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Abstract

This study was designed to investigate the induction of apoptosis during the reperfusion phase following warm liver ischemia in vivo. We evaluated apoptotic bodies (ABs) in sections stained with hematoxylin and eosin (H. E.) and positive hepatocytes in sections stained by the in situ nick end labeling method (TUNEL method) during the reperfusion phase up to 48 h after a 70% liver ischemia for 30 or 60min in duration (30 or 60 min group). The peak number of ABs in H. E.-stained sections was observed at 1 to 3 h in the 30 min group and 3 to 6 h in the 60 min group. The number of ABs gradually fell as the length of the perfusion period increased, and few ABs were observed at 24 and 48 h after reperfusion. A peak number of TUNEL-positive hepatocytes was recognized at 3 h after reperfusion in both groups, after which the numbers decreased gradually. DNA extracted from both groups was electrophoresed on a 1.5% agarose gel. In both groups, a ladder-like pattern over smear pattern was recognized at 3 h after reperfusion. These results show that hepatocyte apoptosis was induced during the early phase of reperfusion after rat liver ischemia morphologically and biochemically, which suggests that hepatocyte apoptosis may be associated with ischemia and reperfusion injury.

KEYWORDS: apoptosis, warm ischemia, reperfusion injury

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Induction of Apoptosis During the Early Phase of Reperfusion after Rat Liver Ischemia

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This study was designed to investigate the induction of apoptosis during the reperfusion phase following warm liver ischemia *in vivo*. We evaluated apoptotic bodies (ABs) in sections stained with hematoxylin and eosin (H. E.) and positive hepatocytes in sections stained by the *in situ* nick end labeling method (TUNEL method) during the reperfusion phase up to 48 h after a 70% liver ischemia for 30 or 60 min in duration (30 or 60 min group). The peak number of ABs in H. E.-stained sections was observed at 1 to 3 h in the 30 min group and 3 to 6 h in the 60 min group. The number of ABs gradually fell as the length of the perfusion period increased, and few ABs were observed at 24 and 48 h after reperfusion. A peak number of TUNEL-positive hepatocytes was recognized at 3 h after reperfusion in both groups, after which the numbers decreased gradually. DNA extracted from both groups was electrophoresed on a 1.5% agarose gel. In both groups, a ladder-like pattern over smear pattern was recognized at 3 h after reperfusion. These results show that hepatocyte apoptosis was induced during the early phase of reperfusion after rat liver ischemia morphologically and biochemically, which suggests that hepatocyte apoptosis may be associated with ischemia and reperfusion injury.

Key words: apoptosis, warm ischemia, reperfusion injury

The liver is damaged by ischemia in liver transplantations or liver operations, and reperfusion after ischemia results in functional impairment. Prolonged ischemia causes massive necrosis in the pericentral area, zone 3 (1) and induces liver failure. Whether liver dysfun-

ction is reversible or causes liver failure depends on the duration of ischemia. But even brief periods of ischemia damage the liver with no morphological evidence of liver cell degeneration.

Recently, another type of cell death, apoptosis, has been found to accompany necrosis during cell injury caused by ischemia. Several studies have shown that apoptotic cell death is associated with renal damage, including acute tubular necrosis after brief periods of renal ischemia (2). In neuroscience, neuronal damage in the hippocampus induced by transient forebrain ischemia may also be associated with apoptotic-type cell death (3, 4). In the liver, it has been reported that apoptotic cell death is associated with liver damage caused by exposure to hepatotoxins (5-8), clamping of the portal vein (9), and short gut syndrome (10).

Therefore, apoptotic cell death might occur during the reperfusion phase after liver ischemia. We investigated the induction of apoptosis during the reperfusion phase following liver ischemia *in vivo*. Apoptotic cell death can be identified morphologically by characteristic 'apoptotic bodies (ABs)', and biochemically by DNA fragmentation, a consequence of the activation of endonucleases (11-13). We evaluated and quantified ABs in liver sections stained with hematoxylin and eosin (H. E.), and positive hepatocytes in sections stained by the nick end labeling method (TUNEL method) (14). Electrophoresis of the DNA extracted from the liver tissue was also examined to reveal apoptosis induction.

