 2.1 Atrophy. Skeletal muscle from the tongue of a young horse. The affected muscle (*) atrophied when blood supply to that portion of the tongue was impaired, due to an arterial embolus. Both muscle bundles and individual myofibers are reduced in size, and there is increased space between fibers. Hematoxylin and eosin stain.

In severe cases, loss of specialized structures may be so extensive that the cell may no longer be recognizable phenotypically. In metabolically active cells that have a high turnover of membrane components, lysosomes filled with solid precipitates of partially-degraded complex lipids and lipoproteins may accumulate over time, forming golden brown lipofuscin and ceroid, the "wear and tear" pigments. Histologically, the cell may be obviously reduced in size, have decreased staining affinity, have an altered shape, or be less differentiated in morphology. Grossly, one may only see a reduction in the overall size or mass of the tissue or organ in which the affected cell is found, with perhaps a softer texture or paler color.

Atrophy at the tissue or organ level, however, can reflect more than just a reduction in cellular mass, for a decrease in cell number can also result in a gross reduction in mass. Atrophy in this case is the result of cell death, either by apoptotic necrosis or oncotic necrosis, leaving behind a tissue which may not only be smaller, but which also may have a variety of altered characteristics, depending on the cause of the atrophy. In cases where cell loss occurred via apoptotic necrosis, there may be macrophages, vacuoles, or granules that are in reality phagolysosomes containing portions of the dead cells in various stages of degradation within nearby tissue. Histologically, these granules may appear as hyalin droplets. Inflammation or scarring is not usually present. Grossly, atrophy associated with reduction in cell numbers due to apoptotic necrosis resembles that observed with a simple reduction in cellular mass without cell death.

By contrast, in cases where oncotic necrosis is the cause of the reduction in cell number, there will be some degree of inflammation, often with resultant scarring. In these situations, the reduction in tissue or organ size may be irregular and distorted—depending on the degree of scar tissue present, firmer than normal and grayish or whitish as a reflection of the scarring.

Hypertrophy

Hypertrophy, by definition, is an increase in mass of a cell, tissue, or organ without cellular proliferation. While less commonly encountered in toxicologic pathology than atrophy or other signs of adaptation, it can nevertheless, on occasions, have significant consequences for the overall well-being of the individual. Classically, hypertrophy is a response to increased metabolic demand for a specialized function provided by the particular cell. At the ultrastructural and histological levels, this translates into an increase in the volume of cytoplasm and an increase in the number

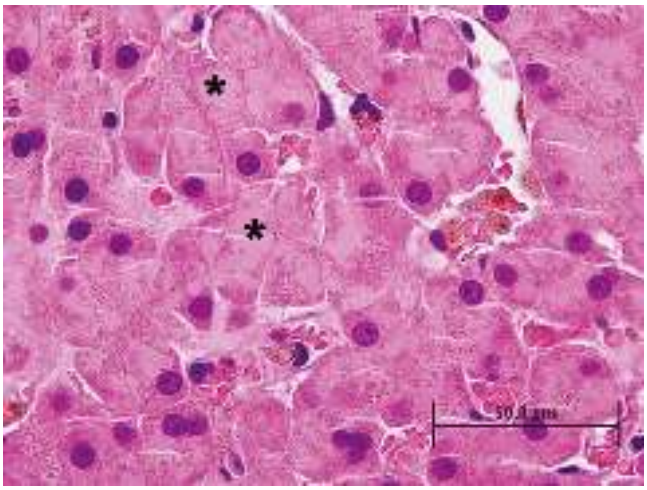

FIGURE 2.2 Pathologic hypertrophy. Liver from a dog with enlarged hepatocytes having increased pale, "waxy" cytoplasm (a*), due to profound increase in smooth endoplasmic reticulum. The animal had been treated for a prolonged period of time with oral phenobarbital to control convulsions. Hematoxylin and eosin stain.

of cell organelles, microfilaments, microtubules, and other specialized structures. Hypertrophy is usually difficult to quantify at the ultrastructural and light microscopic levels without specialized morphometric techniques, but it is usually evident grossly. As with atrophy, weighing an organ and calculating organ to body weight ratios may be the only way to detect subtle forms of hypertrophy. The consequences of hypertrophy are often benign, and merely reflect a physiological response to increased metabolic demand for specialized function. However, there are situations where the increased mass exceeds physiologic limits, and dysfunction of the hypertrophied tissue occurs. Examples of "pathologic" hypertrophy in response to a toxic stimulus do exist, for example, the tremendous hypertrophy of smooth endoplasmic reticulum in the hepatocyte in individuals treated with phenobarbital and other anticonvulsant drugs (Figure 2.2), which in severe cases can lead to loss of other hepatocytic functions.

