“Apoptotic” Myocytes in Infarct Area in Rabbit Hearts May Be Oncotic Myocytes With DNA Fragmentation

Analysis by Immunogold Electron Microscopy Combined With In Situ Nick End-Labeling

Michiya Ohno, MD; Genzou Takemura, MD, PhD; Atsuko Ohno, MD; Jun Misao, MD; Yukihiro Hayakawa, MD; Shinya Minatoguchi, MD, PhD; Takako Fujiwara, MD, PhD; Hisayoshi Fujiwara, MD, PhD

Background—Modes of cell death have been defined morphologically as apoptosis and oncosis. Infarcted myocytes have been reported to show apoptosis, as revealed by DNA fragmentation by DNA ladder and by in situ terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) at the light microscopic level. We investigated whether TUNEL-positive infarcted myocytes have apoptotic or oncotic ultrastructures by using electron microscopic TUNEL, which can simultaneously observe the ultrastructure and DNA fragmentation of the same myocytes.

Methods and Results—Thirty rabbits were divided into 5 groups (n=6 each) that were subjected to a sham operation or to 30-minute ischemia followed by 0-minute, 30-minute, 2-hour, or 4-hour reperfusion of a coronary artery. In the 2- and 4-hour reperfusion groups only, DNA electrophoresis showed a ladder pattern, and the light microscopic TUNEL finding was positive in the nuclei of myocytes localized in the infarcted area (6±2% and 11±3%, respectively). Electron microscopic TUNEL showed that nuclei with a significant accumulation of immunogold particles (indicating an electron microscopic TUNEL–positive result) were observed only in the infarcted myocytes with irreversibly oncotic ultrastructures that were found in the hearts of the 2- and 4-hour reperfusion groups (41±3% and 83±4%, respectively). Irreversibly oncotic myocytes (indicated by swelling, inhomogeneously clumped chromatin in nuclei, dense bodies in mitochondria, and/or ruptured plasma membranes) were also seen in the 0- and 30-minute reperfusion groups, which did not exhibit TUNEL-positive myocytes. There was no evidence of apoptotic ultrastructures in the myocytes.

Conclusions—DNA fragmentation occurs in the myocytes that had already shown irreversibly oncotic, but not apoptotic, ultrastructures with ischemia and/or reperfusion. Therefore, DNA fragmentation itself does not always mean apoptosis, and so-called apoptotic infarcted myocytes may belong to a category of cell death other than apoptosis. (Circulation. 1998;98:1422-1430.)

Key Words: myocardial infarction • apoptosis • myocytes • ischemia • reperfusion

Generally, cell death is morphologically classified as necrosis or apoptosis. After cells undergo an injury that progresses into necrosis, the cellular reaction progresses from an initially reversible phase (prelethal phase) to the early stage of an irreversible phase (point of no return) and finally into an end stage of the irreversible phase (postmortem phase). These 3 phases have been characterized by electron microscopy (EM). However, the term necrosis was originally a general term referring to the final morphological changes that occurred after cell death (postmortem change) and thus does not include the process of cellular reaction toward death, such as the reversible or the early irreversible phase.1–4 The reversible cellular reaction, in particular, cannot be called necrosis. Conversely, many apoptotic cells in vivo show initial shrinkage and pyknosis and finally undergo phagocytosis and secondary degeneration within phagolysosomes and morphological changes similar to those of necrosis (secondary necrosis). Therefore, the 2 main modes of the progression of cellular changes toward death are currently proposed by Majno and Joris as the progress (1) from oncosis to necrosis and (2) from apoptosis to necrosis. This terminology is used in the present study.

