

Induction of DNA Fragmentation in Rat Small Intestinal Smooth Muscle Cells by Ischemia

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Abstract

Injuries caused by ischemia in the small intestine have been widely accepted as resulting in necrosis. The aim of this study was to ascertain whether apoptosis of intestinal smooth muscle cells (ISMCs) also occurs. For this purpose rat small intestine subjected to ischemia was studied. Apoptosis was assessed by the TUNEL method and by the electrophoretic detection of DNA laddering. Necrosis was evaluated by a -smooth muscle actin monoclonal antibody labeling.

ISMCs of the longitudinal layer (LL) were damaged by ischemia, whereas those of the circular layer (CL) were not. ISMCs showing fragmented DNA were first observed after 1.0 hour. Damaged cells showing both DNA laddering and actin labeling were first observed after 3 hours. Agarose gel electrophoresis of DNA confirmed these observations by showing both ladder and smear patterns. Finally, the expression of Bax but not Bcl-2 and Fas in ISMCs increased after ischemia.

The present study demonstrated that rat ISMCs subjected to ischemia exhibit both DNA laddering and actin labeling. Apoptosis appears as the initial form of cell death, followed by necrosis. Enhanced expression of Bax may be implicated in this activation of apoptosis. **Key words:** apoptosis, necrosis, Bax, smooth muscle cell, small intestine, rat.

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The concept of two major distinct modes of death in eukaryotic cells, namely, necrosis and apoptosis, has become widely accepted in recent years [18, 27, 44, 45]. Necrotic cells show damaged organelles, ruptured plasma membranes, and dispersal of cytoplasmic elements into the extracellular space [44, 45]. On the other hand, apoptosis has characteristic structural changes consisting of loss of surface contact with neighboring cells, cell shrinkage with remaining intact organelles, condensation of chromatin to give crescent caps at the nuclear periphery, and eventual fragmentation of both the nucleus and cytoplasm to form apoptotic bodies [44, 45].

Recently, evidence has accumulated indicating that apoptosis is triggered by mild cellular injuries due to hyperthermia [1,39], hypoxia [18,45], hepatic toxins [34], direct acting agents including anticancer drugs [1,24], and ischemia and ischemia/reperfusion [25, 26, 40]. We previously reported that the typical human ischemic myocardial injuries known as coagulation necrosis and contraction band necrosis feature distinct internucleosomal cleavage of DNA [15]. Furthermore, we demonstrated that apoptosis is a major form of intestinal epithelial cell death occurring after short-term ischemia or is-

chemia/reperfusion in the rat jejunum [14], while standard textbooks of pathology state that the intestinal damages induced by circulatory disorders result in necrosis [6, 7]. However, whereas the sequence of development of intestinal mucosal injury after ischemia and ischemia/reperfusion has been extensively studied [5, 12, 32, 33, 37, 42], the changes in intestinal smooth muscle cell (ISMC) injury have not received much attention. In vascular smooth muscle cells (VSMCs), the induction of apoptosis has been reported recently [2, 3, 10, 31, 33], but the situation with ISMCs has remained unclear.

The aim of this study was to ascertain whether apoptosis of ISMCs also occurs. We therefore examined the response of rat ISMCs to ischemia using histological and immunohistochemical approaches, as well as agarose gel electrophoresis.

Materials and Methods

Induction of ischemic injury

Six-wk-old female Wistar rats were used. Pentobarbital sodium solution (30-40 mg/kg) was administered into the intraperitoneal cavity to induce anesthesia and all proce-

dures were performed with the animals breathing spontaneously.

The rats were divided into 2 experimental groups: (1) sham ischemia (n = 10); and (2) ischemia (n = 35). Through a mid line abdominal incision, one of the branches of the superior mesenteric artery (SMA) was occluded for 0.5-, 1.0-, 1.5-, 2.0-, 3.0-, 4.0-, and 5.0-h with an atraumatic vascular clamp. In order to avoid collateral blood supply from right colic and jejunal arteries, we used the modified procedure developed by Megison et al. [14, 28] in which the collateral arcades from the right colic artery and the jejunal arteries proximal to the site of occlusion were ligated. Control animals underwent the same procedure except for the clamping of SMA. After 0.5-, 1.0-, 1.5-, 2.0-, 3.0-, 4.0-, and 5.0-h mesenteric ischemia, or sham ischemia, the small intestines were harvested.

Light and electron microscopical assessment

Two cm segments of samples were immediately fixed in 20% buffered formalin, processed routinely for embedding in paraffin and sectioned serially. Both transverse and longitudinal sections of each samples were stained with H&E for histopathological assessment.

