Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats

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Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats. Am J Physiol Heart Circ Physiol 280: H2726–H2731, 2001.—We investigated the role of cardiomyocyte apoptosis in the remodeling of the left ventricle from 24 h to 12 wk after myocardial infarction in the rat. Infarct size planimetry, quantification of cardiomyocyte apoptosis, terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) methodology, and echocardiography (left ventricular diastolic diameter and ejection fraction) were performed. Sham-operated animals showed low rates of cardiomyocyte apoptosis (0.03%) and no change in diastolic diameter or ejection fraction during the study. Twenty-four hours after infarction, TUNEL positivity was high in the infarct areas (1.4%) and border zones (4.9%). It declined to 0.34% (P < 0.01 vs. sham) at 4 wk and 0.10% at 12 wk in the border zones. In the remote myocardium, cardiomyocyte apoptosis increased to 0.07% (P = 0.03 vs. sham) on day 1 and remained on the same level up to 4 wk. The increase in diastolic diameter 1–4 wk after infarction correlated (r = 0.60, P < 0.01) with cardiomyocyte apoptosis in the noninfarcted myocardium, which quantitatively contributed most (>50%) to the apoptotic cell loss by 4 wk.

Apoptosis is a distinct type of cell death characterized by a series of typical morphological events, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies, and rapid phagocytosis into neighboring cells without induction of inflammatory response (15). The biochemical hallmark of apoptosis is internucleosomal DNA fragmentation (30). Cardio-myocyte apoptosis has recently been shown to occur in ischemic and reperfused myocardium both in animal models (1, 5, 8, 13) and in humans (12, 24). Moreover, apoptosis has been found in viable myocardial areas after MI (3, 21, 24) and in experimental (26) and human ischemic heart failure (17, 19, 25).

To clarify the role of apoptosis in chronic post-MI remodeling, we studied the time course and anatomic distribution of cardiomyocyte apoptosis in rat MI. We assessed the occurrence of cardiomyocyte apoptosis from 24 h to 12 wk after infarction in the infarcted, border zone and remote noninfarcted myocardial regions and compared the proportions of apoptotic myocytes with echocardiographic measures of the remodeling process.

MATERIALS AND METHODS

Experimental MI and tissue sampling. MI was produced by ligation of the left anterior descending coronary artery. Briefly, adult male Wistar rats weighing 350–500 g were anesthetized subcutaneously by using 0.5 mg/kg of medetomidine (Domitor, Orion; Turku, Finland) and with 70–80 mg/kg ip ketamine (Ketalar, Parke-Davis; Barcelona, Spain). The rats were connected to a respirator through a tracheotomy, and the heart was rapidly exteriorized through a left thoracotomy and pericardial incision. The coronary artery was ligated about 3 mm from its origin, the heart was returned to its normal position, and the thorax was closed.

Throughout the operation, the body was maintained at a stable temperature with the use of a thermal plate. The anesthesia was partially antagonized with atipamezole hydrochloride (0.75 mg/kg sc, Antisedan; Orion), and the rats were disconnected from the respirator. The rats were hydrated postoperatively with 10 ml sc of physiological saline and given 0.02 mg/kg sc of buprenorphine hydrochloride (Temgesic; Reckitt and Colman; Hull, UK) twice for analgesia. The control rats underwent the same procedure except for the ligation of the coronary artery (sham operation).

After 24 h, 1 wk, 4 wk, and 12 wk after coronary ligation, the rats were euthanized with CO2 (n = 6–14 rats in each group). The heart was excised and cut into 2-mm transverse slices below the point where the coronary artery was ligated. The myocardial samples were fixed in 4% neutral-buffered formaldehyde and paraffin embedded. Sections of 5-μm thickness were stained with hematoxylin and eosin and with TUNEL methodology to detect apoptosis.

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formalin for 24 h, embedded in paraffin, and cut in 4-μm-thick sections for histology, planimetry, and assessment of apoptosis.