See p 1355

Acute ischemic cell death had been recognized as an example of oncosis, until recently. There is now increasing interest in the possibility that ischemic cell death may also occur through apoptosis; this interest is based on recent

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From the Second Department of Internal Medicine, Gifu University School of Medicine (M.O., G.T., A.O., J.M., Y.H., S.M., H.F.), Gifu, Japan, and the Department of Food Science, Kyoto Women’s University (T.F.), Kyoto, Japan.
Correspondence to Hisayoshi Fujiwara, MD, PhD, Second Department of Medicine, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500, Japan.
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findings using animal models with myocardial infarction and human autopsy hearts. These findings were obtained with the use of DNA fragmentation by in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) at the light microscopic (LM) level and DNA ladder by DNA gel electrophoresis. Moreover, the expressions of proteins associated with apoptosis, such as Bcl-2, Bax, and Fas, have been reported in acute myocardial infarction and have been considered indirect evidence of apoptosis.

The special features of apoptosis shown by EM analysis are shrinkage of the cell, nuclear chromatin condensation, and phagocytosis in the final stage. However, conventional EM analysis has revealed the oncosis of myocytes during ischemia and/or reperfusion but not apoptosis. Therefore, we speculated that DNA fragmentation of the nuclei of myocytes may occur in the process of oncosis during ischemia and/or reperfusion.

Our strategy for testing this hypothesis is to simultaneously investigate the ultrastructure and DNA fragmentation in the same myocytes. In the present study, we examined DNA fragmentation at the EM level with the use of immunogold cytochemistry and in situ nick end-labeling (EM-TUNEL) in rabbit hearts subjected to ischemia and/or graded reperfusion. This method enables the simultaneous evaluation of cellular ultrastructure and DNA fragmentation.

Methods

In the present study, all rabbits received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 8523, revised 1985).

Animal Selection

Japanese white male rabbits each weighing ~2.0 kg were used. None of the rabbits had any clinically evident infections.

Experimental Protocols

Thirty rabbits were divided into 5 groups (n=6 each) that were subjected to a sham operation or to 30-minute ischemia followed by 0-minute, 30-minute, 2-hour, or 4-hour reperfusion of an anterolateral branch of the left coronary artery. DNA fragmentation was detected using TUNEL and agarose gel electrophoresis of DNA. The relationship between the ultrastructural features and DNA fragmentation in the same myocytes in the myocardial infarction hearts was observed by EM-TUNEL.

Experimental Procedures

Rabbits were anesthetized with an intravenous injection of sodium pentobarbital (30 to 40 mg/kg), and additional doses were given when required throughout the experiment. The animals were orally intubated and mechanically ventilated with room air supplemented with a low flow of oxygen by a mechanical ventilator (tidal volume, 25 to 35 mL; respiratory rate, 20 to 30/min). The respirator was adjusted in accordance with the results of a serial arterial blood gas analysis to maintain arterial blood gases within the physiological range. The standard limb leads of the ECG were monitored. The rabbits were reanesthetized, and the incisions were opened. The animals were then killed with an overdose of pentobarbital. Each heart was excised and mounted on a Langendorff apparatus.

The coronary branch was reoccluded, and monastral blue dye (4%, Sigma Chemical Co) was injected from the aorta at 80 mm Hg so that the risk area was identified as the area without blue dye. The atria were separated, the blood in the ventricular chambers was removed, and the ventricular portion below the occlusive site of the coronary artery was transversely cut into 5 slices. In the second slice, for DNA extraction, the ischemic (risk) and nonischemic (nonrisk) areas were isolated with the dye perfusion used as a guide, then frozen in liquid nitrogen, and stored at ~80°C until analysis. The third slice was used for EM-TUNEL. Myocardial samples for EM analysis were further subdivided into outer, middle, and inner thirds. The first, fourth, and fifth slices were fixed with 10% buffered formalin for conventional histology and LM-TUNEL.

DNA Extraction and Gel Electrophoresis

The frozen sections were mechanically homogenized on ice and lysed with lysis buffer containing 10% SDS, 10 mmol/L Tris, and 1 mmol/L EDTA (pH 7.8) and were digested with proteinase K at 200 µg/mL at 37°C for 16 hours. The DNA was purified by extraction with phenol/chloroform and dissolved in TE buffer (10 mmol/L Tris and 1 mmol/L EDTA). The concentration and purity of DNA were determined by the measurement of the optical density at 260 nm and the ratio of optical density at 260 nm to that at 280 nm. DNA (4 µg) was run on 2.0% agarose gel. The DNA was visualized with ethidium bromide.

