Possible Involvement of Apoptotic Death of Myocytes in Left Ventricular Remodeling after Myocardial Infarction

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Abstract: Pathophysiological roles of apoptosis in post-infarction left ventricular (LV) remodeling have not been well characterized. This study showed that TUNEL- or cleaved caspase-3–positive myocytes were identified late after ligation of the left coronary artery in rats, suggesting that apoptotic myocyte death contributed to the morphological change associated with LV remodeling. [The Japanese Journal of Physiology 53: 247–252, 2003]

Key words: left ventricular remodeling, myocardial infarction, apoptosis.

Post-infarction left ventricular (LV) remodeling is the process by which ventricular size, shape, and function are regulated by mechanical, neurohumoral, and genetic factors [1], and is believed to be one kind of adaptive response to infarction [2]. Besides necrotic death, apoptosis or programmed cell death is involved in the loss of myocytes caused by acute myocardial ischemia [3, 4]. However, the functional and/or pathological roles of myocyte apoptosis in post-infarction left ventricular (LV) remodeling have not been well characterized. Recent studies have suggested that the apoptosis of myocytes is involved in post-infarction LV remodeling [5–7]. In this study, we have tried to elucidate whether there is apoptotic death of cardiac myocytes during the process of LV remodeling late after ligation of the left coronary artery of rats, and if so, whether it coincides with the morphological changes to the left ventricle in the post-infarcted heart.

Male Wistar rats 10–13 weeks old and weighing 250 to 350 g were anesthetized with diethyl ether and connected to a respirator through the trachea. The heart was rapidly exteriorized through a left anterior thoracotomy and pericardial incision, and the coronary artery was ligated at 2 to 3 mm from its origin with 5-0 silk suture. The heart was returned to its normal position, and the thorax was closed. After coming out of the anesthesia, the rat was disconnected from the respirator. Another group of rats underwent the same procedure except for ligation of the coronary artery (sham operation). After the operation, the rats were kept for 1 day, 2 weeks, or 6 weeks. Rats that underwent no surgical operations were used in the experimentation as a control.

DNA fragmentation, which is indicative of apoptotic cells, was detected with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for the assessment of apoptosis. In brief, the heart was excised and cut into 3 short-axis slices. After fixation with 4% paraformaldehyde, each slice was stained transversely with 1 to 2 mm, and the TTC-stained myocardial slices were photographed with a digital camera. A cross-sectional area (CSA) was measured by using image analysis software, Photoshop (Adobe Systems, San Jose, CA). The differences in heart weight or the ratio of heart weight to body weight at 1 day, 2 weeks, and 6 weeks (n=4 hearts in each group) between post-infarct and control hearts were analyzed by using a one-way analysis of variance. When a significant F ratio was observed, the post-hoc Scheffe’s test was used to identify significant differences.

The extent of myocardial infarction was quantified by staining with triphenyl-tetrazolium chloride (TTC; Sigma, St. Louis, MO). A heart was excised, weighed, and perfused retrogradely with 2% TTC in a physiological saline solution for 20 min at 37°C, then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The heart was sliced transversely into 11 to 14 slices (1 to 2 mm), and the TTC-stained myocardial slices were photographed with a digital camera. A cross-sectional area (CSA) was measured by using image analysis software, Photoshop (Adobe Systems, San Jose, CA). The differences in heart weight or the ratio of heart weight to body weight at 1 day, 2 weeks, and 6 weeks (n=4 hearts in each group) between post-infarct and control hearts were analyzed by using a one-way analysis of variance. When a significant F ratio was observed, the post-hoc Scheffe’s test was used to identify significant differences.

