Inhibition of Granulation Tissue Cell Apoptosis During the Subacute Stage of Myocardial Infarction Improves Cardiac Remodeling and Dysfunction at the Chronic Stage

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Background—Granulation tissue cells at the subacute stage of myocardial infarction (MI) are eliminated by apoptosis to finally make a scar at the chronic stage. We hypothesized that postinfarct inhibition of apoptosis might preserve myofibroblasts and endothelial cells in granulation and modulate chronic left ventricular (LV) remodeling and heart failure.

Methods and Results—A pancaspase inhibitor, Boc-Asp-fmk (BAF, 10 μmol/kg per day), or vehicle (control) was given to rats with experimental large MI. The treatment was started on the third day after MI and continued until 4-week-old MI. Two weeks later, the apoptosis of granulation tissue cells was significantly reduced and conversely, the cell population was greater in BAF. Twelve weeks later, BAF showed significantly greater survival rates (84% versus 42%) with significantly smaller LV cavity, lower LV end-diastolic pressure and central venous pressure, and higher LV dP/dt, which indicated improvement of LV remodeling and dysfunction. A scar was established in old infarct of control subjects, but in BAF, the infarct wall was thicker because of greater old infarct area, which contained abundant myofibroblasts and vessels. Surprisingly, many of the α-smooth muscle actin–positive myofibroblast-like cells in BAF, making bundles and running parallel to the survived cardiomyocytes, were ultrastructurally mature smooth muscle cells with contractile phenotype. Cardiomyocyte apoptosis in the infarct area was equally rare in each group.

Conclusions—The postinfarct treatment with BAF improved LV remodeling and dysfunction through inhibition of granulation tissue cell apoptosis. These findings imply a new therapeutic strategy against postinfarct heart failure.

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

Large myocardial infarction (MI) causes severe chronic heart failure with left ventricular (LV) remodeling, which is characterized by a ventricular dilation and diminished cardiac performance.1 Chronic heart failure secondary to large MI is one of the most serious and important diseases, and such patients account for approximately half of the candidates for heart transplantation.2 Many factors such as death or hypertrophy of cardiomyocytes, fibrosis, and expression of various cytokines during chronic heart failure are associated with the disease progression,3–6 in addition to the size of acute MI, which is determined within several hours after the attack of MI.7 At present, many researchers believe that cardiomyocyte death caused by apoptosis during chronic heart failure plays an important role in the disease progression.8–10 However, it is mysterious that to our knowledge, there has been no report on the beneficial effect of apoptosis inhibitors on the progression of chronic heart failure. Meanwhile, some researchers including us consider the role of cardiomyocyte apoptosis to be unclear in chronic heart failure because of its rare incidence.11–14 MI tissues transform from dead (infarcted) cardiomyocytes and infiltrated inflammatory cells at the acute stage into scar tissues, with few cell components at the chronic stage through granulation tissue consisting of numerous myofibroblasts with contractile elements and rich vessels at the subacute stage. Previously, we reported that the mechanism of loss of these interstitial noncardiomyocytes in MI tissues is apoptosis.15 Thus, we hypothesized that inhibition of apoptosis at the subacute stage of large MI maintains enhanced granulation tissues and modulate postinfarct ventricular remodeling and dysfunction at the chronic stage.
We report that Boc-Asp-fmk (BAF), a pancaspase inhibitor, intraperitoneally injected during the subacute and early chronic stages of large MI, improves the survival rate and ventricular remodeling at the chronic stage by blocking the effect of granulation tissue cell apoptosis in the rat hearts, in which cardiomyocyte apoptosis is rare. In addition, we found phenotypic alteration of granulation tissue cells into mature smooth muscle cells with contractile phenotype in the extravascular area of old infarct tissues. The new therapeutic concept of inhibition of granulation tissue cell apoptosis but not of cardiomyocyte apoptosis may become one breakthrough in the treatment of patients with chronic heart failure after large MI.

