J Gastroenterol 1995; 30:725–730

Journal of Gastroenterology

Identification of apoptotic hepatocytes in situ in rat liver after lead nitrate administration

Tomoki Nakajima,[1] Takeshi Deguchi,[1] Keizo Kagawa,[3] Hiroshi Hikita,[1] Kazushige Ueda,[1] Tatsuo Katagishi,[1] Tohru Ohkawara,[1] Masamichi Kakusui,[1] Hiroyuki Kimura,[1] Takeshi Okanoue,[1] Kei Kashima,[1] and Tsukasa Ashihara[2]

[1] Third Department of Internal Medicine and [2] First Department of Pathology, Kyoto Prefectural University of Medicine, 465, Kajii-cho, Hirokoji, Kawaramachi, Kamigyo-ku, Kyoto, 602 Japan
[3] Fukuchiyama City Hospital, 231, Atsunaka-cho, Fukuchiyama, Kyoto, 620 Japan

Abstract: Apoptosis plays a major role in the regression of mitogen (lead nitrate)-induced hepatic hyperplasia. We compared the in situ end-labeling (ISEL) technique with the conventional detection of apoptotic bodies in this process. In hematoxylin and eosin (H&E) sections, apoptosis is usually recognizable by the presence of apoptotic bodies (apoptosis phase 2). Although the early phase of apoptosis (apoptosis phase 1) can be detected as a prekaryorrhectic appearance in H&E sections, it is difficult to detect and is easily overlooked. On the other hand, ISEL presents intense staining mainly in phase 1 and weak or negative staining in phase 2. Thus, simultaneous investigation by these two methods in two serial sections is the most reliable way to calculate the incidence of apoptosis and gives us precise information on the stages of apoptosis in situ. Since the colorized signals of ISEL are much easier to detect than apoptotic bodies in H&E sections, ISEL is particularly useful for liver tissues, where the incidence of apoptosis is low.

Key words: apoptosis, in situ end-labeling, lead nitrate, hepatic hyperplasia, tissue kinetics

Introduction

Since the proliferative activity of hepatic tissue is an important factor in the evaluation of liver diseases, various methods have been used to detect proliferating hepatocytes. In addition to conventional methods, such as tritiated thymidine autoradiography[1] and bromodeoxyuridine (BrdU) immunohistochemistry,[2,3] the development of monoclonal antibodies to cell cycle-related proteins has enabled us to easily detect proliferating cells in human liver tissues.[4–6]

However, to determine the precise state of tissue kinetics, cell loss, in addition to cell proliferation, should be evaluated. Apoptotic cell death is now considered to play an important role in maintaining homeostasis in actively proliferating tissues.[7,8]

The terminal features of apoptotic cells have been traditionally recognized as apoptotic bodies showing fragmented nuclei in routine hematoxylin and eosin (H&E)-stained sections.[9,10] However, by light microscopy, nuclear shapes similar to those of apoptotic bodies are occasionally encountered in some liver cells, such as polymorphonuclear lymphocytes, mitotic cells, and some cancer cells with bizarre-shaped nuclei.

Some researchers have speculated on the early morphological features of apoptotic hepatocytes in H&E sections.[10] However, direct evidence of apoptotic processes in such cells has never been demonstrated in situ. A method known as in situ end-labeling (ISEL) has been reported to be useful in detecting apoptotic cells.[11–13] With this method, the terminals of fragmented DNA in apoptotic cells can be labeled by biotinylated deoxy-uridine triphosphate (dUTP) with the specific enzyme terminal deoxynucleotidyl transferase. Using this method in the regression of mitogen-induced hyperplasia in rat liver,[14–16] we measured apoptosis by ISEL and by the conventional detection of apoptotic bodies, and we morphologically confirmed the time course of apoptotic changes.

