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Cardiovascular Research 45 (2000) 651–660

Cardiovascular Research

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Reperfusion induces myocardial apoptotic cell death

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Received 8 April 1999; accepted 10 June 1999

Abstract

Objective: The purpose of the present study was to investigate whether apoptosis is triggered during ischemia (I) and reperfusion (R) and whether I/R-induced apoptosis is correlated with changes in expression of Bcl-2 and Bax. **Methods:** Anesthetized open-chest dogs were divided into two groups. Group I: 7 h of permanent I without R (PI, $n=7$); Group II: 60 min I followed by 6 h R (I/R, $n=8$). Apoptosis was identified as ''DNA ladder'' by agarose gel electrophoresis or confirmed histologically using the terminal transferase UTP nick end labeling (TUNEL) assay. **Results:** Collateral myocardial coronary blood flow during I, confirmed by colored microspheres was comparable in both groups. Although PI caused 72±5% infarct size, very few TUNEL-positive cells were detected in the necrotic area (0.2±0.1% of total normal nuclei), consistent with an absence of DNA laddering. In contrast, the appearance of TUNEL-positive cells was significantly displayed after 6 h R in the necrotic area in I/R group (26±4%, $P<0.001$ vs. PI group), and DNA ladder occurred in all experimental animals, suggesting that myocardial apoptosis is primarily elicited by R. Densitometrically, Western blot analysis showed significant reduction in expression of Bcl-2 (16±1%) and increase in Bax (29±8%) after 6 h R in the necrotic area compared with normal tissue while expression of these two proteins was not changed in the PI group. Polymorphonuclear neutrophil (PMN) accumulation in the necrotic area determined either by immunohistochemistry with anti-CD18 antibody or by myeloperoxidase activity was significantly increased in the I/R group compared to the PI group (358±24 vs. 24±2, mm^2 myocardium, $P<0.01$) and (2.9±0.3 vs. 0.4±0.1, U/100 mg tissue, $P<0.01$). There was a significant linear relationship between CD18-positive PMNs and TUNEL-positive cells ($P<0.05$) in the I/R group. **Conclusions:** These results indicate that (1) PI without R did not induce apoptotic cell death, while two types of cell death, necrosis and apoptosis were found after I/R, (2) the Bcl-2 family may participate in early R-induced myocardial apoptosis, (3) PMN accumulation may play a role in the development of apoptosis.

Keywords: Apoptosis; Necrosis; Reperfusion

1. Introduction

Necrosis and apoptosis are two major distinct types of cell death in cardiomyocytes that have been associated with ischemia and reperfusion [1–5]. Cardiomyocytes undergoing necrosis and apoptosis show characteristic but morphologically and biologically distinct features. Necrosis, also called accidental or pathological cell death [6], is manifested by severe cell swelling or cell rupture, denaturation and coagulation of cytoplasmic proteins, breakdown of cell organelles and significant inflammatory response. Apoptosis, in contrast to necrosis, is genetically controlled programmed cells death. Its chief morphologic features include cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies, no loss of membrane integrity and no inflammatory response [7–9]. Although the mechanisms of necrosis and apoptosis differ, there is overlap between these two processes under pathological conditions. Several recent studies reported that ischemia/reperfusion induces myocardial apoptosis in the ischemic myocardium in vivo [1,5,10,11]. However, whether apoptosis is triggered during ischemia or during reperfusion is still controversial. Gottlieb et al. [1] found

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Time for primary review 25 days.

0008-6363/00/$ – see front matter
PII: S0008-6363(99)00354-5

that the hallmark of apoptosis, nucleosomal ladders of DNA fragments, was detected in ischemic myocardium after 30 min of ischemia and 4 h of reperfusion in the rabbit, but not in ischemic-only myocardium, suggesting that apoptosis may be expressed only during reperfusion. In contrast, Kajstura et al. [10] and Fliss and Gattinger [5] showed that in rat myocardium, apoptosis induced by permanent ischemia can be detected after 2 h of coronary occlusion, and the apoptotic process was also accelerated after 45 min of ischemia followed by 1 h of reperfusion [5], indicating that apoptosis begins either after prolonged ischemia without reperfusion or after a brief period of ischemia followed by reperfusion. The apparent absence of apoptosis in the rabbit heart and the presence of apoptosis in the rat heart after ischemia may be related with species differences. However, it is still not clear whether myocardial apoptosis is triggered by ischemia or ischemia followed by reperfusion in the in vivo dog model.

