Apoptosis of Endothelial Cells Precedes Myocyte Cell Apoptosis in Ischemia/Reperfusion Injury

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Background—Apoptosis contributes to cell loss after ischemia/reperfusion injury in the heart. This study describes the time course and level of apoptosis in different cell types in the intact heart during ischemia/reperfusion injury.

Methods and Results—Isolated Langendorff-perfused rat hearts were subjected to perfusion alone (control) or to 35 minutes of regional ischemia, either alone or followed by 5, 60, or 120 minutes of reperfusion. Sections were stained by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) and propidium iodide and with anti-von Willebrand factor, anti-desmin, or anti-active caspase 3 antibodies; they were then visualized by confocal microscopy. Sections were also examined by electron microscopy. No TUNEL-positive cells were seen in control hearts or hearts exposed to ischemia alone. Early in reperfusion, TUNEL staining was colocalized with endothelial cells from small coronary vessels. Endothelial apoptosis peaked at 1 hour of reperfusion and, at this time, there was clear perivascular localization of apoptotic cardiac myocytes, whose number was inversely proportional to their distance from a positive vessel. After 2 hours of reperfusion, apoptotic cardiac myocytes assumed a more homogeneous distribution. Active caspase 3 labeling was seen independent of DNA fragmentation during ischemia alone, but it colocalized with TUNEL staining over the 3 time points of reperfusion. Immunocytochemical findings were confirmed by electron microscopy and Western blotting.

Conclusions—In the very early stages of reperfusion, apoptosis is first seen in the endothelial cells from small coronary vessels. The radial spread of apoptosis to surrounding cardiac myocytes suggests that reperfusion induces the release of soluble pro-apoptotic mediators from endothelial cells that promote myocyte apoptosis. (Circulation. 2001;104:253-256.)

Key Words: apoptosis ■ endothelium ■ ischemia ■ reperfusion

Apoptosis is a genetically programmed form of cell death that is mediated by the activation of the caspase cascade and results in the cleavage of protein substrates and oligonucleosomal fragmentation of DNA. Apoptosis has become increasingly recognized as one mechanism of cell death during ischemia/reperfusion injury, although the relative contribution of necrosis and apoptosis to total cardiac cell loss remains controversial. Although the specificity of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) for detecting the DNA fragmentation characteristic of apoptotic cells has been questioned, TUNEL-positive (TP) cells are not seen in normal hearts or in the unstained necrotic areas produced by prolonged ischemia. The ability of TUNEL to detect apoptosis specifically is further validated by molecular probes capable of recognizing different aspects of DNA fragmentation, which clearly differentiate apoptosis from necrosis.

DNA fragmentation and the characteristic apoptotic morphology are end stages of the complex set of molecular processes that mediate the apoptotic program. Apoptotic stimuli, such as ischemia/reperfusion injury, activate the caspase cascade and the final cleaved forms of effector caspases, such as caspase 3 (C3), then cleave substrates important in the maintenance of cellular integrity. Therefore, an additional method for assessing apoptosis is to measure the appearance of active caspases, such as C3.

In this study, we determined whether apoptosis in cardiomyocytes and in noncardiomyocytes proceeds with different time kinetics in the isolated, perfused rat heart exposed to ischemia/reperfusion injury.

Methods

Animal Model
Hearts from anesthetized adult male Sprague-Dawley rats were mounted in a Langendorff perfusion apparatus, and ischemia/reperfusion was performed as described previously. Isolated hearts were...
divided into 5 groups (6 hearts per group). Control hearts were continuously perfused. The second group was exposed to 35 minutes of ischemia alone, which was achieved by occlusion of the left coronary artery. After 35 minutes of ischemia, the remaining groups were reperfused for 5, 60, and 120 minutes, respectively. At the end of each experiment, 0.5 mL of Evans Blue solution was slowly infused to delineate the risk area.