Materials and methods

Animal treatment and tissue preparation

A total of 40 male Wistar rats (8 weeks of age, 204 ± 6 g) were fed a semisynthetic diet. Thirty-six of the

Offprint requests to: T. Nakajima
(Received for publication on Nov. 15, 1994; accepted on Mar. 24, 1995)

animals were given lead nitrate (Nacalai, Kyoto, Japan), dissolved in sterilized distilled water (DW), intravenously at a dose of 10 μM/100 g body weight. These 36 animals were divided into nine groups, 4 rats being sacrificed at 12, 24, 36, 48, 72, 96, 120, 168, and 336 h after the injection. As a control study, the other 4 rats were sacrificed without injection. Each animal was intraperitoneally injected with BrdU (40 mg/kg; Sigma, St. Louis, USA) 1 h before sacrifice. After the rats were killed, their body weight and liver weight were determined and the relative liver weight was calculated. The liver was cut into small pieces (0.5–1 cm^3), fixed in 4% buffered paraformaldehyde for 14–17 h at 4°C, and paraffin-embedded. Four-μm-thick serial sections were prepared for H&E staining, BrdU immunohistochemistry, and ISEL.

H&E Staining

The apoptotic index (‰) was determined, this being defined as the incidence of apoptotic bodies in 1000 hepatocytes. Apoptotic bodies were identified according to previously published criteria.[10]

BrdU Immunohistochemistry

After deparaffinization, the sections were incubated with 4 N HCl for 20 min to denature DNA strands, and washed with Tris-buffered saline (TBS). They were then treated with 0.1% trypsin dissolved with $CaCl_2$ in Tris-HCl buffer (pH 7.6) at 37°C for 13 min, immersed in 0.3% H_2O_2 in absolute methanol for 25 min, and preincubated with 2% fetal bovine serum (FBS) diluted in TBS at room temperature for 10 min, with a brief wash in TBS between each step. The slides were then incubated with anti-BrdU antibody (Dako, Tokyo, Japan) diluted 1:100 with TBS at 4°C overnight. We then employed the streptavidin-biotin-peroxidase method, using biotinylated anti-mouse immunoglobulin (DAKO) and peroxidase-conjugated streptavidin (DAKO). Finally, positive nuclei were stained by the peroxidase reaction with 3.3′-diaminobenzidine (Dojin, Kumamoto, Japan) as a substrate. The slides were lightly counter-stained with hematoxylin. The BrdU labeling index (BrdU-LI) was determined, this being defined as the percentage of labeled nuclei in 100 hepatocytes.

In Situ end-labeling (ISEL)

The sections were preheated at 60°C for 30 min and deparaffinized; they were then washed with distilled water (DW) and incubated with Tris-HCl buffer (pH 7.4) for 3 min, after which, proteinase K 20 μg/ml was applied at room temperature and allowed to remain for 15 min. After being washed with tap water, the sections were reacted with 0.3% H_2O_2 in absolute methanol for 25 min. They were then washed with doubled-distilled water (DDW), and immersed in terminal deoxynucleotidyl transferase (TdT) buffer (pH 7.2, 0.1 M potassium cacodylate, 0.2 mM dithiothreitol; Promega, Madison, USA) for 10 min. TdT (0.3 eu/μl; Promega) and biotin-16-dUTP (0.04 nmol/μl; Boehringer Mannheim, Mannheim, Germany) in TdT buffer were added and the sections were incubated in a moisture chamber at 37°C for 90 min. The reaction was terminated in 2 × standard saline citrate (SSC; 300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. The sections were then washed with DDW, and non-specific reaction was blocked by incubation with fetal bovine serum (FBS) diluted in TBS at room temperature for 10 min. The signals were visualized in the same way as for BrdU. After an additional washing with tap water, the sections were counterstained with hematoxylin. The in situ end-labeling index (ISE-LI) was determined, this being defined as the incidence (‰) of labeled nuclei in 1000 hepatocytes. All values were expressed as means ± SD.

Morphological study of apoptotic cells

In practice, it was impossible to obtain precise correspondence for each apoptotic hepatocyte between the 4-μm-thick serial sections subjected to ISEL and those subjected to H&E staining. Therefore, we examined the nuclear staining patterns in H&E sections similar to those of end-labeled cells, and carried out a comparative morphological study.

