Sensitivity to Apoptosis Signal, Clearance Rate, and Ultrastructure of Fas Ligand–Induced Apoptosis in In Vivo Adult Cardiac Cells

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Background—Sensitivity to apoptotic signals, the clearance rate of apoptosis, and the apoptotic ultrastructure have not been studied in cells of the in vivo adult heart.

Methods and Results—To minimize the systemic influence, soluble Fas ligand was injected directly into in vivo rat hearts and livers (as the control) at concentrations of 0, 0.5, 2, and 5 μg/mL (groups C, F0.5, F2, and F5). Apoptotic cardiomyocytes and apoptotic noncardiomyocytes of the heart were identified with similar incidences only in F5. Their incidences peaked at 12 hours after injection (2.0±0.09% in cardiomyocytes) and diminished markedly 24 hours later. Caspase-3 was activated only in F5. Boc-Asp-fmk, a pancaspase inhibitor, inhibited apoptosis, suggesting that the apoptosis sensitivity was regulated upstream of caspase-3. Apoptotic noncardiomyocytes showed typical ultrastructure. In addition to the typical ultrastructure, such as cellular shrinkage, chromatin condensation, and apoptotic bodies, however, apoptotic cardiomyocytes showed unique features: doughnut-like, but not half-moon– or crescent-like, chromatin condensation; frequent plasma membrane rupture even during the early stage; condensed mitochondria with wrinkled cristae inside; the appearance of cytoplasmic lipid-like droplets; and myofibrillar derangement. In the livers, typical apoptosis was induced in hepatocytes and nonhepatocytes of the liver even in the F0.5 group, which were cleared 24 hours later.

Conclusions—Compared with liver cells, cardiomyocytes as well as noncardiomyocytes of the heart are more resistant against the apoptotic signal, but the clearance is similarly rapid (within 24 hours). The ultrastructure of apoptotic cardiomyocytes is unique. These findings provide new insights into the dynamics of cell death in the heart. (Circulation. 2002;105:3039-3045.)

Key Words: apoptosis • myocytes • ligands

The incidence of cardiomyocyte apoptosis is very low in in vivo adult hearts of animals and humans with congestive heart failure and hypertrophy, despite the diffuse expression of various apoptotic signals, such as upregulation of Fas, an increase in the Bax/Bcl-2 ratio, cytochrome c release from mitochondria, etc.1–3 Thus, we suggest that (1) cardiomyocytes may be resistant to apoptosis signals and apoptosis may be induced only when the degree of the signals overcomes the high threshold or that (2) the clearance rate of apoptotic cardiomyocytes may be too rapid in the in vivo beating heart to be easily detected despite their considerable occurrence. At present, however, there is no evidence on this issue. In addition, the very low incidence of apoptosis makes it impossible to precisely analyze the ultrastructure of in vivo cardiomyocyte apoptosis, although we recently reported ultrastructural features of apoptosis in cultured rat adult cardiomyocytes.

Fas (APO-1/CD95) stimulation is one of the most frequently investigated systems inducing typical apoptosis in various cell types, eg, lymphocytes and hepatocytes.4 Systemic injection of the Fas stimulator induces apoptosis in hepatic cells, but not in cardiac cells.5,6 The systemic in vivo injection of higher doses is limited because of its lethal effect through severe liver damage. Thus, we hypothesized that a direct injection of the Fas stimulator into a part of the heart and liver might allow treatment with higher doses without significant effects on the whole heart and liver and that a very high dose of the Fas stimulator would induce both cardiac and hepatic cell apoptosis focally in the injected areas, unlike the case of the usual dose, which induces only hepatic cell apoptosis.

The purpose of the present study was to delineate sensitivity to the apoptotic signal and the clearance rate and
 ultrastructure of apoptosis in in vivo adult cardiomyocytes and interstitial cells of the heart in comparison with those of hepatocytes and interstitial cells of the liver by use of a direct injection method of soluble Fas ligand (sFIL) into adult rat hearts and livers.