Materials and Methods

Treatment of animals. Male Wistar rats (weighing between 230 and 260 g, 7- to 9-week-old) obtained from the Shizuoka Experimental Animal Farm

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(Hamamatsu, Japan) were fed *ad libitum* and then starved for 12h before use. Surgical procedures were performed under ether anesthesia. The liver was approached through a median incision. While maintaining blood flow to the right lobe of the liver, the portal vein and the hepatic artery to the left and median lobes were clamped for 30 or 60 min to induce 70 % liver ischemia. Reperfusion was continued up to 48h after declamping. The rats were sacrificed 0, 1, 3, 6, 12, 24 and 48h after initiating reperfusion, and their livers were sampled. We compared the tissues from the left lobe (ischemic group, 30/60 min) and the right lobe (control group). From 5 to 8 rats were sacrificed at each point.

Histologic examination of the sections.

The liver was immediately fixed in 10 % buffered formalin overnight and embedded in paraffin. Sections of 5 μ m thickness were prepared, which were then deparaffinized and stained with H. E., and also stained by the TUNEL method, as described by Gavrieli *et al.* (14) with minor modifications. Briefly, the sections, pretreated with proteinase K, were nick end labeled with biotinylated poly deoxyuridine triphosphate (d-UTP), introduced by terminal deoxynucleotidyl-transferase (TdT) to the 3'-OH ends of the DNA, and then reacted with avidin-conjugated peroxidase. Finally, treated sections were reacted in 3-3'-diaminobenzidine (DAB). The sections were examined under a light microscope. For each section stained by the H. E. and the TUNEL method, about 100 to 200 high concentration fields were examined (400 $\times$, about 400 to 500 hepatocytes per field, 0.166 mm²), and the numbers of ABs in the H. E. sections and positive hepatocytes in the TUNEL sections were counted (number per square cm).

DNA extraction. Part of the extracted liver tissues was immediately frozen in liquid nitrogen after removal. DNA was extracted from the frozen tissues using a DNA extraction kit (Sepa Gene, Sanko Junyaku Co., Tokyo, Japan). DNA concentrations were evaluated by UV spectrophotometry at 260nm, and 5 μ g of DNA was electrophoresed on a 1.5 % agarose gel. The gel was briefly stained with ethidium bromide, and visualized and photographed under ultraviolet transillumination.

Statistical analysis. All values are expressed as mean $\pm$ SEM. Statistical analyses were performed by non-parametric Wilcoxon's signed rank tests and paired Student's *t*-tests. *P* values less than 0.05 were considered significant.

Results

Histological examination of the sections stained with H. E.

Histological examination of the sections revealed no remarkable changes immediately after reperfusion. One hour after reperfusion, the liver cells were swollen at zones 1 and 2, and characteristically round acidophilic bodies with condensed chromatin, apoptotic bodies (ABs), were observed in the periportal area (zone 1). In the 30 min group 27.3 ± 4.6 ABs/cm², and in the 60 min group 22.1 ± 5.9 ABs/cm² were observed without any inflammatory reactions. Three hours after reperfusion, 26.1 ± 4.3 ABs/cm² in the 30 min group and 33.7 ± 5.9 ABs/cm² in the 60 min group were observed at zones 1 and 2 (Fig. 1). In the sections of the 60 min group, focal necrosis with inflammatory reactions were seen occasionally in zone 2. Six hours after reperfusion, 14.9 ± 2.1 ABs/cm² in the 30 min group and 32.6 ± 5.5 ABs/cm² in the 60 min group were observed. Three to 12h after 60 min of ischemia, ABs were occasionally observed in zone 2. Twelve hours after reperfusion, the number of ABs was gradually reduced, and 24 and 48h after reperfusion, few ABs, necrosis, or inflammatory reactions were observed. Few ABs were recognized in the sections of the control group at any time.

The number of ABs per cm² was analyzed (Fig. 2). As shown in Fig. 2, the number of ABs was significantly higher in the 30 min and 60 min groups 1 and 3h after reperfusion compared with the control group. But no significant differences between the number of ABs in the 30 min group and the 60 min group were found at any time.

Histological examination of the sections stained by the TUNEL method.