REVERSIBLE CELL INJURY

Reversible injury is sometimes referred to as degeneration, although this term has been used in the past to describe other conditions, which may not be reflective of injury. Reversible injury is not enough to kill the cell, even if its specialized function in the body is impaired. It must be remembered, however, that although a cell may be reversibly injured, if the loss of its function is one that is vital for the survival of the individual as a whole, death of the individual may occur even though a single cell has not died. There are two cellular morphologic changes commonly recognized as reflecting reversible injury, although in either case these changes may progress further to those characteristic of cell death. These are cell swelling and fatty change.

Cell Swelling

Cell swelling is an early change that occurs in most types of acute injury, and which may be a prelude to more drastic changes. By light microscopy, the cells in an affected tissue are typically swollen, with compression or displacement of adjacent structures. Staining affinity is often diminished, generally giving the cells a pale or cloudy appearance. Clear spaces or vacuoles may form. These are usually manifestations of dilated endoplasmic reticulum or Golgi. This type of change has, in the past, been termed vacuolar degeneration. Sometimes vacuoles are not present at all in the affected cells; rather, the cytoplasm is diluted and organelles are widely dispersed within the rarefied cytoplasm (Figure 2.3). This change has been termed ballooning degeneration by some. Nuclear changes are often mild or minimal, at least early on, and the nucleus generally occupies a location that is typical for the particular cell type.

Cell swelling occurs when the cell loses its ability to control the movement of ions and water into and out of the cytosol precisely. For the most part, this reflects the influx of sodium and water across the membrane

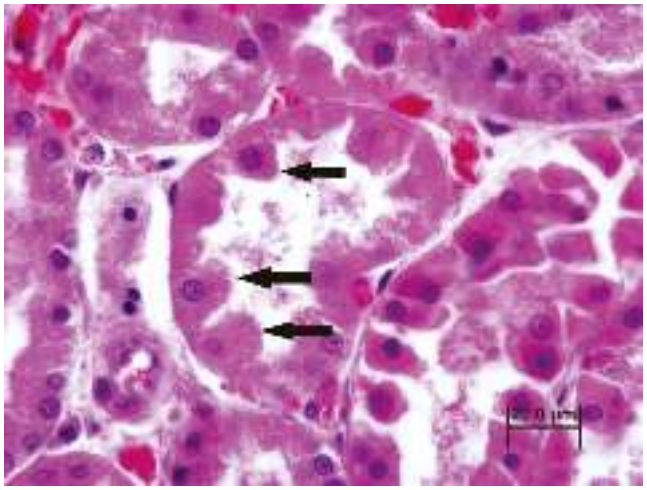

FIGURE 2.3 Cell swelling. Swollen renal tubular epithelial cells from a dog in septic shock. Note the bulging of the apical portions of the cells (arrows), loss of brush border, and elevation of the nuclei from the basal portions of the cells. Hematoxylin and eosin stain.

into the cell, due to altered function or insufficient capacity on the part of membrane Na^+-K^+-ATPases to exchange sodium for potassium at a rate sufficient to maintain water balance. Direct damage to these pumps, inadequate supplies of the essential substrate, ATP, or inability to keep up with the influx of sodium due to direct damage to the plasma membrane itself may also be causes. Vacuoles may form if the Na^+-K^+-ATPases in the endoplasmic reticulum are sufficiently functional to pump at least some of the excess sodium (followed by water) into its lumenal spaces. Contributing to the morphologic changes associated with cell swelling is loss of normal shape, due not only to the influx of water, but also to the influx of calcium into the cell via the diminished capacity or function of the Na^+-Ca^{2+} exchange pumps, which are also dependent on ATP. The dissociation of cytoskeletal elements, and the loss of intercellular connections that result from excessively high free cytosolic calcium levels, lead to additional loss of a shape and a tendency for the cell to assume a spherical shape if anatomically possible.

