Myocardial Cell Death and Apoptosis in Hibernating Myocardium

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Objectives. This study was designed to study apoptosis in hypoperfused hibernating myocardium subtending severe coronary stenosis.

Background. Apoptosis contributes to myocyte death in acute myocardial infarction.

Methods. A left anterior descending coronary artery stenosis was created in 13 pigs and maintained for 24 h (n = 4), 7 days (n = 5) and 4 weeks (n = 4) to reduce coronary blood flow by a mean of 34% with severe regional myocardial systolic dysfunction, as documented by echocardiography. Apoptosis was detected with an in situ end-labeling method and confirmed by “deoxyribonucleic acid laddering” on agarose-gel electrophoresis. The severity of apoptosis was expressed as the percentage of apoptotic myocyte nuclei and nonapoptotic myocardial nuclei.

Results. Myocardial blood flow of the anterior left ventricular wall was reduced from 1.00 ± 0.18 to 0.66 ± 0.21 ml/min per g (p < 0.01), with a severe reduction of anterior regional wall thickening from a mean (±SD) of 39 ± 4% to 9 ± 8% (p < 0.01).

Apoptosis, or programmed cell death, is a distinct type of cell death that differs from necrosis in nature and biologic significance (1–3) and is considered a highly regulated and active process that contributes to biologic homeostasis (4). This type of cell death is most often associated with cells that are progressing through the cell cycle; thus, it has generally been believed that under physiologic conditions, apoptosis does not occur in terminally differentiated normal adult cells such as myocytes (1–4). Recent studies have demonstrated that myocardial cell apoptosis can be induced by a variety of insults, including hypoxemia (5), acute ischemia-reperfusion (6), myocardial infarction (7,8), cardiomyopathic end-stage heart failure (9,10) and myocardial pressure stretch (11). This study was intended to determine whether apoptosis occurs in long-term hypoperfused myocardium, which has been termed myocardial hibernation (12), a functionally and possibly metabolically downregulated protective mechanism against ischemia to preserve myocyte viability.

This study was conducted in a previously established porcine model of myocardial hibernation resulting from reduced coronary blood flow by severe coronary stenosis (13). Because the biologic hallmark of apoptosis is an active fragmentation of genomic deoxyribonucleic acid (DNA) into small oligomers of 180 bp, caused by activation of endogenous endonuclease (2), in this study we applied in situ end-labeling using a digoxigenin-labeled nucleotide and terminal deoxynucleotidyl transferase (TdT) to identify fragmented nuclear DNA, and we used the agarose-gel electrophoresis technique to detect the typical “DNA laddering” to confirm the active fragmentation of genomic DNA into the small oligomers.

Methods

Animal preparation. The study protocol was approved by the Committee on Animal Care at Hartford Hospital, and the “Position of the American Heart Association on Research Animal Use,” adopted by the Association in November 1994, was followed. Thirteen pigs weighing 28 to 48 kg were studied.

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Animal preparations have been described previously in detail (13). General anesthesia was maintained with 0.5% to 1.5% isoflurane with an oxygen–nitrous oxide mixture (40%:60%) and normal arterial blood gases. A midline thoracotomy was performed and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was carefully dissected free over 1 to 2 cm to accept a probe (Transonic Inc.) to measure coronary blood flow. A fixed LAD stenosis was created to reduce the mean LAD coronary blood flow to ~60% of baseline (~40% reduction), as described in our previous report (13). After stabilized stenosis and coronary flow for 1 h, the chest was closed and the animals were allowed to survive for 24 h (n = 4), 7 days (n = 5) or 4 weeks (n = 4).

**Experimental protocol.** Baseline measurements of wall thickening by echocardiography, heart rate, left ventricular (LV) pressure, aortic pressure, regional coronary flow and coronary venous lactate, pH concentration and oxygen content were obtained under stable conditions as described previously (13). In 13 pigs, LAD flow was reduced to ~60% of baseline, and the reduction was maintained for 24 h (n = 4), 7 days (n = 5) or 4 weeks (n = 4). The stability of the coronary stenosis was verified by serial measurements of coronary flow at 15 min, 60 min, 24 h, 7 days or 4 weeks under the same conditions of anesthesia.

In all animals, after maintenance of a stable LAD stenosis for >60 min, the chest was closed in layers, with the pericardium left open. The animals were then allowed to recover in their cages. Aspirin and intravenous heparin were given postoperatively to prevent thrombotic coronary occlusion. After 24 h, 7 days or 4 weeks of LAD stenosis, the animals were restudied and all measurements were repeated under the same anesthesia conditions. The heart was arrested by 10 ml of 10% formalin, embedded in paraffin, sliced into 5-μm sections and normal arterial blood gases. A midline thoracotomy was performed and the heart was suspended in a pericardial cradle. After maintenance of a stable LAD stenosis, the chest was closed and the animals were allowed to survive for 24 h (n = 4), 7 days (n = 5) or 4 weeks (n = 4).