Conventional Histology and LM-TUNEL

The fixed transverse ventricular slices were embedded in paraffin. After deparaffinization and rehydration, two of the third sections were stained with hematoxylin-eosin and Masson’s trichrome. The DNA fragmentation in the third section was determined with the use of an ApopTag in situ apoptosis detection kit (Oncor). The DNA nick was labeled according to the supplier’s instructions, which are based on the method described by Schmitz et al. After the TUNEL, the sections were counterstained by immersing the slides in hematoxylin. Prostate tissue from a rabbit castrated 2 days beforehand was the positive control for the TUNEL reaction.

Cardiomyocytes in the infarcted area were counted by LM analysis. In each specimen, cardiomyocytes with counterstained nuclei were counted in 60 random high-power fields (X400) from the endocardial to epicardium portion in the infarcted areas. Myocytes in which the nucleus was obviously labeled with diaminobenzidine were defined as TUNEL-positive, and they were also counted. The percentage of TUNEL-positive myocytes to ~3000 myocytes with a nucleus in the infarcted tissues was then calculated. This evaluation was carried out independently by 2 persons who were unaware of the experimental protocol.

EM-TUNEL

EM-TUNEL was performed by a method essentially the same as that reported by Migheli et al., but with modifications as follows. Immediately after the animals were killed for study, tissue samples (total, 30 portions per one heart) were taken: 10 from the inner and middle thirds and 10 from the outer third of the center of the risk area and 10 from the center of the nonrisk area; these samples were cut...
into 1-mm cubes and fixed for 4 hours at 4°C in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer. They were postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanol, and embedded in epoxy resin. Thin sections (80 nm) were cut with a diamond knife and collected on bare 300-mesh nickel grids. They were etched by incubation in 10% H2O2 for 10 minutes. The fragmented DNA was labeled on the thin sections using components of the ApopTag kit. After stopping the TdT enzymatic reaction, the grids were incubated with anti-digoxigenin mouse monoclonal antibody (0.4 μg/mL IgG, Boehringer Mannheim) for 30 minutes at room temperature. Next, they were incubated with 15 nm gold–labeled goat anti-mouse IgG (Amersham) at a dilution of 1:50 in PBS for 1 hour at room temperature. They were then washed with PBS, rinsed in distilled water, counterstained with uranyl acetate and lead citrate, and examined in an electron microscope (Hitachi 700). The grids were washed with PBS between each step. The validity of this method was checked by omitting TdT during the procedure as the gold particles did not accumulate in any cells. Thus, the degree of the gold accumulation on the nuclei of the epithelial cells was classified as slight or significant, indicating EM-TUNEL negativity or positivity, respectively (Figures 1A and B). This evaluation was performed in the nuclei of 200 to 300 myocytes in each heart and was carried out independently by 2 persons who were unaware of the experimental protocol.

Definition of DNA Fragmentation Regarding EM-TUNEL
The methodological validity of the EM-TUNEL staining was first evaluated on the sections of castrated rabbit prostate. Fragmented DNA labeled with gold tended to accumulate slightly on the nuclear chromatin in the apparently normal cells; whereas the accumulation was much more than slight, ie, significant, on the condensed chromatin of the epithelial cells with apoptotic ultrastructures (Figure 1). When TdT was omitted during the staining procedure, gold particles did not accumulate in any cells. The degree of the gold accumulation on the nuclei of the epithelial cells was classified as slight or significant, indicating EM-TUNEL negativity or positivity, respectively (Figures 1A and 2). This evaluation was performed in the nuclei of 200 to 300 myocytes in each heart and was carried out independently by 2 persons who were unaware of the experimental protocol.