Recent studies have demonstrated the roles of anti-apoptotic protein, Bcl-2 and proapoptotic protein, Bax in regulating myocardial programmed cell death. A clinical study indicated that positive Bcl-2 protein expression was detected in salvaged myocytes in acute infarction while Bax was overexpressed only in the infarcted area in matured myocardial infarction, suggesting that Bcl-2 may play a role in salvage of ischemic tissue [12]. Furthermore, expression of Bcl-2 protein in the prevention of apoptosis was also demonstrated in isolated rat myocytes [13]. Although an anti-apoptotic effect of Bcl-2 has been demonstrated and linked to alternated myocardial injury, it is not known whether the involvement of Bcl-2 and Bax in the development of program cell death occurs during ischemia or reperfusion after coronary occlusion. Therefore, in the present study, the induction of apoptosis in ischemic myocardium was evaluated in dogs after ischemia and reperfusion, and expression of Bcl-2 and Bax in the area at risk myocardium was determined. In addition, polymorphonuclear neutrophil (PMN) accumulation in ischemic myocardium after permanent ischemia and ischemia/reperfusion was confirmed by immunohistochemistry with anti-CD18 antibody and myeloperoxidase (MPO) activity.

2. Methods

2.1. Surgical preparation of animal

Dogs of either sex weighing 20–30 kg were initially premedicated with an intramuscular injection of morphine sulfate (4 mg/kg). Subsequently, a bolus intravenous injection of sodium pentothal (20 mg/kg) was followed by continuous inhalation of isoflurane (0.5–2%) after endotracheal intubation. Dogs were ventilated with a positive pressure respirator (Narkomed 2A, North American Drager, Telford, PA, USA) using room air supplemented with 100% oxygen. Tidal volume and ventilatory rate were adjusted to maintain PCO_2 between 35 and 45 mmHg, $PO_2 > 100$ mmHg, and pH between 7.35 and 7.45. The left femoral artery was isolated for blood sampling. A left lateral thoracotomy was performed, and the pericardium was widely opened. A micromanometer pressure transducer (MPC-500, Millar, Houston, TX, USA) was inserted into the left internal mammalian artery to measure arterial blood pressure. A catheter was inserted into the left atrium for injection of colored microspheres to measure regional myocardial blood flow by reference sampling method. A 4-0 silk suture was passed below the first branch of the left anterior descending (LAD) coronary artery, and the ends of the tie were threaded through a small plastic (PE120) tube to form a snare for later coronary occlusion. All dogs were then systemically heparinized with 300 U/kg sodium heparin before starting the experiment. The experimental procedures complied with the *Guiding Principles in the Use and Care of Animals* approved by the Council of the American Physiological Society as well as with state and federal regulations. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

2.2. Experimental protocol

After a period of postsurgical stabilization, steady-state baseline hemodynamic measurements were acquired in duplicate and microspheres were injected. All animals were then randomized to one of two groups: (1) Permanent ischemia (PI group, $n=7$): dogs were subjected to 7 h of coronary occlusion without reperfusion; (2) Ischemia/reperfusion (I/R group, $n=8$): after 60 min of LAD occlusion, the ligature was loosened, and the ischemic myocardium was reperfused for 6 h. At the end of the experiment in two groups, tissue samples from the nonischemic and ischemic myocardium were used to quantify tissue myocardial blood flow, infarct size, PMN accumulation by immunohistochemistry and MPO activity, DNA laddering, myocardial apoptosis and expression of Bcl-2 and Bax proteins (see below).

2.3. Hemodynamic measurements

Heart rate and mean arterial pressures were acquired and processed using a videographic program (Spectrum, Wake Forest University, Winston-Salem, NC, USA) [14]. Hemodynamic measurements and regional myocardial blood flow were taken before coronary artery occlusion (control), at the end of 60 min of ischemia, and at the end of permanent ischemia and reperfusion. Hemodynamic data were averaged from no less than 15 beats.

2.4. Regional myocardial blood flow

Regional myocardial blood flow using colored micro-

spheres (15 μm diameter, Triton, San Diego, CA, USA) was calculated using the reference sampling method as previously described [15]. After KOH digestion and filtration trapping of microspheres, tissue and reference blood samples were analyzed with a spectrophotometer (DU7400: Beckman, Fullerton, CA, USA). Blood flow was calculated in the nonischemic and the area at risk myocardium as $\text{RMBF} = (C_T \times F_R / C_F) \times W_T$, where C_T and C_F are the absorbance from dispersed microspheres in the tissue and reference blood samples, respectively. F_R is reference flow-rate (7 ml/min), and W_T is total weight of the tissue sample in grams. Results are expressed as ml/min/g tissue.

