Critical Roles for the Fas/Fas Ligand System in Postinfarction Ventricular Remodeling and Heart Failure

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Abstract—In myocardial infarction (MI), granulation tissue cells disappear via apoptosis to complete a final scarring with scanty cells. Blockade of this apoptosis was reported to improve post-MI ventricular remodeling and heart failure. However, the molecular biological mechanisms for the apoptosis are unknown. Fas and Fas ligand were overexpressed in the granulation tissue at the subacute stage of MI (1 week after MI) in mice, where apoptosis frequently occurred. In mice lacking functioning Fas (lpr strain) and in those lacking Fas ligand (gld strain), apoptotic rate of granulation tissue cells was significantly fewer compared with that of genetically controlled mice, and post-MI ventricular remodeling and dysfunction were greatly attenuated. Mice were transfected with adenovirus encoding soluble Fas (sFas), a competitive inhibitor of Fas ligand, on the third day of MI. The treatment resulted in suppression of granulation tissue cell apoptosis and produced a thick, cell-rich infarct scar containing rich vessels and bundles of smooth muscle cells with a contractile phenotype at the chronic stage (4 weeks after MI). This accompanied not only alleviation of heart failure but also survival improvement. However, the sFas gene delivery during scar tissue phase was ineffective, suggesting that beneficial effects of the sFas gene therapy owes to inhibition of granulation tissue cell apoptosis. The Fas/Fas ligand interaction plays a critical role for granulation tissue cell apoptosis after MI. Blockade of this apoptosis by interfering with the Fas/Fas ligand interaction may become one of the therapeutic strategies against chronic heart failure after large MI. (Circ Res. 2004;95:000-000.)

Key Words: apoptosis, gene therapy, heart failure, myocardial infarction, remodeling

Large myocardial infarction (MI) causes severe chronic heart failure with unfavorable remodeling of the left ventricle (LV), which is characterized by a ventricular dilatation and diminished cardiac function.1 The magnitude of acute MI, which is determined within several hours after an attack of MI,2 is the most critical determinant of subsequent heart failure. However, many other factors, such as late death or hypertrophy of cardiomyocytes, fibrosis, and the expression of various cytokines, are associated with the disease progression.3–6 Cardiomyocyte death resulting from apoptosis during chronic heart failure may play an important role in the disease progression,7–9 although its role is still unclear because of a low incidence.10–13 In contrast, nonmyocytes in the infarct area, such as infiltrating inflammatory cells during the acute stage and granulation tissue cells during the subacute stage of MI, die via apoptosis as we reported previously.14 Granulation tissue in particular contains an abundance of neovascularure, myofibroblasts, and macrophages. We reported recently that inhibition of granulation tissue cell apoptosis by use of Boc-Asp-fmk, a pancaspase inhibitor, significantly improved LV remodeling and heart failure at the chronic stage of MI.15 However, the molecular mechanisms of this apoptosis have not been determined, although a dependency on caspases is recognized.

Fas/Fas ligand interaction is an important trigger for apoptosis in many cell types, particularly cells related to the immune system.16 Because MI ensues inflammation and post-MI granulation tissue contains chronic inflammatory cells, we hypothesize that the Fas/Fas ligand system is involved in the apoptosis of granulation tissue cells. In the present study, we first report that the Fas/Fas ligand system is activated in post-MI granulation tissue cells and significantly influences the postinfarct process. Next, we show that inhibition of the Fas/Fas ligand system by delivery of the gene for soluble Fas, a competitive inhibitor of Fas/Fas ligand interaction,17 during the subacute stage of MI could potentially prevent post-MI heart failure at the chronic stage.

Methods

Experimental MI in Mice

The study was approved by our institutional animal research committee. MI was created in male C57BL/6J wild-type mice and...
syngeneic lpr mice and gld mice (Clea Japan; Shizuoka, Japan) at 12 weeks of age by ligating the left coronary artery as described. In sham-operated mice, the suture was passed but not tied. Animals were killed 2 days, 10 days, 4 weeks, or 10 weeks after surgery.

