5
Measurement of Cell Replication and Apoptosis in Mice

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This chapter focuses primarily on practical methods for assessing cell proliferation and apoptosis in tissue sections from mice. Alternative methods (e.g., biochemical, flow cytometric, molecular) that may also be used to assess cell cycle and apoptotic activity have been covered in the literature (Baserga and Wiebel 1969; Zietz and Nicolini 1978; Hall and Levison 1990; Alison et al. 1994; Amati and Land 1994; Baserga et al. 1994; Gong et al. 1994; Loyer et al. 1994; Piwnica-Worms 1994; Levine and Broach 1995) and will not be presented in this chapter. Information on apoptosis can be found in recent books (Bowen and Bowen 1990; Tomei and Cope 1991; Lavin and Watters 1993; Slyuser 1996). The emphasis in this chapter is on the authors' experience. The approaches and techniques described apply equally to genetically engineered and conventional mice. It is assumed that the ultimate goal of studies on cell growth is assessment of organ or tissue status as influenced by the balance between cell birth and cell death. Thus, in most instances, obtaining measurements of both cell proliferation and cell death parameters is warranted.

CELL PROLIFERATION

Since passage through S-phase is an obligatory step in cell proliferation, this measurement has been frequently used to measure cell proliferation. Passage through S-phase, however, is not absolute proof that the cell will ultimately undergo cell division since cells that have undergone replicative DNA synthesis may arrest prior to undergoing mitosis (Wilke et al. 1988) and subsequently may be removed from the proliferating pool by undergoing apoptosis. For some rodent tissues, such as liver and salivary glands, replicative DNA synthesis may result in increased ploidy without cell division (Carriere 1967; Brodsky and Uryvaeva 1977; Stein and Kudryavtsev 1992; Lin and Allison 1993). This is a frequent occurrence in mouse liver, where the proportion of tetraploid and octaploid cells increases as the animal ages (Fig. 5.1). Despite these potential limitations, calculation of the S-phase labeling index remains a primary means of quantitating the cell proliferative state; thus, S-phase is indicative of cell replication. The ability to

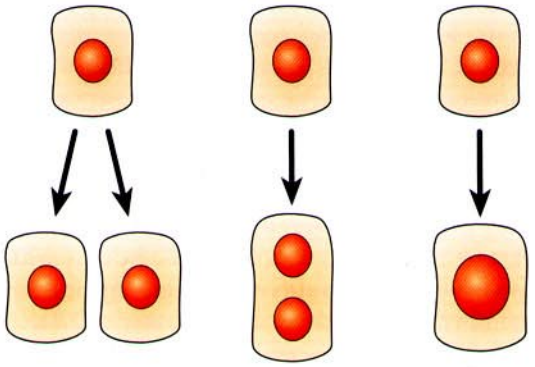

FIGURE 5.1 Replicative DNA synthesis of diploid hepatocytes in the mouse may result in production of two diploid daughter cells, a binucleated original cell that is tetraploid by virtue of having two diploid nuclei, or the original cell with a single tetraploid nucleus. As the mouse ages, the proportion of tetraploid and octaploid hepatocytes increases.

detect S-phase cells in tissue sections allows for correlating the proliferative state in specific target cells with histopathological changes (Goldsworthy et al. 1991).

The identification of S-phase cells involves (1) use of exogenously introduced labeling agents that can ultimately be visualized in tissue sections, (2) staining of intrinsic tissue proteins reflective of cycling cells, or (3) a combination of both approaches. Even if one relies primarily on a single methodological approach, it is reassuring to generate at least a limited amount of data by an alternative method as a means of confirming the primary selected approach or, alternatively, linking to other measurements of the cell cycle such as mitosis.

Exogenous Labeling Agents

Tritiated thymidine (^{3}H-TdR) and bromodeoxyuridine (BrdU) are two frequently used labeling agents for identifying cells undergoing replicative DNA synthesis. When present in the nucleotide pool, these thymidine analogues are incorporated into newly synthesized DNA by substituting for thymidine. The extent of incorporation is dependent upon the amount of labeling agent present as well as the duration of labeling. The amount of labeling agent (the dose) should be sufficient to allow enough incorporation into newly synthesized DNA to permit the ultimate visualization of cells that are in S-phase of the cell cycle but should be below the level of toxicity. High doses of these potentially toxic agents can induce cellular damage resulting in either unscheduled DNA synthesis or overt cytotoxicity with subsequent compensatory enhanced cell proliferation. Either situation could obviously compromise interpretation of a study. The extent of labeling is directly dependent upon the amount of exogenous labeling agent incorporated and the length of S-phase. These two factors will directly affect the intensity of the stain. For example, if labeling agent is present and available throughout a 10-hour period of S-phase, a relatively large amount of the agent will be incorporated into the newly synthesized DNA, providing the cells are in S-phase for a large portion of the 10-hour period. Cells leaving the S-phase compartment just as labeling agent becomes available and cells just entering the S-phase compartment at the end of the 10-hour period will incorporate small amounts of labeling agent and, thus, will be more difficult to score as positive. On the other hand, if the labeling agent is present for only 30 minutes during a 10-hour S-phase, small amounts of labeling agent will be incorporated into newly synthesized DNA, subsequent visualization will be less dramatic, and the number of cells scored as positive will be less. The approximate durations of various phases of the cell cycle for epithelial cells are given in Table 5.1. These times are intended as a relative comparison and do not take into account the observation that cells may remain in G_0 for days or weeks, and cells in the proliferating pool may arrest in G_1 or G_2 (Pederson and Gelfant 1970; Gelfant 1981).

TABLE 5.1. Duration of phases of the cell cycle for rapidly proliferating mouse epithelial cells

Cell cycle phase	Average duration (h)	Range (h)
G_0	24	12–50
G_1	11	3–38
S	10	5–25
G_2	3	1–15
M	1	0.4–1.4

Source: From data in Bisconte 1979 and Wright and Alison 1984.

Tritiated thymidine (^{3}H-TdR) has been used as a labeling agent for decades. Once incorporated into newly synthesized DNA, it can be visualized by covering the tissue section with a photographic emulsion and allowing a sufficient time for the energy emitted by the radioactive thymidine to expose the silver grains in the overlying photographic emulsion. Application and subsequent exposure of the photographic emulsion is carried out in the dark and typically requires days to months to sufficiently expose the emulsion. The emulsion-coated slides are developed, much as photographic film is developed, and then the tissue is stained with a histologic dye, "coverslipped," and visualized microscopically. Positive cells are identified by discrete silver grains that overlie the nucleus of any cell that incorporated the ^{3}H-TdR. Cells are scored as positive when the number of silver grains exceeds whatever background silver grains are present on the section. As an indicator of proper systemic exposure to the ^{3}H-TdR as well as a positive control for the histoautoradiographic technique, it is recommended that a section of small intestine be included on each slide that is to be evaluated. Technical factors and pitfalls in use of ^{3}H-TdR and autoradiography have been reviewed (Bisconte 1979; Maurer 1981; Simpson-Herren 1987). Some advantages and disadvantages of labeling S-phase cells with ^{3}H-TdR are listed in Table 5.2.