2.5. Determination of area at risk and infarct size

After harvesting of the heart, LAD was retired and a catheter was inserted into aortic root. Unisperse blue dye was injected under 100 mmHg perfusion pressure to stain the normally perfused region blue and outline the area at risk. The left ventricle (LV) was cut into transverse slices. The area at risk was separated from the nonischemic zone and incubated for 15 min in 1% solution of triphenyltetrazolium chloride (TTC) at 37°C to differentiate necrotic (pale) from nonnecrotic (red) area at risk The area at risk as a percent of the LV mass (area at risk/LV) was calculated as [(weight of nonnecrotic and necrotic tissue in the risk region)/(total weight of LV)]×100. Area of necrosis as a percent of the area at risk (area of necrosis/area at risk) was calculated as [(weight of necrotic tissue in the risk region)/(total area at risk)]×100.

2.6. Determination of tissue MPO activity

After determining infarct size, tissue samples weighing approximately 0.3 g were taken from the nonischemic and necrotic zones for analysis of MPO activity, a maker of neutrophil accumulation in myocardium. The samples were frozen and stored at −70°C until assayed. The activity of MPO was measured spectrophotometrically at 460 nm (SPECTRAmax, Molecular Devices, Sunnyvale, CA, USA) and expressed as units (U) per 100 mg tissue as previously described [14].

2.7. Tissue preparation

Myocardial tissues (150–200 mg for each) from nonischemic and necrotic zones were isolated by visual inspection after harvesting heart before TTC staining. The tissues for identifying apoptosis were then freshly placed in tubes for DNA isolation, and in molds oriented appropriately for sectioning and embedded in optimal cutting temperature compound (O.C.T., Miles Laboratories), frozen in liquid nitrogen, and stored at −70°C in airtight bags for detection of apoptotic cells using the terminal transferase UTP nick end labeling (TUNEL) method. Cryosections (7 μm) were obtained using a Hacker–Bright cryostat and thaw-mounted onto Vectabond (Vector, Burlingame, CA, USA) coated slides or Fisher-Plus (Fisher) slides, refrozen, and stored at −70°C with desiccant until use.

2.8. DNA isolation and gel electrophoresis

Freshly frozen nonischemic and necrotic myocardium (20–30 mg) was minced in 600 μl of lysis buffer (Puregene DNA Isolation Kit) and were quickly homogenized using 30–50 strokes with a microfuge tube pestle. The tissue was digested with 100 μg/ml of proteinase K (Sigma) at 56°C for 3–4 h and incubated with Rnase A at 37°C for 1 h. After incubation, tissues were precipitated and centrifuged at 13 000 *g* for 5 min. Supernatants containing DNA were precipitated with isopropanol. After centrifugation at 13 000 *g* for 5 min, the resulting DNA pellets were washed with 75% ethanol and dissolved in DNA hydration solution at 260 nm by spectrophotometry. Ten μg of DNA were loaded into 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. DNA electrophoresis was carried out at 80 V for 1 to 2 h. DNA ladders, an indicator of tissue apoptotic nucleosomal DNA fragmentation, were visualized under ultraviolet light and photographed for permanent records.

2.9. In situ detection of cell death

Freshly frozen nonischemic and necrotic myocardium were cut at 6–7 mm thickness, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, and incubated with proteinase K (1 μg/ml) in PBS for 30 min. DNA fragments in the tissue sections were determined using an in situ cell death detection kit (Boehringer Mannheim, Ridgefield, CT, USA). Briefly, the enzyme, terminal deoxynucleotidyl transferase (TdT) was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragments. The signal of TdT-mediated dUTP nick end labeling (TUNEL) was then detected by an anti-fluorescein antibody conjugated with alkaline phosphatase, a reporter enzyme that catalytically generates a red-colored product from Vector Red substrate. The slides were dehydrated in graded alcohols and coverslipped with hematoxylin counterstaining. The slides were washed, dried, and mounted in Permount medium. For each slide, color video images of 280–360 μm fields were captured and digitized by use of a ×25 objective with a Sony DXC-760MD video camera, a RasterOps 24 XLTV video card, and Media Grabber software on a Macintosh Quadra 950 computer. The cells with clear nuclear labeling were defined as TUNEL-positive cells. The apoptotic cells were calculated as percentage of TUNEL-positive cells using the following formula: number of TUNEL-positive cell nuclei/(number of TUNEL-positive cell nuclei + number of total cell nuclei)×100.

