

Rat Cardiomyocytes with TUNEL-Positive Nuclei Induced by Permanent Ischemia Show Increased Plasma-Membrane Permeability Monitored by a Combined Use of TUNEL Method and Lanthanum Ions

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Abstracts

The aim of this study was to clarify whether myocardial cells with DNA fragmentation induced by ischemia show increased plasma-membrane permeability (PMP), focusing on the early lesions of acute myocardial ischemia (AMI). Rat AMI was induced by a permanent occlusion of the left coronary artery. The TUNEL method was used for the demonstration of DNA fragmentation, and lanthanum ions (La) were employed for the increased PMP of cardiomyocytes. The increased PMP of cardiomyocytes having TUNEL-positive nuclei was determined by a combined use of the TUNEL method in frozen sections and electron microscopic identification of intracellular depositions of La.

Seven of 10 cardiomyocytes having TUNEL-positive nuclei revealed the deposition of La 2 hours after ischemia, and 10 of 13 showed La deposition 3 hours after ischemia. Almost all cardiomyocytes labeled with TUNEL stain showed intracellular deposition of La 4 and 6 hours after ischemia, respectively.

The evidence obtained from this experiment demonstrates that almost all cardiomyocytes labeled with TUNEL stain simultaneously show an increase of PMP in the AMI foci. In conclusion, the mechanism of myocardial cell death caused by permanent ischemia is due to a mixed form of apoptosis and necrosis.

Key words: rat myocardial ischemia, TUNEL method, apoptosis, DNA fragmentation, lanthanum ions.

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Apoptosis has been reported to occur after ischemia in cultured neonatal rat cardiomyocytes [14] and after ischemia/reperfusion in rabbit acute myocardial infarction, but never after ischemic injury alone [3]. However, we have reported that the nuclei of human infarcted myocardial cells with the morphological features of necrosis showed DNA fragmentation [5], and also that permanent ligation of the left coronary artery without reperfusion produced DNA fragmentation of rat myocardial cells at the ischemic foci [13]. Kajstura et al. [6] have reported that, in rat acute myocardial infarction, programmed cell death (apoptotic cell death) was the major independent form of cardiomyocyte cell death up to 4.5 - 6 hours after ischemia, and that necrotic cardiomyocyte cell death followed apoptosis. They also demonstrated recently [8] that coronary artery narrowing caused both necrotic and apoptotic cardiomyocyte cell death and that cardiomyocyte necrosis markedly exceeded apoptosis. In

their experiment, as a tracer of the ruptured myocardial cell-membrane they used monoclonal anti-myosin antibody, which was relatively large in molecular size, and they reported that cardiomyocytes showing both DNA strand breaks (apoptosis) and myosin labeling (necrosis) were a prominent aspect of myocardial damage only 6 hours after ischemia [6].

With regard to the PMP of myocardial cells injured by ischemia, a few studies have reported using lanthanum ions ($\text{La}(\text{NO}_3)_3$) (La) as a tracer of PMP [4, 11], since they had smaller molecules and have been successfully used as a tracer for the detection of increased permeability of the plasma membrane of cardiomyocytes [4, 11, 10]. Koba et al. [10] have reported that, when using La, increased PMP of myocardial cells occurred at the ischemic foci in the very early stage of ischemia. They detected increased PMP in 19%, and 30% of myocardial cells at 30 min, and at 1 hour after ischemia, respectively. Though Kajstura et al. [6] have

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detected cardiomyocytes showing both DNA strand breaks (apoptosis) and myosin labeling (necrosis), the tracer of necrosis they used was a monoclonal anti-myosin antibody which, as they pointed out, had a large molecular size, which was a limitation of the method. In this study, therefore, by using La as a tracer of PMP (necrosis) and the TUNEL method to monitor DNA strand breaks (apoptosis), we determined whether the PMP of rat myocardial cells at the ischemic foci is increased, focusing on the early stage of acute myocardial damage from 30 minutes to 6 hours after the induction of ischemia. We also tried to determine whether the membrane permeability of myocardial cells with DNA strand breaks (TUNEL-positive nuclei) increases by a combination of the TUNEL method and an electron microscopic identification of intracellular depositions of La.