Preparation and Staining of Sections
Serial 5-μm sections were cut from paraffin blocks and, after dewaxing and heat-mediated antigen retrieval, were stained with TUNEL reagents, propidium iodide, and anti-desmin antibody, as previously described. Two additional staining reactions were used; in the first, sections stained with TUNEL reagents were incubated with mouse anti-von Willebrand factor antibody (Boehringer Mannheim Biochemica) to identify endothelial cells (EC) and were then counterstained with rhodamine-conjugated anti-mouse secondary antibody (DAKO). In the second reaction, TUNEL-stained sections were incubated with an antibody that specifically recognizes the active form of C3 (Santa Cruz Biotechnology, Inc). After washing, slides were counterstained with rhodamine-conjugated anti-rabbit antibody (DAKO), washed, mounted, and examined by confocal microscopy as described before. Data are expressed as the mean±SD of 12 to 15 high power fields.

Western Blotting and DNA Laddering
Cardiac ventricular cells from each group were homogenized in lysis buffer, electrophoresed on 8% SDS-polyacrylamide gels, and Western blotted with anti-active C3 antibody, as previously described. Oligonucleosomal DNA fragmentation was visualized on 1.2% ethidium bromide–stained agarose gels.

Electron Microscopy
Tissue samples were fixed and embedded for electron microscopy analysis, as previously described, with minimal modifications.

Statistics
Significance was evaluated using ANOVA. P<0.05 was considered significant.

Results
Endothelial Cell and Myocyte Apoptosis
No TUNEL staining was detectable in any cell type in control hearts or after 35 minutes of ischemia alone (Figure 1a). TP cells forming fluorescent rings, representing sections of small coronary vessels, were normally the only positive cells detected after 35 minutes of ischemia followed by 5 minutes of reperfusion (Figures 1b and 1c). The endothelial origin of these cells was confirmed by colocalization of TUNEL positivity with anti-von Willebrand factor antibody staining (Figure 1d). At this time, a few positive vessels sometimes exhibited a small perivascular cuff of TP cardiomyocytes (Figure 1e). With 60 minutes of reperfusion, significantly more EC, including those in larger vessels, had become TP (Figure 1f; P<0.02), and the perivascular ring of TP and desmin-positive cardiomyocytes expanded further from the vessels (Figure 1g). The range and mean diameters of TP vessels with increasing reperfusion times is shown in Figure 2a. Superimposition of a radial grid revealed that the number of TP cardiomyocytes progressively decreased with distance from the vessel after both 5 and 60 minutes of reperfusion (Figure 2b). The proportion of apoptotic cardiomyocytes was increased after 2 hours of reperfusion (P<0.05 compared with 5 and 60 minutes of reperfusion), although the proportion of TP EC had decreased (Figure 1f). At this time, TP cardiomyocytes tended to lose their perivascular localization and assumed a more homogenous distribution (Figure 2b).

Colocalization of TUNEL Positivity With Cleaved C3
No cleaved C3 expression was seen in control hearts, although a few positive cells appeared after ischemia alone (Figure 1f). The highest expression of cleaved C3 in hearts exposed to ischemia alone was observed in vascular cells;
expression was significantly ($P<0.03$) greater than that in cardiomyocytes. In the same sections, no TP cells were detected (Figure 1f). However, after 5, 60, and 120 minutes of reperfusion, TP cells were colocalized with cells staining with the cleaved C3 antibody (Figure 2c). The number of EC positive for cleaved C3 peaked at 60 minutes of reperfusion, whereas the number of positive cardiomyocytes was still increasing (Figure 1f). Similarly, Western blotting showed C3 cleavage in protein extracts from hearts exposed to ischemia alone and to ischemia/reperfusion (Figure 2d) and confirmed that the antibody only recognized the cleaved, active form of C3.