**Echocardiography.** All of the experimental animals underwent echocardiography under anesthesia just before the operation (baseline) and 24 h, 1 wk, and 4 wk after coronary ligation or sham operation. The animals were sedated with medetomidine and placed on a warm thermal plate. The stability of the body temperature was monitored with the use of a rectal probe. The echocardiographic measurements were performed with the use of a 12-MHz ultraband sector probe (SONOS model 5500, Hewlett-Packard; Andover, MA). LV systolic diameter and LV diastolic diameter (LVDD), respectively, were measured in the short-axis M-mode right parasternal projection in a plane below the mitral valves and perpendicular to the LV (27). An average of five measurements were used to measure LVDD and to calculate ejection fraction (EF). The coefficients of variation for repeated measurements of LV systolic diameter and LVDD were 0.027 and 0.046, respectively.

**Histology and planimetry of infarct size.** The presence of either signs of acute MI (eosinophilia, karyolysis, and leukocyte infiltration) or collagen scars compatible with an old infarction was analyzed by examination of Van Gieson-stained transverse LV sections. Infarct size was determined planimetrically as the ratio of infarcted tissue or scar to the length of the entire LV endocardial circumference as described previously (23). Infarcts were classified as small (4–7 mm), moderate (7–10 mm), or large (>10 mm). Hearts that showed no histological signs of infarction were not included in the study (n = 7).

**Assessment of apoptosis.** Apoptotic cardiomyocytes were detected with a terminal deoxynucleotidetransferase-mediated dUTP nick-end labeling (TUNEL) assay as previously described (6, 24, 25). In brief, myocardial tissue sections were heated in sodium citrate solution and digested with proteinase-K to expose the DNA. DNA strand breaks were labeled with the use of terminal transferase enzyme with dUTP molecules conjugated to alkaline phosphatase and visualized immunohistochemically. The assay was standardized with the use of serial sections treated with DNase I (1 U/ml for 30 min at 37°C) to induce the formation of DNA strand breaks (positive control of apoptosis). The development of the immunohistochemical staining was monitored by microscopy, and the reaction was interrupted at the moment when intense positive signal appeared in the corresponding DNase I-treated section (25).

Analysis of apoptosis was performed in one LV section obtained from the sample that showed maximal infarct size. The number of apoptotic cardiomyocytes was counted in the whole LV under light microscopy with an ocular grid. The cardiomyocyte origin of the apoptotic cells was identified by the presence of myofilaments surrounding the nucleus. The amounts of apoptotic cardiomyocytes were expressed as the proportion of the TUNEL-positive cardiomyocyte nuclei from the total number of cardiomyocyte nuclei, which was obtained by multiplying the density of cardiomyocyte nuclei in the serial DNase I-treated control section times the area of the section. The density of cardiomyocyte nuclei was always counted in at least four representative microscopic fields in each region of interest. To verify that all cardiomyocyte nuclei were labeled in DNase I-treated sections, we compared the nuclear densities obtained in DNase I-treated sections and Hoechst 33258-stained serial sections (n = 10). We did not find significant difference in the average nuclear density obtained with these two methods [887 ± 200 vs. 773 ± 98 (SD)]. The proportion of apoptotic cardiomyocytes was counted in the infarcted tissue, in the tissue bordering infarction (1-day infarct), and in the remote noninfarcted myocardium. The myocardium extending 0.5–1.0 mm from the infarcted tissue or infarct scar was considered to represent the border zone myocardium. To avoid contamination of the remote myocardium with border zones, a myocardial area extending ~1–2 mm from the border zone area was not included in the statistical analysis. The rest of the LV was considered to represent the remote myocardium. To assess the anatomical distribution of apoptotic cells quantitatively, we determined the relative proportions of the infarcted, border zone, and remote myocardial regions of the total area of the LV in the analyzed section.

**Statistical analysis.** Quantitative results were calculated as means ± SD. The differences in the amounts of apoptotic cardiomyocytes between groups were compared using one-way ANOVA and Bonferroni’s method (SPSS Software; Chicago, IL). Echocardiography measurements between baseline and end point were compared in each group with the use of Student’s t-test for paired data. Pearson’s correlation coefficients were calculated to compare the amounts of TUNEL-positive cells and echocardiography measurements.