Materials and Methods

Animals

Animals were cared for and maintained in accordance with the guidelines of the National Institute of Health (Bethesda Md. USA).

Male rats (Wistar strain, 6-weeks-old) purchased from Japan SLC, Inc. (Shizuoka, Japan) were used.

Experimental protocol

Acute myocardial infarction (AMI) was induced by the method described previously [13, 12]. In brief, after being anesthetized with an intraabdominal injection of sodium pentobarbital (35 mg/kg), male rats (120-130 g) were fitted with an endotracheal tube and placed on controlled respiration with positive pressure on inspiration. The chest was then opened through the third intercostal space. The heart was exteriorized quickly, and a 5-0 nylon ligature was placed under the visualized proximal segment of the left coronary artery (LCA). The ligature was then permanently ligated. Rats were then placed on controlled respiration until constant spontaneous respiration resumed. Those subjected to the same procedure but without coronary artery ligation were used as control animals.

Myocardial tissue preparation

Under ether anesthesia, each group of 3 rats was exsanguinated, and the hearts were removed at intervals of 30 minutes, 1, 2, 3, 4 and 6 hours after ischemia. After the hearts were sliced horizontally, the ischemic portion was selected and cut into two slices, one of which was fixed with 10% neutral buffered formalin at room temperature (RM) for 24 hours, dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin.

Identification of increased permeability of myocardial cell-membrane

The second slice was cut into small pieces for electron microscopic examination. After being fixed with 4% glutaraldehyde in 0.1 M cacodylate buffered solution containing 1.3% La (NO₃)₃(La) [4], pH 7.4, for 2 hours, small pieces of the specimens were washed thoroughly with 0.1 M cacodylate buffered solution containing 1.3% La. After the pieces thus treated were postfixed for 2 hours with 1% osmium tetroxide in 0.1 M cacodylate buffered solution without added La, they were rinsed in PBS, dehydrated in alcohol, embedded in Epon 812, cut into ultrathin sections, and examined electron microscopically. The frozen sections (8 mm thick) of the remaining small pieces were stained with TUNEL method. The frozen sections stained with TUNEL were embedded in Epon 812 on the slide glasses for electron microscopic identification of La.

TUNEL method

DNA nick end labeling was performed to demonstrate DNA fragmentation. The staining method originally reported by Gavrieli et al. [2] was applied. In brief, after being deparaffinized and washed in double distilled water (DDW), the sections were incubated with 20 mm/ml proteinase K (PK) for 15 min at room temperature (RT), and then washed four times in DDW for 2 min. After the endogenous peroxidase was inactivated by covering sections with 2% H₂O₂ for 5 min at RT, the sections were rinsed in DDW, and immersed in TdT buffer (30 mM Tris-HCl buffer, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (0.3 e.u./ml) and biotinylated dUTP in TdT buffer were added to cover the sections, which were then incubated in a humid atmosphere at 37°C for 60 min. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at RT. The sections were rinsed in DDW, covered with a 2% aqueous solution of BSA for 10 min at RT, rinsed again in DDW, and immersed in PBS for 5 min. Sections were then covered with streptavidin peroxidase, incubated for 10 min at 37°C, washed in DDW, immersed for 5 min in PBS, and stained with DAB (3,3'-diaminobenzidine tetrahydrochloride) for about 30 min at RT.