2.10. Immunohistochemistry

Immunohistochemistry for identifying expression of common β-subunits of adherence-promoting glycoprotein (CD18) on PMNs was performed as previously described [16]. After fixing in acetone and air drying, the sections were blocked for endogenous peroxidase with 0.3% H_2O_2/methanol, and then treated with 1% gelatin in PBS for blocking nonspecific binding. The sections were incubated with monoclonal anti-PMN antibody (R15.7, 10 mg/ml, a gift from Dr. Robert Rothlein, Boehringer-Ingelheim, Ridgefield, CT, USA) for 1–2 h. The slides were then washed in PBS, and incubated with a 1/400 dilution of biotinylated horse anti-mouse IgG (Vector). The sections were stained using the ABC-peroxidase kit (Vector) and substrated with 3,3′-diaminobenzidine tetrahydrochloride (Sigma). The slides were dehydrated in graded alcohols and Americlear and coverslipped with or without hematoxylin counterstaining. Single- and double-label immunohistochemistry experiments were controlled by either elimination of the primary antibody or incubation of the tissue with a nonimmune IgG. To identify whether apoptotic cells were PMNs, double immunohistochemical analysis was performed by staining first with TUNEL followed by anti-CD18 antibody for PMNs.

2.11. Western blot analysis of Bcl-2 and Bax proteins

Total proteins were extracted from normal and ischemic zones of LV. One hundred mg heart tissue were placed in 500 μl lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate: 0.1% sodium dodecyl sulfate [SDS] in 1XPBS) containing inhibitors, and homogenized at 4°C for 20 s, incubated on ice for 2–3 h, and then centrifuged twice at 16 000 *g* for 20 min. Protein concentration was measured by the DC Protein Assay (BioRad). Fifty to eighty μg of total protein were mixed with loading buffer (5% beta-mercaptoethanol, 0.05% bromophenol blue, 75 mM Tris–HCl, pH 6.8, 2% SDS and 10% glycerol), boiled for 2 min, and loaded onto 4–20% gradient SDS–polyacrylamide gel using Mini Protean II Dual Stab Cell (BioRad). Proteins were transferred to nitrocellulose filters in the presence of glycine/methanol transfer buffer (20 mM Tris base, 0.15 M glycine, 20% methanol) in Mini Protean II Transfer system (BioRad). Nitrocellulose filters were blocked with 5% milk in 1×TBS-T buffer (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. Membrane was subsequently exposed to rabbit polyclonal anti-rat Bcl-2 and rabbit polyclonal anti-rat Bax (Pharmingen, San Diego, CA, USA) at 1:1000 concentration in 5% milk in TBS-T for 1 h, respectively. Bound antibody was detected by horseradish peroxidase conjugated anti-rabbit IgG. Finally, enhanced chemiluminescence (ECL) detection reagents were employed to visualize peroxidase reaction products (Amersham). Bcl-2 protein was detected as a 29-kilodalton (kDa) band, and Bax protein was detected as a 26-kDa band using molecular weight marker bands. The intensities of bands were determined by the National Institute of Health (NIH) image program.

2.12. Statistical analysis

A one-way analysis of variance (ANOVA) followed by Duncan's post hoc test was used to analyze group differences such as PMN localization and infarct size. Hemodynamic data and other time-dependent determination were analyzed by repeat measure ANOVA. A $P<0.05$ was considered significant. Results were reported as mean and standard error of the mean (SEM).

3. Results

3.1. Hemodynamics

Hemodynamic data for heart rate and mean arterial pressure in the two groups are shown in Table 1. There were no significant differences in any measured variables between the two groups at baseline. Coronary occlusion was associated with an increase in heart rate in both groups, but it did not reach to significant difference compared with control. However, heart rate was significantly increased at the end of the experiment in both groups. There was no change in mean arterial pressure during the experiment in the two groups.

3.2. Regional myocardial blood flow

Distribution of transmural myocardial blood flow to the nonischemic and ischemic zones is shown in Fig. 1. During LAD occlusion, blood flow in the nonischemic myocardium in both groups remained unchanged while blood flow in the area at risk myocardium was reduced by approximately 98% from baseline value**.** There was no difference between the two groups in blood flow at the end of 7 h and 60 min of coronary occlusion in the necrotic area, excluding the role of collateral blood flow in developing cell death during ischemia.

Table 1
Hemodynamic data during the course of experiment[a]

	PI group		I/R group	
	HR	MAP	HR	MAP
Control	100±5	74±5	91±11	71±3
Ischemia	138±4	77±4	114±1	67±5
End	148±3*	69±6	145±10*	66±2

* $P<0.05$ vs. Control in each group.

[a] Ischemia, 1 h into coronary occlusion; End, 7 h of ischemia at PI group and 6 h of reperfusion at I/R group. HR, heart rate (beats/min); MAP, mean arterial pressure (mmHg). Values are mean±SEM.