Statistical Analysis
The numbers of hearts with a DNA ladder and TUNEL-positive myocytes among the groups were assessed by χ2 analysis. The percentages of TUNEL-positive nuclei among the groups were assessed by 1-way ANOVA. If significant differences were present, paired comparisons among groups were performed using the Student-Newman-Keuls test. A probability level of P<0.05 was accepted as significant. Values are mean±SEM.

Results
DNA Gel Electrophoresis
Neither the ladder nor smear pattern was observed in any tissues from the risk and nonrisk areas of the sham-operated, 30-minute ischemia only, or 30-minute reperfusion groups or in any tissues from the nonrisk area of the 2-hour or 4-hour reperfusion groups. However, the typical ladder pattern of DNA degradation was seen in the tissues from the risk area in the 2-hour and 4-hour reperfusion groups (Figure 3 and Table 1).

Conventional Histology and LM-TUNEL
Conventional LM analysis was performed using hematoxylin-eosin and Masson’s trichrome stain. Microscopically, in hearts subjected to 30-minute ischemia only, 30-minute reperfusion, and 2-hour reperfusion, the infarcted areas were unclear. In the hearts subjected to 4-hour reperfusion, infarcted areas with deep red–stained dead myocytes with and without contraction bands, inflammatory cells, and/or hemorrhage were mainly localized in the inner and middle thirds of the left ventricular wall within the risk area in all hearts, but they were rarely observed in the outer third.

In the sections of castrated prostate that were used as a positive control for the LM-TUNEL staining, TUNEL-positive nuclei were observed in some of the epithelial cells. Myocytes with TUNEL-positive nuclei were observed in the 2-hour and 4-hour reperfusion groups but not in the sham-operated, 30-minute ischemia only, or 30-minute reperfusion groups (Figure 4 and Table 2). The LM-TUNEL–positive cells were distributed in the infarcted areas. The mean percentage of LM-TUNEL–positive myocytes within the infarcted areas was 6±2% in the 2-hour reperfusion group and 11±3% in the 4-hour reperfusion group (Table 2).
Figure 2. Electron photomicrographs of myocytes stained by EM-TUNEL method. In lower panels, the nucleus (N) shown in upper panels is presented with a higher magnification and fainter printing for clearer documentation of immunogold particles. A, Normal myocyte taken from sham-operated rabbit is shown in upper panel and has a normal ultrastructure. In lower panel, the myocyte has a slight accumulation of immunogold particles on heterochromatin in nucleus. Distribution of gold particles was similar to that seen in normal prostatic cells in Figure 1A, and degree of distribution was as slight as that observed in normal cells (ie, EM-TUNEL-negative). Note that in the lower panel, glycogen granules (indicated by arrows) can be easily distinguished from immunogold particles (indicated by arrowheads) because of differences in electron density between them, both of which look identical with normal printing in upper panel. B, Myocyte subjected to 30-minute ischemia followed by 30-minute reperfusion. Glycogen granules have disappeared. Nuclear chromatin is clumped in various sizes and randomly scattered. Mitochondria (Mt) are swollen and contain amorphous dense bodies. Disruption of their cristae is also noted. Myofibrils (Mf) are abnormally contracted and partially disrupted. These findings indicate irreversible oncosis of the myocyte. As shown in lower panel, gold particles are accumulated on clumped chromatin, but the degree is as slight as that seen in normal prostatic cells or in normal myocytes (ie, EM-TUNEL-negative). C, Myocyte subjected to 30-minute ischemia followed by 4-hour reperfusion. A red blood cell (RBC) invades the myoplasm, indicating rupture of the plasma membrane of the myocyte. Thus, the ultrastructural changes showing irreversible oncosis are more distinct but basically similar to those shown in panel B. In contrast, accumulation of gold particles is conspicuous (ie, EM-TUNEL-positive) on nucleus shown in lower panel. Bars = 1 μm.
DNA Fragmentation in Ischemic and Reperfused Myocytes