Results

As shown in Fig. 1, relative liver weight (g/100 g body weight) increased until day 5, and then gradually decreased to the control level.

BrdU-LI, as shown in Fig. 2, showed a rapid increase during the first 36 h but returned to the control level by day 5. Labeled cells were distributed mainly in the periportal areas.

The incidence of apoptotic bodies recognized on H&E sections gradually increased from day 1 until day 3, and then increased rapidly, reaching a maximum on day 4, followed thereafter by a gradual decrease (Fig. 3a). These bodies often appeared as small clusters but never showed specific distribution.

Sequential changes in ISE-LI (Fig. 3b) were similar to those in the apoptotic bodies. Figure 3c shows the significant correlation between the apoptotic index and ISE-LI. ISE-LI was lower than the apoptotic index. Furthermore, the end-labeled cells appeared as small

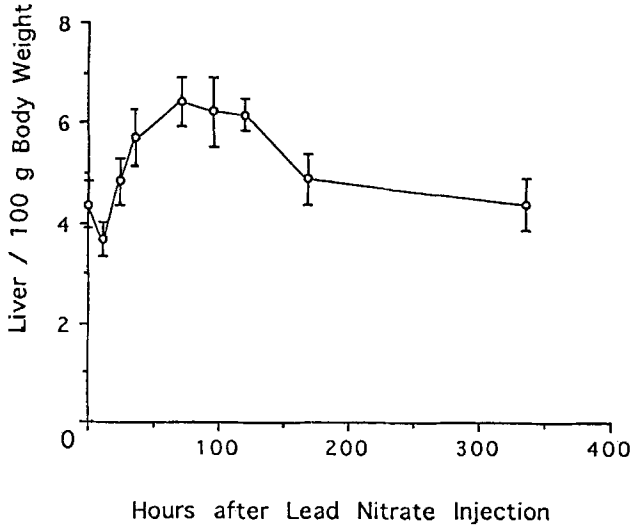


Fig. 1. Sequential changes in relative liver weight (g/100 body weight) after lead nitrate administration. Increases were observed until day 5, with a subsequent gradual decrease to the control level

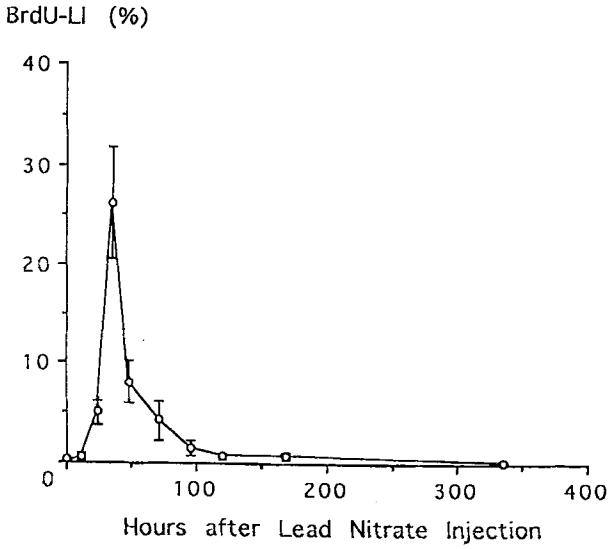
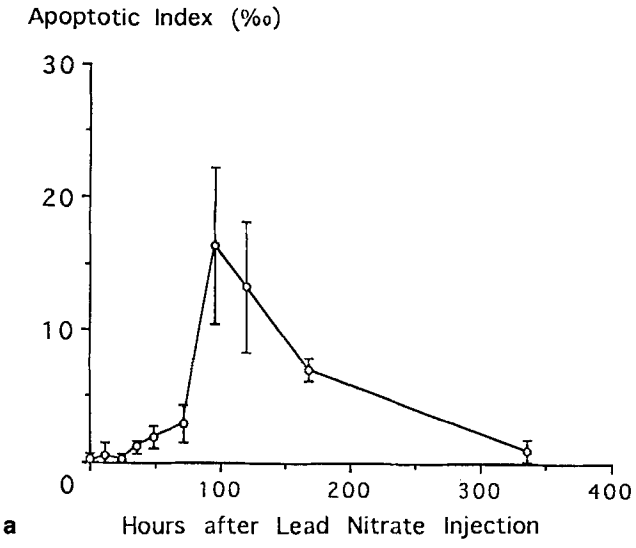
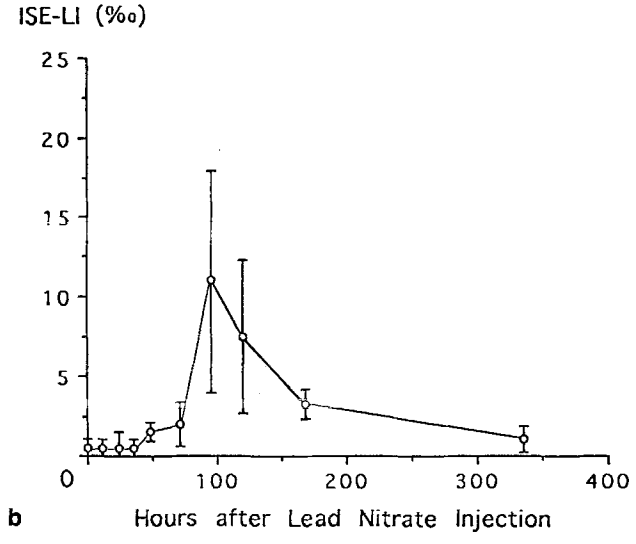


