Progressive left ventricular remodeling and apoptosis late after myocardial infarction in mouse heart

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Received 12 August 1999; accepted in final form 11 January 2000.

AFTER MYOCARDIAL INFARCTION (MI) there is progressive myocardial remodeling characterized by left ventricular (LV) dilation, contractile dysfunction (30, 33), myocyte hypertrophy (19), and altered expression of contractile (39), calcium-handling (37), and extracellular matrix proteins (38). In mice (4, 25), there is evidence that apoptosis of cardiac myocytes occurs transiently during the first several days after MI in the ischemic area but not in myocardium remote from the area of ischemic injury. However, apoptosis of cardiac myocytes has been observed in LV myocardium from patients with chronic cardiomyopathy late after MI (28), leading to the thesis that myocyte apoptosis contributes to the chronic progression of myocardial failure (2).

Therefore, an important unresolved question is whether myocyte apoptosis occurs late (i.e., months) after MI in tissue remote from the ischemic injury. Several factors that may be present in remodeling myocardium (6, 12, 17, 23, 40) have been shown to stimulate myocyte apoptosis in vitro, including increased mechanical strain (11), neurohormones (13), reactive oxygen species (1, 35), and inflammatory cytokines (21). Because these potential stimuli for apoptosis are not restricted to the infarct, per se, it is possible that late remodeling is associated with apoptosis in myocardium remote from the area of ischemic damage.

The goal of this study was to test the hypothesis that LV remodeling late after MI is associated with apoptosis in myocardium remote from the infarct and, if so, to determine the temporal course of apoptosis and its relationship to the progression of structural and functional remodeling. Accordingly, we determined LV dilation and maximal LV contractile function using an isovolumic balloon-in-LV Langendorff technique and measured myocyte apoptosis by terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) in hearts from mice 1, 4, and 6 mo after coronary artery ligation.

METHODS

Myocardial infarction. MI was induced in male CD-1 mice (Charles River) by ligation of the left coronary artery as previously described (15, 26). The mice were 8–10 wk old and weighed 30–32 g. The animals were killed and studied at 1, 4, and 6 mo after MI. The protocol was approved by the Institutional Animal Care and Use committee at Boston University School of Medicine.

Langendorff preparation. LV volume and function were determined using an isolated, isovolumic red cell-perfused Langendorff heart preparation as previously described (15). After 20 min of baseline perfusion at a systolic pressure of 80–100 mmHg and a pacing rate of 7 Hz (420 beats/min), the intracoronary balloon volume was set to the lowest volume at which minimal LV pressure tracings could be recorded. This volume (Vₒ) was defined as the “zero” volume. Systolic and diastolic pressure-volume relationships were established by increasing the LV balloon volume in 5-μL increments using...
an air-tight Hamilton syringe. LV functional measurements were obtained 1–2 min after each increment in volume when a new steady state was reached. The LV volume was increased up to a value (V_{max}) at which peak LV developed pressure (LVDP) was reached and a further increase in balloon volume led to a decrease in peak LVDP.

After pressure-volume curves were obtained, the heart was perfused with saline for a few minutes to clear red cells from the LV drain. With the LV balloon volume set to an end-diastolic pressure of 5 mmHg, the heart was arrested in diastole with KCl and perfusion-fixed with 10% buffered Formalin at a coronary perfusion pressure of 80 mmHg. After 3 days of fixation, the balloon was removed, excess Formalin was blotted, and the heart was weighed.