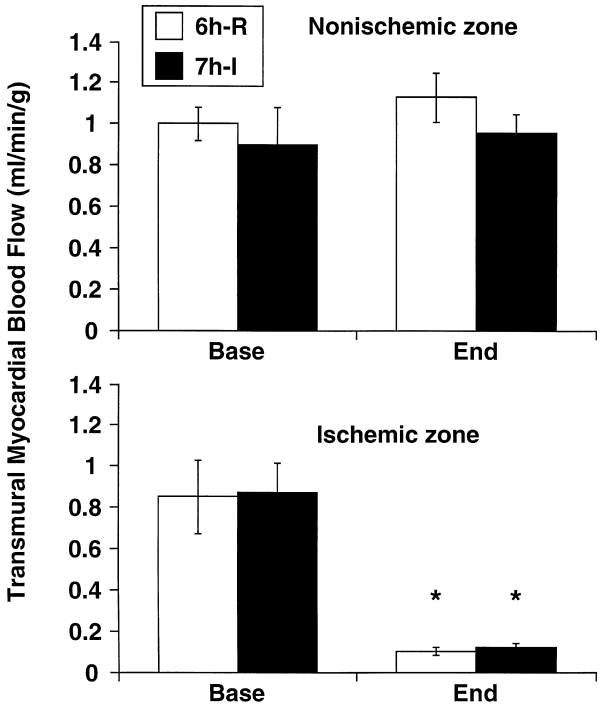

Fig. 1. Bar graph showing regional transmural myocardial blood flow in the nonischemic zone (top panel) and ischemic zone (bottom panel) after 1 h of LAD occlusion and 6 h of reperfusion (6h-R) or 7 h of LAD occlusion without reperfusion (7h-I). Bars represent group mean; brackets indicate SEM. * $P<0.05$ vs. baseline values for each group.

3.3. Area at risk and infarct size

The area placed at risk by coronary occlusion expressed as a percent of the left ventricular mass (area at risk/LV), and the area of necrosis expressed as a percent of the area at risk (area of necrosis/area at risk) are shown in Fig. 2. Area at risk/LV was comparable in both groups ranging between 24 and 31% in the PI group and 25–30% in the I/R group. Area of necrosis/area at risk averaged 72±5% at the end of 7 h of ischemia in the PI group while area of necrosis/area at risk averaged significantly less (26±2%) in the I/R group.

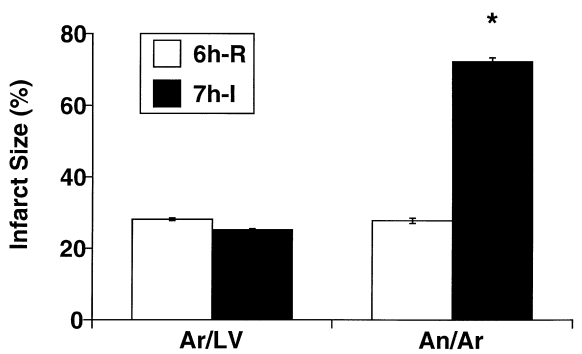

Fig. 2. Bar graph showing the size of the area at risk relative to the left ventricle (Ar/LV) and infarct size data expressed as a percent of the area at risk (An/Ar) after 1 h of LAD occlusion and 6 h of reperfusion (6h-R) or 7 h of LAD occlusion without reperfusion (7h-I). Bars represent group mean; brackets indicate SEM. * $P<0.05$ vs. 6h-R group.

3.4. DNA fragmentation

DNA fragmentation of myocytes in the nonischemic and necrotic areas in both groups is shown in Fig. 3. Seven h of permanent LAD occlusion and 1 h of LAD occlusion followed by 6 h of reperfusion did not cause visible DNA ''ladders'' in the nonischemic zone. However, DNA ladders were clearly detected in the necrotic zone of all hearts in the I/R group, indicating the presence of apoptotic internucleosomal DNA fragmentation. There was no ''smear'' background pattern found, which has been suggested to indicate random DNA fragmentation involved in necrotic cell death [2,17]. No visible DNA ''ladders'' were found in the PI group, consistent with an absence of TUNEL-positive cells in the necrotic area of this group (see below).