Fig. 2. Sequential changes in bromodeoxyuridine-labeling index (*BrdU-LI*) after lead nitrate administration. A rapid increase was observed during the first 36 h, with a subsequent decrease to the control level by day 5





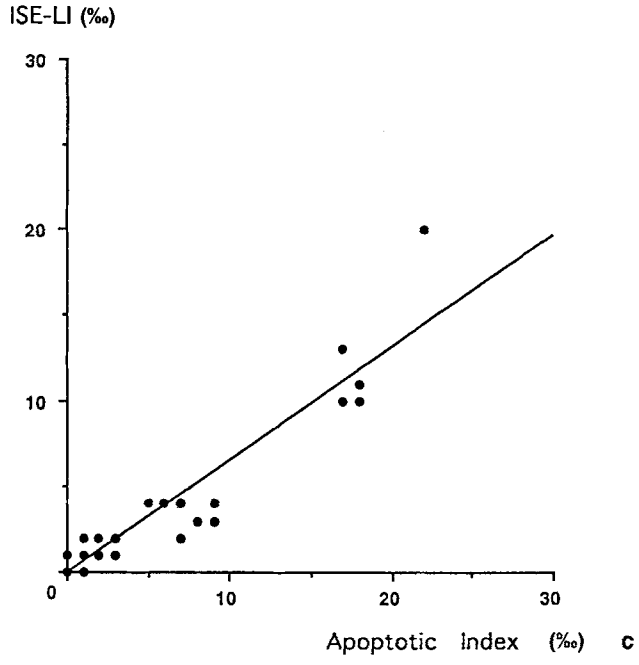


Fig. 3a–c. Sequential changes in **a** apoptotic index and **b** in situ end-labeling index (*ISE-LI*), after lead nitrate administration. The apoptotic index gradually increased from day 1 until day 3, and then increased rapidly, reaching a maximum on day 4. A gradual decrease was observed thereafter. The ISE-LI showed similar sequential changes. **c** There was a significant correlation between the apoptotic index and ISE-LI in this experiment ($y = -0.164 + 0.664\,x$; $r = 0.942$; $n = 40$)