For electron microscopy, small pieces of small intestine including both longitudinal (LL) and circular layer (CL)

were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 mol phosphate buffer (PB) (pH 7.4) for 2 h. After rinsing three times with PB, samples were then stored in PB for 24 h. The tissues were then postfixed in 1% osmium tetroxide in Millonig's buffer (pH 7.4) for 2 h, dehydrated in increasing ethanol concentrations, cleared in QY-1 and embedded in WE812. Semithin sections (~ 1.0 μ m thick) were stained with toluidine blue. Ultrathin sections of areas of interest were cut on a ultramicrotome, and double stained with uranyl acetate and lead citrate before examination with a Hitachi H7000 electron microscope at 75KV[14].

Immunohistochemical detection of DNA fragmentation and quantitative analysis of DMA fragmented cells

The terminal deoxynucleotidyl-transferase-mediated d-UTP-biotin nick end labeling (TUNEL) method was used to demonstrate DNA fragmentation (internucleosomal cleavage of DNA) and apoptotic bodies. The staining method applied was identical to that described in our previous studies [11, 14, 15]. The thymus of a 4-wk-old mouse, sacrificed 6 hours after sublethal irradiation with a

Co source at 600 cGy, was used as a positive control. As a negative control biotin-16-dUTP was not added. Quantitative analysis of TUNEL positive cells was performed.

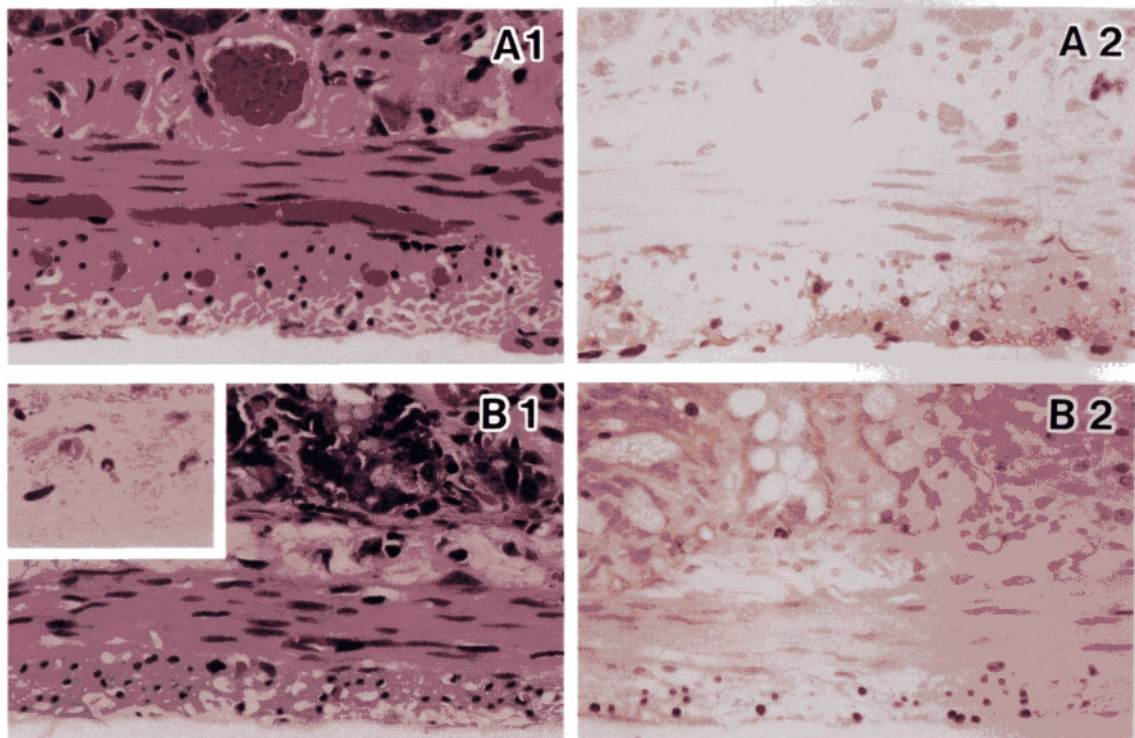


Figure 1. Photographs of transverse sections of smooth muscle cells after ischemic injury. (A 1): Smooth muscle cells of LL after 2.0-h ischemia show both pyknotic nuclei and degenerated cytoplasm. (B1): Note both thinner layer of LL and severely damaged muscle cells after 4.0-h ischemia. Inset shows pyknotic and karyolytic nuclei. (A2): Some of the cells in LL but not CL in a semi-serial section are TUNEL positive. (B2): Similar staining pattern with TUNEL as shown (A2). (A. B1) are stained with H&E and (A. B2) with the TUNEL method. Magnification, X80 and X120 (B1 inset).