Quantitative analysis of TUNEL-positive cells and La-deposited cells

In brief, TUNEL-positive cells in three fields of infarcted areas were calculated in three experimental rats. The percentages of TUNEL-positive nuclei among the total number of nuclei at the ischemic foci were calculated at 0.5, 2, 3, 4 and 6 hours after the initiation of ischemia. At least 150 of both La-deposited and non-deposited cells were also counted under the electron microscope in each group. The percentage of La-deposited cells among total cells at the ischemic foci were also

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calculated at 0.5, 2, 3, 4, and 6 hours after the induction of ischemia. Paired *t* tests were performed to detect significant differences.

Combination of TUNEL method and electron microscopic identification of intracellular deposition of La

In order to clarify whether or not the permeability of the plasma membrane of myocardial cells with TUNEL-positive nuclei increases, a combination study using the TUNEL method and electron microscopic identification of intra-myocardial cell depositions of La was done. In brief, small pieces of cardiac tissue fixed with 4% glutaraldehyde in 0.1 M cacodylate buffered solution containing 1.3% La, pH 7.4, for 2 hours were cut into 8 micron-thick frozen sections and stained with the TUNEL method. After the TUNEL-positive cells was identified on frozen sections with a light microscope (Fig. 1), the frozen sections on slide glasses were then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffered solution without La, rinsed in PBS, dehydrated in alcohol, and embedded in Epon 812. Ultrathin sections were

made and examined with a Hitachi 7000 electron microscope at 75 KV.

Results

Microscopic findings of hematoxylin and eosin (H&E) stain and TUNEL method

Thirty minutes to 4 hours after the induction of ischemia, no remarkable changes were observed in the ischemic area by H&E staining. However, by TUNEL staining, positive nuclei were identified in a limited number of myocardial cells in the ischemic subendocardial area 2 hours after the induction of ischemia. Six hours after ischemia, myocardial cells showed deep eosinophilia of the cytoplasm in H & E sections, while in TUNEL-stained sections most of the myocardial cell nuclei corresponding to those showing deep eosinophilic cytoplasm in H&E sections were positively stained.

A quantitative analysis of TUNEL-positive cells at ischemic foci

At the ischemic foci, $17.9\% \pm 1.9$ of cells showed positive with TUNEL stain at 2 hours after ischemia. With