Bromodeoxyuridine (BrdU) has been used in recent years in lieu of ^{3}H-TdR and has been reported to yield quantitatively similar results (Lanier et al. 1989; Eldridge et al. 1990). Once incorporated into newly synthesized DNA, the label can be visualized immunohistochemically using commercially available anti-BrdU antibody. The antibody binds to single-stranded DNA. Positive cells are identified by chromogen-staining of the nuclei. There should be virtually no background staining with this procedure. Setting the threshold for

TABLE 5.2. Advantages and disadvantages of exogenously adminstered labeling agents

Tritiated thymidine (^{3}H-TdR)
Advantages
Well-established procedure
Sensitive
Lower-end cutoff is clear
Automated morphometric grain count possible
Disadvantages
Long emulsion exposure times
Radioactive containment issues
Potential toxicity
Difficult to assess tissue morphology
Bromodeoxyuridine (BrdU)
Advantages
Well-established procedure
Rapid procedure
As sensitive as ^{3}H-TdR
No radioactive disposal costs
Disadvantages
Poorly defined lower-end cutoff for positive cells
Potential toxicity

when to score weakly stained nuclei as positive represents a shortcoming of this immunohistochemical approach. It is recommended that a piece of small intestine from each animal be included on each slide to serve as a control to indicate appropriate delivery of the BrdU and to simultaneously show the sensitivity of the immunohistochemical technique. Small intestine has a high intrinsic rate of cell proliferation and should always be positive even with short exposure to BrdU. The current BrdU staining method used at the National Institute of Environmental Health Sciences can be found on the Internet [*http://dir.niehs.nih.gov/dirlep/immuno.html*]. The advantages and disadvantages of labeling S-phase cells with BrdU are listed in Table 5.2.

Intrinsic Measures of Cell Proliferation

Counting mitotic figures in tissue sections has long been used as a measure of cellular proliferative activity. This is generally done on hematoxylin and eosin stained histopathological sections, and the results reflect the percentage of cells undergoing morphologically recognizable mitosis at the moment the tissue is harvested. Since the duration of mitosis represents a relatively transient portion of a cell's proliferative cycle (see Table 5.1), only a few cells may be in the mitotic phase at a particular moment in time. Thus, measurement of the mitotic index is relatively insensitive. One way to increase the sensitivity of mitotic index measurements is to administer a stathmokinetic agent such as colcemid or vinblastin an hour or two before collecting tissue (Tannock 1965; Wright and Appleton 1980; Alabi and Alison

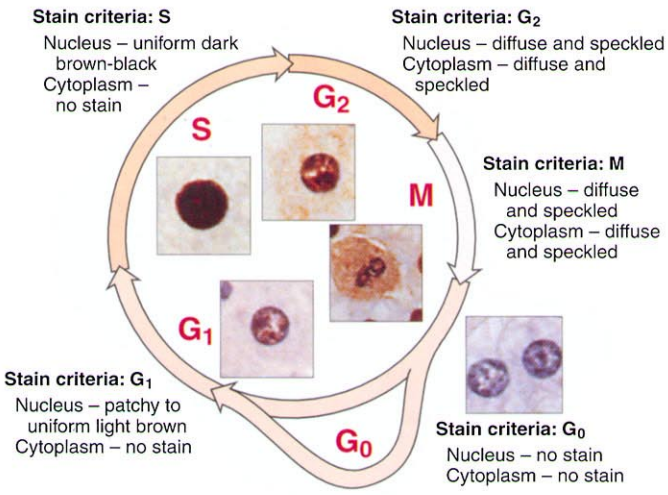

FIGURE 5.2 Characteristic features of PCNA-stained hepatocytes in different phases of the cell cycle.

1984). Colcemid, a mitotic spindle poison, maintains any cells undergoing mitosis in metaphase, allowing accumulation of recognizable mitotic cells and, thereby, increasing the sensitivity of this measure of cell proliferative activity. Arrested metaphase cells begin to undergo visible degenerative changes 60 to 90 minutes following administration of colcemid (Wright and Appleton 1980). Thus, care should be taken to harvest tissues between 1 and 2 hours after administration of this compound.

Proliferating cell nuclear antigen (PCNA) is a 36,000–molecular weight auxiliary protein of DNA polymerase delta, an enzyme vital for DNA replication (Kurki et al. 1986; Bravo et al. 1987). PCNA is differentially expressed during different phases of the cell cycle. Synthesis begins in late G_1 of the cell cycle and peaks during S-phase. It is a relatively stable protein found in all tissues. Commercially available antibodies allow for immunohistochemical staining of PCNA. For many tissues, intensity and cellular localization of staining allows for classifying cells into G_1, S, G_2, and M fractions (phases) of the cell cycle (Figs. 5.2, 5.3). The PCNA labeling index is a measure of cell cycle activity at the time the tissue was harvested, taking into account the ~18-hour half-life of PCNA in tissue. The PCNA S-phase index is similar to a 30-minute pulse dose BrdU or ^{3}H-TdR S-phase index (Eldridge et al. 1993; Foley et al. 1993). Immunohistochemical procedures for PCNA staining have been described (Greenwell et al. 1991, 1993; Foley et al. 1993). The current procedure used at the National Institute of Environmental Health Sciences can be found on the Internet [*http://dir.niehs.nih.gov/dirlep/immuno.html*]. The advantages and disadvantages of PCNA as a measure of cell cycle activity are presented in Table 5.3.

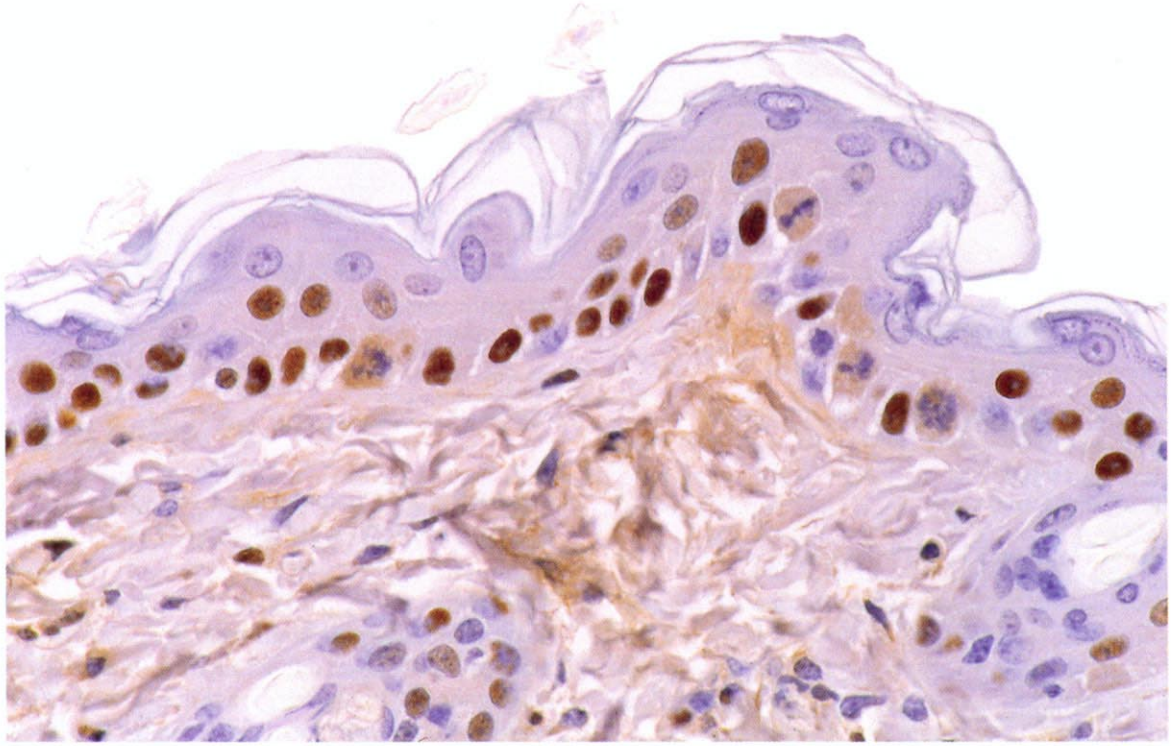

FIGURE 5.3 Section of mouse skin stained for PCNA. Darker brown basal cells are considered to be in S-phase, while faintly staining nuclei are in G_1. Diffuse, speckled cytoplasmic staining is present in mitotic epidermal cells.