Cell swelling is not lethal *per se*, and may indicate relatively mild injury; however, cells that are severely injured or lethally injured usually also go through a phase of swelling. Therefore, it must be remembered that lethally injured cells that were fixed in the early stages of death may be interpreted as only being mildly injured, when in fact they are severely damaged. The location of the cell swelling must also be considered when assessing the consequences of cell swelling. Cell swelling in the myocardium secondary to poor vascular perfusion will at some point lead to separation of actin-myosin microfilaments and alter contraction. Ion shifts will also occur that affect depolarization—with serious consequences even if the myocardial cells are reversibly injured. Swelling of astrocytes in the brain during hyperammonemia during liver failure can have tremendous functional consequences, even if no lethal injury has occurred. By contrast, cell swelling in the liver can be quite marked, with few long-term consequences if the injurious stimulus is removed.

Fatty Change

Fatty change is a second manifestation of reversible injury that is often observed in cells that metabolize large quantities of lipids for energy, in particular the liver, but also myocardium and renal tubular cells. Although conditions other than injury can lead to intracellular accumulation of lipid, damage to certain organelles can also lead to fatty change. Direct damage to membranes in cells with a high flux of triglycerides can lead to excessive build-up of triglyceride within the cytoplasm, due to impaired capability to re-export triglycerides. Hypoxia or damage to mitochondria within a cell can lead to insufficient β-oxidation of triglycerides and accumulation of unmetabolized triglycerides within the damaged mitochondrion, and also within the cytoplasm. Damage to protein synthetic machinery in endoplasmic reticulum by the direct action of toxins or hypoxia can result in decreased synthesis of apoproteins for lipid transport, and decreased synthesis of oxidative enzymes for β-oxidation of fatty acids, with the resultant storage of unmetabolized and/or untransported lipids within the cytoplasm. Swelling of fat-laden cells can progress to the point of occluding blood supply or can simply crowd out other organelles needed for other essential cellular function, leading to cell death.

Ultrastructurally, fatty change is characterized by accumulation of amorphous, moderately electron-dense cytoplasmic inclusions free within the cytosol but often associated with proliferative or mildly dilated endoplasmic reticulum. The inclusions can be small and dispersed—often associated with acute, rapidly developing injury—or quite large, occupying the entire central area of the cell and pushing organelles peripherally. This type of "macrovesicular" fatty change has been linked to more slowly-developing toxic injury. Histologically, lipid-laden cells are swollen and clear, with numerous, clearly defined round spaces (vacuoles) or one large central vacuole compressing cytosol and nucleus around the periphery of the cell (Figure 2.4). Due to conventional processing techniques that wash out triglycerides and other

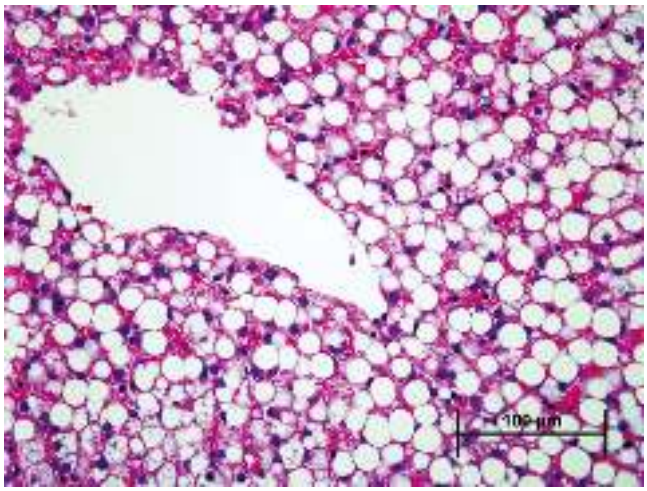

FIGURE 2.4 Fatty change in the liver from a goat with pregnancy toxemia. The vacuoles are large and displace other cellular structures to the periphery of the cell. Hematoxylin and eosin stain.

lipids, frozen sections must be used to preserve the fat in place, followed by special stains like Oil Red O, Sudan black, or osmium tetroxide, to stain lipids orange or black. This is occasionally done to distinguish lipid-filled vacuoles from other types of vacuoles, in particular those filled with water. The gross appearance of fatty liver is a classic lesion in pathology, with its orange or yellow coloration, reticulated pattern and friable, often greasy, texture. Lipid accumulations in non-hepatic tissues are less visible, but can be noted occasionally in the myocardium under conditions of hypoxia, usually in the myocytes bordering an infarctive lesion. This is due to damage to the β-oxidation pathways in the damaged mitochondria. It must be remembered, however, that not all fatty change is due to toxic injury. The kidney, for example, will accumulate triglycerides when there is hyperlipidemia during the course of diabetes mellitus. Macrophages will often accumulate lipids when involved in inflammatory lesions, where large amounts of lipid released from dead or dying cells must be taken up, digested, and metabolized. This a common change when there is degeneration or death of tissue in the CNS, especially in the lipid-rich white matter.