3.5. Detection of apoptotic cells

The TUNEL staining was performed in the necrotic area in both groups. No apoptotic cells were found in the nonischemic zone in either group (Fig. 4A). Although infarct size in the PI group after 7 h of ischemia averaged 72±5%, very few TUNEL-positive cells were detected in the necrotic area (0.2±0.1% of total nuclei, Fig. 4B), suggesting that necrotic cells are not stained by the TUNEL staining in this setting. However, TUNEL-positive cells expressed as the percent of total nuclei were significantly increased in the necrotic area in the I/R group

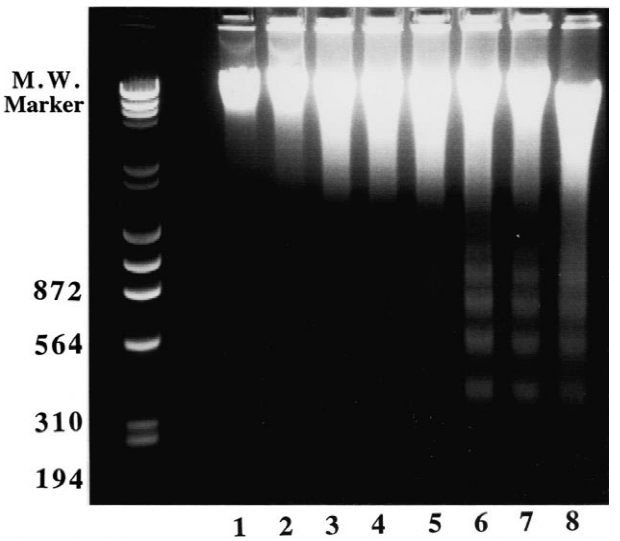

Fig. 3. DNA fragmentation using agarose gel electrophoresis. Lanes 1 and 2 represent normal tissue; lanes 3, 4 and 5 represent necrotic tissue after 7 h of LAD occlusion without reperfusion; lanes 6, 7 and 8 represent necrotic tissue after 1 h of LAD occlusion and 6 h of reperfusion. The figure is representative of at least six separate experiments.