TABLE 5.3. Advantages and disadvantages of PCNA immunohistochemistry as a method for assessing cell proliferation

Advantages
Does not require administration of an exogenous labeling agent
Works on archival fixed samples
Provides rapid results for evaluation
Allows for identification of cells in various phases of the cell cycle
Disadvantages
May not be useful in tissues with slow cell turnover
May not be reliable in transformed cells (potentially influenced by perturbations in growth factors, oncogenes, DNA repair, etc.)
May miss early or late proliferation spikes (e.g., snapshot view only)

TABLE 5.4. Examples of intrinsic tissue capacity for cell replication

High
Skin
Esophagus
Intestine
Bone marrow
Medium
Liver
Salivary glands
Bone and cartilage
Kidney
Low
Neurons
Cardiac muscle
Skeletal muscle

Another intrinsic cell proliferation marker, Ki-67 (MIB-5), has recently been shown to yield acceptable immunohistochemical staining in rodent tissues (Gerlach et al. 1997; Ito et al. 1998; Reed et al. 1998). Ki-67 provides a proliferative index by labeling all cells in G_1, S, G_2, and M fractions. While we have no hands-on experience with this intrinsic marker of cell proliferation, interested investigators are encouraged to consult these relevant references.

Considerations in Selecting Methods for Identifying Proliferating Cells

Different tissues have different inherent rates of cell proliferation (Table 5.4). Normal small intestinal epithelium, for example, is completely replaced by newly pro-

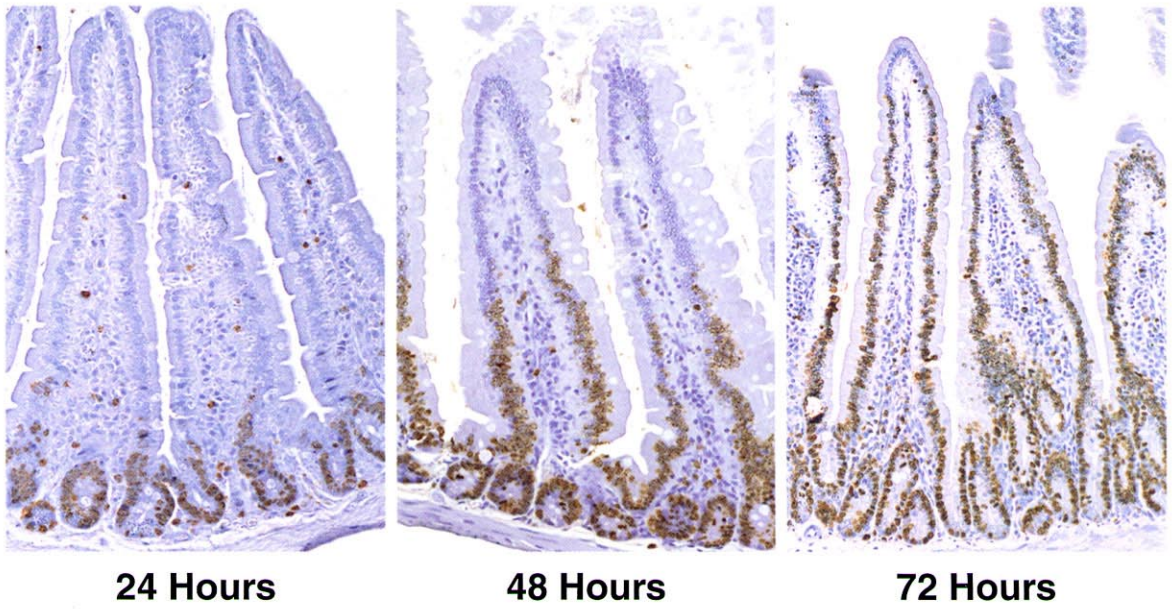

FIGURE 5.4 Immunohistochemical staining of incorporated BrdU in the small intestine of the mouse following 24, 48, or 72 hours of administration by osmotic minipump. While predominantly crypt epithelium is labeled during 24 hours of BrdU administration, progressively more of the villous epithelium becomes labeled with longer periods of continuous administration.

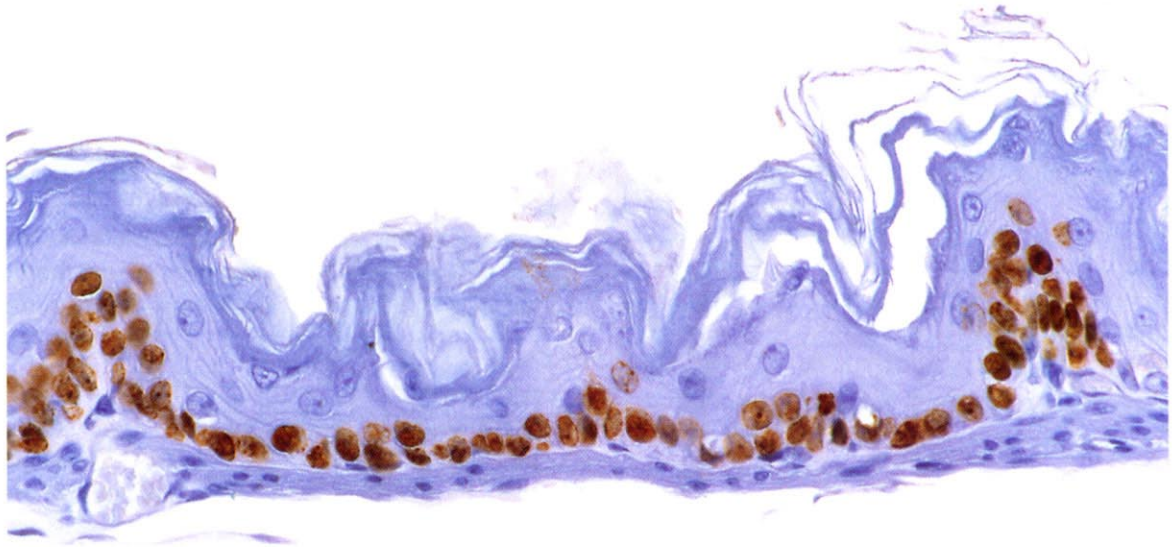

FIGURE 5.5 The entire basal layer of the forestomach mucosal epithelium is labeled following 24 hours of continuous BrdU administration.