Hepatocytes stained positively by the TUNEL method were observed more extensively in zones 1 and 2 than ABs in the H. E. stained sections (Fig. 3). One hundred to 200 high concentration fields (400 $\times$, about 400 to 500 hepatocytes per field) were examined in the sections stained by the TUNEL method, and the number of TUNEL-positive hepatocytes per cm² was counted (Fig. 4). The peak in number of TUNEL-positive hepatocytes was observed at 3h after reperfusion in the both groups (30 and 60 min groups), and then the number gradually decreased. From 1 to 48h, a significant increase in the number of TUNEL-positive hepatocytes was recognized in the both groups compared to the control group. There was no

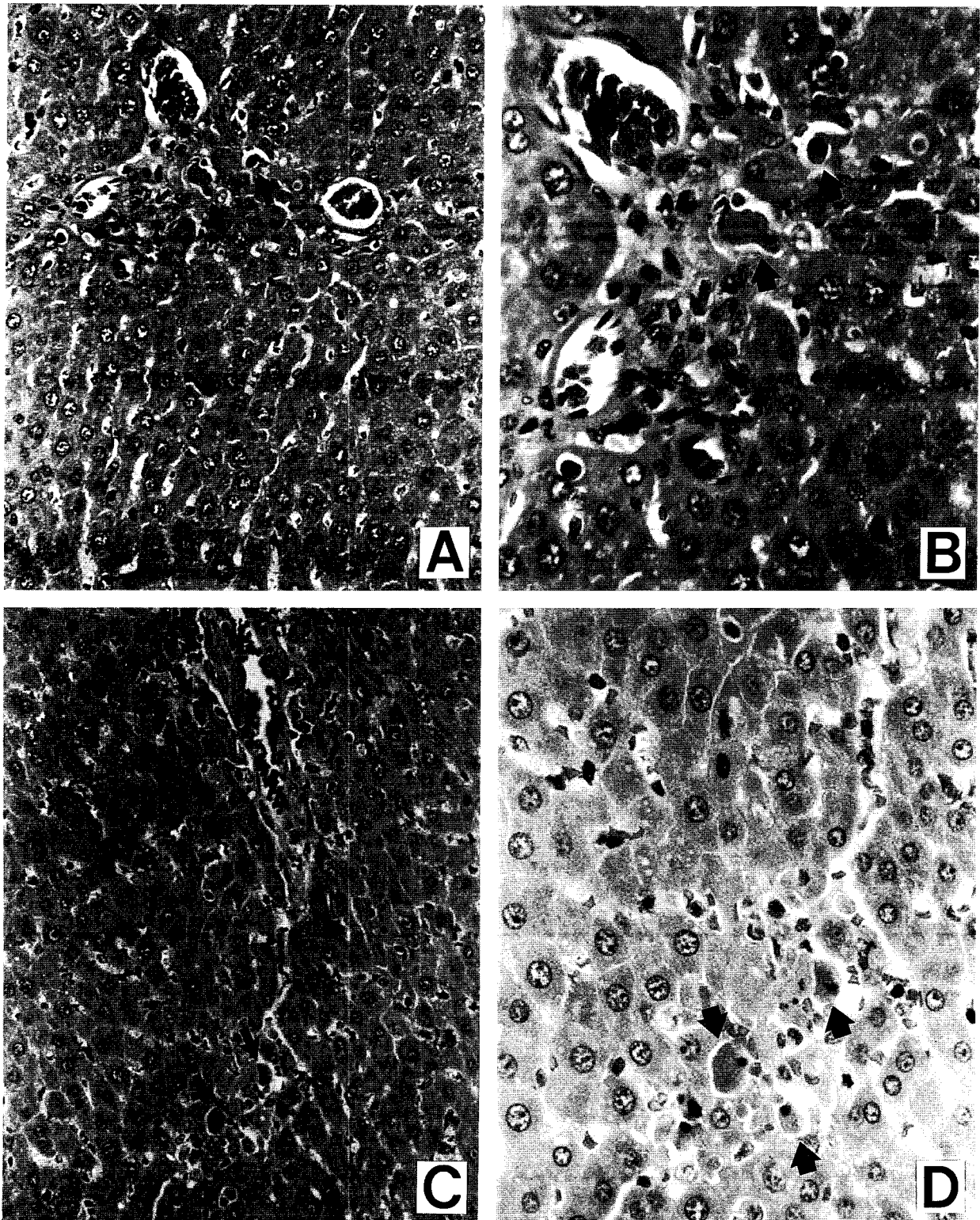


Fig. 1 Light micrographs of liver sections stained with hematoxylin and eosin (H. E.) 3h after 30 or 60 min of liver ischemia. Arrows indicate