Methods

Operative Procedure
Male Wistar rats (Chubu Kagaku, Nagoya, Japan) weighing 240 to 260 g were anesthetized with sodium pentobarbital (50 mg/kg IP), intubated, and ventilated with a volume-cycled small-animal respirator. The chest was opened via a left thoracotomy, the pericardium was opened, and the heart was exposed. Direct intramuscular injection of sFIL (Oncogene) at 3 different concentrations was performed on the anterior left ventricular wall of the rat heart with a 27-gauge needle: group F0.5 with 0.5 μg/mL, group F2 with 2 μg/mL, and group F5 with 5 μg/mL sFIL. Then the chest was closed. The animals were killed 1, 3, 6, 12, 24, or 48 hours later (n = 8 each).

We also had a solvent injection group (group C, n = 8) and a group with sFIL at a dose of 5 μg/mL plus a pancaspase inhibitor, Boc-Asp-fmk (BAF, Enzyme Systems Products), at a dose of 2 mmol/L (group BAF, n = 8). The hearts were removed, and 5 hearts from each group were divided into 2 portions: the specimens were fixed with 10% buffered formalin for histology, terminal dUTP nick end-labeling (TUNEL) assay, Taq polymerase–based in situ ligation assay (Taq assay), and immunohistochemistry and were immediately frozen for DNA and protein extraction. The other 3 hearts from each group were used for transmission electron microscopy after perfusion fixation.

The liver is considered more sensitive to Fas-induced apoptosis than the heart. Thus, in a separate experiment using anesthetized rats, we performed a direct injection of sFIL into the rat liver via a 27-gauge needle. The concentrations of the reagents were as in the heart: normal dose 0.5, high dose 2, and very high dose 5 μg/mL. After 3, 6, 12, or 24 hours, the liver tissue was processed for light and electron microscopy. For controls, the liver was injected with a solvent.

TUNEL Assay
TUNEL assay was performed in deparaffinized sections (4 μm thick) with an ApopTag kit (Intergene) according to the supplier’s instructions. Aminoethylcarbazole was used as a chromogen. Sections were counterstained with hematoxylin. Prostate tissue from a rabbit castrated 2 days before the study was the positive control for the TUNEL reaction. Forty areas at a high magnification (400) were randomly chosen in the anterior wall of the left ventricle of each heart where vehicle or sFIL was injected, and the percentages of TUNEL-positive cells were determined per section by 2 persons blinded to treatments.

α-Sarcomeric Actin Immunohistochemistry
Combined With TUNEL
Sections of heart tissue were stained first with TUNEL as described above, but the chromogen was diaminobenzidine substrate. They were subjected to a second immunohistochemistry with Vectastain Elite ABC system (Vector Laboratories) with a second primary antibody against α-sarcomeric actin (αSr-1, diluted at 1:50, Dako Japan) and visualized with VIP substrate (Vector).

Taq Assay
The deparaffinized 4-μm-thick sections of heart tissue were subjected to Taq assay using the DNA fragments labeled with digoxigenin according to a method previously reported.

DNA Extraction and Electrophoresis
DNA was extracted from frozen samples of heart tissue and electrophoresed as previously described.

Caspase-3 Activity
Activity of caspase-3 was detected with the caspase-3 colorimetric protease assay kit (MBL).

Subsequent Immunohistochemical Staining for Proliferating Cell Nuclear Antigen
The TUNEL-stained sections were subsequently stained for proliferating cell nuclear antigen (PCNA). The method was based principally on that previously reported by Kockx et al.

Figure 1. Light photomicrographs of rat hearts (A1 and A2) and livers (B1 and B2) stained with hematoxylin-eosin. Magnification ×400. A1, Control heart from group C. A2, Heart from group F5 12 hours after sFIL injection. Pyknotic nuclei and nuclear fragmentation are evident in some cardiomyocytes and noncardiomyocytes in group F5 (arrows). B1, Control liver tissue. B2, Liver sample from a rat treated with 0.5 μg/mL sFIL. Apoptotic hepatic cells accompanied by pyknotic and fragmented nuclei are abundant (arrows).