**Recombinant Adenoviral Vectors**
Replication-incompetent adenoviral vector that ubiquitously and strongly expresses a chimeric fusion protein of extracellular region of mouse Fas and the Fc region of human IgG, (mFas-Fc), that is, soluble Fas (sFas), was generated as follows. Adenoviral vector plasmid pAd-sFas, which comprises the cytomegalovirus immediate early enhancer, a modified chicken β-actin promoter and the extracellular region of mouse Fas (sFas) cDNA (Ad.CAG-sFas) was constructed by the in vitro ligation method (gift from Dr Mark A. Kay, Stanford University School of Medicine, California) as described previously. Plasmid pFAS-FcII was generously provided by Dr S. Nagata (Osaka University Graduate School of Medicine, Japan). Control Ad-LacZ was prepared as reported previously.

On day 3 of MI, the sFas gene or LacZ gene was systemically delivered to mice by injection of Ad.CAG-sFas or Ad-LacZ (1×10⁹ plaque-forming units/mouse) into the hindlimb muscles.

**Measurement of the sFas Level in Plasma**
The plasma concentration of sFas was measured by detecting human IgG-Fc using an ELISA kit (Institute of Immunology).

**Physiological Studies**
Echocardiograms were recorded with an echocardiographic system (Aloka) equipped with a 2.5-MHz imaging transducer at 4 or 10 weeks after MI. After cardiac echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 407; Millar Instruments) and advanced into the aorta and then into the LV for recording pressure and ±dP/dt.

**Histological Analysis**
After measurements, hearts were removed and cut into 2 transverse slices, and the basal specimens were fixed with 10% buffered formalin and embedded in paraffin. Sections 4-μm thick were subjected to the assay. Sham-operated hearts 10 days after surgery, hearts with 10-day-old MI, normal thymus, and mammary tissue of mice were used as the positive controls. Sections were observed under a light microscope (H700; Hitachi).

**Statistical Analysis**
Values are shown as mean±SEM. Analyses of survival after the third or tenth day after MI were performed using the Kaplan–Meier method with the log-rank Cox–Mantel method. The significance of differences was evaluated with Student t test, and a difference at *P*<0.05 was considered significant.

**Results**
Expression of Fas and Fas Ligand in Granulation Tissue Cells During MI
We first examined the expression of Fas and Fas ligand in granulation tissue of the heart at the subacute stage of MI (10 days after MI) and 10 days after sham operation; n=5 each). Western blot analysis of the cardiac tissue revealed an augmented expression of Fas and Fas ligand in the heart with MI (Figure 1A). Under a confocal microscope, Fas was identified on the plasma membrane of endothelial cells (Fas positivity 81±2.9% of the endothelial cells), vascular smooth muscle cells (69±2.0%), extravascular myofibroblasts (45±2.9%), macrophages (79±3.2%), and leukocytes (73±3.6%), whereas Fas ligand was found only on the plasma membrane of leukocytes (Fas ligand positivity 21±2.7% of the CD45-positive cells; Figure 1B). Under the present staining conditions, neither Fas nor Fas ligand was detected on the surface of cardiomyocytes, even at the border of the infarct area (Figure 1B).

Apoptosis was detectable by TUNEL assay in noncardiomyocytes of granulation tissue but never in cardiomyocytes. We failed to detect a ladder pattern on DNA gel electrophoresis in the tissue from hearts with 10-day-old MI (data not shown). This failure was compatible with previous reports and was probably attributable to the relatively low incidence of apoptotic cells. Electron microscopy confirmed this finding, being compatible with previous studies.

**In Situ Nick End-Labeling (TUNEL) and DNA Gel Electrophoresis**
The TUNEL assay was performed in sections using an ApopTag kit (Intergene) principally according to the instructions of the supplier. Mammary tissue of mice was used as the positive control.

DNA extraction from cardiac tissue and subsequent electrophoresis were performed as reported previously.

Electron Microscopy
Two to 3 animals in each group were used exclusively for transmission electron microscopic examinations after the hemodynamic examination. After perfusion fixation with phosphate-buffered 2.5% glutaraldehyde, pH 7.4, for 30 minutes, they were immersion-fixed in the same fixative overnight, postfixed with 1% osmium tetroxide for 1 hour, dehydrated through a graded series of ethanol, and embedded in Epon medium. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in an electron microscope (H700; Hitachi).