the apoptotic bodies (ABs) at the acinar zones 1 and 2. **A:** 30 min group, $\times 200$; **B:** 30 min group, $\times 400$; **C:** 60 min group, $\times 200$; **D:** 60 min group, $\times 400$.

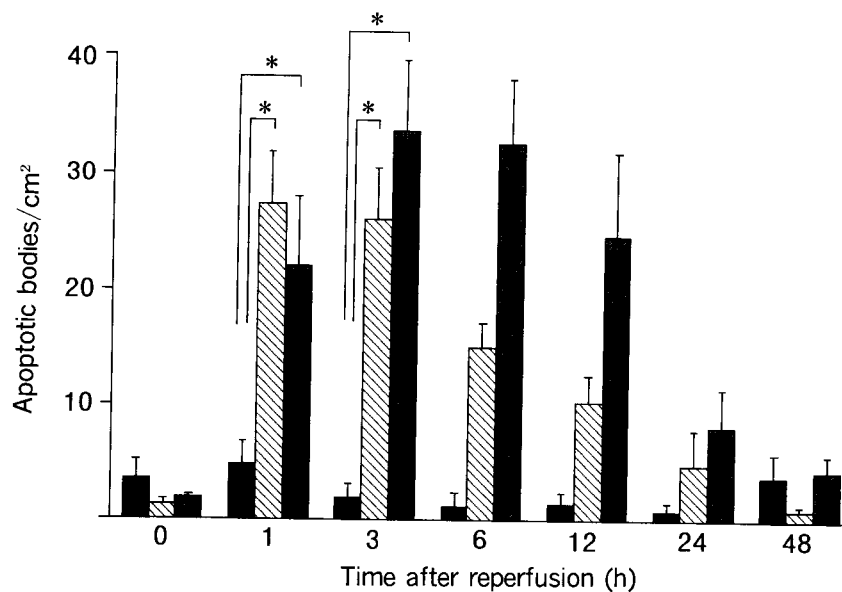


Fig. 2 The number of ABs at the sections stained with H. E. after 30 or 60 min of liver ischemia. The values are expressed as the mean $\pm$ SEM of 5 to 8 animals. An asterisk indicates a significant difference from the control ($*P < 0.05$; non-parametric Wilcoxon's signed rank test). Significant increases in the number of ABs were observed 1 and 3h after reperfusion in the 30min and the 60min groups as compared with the control group. Between the 30min and 60min groups, no significant differences in the number of ABs were observed at any point. (■) Control group; (▨) 30 min group; (■) 60 min group. H. E. and ABs: Shown in the legend to Fig. 1.