Figure 2. Apoptotic indices of cardiomyocytes (A1), cardiac interstitial cells (A2), hepatic parenchymal cells (B1), and hepatic interstitial cells (B2). *Significant difference at P<0.05 vs corresponding control values.
Transmission Electron Microscopy

The heart was cannulated through the aorta with a polyethylene tube and perfusion-fixed at a pressure of 80 mm Hg for 30 minutes with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Tissue samples were then cut into 1-mm cubes and fixed again overnight at 4°C in the fixative. They were postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanol, and embedded in epoxy resin. Thin sections (80 nm) were examined under an electron microscope (H-700, Hitachi).

Statistical Analysis

Values are shown as mean±SD. The significance of differences in the findings was evaluated by 1-way ANOVA followed by the

| Characteristics of the Apoptotic Ultrastructure of In Vivo Adult Cardiomyocytes Compared With Other Cell Types |
|-------------------------------------------------|-----------------|-----------------|
|                                                  | Heart           | Heart           |
|                                                  | Cardiomyocytes  | Interstitial Cells |
|                                                  | Hepatocytes     | Interstitial Cells |
| Condensed nuclear chromatin                      |                 |                 |
| Half-moon- or crescent-like                      | –               | +               |
| Doughnut-like                                    | +               | –               |
| Nuclear fragmentation                             | +               | +               |
| Cellular shrinkage                                | +               | –               |
| Rupture of plasma membrane                       | + + +           | ±               |
| Mitochondrial condensation with wrinkled cristae | +               | –               |
| Cytoplasmic lipid-like droplets                  | +               | –               |
| Myofibrillar derangement                          | + NA            | NA              |
| Cellular fragmentation (apoptotic body formation) | +               | +               |

NA indicates not applicable.
According to the double immunohistochemistry, the TUNEL-positive cells in group F5 consisted of not only myocytes but also nonmyocytes (Figures 2 and 3). Apoptotic indexes of myocytes and those of nonmyocytes based on TUNEL appeared at 3 hours (0.062±0.006% and 0.067±0.006%) and peaked at 6 hours (1.5±0.011% and 1.9±0.22%) and 12 hours (2.0±0.09% and 3.2±0.11%) after injection, whereas most of them disappeared at 24 hours (0.10±0.008% and 0.21±0.006%), and no positive cells were seen 48 hours later. BAF treatment significantly reduced TUNEL positivity (0.017±0.003% in cardiomyocytes at 12 hours). Cells positive for in situ ligation assay were observed in group F5 in similar proportions but were not seen in the other groups (Figure 3). A clear ladder pattern of DNA was confirmed on gel electrophoresis only in group F5 but was not observed in other groups (Figure 3). In group F5, the ladder was seen only at 6 and 12 hours after injection. Combined treatment with BAF (group BAF) suppressed the ladder. Apoptosis of hepatic parenchymal cells was induced by all doses of sFL in similar proportions of nearly 20% as early as 3 hours after injection. It was similarly observed 6 and 12 hours later but disappeared 24 hours later (Figure 2). A significantly higher incidence of interstitial cell apoptosis of myocardium. Bars=1 μm. A, Normal cardiomyocyte of group C, containing a normal nucleus (N). B through D, Apoptotic nuclei observed in cardiomyocytes of group F5. Nucleus of B shows glossy and well-demarcated condensation of chromatin with a doughnut-like shape. Nucleus of C not only has condensed chromatin but also shows conspicuous deformity. In D, nuclear fragmentation (arrows) is apparent. E and F, Budding and apoptotic body formation in cardiomyocytes of group F5. Arrowheads surround structures of interest. G, Fragments of degenerated cardiomyocytes are being phagocytized by a macrophage (Mφ), seen 24 hours later in group F5. Inset, upper right, highly magnified view of boxed area to show myofibrils (Mf).
the liver (1.8±0.12%) was observed only with the very high dose of sFL 6 hours later. At 12 hours later, however, it was induced by all doses of sFL and then disappeared 24 hours later (Figure 2). The incidence was far less frequent than that of parenchymal cell apoptosis.

Caspase-3 Activation
There was a significant increase in caspase-3 activity in the heart 6 hours after Fas stimulation that peaked at 12 hours (Figure 3D). The activity (in milli–optical density/mg protein) was 445±32 at 6 hours and 1083±156 at 12 hours; the baseline value at 0 hours was 221±74. There was no significant increase in activity in the other groups.