In the sham-operated group, the ultrastructure of myocytes revealed by EM-TUNEL showed rich glycogen granules in the cytoplasm and dispersed chromatin in the nuclei. Immunogold particles were slightly accumulated on the nuclei of all cells of the sham-operated group but never on other subcellular organelles, cytoplasm, or interstitium (Figure 2A). In the group subjected to 30-minute ischemia only, in which the infarcted areas were unclear at the LM level, the representative ultrastructure of myocytes in the myocardium obtained from the inner and middle thirds of the left ventricular wall within the risk area showed marked swelling, a marked decrease of glycogen granules, swollen mitochondria with disrupted cristae and amorphous dense bodies, disrupted cytoplasmic membranes, markedly marginalized and clumped chromatin in the nuclei, and stretched fibers (widening of the I bands), indicating irreversibly oncotic changes. In contrast, the representative ultrastructural features of the myocytes in the myocardium obtained from the outer third of the left ventricular wall within the risk area included slight swelling of glycogen granules, mild swollen mitochondria without amorphous dense bodies, and mildly marginalized and clumped chromatin in the nuclei, and stretched I bands, indicating reversibly oncotic changes. In the group subjected to 30-minute reperfusion, in which the infarcted areas were unclear at the LM level, the findings of irreversible oncosis were present in most of the myocytes in the myocardium obtained from the inner and middle thirds of the left ventricular wall within the risk area. That is, we observed grossly swollen mitochondria with many amorphous dense bodies, frequently disrupted cytoplasmic membranes, marked clumped chromatin materials that were variously sized and randomly dispersed in the nuclei, the disappearance of glycogen granules, and the formation of numerous contraction bands of myofibers. The findings for the myocytes in the myocardium obtained from the outer third of the left ventricular wall within the risk area were similar to the findings for the myocytes in the group subjected to 30-minute ischemia only. However, the degree of gold accumulation on the nuclei of ischemic myocytes with reversible and irreversible oncosis in each heart from the groups subjected to 30-minute ischemia only and 30-minute reperfusion was as slight as that seen in the nuclei of the normal myocytes of the sham-operated group (Figure 2B and Table 3).

In the 2-hour and 4-hour reperfusion groups, the findings of irreversible oncosis in most of myocytes of the inner and middle thirds of the left ventricular wall within the risk area became more marked. A significant accumulation of immunogold on the nuclei similar to that observed in the apoptotic epithelial cells of the prostate tissues used as a positive control was seen in many myocytes with irreversible oncosis but not in myocytes with reversible oncosis in each heart of the 2-hour and 4-hour reperfusion groups (Figure 2C and Table 3). The findings of myocytes in the myocardium obtained from the outer third of the left ventricular wall within the risk area were similar to those of the 30-minute ischemia only group. The accumulation of gold in these myocytes was slight, similar to that in the normal myocytes of the sham-operated group. There was no evidence of apoptotic ultrastructures in the myocytes in any hearts of the groups subjected to ischemia and/or reperfusion.

In the inner or middle thirds of the left ventricular wall within the risk area, most of the myocytes (>90%) showed ultrastructurally irreversible myocytes in the groups subjected to 30-minute ischemia only and to 30-minute ischemia along with 30-minute, 2-hour, and 4-hour reperfusion. Nuclei with a significant accumulation of immunogold, indicating EM-TUNEL–positive myocytes, were seen only in myocytes with irreversible oncosis. The mean percentage of EM-TUNEL–positive myocytes in the inner and middle thirds of the left ventricular wall within the risk area with a significant accumulation of immunogold on the nuclei was 0% each in the 30-minute ischemia only and 30-minute reperfusion groups, 41±3% in the 2-hour reperfusion group, and 83±4% in the 4-hour reperfusion group (Table 3).