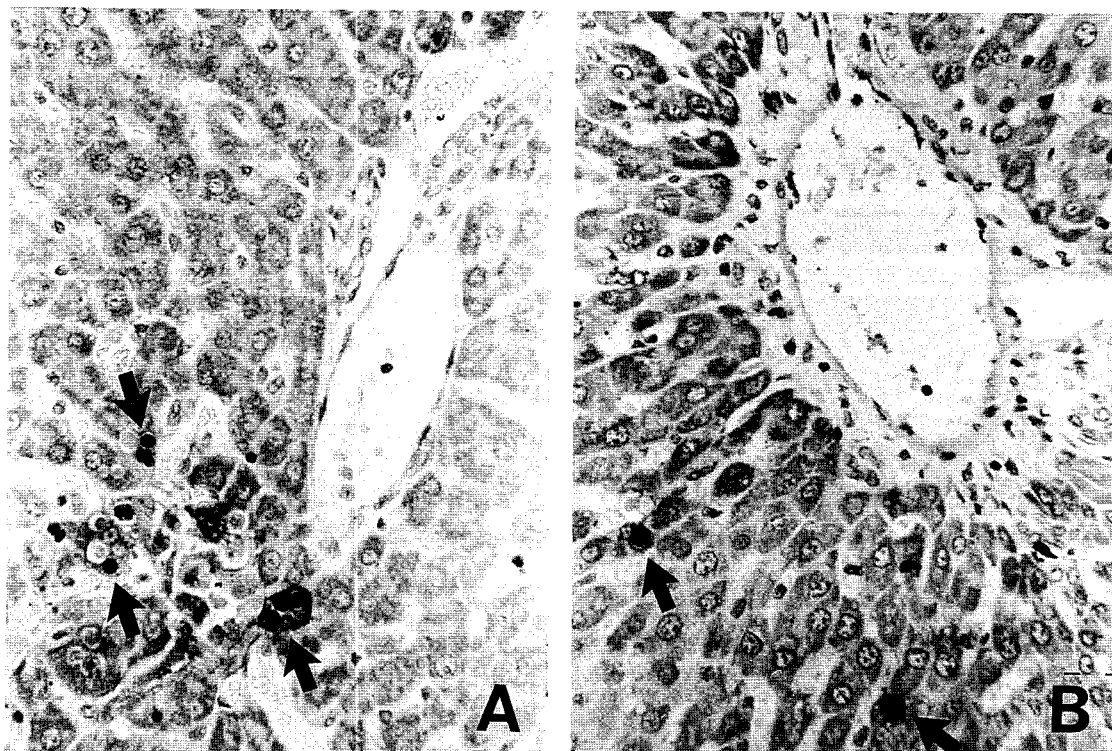


Fig. 3 Light micrographs of liver sections stained by the TUNEL method 3h after 30 or 60 min of liver ischemia. Arrows indicate positive hepatocytes revealed by the TUNEL method. A: 30 min group, $\times 400$; B: 60 min group, $\times 400$.

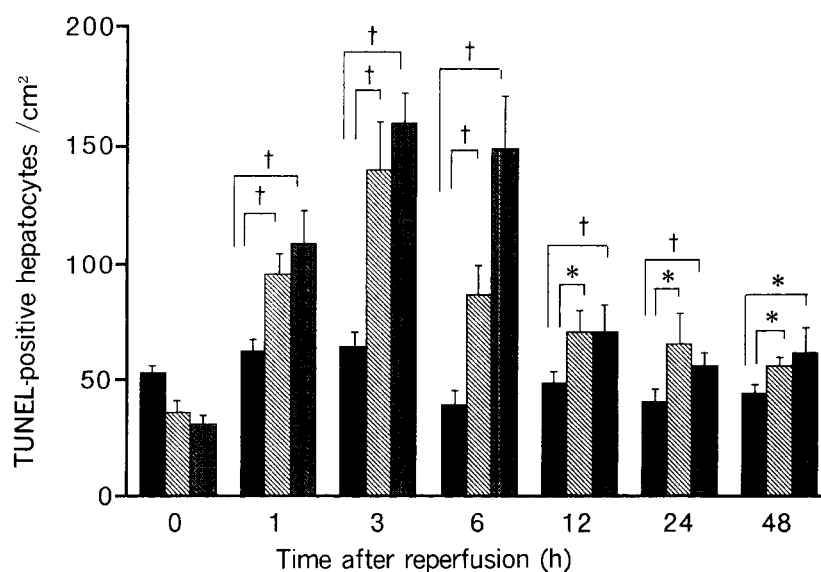


Fig. 4 The number of TUNEL-positive hepatocytes in liver sections after 30 or 60 min of liver ischemia. The values are expressed as the mean $\pm$ SEM of 5 to 8 animals. An asterisk and a cross indicate a significant difference (* $P < 0.05$, † $P < 0.01$; paired Student's *t*-test). Significant increases in the number of TUNEL-positive hepatocytes were seen between the 30 min and 60 min groups and the control group from 1 to 48 h after declamping, but no significant differences in the numbers of TUNEL-positive hepatocytes were seen between the 30 min and the 60 min ischemic groups at any time point. (■) Control group; (▨) 30 min group; (■) 60 min group.