liferating cells in approximately 72 hours (Fig. 5.4). Lymphoid and myeloid tissue may, likewise, maintain an inherently high rate of proliferation. The entire population of basal cells in the rodent forestomach is labeled following a 24-hour dosing with BrdU (Fig. 5.5). Other tissues in a normal state, such as liver and kidney, have a slow rate of normal cell proliferation, on the order of weeks or months. Neural tissue or skeletal muscle has yet a slower rate of cell proliferation. Because of different inherent rates of cell proliferation between tissues, a given labeling technique is unlikely to be ideal for all tissues. While a pulse dose of BrdU would identify a quantifiable number of S-phase cells in the small intestinal crypts, only a few hepatocytes or renal tubule cells would be labeled by the same pulse dose, making it difficult to generate a sensitive S-phase

count for these latter tissues. For obtaining cell proliferation rates from tissues with an inherently slower rate of proliferation than intestine, multiple injections of an exogenous labeling agent or continuous administration of an exogenous labeling agent for several days may be necessary to generate a measurable S-phase labeling index (LI). Some tissues, such as liver, are conditionally highly proliferative. Comparison of LIs in control animals, where cell proliferation is low, to those in animals exposed to a hepatocytotoxic agent or a hepatomitogenic agent, where cell proliferation would be conditionally high, requires selection of a labeling interval that will capture data from both groups of animals. In this example with liver, a pulse dose of exogenous labeling agent might be adequate for the treated group but would be insufficient for a reliable measure of cell proliferation in the control group. Continuous labeling for 24 to 72 hours would yield sufficiently sensitive results in both control and treated groups. This type of approach would also allow for reasonable control values that would permit examination of circumstances that lead to inhibition of cell proliferation (e.g., diet restriction).

In situations where an exogenous labeling agent cannot be administered, such as when using archival samples, immunostaining of PCNA is a reasonable choice for tissues that have a moderate to high cell proliferation rate. The PCNA S-phase LI should be approximately equivalent to a BrdU pulse dose LI. It is always possible to stain for PCNA in situations where the animal has had prolonged exposure to an exogenous labeling agent such as BrdU. In such situations, the BrdU S-phase LI can be acquired from tissues with a slower turnover (e.g., liver, kidney) while the PCNA S-phase LI can be acquired from tissues with a rapid turnover (e.g., intestine, forestomach).

Methods for Administration of Exogenous Labeling Agents

SINGLE OR MULTIPLE INJECTIONS

The most common method for administration of labeling agents such as ^{3}H-TdR or BrdU is by intraperitoneal injection in a saline vehicle. The labeling agent is rapidly absorbed and incorporated into cells undergoing replicative DNA synthesis. It is fairly traditional for investigators to inject their animals with ^{3}H-TdR or BrdU 2 hours prior to necropsy. While this is practical and allows time to complete the injections and prepare for the necropsy, it should not be assumed that there is continuous labeling of S-phase cells during the entire interval between injection and necropsy. The availability of injected ^{3}H-TdR or BrdU for incorporation into replicating DNA is approximately 20–30 minutes (Hellman and Ullberg 1986; Wynford-Thomas and Williams 1986; deFazio et al. 1987; Boswald et al. 1990). Following subcutaneous injection of ^{3}H-TdR, the maximum concentration in the blood appears within minutes and subsequently decreases nearly exponentially with a half-life of 10 to 20 minutes (Carlsson et al. 1979). Once labeled, the cells will retain their label for several hours or days in rapidly dividing cells and for months in slowly dividing cells (Ward et al. 1991), with progressively diminished label retention as labeled cells divide into daughter cells. If a somewhat longer interval of labeling is necessary, multiple intraperitoneal injections may be given at selected intervals. Subcutaneous injections also can be used and should result in a somewhat slower absorption of the labeling agent, particularly if the vehicle is corn oil. Recommended injection doses of ^{3}H-TdR and BrdU for mice are 1 μCi/g body weight and 50 mg/kg body weight, respectively (Table 5.5).

OSMOTIC MINIPUMPS

Administration of labeling agents for 24 hours or longer, even up to 2 weeks or longer, can be achieved by using surgically implanted osmotic minipumps. These devices are available in different sizes and with different pumping rates [*http://www.alzet.com*]. They allow for the continuous slow administration of the labeling agent and are ideal for tissues with a low intrinsic rate of cell proliferation, where continuous administration of labeling agent is critical. The use of an anesthetic and the surgical procedure associated with implantation may be accompanied by some unwanted physiological effects (Wyatt et al. 1995) and, thus, may not be appropriate for some research. The recommended concentration for continuous dosing of mice with ^{3}H-TdR is 1 mCi/ml with a specific activity ranging from 25 to 90 mCi/mmol, and for continuous dosing of mice with BrdU, 30 mg/ml when the rate of delivery is 1 μl/h (Table 5.5).

In a study comparing the prolonged administration of BrdU by osmotic minipump and slow-release pellets, it was shown that prolonged administration by subcutaneously implanted pellets may be associated with toxic effects that could either increase or decrease cell proliferation rates (Weghorst et al. 1991). The authors suggest that slow-release pellets may not provide a constant rate of BrdU release and suggest that minipump administration is preferable for prolonged BrdU administration. Caution is recommended in interpreting data from prolonged BrdU administration; rigorous preliminary studies are needed to establish nontoxic doses.

DRINKING WATER ADMINISTRATION

We have recently explored the merits of BrdU administration in the drinking water for toxicology-based cell

TABLE 5.5. Recommended dosage of exogenous labeling agents for mice

	^{3}H-TdR	BrdU	Colcemid
Pulse dose	1 μCi/g BW	50 mg/kg BW	1–4 mg/kg BW
Continuous administration			
Osmotic pump	Specific activity of 25–90 mCi/mmol	NA	NA
	Concentration of 1 mCi/mL	Concentration of 30 mg/ml	NA
	Delivery rate of 1 μL/h	Delivery rate of 1 μL/h	NA
Drinking water	NA	Concentration of 0.2 mg/ml	NA

proliferation analysis in mice (Ton et al. 1997). The periodic drinking habits of rodents, particularly during the evening hours, should provide the opportunity for long-term intermittent labeling of S-phase cells. Under the assumption that drinking habits are relatively consistent between groups, comparison of treated versus controls, or transgenic versus wild-type animals, is possible using dose water for administration of BrdU. Should there be a difference in water consumption between the animal groups being compared, measurement of water consumption will provide an estimate of the amount of BrdU consumed over a finite period of time.

While results from drinking water administration of BrdU are not expected to be quantitatively similar to those of continuous administration via osmotic minipump, the use of dosed water for administration is practical and cost-effective and permits comparison of results between groups of animals (Ton et al. 1997). Thus, relative comparisons would be valid, but an LI from a dosed water study would not be as exact as that from administration by osmotic minipump. Experiments requiring more precise labeling data necessitate use of osmotic minipumps. As a further caution in adopting the dosed water route of delivering BrdU, there is some evidence that metabolism may be sufficiently rapid to preclude accurate labeling of proliferating immune cells (Jecker et al. 1997). The implication here is that rapid metabolism of orally administered BrdU may prevent sufficient labeling agent from reaching lymphoid and bone marrow cells such that BrdU incorporation is below a level necessary for immunohistochemical detection.