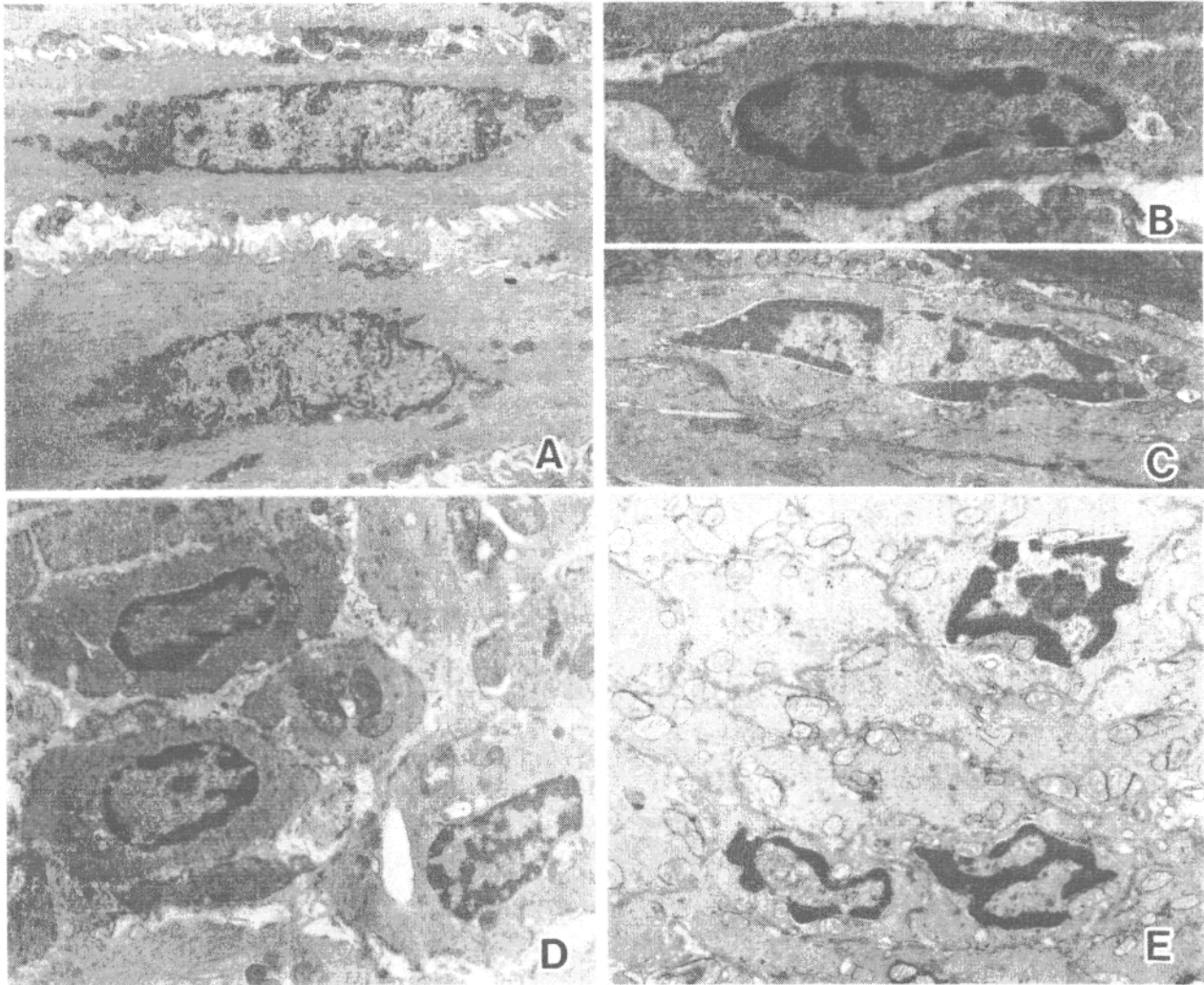


Figure 2. Electron microphotographs of smooth muscle cells after sham ischemia (A) and 2.0-h (B, D) and 4.0-h ischemia (C, E). The smooth muscle cells in (A) are normal in appearance, while condensation of chromatin under the nuclear membrane (B, longitudinal and D, transverse section) is evident in 2.0-h ischemia. However, no typical apoptotic features, like condensation of chromatin to give crescent caps at the nuclear periphery, apoptotic body formation, and phagocytosis by neighbors are apparent. More condensed chromatin are observed after 4.0-h ischemia (C, longitudinal and E, transverse section). Magnifications, X4,000 (A), X10,000 (B), X7,000 (C), X6,000 (D), and X9,000 (E).