PCNA Staining
All TUNEL-positive cardiomyocytes in group F5 were negative for PCNA (Figure 3E).

Electron Microscopic Findings
In general, the modes of cell death are ultrastructurally classified into 4 forms.11 First, oncosis (necrosis) is defined as cell death with ruptured plasma membrane, cytoplasmic swelling, swollen mitochondria with amorphous dense bodies, and clumped chromatin in the nucleus. Second, typical apoptosis without degeneration shows cytoplasmic shrinkage; nuclear chromatin with well-defined, extensive, and glossy condensation; fragmented nuclei; budding or blebbing on the cell surface; apoptotic bodies; and unruptured plasma membrane. Third, apoptosis with secondary degeneration is indicated by cell death with apoptotic nuclear condensation but with ruptured plasma membrane. The fourth is severely degenerated cells in which differentiation between oncosis and apoptosis is impossible.

Features of apoptotic ultrastructure are summarized in the Table. Electron microscopy demonstrated cardiomyocytes with the features of typical apoptosis in the group F5 hearts at 6 and 12 hours after sFL injection (Figures 4 and 5). Condensed chromatin, however, showed a doughnut-like (Figures 4 and 5) but not half-moon– or horseshoe-like shape as seen in cultured neonatal cardiomyocytes8 or other cell types such as hepatocytes and interstitial cells of the heart and liver. Apoptotic cardiomyocytes contained abundant lipid-like structures in the cytoplasm, and many of the mitochondria were markedly condensed, with wrinkled cristae inside (Figures 4C and 4C'). Ultrastructural changes of apoptotic mitochondria were in contrast to those of mitochondria of oncocytic cardiomyocytes, which are known to be electron-lucent and swollen and contain flocculent dense bodies.12 Apoptotic cardiomyocytes with secondary degeneration were also seen, however (Figure 4). In group F5 at 6 hours, the percentages of apoptotic cardiomyocytes without and with secondary degeneration and oncocytic cardiomyocytes were 0.40±0.20%, 1.8±0.8%, and 0.73±0.12%, respectively. Severely degenerated cardiomyocytes were observed 24 and 48 hours later, which were being phagocytized by macrophages and lymphocytes (Figure 5G). Apoptotic figures were also observed in nonmyocytes, such as capillary endothelial cells, pericytes, and fibroblasts (Figure 4B). The myocardium of groups C, F0.5, F2, and BAF did not contain cells with apoptotic ultrastructure.

Apoptosis was observed in the liver tissue treated with sFL (Figure 6). The apoptotic nuclei showed typical half-moon– or horseshoe-like shapes. Many apoptotic cells, however, were affected by various grades of secondary degenerative changes. Their plasma membranes were ruptured, and in severe cases, nuclear lysis had occurred.
Discussion

Methodological Considerations

An agonistic Fas antibody, an inducer of apoptosis, at a dose of 10 μg induces apoptosis in the liver when systemically injected into 5-week-old BALB/c mice (body weight, 14 to 19 g) but not in the heart.5,6 This dosage corresponds approximately to the concentration of 0.5 μg/mL of sFL at the molecular concentration, because the molecular weight is 45 kDa for the Fas antibody and 35 kDa for sFL. Thus, we tried direct intramuscular injection of sFL at usual (0.5 μg/mL), high (2 μg/mL), and very high (5 μg/mL) doses to rat hearts and livers. The present findings confirmed that apoptosis was induced in liver cells treated with the usual dose.

Previously, we reported that a positive TUNEL reaction does not always mean apoptosis, because some living hypertrophied cardiomyocytes with bizarrely shaped nuclei and a positive PCNA reaction show a positive TUNEL reaction in hypertrophied hearts.7 In the present study, because we used hearts without hypertrophy, we did not observe the hypertrophied cardiomyocytes with bizarrely shaped nuclei or PCNA-positive cardiomyocytes. In addition, only tissues obtained from hearts containing TUNEL-positive cardiomyocytes showed a positive reaction to the in situ ligation assay, a positive DNA ladder, and apoptotic ultrastructure. The incidence was similar between TUNEL, in situ ligation, and apoptosis was induced in liver cells treated with the usual dose.