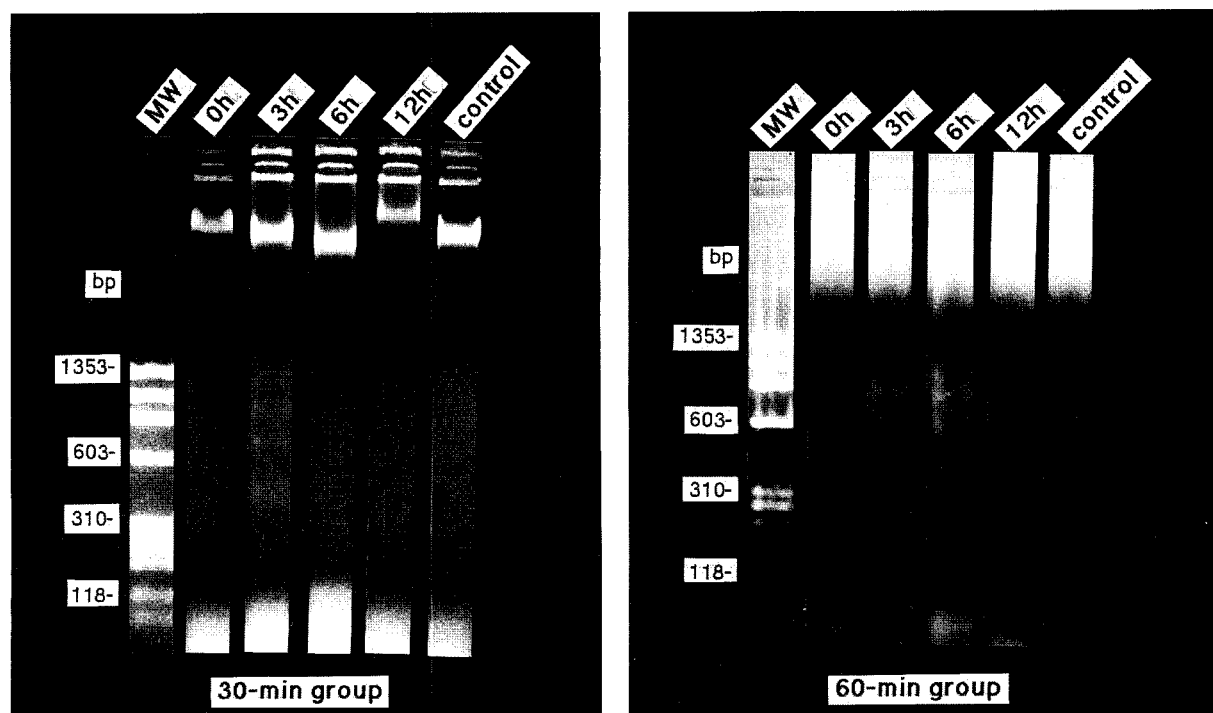


Fig. 5 Electrophoretic analysis of DNA extracted from the tissues of the 30 min group and 60 min group sampled at 0, 3, 6, 12 h after reperfusion. MW: Molecular weight standards. Control: DNA extracted from non-ischemic liver. A ladder-like pattern was seen at 3 h after reperfusion in the 30 min group, and at 3 and 6 h in the 60 min group. This pattern gradually diminished thereafter.

significant difference in the number of TUNEL-positive hepatocytes between the 30 min group and the 60 min group.

Electrophoresis of DNA. The DNA extracted from the liver tissues of the 30 min and 60 min groups was electrophoresed on a 1.5 % agarose gel. The ladder formation of DNA electrophoresed is a characteristic sign of apoptosis, which produces small double stranded fragments of DNA that migrate in a ladder-like pattern (multiples of 180 to 200bp) after electrophoresis (14). A ladder pattern over a smear pattern was recognized 3 h after reperfusion in the 30 min group and 3 and 6 h in the 60 min group. At that time, peak number of TUNEL-positive hepatocytes in both groups was observed. This pattern gradually diminished thereafter (Fig. 5).