In brief, photographs of six fields of antimesenteric regions of each transverse section were taken randomly at a magnification of X 50 in three experimental and control rats. Then, photographs were enlarged three times to give a final magnification of X150. Mean $\pm$ SD values for the percentages of positive nuclei among the total number of nuclei in LL were calculated and paired t tests were performed to detect significant differences.

Evaluation of necrosis using α -smooth muscle actin monoclonal antibody labeling

Rats were injected i.v. with 600 μ g of monoclonal antibody specific for α -smooth muscle actin (α -SMA) (clone 1A4). Animals that were to be killed at 1.0-, 1.5-, 2.0-, 3.0-, 4.0-, and 5.0-h after SMA occlusion were injected 1 hour

before surgery. Samples were frozen in OCT compound and frozen sections of small intestine, 4.0 (im thickness of transverse section, were fixed for 4 minutes in acetone at room temperature (RT), rinsed in phosphate buffer saline (PBS), and incubated with peroxidase conjugated anti-mouse IgG for 30 minutes at RT. Sections were then washed in PBS and developed in diaminobenzidine (DAB)-H₂O₂, postfixed in formalin for 5 minutes, and counter stained with methyl green.

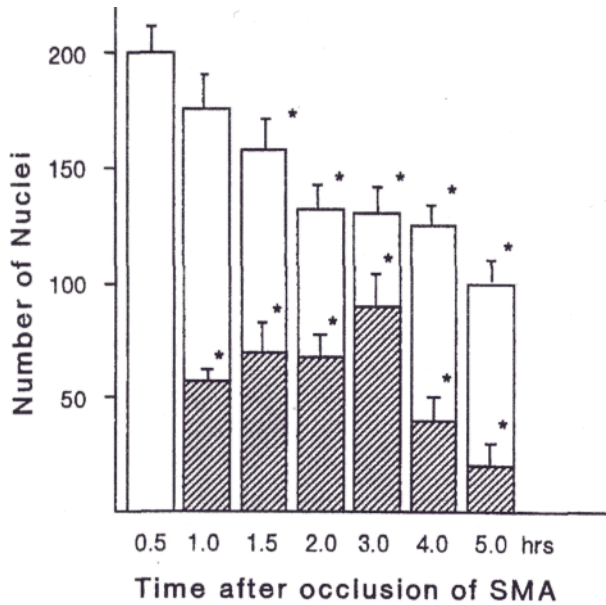
DNA electrophoretic analysis of ISMCs in agarose

DNA extraction and electrophoresis were performed by the methods described by Facchinetti et al. [8,14,15] DNA of ISMCs of SMA occluded rats at 1.0, 2.0, 3.0, 4.0, and 5.0 hours was examined. In brief, epithelial cells were

scraped off with a blade to obtain the smooth muscle layer. Then the tissues were cut into small pieces to obtain DNA as described previously [14, 15].

Immunohistochemical localization of Fas, Bcl-2, and Box in ISMCs

For assessment of the effects of ischemic injury tissue



sections mounted on poly-L-lysine-coated slides were deparaffinized in xylene. After dehydration in ethanol for 5 min, slides were heated in a microwave in 10 mmol sodium citrate (pH 6.0) buffer for 25 min and then incubated in 0.3% (v/v) H₂O₂ in methanol for 20 min. After washing twice in PBS for 10 min, tissue sections were preblocked using an avidin/biotin blocking kit (Vector Laboratory) and incubated for 60 min at RT with anti-Bcl-2 (N-19), Bax (P-19), and Fas (M-20) rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, California USA) at a 1:100 dilution. After washing with PBS, the tissue sections were incubated for 40 min with biotinylated goat anti-rabbit IgG, washed and incubated for 10 min with peroxidase conjugated-streptavidin, developed in DAB-H₂O₂ and counterstained with methyl green.

Figure 3. Number of TUNEL positive nuclei in LL at various time periods of ischemia. The number of the nuclei in a given area of LL decreases and those of TUNEL positive cells increases. Significant decrease of total nucleus number after 1.5-h ischemia and increase of TUNEL positive nucleus number from 1.0-h to 3.0-h ischemia are observed, respectively. However, TUNEL positive nuclei decreases thereafter. * $P < 0.01$ compared with the control value.