Regional coronary blood flow was measured using a cuff flow probe connected to a transonic flowmeter (Transonic Inc.). At the conclusion of each experiment, the flowmeter was calibrated against a known flow rate to ensure accuracy. Methylene blue was injected into the LAD or circumflex coronary artery to stain the tissue supplied by the vessel. The stained tissue was dissected and weighed to determine the regional myocardial mass perfused by the stenotic coronary artery. Coronary blood flow is expressed as ml/min per g of wet tissue.

Arterial and coronary venous blood samples were obtained anaerobically in cold, dry syringes containing heparin fluoride to inhibit glycolysis. Samples were stored in ice and processed immediately after the experiment. Plasma lactate content was measured using the enzymatic method. Regional myocardial lactate production/consumption was calculated as described previously (13).

**Pathologic and immunohistochemical evaluations.** At the conclusion of the study, methylene blue was injected distally into the stenotic LAD to delineate the area at risk. The LV was cross sectioned at 0.5-cm intervals from apex to base. The area at risk (area stained with methylene blue) was dissected and weighed. The LV sections of the normal region and the area at risk were then separatedly immersed in 0.09 mol/liter sodium phosphate buffer (pH 7.4) containing 1.0% TTC for 10 to 15 min at 37°C to identify myocardial necrosis. Myocardium with deep red staining by TTC was considered viable, and unstained myocardium by TTC was deemed infarcted or fibrotic. In pigs with patchy infarction (not stained by TTC), the total surface area, infarct-related area and normal area of each LV section in regions supplied by the LAD (stained by methylene blue) were traced on transparent paper. The infarct size for each pig was calculated by integrating infarct-related areas from all LV sections and expressing them as a percentage of the total area at risk (13). All LV sections, including areas with patchy infarction by TTC, were then fixed with 10% formalin, embedded in paraffin, sliced into 5-μm sections and stained with hematoxylin–eosin and trichrome for light microscopic examination. Histologic study of the LAD-perfused regions and normal regions was performed by a cardiac pathologist (J.T.F.) who had no knowledge of the origin of the samples.

**In situ detection of apoptotic cells.** A method of end-labeling mediated by TdT (ApopTag kit, Oncor) was used for in situ detection of apoptosis at the level of a single cell. The labeling target of the ApopTag kit is the multitude of new 3‘-OH DNA ends generated by DNA fragmentation and typically located in nuclei and apoptotic bodies. The principle of this method is to catalytically add residues of digoxigenin-labeled nucleotide to the DNA by TdT (15), an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3‘-OH ends of double- or single-stranded DNA. Anti-digoxigenin antibody conjugated to peroxidase is then used with chromogen substrate to generate an immunohistochemical signal of the presence of DNA fragmentation, indicating apoptosis. All of the end-labeling experiments were completed before the addition of chromogen substrate.
performed multiple times according to the manufacturer’s recommendations so that the results could be standardized. Paraffin-embedded myocardial tissue sections (6 μm) were layered onto glass slides (Superfrost Plus, Baxter), which were then deparaffinized in xylene two times for 5 min each, rehydrated in graded dilutions of ethanol, equilibrated in protein-digesting enzyme buffer and then treated with 20 μg/ml of proteinase K (Sigma Chemical Co.) for 20 min at room temperature. After several washes in deionized water, the slides were quenched in 2% to 3% hydrogen peroxide before exposure to TdT and the digoxigenin-labeled nucleotide (37°C, 60 min). After several washes, the tissue sections were incubated with antidigoxigenin antibody conjugated to a peroxidase enzyme and signal detected using diaminobenzidine as a chromogen. Subsequently, tissue sections were counterstained with ethyl green or eosin before coverslipping. Four to seven sections of each LV were stained and examined. Sections were first examined under light microscopy at a magnification of ×20, whereas ×40 and ×100 magnifications were used to confirm apoptotic staining detected at the low magnification. Quantitative counting of apoptotic myocytes with nuclear staining was performed at subendocardial, mid-myocardial and epicardial layers. For each layer, 10 randomly chosen fields at a magnification of ×20 were examined, and in each field all myocytes with positive and negative nuclei labeling for apoptosis were counted. The extent of apoptosis was expressed as a percentage of nonapoptotic myocytes. Nonmyocardial cells and cells at the edges of the tissue section were not counted.

Positive control staining for apoptosis was performed using sections of rat mammary glands obtained on the fourth day after weaning (Oncor) and 2 h after acute myocardial infarction in pigs. Negative control staining consisted of LV myocardial sections from four sham-operated pigs without coronary stenosis.