**RESULTS**

**Myocardial infarction.** Histologically, there was either an area of necrotic myocardium (day 1 after operation) or a collagenous infarct scar (1–12 wk after the operation) in 80% of the rats that underwent coronary artery ligation. Planimetrically, there were small, moderate, and large infarctions at a rate of 41, 28, and 31%, respectively. The sizes were equally distributed at all time points.

**Cardiomyocyte apoptosis.** Apoptotic cells were rarely found in the sham-operated animals with the use of the TUNEL assay. In contrast, after coronary artery ligation, the apoptotic cells were much more numerous. TUNEL-positive inflammatory cells and interstitial cells were frequently observed in the infarcted areas 24 h after coronary ligation and among the scar tissue 1–12 wk after infarction. In contrast, very few scattered TUNEL-positive inflammatory or interstitial cells were found in the border zones of infarct scars and the remote myocardium. Also, TUNEL-positive cardiomyocytes were numerous 24 h after coronary ligation in the infarcted tissue (Fig. 1A). At later time points, we observed scattered TUNEL-positive cardiomyocytes in the border zones adjacent to infarct scars and in the remote myocardium (Fig. 1, B and C), whereas they were absent among the scar tissue. The TUNEL-positive cardiomyocytes contained condensed nuclei, which is a typical feature of cells undergoing apoptosis (Fig. 1, A and B).

Quantitatively, the percentage of apoptotic cardiomyocytes in the myocardium of sham-operated rats was on average only 0.03 ± 0.02% and remained stable during the study (Fig. 2). Compared with sham-operated rats, significantly higher percentages were found after ligation of the coronary artery. On day 1 after ligation, an average of 4.93 ± 2.51% of TUNEL-positive cardiomyocytes were found in the border zones of infarcted myocardium. Moreover, clusters of positive cells were found among the necrotic myocytes in the
central infarcted areas (average 1.44 ± 0.99%). Apoptosis remained high in the myocardium bordering infarct scars at 1 wk (0.23 ± 0.09, \( P = 0.01 \)), 4 wk (0.34 ± 0.20%, \( P < 0.01 \)), and even 12 wk (0.10 ± 0.05%) after infarction. The percentages of apoptotic cardiomyocytes tended to be higher in the border zone areas of moderate- and large-sized infarctions than in the border zones of small-sized infarctions at 24 h (3.56 vs. 3.36%) and 1 wk (0.29 vs. 0.18%) after infarction. The percentages were similar 4 and 12 wk after infarction at 0.33 versus 0.37% and 0.10 versus 0.09%, respectively.

In the remote noninfarcted myocardium, apoptosis significantly increased already on day 1 after coronary occlusion (0.07 ± 0.03%, \( P = 0.02 \)) and again 4 wk after infarction (0.09 ± 0.05%, \( P < 0.01 \)) when compared with controls (Fig. 2). Again, the percentages of apoptosis in rats with large- and moderate-sized infarctions tended to be higher than in those with small infarctions at 24 h (0.08 vs. 0.04%), 1 wk (0.05 vs. 0.02%), and 4 wk (0.10 vs. 0.07%). However, 12 wk later, infarction percentages were similar (0.04%) in all sizes. There were no predilection sites of apoptotic cells other than the border zone area.

Relative distributions of apoptotic cells in infarcted border zone and remote areas. To further analyze the quantitative distribution of apoptosis in the remote, border zone and infarcted areas, we determined the proportion of each region from the total area of the LV section. We then calculated the distribution of apoptotic cells between these areas. On day 1 after coronary artery ligation, most of the TUNEL positivity occurred in the infarcted and BZ tissues (cross hatched and solid bars, respectively) but was also increased in the remote noninfarcted myocardium (hatched bars) when compared with the sham-operated animals (open bars). Apoptosis remained high thereafter in the BZ of the infarction scars until 4 wk after MI. Four weeks after MI, apoptosis was also increased in the remote noninfarcted myocardium. Note logarithmic y-axis scale in A. *\( P < 0.05 \); **\( P < 0.01 \).