**Electron Microscopy**

By qualitative electron microscopic analysis, typical features of apoptosis were not seen in EC or cardiomyocytes after ischemia alone (Figure 2f), but they were apparent in EC after 5 minutes of reperfusion (Figure 2g). Cardiomyocytes with the characteristic apoptotic morphology were observed together with apoptotic EC after 60 minutes of reperfusion.

**Discussion**

This study shows that apoptosis after ischemia/reperfusion proceeds in a cell- and time-dependent manner. Ischemia alone is not sufficient to complete the apoptotic death of myocyte and nonmyocyte cells assessed by TUNEL and electron microscopy. After 5 to 60 minutes of reperfusion, EC within initially small, but later larger coronary vessels become TP and are associated with a perivascular cuff of TP cardiomyocytes of progressively increasing radius from the vessel with time. By 2 hours of reperfusion, the proportion of TP EC had decreased, and the distribution of the positive cardiomyocytes had become more homogenous.

Although no TP cells were seen after ischemia alone, some EC were stained with an antibody that recognizes the cleaved form of C3. This suggests that ischemia without reperfusion can initiate the molecular pathway of apoptosis, although reperfusion is required to complete the DNA fragmentation and morphological changes characteristic of an end-stage apoptotic cell. The colocalization of cleaved C3 with TUNEL positivity after reperfusion would support this interpretation. This requirement for reperfusion in completing the apoptotic program is in accordance with several previous studies, although the ex vivo data reported here require confirmation in vivo. As with TUNEL labeling, the number of EC expressing active C3 peaked at 60 minutes, when the number of positive cardiomyocytes was still increasing.

The demonstration that EC apoptosis precedes that of cardiomyocytes has 2 important implications. First, EC DNA fragmentation after short periods of reperfusion may follow the release into the restored circulation of a mediator(s) generated during ischemia that is necessary to complete the apoptotic process. Second, the radial distribution of apoptotic cardiomyocytes seen 5 and 60 minutes after reperfusion may reflect the diffusion into the myocardium of soluble apoptotic mediators from damaged EC. Several candidate mediators for the paracrine apoptosis of cardiomyocytes may be postulated, including those that ligate a death receptor (such as tumor necrosis factor-$\alpha$ or Fas ligand) and those that damage mitochondria (such as free radicals). The potential involvement of soluble factors in apoptosis after ischemia/reperfusion injury suggests that strategies based on their scavenging or inhibition may allow endothelial cell rescue and protect the myocardium.
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CORRECTION

Correction to: Apoptosis of Endothelial Cells Precedes Myocyte Cell Apoptosis in Ischemia/Reperfusion Injury

As part of an investigation by the University College of London (UCL), concerns were raised regarding certain figures in three American Heart Association journals. To address these concerns, the authors of these articles have prepared the following corrections:

For the article by Scarabelli et al in Circulation (Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischaemia/reperfusion injury. Circulation 2001;104:253–256.), concerns were raised regarding Figure 2d, which was used inadvertently in a subsequent publication. To avoid any misunderstanding, the authors have corrected the panel with a replicate Figure performed at much the same time as the original but in a different laboratory.

For the article by Lawrence et al in Circulation (KATP Channel gene expression is induced by urocortin and mediates its cardioprotective effect. Circulation, 2002; 106: 1556–1562.), to address the concerns raised about Figure 3a, the first author, Dr Lawrence, repeated the experiment, reproduced the induction of the KATP channel with an appropriate actin control, and corrected the panel.

For the article by Scarabelli et al in Circulation Research (Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischaemia-reperfusion injury. Circ Res. 2002;90:745–748.), to address the concerns raised about Figure 2d, the authors completed two new distinct sets of experiments. As the same loading control was used twice for both Figure 2c and Figure 2d, the authors repeated both experiments, using a caspase 8 inhibitor (Figure 2c) and a caspase 9 inhibitor (Figure 2d), respectively and corrected these panels.

The authors apologize for these errors, which have been corrected in the online version of each article.

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