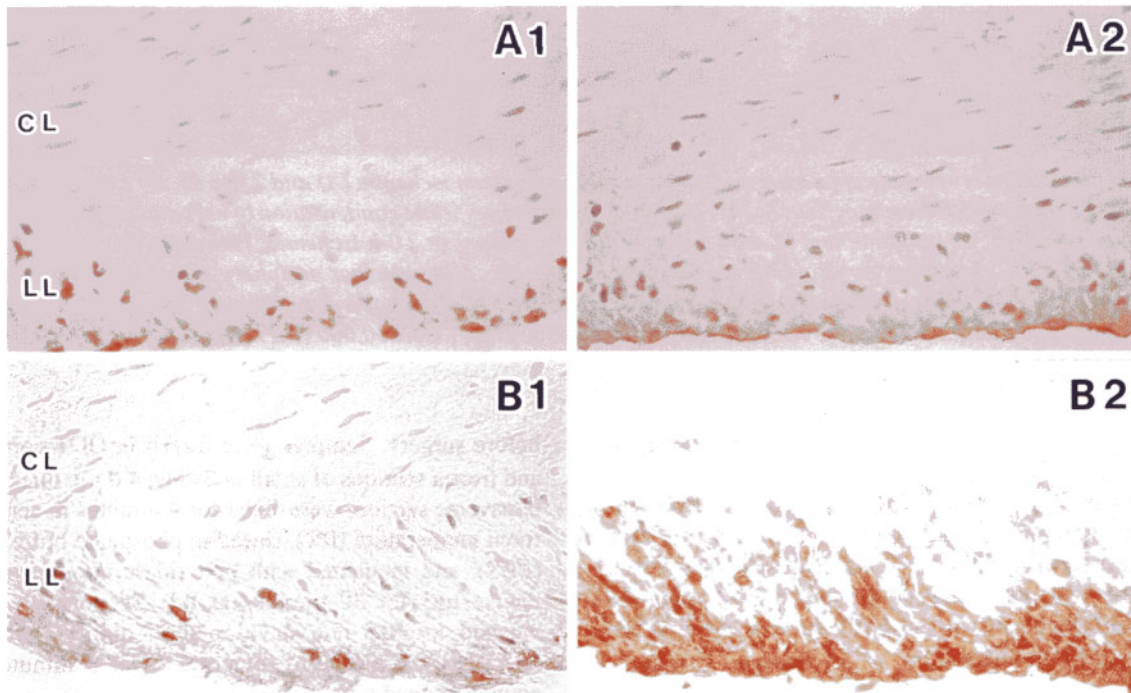


Figure 4. Photographs of transverse sections of smooth muscle after 2.0-h ischemia (A1, 2) and 4.0-h ischemia (B1, 2). (A, B): TUNEL positive cells in LL after 2.0-h (A1) and 4.0-h ischemia (B1). In contrast no positive cells are appeared in CL (A, B1). Some of the smooth muscle cells in LL are labeled with anti- α SMA antibody after 4.0-h ischemia (B2), while those cells after 2.0-h ischemia are not (A2). (A1, B1) are stained with the TUNEL method and with anti- α SMA antibody (A2, B2) immunohistochemically. Magnifications, X100 (A-C) and X65 (D).

Results

Light and electron microscopical findings

In areas of antimesenteric regions, which showed epithelial destruction, ISMCs of LL showed pyknotic or condensed nuclei and degenerated cytoplasm after 1.0-h ischemia. After 2.0-h ischemia, more destructive changes were observed (Fig. 1 A1), whereas those in CL appeared normal. After 4.0-h ischemia, severe damage was observed, with thinner LL (Fig. 1 B1). Severely affected areas exhibited karyolysis and karyorrhexis, considered as necrotic features (Fig. 1 B1 inset), without any of the characteristic morphological features indicative of apoptosis like shrinkage of cells, compaction and segregation of chromatin against the nuclear envelope, nuclear fragments, and apoptotic bodies or phagocytosis by neighbors. However, the electron microscopic study demonstrated condensation of chromatin under the nuclear membrane after 2.0-h ischemia (Fig. 2B, D), which was not evident after sham ischemia (Fig. 2 A). More condensed chromatin was observed after 4.0-h ischemia (Fig. 2 C, E).