Methods

Experimental MI

We used rats with MI caused by coronary ligation for 60 minutes followed by reperfusion. This model presents a large acute infarct occupying ≥40% of the LV wall and subsequent heart failure.1 Male Wistar rats weighing 250 to 300 g (Chubu Kagaku, Nagoya, Japan) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), intubated, and ventilated with a volume-cycled small-animal respirator. The heart was exposed through a left thoracotomy, and the left coronary artery was occluded with a Prolene 6-0 suture and reperfused after 60 minutes of ligation. Myocardial ischemia was confirmed by regional cyanosis and electrocardiographic change (ST elevation). Reperfusion was confirmed by myocardial blush over the risk area after releasing ligation. In addition, reperfusion arrhythmia was helpful, which usually appeared ∼10 minutes after successful ischemia-reperfusion. Positive end-expiratory pressure was applied to fully inflate the lungs, and the chest was closed in layers. MI was made in 35 rats, in which coronary ligation was confirmed by recording pressures and the maximal rate of the rise in LV pressure (+dP/dtmax). All tracings were recorded on a physiological recorder, and the heart rate was obtained from an arterial pressure tracing.

Histology

After measurements, all surviving rats were killed at 12 weeks after making MI, and the hearts were removed. The hearts were cut into two transverse slices, and the basal specimens were fixed with 10% buffered formalin. They were dehydrated and embedded in paraffin. After deparaffinization, 4-μm-thick sections were stained with hematoxylin-eosin and Masson’s trichrome. Quantitative assessments were performed with the use of a multipurpose color image processor (LUZEX F, Nireco).

In Situ DNA Nick End-Labeling Assay

In situ DNA nick end-labeling (TUNEL) assay was performed in deparaffinized 4-μm-thick sections with an ApopTag kit (Intergene), as we previously reported.15

Electron Microscopy

Two hearts from each group of 2-week MI and 3 from each group of 12-week MI were cannulated through the aorta and perfusion-fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 30 minutes, followed by immersion in the same fixative overnight at 4°C. They were cut into 1-mm cubes and postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanol, and embedded in epoxy resin. Thin sections (80 nm), double-stained with uranyl acetate and lead citrate, were examined under an electron microscope (H-800, Hitachi).

Statistical Analysis

Values are given as mean±SD. Analyses of survival after the third day after MI were carried out by the Kaplan-Meier method with the log-rank Cox-Mantel method. The significance of differences in the findings was evaluated by 1-way ANOVA followed by the Newman-Keuls’ multiple comparison test. A difference of P<0.05 was considered significant.

Results

Blockade of Postinfarct Granulation Tissue Cell Apoptosis by Pancaspase Inhibitor

We first investigated whether administration of a pancaspase inhibitor, BAF, inhibits apoptosis of granulation tissue cells at the subacute stage of MI (2 weeks after infarction) in these animals. Intraperitoneal injection of BAF (10 μmol/kg), once per day from the third to fourteenth days after MI, significantly reduced the incidence of TUNEL-positive cells in the infarct area consisting of granulation tissue (Figure 1, A, B, and D); the apoptotic index in the 2-week-old infarct area of BAF-treated rats was 0.69%, compared with 1.9% for vehicle (DMSO)-treated rats (P<0.05). The noncardiomyocyte population in the infarct area was greater in BAF-treated rats (8909±873 cells/mm²) than in vehicle-treated rats (6219±582 cells/mm²) (Figure 1F). A positive reaction to the

vena cava for recording arterial and central venous pressure, respectively. The aortic catheter was then advanced into the left ventricle for recording pressures and the maximal rate of the rise in LV pressure (+dP/dtmax). All tracings were recorded on a physiological recorder, and the heart rate was obtained from an arterial pressure tracing.

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active form of caspase-3 was observed in the granulation tissue cells, many but not all of which presented apoptotic morphology (Figure 1C), and the incidence of the cells positive for the active form of caspase-3 was significantly reduced in the BAF-treated rats (Figure 1E), confirming the inhibitory effect of BAF on caspase-3 activation. Electron microscopy revealed typical ultrastructural features of apoptosis of capillary endothelial cells, myofibroblasts, and macrophages in granulation tissue of each group (Figure 1, G, H, and I). A smaller incidence of apoptotic cells in BAF-treated rats was also confirmed by electron microscopy. These findings suggest that apoptosis of postinfarct granulation tissue is caspase-3–dependent and that the inhibition results in an increase in the granulation tissue cell population.