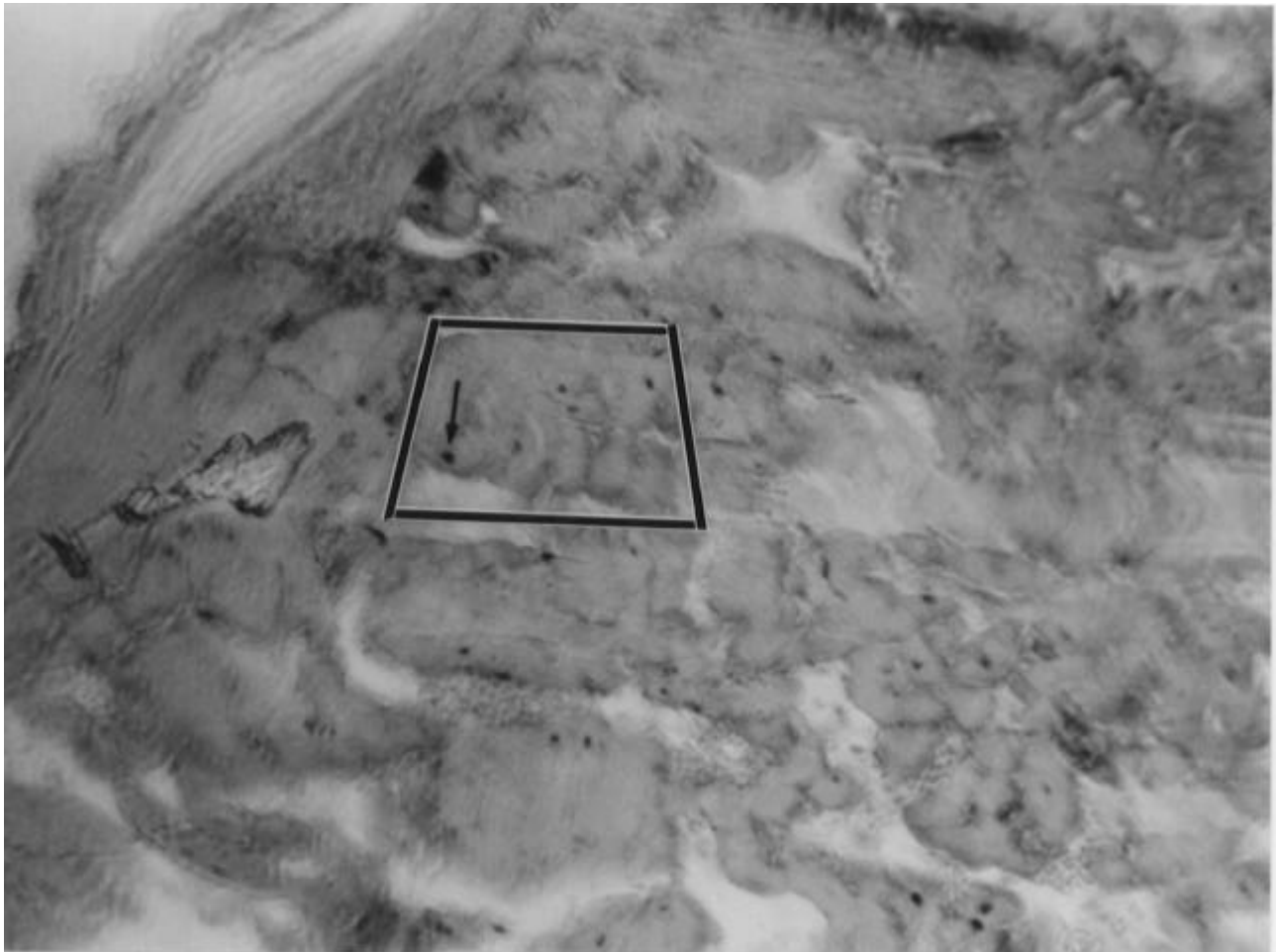


Figure 1. This shows a frozen section of the rat ischemic myocardial tissue which, after being fixed with 4% glutaraldehyde containing 1.3% La, was stained with the TUNEL method and embedded in Epon 812. Quadrangular area was examined by electron microscope. Arrow indicates TUNEL-positive nuclei. (x25)

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time TUNEL-positive nuclei increased thereafter, reaching $54.9\% \pm 2.0$ at 6 hours after ischemia (Table 1).

Identification of La by electron microscope and quantitative analysis

Portions of myocardial tissues corresponding to those examined with the light microscope for both H & E and TUNEL stain were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffered solution containing 1.3% La, pH 7.4, for 2 hours, and then studied electron microscopically. Even 30 min after the onset of ischemia, 26.7% of myocardial cells in the ischemic area showed depositions of La. Percentages of La-deposited cells increased with time, reaching the more than 90% at 4 and 6 hours (Fig. 2a) after ischemia (Table 1). Percentages of La-deposited cardiomyocytes are significantly larger than those of TUNEL-positive nuclei in ischemic foci. In the control group, La was only visible in the intercellular space between two adjacent normal myocardial cells (Fig. 2b).

A quantitative analysis of PMP of myocardial cells with TUNEL-positive nuclei

The increased permeability of the plasma membrane of myocardial cells having TUNEL-positive nuclei at the ischemic foci was examined by electron microscope at 2,

3, 4 and 6 hours after the initiation of ischemia. Two hours after ischemia, 7 of 10 myocardial cells having TUNEL-positive nuclei revealed intracellular depositions of La (Fig. 3a), whereas the remainder did not. Three hours after ischemia, 10 of 13 myocardial cells having TUNEL-positive nuclei showed La depositions in myocardial cells (Fig. 3b), and the remainder did not. Almost all myocardial cells stained positive with the TUNEL method showed intracellular depositions of La at 4 and 6 hours after ischemia (Fig. 3c), respectively.