IRREVERSIBLE INJURY

Cell injury is any disruption that results in the loss of a cell's or tissue's ability to maintain homeostasis, normal or adapted; in other words, the cell can no longer regulate its environment within physiologic limits. The "point of no return" at the biochemical level for an injured cell has been much debated and researched over recent years, with the slowly emerging understanding that the mitochondrial permeability transition, leading to leakage of the electron transport chain enzyme cytochrome c into the cytosol, may be the final step in both (oncotic) necrosis and apoptotic necrosis (apoptosis). Determining the "point of no return" morphologically at the ultrastructural and histologic levels is even more difficult, particularly for histologic assessment, and a variety of other considerations must enter into any evaluation of a lesion where a judgement regarding the reversibility of the lesion must be made. This is in large part due to the lag time between the biochemical events leading to injury and the morphologic manifestations of these biochemical disruptions. In the case of ultrastructural change, the lag may be only minutes or hours, but for histologic manifestations to appear, it may be hours, or in some cases days, for a change to become apparent. Grossly, the appearance of a lesion may be even longer. Because of this lag other lines of evidence—morphological and non-morphological—must be utilized to reach a conclusion. Such things as the presence of an inflammatory reaction, the duration of the injurious stimulus (if known), the clinical signs in the affected individual, clinical pathology changes (if available), even the type of cell involved and the tissue affected, may have to enter into the equation before a conclusion of irreversible injury can be made.

Irreversible injury that leads to death of the cell in the living organism is termed necrosis. If the process is uncontrolled, the result is sometimes termed "oncotic" or "accidental" necrosis or simply "necrosis." If the process of cell death is tightly regulated and orderly, the process has been termed "apoptotic" necrosis or simply "apoptosis." In either case, the cell after death will ultimately be degraded and dissolved, disappearing permanently. In both forms, the exact point of "death" is disputed, but the morphologic changes that precede, or follow, the "point of no return" can be identified, and conclusions drawn as to mechanism or etiology (Table 2.1). It must be emphasized; over and over again, that the term necrosis encompasses not only the actual occurrence of cell death in the living organism, but also the degenerative changes that follow the death process. The degenerative changes that follow death of the cell are often the most important part of identifying lethal cell injury morphologically, especially in cases of oncotic necrosis. Generally, it is these secondary changes that are most obvious grossly, or histologically. It must also be remembered that postmortem autolysis involves similar processes, and that these changes occur in all cells after death of the entire organism. Fortunately, it is the reaction of the surrounding tissues to the dead cells that usually allows the pathologist to distinguish antemortem cell death from the postmortem autolytic changes that follow the death of the individual.

Oncotic Necrosis

The initial ultrastructural changes that occur in oncotic necrosis (Figure 2.5A) are frequently the same as those that follow reversible cell injury; there is typically cell swelling with rarefaction of the cytosol due to the influx of water, dilation of the endoplasmic reticulum, loss or deformation of specialized surface features and rounding of the cell. The ultrastructural changes precede the histologic manifestations of injury by a long interval of hours to days, but they are nevertheless important to be aware of since they give valuable clues to the ultimate morphologic manifestations of lethal injury as observed histologically.