In contrast, cardiomyocyte apoptosis was substantially absent in the hearts on the third day of MI and in those with 2-week-old MI in both vehicle-treated and BAF-treated groups in TUNEL-stained preparations and under an electron microscope. Within the infarct area on the third day of MI, surviving cardiomyocytes were rarely observed (Figure 1J). Moreover, the proportion of cardiomyocytes in the infarcted segment was very small and not significantly different between the groups: 0.055±0.034% in the control group and 0.047±0.022% in the BAF-treated hearts (Figure 1, K, and L). These findings clearly deny the presence of ongoing apoptosis of cardiomyocytes during the late acute and subacute stage of MI in the present model and exclude the possible influence of BAF on the apoptosis of the cardiomyocytes left in the infarct area.

Survival Rate and Cardiac Function at the Chronic Stage of MI
Administration of BAF, begun at the third day after infarction and continued for the first 4 weeks, increased the number of surviving rates at 12 weeks after the onset of infarction; the 12-week survival rate of postinfarct rats treated with BAF was 84%, compared with 42% for vehicle-treated rats ($P<0.05$) (Figure 2).

**Figure 1.** BAF significantly prevented caspase-3 activation and apoptosis of granulation tissue cells 2 weeks after MI. Immunohistochemical preparations are shown for TUNEL staining (A and B) and the active form of caspase-3 (C) of granulation tissue of left ventricles from vehicle-treated (A and C) and BAF-treated rats (B). Bar graphs show percentages of the apoptotic index (D) and of caspase-3–positive cells (E) and total cell number (F) of the infarct area of each group. Electron photomicrographs show apoptosis of an endothelial cell (G), myofibroblast (H), and macrophage (I) found in the infarct areas of the vehicle-treated rats 2 weeks after MI (J and K). Immunohistochemistry for myoglobin in tissue sections of hearts on the third day of MI (J) and in BAF-treated hearts with 2-week-old MI is shown (K). Asterisk in J indicates necrotic myocardium; arrows in K indicate surviving cardiomyocytes in infarct area. Bar graph in L shows proportion of surviving cardiomyocytes in the 2-week-old infarct area of each group. Scale bars, 10 μm in A, B, and C; 1 μm in G through I; and 5 μm in J and K.

**Figure 2.** Survival curves of post-MI rats. Treatment was started on third day of MI and continued until 4 weeks after MI.
At 12 weeks after the onset of MI, vehicle-treated rats showed severe LV remodeling with marked enlargement of the LV cavity and thinning of the infarct wall (Figure 3, A and B) and signs of decreased cardiac function (Figure 3C): lower LV fractional shortening (%FS) and lower $\frac{dp}{dt}_{\text{max}}$ (Figure 3D), a higher LV end-diastolic pressure (Figure 3E), and an increased central venous pressure (Figure 3F). Administration of BAF improved each of these conditions, indicating the improvements of postinfarct remodeling and cardiac function. Systemic blood pressure and heart rate were similar between the vehicle-treated and BAF-treated groups. Treatment with the same dose of BAF for 4 weeks in rats did not cause any hemodynamic alteration or morphological change in the hearts, compared with vehicle treatment ($n=5$ each) (data not shown).