**Infarct size.** After fixation, the LV was embedded in paraffin, and sections were taken at the apex, midcavity, and base and stained with trichrome Masson. Infarct size was measured using the ApoAlert CPP32/Caspase-3 assay kit (Clontech). Samples of ventricular myocardium remote from the infarct was measured in these sections by planimetry from digital images taken at low magnification using SigmaScan Pro image analysis software (version 4.0; Jandel Scientific, San Rafael, CA). The number of TUNEL-positive myocyte nuclei in the myocardium remote from the infarct was counted manually in three sections (apex, midcavity, base). Only TUNEL-positive nuclei that appeared to be located in cardiac myocytes were counted. The total area of myocardium remote from the infarct was measured in these sections by planimetry from digital images taken at low magnification using SigmaScan Pro image analysis software (version 4.0; Jandel Scientific, San Rafael, CA). The number of nuclei per unit area was expressed as the weight of the infarct/peri-infarct sample to the whole LV (i.e., before removal of the infarct). Only samples from hearts with infarcts ≥30% were used. mRNA was extracted and subjected to Northern hybridization as previously described (8) using a full-length cDNA for rat prepro-Atrial natriuretic peptide (ANP). Blots were exposed and developed with an oligonucleotide complementary to 18S rRNA (8).

**Northern hybridization.** Mice were killed at 1, 4, and 6 mo after MI, the atria and right ventricle were removed, and the LV was weighed. The infarct was carefully dissected along with a 0.5- to 1-mm rim of normal-appearing tissue (peri-infarct region) to yield samples referred to as "infarct/peri-infarct" and "remote," which were weighed and rapidly frozen in liquid nitrogen. Infarct size for these hearts was expressed as the weight ratio of the infarct/peri-infarct sample to the whole LV (i.e., before removal of the infarct). Only samples from hearts with infarcts ≥30% were used. mRNA was extracted and subjected to Northern hybridization as previously described (8) using a full-length cDNA for rat preproatrial natriuretic peptide (ANP). Blots were exposed to X-ray film and quantified by laser densitometry using Molecular Analyst software (Bio-Rad, Hercules, CA). mRNA levels were normalized by reprobing with an oligonucleotide complementary to 18S rRNA (8).

**Table 1. Heart and body weights**

<table>
<thead>
<tr>
<th></th>
<th>1 Mo</th>
<th></th>
<th>4 Mo</th>
<th></th>
<th>6 Mo</th>
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<tr>
<td>Heart weight, mg</td>
<td>18.0 ± 0.01</td>
<td>23.5 ± 1.6</td>
<td>23.9 ± 0.7</td>
<td>27.0 ± 1.3</td>
<td>24.6 ± 6.5</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>40.8 ± 0.6</td>
<td>40.7 ± 1.0</td>
<td>49.1 ± 1.9</td>
<td>46.5 ± 1.0</td>
<td>47.7 ± 1.4</td>
</tr>
<tr>
<td>Heart/body weight, mg/g</td>
<td>4.40 ± 0.1</td>
<td>5.79 ± 0.5</td>
<td>4.89 ± 0.21</td>
<td>5.82 ± 0.29</td>
<td>5.18 ± 0.12</td>
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Values are means ± SE; n = no. of hearts per group. MI, myocardial infarction. *P < 0.05; †P < 0.001 vs. sham.
mRNA (see Northern hybridization). Samples were finely ground under liquid nitrogen using a mortar and pestle. Samples of 20–40 mg of tissue were lysed using the cell lysis buffer from the kit using 100 μl of buffer per 10 mg of tissue and were left on ice for 20 min. Samples were centrifuged at 10,000 g for 3 min, and CPP32 (cysteine protease protein-32) activity was measured in 50-μl volumes of supernatant per the manufacturer’s instructions. Protein concentration in the same samples was measured using the Bradford assay, and units of caspase activity were calculated per microgram of protein.

Statistical analysis. Differences in pressure-volume relationships were tested by two-way analysis of variance (ANOVA) for repeated measures. When two-way ANOVA indicated a significant (P < 0.05) difference, data were further examined by the method of least significant differences. Group differences for a single variable were tested by Student’s unpaired t-test (two-tailed). A P value of < 0.05 was considered significant. All data are presented as means ± SE.

RESULTS

Myocardial infarction. A total of 140 mice were studied. Of the 122 mice that underwent coronary artery ligation, ~50% died in the first 24 h after surgery. In the surviving mice, the 6-mo survival rate as assessed by Kaplan-Meier analysis was 72 ± 7% (vs. 100% in sham-operated mice).