Selecting Doses of Exogenous Labeling Agents

While low levels of ^{3}H-TdR and BrdU do not apparently interfere with the cell cycle, there is concern that high doses of these agents may be toxic, resulting in enhanced LIs (Maurer 1981; Wynford-Thomas and Williams 1986; Goldsworthy et al. 1992). Recommended doses that do not result in toxicity are presented in Table 5.5.

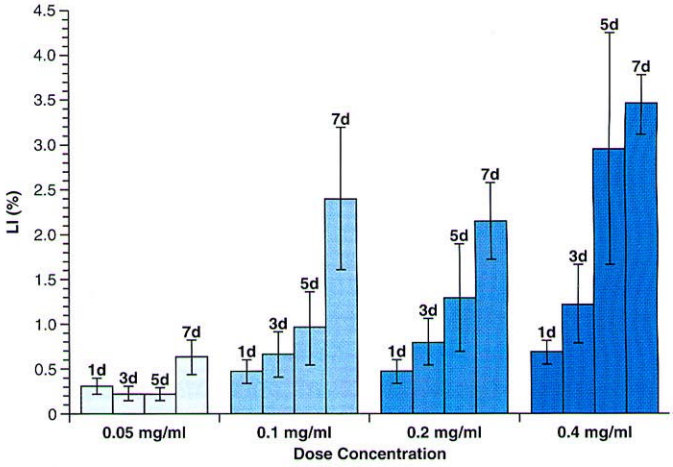

FIGURE 5.6 The hepatocyte labeling index in mice receiving different concentrations of BrdU in drinking water for 1, 3, 5, or 7 days. Bars represent means. Error bars are standard deviations.

In an effort to determine the lowest concentration of BrdU in drinking water that will result in acceptable immunostaining, mice were given 0.05, 0.1, 0.2, and 0.4 mg of BrdU/ml of drinking water for up to 1 week. Immunostaining of liver sections and determination of LI are presented in Fig. 5.6. Based on these results, we recommend a concentration of 0.1 to 0.2 mg BrdU per ml for drinking water administration in mice. Ton et al. (1997) compared liver labeling indices following BrdU administration in drinking water with those following osmotic minipump administration.

In studies conducted in our laboratory, aqueous solutions of BrdU were found to be stable for 35 days at room temperature or 4°C at a concentration of 0.05 mg/ml. Samples stored in clear or amber drinking water bottles exposed to light and air for 7 days showed no significant loss of BrdU. The dose analysis method was validated over a concentration range of 0.0409 to 0.50 mg/ml. It is noteworthy that blood and liver samples taken at

necropsy at 9 a.m. had minimal to no detectable levels of free BrdU when BrdU was administered in drinking water at 0.05 to 0.4 mg/ml (unpublished observation). However, adequate BrdU immunostaining was observed in liver sections.

Counting/Measurement of Indices

One of the most important considerations in determining mitotic or labeling indices relates to generation of the denominator data for the calculation:

$$\text{Mitotic index (MI)} = \frac{\text{Number of mitotic cells}}{\text{Total number of cells examined (mitotic + nonmitotic)}}$$

$$\text{Labeling index (LI)} = \frac{\text{Number of labeled cells}}{\text{Total number of cells examined (labeled + nonlabeled)}}$$

The generation of denominator values for the above calculations is labor-intensive but is required for precise estimation of indices, especially when investigating potential dose responses. It is generally acceptable to count 1000 to 2000 cells for estimating LI.

For a relatively solid tissue such as the liver, when the cellular density is similar in all portions of the hepatic lobule, one labor-saving approach in generating an MI is to determine the average number of hepatocytes per microscopic field and then simply to count the number of mitoses per microscopic field and calculate the MI as follows:

$$\text{MI} = \frac{\text{Total number of mitoses in all microscopic fields examined}}{\text{Number of microscopic fields examined} \times \text{Average number of hepatocytes per microscopic field}}$$

Simply counting mitotic figures per microscopic field without determining the denominator is rapid and may be a reasonable first estimate of relative mitotic rates between groups of animals. If the cellular density per microscopic field of view is essentially identical between the groups of animals being compared, then counting mitotic figures per microscopic field or per square millimeter area for solid tissues is certainly reasonable. Counting mitoses per microscopic field has also been used for decades by pathologists when grading neoplasia (Quinn and Wright 1992). To generate the data, one only has to settle on the number of microscopic fields or the square area to examine. Since the mitotic rate is generally low in most nonneoplastic tissues, it is often necessary to examine many microscopic fields when generating mitotic counts. We recommend counting at least 5000 cells (or the representative number of microscopic fields) to determine a mitotic index.

Although the S-phase LI is traditionally generated by counting both the numerator (labeled cells) and the denominator (total labeled and unlabeled cells) and performing the above calculation, some alternatives to ease the burden of laborious counting have been used in different laboratories and may be applicable in specific situations. One alternative is to count the total number of cells in four to six microscopic fields and to use these counts to calculate the average number of cells per microscopic field. Subsequently, the labeled cells in many microscopic fields can be counted in relatively rapid fashion, and the labeling index can be calculated as follows:

$$\text{Labeling index} = \frac{\text{Number of labeled cells}}{\text{Total number of microscopic fields examined} \times \text{Average number of cells per microscopic field}}$$

This approach works well for solid tissues, such as liver or endocrine tissues, where there is uniform density of cells throughout the organ and between the groups being compared. If there were centrilobular hepatocyte hypertrophy in one group, this approach would not be appropriate since the cellular density would vary between successive random microscopic fields and hypertrophy is not expected to be present in the control group.

Another method for estimating the labeling index that is especially useful for mucosal and epidermal surfaces is the unit length labeling index (ULLI). The ULLI is defined as the number of labeled cells per millimeter of lining epithelium. This measurement involves counting the labeled nuclei along a measured length of basement membrane. This methodology has been described in detail for nasal epithelial surfaces (Monticello et al. 1990). ULLI comparisons between animal groups should also take into consideration histopathological alterations that further clarify any quantitative response data. For example, comparison of two basically similar mucosal surfaces (Fig. 5.7A, B) using the ULLI approach is a useful measurement. However, if some of the groups have a hyperplastic or thickened mucosal surface where the proliferating pool of cells extends considerably beyond the basement membrane (Fig. 5.7C) or a dysplastic epithelium (Fig. 5.7D), then simply reporting the ULLI does not adequately reflect the pathophysiological process being observed. Some indication of relevant tissue alterations in the population being counted should accompany the ULLI.