**Immunohistochemical Analysis**
The sections, 4-μm-thick deparaffinized sections or 8-μm-thick cryosections from the apical half of the ventricle, were incubated with anti-Fas antibody, anti-Fas ligand antibody, anti-Flk-1 antibody (Santa Cruz Biotechnology), anti-α-smooth muscle actin (SMA) antibody (Sigma), anti-CD45 antibody (Pharmingen), antimacrophage antibody (F4/80; Biomedicalcs AG), or anti-cardiac myosin heavy chain antibody (Santa Cruz Biotechnology). The ABC kit (DAKO) was used for the immunostaining of the deparaffinized sections with diaminobenzidine as the chromogen. For immunofluorescence of cryosections, Alexa Fluor 568 and 488 (Molecular Probes) were the secondary antibodies. Nuclei were counterstained with hematoxylin or Hoechst 33342. Sections were observed under a light, or confocal, microscope (LSM510; Zeiss).
Attenuated Postinfarction Heart Failure in Mice with Nonfunctioning Fas and Fas Ligand

MI was induced in animals with a nonfunctioning Fas (lpr strain: fas-/-; n=10), those with a nonfunctioning Fas ligand (gld strain: fas ligand-/-; n=10), and in the syngeneic control mice (C57BL/6J strain; n=10). The lack of Fas and Fas ligand was confirmed, respectively, in the hearts of the lpr strain and the gld strain mice 10 days after MI (data not shown). On the basis of the TUNEL assay, granulation tissue cell apoptosis in surviving mice with 10-day-old MI...
To check the possible difference in magnitude of acute MI between the control and nonfunctioning Fas/Fas ligand mice, we histologically measured the acute infarct size 2 days after MI (n=6 each). There was no difference in the percentage of MI in LV area among the groups (Figure 2C). Also, there was no difference in the degree of acute inflammatory cell infiltration at the periphery of the 2-day-old infarct area (Figure 2C).

**Inhibition of Granulation Tissue Cell Apoptosis by sFas, an Inhibitor of Fas-Mediated Apoptosis**

MI was induced in 12-week-old male C57BL/6J mice, and Ad.CAG-sFas (10⁸ pfu/mouse) was delivered systemically through injection into the hindlimbs on the third day after MI (n=10) when cardiomyocyte necrosis was already completed. The control gene was LacZ cDNA (Ad.CAG-LacZ; n=10).

In the sFas gene–delivered mice, the plasma level of exogenous sFas reached 51.0±11.0 µg/mL and 80.7±4.7 µg/mL, respectively, 3 and 7 days after the injection (6 and 10 days after MI), when the infarct area consisted of granulation tissue (Figure 3A); these levels might be sufficiently high when considering that in humans, the normal level of plasma sFas is ~2 ng/mL.²⁴ However, the exogenous sFas was undetectable in the plasma at 4 weeks after MI. We confirmed expression of exogenous sFas by Western blotting for human IgG in the hindlimb muscles injected with Ad.CAG-sFas 7 days earlier, but it was not detected in those treated with Ad-LacZ (Figure 3B).

The sFas gene treatment significantly reduced the incidence of TUNEL-positive cells in the infarct area consisting of granulation tissue (Figure 3C); the apoptotic index on the basis of TUNEL in the infarct area of the treated mice 10 days after MI was 0.24±0.09% compared with 2.0±0.18% for the control mice. Active forms of caspase-8 and caspase-3 were detected not in the sham-operated mouse hearts but in the hearts with 10-day-old MI. However, these signals were apparently attenuated in the hearts treated with the sFas gene (Figure 3D). The noncardiomyocyte population in the infarct area was significantly greater in the sFas-treated mice (569±67 cells/high-power field [HPF]) than in the LacZ-treated mice (324±19 cells/HPF; Figure 3C). The number of vessels, the %area of myofibroblasts, and the number of macrophages in the infarct was significantly greater in the sFas-treated group than in the LacZ-treated group; vessels (vessels/HPF) 231±19 versus 168±16, P=0.0022; myofibroblasts (%) 31.5±6.4 versus 17.8±2, P=0.0075; and macrophages (cells/HPF) 7.5±0.66 versus 4.5±0.38, P=0.0024. These findings suggest that the inhibition of apoptosis through the blocking Fas/Fas ligand interaction resulted in preservation of the postinfarct granulation tissue cell population.