Immunohistochemical detection of DNA fragmentation and quantitative analysis of DNA fragmented cells

The TUNEL method demonstrated some nuclei of LL with positive staining after 2.0-h (Fig. 1 A2) and 4.0-h ischemia (Fig. 1 B2). On the other hand, neither control ISMCs nor those in the CL after ischemia (Fig. 1 A2, B2) showed any TUNEL positive nuclei. Positive reactions were first observed at the antimesenteric area after 1.0-h ischemia. The number of the nuclei in a given area of LL decreased significantly ($P < 0.01$) after 1.5-h ischemia and those of TUNEL positive cells increased significantly ($P < 0.01$) from 1.0-h to 3.0-h, then decreased during the experiments (Fig. 3) (Table 1).

Table 1. Histological and immunohistological findings of smooth muscle cells subjected to ischemia.

	Degenerated cytoplasm and pyknotic nuclei	TUNEL	α -SMA
Ischemia at			
0.5-h	-	-	-
1.0-h	+	+	-
2.0-hrs	+	+	-
3.0 to 5.0-hrs	+	+	+

+, Damaged cells or positive cells are observed.
-, Not observed.

Evaluation of necrosis using α -smooth muscle actin monoclonal antibody labeling

TUNEL positive cells were observed at 2.0-h ischemia (Fig. 4 A1), while these cells were not labeled with anti-SMA antibody (Fig. 4 A2). Labeled ISMCs in LL were detected first after 3.0-h ischemia (Fig. 4 B2), whereas those of CL were not. Both apoptotic and necrotic cells in LL were observed thereafter (Fig. 2 B1) (Table 1).

DNA electrophoretic analysis of ISMCs in agarose

DNA fragmentation in samples of small intestine obtained from sham ischemia and ischemia groups was studied by agarose gel electrophoresis [8, 14]. Multiples of 180- to 200-bp subunits were detected at 1.0- (Fig. 5A lane 1) and 2.0-h (Fig. 5A lane 2) after SMA occlusion. In contrast, the diffuse pattern of DNA degradation was not detected both in these groups (Fig. 5 A) and control animals (Fig. 5B lane 1)). Conversely, nucleosomal cleavage at

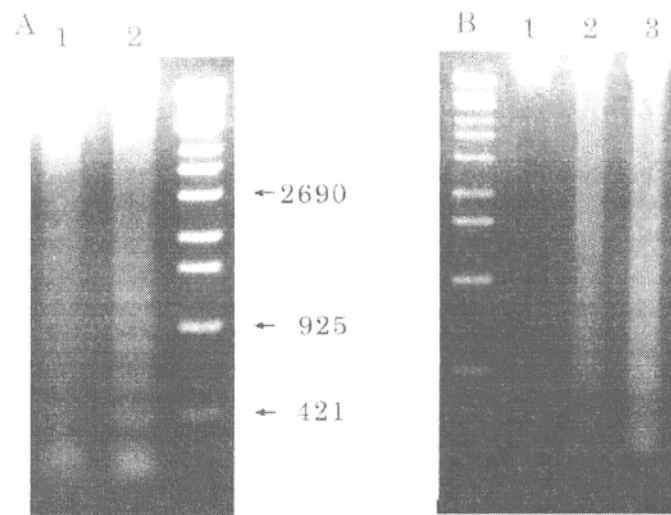


Figure 5. Agarose gel electrophoresis of DNA extracted from smooth muscle cells after 1.0-h (A, lane 1), 2.0-h (A, lane 2), 3.0-h (B, lane 2), 4.0-h ischemia (B, lane 3), and sham ischemia (B, lane 1). Both 1.0-h (A, lane 1) and 2.0-h (A, lane 2) ischemia show DNA ladder. On the other hand, both 3.0-h (B, lane 2) and 4.0-h (B, lane 3) are associated with ladder as well as smear patterns, in contrast to the sham ischemia case where both are lacking (B, lane 1).