In control, intracellular depositions of La were not observed (Fig. 3d).

Discussion

It has been widely accepted that apoptosis and necrosis are two forms of cell death with clearly distinguishing morphological and biochemical features [9, 15, 16]. Recent accumulating evidence indicates that the programmed cell death of cardiomyocytes plays an important role during the postnatal development of the heart [7] as well as during the occurrence of ischemia/reperfusion injury to the myocardium [3]. Furthermore, we previously reported that the nuclei of human infarcted myocardial cells having the morphological features of necro-

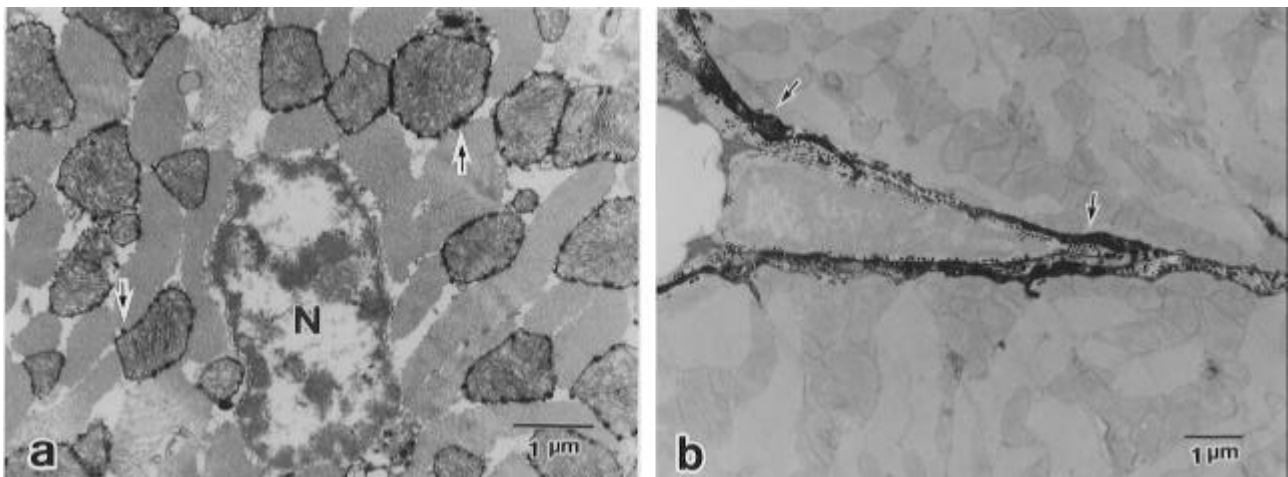


Figure 2. (a) Ultrastructure of the rat ischemic myocardial tissues which were fixed with 4% glutaraldehyde containing 1.3% La and postfixied with 1% osmic acid. Depositions of La (arrows) were found around the mitochondria. (Six hours after the onset of ischemia. This section was stained with uranyl acetate and lead citrate. N: nucleus). (b) Ultrastructure of the normal control rat myocardial tissues which were treated with the same procedures as the ischemic rat myocardial tissues. La were observed only in the intercellular space (arrows) at two adjacent normal myocardial cells. This section was stained with uranyl acetate and lead citrate.

Table 1. Frequency of lanthanum and TUNEL-positive cells at various time periods of ischemia.

	30 min (n = 1)	1 hr (n = 1)	2 hr (n = 3)	3 hr (n = 3)	4 hr (n = 3)	6 hr (n = 3)
TUNEL (%)			17.9 ± 1.9	26.2 ± 3.7	38.1 ± 2.1	54.9 ± 2.0
Lanthanum (%)	26.7	44.3	66.8 ± 5.0	81.5 ± 3.9	97.3 ± 2.8	99.5 ± 0.8

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling. Lanthanum: Indicator of permeability of myocyte plasma membrane.

