Mechanisms by Which Late Coronary Reperfusion Mitigates Postinfarction Cardiac Remodeling


Abstract—Although recanalization of the infarct-related artery late after myocardial infarction (MI) is known to reduce both cardiac remodeling and mortality, the mechanisms responsible are not yet fully understood. We compared infarcted rat hearts in which the infarct-related coronary artery was opened 24 hours after infarction (late reperfusion [LR] group) with those having a permanently occluded artery. Left ventricular dilatation and dysfunction were significantly mitigated in the LR group 1, 2, and 4 weeks post-MI. Attributable, in large part, to the greater number of cells present, the infarcted wall was significantly thicker in the LR group, which likely reduced wall stress and mitigated cardiac dysfunction. Granulation tissue cell proliferation was increased to a greater degree in the LR group 4 days post-MI, whereas the incidence of apoptosis was significantly lower throughout the subacute stage (4 days, 1 week, and 2 weeks post-MI), further suggesting preservation of granulation tissue cells contributes to the thick, cell-rich scar. Functionally, myocardial debris was more rapidly removed from the infarcted areas in the LR group during subacute stages, and stouter collagen was more rapidly synthesized in those areas. Direct acceleration of Fas-mediated apoptosis by hypoxia was confirmed in vitro using infarct tissue-derived myofibroblasts. In salvaged cardiomyocytes, degenerative changes, but not apoptosis, were mitigated in the LR group, accompanied by restoration of GATA-4 and sarcomeric protein expression. Along with various mechanisms proposed earlier, the present findings appear to provide an additional pathophysiological basis for the benefits of late reperfusion. (Circ Res. 2008;103:98-106.)

Key Words: apoptosis ■ heart failure ■ myocardial infarction ■ remodeling ■ reperfusion

Large myocardial infarctions (MIs) cause severe chronic heart failure, with adverse remodeling of the left ventricle characterized by cavity dilatation and diminished cardiac performance.1 The most critical determinants of subsequent heart failure are the magnitude of the acute MI, which can be determined within several hours of an attack,2 and recanalization of the infarct-related artery, which, if performed early enough for myocardial salvage, reduces the size of the acute infarct, prevents subsequent heart failure, and improves prognosis.3 In addition, the “open artery hypothesis” formulated by Kim and Braunwald proposes that late reperfusion, beyond the window for myocardial salvage, also reduces left ventricular remodeling and decreases mortality.4,5 This proposal is supported by much experimental and clinical evidence, and a number of possible mechanisms by which an open infarct-related artery could confer benefit in ways other than by salvaging ischemic myocardium have been proposed. For instance, when the importance of the early inflammatory response needed to initiate infarct scar formation is considered,6 restoration of blood flow and allowing an influx of inflammatory cells into the infarct may improve healing of the infarcted tissue and prevent ventricular remodeling.4 In addition, an open, blood-filled infarct-related artery and vascular bed may provide a supporting scaffold that helps maintain the structural integrity of the necrotic myocardium and limits infarct expansion and ventricular remodeling.7 Another proposed mechanism suggests that late reperfusion elicits intramyocardial hemorrhage, edema, and contraction band necrosis, within which sarcolemmal tubes persist and prevent collapse of the necrotic tissue.8 Late revascularization of “hibernating” myocardium present within the perinfarct region is also a possible benefit of a patent infarct-related artery.9 Collagen turnover is reportedly more pronounced in patients with an occluded infarct-related artery, and thus prevention of interstitial collagen turnover may be another beneficial effect.10 Finally, a recent study reported a significantly higher rate of apoptosis among cardiomyocytes in patients with persistent occlusion of the infarct-related artery.11 This increased apoptosis was apparent well beyond the acute phase of MI, and those investigators proposed that late
reperfusion might inhibit the apoptotic loss of salvaged cardiomyocytes, thereby preventing the progression of heart failure.