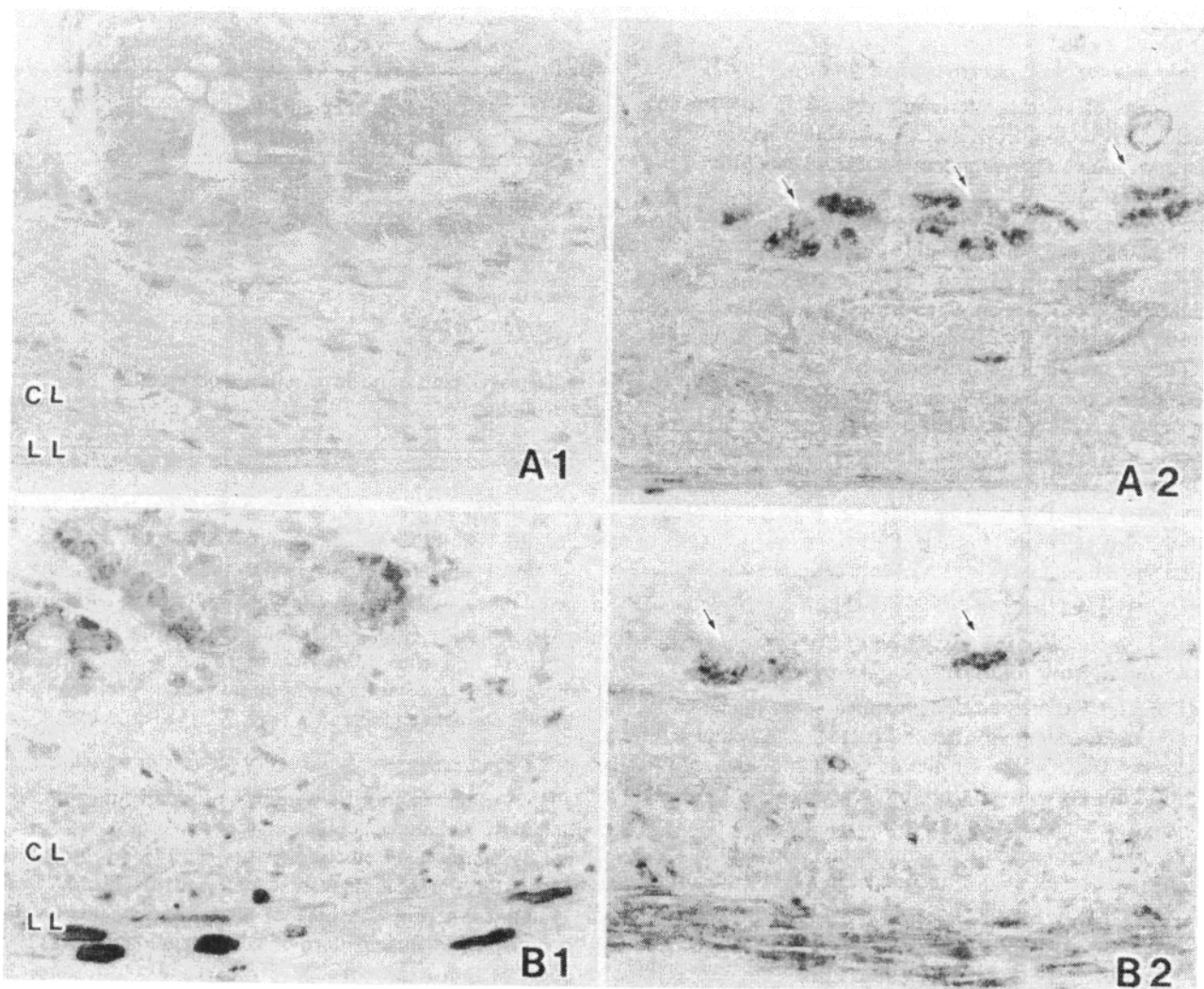


Figure 6. Immunohistochemical detection of Bax in smooth muscle cells after 3.0-h ischemia. In the normal case, Bax was expressed in the Paneth cells (4-), absorptive epithelial cells, and ISMCs (A2, B2). Enhanced expression of Bax in LL was observed (B2), where TUNEL positive cells were observed (B1), in contrast the expression of Bax was not altered (A2), where TUNEL positive cells were not observed (A1). (A1, B1) are stained with the TUNEL method and with anti-Bax antibody (A2, B2) immunohistochemically. Magnification, X80.

3.0- (Fig. 5B lane 2) and 4.0-h (Fig. 5B lane 3) after ischemia was associated with diffuse pattern of DNA damage, indicating that apoptosis and necrosis were both simultaneously present.

Expression of Fas, Bcl-2, and Bax in ISMCs after ischemia

In the normal case, neither Fas nor Bcl-2 was expressed in ISMCs, in contrast Bax was expressed in the Paneth cells, absorptive epithelial cells, and ISMCs (Fig. 5 A2) [13, 20, 21, 23]. The expression of Fas and Bcl-2 was not altered after 3.0-h ischemia (data not shown), in contrast Bax was expressed intensively in LL (Fig. 5 B1), where TUNEL positive cells were observed (Fig. 5 B2).