**Discussion**

The present study revealed that in rabbit hearts subjected to ischemia and/or reperfusion, definite DNA fragmentation was

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**TABLE 1. Ladder Pattern of DNA Fragmentation in Tissue From Risk Area in Myocardium**

<table>
<thead>
<tr>
<th>Treatment/Groups</th>
<th>n</th>
<th>No. of Hearts With DNA Ladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>30-min ischemia, no reperfusion</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>30-min ischemia, 30-min reperfusion</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>30-min ischemia, 2-h reperfusion</td>
<td>6</td>
<td>5*</td>
</tr>
<tr>
<td>30-min ischemia, 4-h reperfusion</td>
<td>6</td>
<td>6*</td>
</tr>
</tbody>
</table>

*P<0.05 vs group subjected to sham operation.
detected ultrastructurally by EM-TUNEL in myocytes with irreversible oncosis.

**Acute Ischemic Myocyte Death and Apoptosis**

Apoptosis is defined by 2 independent hallmarks: (1) a characteristic condensation of chromatin, no rupture of cell membrane, and cell fragmentation into small membrane-bound vesicles as a morphological marker and (2) endonuclease-activated fragmentation of internucleosomal DNA to multiple 180- to 200-bp fragments as a biochemical marker.

No previous reports had documented any ultrastructural evidence of apoptotic changes in cardiomyocytes after ischemia/reperfusion; the previous findings were exclusively based on a biochemical marker (DNA fragmentation shown on gel electrophoresis and by LM-TUNEL). This is in contrast to the reports on familial heart block in humans and on chronic heart failure in dogs, which described the ultrastructure of apoptotic changes in cardiomyocytes. Gottlieb et al described the different ultrastructural features of the nucleus between reperfused myocytes and permanently ischemic myocytes. Those nuclear ultrastructural changes in reperfused myocytes are specific not for apoptotic changes but for oncocytic changes of myocytes with reperfusion damage.

Kajstura et al observed a positive TUNEL reaction at the LM level in ischemic rat myocytes that were labeled and in those that were not labeled by anti-myosin antibody and speculated that the unlabeled myocytes had intact cytoplas-
mic membranes and that the changes were thus not oncotic but apoptotic changes. However, their method has problems regarding sensitivity for the detection of cells with ruptured membranes (irreversibly oncotic change) because of the relatively large molecular weight of the antibody and problems regarding the lack of ultrastructural observations in myocytes.

In our present investigation, the DNA ladder pattern and TUNEL-positive myocytes at the LM level were not seen in the sham-operated, 30-minute ischemia only, or 30-minute reperfusion groups but were present in the 2-hour and 4-hour reperfusion groups. The data for the sham-operated, 30-minute ischemia only, and 4-hour reperfusion groups confirmed the data for the rabbit groups studied by Gottlieb et al.5 In the present 30-minute ischemia only and 30-minute, 2-hour, and 4-hour reperfusion groups, however, the ultrastructures of myocytes were representative of reversible or irreversible oncotic changes. There was no evidence of myocytes with apoptotic ultrastructures in any of the hearts of these groups. EM-TUNEL–positive nuclei with a significant gold accumulation in rat myocytes were representative of reversible or irreversible oncotic changes. These larger fragments appeared transiently; they had disappeared in the 30-minute ischemia only or 30-minute reperfusion group;

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Cellular damage of myocytes in ischemia and reperfusion. Cellular damage of infarcted myocytes proceeds from reversible and irreversible oncotic changes to irreversible oncotic changes with or without DNA fragmentation. In the progression from irreversible oncotic changes without DNA fragmentation to irreversible oncotic changes with or without DNA fragmentation, the thick arrow indicates that most of the myocytes may have a significant accumulation of immunogold particles on nuclei, indicating DNA fragmentation.

they were found only in myocytes with irreversibly oncotic changes in the 2-hour and 4-hour reperfusion groups. In the 4-hour reperfusion group, the incidence of these nuclei was increased, and most of the myocytes with irreversible oncotic (83%) showed a significant gold accumulation on the nuclei. Thus, the damage of myocytes in ischemia and/or reperfusion proceeded from reversible oncotic to irreversible oncotic changes without DNA fragmentation and then to irreversible oncotic changes with or without DNA fragmentation (Figure 5). Therefore, so-called apoptotic myocytes may belong to a category other than apoptosis.