Infarcted tissue is highly dynamic and shows remarkable changes during the course of healing\textsuperscript{12,13}: necrotic tissue is infiltrated by inflammatory cells during the acute stage of MI; granulation tissue forms during the subacute stage; and scar tissue forms during the chronic stage. Most cellular components that infiltrate and proliferate within an infarct, including inflammatory and granulation tissue cells, disappear via apoptosis during the subacute and chronic stages.\textsuperscript{14,15} In that regard, we previously reported that inhibition of apoptosis among granulation tissue cells during the subacute stage of MI alters infarct tissue dynamics, making the infarct scar thicker and rich in preserved cellular components. Most likely by attenuating wall stress, such effects mitigate the adverse effects that infiltrating cells might have on the myocardium. These results imply that late reperfusion might inhibit the apoptotic loss of salvaged cardiomyocytes, thereby preventing the progression of heart failure.

Materials and Methods

This study was approved by our Institutional Animal Research Committee. MI was induced in male Wister rats weighing 250 to 300 g (Chubu Kagaku, Nagoya, Japan) by ligating the left coronary artery as previously described.\textsuperscript{16,17} In sham-operated animals, the suture was passed around the artery but not tied. Twenty-four hours later, the hearts of the surviving rats were reperfused, and the rats were randomly assigned to a permanent occlusion (PO) group, in which the coronary artery ligation was maintained, or a late reperfusion (LR) group, in which the ligature was completely removed. The left ventricular peak systolic pressure was similar between the groups. Examination at autopsy confirmed the absence of perfusion in the infarcted areas of all hearts in the LR group, ie, hearts in the LR group showed no significant difference between the groups (f test).

Results

Acute MI

To characterize the acute myocardial infarcts induced for this study, we intravenously injected rats with the fluorescent indicator DiOCl\textsubscript{3} 24 hours after they were assigned to the PO (n=7) or LR (n=6) group. This enabled us to confirm perfusion of vessels in the infarcted areas of all hearts in the LR group and the absence of perfusion in the infarcted areas of all hearts in the PO group (Figure 1A). The size of the infarcted area expressed as a percentage of the total left ventricular area did not differ between the 2 groups 48 hours post-MI (PO, 41±7.2%; versus LR, 40±5.6%; P=NS) (Figure 1B).

Cardiac Function and Histology During the Chronic Stage of MI

Rats in the PO and LR groups (n=18 each) were followed up 4 weeks post-MI. The survival rate was 100% in both groups at euthanasia. Echocardiography and cardiac catheterization revealed that postinfarction left ventricular dysfunction (both systolic and diastolic) and remodeling were significantly attenuated in the LR group, ie, hearts in the LR group showed greater fractional shortening, a less dilated left ventricular cavity, a thicker ventricular septum, smaller left ventricular diastolic average wall stress, and better ±dP/dt (Figure 2). The left ventricular peak systolic pressure was similar between the groups. Examination at autopsy confirmed the smaller left ventricular cavity in hearts receiving late reperfusion (Figure 3A). The area of the infarct scar as a percentage of the total left ventricular area did not significantly differ between the groups (22.2±3.7% in the PO group versus 23.4±2.6% in the LR group, P=NS). We noted, however,
that the segmental length of the infarct scar was shorter and the scar was thicker in the LR group (Figure 3B).

Histological examination revealed a significantly greater cell population in the infracted areas of LR hearts, which immunohistochemical analysis showed to be made up of CD31-positive endothelial cells and \( \text{HSPC251} \)-positive myofibroblasts (Figure 4). The macrophage population did not differ between the groups. Most myofibroblasts were oriented with their long axis parallel to the coronal plane of the infarct scar.

The surviving cardiomyocytes adjacent to the infarct scar showed degenerative changes, including cytoplasmic vacuol-
Collagen fibers appeared earlier and were stouter in the infarcted tissue of the LR group and were distributed in a reticulated manner among the infiltrated cells (percentage of collagen area: 1.3±0.039% in PO versus 1.9±0.096% in LR 4 days post-MI; \( P<0.05 \)) (supplemental Figure IB). The larger cell population and greater collagen content was observed in the LR group throughout the subacute stages (Figure 5B). These findings suggest that late reperfusion accelerates the post-MI healing process, during which the greater population of the infiltrating cells might play a significant role in synthesizing collagen and scavenging necrotic myocardium.