Isolation and detection of fragmented genomic DNA. Extraction of DNA was performed using a nonorganic DNA extraction kit (Oncor). Eight fresh-tissue specimens from the ischemic or nonischemic portion of the myocardium in two pigs were minced in 1 ml of 1× phosphate-buffered saline with sterile scalpel blades before further processing, according to the recommendations of the manufacturer. Isolated nuclei were then digested with proteinase and protein precipitate recovered by centrifugation. The DNA was precipitated from the supernatant using 2 vol of ethanol. The DNA was then suspended in 150 μl of suspension buffer, and concentrations were determined spectrophotometrically. Electrophoresis was performed at 180 volt-hours on 2% agarose-gel plates in 1× Tris, boric acic, ethylenediaminetetraacetic acid buffer. The DNA was visualized by ethidium bromide staining and exposure to ultraviolet light.

Statistical analysis. All parametric data were expressed as mean value ± SD. One-way analysis of variance was used to compare parametric data among different stages using a commercially available statistical software package (RS1, BBN Software Company). If there was a statistical difference by analysis of variance, a paired t test was used to examine parametric data between two stages. A paired t test was also used to examine parametric data between normal control and hibernating regions. Corrections for multiple comparisons were also applied using Tukey honestly significant difference test (HSD) where applicable. Linear regression analysis was used to examine the correlation between regional coronary flow and percent myocardial cell apoptosis. A p value ≤0.05 was considered significant.

Results

Hemodynamic data, metabolism and regional LV function. Mean regional coronary blood flow was reduced from 1.00 ± 0.18 to 0.66 ± 0.21 ml/min per g of myocardium immediately after creation of the LAD stenosis and remained at 0.65 ± 0.26 ml/min per g of myocardium at 24 h, 7 days or 4 weeks. On average, coronary flow was reduced by 34% immediately after creation of the stenosis, and remained the same at the end of the experiment (n = 4 for 24 h, n = 5 for 7 days or n = 4 for 4 weeks). Mean blood pressure (81 ± 11 mm Hg) did not change significantly. Heart rate was slightly but not significantly higher at the end of the experiment (105 ± 11 beats/min at baseline, 114 ± 12 beats/min immediately after stenosis and 122 ± 14 beats/min at 24 h, 7 days or 4 weeks after stenosis; p > 0.05). If coronary flow was corrected by heart rate (flow/heart rate), the average flow reduction was 40% immediately after stenosis and 46% at the end of the experiment. Regional anterior wall thickening (hibernating region) decreased from 39 ± 4% at baseline to 9 ± 8% immediately after creation of the stenosis, and was 10 ± 9% at the end of the experiment. The inferior wall thickening (the control region) increased slightly from 38 ± 5% at baseline to 41 ± 4% at the end of the experiment (p < 0.05). The arterial-coronary venous lactate extraction decreased from 35 ± 25% at baseline to 10 ± 30% (p < 0.01) immediately after the creation of the stenosis and recovered to 20 ± 18% at 24 h, 7 days and 4 weeks.

Pathologic changes. Minimal patchy subendocardial infarction was found in eight of the 13 pigs. Five of eight infarctions could be seen by TTC staining and were confirmed by light microscopy, occupying 3% to 6% of the area at risk. In three pigs, patchy subendocardial infarction was seen only under light microscopy. Five pigs did not have any evidence of infarction.

Immunohistochemical findings. In all experimental pigs, positively stained myocytes indicating apoptosis were detected in the hibernating regions supplied by the stenotic LAD with the in situ end-labeling method. Apoptosis was patchy and seen predominantly in the subendocardial regions (9.8 ± 4.6%, Fig. 1) of the hibernating region. Apoptosis was rare in the subepicardial region (0.32 ± 0.45% [range 0% to 1.2%]) of the hibernating region. If apoptotic cells in the subendocardial and subepicardial myocardium were averaged for each of the pigs, there were 4.8 ± 2.3% apoptotic myocytes (range 2.2% to 9.5%). Apoptosis was observed both in the regions adjacent to the patchy fibrosis or in the regions distant from fibrosis (Fig. 2). Apoptosis was observed mostly in myocytes where muscle striations were noted. There was no difference in the number
of apoptotic cells in the hibernating region with different durations of hibernation at 24 h (5.67 ± 1.56%, n = 4), 7 days (4.63 ± 2.83%, n = 5) and 4 weeks (3.22 ± 0.71%, n = 4). However, a large number of animals are needed to determine whether the number of cells dying of programmed cell death increases after time, stabilizes or eventually decreases with the duration of hibernation. No apoptosis was noted in the normal control region (inferior wall of the LV).

The severity of apoptosis (x, percentage of cells with apoptosis) correlated significantly with the severity of myocardial hypoperfusion (y) \((y = 7.59x + 9.58, r = 0.75, p < 0.01)\). Apoptosis was more severe in pigs with patchy infarction (5.4 ± 1.74%) than in pigs without patchy infarction (2.65 ± 0.83%, \(p < 0.05\)). No apoptosis was found in sham-operated control pigs.