Internal nucleosomal DNA fragmentation was reported to be caused by the activation of Ca²⁺- and Mg²⁺-dependent nuclease, which may be indistinguishable from DNase I.27.28 One possible explanation of the mechanism underlying the appearance of DNA fragmentation in oncotic myocytes is that the activation of endonuclease or the dying cascade caused by oncotic cell death due to ischemia/reperfusion is in part the same as in apoptotic cell death. This idea is supported by the findings that not only the apoptotic cell death but also the oncotic cell death of KCN-treated PC12 cells (a pheochromocytoma cell line) were blocked by Bcl-2, an inhibitor of apoptotic cell death,29 and that the overexpression of Bcl-2 in rat myocytes prevents the DNA fragmentation of myocytes during ischemia/reperfusion.30 The issues concern the definition of apoptosis; whether the existence of DNA fragmentation is specific for apoptosis should be further elucidated. In addition, it remains to be determined whether the progression of ischemic cellular damage from reversible oncotic to irreversible oncotic changes with DNA fragmentation can be blocked by the inhibition of the early apoptotic biochemical process before the appearance of DNA fragmentation.

DNA fragments of >1 kbp of high molecular weight were observed in the present 30-minute ischemia only and 30-minute reperfusion groups, where neither the DNA ladder nor a positive TUNEL reaction was seen. These larger fragments appeared transiently; they had disappeared in the 2-hour reperfusion groups. Thus, these DNA fragments may be a precedent of the DNA ladder even in oncotic myocytes, but the pathophysiology remains unknown.

**Methodological Limitations**

Compared with the tissue areas observed by LM analysis in the present study, the tissue areas observed by the EM

### TABLE 2. DNA Fragmentation in Myocytes Revealed by TUNEL at LM Level

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>No. of LM-TUNEL–Positive Hearts</th>
<th>LM-TUNEL–Positive Myocytes, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>30-min ischemia, no reperfusion</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>30-min ischemia, 30-min reperfusion</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>30-min ischemia, 2-h reperfusion</td>
<td>6</td>
<td>5*</td>
</tr>
<tr>
<td>30-min ischemia, 4-h reperfusion</td>
<td>6</td>
<td>6*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *P<0.05 vs group subjected to sham operation.

### TABLE 3. DNA Fragmentation in Myocytes Revealed by TUNEL at EM Level

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>No. of EM-TUNEL–Positive Hearts</th>
<th>EM-TUNEL–Positive Myocytes, %</th>
</tr>
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<tbody>
<tr>
<td>Sham operation</td>
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<td>30-min ischemia, no reperfusion</td>
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<td>0</td>
</tr>
<tr>
<td>30-min ischemia, 30-min reperfusion</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>30-min ischemia, 2-h reperfusion</td>
<td>6</td>
<td>6*</td>
</tr>
<tr>
<td>30-min ischemia, 4-h reperfusion</td>
<td>6</td>
<td>6*</td>
</tr>
</tbody>
</table>

A significant accumulation of immunogold on a nucleus is defined as EM-TUNEL–positive. Values are mean ± SEM. *P<0.05 vs group subjected to sham operation.
analysis were considerably small, although multiple tissues were taken from the myocardium. Therefore, we cannot exclude the possibility that the EM-TUNEL analysis may have overlooked a very small number of truly apoptotic cells, although all of the myocytes with TUNEL-positive nuclei showed irreversible oncotic changes by EM-TUNEL.