Discussion

Cell death has two distinct modes, apoptosis and necrosis (7, 9, 16). Necrosis is a degenerative phenomenon accompanied by massive inflammation and chemical damage. In contrast, apoptosis is thought to be an active and physiological process characterized by acidophilic bodies, chromatin condensation, and DNA fragmentation resulting from the activation of endonuclease during embryogenesis (17–20), metamorphosis, and regulation after hyperplasia (21, 22). Recently, it has been reported that apoptosis is induced not only under physiological circumstances, but also under conditions of cell injury, such as exposure to toxic agents (23, 24). In the liver, several studies have shown that both necrosis and apoptosis are induced by dimethylnitrosamine, thioacetamide, heliotrine and many other chemicals (5–8).

In this study, we showed that apoptotic cell death was induced 1 to 12 h after 30 or 60 min of liver ischemia both morphologically and biochemically. In the sections stained with H. E., 1 to 12 h after reperfusion, ABs were observed, which almost completely disappeared 24 h after reperfusion. This might indicate that the apoptotic cycle is as comparatively brief as the 3 to 4 h period suggested by Wyllie *et al.* (16) and Bursch *et al.* (32).

We observed fewer ABs than we expected (4 to 28 ABs per cm²). The formation of ABs is part of the process of apoptosis, and it is hard to detect ABs because they disappear within a few hours (16, 32). However the TUNEL method effectively detects apoptosis because the TUNEL method can detect not only apoptotic cells as defined histologically, but also morphologically intact cells

going through the process of apoptosis.

TUNEL stands for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling. This method is based on the specific binding of TdT to the 3'-OH ends of DNA stands fragmented by apoptosis (14). However, cells dividing, entering or in the later stages of necrosis can be positively stained by this method (33). At this point, only a small amount necrosis was detected in the sections stained with H. E., even in the 60 min group, and the number of positive hepatocytes detected by the TUNEL method in regenerating liver sections for 24 h after 70 % hepatectomy did not increase (data not shown). In addition, only the positive hepatocytes which had clearly stained nuclei and intact cytoplasm were counted. The number of TUNEL-positive hepatocytes correlated significantly with the number of ABs ($y = 4.353x + 9.022$, y : the number of ABs, x : the number of TUNEL-positive hepatocytes, $r = 0.639$, $P < 0.01$). Therefore, TUNEL-positive hepatocytes indicate the number of hepatocytes collectively going through apoptosis in this study.

Under physiological circumstance, such as regression after hyperplasia, induction of hepatocyte apoptosis is observed in the mid-zone and pericentral areas (34). But in this study, hepatocyte apoptosis was observed in the periportal and mid-zone area. Suematsu *et al.* reported that cell death induced by oxidative stress during low-flow hypoxia was restricted to the periportal area early on, and then to the mid-zone region (35). The localization of cell death in their report partially corresponds to the localization of apoptotic cell death observed in this study. Oxidative stress upon reoxygenation of the hepatocytes might induce apoptotic cell death in the ischemia/reperfusion injured liver. A current theory suggests that superoxide anions (25, 26) and cytokines, such as tumor necrosis factor- α (TNF- α) (27), participates in reperfusion injury. Recent *in vitro* studies have also shown that superoxide anions and TNF- α induce apoptosis in a variety of cell types (28–31). These mediating agents are thought to be inducers of hepatocyte apoptosis. Further investigation is needed to determine which mediators primarily induces hepatocyte apoptosis in rat ischemia/reperfusion model.

DNA fragmentation was revealed by electrophoresis, and we discovered that apoptosis was induced during the reperfusion phase following liver ischemia. Recently, it was reported that the ladder-like pattern of DNA was observed after electrophoresing DNA extracted from

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necrotic tissues, and thus, this might not always be indicative of apoptotic cell death (36). However, at present, we assume that the ladder-like pattern is indicative of apoptotic cell death. In this study, we used the H. E. staining method and TUNEL method to give clear physiological evidence of apoptotic cell death. We showed that apoptosis is induced in the early stages of reperfusion after liver ischemia, and that apoptosis may be associated with ischemia/reperfusion injuries which occur following liver ischemia.

In conclusion, the present study showed that hepatocyte apoptosis is induced during the early phase of reperfusion following rat liver ischemia morphologically and biochemically, and this suggests that hepatocyte apoptosis may be associated with ischemia/reperfusion injuries after liver ischemia.

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