We then examined cell proliferation and apoptosis, both of which could affect the cell population. The incidence of Ki-67–positive cells was significantly greater in the LR group 4 days post-MI but was found to be similar in the 2 groups, thereafter (Figure 6A). The incidence of TUNEL-positive cells was always greater in the PO group (Figure 6B). Granulation tissue cell apoptosis is reportedly Fas- and caspase-3–dependent,16,17 and our Western blotting showed expression of both Fas and Fas ligand to be upregulated in all hearts 1 week post-MI, but their levels were significantly lower in the LR group than the PO group (supplemental Figure IIA). Furthermore, the degree of caspase-3 activation was significantly lower in the LR group (supplemental Figure IIB). We also found many granulation tissue cells that were positive for both TUNEL and activated caspase-3 in the 2 groups (supplemental Figure IIC), but the incidence was significantly smaller in the LR group. These findings suggest that late reperfusion not only promotes proliferation of

**Figure 4.** Representative histological and immunohistochemical preparations of the infarcted, border, and noninfarcted areas of hearts collected from PO and LR rats 4 weeks post-MI. A and F, Hematoxylin/eosin stain. B, Anti-CD31 immunostain. C, Anti–α-smooth muscle actin immunostain. D, Antimacrophage stain. E, Graphs showing comparisons of the morphometric data from the groups. HPF indicates hours postfertilization. G, Double immunostaining for MHC (red) and laminin (green), followed by nuclear staining with Hoechst (blue). H, Sirius red stain. I, Graph showing a comparison of percentage of collagen in the noninfarcted areas in the groups. Bars=20 μm; \#P<0.05 vs the PO group (t test).
granulation tissue cells but also protects those cells from apoptotic loss, which likely explains the greater abundance of cells in the infarct scars in the LR group during the chronic stage of MI.

The attenuation of infarct expansion suggests late reperfusion mitigates this apoptosis.\(^{11}\) To address that issue, we evaluated apoptosis among cardiomyocytes in the border zone 1 and 4 weeks post-MI using the same histological preparations used to examine the infarct scar. We rarely observed TUNEL-positive cardiomyocytes in the border zone of hearts from either the PO (0.013±0.009% 1 week post-MI and 0.013±0.013% 4 weeks post-MI) or LR (0.008±0.008% 1 week post-MI and 0.005±0.005% 4 weeks post-MI; \(P=\text{NS}\) versus the PO group) group (supplemental Figure IVA). Furthermore, we detected no cardiomyocytes positive for both TUNEL and activated caspase-3 in either group (supplemental Figure IVB). Electron microscopy with TUNEL revealed degenerative, but never apoptotic, cardiomyocytes in both groups, with Immunogold labeling indicating fragmented DNA in the nuclei (supplemental Figure IVC). The nuclei were bizarrely shaped and contained diffusely clumped heterochromatin (“hypertrophied nuclei”).\(^{21}\) Such cardiomyocytes also have been observed in hearts with dilated and reperfusion-reperfusion.

Salvaged Cardiomyocytes After MI

It has been proposed that loss of surviving cardiomyocytes attributable to apoptosis contributes to the progression of postinfarction heart failure\(^{20}\) and, more importantly, that late reperfusion mitigates this apoptosis.\(^{11}\) To address that issue, we evaluated apoptosis among cardiomyocytes in the border zone 1 and 4 weeks post-MI using the same histological preparations used to examine the infarct scar. We rarely observed TUNEL-positive cardiomyocytes in the border zone of hearts from either the PO (0.013±0.009% 1 week post-MI and 0.013±0.013% 4 weeks post-MI) or LR (0.008±0.008% 1 week post-MI and 0.005±0.005% 4 weeks post-MI; \(P=\text{NS}\) versus the PO group) group (supplemental Figure IVA). Furthermore, we detected no cardiomyocytes positive for both TUNEL and activated caspase-3 in either group (supplemental Figure IVB). Electron microscopy with TUNEL revealed degenerative, but never apoptotic, cardiomyocytes in both groups, with Immunogold labeling indicating fragmented DNA in the nuclei (supplemental Figure IVC). The nuclei were bizarrely shaped and contained diffusely clumped heterochromatin (“hypertrophied nuclei”).\(^{21}\) Such cardiomyocytes also have been observed in hearts with dilated and reperfusion.