The nuclear chromatin was slightly labeled with immunogold even in the myocytes with a normal ultrastructure. It is possible that a small amount of cleaved DNA was present even in the normal myocytes or that little cleavage of DNA occurred as an artifact product during tissue processing. The incidence of myocytes with a significant immunogold accumulation on the nuclei examined by EM-TUNEL was ≈7-fold higher than that of the myocytes with LM-TUNEL–positive myocytes in all groups. This discrepancy may be explained by the difference of sensitivity to DNA fragmentation between EM-TUNEL and LM-TUNEL.

In the present study, the demarcation between the risk area and nonrisk area was clear, as shown by monastral blue dye in all groups. Infarcted areas were confirmed by LM findings using hematoxylin-eosin and Masson’s trichrome stainings and were localized in the inner and middle thirds of the left ventricular wall within the risk area in the hearts subjected to 30-minute ischemia followed by 4-hour reperfusion. However, when the methods described above were used, the entire infarcted areas were not clear in the hearts subjected to 30-minute ischemia only and to 30-minute ischemia followed by 30-minute and 2-hour reperusions. On the other hand, EM analysis can detect myocytes with irreversible cellular damage, ie, infarcted myocytes, in the sampled tissues even after only 30 minutes of ischemia or 30 minutes of ischemia followed by 30-minute and 2-hour reperusions, but this damage cannot be detected in the entire infarcted areas because of the multiple but small sampling areas. Therefore, at present, there is no consensus as to whether the size of the myocardial infarct can expand somewhat during reperfusion after ischemia (so-called reperfusion injury) or is determined only by cellular damage during ischemia. If the former is true, the infarcted areas after only 30 minutes of ischemia or 30 minutes of ischemia followed by 30-minute and 2-hour reperusions, but this damage cannot be detected in the entire infarcted areas because of the multiple but small sampling areas. Therefore, at present, there is no consensus as to whether the size of the myocardial infarct can expand somewhat during reperfusion after ischemia (so-called reperfusion injury) or is determined only by cellular damage during ischemia. If the former is true, the infarcted areas after only 30 minutes of ischemia or 30 minutes of ischemia followed by 30-minute and/or 2-hour reperusions may be smaller than the infarcted areas after the 4-hour reperfusion. Therefore, we cannot exclude the possibility that the infarcted areas were missed in the groups subjected to 30-minute ischemia only or to subsequent 30-minute and 2-hour reperfusion despite the same sampling sites as in the group subjected to 4-hour reperfusion. However, in either case, the EM analysis of the 30-minute ischemia only or subsequent 30-minute and 2-hour reperfusion groups, as well as the 4-hour reperfusion group, clearly showed that most of the myocytes had irreversible cellular damage of oncotic type in the tissues obtained from the inner and middle thirds of the left ventricular wall within the risk area. It also showed the presence of myocytes with reversibly oncocytic changes in the tissues obtained from the outer third. These findings indicate that considerable infarcted areas were present in the inner and middle thirds of the left ventricular wall within the risk area in the 30-minute ischemia only or subsequent 30-minute and 2-hour reperfusion groups and that most of the tissue samplings for the EM analysis were performed in the infarcted areas. There was no evidence of EM-TUNEL–positive nuclei in any of myocytes within the entire risk areas of the 30-minute ischemia only and subsequent 30-minute reperfusion groups. However, in the 2-hour and 4-hour reperfusion groups, myocytes with EM-TUNEL–positive and LM-TUNEL–positive nuclei were seen in the infarcted tissues within the risk areas. Thus, we believe that the data provided by EM-TUNEL are reliable for the 30-minute ischemia only or subsequent 30-minute and 2-hour reperfusion groups as well as for the 4-hour reperfusion group.

Conclusion
EM-TUNEL revealed that DNA fragmentation was present in myocytes with irreversible oncosis. So-called apoptotic myocytes may therefore belong to a category other than apoptosis.

Acknowledgments
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References


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Michiya Ohno, Genzou Takemura, Atsuko Ohno, Jun Misao, Yukihiro Hayakawa, Shinya Minatoguchi, Takako Fujiwara and Hisayoshi Fujiwara

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