Fig. 3. Relative proportions of apoptotic cardiomyocytes in the remote, BZ, and MI areas. On day 1 after infarction, majority of apoptosis occurred in the MI and BZ areas (open and solid bars, respectively). Thereafter, the relative proportion of apoptotic cells found in the remote noninfarcted tissue (hatched bars) significantly increased when compared with the BZ tissue (solid bars). **\( P < 0.01 \).
54\% in 4 wk \((P < 0.01 \text{ vs. } \text{day 1})\), and 70\% in 12 wk \((P < 0.01 \text{ vs. } \text{day 1})\) after infarction. Thus, although the percentage of apoptotic cells was higher in the border zone areas, the remote areas dominated the actual amount of apoptosis from week 4 after infarction.

**Apoptosis, ventricular function, and ventricular diameter.** To characterize the changes in ventricular function and geometry after MI, we measured EF and LVDD with the use of echocardiography preoperatively and at 24 h, 1 wk, and 4 wk after the operation (Fig. 4). The sham-operated animals showed only minor changes in EF during the study (Fig. 4A). In contrast, EF decreased already 24 h after coronary occlusion (63 vs. 51\%, \(P = 0.01\)) and remained low thereafter. Sham-operated animals did not show significant increases in the LVDD (Fig. 4B). After coronary ligation, the ventricular diameters were highly variable (from 7.5 to 11.1 mm). The average ventricular diameter gradually increased and was 12\% higher compared with baseline at 4 wk after infarction (\(P = 0.01\)).

We did not find any correlation between the EF and the amount of apoptosis either in the border zones or remote myocardium after infarction. Neither was there a correlation between the amount of apoptosis in the border zone areas and the change in the ventricular diastolic diameter. In contrast, the amount of apoptosis in the remote myocardium was related to the increase of LVDD (\(r = 0.60; P < 0.01\)) (Fig. 5B). The high degree of ventricular enlargement was associated with a high percentage of apoptosis.

**DISCUSSION**

Occlusion of a major coronary artery in the rat is a well-characterized animal model of acute MI and its chronic sequelae, including congestive heart failure (19, 22, 23, 28). Previous studies in this model have shown that internucleosomal DNA fragmentation and TUNEL-positive cardiomyocytes can be found in the central ischemic areas in the acute phase of infarction (5, 13). Moreover, activated forms of caspases (2), the key executioners of apoptotic cell death, have been found in myocytes in this situation. Increased expression of the apoptosis-activating Fas receptor (13) and increased ratio of a proapoptotic protein Bax to an antiapoptotic protein Bcl-2 have been suggested as potential mediators of myocyte apoptosis in this model (3).

On the basis of quantification of TUNEL positivity, apoptosis has been suggested to be the major form of cell death during the first hours of evolution of MI (5, 13). Apoptosis has also been found to a smaller extent in the viable myocardium of the LV free wall (3). In this area, apoptosis peaked on \textit{day 1} after infarction but continued at a low rate (0.02 apoptotic nuclei per 100 cardiomyocytes) until 4 wk after infarction (3). In contrast, the remote myocardium of the interventricular septum showed constantly low rates of apoptosis (<0.01 apoptotic nuclei per 100 cardiomyocytes) (3). We extend the previous findings of Cheng et al. (3) by showing that the occurrence of cardiomyocyte apoptosis in
the viable border zones of infarct scars remains high (5- to 12-fold compared with controls) even until 12 wk after experimental MI. Compared with sham-operated rats, apoptosis is also increased 2.5-fold in the entire remote noninfarcted tissue 24 h–4 wk after infarction. Moreover, we found that the amount of TUNEL-positive cardiomyocytes in the noninfarcted tissue correlated with the degree of ventricular enlargement, as measured with echocardiography at 1 and 4 wk after infarction. By 4 wk, as much as 54% of the TUNEL-positive cardiomyocytes resided in this large segment of the LV.