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hypertrophic cardiomyopathies and in hypertensive hearts and are known to give false-positive TUNEL reactions.\(^{22}\)

Electron microscopic examination of hearts 4 weeks post-MI revealed degenerative, but not apoptotic, changes. These included myofibrillar disorganization and loss, vacuolization, and marked accumulation of mitochondria with deformity or degeneration (mitochondriosis) in cardiomyocytes at the border zone in hearts from both groups, but the changes appeared less severe in the LR group (Figure 7A).

Morphometric analysis at the electron microscopic level revealed that the percentage volume comprised of myofibrils in cardiomyocytes was significantly greater in the LR group (43±1.5\% vs PO group (35±1.5\%, \(P<0.05\)) (Figure 7A), indicating less severe degeneration in cardiomyocytes in the LR group. This finding prompted us to compare expression of GATA-4, a transcription factor that stimulates expression of important sarcomeric proteins, such as MHC and troponin I,\(^{23,24}\) in the 2 groups. We found that immunohistochemical staining for GATA-4 was more intense in the nuclei of cardiomyocytes in the LR group, and Western blotting confirmed greater expression of GATA-4 in the LR group (Figure 7B).

**In Vitro Effects of Hypoxia on Granulation Tissue Cells**

Because we found that late reperfusion suppressed apoptosis among granulation tissue cells, we also tested whether hypoxia exerts a direct effect on the incidence of apoptosis among granulation tissue cells in vitro. We found that culture for 24 hours under hypoxic conditions (1\% O\(_2\)) had no effect on the incidence of apoptosis among infarct tissue-derived myofibroblasts. On the other hand, hypoxia accelerated Fas-mediated apoptosis when the cells were pretreated with Fas ligand plus actinomycin D (Figure 8). There was no difference in the apoptotic responses between myofibroblasts obtained from the infarcted area of a heart in the PO group (Figure 8) and those from a heart in the LR group (data not shown). This is likely because after 24 hours in culture under the same conditions, the phenotypes of the cells were similar.

**Discussion**

**Antiapoptotic and Proliferative Effects of Late Reperfusion on Granulation Tissue Cells**

Increased wall thickness without reduction of infarct scar area is reportedly among the morphological characteristics of postinfarction hearts receiving late reperfusion.\(^{4,5,25,26}\) Our results confirm those findings. On the other hand, the historical characteristics of the thickened wall had not been well described. In the present study, hearts from the LR group showed a greater abundance of cells within the infarct scar than those from the PO group, which likely contributed to the thickening of the infarcted wall in LR hearts. The majority of these cells were myofibroblasts and endothelial cells, which are also the main cellular components of granulation tissue at the subacute stage of MI. That prompted us to compare the cellular dynamics of granulation tissue cells in LR and PO hearts. We found that the proliferation rate was greater during the early subacute stage in the LR group, whereas the
incidence of apoptosis was significantly lower throughout the subacute stages. Moreover, hypoxic conditions directly accelerated Fas-mediated apoptosis in infarct tissue-derived cultured myofibroblasts. Taken together, these findings suggest that late reperfusion stimulated proliferation of granulation tissue cells and protected the cells from apoptosis, enabling them to contribute to the formation of a thick infarct scar that was rich in cellular components. In addition, collagen fibers appeared earlier and were stouter in the infarcted tissue in the LR group, and myocardial debris disappeared earlier. This suggests that late reperfusion accelerated the healing process after MI, perhaps because the larger population of infiltrating cells, is better able to synthesize collagen and scavenge necrotic myocardium.

According to Laplace’s law, wall thickness is a key determinant of left ventricular wall stress, ie, wall stress is proportional to the cavity diameter and intracavity pressure and inversely proportional to the wall thickness. Consequently, wall stress increases as wall thickness decreases and accelerates cavity dilatation, which in turn further increases wall stress (vicious relationship). Because increased wall stress promotes both overload and ischemia in myocardial tissue, a thick infarct scar could be a significant factor, reducing the load on postinfarction hearts.

In addition to the greater scar thickness, the infarct segmental length was shorter in the LR group, which may be attributable to the lower wall stress in this group. On the other hand, we also noted an abundance of myofibroblasts in the infarcted area of LR hearts, and these cells are known to play a key role during wound healing, contracting the wound and producing collagen. Because the long axis of the myofibroblasts was oriented parallel to the coronal plane within the infarct scar, we presume that they contribute to the shrinkage of the infarct in the coronal direction. In addition, the greater number of small vessels may contribute to maintaining the activity of cells in the old infarct tissue.