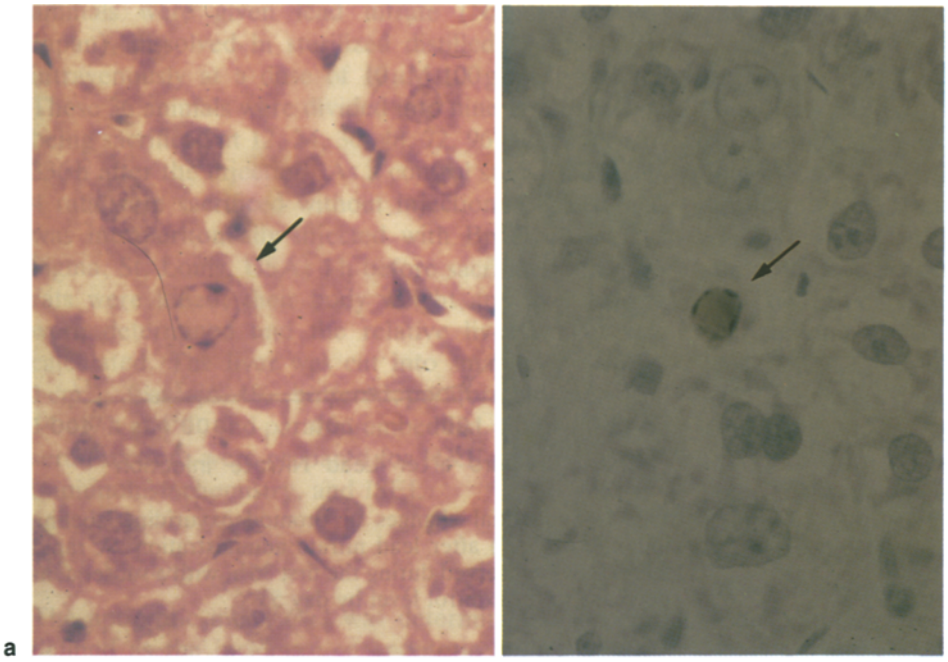


Fig. 4. a A prekaryorrhectic hepatocyte (*arrow*) shows eosinophilic cytoplasm slightly detached from the neighboring cells and chromatin condensation adjacent to the nuclear membrane. (H&E, ×200). **b** Intense staining is observed in the nucleus of such a hepatocyte (*arrow*). (ISEL, ×200)

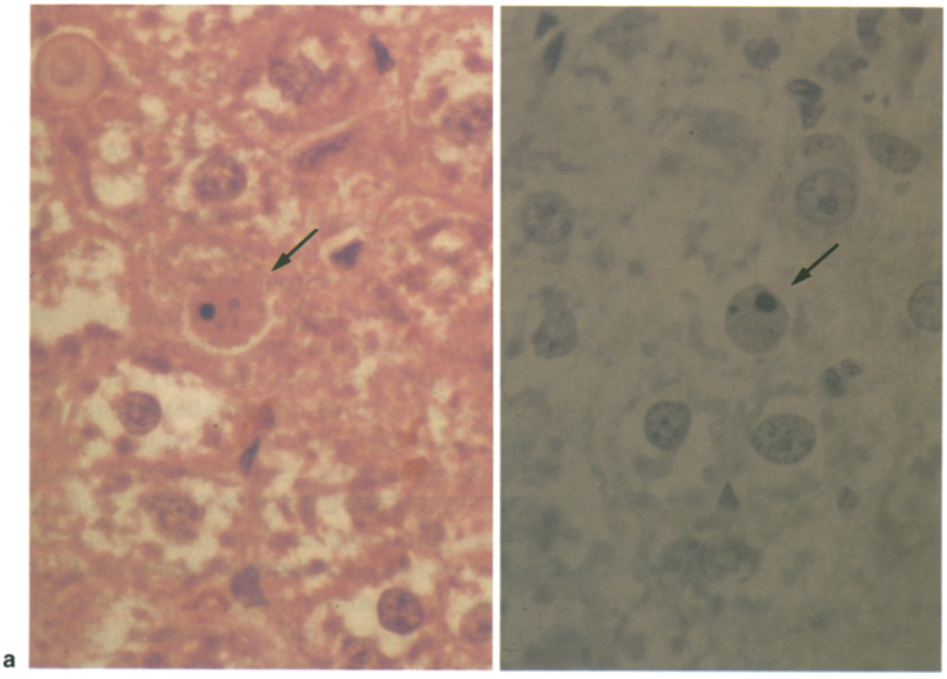


Fig. 5. a An apoptotic body (*arrow*) showing fragmented nucleus isolated from the neighboring cells. (H&E, ×200). **b** Such apoptotic bodies (*arrow*) were negative or weakly stained for ISEL. (ISEL, ×200)

clusters but never showed specific tissue distribution as in the case of the apoptotic bodies.