Significance of apoptosis in the acute stages of MI. As in previous human (12, 24) and experimental studies (1, 5, 8, 13), we found numerous TUNEL-positive cells in the infarcted and border zone regions on day 1 after infarction. The majority of the cells showed condensed nuclei, which is a typical feature of apoptosis (15). The significance of apoptosis compared with other types of cell death remains controversial in the acute stages of infarction. Only necrotic morphology of cell death has been found in two previous studies (8, 18) on acute ischemia-reperfusion. Moreover, a large proportion of TUNEL-positive myocytes have necrotic membrane damage, as demonstrated by uptake of myosin antibody from 6 h after coronary occlusion (13). A possible explanation for this could be that apoptosis and necrosis share common biochemical pathways in the early stages of the process (23). Apoptosis could even serve as a precursor of secondary necrosis (7, 15). In this study, we were interested in clarifying the role of apoptosis at later time points after infarction. At this time, only apoptotic inflammatory and interstitial cells were found among the infarct scars, whereas the apoptotic cardiomyocytes were observed at the border zone and remote areas.

Apoptosis and post-MI remodeling. Both experimental (3) and clinical studies (21) have shown that cardiomyocyte apoptosis is not limited to the acute stages of MI but remains increased in the viable myocardium adjacent to infarction. Ongoing cardiomyocyte apoptosis has also been shown to occur in areas supplied by partially occluded coronary artery in the human hibernating myocardium (4) and in experimental (26) and human ischemic chronic heart failure (17, 19, 25). Necrotic cardiomyocytes have not been found by antomyosin labeling in the later stages of infarction in either border zones or remote areas (3). In the border zones of infarct scars, we did not find correlation between the amount of apoptosis and ventricular enlargement. This does not mean that apoptosis in these areas could not contribute to ventricular remodeling but that the process may be patchily distributed (24) and regulated dynamically by repetitive ischemia-reperfusion. Thus the kinetics of apoptosis may be such that the estimation of the total amount of apoptosis occurring in the time course is difficult on the basis of TUNEL-positivity quantified only at a limited number of time points.

In contrast to the border zone areas, we found that the amount of apoptosis in the remote myocardium correlated with an increase in the ventricular diameter 1 and 4 wk after infarction. This provides evidence that apoptosis in this region plays a role in post-MI ventricular remodeling and thus may contribute to the development of congestive heart failure. Actually, we calculated that by 4 wk after infarction the majority of apoptosis occurs in this region due to the large volume of the remote myocardium when compared with the border zone (Fig. 3).

There are several possible triggers of apoptosis after MI in the remote myocardium. It has been proposed that stretch and tension of ventricular wall due to increased filling pressure could have caused apoptosis in rats with large infarctions (3). Notably, β-adrenoceptor blocking agents have reduced cardiomyocyte apoptosis in such experiments (31). In the present study, the average size of infarction was moderate and did not cause severe, progressive impairment of ventricular function, as measured with echocardiography. The fact that there was no correlation between the amount of apoptosis and the decrease in EF may be partly due to the technical limitations of assessing the overall function of the ventricle with the use of single plane M-mode echocardiography.

Some paracrine mediators that are actively produced in the remote myocardium after infarction in rats, such as tumor necrosis factor-α (11) and angiotensin II (10), have been shown to induce cardiomyocyte apoptosis in vitro (14, 16). Recently, angiotensin-converting enzyme inhibitors have been shown to decrease apoptosis in the border zones of infarct scars in a dog model of ischemic heart failure (9). However, the contributions of afterload reduction and direct action on cardiac tissue angiotensin production to the attenuation of remodeling by angiotensin-converting inhibitors remain obscure (29).

In conclusion, we have shown that cardiomyocyte apoptosis occurs after MI in the rat continuously over an extended period of time both in the viable border zones of infarct scars and in the remote noninfarcted myocardium. In the remote myocardium, apoptosis correlates with ventricular enlargement and thus plays a role in the postinfarction remodeling in this model.

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REFERENCES