MMPs are a family of enzymes that catalyze the degradation of extracellular matrix and are believed to play important roles in heart disease. MMPs play a crucial role in the complex interplay between inflammatory and vascular cells, fibroblasts, and cardiomyocytes, which can result in myocardial protection or destruction, depending on the etiology of the cardiac damage. In the present study, downregulation of MMP-2 and MMP-9 was noted in infarcted tissue from the LR group, where fibrosis was less developed and the infarct segment was less elongated. Although their activities remain to be confirmed because the antibodies for MMPs used here detect only their proforms, our findings suggest late reperfusion modulates MMP activation in the infarcted tissue, which could affect the infarct tissue dynamics. Clarification of the roles of MMPs during the healing process after MI, however, awaits further study.

**Late Reperfusion Protects Salvaged Cardiomyocytes From Degenerative Changes but Has No Effect on the Incidence of Cardiomyocyte Apoptosis**

It has been suggested that cardiomyocyte apoptosis contributes to the progression of heart failure stemming from various heart ailments. On the other hand, some investigators, including ourselves, are uncertain about the impact of cardiomyocyte apoptosis in chronic heart failure. This is because, in
contrast to granulation tissue cell apoptosis,15,16 apoptosis among cardiomyocytes appears to be so rare that there is no ultrastructural evidence of its occurrence.32–35 Baldi et al reported seeing apoptosis among salvaged cardiomyocytes during both the subacute and chronic stages of human MI.20 Moreover, the same group reported a lower incidence of such apoptosis in patients with a patent infarct-related artery than in those with an occluded artery and suggested that a reduction in the incidence of apoptosis among salvaged cardiomyocytes was a beneficial effect of late reperfusion.11 By contrast, our present findings do not support the notion that cardiomyocyte apoptosis plays a major role in post-MI hearts. Furthermore, we suggest that the incidence of TUNEL-positive cardiomyocytes reported by Baldi and colleagues is unreasonably high (>35% of salvaged cardiomyocytes at the border zone)11,20, such a high rate of cardiomyocyte loss would totally extinguish the salvaged myocardium within weeks. Evidence indicates that assays for DNA breaks, TUNEL in particular, are unreliable methods for detecting apoptotic cells, if used alone.35 Baldi and colleagues did count TUNEL-positive cardiomyocytes coexpressing caspase-3,11,20 but the antibody they used detects not only cleaved caspase-3 but also its precursor, which is ubiquitously expressed in all cells, making their evaluation of apoptosis no more reliable than if it had been based on TUNEL alone. In the present study, Western blotting clearly showed that the level of activated caspase-3 was indeed higher in the PO group, but the immunolabeling occurred exclusively in nonmyocytes within the injured area, not in salvaged cardiomyocytes in the same histological sections. Most importantly, ultrastructural evidence of cardiomyocyte apoptosis, which is the gold standard for diagnosis,36 is still lacking in actual cases of heart disease.35 By contrast, ultrastructural evidence of apoptosis among granulation tissue cells has been well documented.15,36 Although in a more recent study Abbate et al showed an electron micrograph of a section of infarcted heart containing what they claimed was an apoptotic cardiomyocyte, the cell shown was not apoptotic, nor even a cardiomyocyte.37

Instead of apoptosis, we noted a significant difference in the degree of degenerative changes in salvaged cardiomyocytes in the PO and LR groups. Degenerative changes characterized by myofibrillar loss and increased numbers of subcellular organelles (eg, mitochondria) were conspicuous in cardiomyocytes from PO hearts, whereas those from LR hearts showed significantly fewer degenerative changes. Importantly, expression of GATA-4, a transcription factor stimulating MHC and troponin I expression, was weaker in PO hearts than in LR hearts. Moreover, our in vitro experiment showed that hypoxia directly downregulates GATA-4 expression, leading to lower MHC levels in cultured cardiomyocytes, and that subsequent normoxia restores expression of both GATA-4 and MHC. We therefore suggest that restoration of contractile proteins in cardiomyocytes following reperfusion likely contributed to the improved global left ventricular systolic function seen in the LR group.