Low Sensitivity of Cardiomyocytes and Noncardiomyocytes to Apoptotic Signal

The present study revealed that apoptosis was induced in hepatocytes and nonhepatocytes of the liver when the usual dose of sFL was used, but only the very high dose induced apoptosis in cardiomyocytes and noncardiomyocytes of the heart. This suggests that resistance against the Fas signal is observed not only in cardiomyocytes but also in noncardiomyocytes of the heart. This finding of cardiomyocytes supports previous studies that reported that cardiomyocyte apoptosis is rare even in hypertrophic failing hearts. We previously reported, however, that infiltrated and proliferated noncardiomyocytes appearing in the infarct area after myocardial infarction easily undergo apoptosis (are highly sensitive to apoptosis) and are cleared via apoptosis to make a scar.13 Apoptosis of such noncardiomyocytes after myocardial infarction might be regulated differently from that of the resident noncardiomyocytes of the normal heart of the present study. The extracardiac origin of some of those regenerated noncardiomyocytes, such as endothelial cells, after infarction as reported recently might support this notion.14

In the heart, caspase-3 was activated only by the very high dose of sFL (group F5), and BAF, a pancaspase inhibitor, prevented its activation and apoptosis. This suggests that the site determining the low sensitivity of cardiac cells to Fas stimulation is present upstream of caspase-3.

Clearance Rate of Apoptosis of Cardiomyocytes and Noncardiomyocytes of the Heart

The present study revealed that the time interval between Fas stimulation and the appearance of apoptosis was 3 to 6 hours in cardiomyocytes and noncardiomyocytes of the heart, 3 hours in hepatocytes, and 6 to 12 hours in nonhepatocytes of the liver. In each cell type, however, the incidence of apoptosis suggested by DNA fragmentation and apoptotic morphology peaked 12 hours after Fas stimulation and diminished markedly 24 hours later.

Apoptotic DNA fragmentation occurs by activation of DNase, such as caspase-3–dependent DNase, via caspase-3 activation and apoptotic morphology via activated proteases, such as apoptotic chromatin condensation inducer, in the nucleus (Acinus) via caspase-3 activation.15,16 The time interval from the initial Fas stimulation to the appearance of apoptosis (DNA fragmentation and apoptotic morphology) depends on the organs and/or cell types, whereas the time interval from the appearance to the disappearance of apoptotic cells may be constant and short within 24 hours, independent of organs and cell types.

Ultrastructural Features of Apoptosis of Cardiomyocytes and Cardiac Interstitial Cells

Although the present study showed typical and secondarily degenerated apoptotic ultrastructure of in vivo adult cardiomyocytes that are common to those seen in other cell types,11 we also observed different findings from other cell types, as summarized in the Table. These ultrastructures were strikingly similar to those of in vitro adult cardiomyocytes reported previously.17 Secondary degeneration with plasma membrane rupture was more conspicuous, however, in apoptotic in vivo adult cardiomyocytes than in those seen in apoptotic in vitro cardiomyocytes. This phenomenon may reflect the specific characters of cardiomyocytes, which continually contract and relax. Fas stimulation, however, caused a similar finding (conspicuous plasma membrane rupture) in hepatocytes. Further studies are needed to determine which factors contribute to massive postapoptotic secondary necrosis.

Clinical Implications

The present study revealed the low sensitivity of in vivo adult cardiomyocytes to the apoptotic signal and its rapid clearance (within 24 hours). It is generally considered that proliferation is rare in cardiomyocytes of hearts with chronic heart failure, although the presence of cardiomyocyte proliferation was recently suggested in hearts after myocardial infarction.18 Instead, compensatory hypertrophy of cardiomyocytes is observed in hearts with abnormal cardiac function. Therefore, the low sensitivity of cardiomyocytes to the apoptotic signal observed in the present study would be one protective mechanism to preserve the number of cardiomyocytes as the cell type with no or rare proliferation.

The incidence of apoptotic cardiomyocytes is very low in hearts with chronic heart failure.1 The present study has shown that the clearance rate of apoptotic cardiomyocytes is <24 hours (1 day). Therefore, despite the very low incidence of cardiomyocytes resulting from low sensitivity and rapid clearance, apoptosis may make a significant contribution to the progression of chronic heart failure.
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