Coronary artery ligation in the mouse results in transmural infarction and subsequent scar formation. Infarct size ranged from 10% to 50%. The extent of LV remodeling is related to MI size (29, 31, 32), and therefore infarcts involving >30% of the LV were excluded from further analysis. Of the 33 hearts with infarcts ≥30%, infarct size averaged 40 ± 3, 35 ± 2, and 40 ± 3% (P = not significant), respectively.

Table 2. Left ventricular systolic pressure at an end-diastolic pressure of 5 mmHg or at peak systolic pressure

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>MI</th>
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<tr>
<td>LV systolic pressure, mmHg</td>
<td></td>
<td></td>
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<tr>
<td>LVEDP (5 mmHg)</td>
<td>105 ± 3</td>
<td>78 ± 7†</td>
</tr>
<tr>
<td>Peak</td>
<td>147 ± 5</td>
<td>145 ± 8</td>
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Values are means ± SE. LV, left ventricular; LVEDP, LV end-diastolic pressure. *P = 0.064; †P < 0.01; ‡P < 0.001 vs. sham.

Fig. 2. A: mean curves describing LV developed pressure vs. volume in mice 1, 4, and 6 mo post-MI and their sham-operated controls. The curves are shifted progressively rightward and downward, and the peak developed pressure declines progressively at 4 and 6 mo (*P < 0.05 vs. sham; **P < 0.01 vs. sham). The numbers of hearts in each group are as reported in Fig. 1. B: peak LV developed pressure at 1, 4, and 6 mo post-MI normalized to the LV volume that yielded the maximum developed pressure (referred to as Vmax). The curves were shifted rightward in infarcted hearts at 4 and 6 mo (***P = 0.003 vs. shams; ****P = 0.001 vs. shams). The numbers of hearts in each group are as reported in Fig. 1.
**Heart weight.** Body weight increased between 1 and 4 mo in both the MI and sham groups, reflecting normal age-related growth (Table 1). There was no difference in body weight between MI and sham groups at 1, 4, or 6 mo. Heart weight likewise increased between 1 and 4 mo but was greater in the MI group at each time point, with or without normalization to body weight. Neither ascites nor pleural effusion was found at death in any animal, and ratios of wet to dry lung and liver weights were similar in the MI and sham groups (data not shown).

**LV volume and function.** LV size and function were measured using the isovolumic, blood-perfused (balloon-in-LV) Langendorff preparation paced at 7 Hz (15). The LV end-diastolic pressure-volume relationship was shifted rightward (vs. sham) at 1, 4, and 6 mo, and the degree of rightward shift increased progressively from 1 to 6 mo (Fig. 1).

LV systolic pressure at a LV end-diastolic pressure (LVEDP) of 5 mmHg was lower in MI (vs. sham) hearts at 1, 4, and 6 mo, whereas LV peak systolic pressure in MI hearts was decreased only at 6 mo (Table 2). In MI (vs. sham) hearts at 1, 4, and 6 mo, the LVDP was decreased and shifted rightward over the entire LV volume range tested (Fig. 2A). Because LV volumes varied in the MI groups, the LVDP for each heart was normalized to the volume at the maximal developed pressure (V_{max}) as described by Stromer et al. (36) (Fig. 2B). With this normalization for volume, peak LVDP in MI hearts decreased progressively between 1 and 6 mo. In sham-operated mice, the peak LVDP was similar at 1, 4, and 6 mo (109 ± 5, 120 ± 5, and 119 ± 1 mmHg, respectively). Peak LVDP decreased progressively from 1 to 6 mo in the infarcted hearts (P < 0.0001 by log rank test). At 6 mo, peak LVDP was decreased compared with shams (P = 0.004). The relationship between peak LVDP and LVEDP was likewise preserved at 1 mo and decreased progressively at 4 and 6 mo (data not shown).