Discussion

It is well known that the small intestine subjected to ischemia alone or ischemia/reperfusion becomes injured, resulting in necrosis [5, 12, 32, 33, 37, 42]. The present study provided the first evidence that two distinct modes of cell death, that is apoptosis and necrosis, are involved in the destruction of ISMCs. Both fragmented and degraded DNA were confirmed by the morphological feature of chromatin condensation, TUNEL method, and DNA agarose gel electrophoresis, respectively, despite the lack of some of the characteristic histological features of apoptosis, such as condensation of chromatin to give crescent caps at the periphery, cytoplasmic blebbing, and apoptotic bodies.

Damage to the muscular coat of the intestine was reported earlier [33, 42] with pyknotic nuclei observed as early as 1.0-h ischemia. In this study, a decrease of smooth

muscle cell number in LL was demonstrated after SMA occlusion. The fact that injury was limited to ISMCs in LL is in line with the dynamics of blood supply to the musculature. Namely, the SMA reaches one side in the mesentery, runs into the serosa, and breaks up into large branches that penetrate the muscularis externa then enter the submucosal layer, where they form a large plexus. The smooth muscle of the CL thereby is well supplied with blood, whereas the LL occupies a peripheral site [9].

Recently, Kajstura et al. reported that apoptotic and necrotic myocyte cell deaths are involved in the destruction of myocardium subjected to ischemia [17]. Apoptosis is the major initial form of myocardial damage and necrotic cell death mostly follows apoptosis. DNA strands breaks are clearly demonstrated by the TUNEL method and agarose gel electrophoresis. In the present study, we also revealed that apoptotic and necrotic cell deaths were involved in the destruction of ISMCs subjected to ischemia in the rat and that apoptosis was also the initial form of cell death. However, typical morphological features of apoptosis were lacking in their study [17] and here. Similar findings were obtained in our previous study of human myocardial infarction cases [15]. On the other hand, apoptosis of vascular smooth muscle cells (VSMCs) has been reported in *in vitro* model [2, 3, 10, 31] and ischemia in rat brain clearly demonstrates the characteristics features of apoptosis [25, 26, 40]. The reason for the difficulty in detecting the apoptotic features in ISMCs is not clear. However, clumping at the nuclear membrane and in the center of there nuclei, as found here, has been described, in various cell types, as an early sign of apoptosis [19, 43].

Schulze-Osthoff et al. reported that enucleated cells incubated with anti APO-1 antibody revealed the key morphological features of apoptosis [35]. Namely, DNA degradation and nuclear signaling are not required for induction of apoptotic cell death. Furthermore, Sun et al. reported that endonuclease activation in apoptosis is neither necessary nor sufficient to induce chromatin condensation, and that DNA fragmentation and chromatin condensation may be triggered through separate pathways during apoptosis [38]. Thus, ischemia could induce the DNA degradation in ISMCs, not induce any apoptotic features or ISMCs may lack the pathways of induction of apoptotic features, such as blebbing of cytoplasm and apoptotic bodies.

While hypoxia is well known to produce cell necrosis, recent reports suggested that it may also activate apoptotic processes in cardiac myocytes [17], hepatocytes [16], colon adenocarcinoma cells [46], T-lymphoma cells [29], and renal tubular cells [4, 36]. Rapid DNA fragmentation due to hypoxia and reoxygenation may involve expression of Fas [17], upregulation of c-myc, ref-1 [46] and c-fos genes [29], and activation of endonuclease [36]. Recently, Bennett reported that apoptosis in rat VSMCs is induced by c-myc and E1A adenovirus and blocked by expression of Bcl-2 [3]. Under normal conditions, the expression of both Bcl-2 and Bax is confined to crypt epithelial cells [13,

20], while Fas is found in the cytoplasm of villous epithelial cells except for the Paneth cells [23]. The present study of Fas, Bcl-2, and Bax expression in damaged ISMCs demonstrated that only Bax was altered in ISMCs, clear increase being observed. In the control ISMCs, neither Bcl-2 nor Fas was expressed. The Bcl-2 protooncogene product is able to repress apoptosis and its deficiency is coupled with extensive cell death [22, 41]. Bcl-2 forms heterodimers with other members of the Bcl-2 protein family including Bax, which in contrast promotes apoptosis. If Bax homodimers predominate, cell death will occur; if there are only Bcl-2/Bax heterodimers, the cell will survive [30]. Thus, the enhanced expression of Bax in ISMCs may be involved in the activation of apoptosis.

In conclusion, the present study demonstrated that rat ISMCs subjected to ischemia exhibit nuclear DNA fragmentation and cell death. Apoptosis is the initial form of cell death produced by occlusion of the superior mesenteric artery, whereas necrotic cell death follows apoptosis. Enhanced expression of Bax may be implicated in the activation of apoptosis.

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