**Improvement of Postinfarction LV Remodeling and Heart Failure by the sFas Gene Delivery**

The influence of the sFas gene therapy was examined 4 weeks after MI (sFas gene [n=8] and LacZ gene [n=6]). At the chronic stage of MI, the LacZ-treated mice showed severe LV remodeling with a marked LV dilatation accompanying a thin infarct segment and signs of decreased cardiac function:
decreased LV%FS and ±dP/dt; and an increased LV end-diastolic pressure. Gene delivery on the third day of MI resulted in a significant improvement of each of these conditions (Figure 4). Systemic blood pressure and heart rate were similar between the LacZ-treated and sFas-treated groups. Treatment with the sFas gene in normal mice did not cause any hemodynamic alteration or morphological change in the hearts compared with the LacZ treatment (n=5 each; data not shown).

Necropsy of the hearts of mice at 4 weeks after MI revealed a severely dilated LV cavity with a thin infarct wall in the LacZ-treated group. However, this unfavorable LV remodeling appeared attenuated in the sFas-treated group (Figure 5A). The absolute infarct size and proportion of infarct area to total LV area were similar between the LacZ-treated and sFas-treated mice at 4 weeks after MI (Figure 5B). Interestingly, the wall thickness of the infarct segment was greater, whereas the inner circumferential length of the infarct seg-

**Figure 3.** Effects of sFas gene delivery on granulation tissue cell apoptosis during the subacute stage of MI. A, Time course of exogenous sFas levels in the plasma of mice. ● indicates sFas gene-treated mice; ○, LacZ gene-treated mice (n=6 each). *P<0.05 compared with the value for the LacZ gene-treated mice at the corresponding time point. B and C, Photomicrographs of granulation tissue used for TUNEL assays: left, LacZ gene-treated mouse heart; right, sFas gene-treated mouse heart. Arrows indicate TUNEL-positive cells. Bars=10 μm. Graphs showing the apoptotic index on the basis of TUNEL (right) and cell population of granulation tissue (left) of each group. *P<0.05. D, α-tubulin was a loading control.
ment was smaller in the sFas-treated mice (Figure 5B). This indicated that the remodeling of the infarct wall expanding in the coronal directions was significantly suppressed in the sFas-treated mice.

The 4-week-old infarct area of the LacZ-treated mice was replaced by fibrous scar tissue (Figure 6A). However, that of the sFas-treated mice contained not only collagen fibers and fibroblasts but also many small vessels and abundant extravascular α-SMA–positive cells (myofibroblasts). The population of noncardiomyocytes in the old infarct area was significantly greater in the sFas-treated mice (390 ± 9 cells/HPF in the sFas group versus 259 ± 9 cells/HPF in the control) and so was that of vessels (165 ± 7 vessels/HPF in the sFas group versus 114 ± 7 vessels/HPF in the control; Figure 6A). The percent area of extravascular α-SMA–positive cells was significantly greater in the sFas-treated group (18 ± 1.7%) than in the LacZ-treated group (7.6 ± 1.6%). Some α-SMA–positive cells accumulated and formed bundles running parallel to the surviving cardiomyocytes. Such bundles were not observed in the infarct wall of the LacZ-treated mouse. However, macrophages were scarce even in the infarct area of the sFas-treated mouse hearts, and the incidence (1.7 ± 0.62

**Figure 4.** Effects of sFas gene delivery on hearts at the chronic stage of MI (4 weeks after MI). Graphs show anatomical and hemodynamic data obtained from echocardiography and cardiac catheterization. *P < 0.05 compared with LacZ gene-delivered mice. LVEDD indicates LV end-diastolic dimension; LVESD, LV end-systolic dimension.