**ANP mRNA expression.** Prepro-ANP mRNA was measured by Northern hybridization as a marker of fetal gene expression (Fig. 3A). Infarcted hearts were dissected into two parts, one consisting of the infarct with the peri-infarct rim and the other consisting of the remaining noninfarcted (i.e., remote) LV. In noninfarcted LV, ANP mRNA was not increased (vs. shams) at 1 mo but was increased 7- and 4-fold at 4 and 6 mo, respectively (Fig. 3B). In the infarct/peri-infarct sample, ANP mRNA was increased 10,- 10,- and 9-fold at 1, 4, and 6 mo, respectively.

**TUNEL staining, nuclear morphology, and caspase activity.** Cross sections from the LV apex, midcavity, and base of each heart were stained by the in situ TUNEL technique (Fig. 4A). There were rare TUNEL-positive nuclei in sham-operated animals. In myocardium remote from the infarct, the number of TUNEL-positive cells appeared to be increased at all time points. High-power microscopy demonstrated that TUNEL-positive staining was localized primarily to nuclei of myocytes, which had evidence of chromatin condensation by Hoechst staining (Fig. 4A). This impression was confirmed by confocal microscopy in selected sections. TUNEL-positive cells appeared to be randomly distributed throughout the remote myocardium.

The TUNEL-positive myocytes in myocardium remote from the infarct were counted and normalized per area of myocardium and per 10^5 total nuclei (identified by Hoechst 33258 staining in the same sections). Infarcted hearts were dissected into two parts, one consisting of the infarct/peri-infarct and remote regions compared with age-matched sham-operated hearts (*P < 0.05 vs. shams; **P < 0.001 vs. shams; n = 4 hearts for each group).

**DISCUSSION**

In the mouse heart, myocyte apoptosis occurs transiently in the immediate area of ischemic damage for up to several days after MI but not in myocardium remote from the area of ischemia (4, 25). The major
new finding of our study is that late (i.e., 1–6 mo) after MI, chronic LV remodeling with chamber dilation and impaired systolic pump function is temporally associated with increased myocyte apoptosis in myocardium remote from the area of initial ischemic damage.

Structural and functional remodeling. Post-MI LV remodeling consists of at least two temporal phases (32). During the early phase (first few days), there is expansion of the infarcted tissue, leading to early LV chamber dilation and increased mechanical stress on the remaining viable myocardium. During the late phase (subsequent weeks to months), there is progressive remodeling of the remote areas of the LV characterized by further chamber dilation, myocardial hypertrophy, reexpression of a fetal phenotype, and progressive deterioration in systolic pump function.

We found that in the mouse between 1 and 6 mo after a moderate to large MI, there is progressive remodeling with LV dilation and reduced systolic pump function. This finding is consistent with observations in the rat (31). Remodeling was associated with hypertrophy of myocardium remote from the MI as evidenced by increases in LV weight and wall thickness. Likewise, there was increased expression of mRNA for ANP, a fetal gene that is reexpressed in hypertrophied myocardium (14, 22). ANP mRNA was increased in both the infarct/peri-infarct and remote LV samples. The increase was greater in the sample consisting of the infarct/peri-infarct margin, in agreement with studies (22) in rats that have shown the highest levels of ANP expression to be in the peri-infarct border zone (22), where wall stress is highest (3).

The isovolumic blood-perfused Langendorff preparation with a balloon in the LV was used to assess LV size and contractile function (15, 20). An advantage of this method is that it allows LV size and function to be measured over a range of LV filling pressures and, thus, provides a full reflection of both chamber dilation and maximal systolic pump capacity (18).

Compared with age-matched sham-operated mice, LV chamber volume in infarcted mice increased progressively from 1 to 6 mo, suggesting that LV dilation is time dependent. LV dilation was associated with a parallel progressive rightward shift in the relationship between LV volume and developed pressure and with a decrease in maximum LVDP at any given volume. The latter is particularly important because LV size in the infarcted mice was larger than in sham-operated mice and varied considerably because of the range of infarct sizes that is inherent in this model.