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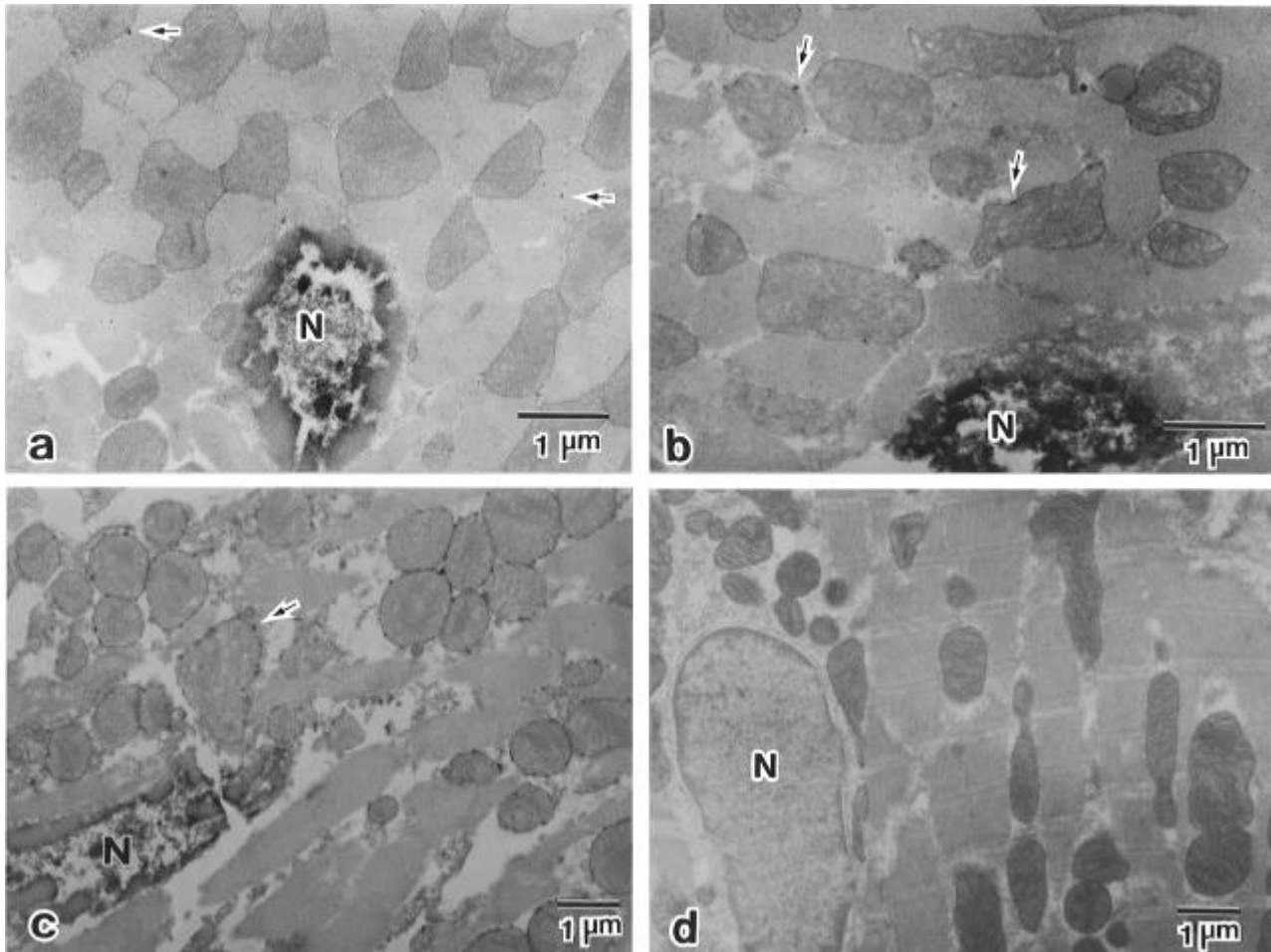