**Figure 5.** Effects of sFas gene delivery on hearts at the chronic stage of MI (4 weeks after MI). A, Transverse sections of hearts with 4-week-old MI; hematoxylin-eosin stain. Bar = 1 mm. B, Graphs showing the absolute infarct area (mm²); the percentage of the infarct area in total LV area (%); the length of the infarct segment (the inner circumferential length; μm); and the thickness of the infarct wall. *P < 0.05 compared with the LacZ gene-delivered mice.
cells/HPF) was similar to that in the LacZ-treated mice (1.3±0.56 cells/HPF; \(P=0.6964\)). The size of cardiomyocytes in the noninfarct area, which was measured as the transverse diameter, was significantly greater in the LacZ-treated mice (17.7±0.3 \(\mu\)m) than sFas-treated (14.0±0.7 \(\mu\)m) mice, suggesting that the compensatory hypertrophy of cardiomyocytes was more developed in the LacZ-treated mice. There was no special difference in thickness or in the degree of fibrosis of the noninfarct LV wall between the groups. No histological abnormality was found in the extracardiac organs such as lungs, liver, intestines, and kidneys of the sFas-treated mice.

Under an electron microscope, 4-week-old infarct areas of LacZ-treated mouse hearts contained fibroblasts/myofibroblasts (mostly fibroblasts), scanty small vessels, and very few macrophages that were surrounded by massive collagen fibrils, being consistent with a scar tissue. However, those of the sFas-treated hearts contained more abundant cell components. They showed not only numerous fibroblasts/myofibroblasts and small vessels but also mature smooth muscle cells with the contractile phenotype. These smooth muscle cells made bundles in the extravascular areas. The cytoplasms of the smooth muscle cells were tightly filled with thin filaments and contained many dense bodies (Figure 6B). The bundles of such smooth muscle cells were identical to the mass of \(\alpha\)-SMA-positive cells that had appeared under the light microscope.

**Influence of the sFas Gene Delivery on Postinfarction Survival**

Using other litters of mice that were alive on the third day of MI (\(n=40\)), the survival was followed up for a period of 10 weeks. Eighteen mice underwent the sFas gene therapy and 22 the LacZ gene therapy. The survival rate was 55\% in the control and 83\% in the sFas-treated group at 10 weeks after MI (\(P=0.0834\); Figure 7). Although the difference was not significant, it was notable that in case of the sFas treatment, mice were all alive when survived for the first 10 days after MI. Thus, when the survival was evaluated later than 10 days after MI, the survival of...
sFas-treated mice was significantly better than that of the LacZ-treated mice ($P=0.0389$; Figure 7).

The echocardiographic and hemodynamic evaluations of the surviving mice revealed that the beneficial effects of sFas gene delivery on post-MI cardiac function were preserved, even up to 10 weeks after MI (Figure 8). The necropsy study revealed that the greater MI wall thickness and smaller MI segmental length in the sFas-treated group was preserved (Figure 8). These findings indicate that the effect of sFas gene therapy persisted for many weeks, even after the exogenous sFas level had become undetectable.

**Ineffectiveness of the sFas Gene Delivery During the Chronic Stage of MI**

In further experiments, we checked whether inhibition of granulation tissue cell apoptosis is really responsible for the beneficial effects on post-MI heart failure. For this purpose, the sFas gene therapy was started at a more chronic stage of MI when granulation tissue has already disappeared; the sFas or LacZ gene (n=10 each) was delivered to mice with a 6-week-old MI that consisted not of granulation tissue but of scar tissue, and these mice were examined an additional 4 weeks later (10 weeks after MI). This time, we found no difference in functional and pathological parameters between the sFas-treated and LacZ-treated groups (Figure 8). These results clearly indicate that the preventive effect of the sFas gene therapy on heart failure is attributable to inhibition of granulation tissue cell apoptosis.