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slice was embedded in a paraffin block and cut into 5 μm sections transversely. Myocardial tissue sections were heated in sodium citrate solution and digested with proteinase-K to expose the DNA. The DNA strand breaks were labeled with the use of a terminal transferase enzyme with dUTP molecules conjugated to alkaline phosphatase and visualized immunohistochemically. Sections were then photographed with a digital camera through a light microscope. The number of apoptotic cells was determined by selectively counting the TUNEL-positive cardiomyocyte nuclei in LV sections 5 μm thick. To detect that a TUNEL-positive cell was a myocyte, the Masson-Goldner stain was performed on similar slices to those used for the TUNEL assay. The heart section that was cut into 5 μm was stained with haematoxylin solution for 5 min. After a 5 min rinsing in running tap water and with 1% acetic acid for 30 s, the section was stained Masson solution (Goldner I) (fuchsin acid [Wako, Japan] 33 mg, xylinid-ponceau [Sigma] 66 mg, azophloxin [Waldeck Division Chroma, Germany] 10 mg, acetic acid 280 μl, and distilled water 99.72 ml) for 10 min. The section was then rinsed in 1% acetic acid for 30 s and stained by using phosphomolybdic acid Orange G solution (phosphomolybdic acid [Sigma] 3 g, orange G [Sigma] 2 g, distilled water 100 ml) for 1 min. After a rinsing in 1% acetic acid for 30 s, the section was stained with haematoxylin solution for 5 min. After a 5 min rinsing in running tap water and with 1% acetic acid for 30 s, the section was stained.

Immunohistochemical analysis using anti-cleaved caspase-3 antibody, an activated form of caspase-3, was also performed to investigate whether the activation of caspase-3 was involved in the process of LV remodeling. In brief, following standard histological processing and paraffin embedding, the heart was cut into 5-μm sections transversely. The sections were washed twice with xylene for 5 min each, and washed with 100, 100, 90, 80, and 70% ethanol each for 3 min. After the sections were rinsed in PBS, endogenous peroxidase activity was inhibited by treatment with 0.3% H2O2 in methanol for 20 min. The sections were then incubated with 1% normal goat serum for 30 min. After a rinsing with PBS, the primary antibody, an anticleaved caspase-3 (1: 50 dilution; Cell Signaling Technology, Beverly, MA), was applied for 12 h at 4°C, then for 60 min at 37°C. The sections were next rinsed with PBS and incubated with the secondary antibody, an anti-rabbit IgG (New England Biolabs, Inc., Beverly, MA), for 50 min. They were incubated with A, B Complex (Vector Laboratories, Inc., Burlingame, CA) for 40 min after a rinse with PBS, and staining was then visualized by the use of diaminobenzidine (DAB, Sigma).

A Western blotting analysis was then performed to confirm the specificity of the anti-cleaved caspase-3 antibody used in this study. LV cells separated from a heart were homogenized in homogenization buffer (10 mM Tris-HCl [pH 7.5], 25% glycerol, 0.82 M NaCl, 1 mM Na3VO4, 50 mM NaF, 1.5 mM MgCl2, 0.5 mM ethylenediamine tetraacetic acid [EDTA], 2 mM sodium pyrophosphate, 0.5 mM phenylmethylsulphonyl fluoride [PMSF], 0.5 mM dithiothreitol [DDT], 1.25 μg/ml of pepstatin A, 10 μg/ml of leupeptin, 2.5 μg/ml of aprotonin, and 0.1% Triton X). The homogenates were analyzed by Western blotting by loading 50 μg protein/lane. The following conditions were used for binding the primary antibody, an anticleaved caspase-3 (1: 1,500 dilution) or an antiparaffin IgG, was diluted 1: 2,000.

Figure 1 shows an example of TTC-stained hearts of control (A), at 1 day (24 h) (B), at 2 weeks (C), and at 6 weeks (D) after a ligation of the left coronary artery, and at 6 weeks after the sham operation (E). Myocardial regions unstained with TTC show signs of necrotic cell death. In the heart at 1 day after ligation of the coronary artery, an unstained region was consistently observed in the infarct (Fig. 1B). However, few such regions were observed in the hearts at 2 weeks (C) and 6 weeks (D) after ligation. The wall of the left ventricle became thin, and dilatation of the LV occurred at 2 and 6 weeks (C, D) after the ligation. In contrast, the unstained regions and the detectable morphological changes in the left ventricle were not present in the sham-operated rat heart at 6 weeks after the operation (E).