Cardiac Pathology at the Chronic Stage of MI

Twelve weeks after operation, the body weight of the BAF-treated rats was greater than that of the vehicle-treated rats (397±8 g versus 367±9 g), whereas heart weight was significantly smaller in the BAF-treated group than in the vehicle-treated group (1.26±0.05 g versus 1.34±0.07 g). Since the sham-operated rats treated with vehicle showed significantly greater body weight (449±10 g) than the rats with MI and subsequent heart failure, the low body weight of the rats with MI may have resulted from cardiac cachexia. As a result, the heart weight–to–body weight ratio was significantly decreased in the BAF-treated rats (Figure 4, A and B, and I). Both the absolute area and the percentage of old infarct size to the whole LV wall was significantly greater in the BAF-treated rats (10.5±1.1 mm² and 30.2±2.1%) compared with those of the vehicle-treated rats (8.4±1.6 mm² and 24.6±5.5%) (Figure 4L). The LV wall with old MI was thicker in BAF-treated rats than in vehicle-treated rats (Figure 4J). The old infarct area of vehicle-treated rats was replaced by fibrous scar tissue containing scanty fibroblasts and vessels (Figure 4, C and E). However, that of BAF-treated rats contained not only collagen fibers and fibroblasts but also abundant extravascular α-smooth muscle actin–positive cells (myofibroblasts) and many small vessels (Figure 4, D, F, K, and N). Noncardiomyocyte cell and vessel populations of the old infarct area were nearly 2-fold and nearly 3-fold greater in BAF-treated rats (5418±745/mm² and 33.6±15.5/mm²) compared with that in vehicle-treated rats (2581±418/mm² and 12.8±6.6/mm²) (Figure 4, C and D). In general, acute infarct size is determined within several hours after reperfusion after ischemia. In the current study, the BAF treatment was started on the third day after the onset of MI. Therefore, the greater size of the old infarct and thickening of the infarct wall segment in the BAF-treated group were due to an increase in the granulation tissue of the infarct by inhibition of apoptosis. Some of the myofibroblasts accumulated and formed bundles running parallel to the surviving cardiomyocytes. These bundles were never observed in the infarct wall of vehicle-treated rats. Endothelial cells were also abundantly preserved in the infarct area of the BAF-treated rat hearts (Figure 4, G and O). However, macrophages, which were one of the major cell components of granulation tissue, were few even in the infarct area of BAF-treated rat hearts, and the incidence was similar to that of vehicle-treated rat hearts (Figure 4, H and P). The size of surviving cardiomyocytes measured as the transverse diameter was significantly greater in the vehicle-treated (23.1±3.1 μm) than in the BAF-treated (19.2±2.7 μm) rats (Figure 4M). On the other hand, there was no special difference in thickness (Figure 4J) and in the degree of fibrosis of the noninfarcted LV wall between the groups. No histological abnormality was found in the extracardiac organs such as lungs, liver, intestines, and kidneys of the BAF-treated rats.
Basic ultrastructural features of 12-week-old infarct areas of the vehicle-treated rat hearts were fibroblasts, scanty small vessels, and few myofibroblasts that were surrounded by massive collagen fibrils. However, those of the BAF-treated rat hearts were abundant with myofibroblasts and small vessels. Inflammatory cells such as macrophages were extremely few in 12-week-old infarct areas of both groups. Surprisingly, light microscopic myofibroblasts with α-smooth muscle actin, which made bundles in the extravascular areas and were arranged parallel to the running of the survived cardiomyocytes around the infarct area of the BAF-treated rats, were (by electron microscopy) mature smooth muscle cells with the contractile phenotype. The cytoplasm of the smooth muscle cells was tightly filled with thin filaments and contained many dense bodies (specific structures for contractile phenotype smooth muscle cells(19)) (Figure 5A). Myofibroblasts are indistinguishable from the synthetic phenotype smooth muscle cells at electron microscopic levels (Figure 5B). Considering this and the time course of the appearance, it is possible that these smooth muscle cells with the contractile phenotype might have been transdifferentiated from the myofibroblasts in the infarct area, although there has been no report on transdifferentiation from cardiac myofibroblasts or synthetic phenotype smooth muscle cells into contractile phenotype smooth muscle cells.