Late myocyte apoptosis. The mouse MI model has been used to characterize the mode of cell death during the first 7 days after MI (4, 24). In mice 7 days after MI, Li et al. (25) found that LV chamber dilation was associated with evidence of both necrosis and apoptosis.
of cardiac myocytes in the infarct/peri-infarct region, but not in the septum, which is remote from the area of ischemic damage. Furthermore, they found that, in mice that overexpress insulin-like growth factor-I, there was less apoptosis, less extensive remodeling, and better LV function. During the first 72 h after MI, Bialik et al. (4) also found evidence of myocyte apoptosis that was restricted to the area of hypoxic myocardium. Likewise, in rats apoptosis is localized to the infarct and peri-infarct regions early after MI, with little to none in areas remote from the infarct (10).

We found that late after MI, there was a progressive increase in the number of apoptotic myocytes in myocardium remote from the area of ischemic damage. The assessment of apoptotic myocytes was based primarily on TUNEL staining, which detects double-stranded DNA breakage. TUNEL staining may also identify necrotic cells (7, 16). We observed that TUNEL-positive cells had condensation of nuclear chromatin, which is typical of apoptosis rather than oncocytic necrosis. Although we cannot exclude a contribution from necrotic cells, myocyte necrosis has not been associated with remodeling of remote myocardium. The observed increase in the frequency of apoptotic cells suggests an increased rate of apoptosis. This conclusion depends on the untested, but reasonable, assumption that the duration of time that TUNEL-positive cells are visible remains constant. The observed increase in caspase-3 activity at 6 mo after MI, a key intermediate in the activation of the apoptosis in many cell types (9) including myocytes (5), is consistent with the conclusion that apoptosis is increased in the remote myocardium late after MI.

Survival. The infarcted mice had a reduced survival over 6 mo of this study. Overt heart failure and organ congestion were not found in mice that survived to 6 mo, suggesting that the more severely affected animals had died before that time. We have observed that some mice carried beyond 6 mo experience rapid deterioration and death associated with respiratory distress and increased lung water, suggesting that animals that are compensated at 6 mo may subsequently progress to overt heart failure. Thus the animals included in our analysis may have less LV dysfunction and apoptosis than those that died before euthanization.

Implications. These observations raise several important questions. First, what is the cause of increased apoptosis in remote myocardium? In vitro, several factors that may be relevant to chronic remodeling can stimulate myocyte death by apoptosis, including mechanical stress (11), angiotensin (23), inflammatory cytokines (34), norepinephrine (13), and oxidative stress (1). The progressive increase in the number of apoptotic myocytes suggests that the stimulus for apoptosis (e.g., mechanical stress) increases with time after MI. We did not determine which factor(s) might be stimulating apoptosis late post-MI. However, the estimated LV diastolic wall stress (27) was ~2–4 kdyn/cm² in shams (assuming an LVEDP of 2–5 mmHg) and increased by ~5–10-fold at 6 mo post-MI (assuming an LVEDP of 15 mmHg) (data not shown). These values are similar in magnitude to the mechanical stress that has been shown to cause apoptosis of cardiac myocytes in vitro (11). Thus increased mechanical strain may have been a stimulus for apoptosis late post-MI.

Second, does late myocyte apoptosis contribute to LV remodeling and dysfunction? The strong correlation between the frequency of apoptotic myocytes and the decrement in maximal developed pressure is consistent with this possibility. However, it is also possible that apoptosis is a marker for, rather than the cause of, worse LV function. This model should allow the use of genetically altered mice to address these and other important questions about the role of apoptosis in post-MI remodeling.

We gratefully acknowledge the help of Nirva D. Kapasi and Michael R. Winter with data analysis. This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-52320, HL-42539, and HL-61639 (to W. S. Colucci) and HL-03878 (to D. B. Sawyer); a Postdoctoral Fellowship from the American Heart Association (AHA), Massachusetts Affiliate (to F. Sam); and a Beginning Grant-in-Aid from the AHA, Massachusetts Affiliate (to D. B. Sawyer).

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