The change in heart weight (HW) associated with LV remodeling was then analyzed (Fig. 2). The HW in post-infarct rats significantly increased at 2 and 6 weeks after ligation of the left coronary artery (Fig. 2A) in comparison with the control. To confirm that the infarction-induced increase in HW was not due to an increase in total body size in the 2 or 6 weeks of the observation period, we then analyzed the change in the heart-weight to body-weight ratio (HW/BW). These ratios also increased significantly at 2 and 6...
weeks after ligation of the coronary artery (Fig. 2B), similar to the change in the HW. We then analyzed the change in the cross-sectional areas (CSA) of hearts after the ligation (Fig. 2C). The CSA had significantly increased at both 2 and 6 weeks. These results reflected the morphological changes of the heart associated with LV remodeling in response to infarction and were almost the same as those reported previously [5].

We then investigated whether apoptotic death was involved in the post-infarction LV remodeling (Fig. 3). Cross sections of each heart were stained by the in situ TUNEL technique (Fig. 3A). Only a few TUNEL-positive cells were in the hearts at 1 day after ligation of the coronary artery. In contrast, many TUNEL-positive myocytes were identified in the infarcted area and at sites distant from the infarct at 2 weeks after the ligation. In hearts at 6 weeks, the number of TUNEL-positive myocytes again decreased compared with that at 2 weeks after the ligation. TUNEL-positive myocytes were selectively counted by referring to Masson-Goldner-stained sections (Fig. 3B), and the counts were analyzed statistically (Fig. 3C). A significant increase was observed in the number of TUNEL-positive myocytes at both 2 and 6 weeks after ligation of the coronary artery. However, the number had significantly decreased at 6 weeks compared with 2 weeks.

We then investigated whether the activation of caspase-3, a crucial enzyme for executing apoptotic cell death [8], was responsible for post-infarction LV remodeling (Fig. 4). Immunostaining using an anti–cleaved caspase-3 antibody, an activated form of caspase-3, was performed on another cross-section of the
heart used for *in situ* TUNEL staining (Fig. 4). Cleaved caspase-3–positive cells were identified in almost the same area in which TUNEL-positive myocytes were identified in the post-infarct hearts. Furthermore, the time course of the change in the number of cleaved caspase-3–positive myocytes after the ligation was almost the same as that in the number of TUNEL-positive myocytes (data not shown). We further confirmed the specificity of the anti–cleaved caspase-3 antibody used here by Western blotting, using either anti–cleaved caspase-3 antibody or anti–caspase-3 antibody (Fig. 4B). Anticaspase-3 antibody is effective on both caspase-3 (35 kDa) and an activated form of caspase-3 (17–20 kDa). In contrast, anti–
cleaved caspase-3 antibody is effective only on an activated form of caspase-3. Western blotting using anti–cleaved caspase-3 antibody showed that an activated form of caspase-3 was clearly identified in the post-infarct hearts, but caspase-3 was not, suggesting that the anti–cleaved caspase-3 antibody used in this study was specific to an activated form of caspase-3 (Fig. 4B, right).

This study has revealed that the apoptotic death of cardiac myocytes occurred long after a ligation of the left coronary artery, most notably at 2 weeks. Post-infarction LV remodeling is characterized by an increase...
in heart weight and by morphological changes to the heart. It has been generally accepted that cardiac myocytes are terminally differentiated and lose the ability to regenerate [9]. Many myocytes die mainly via necrosis after acute myocardial ischemia. Thus, myocardial infarction results in a decrease in contractile force as a result of a loss of myocytes. The surviving myocytes are hypertrophied probably to compensate for the infarction-induced decline in cardiac function. Thus LV remodeling seems to be an adaptive response of the heart to infarction [2]. In this study, the apoptotic or programmed death of cardiac myocytes was observed to nearly coincide with the infarction-induced morphological change of the heart, suggesting that apoptosis is critically involved in the process of post-infarction LV remodeling of the heart.

The authors thank Dr. Tomiyasu Koyama, professor emeritus of Hokkaido University, for valuable advice on the procedure for cardiac surgery. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan 12480256 to KK, and by a grant from the Clark Commemorative Foundation, Hokkaido University to TS.

REFERENCES