Discussion
There are several possible mechanisms that explain the improvement of postinfarct remodeling and cardiac function by the treatment with BAF. The first is the increase in the α-smooth muscle actin–positive cells, collagen, and small vessels as the result of inhibition of granulation tissue cell apoptosis. Myofibroblasts can generate collagen fibers and have actin filaments as a contractile element. The bundles of the smooth muscle cells with the contractile phenotype, running parallel to surviving cardiomyocytes in the extravascular area, may induce improvements in the contractility. The thickened infarct wall, consisting of the increased cell components and collagen, can improve the infarct wall motion by decreasing wall stress.20 In addition, the increase in small vessels may contribute to maintain the activity of the increased cells in the old infarct tissue. Inhibition of cardiomyocyte apoptosis by BAF is not considered important because of the lack of TUNEL-positive cardiomyocytes even in the vehicle-treated hearts. This seems inconsistent with the recent study by Baldi et al.17 reporting that in human hearts from patients who died 12 to 62 days after MI, high-grade apoptosis of cardiomyocytes was present in the later phases of post–acute MI. However, there are major differences in the methods between that study and ours: (1) humans versus rats; (2) most of the subjects (14 out of 16) in their study had persistently occluded infarct-related arteries, whereas we used the ischemia-reperfusion model. The same group more recently reported significantly reduced cardiomyocyte apoptosis in human hearts with reperfusion21; (3) they used autopsy specimens susceptible to laboratory artifacts, including false-positive TUNEL reaction. Compensatory hypertrophy of cardiomyocytes was independent of the beneficial effects in the BAF-treated group because the cardiomyocyte size was smaller in the BAF-treated hearts than in the vehicle-treated hearts.

Previously, we reported apoptosis of infiltrated leukocytes 2 days after MI and apoptosis of myofibroblasts and vessels 2 weeks after MI.15 In general, infiltrated leukocytes are considered to enhance damage of cardiomyocytes. Therefore, the treatment with BAF was started on the third day and continued until 4 weeks after MI. Interestingly, despite quitting BAF, the α-smooth muscle actin–positive cells and vessels in the infarct area were significantly preserved even at 12 weeks after MI. This suggests that the cells that could have
once escaped from such a temporal proapoptotic environment (granulation tissue as chronic inflammatory focus) by an antiapoptotic treatment (BAF in this study) may keep living even after stopping the treatment. On the other hand, inflammatory cells including lymphocytes and macrophages were not resistant against apoptosis and thus not preserved during the chronic stage of MI in the current study. Inflammatory cells are known to show active proapoptotic interactions through death ligands and receptors. Speculatively, apoptotic mechanisms may be differentially regulated in these cell types that may have higher sensitivity to apoptotic stimuli compared with the other preserved cells.

At present, many patients have large-sized infarcts followed by heart failure despite the reperfusion therapy. The current findings that the temporal treatment by a caspase inhibitor after MI in the reperfused hearts resulted in beneficial effects in cardiac remodeling and dysfunction may imply a novel therapeutic strategy against progressive chronic heart failure subsequent to MI.

Acknowledgments

This study was supported in part by grants-in-aid for scientific research 11670668, 12670704, and 13470143 from the Ministry of Education, Science, and Culture of Japan. We thank Akiko Tsujimoto and the staff of Kyoto Women’s University (Koari Abe, Keiko Uotzu, Kazumi Ohara, Hitomi Takagaki, Machiko Mizutani, and Miyuki Morikawa) for technical assistance.

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Figure 5. Electron photomicrographs of infarct area of 12-week postinfarct rats treated with BAF. Bundles of smooth muscle cells with contractile phenotype (A) were found in extravascular areas and thus were not vascular smooth muscle cells. Right panel is a highly magnified photograph of squared portion of the left panel (A), showing densely packed myofilaments and dense bodies (arrows) in cytoplasm. Myofibroblast (B) is ultrastructurally identical to a synthetic phenotype smooth muscle cell. Inset of A shows high magnification of squared portion of the left panel (B), presenting well-developed subcellular organelae (mitochondria, Golgi apparatus, and endoplasmic reticulum) and scanty myofilaments at the periphery. Scale bars indicate 1 μm.
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_Circulation_. 2003;108:104-109; originally published online June 23, 2003; doi: 10.1161/01.CIR.0000074225.62168.68

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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