Hepatocytes intensely positive on ISEL (Fig. 4b) showed eosinophilic cytoplasm slightly detached from neighboring hepatocytes on H&E sections (Fig. 4a). On the other hand, most typical apoptotic bodies on H&E sections (Fig. 5a) were negative or weakly positive on ISEL (Fig. 5b).

Discussion

Columbano et al.[15] first reported that a single dose of lead nitrate, a mitogen for hepatocytes, induced temporary liver cell hyperplasia and that apoptosis played an important role during the regression process. Hikita et al.[16] later elucidated this phenomenon. In the present study, we found that the sequential changes in

relative liver weight occurred in two stages; a temporary increase in proliferative activity during the first few days, and the apoptotic process thereafter. Thus, the tissue kinetics after lead nitrate administration were clearly demonstrated by the sequential changes in BrdU-LI, apoptotic index, and ISE-LI.

Several methods are employed to detect apoptosis. One method involves the detection of a landmark of apoptosis, the presence of nucleosomal DNA fragments, which can be observed as a typical "ladder" on agarose gel electrophoresis.[17,18] However, this method provides neither information regarding the tissue localization of apoptotic cells nor a way to quantify the apoptosis. The ISEL method is based upon direct and specific labeling of DNA breaks in situ,[12–14] enabling us to solve these problems.

Morphological alterations during the apoptotic process, identified by electron microscopy, occur in three phases.[19,20] Phase 1 is characterized by chromatin condensation into crescent caps at the nuclear periphery, nucleolar disintegration, and reduction in nuclear size. Compactness of cytoplasmic organelles and dilatation of the endoplasmic reticulum are also observed. In phase 2, the main features are budding and separation of the nucleus and cytoplasm into small membrane-bound apoptotic bodies. When hepatocytes become apoptotic bodies, they may be phagocytosed by neighboring hepatocytes or macrophages. In phase 3, there is progressive degeneration of subcellular structures.

Based upon the above morphological reports,[19,20] we morphologically confirmed the time course of apoptotic changes in hepatocytes with H&E-staining and ISEL in serial sections. In the H&E sections, apoptosis is usually recognized in phase 2 by the presence of apoptotic bodies. Although some researchers have reported that apoptosis in phase 1 can be detected as a prekaryorrhectic appearance in H&E sections, such apoptotic hepatocytes are very difficult to detect and are easily overlooked. ISEL, on the other hand, presents intense staining mainly in phase 1 and weak or negative staining in apoptotic bodies (phase 2). Since the duration of phase 1 is extremely short,[21] the incidence of prekaryorrhectic hepatocytes is fairly low compared with that of apoptotic bodies. As a result, we found that ISE-LI was lower than the apoptotic index. Thus, we conclude that simultaneous investigations by these two methods in serial sections is the most reliable way to calculate the incidence of apoptosis and to yield precise information on the stages of apoptosis in situ.

One possible reason for these apoptotic bodies being negative or weakly positive for ISEL is that nuclear chromatin condensation, as seen in H&E sections, decreases the accessibility of TdT and dUTP to fragmented DNA terminals. A previous study reporting that ISE-LI was greatly influenced by the degree of the pretreatment with proteolytic digestion supports such a hypothesis.[13,14] We also pretreated tissues with pepsin to enhance the positive identification of all apoptotic bodies, as was done in a previous study,[12] but we found that nonspecific reactions were intensified in control rats.