TABLE 2.1 Necrosis versus apoptosis

Characteristic	Necrosis	Apoptosis
Gross changes	Grossly evident with disruption of normal tissue structure and detail, scarring if long term	Minimal or atrophy without scarring
Histologic changes	Whole fields of cells affected	Individual cells scattered throughout the affected tissue
	Hypereosinophilia	Hyperbasophilia or hypereosinophilia
	Loss of cell borders with irregular fragmentation	Formation of round bodies, often within a "halo"
	Irregular chromatin clumping, pyknosis, karyorhexis and/or karyolysis; rupture of nuclear envelope	Chromatin condensation into "caps" or "crescents," within round nuclear bodies; preservation of nuclear envelope
Ultrastructural changes	Swelling and loss of surface structures with "blebbing" and loss of apical portions of cytoplasm	Condensation, followed by rapid "zeiosis" (budding)
	Rarefaction of cytoplasm, followed by condensation after death	Condensation of cytoplasm, followed by rarefaction after ingestion by phagocytes
	Swelling and loss of organellar integrity	Preservation of organellar integrity
	Low amplitude swelling of mitochondria, followed by high amplitude swelling and rupture	Preservation of mitochondrial ultrastructure
	Rupture and degradation of internal and external membranes, with bursting of the cell	Preservation of internal and external membranes, with preservation of membrane around apoptotic bodies
	Irregular clumping and degradation of chromatin; rupture of nuclear envelope	Migration of uniformly-degraded chromatin to margins of nuclear envelope; preservation of nuclear envelope
Sequelae	Release of intracellular enzymes into extracellular millieu	Retention of intracellular enzymes within the apoptotic bodies
	Release of pro-inflammatory cell breakdown products	No release of pro-inflammatory products
	Ingress of neutrophils, followed by macrophages	Ingestion by adjacent cells, or by tissue macrophages
	Active inflammation with scarring	Atrophy with stromal collapse, but *no* scarring

Plasma membrane changes are among the first changes observed after injury, and are characterized by loss of surface specialization with disappearance of microvilli and swelling of cilia swell. Intercellular attachments break down, and the injured cell may detach from neighbors because of separation of gap junctions, dissolution of the terminal web of cytoskeletal filaments, and degradation of maculae densae and zonulae adherentes that are supposed to adhere the affected cell to its neighbors and to its surrounding stroma. Cytoplasmic "blebs" or outpouchings may form on the surfaces of injured cells. These may actually detach from the surface of the swollen cells, and float away into the interstitial space, to eventually lyse and release their contents. As swelling continues, the plasma membrane will ultimately rupture. Portions of partially degraded, insoluble membranes will eventually aggregate into laminated structures termed "myelin whorls."

Mitochondria are perhaps the most dramatically affected organelles during the process of oncotic necrosis, first undergoing a form of swelling termed "low amplitude" swelling, as ATP is progressively depleted. This change is typified by swelling of the outer compartment as water and electrolytes are lost

FIGURE 2.5 Morphologic changes associated with oncotic (A); and apoptotic (B); necrosis in a "prototypical" secretory epithelial cell. (C indicates a cilium, ER represents rough endoplasmic reticulum, G indicates Golgi apparatus, M signifies a mitochondrion, Ma represents a macrophage, MV indicates microvillous brush border, N signifies the nucleus, Ne indicates a neutrophil and S represents the smooth endoplasmic reticulum.) The changes represented in A: (1) Toxic stimulus affecting the entire population of cells; (2) Initial swelling with swelling of microvilli and cilia, low amplitude swelling of mitochondria (*), clumping of chromatin and dilation of endoplasmic reticulum cisternae;