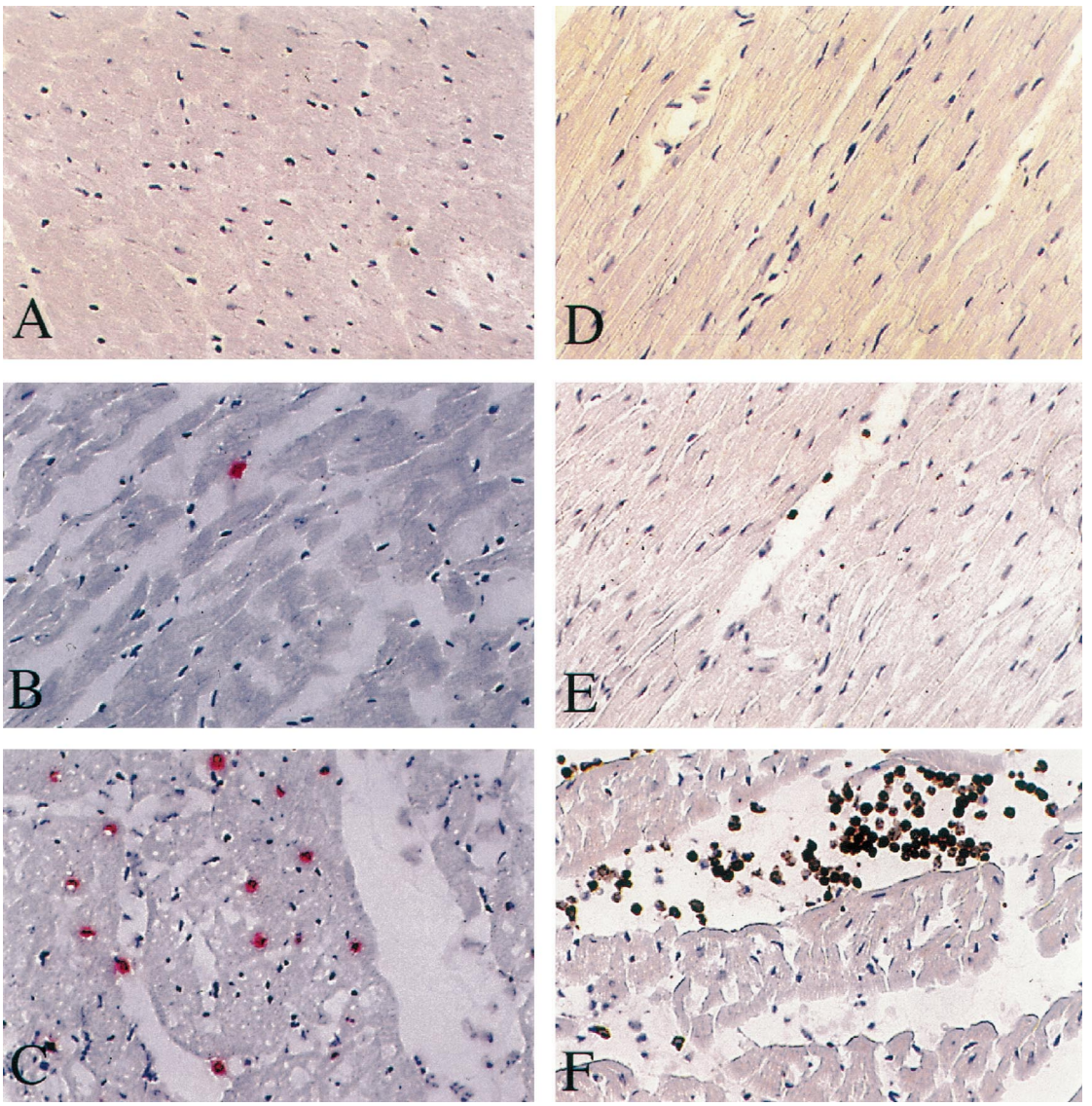

Fig. 4. Detections of apoptotic myocytes using the terminal transferase UTP nick end labeling (TUNEL) technique and PMN accumulation using immunohistochemistry with anti-CD18 antibody in nonischemic zones (A and D), necrotic zones (B and E) after 7 h of LAD occlusion without reperfusion and necrotic zones (C and F) after 1 h of LAD occlusion and 6 h of reperfusion. Red and brown staining indicate apoptotic myocytes and accumulated PMNs, respectively (magnification ×400). Apoptotic myocytes and accumulated PMNs were not detected in the nonischemic zone after ischemia and reperfusion. Figures are representative of at least six separate experiments.

(26±4%, $P<0.01$) (Fig. 4C) compared with the PI group. These results were consistent with the appearance of DNA ladders in the necrotic zone in the I/R group. To exclude the possibility that apoptotic cell death may also occur in the early phase of ischemia due to its dynamic processing nature, TUNEL staining was performed on hearts from two dogs at the end of 1 h ischemia without reperfusion. No clear TUNEL staining was detected from these tissue sections, further supporting that apoptosis is a reperfusion-triggered phenomenon.

3.6. Detection of PMN accumulation

PMN accumulation in the necrotic myocardium in both groups was detected by immunohistochemistry with anti-PMN CD18 antibody, R15.7 and MPO activity. In both groups, CD18-positive PMNs were absent in the nonischemic tissue (Fig. 4D). Few CD18-positive cells (mm^2 myocardium) were found in the necrotic area in the PI group (24±2.8, Fig. 4E), however, ischemia–reperfusion significantly increased CD18-positive cells in the necrotic myocardium to 358±24 ($P<0.01$ versus PI group, Fig. 4F). Consistent with the increase in numbers of positive CD18 cells in the necrotic zone, MPO activity (U/100 mg tissue) was significantly greater in the I/R group versus the PI group (2.9±0.3 vs. 0.4±0.1, $P<0.01$). There was a significant linear correlation between TUNEL-positive cells and CD18-positive cells ($y = 2.486 + 0.067x$, $r =$

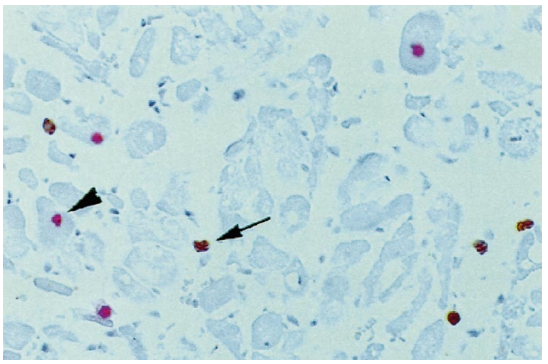

Fig. 5. Double staining using the terminal transferase UTP nick end labeling (TUNEL) technique and immunohistochemistry with anti-CD18 antibody on the same tissue slide after 1 h of LAD occlusion and 6 h of reperfusion (magnification ×400). Brown staining (arrow) indicates PMNs and red staining (arrow head) indicates apoptotic myocytes, respectively. An absence of TUNEL-positive PMN suggests that apoptotic cells are not PMNs. Figures are representative of at least four separate experiments.

0.869, $P<0.05$). To confirm whether TUNEL-positive cells also included PMNs, double staining with TUNEL and anti-CD18 was performed on the same slide. Few CD18-positive cells were stained by the TUNEL method (Fig. 5), suggesting that TUNEL-positive cells are myocytes.