In general, the duration of the apoptotic process is estimated to be as short as 2–3 h.[21] Moreover, unlike rapidly sloughing tissues such as the gastrointestinal mucosa, the liver consists of slowly renewing hepatocytes, and the incidence of apoptosis is extremely low.[11,16] Therefore, ISEL is useful for detecting apoptotic cells in hepatological microscopic fields, since the colorized signals of ISEL are much easier to detect than the apoptotic bodies in H&E sections.[12]

Of particular interest, one of the most useful areas for ISEL lies in the relative quantification of apoptotic cell death in hepatocellular carcinomas (HCCs), in which the identification of apoptotic nuclei may be extremely difficult because of the presence of many polymorphic nuclei in the tumor[13] and because of the severe cellular atypism in the cancer cells. The evaluation of tumor cell death should provide more precise information on the cell kinetics of HCCs. We are currently investigating the relationship between proliferative activity and apoptotic cell death in HCCs in association with their clinical features.

References

1. Leevy CM. In vitro studies of human hepatic DNA synthesis in percutaneous liver biopsy specimens. J Lab Clin Med 1963; 61:761–779.
2. Gratzner HG. Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science 1982;218:474–476.
3. Hamada S. A double labeling technique combining ^{3}H-thymidine autoradiography with BrdU immunohistochemistry. Acta Histochem Cytochem 1985;18:267–270.
4. Namikawa R, Ueda R, Suchi T, et al. Double immunoenzymatic detection of surface phenotype of proliferating lymphocytes in situ with monoclonal antibodies against DNA polymerase alpha and lymphocyte membrane antigens. Am J Clin Pathol 1987;87:725–731.
5. Hall PA, Richards MA, Gregory WM, et al. The prognostic value of Ki-67 immunostaining in non-Hodgkin's lymphoma. J Pathol 1988;154:223–235.
6. Garcia RL, Coltrera MD, Gown AM. Analysis of proliferating grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. Comparison with flow cytometric analysis. Am J Pathol 1989;134:733–739.
7. Cattoretti G, Becker MHG, Key G, et al. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 1992;168:357–363.
8. Kerr JFR, Searle J, Bishop CJ. Apoptosis: A distinctive mode of cell death that plays an opposite role to mitosis in cell population kinetics. Australas Radiol 1979;23:192–201.

9. Cotter TG, Lennon SV, Glynn JG, Martin SJ. Cell death via apoptosis and its relationship to growth, development and differentiation of both tumor and normal cells. Anticancer Res 1990;10:1153–1159.
10. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239–257.
11. Bursch W, Taper HS, Lauer B, Schulte-Hermann R. Quantative histological and histochemical studies on the occurrence and stages of controlled cell death (apoptosis) during regression of rat liver hyperplasia. Virchows Arch [A] 1985;50:153–166.
12. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493–501.
13. Wijsman JH, Jonker RR, Keijzer R, et al. A new method to detect apoptosis in paraffin sections: In situ end-labeling of fragmented DNA. 1993;41:7–12.
14. Ansari B, Coates PJ, Greensein BD, Hall PA. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. J Pathol 1993;170:1–8.
15. Columbano A, Ledda GM, Sirigu P, et al. Liver cell proliferation induced by a single dose of lead nitrate. Am J Pathol 1983; 110:83–88.
16. Hikita H, Kagawa K, Okanoue T, et al. Cell analysis of liver hyperplasia induced by lead nitrate (in Japanese). Acta Hepatol Jpn 1990;31:653–659.
17. Duke RC, Chervenak R, Cohen JJ. Endogenous endonuclease-induced DNA fragmentation: An early event in cell-mediated cytolysis. Proc Natl Acad Sci USA 1983;80:6361–6365.
18. Arends MJ, Morris RG, Wyllie AH. Apoptosis:; The role of the endonuclease. 1990;136:593–608.
19. Wyllie AH, Morris RG, Smith AL, Dunlop D. Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathold 1984;142:67–77.
20. Wyllie AH. Apoptosis. Immunology 1988;1:192–196.
21. Bursch W, Paffe S, Putz B, et al. Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. Carcinogenesis 1990;11:847–853.