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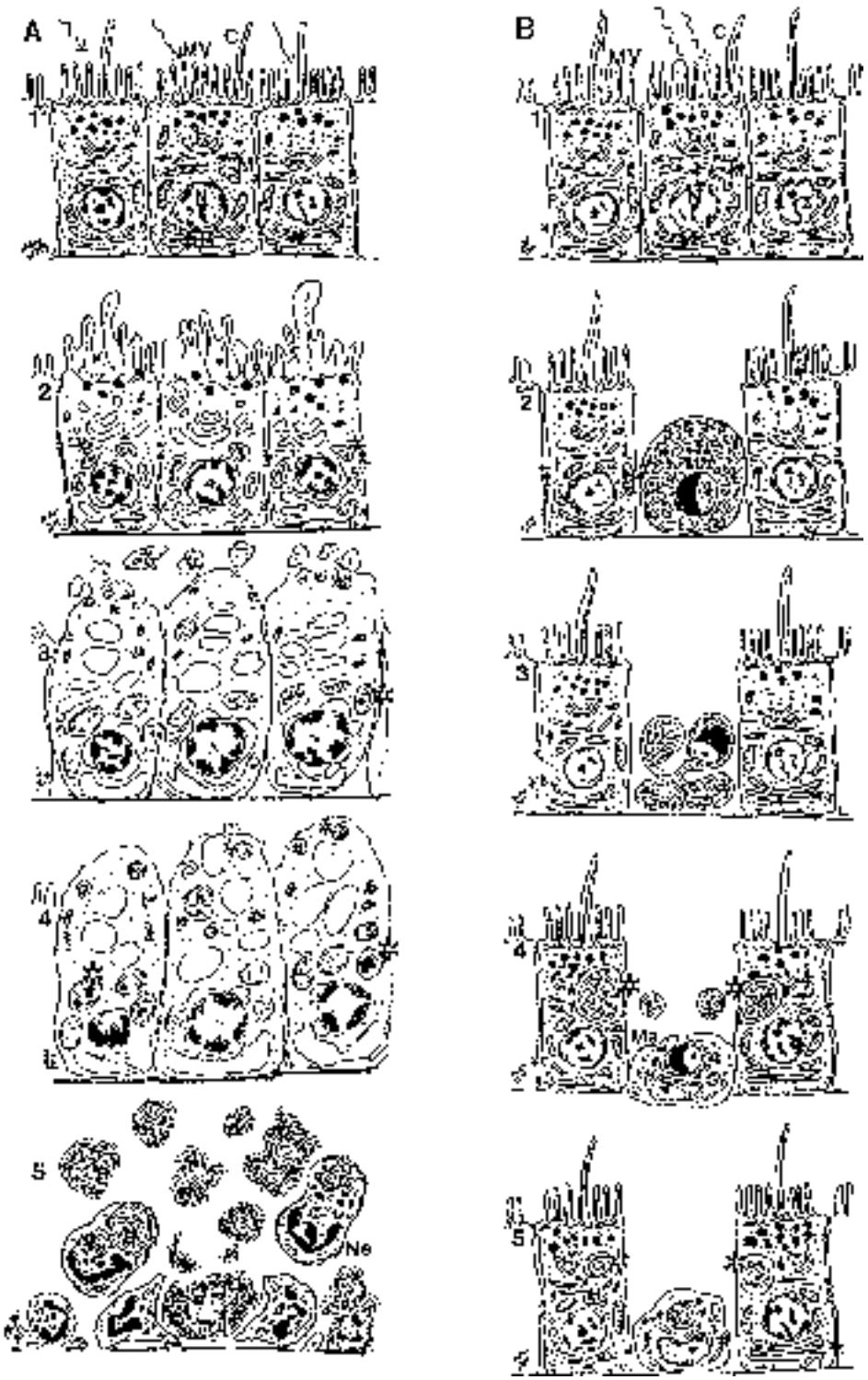

FIGURE 2.5 (3) Continued swelling of cells, with loss of microvilli and cilia, "blebbing" with loss of bits of superficial cytoplasm (*), high amplitude swelling of mitochondria (*), further clumping of chromatin, further dilation of endoplasmic reticulum cisternae and detachment of ribosomes; (4) Rupture of plasma membrane and internal membranes, including the nuclear membrane, further condensation of chromatin, myelin whorls (*) and flocculent densities within burst mitochondria (*); (5) Condensation of cellular remnants and ingestion of cellular debris by neutrophils. The changes represented in B: (1) Reception of an apoptotic stimulus by a single cell in the population; (2) Abrupt condensation of cytoplasm, shrinkage and rounding of the cell with preservation of organellar morphology and condensation of chromatin into a homogenous cap at one pole of the nucleus; (3) Budding of the cell into membrane bound bodies containing intact organelles; (4) Ingestion of the apoptotic bodies by tissue macrophage and adjacent epithelial cells(*); and (5) Digestion of the apoptotic bodies by the macrophage and adjacent epithelial cells (*).

from the inner compartment and pass into the intermembranous space. The result is early condensation of the inner compartment, but this does not necessarily indicate irreversible injury. Inclusions may form, either due to precipitation of small, very electron-dense calcium phosphate crystals as internal calcium homeostasis is lost, or larger, more electron-dense "flocculent densities," composed of partially degraded protein and membrane elements as they accumulate. On further severity or persistence of the injury, mitochondria will undergo high amplitude swelling, with massive swelling of both inner and outer compartments and large-scale accumulation of precipitated mineral and protein, a sure sign of impending doom for both the organelle and the cell. It is at the point where the inner compartment swells and the outer compartment swells to the point of bursting that the mitochondrial permeability transition, considered by many the biochemical "point of no return" in the process of cell death, occurs. The particles on the inner membrane responsible for ATP production become detached, preventing any further ATP production. The accumulation and precipitation of Ca^{2+} salts is especially prominent if some degree of blood flow is present. Soon after the permeability transition, the outer mitochondrial membrane ruptures. At this point, the swollen mitochondrion may resemble a partially double-walled vacuole containing bits of precipitated membrane.