**Discussion**

In the present study, we suggested a significant role for Fas/Fas ligand interaction in the apoptosis of granulation tissue cells in myocardial infarct areas at the subacute stage of MI. Granulation tissue cells disappear naturally via apoptosis to eventually make a scar tissue. The present study revealed that suppression of granulation tissue cell apoptosis by interfering with the Fas/Fas ligand interaction through sFas gene delivery resulted: anatomically, in attenuation of unfavorable remodeling of the LV; and functionally, in amelioration of cardiac dysfunction at the chronic stage of MI. Post-MI survival during the chronic stage (later than the subacute stage) was also affected by the treatment. The results of the treatment were interesting, especially in terms of cardiac structure at the chronic stage: a thickened infarct wall developed containing abundant cellular components.
such as vessels and α-SMA-positive cells, part of which were found to be contractile phenotype smooth muscle cells under an electron microscope. The following are mechanistic considerations of the beneficial effects of the inhibition of granulation tissue cell apoptosis. First, the influence of infarct tissue geometry may be most important (ie, the shortening of the infarct segment length and increase in the infarct wall thickness). Contraction of the infarct tissue contributes to the suppression of ventricular dilatation. Because wall stress is proportional to the cavity diameter and inversely proportional to the wall thickness (Laplace’s law),26 and because wall stress and ventricular remodeling (dilatation) have a vicious relationship, accelerating each other, it is conceivable that such an alteration of infarct tissue geometry would bring a marked benefit of improving the hemodynamic state. Also, a smaller aneurysm has a lesser effect on cardiac function. Second, bundles of smooth muscle cells with a contractile phenotype in the infarct area, running in parallel with the surviving myocytes, might aid the global contractility of the LV. Third, the preservation of vessels might relieve ischemia in the surviving tissue. On the other hand, inhibition of cardiomyocyte apoptosis was not considered important because of the lack of TUNEL-positive cardiomyocytes during the subacute stage of MI, even in the LacZ-treated hearts. Compensatory hypertrophy of cardiomyocytes was independent of the beneficial effects because the cardiomyocytes were smaller in the sFas-treated than LacZ-treated hearts.

Although we showed the beneficial effect of the inhibition of granulation tissue apoptosis after MI, it should be cautioned that the benefit was evident in cases with large, transmural infarcts; the outcome would be unknown if the therapy were applied to cases with subendocardial infarction. In the present study, we suggested that the apoptosis of each cell type of postinfarction granulation tissue is, at least in part, Fas dependent. However, macrophages continued to die, whereas vascular endothelial cells and myofibroblasts, having escaped a strong proapoptotic environment (granulation tissue as an inflammatory focus) by antiapoptotic treatment (sFas gene therapy), might continue to live until later. Speculatively, macrophages may have a higher sensitivity to apoptotic stimuli compared with the other preserved cells because inflammatory cells generally show very active pro-apoptotic interactions through death ligands and receptors.16

Fas was not immunohistochemically detected in cardiomyocytes under the present staining conditions. However, immunohistochemical negativity does not always deny the slight expression of an antibody because the sensitivity depends on the staining conditions. Several previous reports have shown immunohistochemically Fas expression in the cardiomyocytes of rats27,28 and of humans.29 Thus, it may be possible that our immunostaining method for Fas was less sensitive compared with those used in the previous studies. Notwithstanding, we detected Fas expression in the granulation tissue cells. This fact indicates that Fas expression in granulation tissue cells is definitely stronger than that in cardiomyocytes and suggests that the role of the Fas/Fas interaction in granulation tissue cells may be more significant than that in cardiomyocytes.

Postinfarct heart failure affects nearly half of all candidates for cardiac transplantation30 and is one of the most serious clinical problems to be overcome in cardiovascular medicine. Recently, we reported that the inhibition of granulation tissue cell apoptosis by a pancaspase inhibitor after MI had beneficial effects on cardiac remodeling and dysfunction at the chronic stage of MI. The present study confirmed this therapeutic concept. However, because most of the apoptosis in a physiological setting is considered caspase dependent,31 the systemic suppression of caspases may potentially have unfavorable effects on healthy organs. Actually, caspase 3-deficient homozygous mice undergo embryonic death.32 On the basis of these facts, inhibition of the Fas/Fas ligand interaction may be a more specific way to apoptosis inhibition than inhibition of caspases. Our findings may warrant a therapeutic trial against postinfarction heart failure, which could be performed even during the subacute stage of MI in patients who have a large MI because the chance of reperfusion therapy during the acute stage has been lost. Thus, we expect the “inhibition of granulation tissue cell apoptosis” to become a novel therapeutic regimen that is prophylactic against chronic heart failure after large MI.

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