Figure 3. Electron microscopic detection of plasma-membrane permeability (PMP) of myocardial cells with TUNEL-positive nuclei. (a): 2, (b): 3 and (c): 6 hours after the onset of ischemia. Arrows indicate depositions of La which mainly located around mitochondria. (d): The normal control rat myocardial tissues which were treated with the same procedure as the ischemic rat myocardial tissues. No depositions of La were observed in the cytoplasm of myocardial cells. All sections were stained with lead citrate. N: nucleus.

sis (including both typical coagulation necrosis and contraction band necrosis) showed DNA fragmentation [5]. We also reported that permanent ligation of the left coronary artery without reperfusion produces DNA fragmentation of rat myocardial cells at the ischemic foci [13].

Kajstura et al. [6] reported that, by using both the TUNEL method for apoptosis and the anti-myosin antibody labeling method for necrosis, programmed myocardial cell death (apoptosis) appeared 2 hours after the induction of ischemia, that apoptosis was the major independent form of cardiomyocyte cell death up to 4.5-6 hours after coronary artery ligation, and that necrotic cardiomyocyte cell death follows apoptosis. Recently they [8] also reported that coronary artery narrowing caused both necrotic and apoptotic cardiomyocyte death. Necrosis is characterized by the loss of the plasma membrane integrity which remains intact in apoptotic cells [1]. Accordingly, if myocardial cell-injury caused by ischemia is due solely to the mechanism of apoptosis, the PMP of myocardial cells remains intact at an early phase of is-

chemia (2-6 hours after induction). In our study, the permeability of the plasma membrane was assessed by colloidal lanthanum, which has an average particle size of 40 Å, smaller than that of monoclonal anti-myosin antibody, and which penetrates spaces as small as 20 Å and has been successfully used as a marker for plasma membrane injury [4, 11, 10]. By using La as a tracer of PMP, we demonstrated that myocardial cells in the ischemic area showed increased permeability at a very early stage. The data obtained from our experiment were compatible with those by Koba et al. [10] who have reported that in ischemic rat hearts the deposition of lanthanum was found in 19% of ischemic cardiomyocytes after 30 min, and in 30% after 60 min of the initiation of ischemia. According to Kajstura et al. [6], cardiomyocytes showing both DNA strand breaks (apoptosis) and myosin labeling (necrosis) was a prominent mode of cell death only 6 hours after ischemia. However, in this experiment, we demonstrated that the PMP of almost all myocardial cells having TUNEL-positive nuclei showed increased plasma-

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membrane permeability from 2 to 6 hours after ischemia, meaning that DNA strand breaks and increased PMP occurred simultaneously at identical myocardial cells in the ischemic foci. This finding was made possible by the use of La tracer used in this experiment, the molecular size of which is smaller than that of monoclonal anti-myosin antibody, and which penetrates spaces as small as 20 Å. In only a small number of the myocardial cells with TUNEL-positive nuclei was La deposition not detected. These cells, theoretically, might be pure apoptotic cells. However, some limitations exist in the use of the electron microscope for identifying the intracellular deposition of La, since some La-deposited cells might be regarded as La-non-deposited cells by electron microscopic examination, i.e., myocardial cells without deposition of La might not necessarily be true apoptotic cells.

The data obtained from our experiment indicate that almost all myocardial cells having TUNEL-positive nuclei simultaneously show an increase in PMP in the acute ischemic foci.

This strongly suggests that the mechanism of myocardial cell death induced by ischemia is due to two separate processes affecting identical myocardial cells in the ischemic foci, and that the mechanism of cell death is not due solely to either apoptosis or necrosis, but to a combination of both.

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