As mitochondria are undergoing the changes that ultimately lead to the death of the cell, efflux of water into the cytosol continues, and there is dilation and fragmentation of the endoplasmic reticulum in an attempt to eliminate the excessive accumulation of water from the expanding cytosol. Endoplasmic reticulum often dilates so much that it forms water- and ion-filled, electron lucent cisternae which are observable as vacuoles by light microscopy. Eventually, ribosomes detach from the rough endoplasmic reticulum, and protein synthesis is no longer possible. After rupture of the cell membranes (both external and internal), protein degradation begins in earnest, especially after lysosomes begin to release their enzyme contents. At this point, the remnants of cytoplasm become denser and actually shrink. This process of swelling during the process of death, followed by condensation afterwards, is characteristic of oncotic necrosis and in marked contrast to that which occurs in apoptotic necrosis, where the opposite pattern is observed.

Nuclear changes associated with irreversible cell injury leading to oncotic necrosis are manifested morphologically mainly by changes in the morphology of chromatin. Nuclear chromatin clumps along the nuclear membrane and loses the distinction between euchromatin and heterochromatin, because of the drop in pH that typically occurs during the progression of oncotic necrosis. Initially, there may be shrinking and condensation of the nucleus and its chromatin as the swollen cytosol and enlarged perinuclear space impinge on the nucleus, creating the morphologic change known as pyknosis. Eventually the nuclear membrane will break down and rupture, as the nucleus itself swells with dispersion of aggregated bits of chromatin attached to the fragmented membranes. This produces the changes characteristic of karyorhexis. Eventually the entire mass of chromatin, nucleoplasm, and nuclear membrane become sufficiently degraded to fade from view, a morphologic change termed karyolysis.

After the cell is "dead" functionally, lysosomes begin to swell and release enzymes into the cytosol. "Autolysis," as traditionally described, begins at this point. Lysosomal membranes are relatively resistant to damage and degradation, and in most cases of toxic injury release their contents late in the process. The cases where activation of lysosomes is the primary trigger in oncotic necrosis are relatively few, for example in copper toxicity, in which the large amounts of copper that accumulate in lysosomes eventually cause their rupture, with release of enzymes and highly-oxidative forms of copper into the cell. Whatever the situation, after release of lysosomal enzymes, uncontrolled degradation of the cell begins. It is at this point that the cell usually can be identified as "necrotic" by light microscopy.

Once the point of no return in the process of oncotic necrosis has occurred, and release of lysosomal enzymes has occurred, degradation of cell components becomes widespread, and the lesion that is easily recognized as "necrosis" becomes manifest via the light microscope. The morphologic changes are relatively stereotypical, although there are variations between tissues, due to biochemical, functional, and morphologic peculiarities. In most cases, the cytoplasm becomes hypereosinophilic and hyalinized, due to degradation of proteins, releasing reactive groups that can interact with eosin, and due to degradation of normally basophilic ribosomal RNA. Occasionally, eosinophilic or basophilic cytoplasmic granules, representing swollen or mineralized mitochondria, respectively, may be observed. As degradation processes continue, the cytoplasm becomes "moth-eaten" and fragmented. In some tissues, especially where there is a large flux of calcium in and out of the tissue, there may be a very marked degree of calcification. These tissues may be replete with basophilic crystals, giving the tissue a bluish, stippled, fragmented, or even crystalline appearance. As mentioned above,

there are nuclear changes that are characteristic of oncotic necrosis—pyknosis, karyorhexis, or karyolysis (Figure 2.6). These changes do not necessarily follow in sequence, and one or all can be observed in a necrotic tissue. The response of surrounding tissues to cells that have undergone oncotic necrosis will be described below.