Measurement of cell proliferation in lesions often represents a significant challenge. In some instances the labeling index is so high that accurate counts are difficult (Fig. 5.8). Hyperplastic and neoplastic lesions may

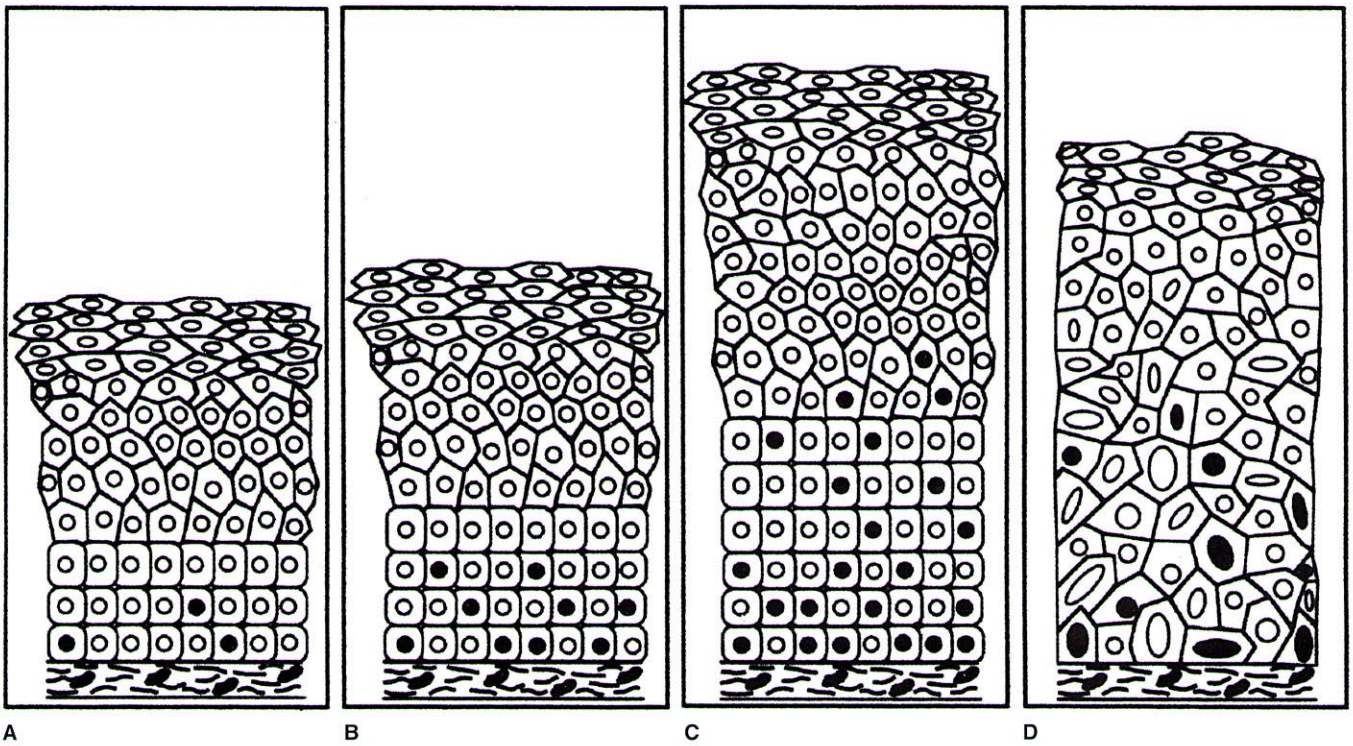

FIGURE 5.7 Diagrammatic representation of normal (A), minimally hyperplastic (B), markedly hyperplastic (C), and dysplastic (D) mucosal surfaces with black nuclei in labeled cells. Comparison of the unit length labeling index (ULLI) between normal and abnormal mucosal surfaces should be accompanied by a description of the histopathologic changes.

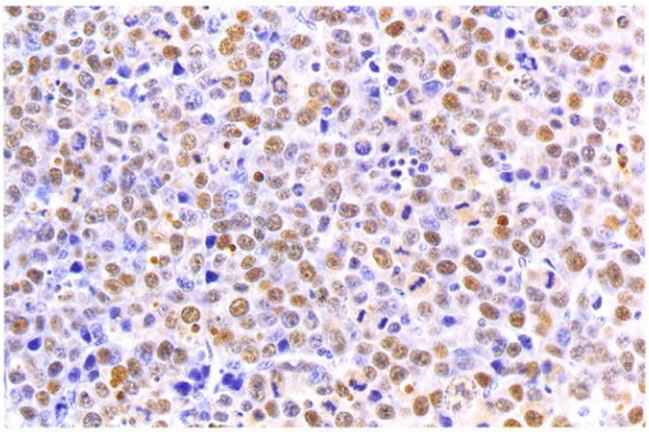

FIGURE 5.8 Immunohistochemical stain for PCNA in a highly proliferative mouse liver lesion. Over 90 percent of the cells are immunopositive.

be characterized by cellular crowding, pleomorphism, dysplasia, or other tissue alterations (Fig. 5.9), making it difficult to establish uniform guidelines for quantitation of a labeling index. The situation may be further complicated by patchy staining within lesions and lack of a distinct boundary between the hyperplastic/neoplastic lesion and the surrounding normal parenchyma. When the difference in labeling is dramatic, a photomicrograph may be more communicative of the ongoing process than laboriously obtained LI comparisons between lesions and "nonlesioned" parenchyma. Reporting of any quantitative data on labeling indices should include appropriate documentation of histopathological features of the lesions.

Variability and Study Design Issues for Cell Proliferation Measurements

Since different tissues have different intrinsic rates of cell turnover (Table 5.4), the target tissue of interest will influence the optimal duration for administering the labeling agent. In general, pulse doses of labeling agent are appropriate for rapidly proliferating tissues. For tissues such as liver or kidney, continuous administration of a labeling agent for approximately 7 days is usually ideal for generating reliable LIs in the absence of proliferative lesions. Administration of labeling agents for 3 to 5 days may also be appropriate for these tissues and possibly for some of the more rapidly proliferating tissues. However, continuous labeling for 3 to 5 days would essentially label all cells in the intestinal tract,

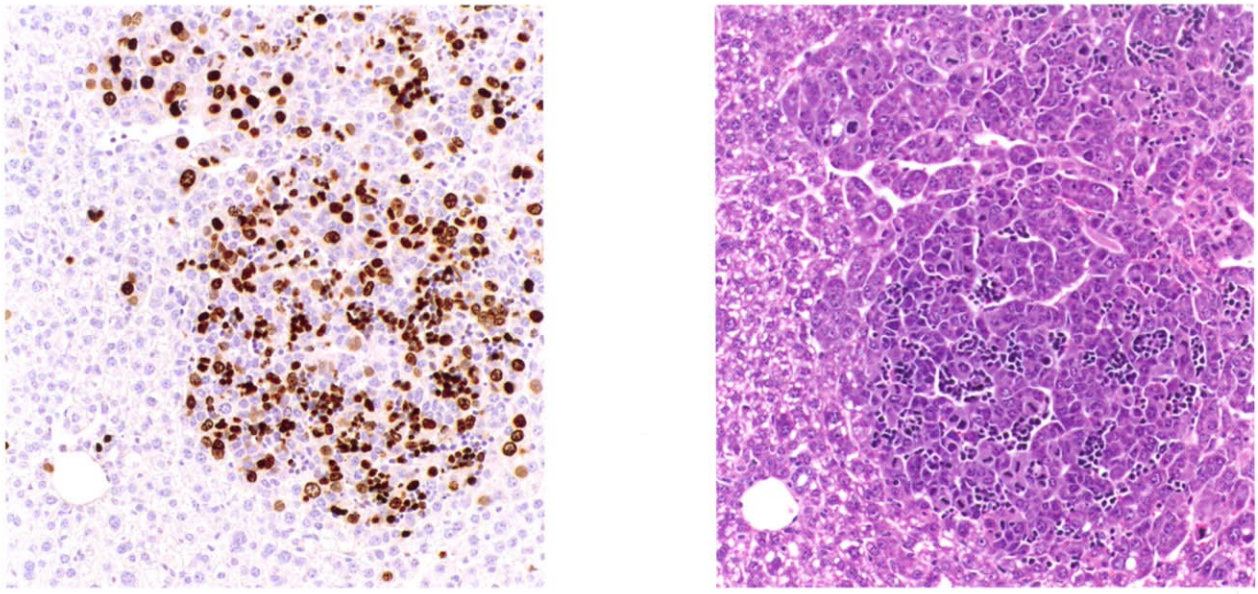

FIGURE 5.9 A proliferative focus of hepatocytes from a mouse carrying the SV40 large T antigen under the influence of an albumin promoter. The left panel is stained with hematoxylin and eosin, and islands of darkly stained hematopoietic cells are present in the focus of proliferating hepatocytes. In the right panel, stained for BrdU following a pulse labeling dose, determining an LI for hepatocytes is complicated by the presence of the labeled hematopoietic cells.

thereby precluding the possibility of detecting any increase in cell proliferation state. In such a situation, use of a pulse dose of BrdU or employing PCNA immunostaining would be preferred for assessing the intestine, while immunostaining for BrdU following a 3-, 5-, or 7-day continuous administration would be the method for assessing more slowly proliferating tissues.