Conclusions and Clinical Considerations
Along with the various mechanisms proposed in earlier reports,6–10 the phenotypic changes described here provide novel insight into the pathophysiology underlying the beneficial effects of late reperfusion of the infarct-related artery on cardiac function. Furthermore, our findings suggest that late reperfusion may be most effective if accomplished during the granulation tissue phase after MI, which may justify late reperfusion therapy in clinical settings.

In contrast to earlier findings, however, 2 recent clinical studies from the same authors reported an absence of clinical benefits of late reperfusion in post-MI patients.38,39 A possible explanation for this discrepancy relates to both the magnitude of the acute MI and the heterogeneity of the timing of the late reperfusion in those studies. The time at which reperfusion was accomplished varied from 3 to 28 days after MI in both studies.38,39 Such within-group heterogeneity makes it difficult to resolve differences between groups. Moreover, solid transmural infarcts developing an aneurysm, such as those caused by the experimental protocol in present study, are relatively rare in human MIs, probably because of collateral development. In the case of small and nontransmural MIs, the beneficial effects of late reperfusion may be far smaller, given their mechanisms. For those reasons, clinical trials using more homogenous patient populations would be desirable in the future.

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Disclosures
None.

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**Mechanisms by which Late Coronary Reperfusion Mitigates Postinfarction Cardiac Remodeling**


**Methods**

*Experimental MI.* This study was approved by our Institutional Animal Research Committee. MI was induced in male Wister rats weighing 250 to 300 g (Chubu Kagaku, Nagoya, Japan) by ligating left coronary artery as previously described.\(^1\) In sham-operated animals, the suture was passed around the artery but not tied. Twenty-four hours later, the chests of the surviving rats were reopened, and the rats were randomly assigned to a permanent occlusion (PO) group, in which the coronary artery ligation was maintained, or a late reperfusion (LR) group, in which the ligature was completely removed. The chests were then closed again, and the rats had free access to standard laboratory food and tap water. Rats were sacrificed with overdose of pentobarbital 48 h (PO, n=7 and LR, n=6), 4 days (PO, n=6 and LR, n=6), 1 week (PO, n=7 and LR, n=6), 2 weeks (PO, n=7 and LR, n=7), or 4 weeks (PO, n=18 and LR, n=18) after the first surgery.

*Visualization of Perfused and Anatomical Vessels.* To determine whether the vessels in the infarcted area was perfused, fluorescent dye 3,3-diheptyloxy carbocyanine [DiOC\(_{7}(3)\)] was injected to rats at 48 hours after the onset of MI, as described previously.\(^2\) DiOC\(_{7}(3)\) has been shown to enable visualization of perfused vasculature.
by preferentially staining cells immediately adjacent to perfused vessels. Briefly, the dye was injected intravenously (1.0 mg/kg in 75% DMSO) 1 min before the heart was removed and rapidly frozen. Cryosections were then cut to a thickness of 5 µm, and the DiOC7(3) fluorescence was imaged to map the location of the perfused vessels. Thereafter, the slides were fixed for 5 min in cold acetone, air-dried and immunostained with anti-rat CD31 antibody (Dako) to visualize anatomical vessels. The secondary antibody was Alexa 568 (Molecular Probes). Sections were then counterstained with Hoechst 33342 and observed under a fluorescence microscope (BZ-8000, Keyence).

**Physiological Study.** For echocardiography and cardiac catheterization, animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). Echocardiograms were recorded before treatment and at sacrifice using a Vevo770 echocardiographic system (Visualsonics) equipped with a 30-MHz imaging transducer. Following echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 671, Millar Instrument) that was advanced into the aorta and then into the left ventricle for recording pressure and maximal and minimal dP/dt (±dP/dt). Left ventricular diastolic average wall stress was calculated by the previously reported method.