Gross changes associated with oncotic necrosis are variable and very much depend on the tissue in which the injury occurs, the etiology of the injury, and the response of surrounding tissues. In some cases, affected tissue may be paler than surrounding tissue and somewhat reduced in volume, in particular if blood flow to the affected area has been compromised or cut off completely. If blood supply is still intact or has been restored after the lethal insult, the tissue may be swollen, engorged with blood, darker than normal and soft. On occasion, there is a central pale area surrounded by a zone of red tissue—a vascular response to the dead tissue. The gross and histologic manifestations of oncotic necrosis have been classified into several categories in the past, three of which will be briefly described. With "coagulation necrosis," the general structural organization of the tissue is still discernible, although specific details are lost. Histologically, "shadows" of dead cells can be ascertained. This type of change is most often observed when blood supply is cut off, and inflammatory cells have not had a chance to move in and clean up the dead cells, but it may also be seen in tissues where cells contain few lysosomes and the autodegradation of dead cells proceeds at a slow pace. Liquefaction necrosis is more often seen in cases where infectious rather than toxic agents have caused the injury. Often there is a severe acute inflammatory response, with large numbers of neutrophils. The neutrophils spill out their degradative enzymes into the affected tissues, creating a lesion that is soft and liquefied. Some tissues, particularly the central nervous system, undergo liquefactive necrosis as a matter of course whenever there is large-scale oncotic necrosis, leading to resultant softening or malacia. Caseous necrosis is confined almost entirely to situations where persistent infectious agents or foreign material are involved. In this case, the necrotic tissue that is whitish, pale yellow or pale green, and pasty.

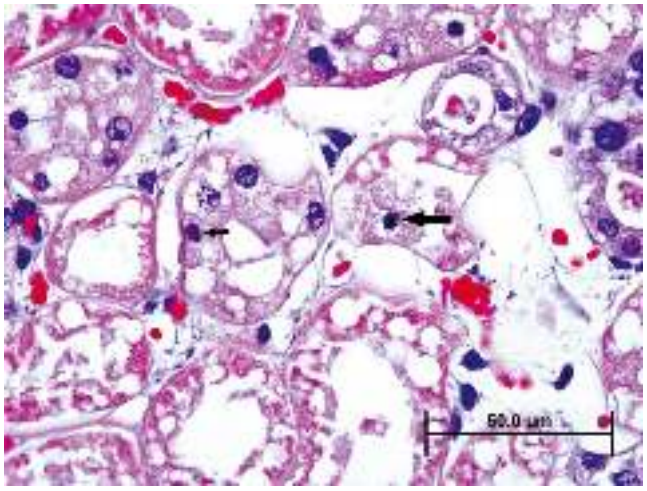

FIGURE 2.6 Oncotic necrosis. Nuclear and cytoplasmic changes in renal tubular epithelium from a cat with lily toxicosis. The entire tubular profile is affected, with loss of cellular detail and fragmentation of cytoplasm. Nuclear changes consistent with pyknosis (large arrow), karyorhexis (small arrow) and karyolysis are present. Hematoxylin and eosin stain.

Apoptotic Necrosis

Apoptotic necrosis is a common type of cell death that has had a variety of names in the past, including "single cell necrosis," "programmed cell death," "cell suicide," "necrobiosis," and "apoptosis," to name just a few. Morphologically, apoptotic necrosis is usually much harder to detect than oncotic necrosis, due its rapid progression once triggered and the rapid disposition of the dead cells via ingestion by adjacent cells or resident macrophages. In addition, only small numbers of cells at any one time undergo this process in most situations. The causes and biochemical events leading to this very orderly and highly regulated form of cell death have been the focus of intense research in the past decade and a half.

Ultrastructurally, apoptotic necrosis has features that are quite distinct from oncotic necrosis (Figure 2.5B). Initial dilation of the endoplasmic reticulum may be observed, but this is indicative of active pumping of ions and water into the cisternae as a prelude to condensation of the cytoplasm. As the cytosol becomes denser and organelles cluster closer together, "zeiosis" or budding off of portions of the cell into spherical membrane-bound fragments frequently occurs. It must be emphasized that the integrity of the plasma membrane is preserved during this process, even after dispersal of the cell fragments. The organelles within the rounded "apoptotic bodies" are well preserved and may even be functional. Most strikingly there is preservation of mitochondrial morphology, and even maintenance of ribosomal attachment to the rough endoplasmic reticulum. Nuclear changes unique to apoptotic necrosis can occur prior to, during, or after the cytoplasmic changes. These changes are characterized by preservation of the nuclear envelope, segregation of the nucleolus from chromatin, and the uniform