Cell proliferation is generally cyclical, with important circadian rhythms that could significantly influence study data (Barbason et al. 1995), especially when using pulse doses of labeling agents. Hormonal cyclicity (Fujii et al. 1985; Oishi et al. 1993) is well-known to influence proliferation and cellular differentiation in the reproductive tract and liver. Cyclicity is also influenced by environmental factors such as room lighting, feeding schedules, and fluctuating corticosterone levels (Leduc 1949; Llanos et al. 1971; Schulte-Hermann and Landgraf 1974; Hume and Thompson 1990). Mouse tongue epithelium has a declining S-phase LI from 0900 to 1500 hours and an increase in LI from 2100 to 0300 hours with associated change in duration of S-phase from approximately 6 to 9 hours, respectively (Hume and Thompson 1990).

It is theoretically possible that certain treatment regimens could shift the normal circadian cycle, thereby making valid comparison between treated and control animals dependent on appropriate timing of pulse doses of exogenous labeling agents. Dealing with potential problems associated with cyclicity turns out to be most problematic for those tissues with a relatively rapid intrinsic rate of proliferation that are being assessed by a pulse dose of labeling agent. Thus, if the maximum rate of cell proliferation in the esophagus, stomach, or intestine, for example, is at 10 a.m., pulse dosing in the late afternoon will miss the peak of the circadian cycle and will result in a lower and less sensitive assessment of the target cell proliferative state. Critical experiments for such tissues may require some preliminary pilot studies to determine the optimal time for pulse dosing. For tissues with lower intrinsic rates of cell proliferation, such as the liver and kidney, continuous administration of labeling agent over a 3- to 5-day period will capture the peaks as well as the valleys of any circadian cyclicity and, thus, will yield sensitive and accurate assessments of cell proliferative states.

Study design factors that influence cell proliferation results include the number of animals used per time point, the age of the animals, and the number of cells counted. Since there is intrinsic biological variability between animals, it is recommended that at least 8 to 10 animals be used per sampling time point. Using fewer animals may result in enough variability to obscure any potential treatment effects, particularly for more subtle increases or decreases in cell proliferation. Younger animals that are still in the rapid growth phase may give more erratic results depending upon whether the individual animals are sampled during a growth burst or if the experimental conditions happen to retard growth. For many situations, the best cell proliferation results in rodents will be obtained from animals that are 10 weeks

of age or older. Extremely old animals generally have a constellation of spontaneous degenerative and inflammatory changes that could adversely affect and confound cell proliferation analysis, and their use should be avoided if possible. The National Toxicology Program does not generally perform cell proliferation measurements in rodents older than 18 months for this reason, although exceptions are sometime made depending upon the question being asked. Other variables that should be considered in the design of cell proliferation studies include treatment regimen, route and dose of chemical exposure, species, strain, sex, diet, environment, target cell population, method of quantitation, and statistical approaches (Goldsworthy et al. 1991).

Perturbations in PCNA synthesis may occur as a consequence of cellular transformation. Transformed cells and tumors generally synthesize high but variable levels of PCNA (Matthews 1989; Hall et al. 1990) depending upon the mechanism of transformation. Cells expressing oncogenes of SV40 and adenovirus 5 have been characterized as having "runaway PCNA synthesis" (Matthews 1989). PCNA is involved in DNA repair, and since many tumors have active ongoing DNA repair, PCNA could be up regulated in nonproliferating tumor cells (van Diest et al. 1998).

APOPTOSIS

Apoptosis is a form of cell death with distinctive morphological features. It has been documented as single-cell death in tissue sections by pathologists for decades but in the past 15 to 20 years has received renewed interest with the publication of information related to the molecular mechanisms of this type of cell death (Corcoran et al. 1994; Uchiyama 1995; Slyuser 1996). Many contemporary references underscore the dynamics of apoptosis during experimental carcinogenesis and the constellation of molecular changes that accompany apoptosis (Goldsworthy et al. 1996a, 1996b; Slyuser 1996; Harmon and Allan 1997; Staunton and Gaffney 1998; Levin et al. 1999; Hall 1999). Apoptosis is a form of active cell death, requiring gene transcription and mRNA production (Staunton and Gaffney 1998). It is an important factor in embryonic organogenesis, in aging, and in the pathogenesis of cancer. The apoptotic process that occurs during embryogenesis is often termed "programmed cell death." The popularity of apoptosis in research and the casual use of this term in recent years has led to considerable confusion over the distinction between necrotic cell death versus apoptotic cell death. Characteristics of apoptosis and differences between apoptosis and necrosis have been reviewed (Columbano 1995; Majno and Joris 1995; Levin et al. 1999). Apoptotic cell death frequently accompanies necrotic cell death and is often elevated in rapidly growing tissues such as neoplasms. The reason that untreated cancers continue to grow despite the presence of ample numbers of apoptotic cells is that the balance between cell proliferation and apoptotic cell death is skewed in favor of cell proliferation. A primary goal in cancer radiation and chemotherapy is induction of tumor regression as a consequence of the cancer cells undergoing apoptotic cell death (Cotter et al. 1992; Glynn et al. 1992; Martin and Green 1994; Anand et al. 1995).

Morphologic Features of Apoptosis

While there are distinct ultrastructural features characteristic of apoptosis, electron microscopy is often not a practical means for assessing apoptosis in tissues. The criteria for identification of apoptosis have been well defined (Kerr et al. 1972; Wyllie et al. 1980). Histomorphologic features of apoptosis as seen in standard hematoxylin- and eosin-stained sections include deletion of single cells; cell shrinkage with condensation of cytoplasm; condensation of chromatin into crescents, smooth masses, or beads in apposition to the nuclear membrane, followed by nuclear fragmentation; cytoplasmic membrane "blebbing" with ultimate pinching off of pieces of the cell (apoptotic bodies), some of which may contain condensed bits of chromatin; phagocytosis and digestion by adjacent normal cells; and absence of an inflammatory response. Extracellular as well as intracellular phagocytosed apoptotic bodies frequently are surrounded by a narrow clear halo (Staunton and Gaffney 1998). The histomorphologic features of apoptosis vary depending upon the inciting cause, the specific tissue, and the concomitant presence of necrosis. Apoptotic cells and bodies that occur along mucosal and luminal surfaces may be primarily lost by exfoliation. It is noteworthy, however, that delayed fixation for intervals as brief as 15 minutes has been reported to significantly increase the number of visible apoptotic cells (Hall et al. 1994).