Electrophoresis. In two pigs with myocardial hibernation for 24 h and 7 days, electrophoresis of the subendocardial myocardium of the hibernating region was performed. Not all samples showed “DNA laddering.” This “DNA laddering” was
found in two of four samples in one pig and in one of four samples in the second pig (Fig. 3), indicating a patchy distribution of apoptosis in the hibernating region. No “DNA laddering” was observed in normal control regions.

**Discussion**

The results of this study provide evidence that ongoing myocyte apoptosis is present in hypoperfused, hibernating myocardium resulting from a severe LAD stenosis of 24 h to 4 weeks in duration. Based on clinical data, myocardial hibernation has been hypothesized to be the “smart heart” mechanism that protects the myocardium against infarction from acute or chronic sublethal ischemic attacks by an undefined mechanism of functional downregulation (12). Experimental studies conducted in our laboratory as well as in that of others have produced animal models of myocardial hibernation with severe regional LV dysfunction with no or minimal infarction by mildly or moderately reduced rest coronary flow (13, 16–18). These experimental studies support the notion that the myocardium subtending severe coronary stenosis reduces its function and oxygen consumption in attempting to match the reduced coronary blood flow or oxygen supply, and thus prevents a large region of myocardial necrosis. The results of this study suggest that the functional downregulation may not be adequate to prevent a gradual myocyte death through the mechanism of programmed cell death, or apoptosis.

**Factors that may be related to apoptosis in hibernating myocardium.** Myocardial systolic dysfunction occurs within 1 min of the reduction of coronary flow, whereas apoptosis is detectable only after 60 min of total coronary occlusion. Therefore, it does not appear that apoptosis is a major cause of myocardial systolic dysfunction in this model. Nonetheless, the possibility that reduced systolic function with increased systolic wall stress and subsequent myocyte stretching induces apoptosis is suggested by the observation that myocyte stretching can induce apoptosis in a single-cell in vitro study (11). An in vitro study using cell culture technique has shown that hypoxia induces apoptosis (5). Although there is only a mild to moderate reduction of rest transmural coronary flow in the hibernating myocardium, the reduction in subendocardial flow may be more severe, leading to cellular ischemia and apoptosis in this model. This notion is supported by the facts that myocardial apoptosis occurred almost exclusively in the subendocardial layer of hibernating myocardium and correlated with regional coronary flow reduction in this study. Inhomogeneous perfusion in the ischemic myocardium may be responsible for the patchy distribution of apoptotic cells.

**Critiques of the pig model and techniques for detecting apoptosis.** Coronary flow was not continuously monitored. Whether the apoptosis is induced by episodes of more severe ischemia superimposed on already reduced coronary flow in the hibernating region or as a result of long-term flow reduction cannot be determined from this study. However, a total thrombotic coronary occlusion was not observed in any animal in the present study. In a previous study in our laboratory with the same pig model, 24-h monitoring of coronary flow did not show coronary occlusion due to spasm, although there was a significant variation in the degree of coronary flow reduction, from 59% to 25% (13). There is a possibility that myocardial cell coagulative necrosis may also have fragmented DNA that may be stained positive with this staining technique (7,8). However, positively stained fragmented nuclear DNA was observed in myocytes with intact cell membranes (Fig. 1B) and was not only in myocytes adjacent to the fibrotic region, but also in those regions free of inflammatory cells and distant from the fibrosis (Fig. 2). It is unlikely that these cells are necrotic myocytes, which usually have ruptured cell membranes with an acute inflammatory process. Kajstura et al. (8) have shown that myocardial cell apoptosis is almost completed within 24 h and necrosis is completed within 7 days of coronary occlusion. It is unlikely that positively stained apoptotic cells in the 7-day and 4-week hibernating myocardium are from the early reduction in coronary flow within the first 24 h. Chronic ischemia with or without episodes of more severe ischemia may be responsible for the apoptotic cells detected at these late periods. Electron microscopy was not used to verify if true apoptotic bodies were present in this study.

**Clinical implications.** Because apoptotic cell death may be completed within 24 h, the fact that apoptosis was observed not only at 24 h but also at 7 days and 4 weeks of myocardial hibernation and hypoperfusion suggests ongoing cell death in the hibernating myocardium. As we discussed in our previous study (13), morphologic changes in the 24-h pig model of myocardial hibernation appear less severe than those seen in patients in whom significant fibrosis is usually found in regions of long-term myocardial hibernation. This study suggests that the ongoing myocardial cell death may be in part responsible for myocyte loss and increased fibrosis in long-term hibernating LV regions. This also raises the possibility that apoptosis is responsible for ischemic cardiomyopathy. Further studies to elucidate these issues are needed.
References