**Histology.** For histological and immunohistochemical examination, hearts were perfused retrogradely through the aorta with 10% buffered formalin. They were then cut into two transverse slices, and the basal specimens were dehydrated and embedded in paraffin. After deparaffinization, 4-µm-thick sections were stained with hematoxylin-eosin, Masson’s trichrome and Sirius red F3BA (0.1% solution in saturated aqueous picric acid, Aldrich). Morphometric analyses of the infarct area, fibrosis and immunopositive cells were carried out using a multipurpose color image processor (LUZEX F, Nireco). The sizes of the infarcted and fibrotic areas were
measured by searching the entire ventricle and were expressed as percentages of the total left ventricular area.

**Immunohistochemistry.** Sections were incubated with a primary antibody against α-SMA (1A4, 1:200 dilution; DAKO Japan), CD31 (1:500 dilution; DAKO Japan); macrophage antigen (ED-1, 1:100 dilution; Serotec), laminin (1:100 dilution; Progen), Ki-67 (1:15 dilution; DAKO Japan), the active form of caspase-3 (1:100 dilution; Chemicon), matrix metalloproteinase-2 (MMP-2, 1:50 dilution), or MMP-9 (1:50 dilution; both from Daiichi Fine Chemical Co.). A Vectastain Elite ABC system (Vector Laboratories) was then used to label the sections; diaminobenzidine served as the chromogen, and the nuclei were counterstained with hematoxylin. Sections incubated with anti-laminin antibody were labeled with an Alexa 488-conjugated secondary antibody (Molecular Probes) then stained with rhodamin-phalloidin (Molecular Probes), and counterstained with Hoechst 33342.

*In situ* DNA nick end-labeling (TUNEL) assays were carried out with deparaffinized, 4-µm-thick sections using an ApopTag kit (Intergene) according to the supplier’s instructions. For double TUNEL and immunofluorescent labeling of the active form of caspase-3, sections were respectively labeled using Fluorescein-FragEL™ (Oncogene) and a primary antibody against the active form of caspase-3 that was subsequently labeled with an Alexa 568-conjugated secondary antibody. Finally the sections were counterstained with Hoechst 33342.

Quantitative assessments of cell population, vessel population, %α-SMA-positive area, %macrophage population, fibrotic area, %Ki-67-positive cells, and %TUNEL-positive cells were performed on 20 randomly chosen HPF in each section with a LUZEX F multipurpose color image processor (Nireco).

**Electron Microscopy.** Cardiac tissue was quickly cut into 1 mm cubes, immersion
fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) overnight at 4°C, and postfixed in 1% buffered osmium tetroxide. The specimens were then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections (90 nm), double-stained with uranyl acetate and lead citrate, were examined under an electron microscope (H-800, Hitachi). We performed a morphometric analysis under an electron microscope using the method previously described. A uniform sampling of 20 electron micrographs, 10 with myofibrils oriented longitudinally and 10 with myofibrils sectioned transversely, was utilized for the morphometric assay of each group. Five random fields, micrographed at 10,000x from each of 5 tissue blocks were printed at a final magnification of 30,000x and analyzed on composite grids as described previously, to calculate the volume fraction of myofibrils within a cardiomyocyte.

**TUNEL at the Electron Microscopic Level (EM-TUNEL).** EM-TUNEL was carried out as previously described, after which the specimens were observed under a Hitachi 700 electron microscope (Hitachi, Japan).

**Western Blotting.** Proteins (100 µg) extracted from hearts or cultured cells (n=3 to 5 from each group) were subjected to 14% polyacrylamide gel electrophoresis and then transferred onto PVDF membranes. The membranes were then probed using primary antibodies against Fas, Fas ligand (both from BD Biosciences), caspase-3 (Cell Signaling), MMP-2, MMP-9 (both from Daiichi Fine Chemical Co.), GATA-4, myosin heavy chain (MHC) and troponin I (all from Santa Cruz), after which the blots were visualized using chemiluminescence (ELC, Amersham). α-Tubulin (analyzed using an antibody from Santa Cruz) served as the loading control.