Alternative Methods for Documenting Apoptosis

Alternative methods for assessing apoptotic cell death are all fundamentally based on intrinsic features of tissues. Molecular assays based on demonstrating nucleosome-size fragments of cleaved DNA by electrophoresis (DNA ladders), immunohistochemical staining of growth factors, or in situ end-labeling of fragmented DNA are popular (Goldsworthy et al. 1996b). While clearly defined DNA ladders are reasonable assays if there is extensive apoptosis, they are less satisfactory when there are only a few apoptotic cells in a tissue comprised

of predominately normal cells. Furthermore, in situ end-labeling is not specific for apoptosis and has been shown to also occur in necrotic cell death (Grasl-Kraupp et al. 1995; Mundle 1995). Histomorphologic features of apoptotic cells are sufficiently distinctive (see above), and the most widely accepted standard for assessing apoptosis is based on quantitation of apoptotic bodies in histologic sections stained with hematoxylin and eosin (Figs. 5.10, 5.11; Potten 1996). Use of in situ end-labeling, use of flow cytometry on cell suspensions, or demonstration of DNA ladders can be helpful in confirming apoptosis observed in standard histological tissue specimens.

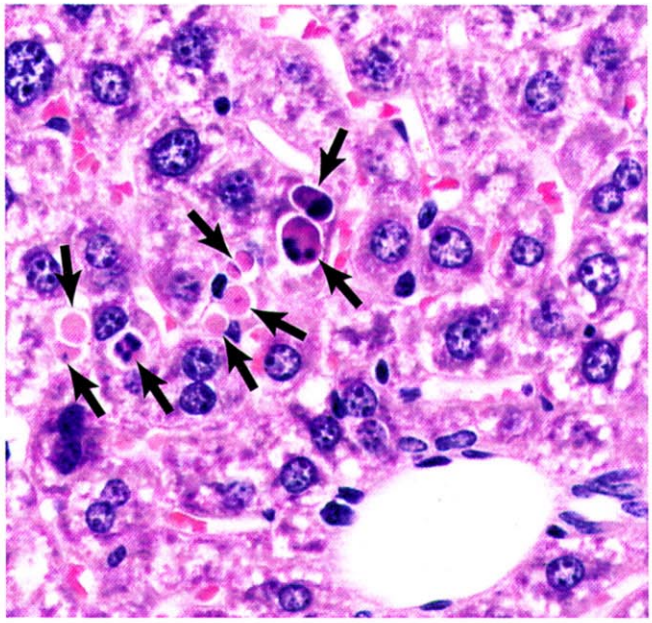

FIGURE 5.10 Hematoxylin- and eosin-stained mouse liver with numerous apoptotic bodies (*arrows*).

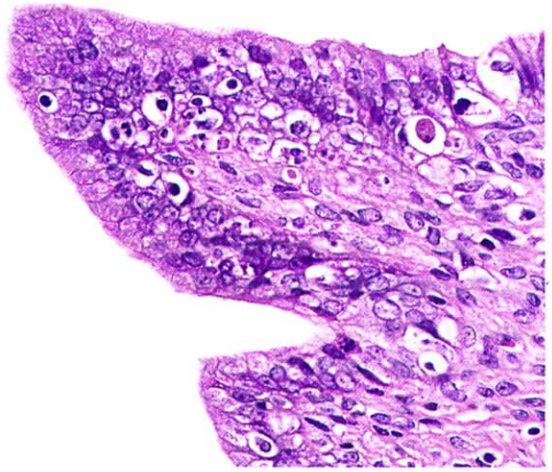

FIGURE 5.11 Hematoxylin- and eosin-stained mouse uterine mucosa with apoptotic bodies surrounded by clear halos.

Counting/Measuring Apoptosis

Quantitation of apoptotic indices is labor-intensive because generally large numbers of normal cells need to be examined just to find a few apoptotic cells. It is common to count 5000 to 10,000 hepatocytes just to find a single or a few apoptotic cells in normal mouse liver (Goldsworthy et al. 1996b). Since most investigations require comparison of the relative apoptotic state between treated and control animals, between tissue lesions and normal tissue, or between wild-type and genetically modified mice, a considerable amount of time will be required to generate appropriate apoptotic indices. Furthermore, just as generating cell proliferation counts alone does not adequately address total tissue dynamics, an apoptotic index without a corresponding assessment of cell proliferation may not correctly depict important tissue dynamics.

Quantitation of apoptosis typically involves generation of an apoptotic index (AI). The AI is defined by the number of apoptotic bodies divided by the total number of nuclei counted. Since a single apoptotic cell may give rise to multiple apoptotic bodies, estimation of an apoptotic cell index is based upon the number of apoptotic bodies observed and their proximity to each other. As a rule of thumb in the mouse liver, a maximum of four apoptotic bodies in close proximity would be considered to be derived from a single apoptotic cell, while apoptotic bodies separated from each other by more than two hepatocytes would be considered to be derived from two different apoptotic cells (Goldsworthy et al. 1996b). Similar to the situation with generating LIs, shortcuts to generating denominator value for an AI (e.g., number of apoptotic bodies per square millimeter of tissue, using the average number of cells per field for estimating the denominator) might sometimes be warranted.

Variability and Study Design Issues for Apoptosis Measurements

Although not as well defined or understood as cell proliferation cyclicity, apoptosis may also be subject to circadian cyclicity (Potten 1996). This cyclicity is dramatically demonstrated in the female mouse reproductive system, where apoptosis is high in ovarian follicles undergoing atresia, or in uterine epithelium during the

estrous cycle. In rats, the feeding schedule appears to synchronize apoptosis during the day-night cycle (Bursch et al. 1984, 1986). Sensitive measurement of apoptosis, much like the case for measurement of mitosis, is further compromised by the fact that the duration of morphologically visible apoptosis is relatively short. Visible apoptosis is estimated to last approximately 4 hours (Bursch et al. 1990; Goldsworthy et al. 1996b); however, there is a wide variability in reported duration of visible apoptosis (Potten 1996) with suggested estimates as short as 1 hour (Coles et al. 1993). In some regards, the balance between cell birth and cell death might best be assessed by comparison of the mitotic and apoptotic indices (Arends et al. 1994), since both measures are of relatively similar duration in terms of visibility in stained tissue sections. But even with attempts to achieve both measurements, temporal difference in peak activity may require sampling at different intervals to first measure the increase in cell proliferation and subsequently to measure the peak of apoptosis several hours later.

The interanimal variability in apoptotic indices is likely to be considerably larger than that for some of the more sensitive measures of cell proliferation. This has been demonstrated for mouse hepatocytes (Goldsworthy et al. 1996b) and is expected to be true for other tissues as well. This is probably attributable to the short duration of apoptosis and to the fact that not all animals in a given group will be synchronized with respect to peak apoptosis. Based upon our experience with mouse liver, we recommend that greater than 10 animals per group be used for accurate assessment of apoptosis.

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Web Sites

Immunohistochemistry procedures for PCNA and BrdU
http://dir.niehs.nih.gov/dirlep/immuno.html

In situ end-labeling (TUNEL) for apoptosis
http://dir.niehs.nih.gov/dirlep/ish.html

Osmotic minipumps
http://www.alzet.com