**In Vitro Experiments.** We carried out an in vitro study using myofibroblasts, one of
the cell types appearing in granulation tissue, to examine the direct effects of hypoxia on cells within the myocardial infarct. MI was induced in rats, and 7 days later cardiac myofibroblasts were collected from the infarcted areas of hearts in the PO (n=3) and LR (n=3) groups as previously described, with some modification. The cells were then incubated for 24 h under normoxic (95% air-5% CO₂) or hypoxic (a mixture of 1% O₂, 94% N₂, and 5% CO₂) conditions. Hypoxia was introduced by exchanging the medium with ischemia-mimetic solution and placing the dishes in a hypoxia chamber. In some cases, a mixture of Fas ligand (0.1 µg/mL, Calbiochem) and actinomycin D (0.05 µg/mL, Sigma) was applied for 24 h to induce apoptosis under the normoxic or hypoxic conditions.

**MTT Assay.** The survival rate among cultured cells was determined using the MTT (3-[4,5- dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) method.

**Statistical Analysis.** Values are shown as means ± SEM. The significance of differences in the findings was evaluated using t tests or one-way ANOVA followed by the Newman-Keul's multiple comparisons test. Values of p<0.05 were considered significant.
References


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Supplementary Figure I

**Legend for Supplementary Figure I**

Infarcted areas in hearts 4 days post-MI.  (A) Photomicrographs showing necrotic myocardium – i.e., myocardial debris (MD) in the infarcted area in PO and LR hearts.  
Left panels show highly magnified photomicrographs of the boxed area in the right panels.  Masson’s trichrome stain.  Bars, 1 mm.  (B) Collagen fibers in the infarcted area of PO and LR hearts.  Sirius red stain.  Bars, 20 µm.
Supplementary Figure II

**Legend for Supplementary Figure II**

Apoptotic signals in granulation tissue in hearts 1 week post-MI.  (A) Western blotting with densitometric analysis of Fas and Fas ligand in hearts from the PO and LR groups.  (B) Western blotting with densitometric analysis of caspase-3 (inactive and active forms) in hearts from the PO and LR groups.  *p<0.05 vs. the sham operated group;  #p<0.05 vs. the PO group (one-way ANOVA).  (C) Representative immunofluorescence images showing the distribution of TUNEL (green) and the active form of caspase-3 (red) in granulation tissue cells in a heart from a PO rat (left panels).  Nuclei were stained with Hoechst (blue).  The arrows indicate double-positive cells.  Right panels show a negative control specimen in which TdT enzyme and the primary antibody against the active form of caspase-3 were omitted.  Bars, 10 µm.
Supplementary Figure III

Legend for Supplementary Figure III

MMP expression.  (A) Western blotting with densitometric analysis of MMP-2 and MMP-9 in hearts from the PO and LR groups.  *p<0.05 vs. the sham operated group; #p<0.05 vs. the PO group (one-way ANOVA).  (B) Immunohistochemical preparations for MMP-2 and MMP-9 in the infarcted area of PO and LR hearts with 1-week-old MIs. Immunostaining of both MMP-2 and MMP-9 appears to be weaker in the LR preparations. Bars, 20 μm.
Supplementary Figure IV

(A) Graph comparing the incidences of TUNEL positivity in PO and LR hearts. There was no significant difference between the groups at either time point (t test). (B) Immunofluorescence images of salvaged cardiomyocytes showing TUNEL-positivity (green) without immunopositivity for the active form (cleaved) of caspase-3 (red) in a heart from a PO rat. Nuclei were stained with Hoechst (blue). The arrow indicates a

Legend for Supplementary Figure IV

Examination of apoptosis among salvaged cardiomyocytes 1 and 4 weeks post-MI. (A) Graph comparing the incidences of TUNEL positivity in PO and LR hearts. There was no significant difference between the groups at either time point (t test). (B) Immunofluorescence images of salvaged cardiomyocytes showing TUNEL-positivity (green) without immunopositivity for the active form (cleaved) of caspase-3 (red) in a heart from a PO rat. Nuclei were stained with Hoechst (blue). The arrow indicates a
TUNEL-positive, but cleaved caspase-3-negative, cardiomyocyte. Bar, 10 µm. (C) EM-TUNEL of a sham operated (upper) and PO heart 4 weeks post-MI (lower). The cardiomyocyte from the PO heart shows a bizarrely shaped nucleus containing diffusely clumped heterochromatin (so-called hypertrophied nucleus) on which immunogold particles indicating TUNEL positivity are accumulated. Bars, 1 µm. The boxed areas in the left panels are highly magnified in the right panels, where they are lightly printed to highlight the immunogold particles.