3.7. Expression of Bcl-2 and Bax proteins

Bcl-2 and Bax protein extracts by Western blot analysis isolated from necrotic myocardium are shown in Figs. 6 and 7. Bcl-2 and Bax were present in the nonischemic zone in both groups. Densitometrically, 1 h of ischemia and 6 h of reperfusion was significantly associated with less expression of Bcl-2 by 16±1% and greater expression of Bax by 29±8% compared with nonischemic tissue, respectively, $P<0.05$. However, expression in these two proteins was not changed in necrotic myocardium after 7 h of ischemia without reperfusion compared with nonischemic tissue.

4. Discussion

The results of the present study indicate that, in dogs, apoptosis defined by DNA fragmentation and in situ TUNEL labeling only appeared in myocardium subjected to a brief period of ischemia followed by reperfusion, and not in ischemic tissue without reperfusion. Lack of apoptotic cells in necrotic myocardium after permanent ischemia suggests that cell death is mainly mediated by a necrotic pathway. Experiments in both groups lasted 7 h, therefore, the effect of time course on development of apoptotic cell death can be excluded. Changes in expression of Bcl-2 and Bax proteins in the I/R group were consistent with appearance of apoptotic mechanisms. Accumulated neutrophil may be involved in triggering apoptosis during the early phase of reperfusion.

The two popular methods, DNA laddering by agarose gel electrophoresis and histochemical visualization of nuclear DNA fragments by the TUNEL staining have been widely used to define apoptosis in rat, rabbit, dog and human myocardium [1,2,5,10,18–20]. Cell death by apoptosis is characterized biochemically by internucleosomal DNA fragmentation, causing DNA laddering on agarose gel electrophoresis coupled with histochemical detection of double-stranded DNA fragmentation by the TUNEL method. However, there are some recent reports suggesting that the appearance of both DNA laddering and TUNEL-positive cells are not absolutely specific for apoptosis because some of the live and necrotic cells may be occasionally positively stained by the TUNEL method [17,21]. In the present study, the absence of TUNEL-positive cells in the nonischemic zone in both groups confirmed that live cells are not stained by the TUNEL method. In addition, the lack of TUNEL-positive cells in the necrotic zone in the permanently ischemic group indicated that TUNEL-positive cells in the ischemia/reperfusion group are not necrotic cells. Furthermore, an absence of the diffuse smear pattern of random DNA breakdown on gel electrophoresis in the present study, which may occur in necrosis [2,21], also supported that TUNEL-positive cells are not necrotic cells. Although there is an issue that identification

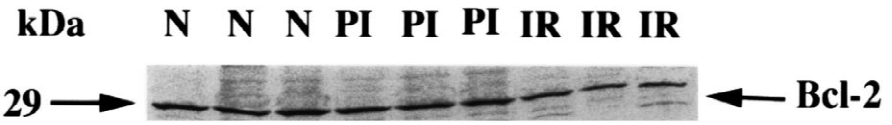

Fig. 6. Expression of Bcl-2 protein was visualized in nonischemic (N) and necrotic myocardium by Western blot analysis after 7 h of LAD occlusion without reperfusion (PI) and 1 h of LAD occlusion and 6 h of reperfusion (I/R). Figures are representative of at least four separate experiments.

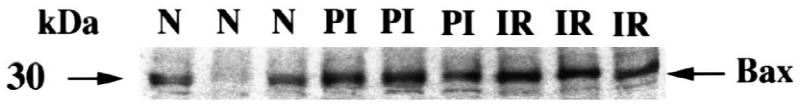

Fig. 7. Expression of Bax protein was visualized in nonischemic (N) and necrotic myocardium by Western blot analysis after 7 h of LAD occlusion without reperfusion (PI) and 1 h of LAD occlusion and 6 h of reperfusion (I/R). Figures are representative of at least four separate experiments.

of myocardial tissue DNA fragmentation may also include other cell types [7,21], such as infiltrated neutrophils, the absence of TUNEL-positive neutrophils confirmed by double staining in the present study, suggested that DNA fragmentation on gel electrophoresis was associated with mostly myocyte DNA fragmentation. This observation is consistent with previous reports which have reported the absence of neutrophil apoptosis in cats and dogs after ischemia/reperfusion, and in human infarct myocardium using in situ nick end labeling (ISEL) staining [22,23]. Therefore, the present results clearly show that myocyte apoptosis is primarily triggered after ischemia followed by reperfusion but not by ischemia alone. It could be possible that the ischemic insult elicits a process for apoptosis to occur but this pathological process is not completed due to the depletion of ATP supply during ischemia [9,20,24]. There is no convincing conclusion to explain mechanisms of reperfusion-induced apoptosis. However, the inflammatory responses that ensues during reperfusion appears to contribute significantly to the triggering in expression of apoptosis [1,5,11].

Using TUNEL staining and electron microscopy, Ohno et al. [25] recently reported that TUNEL-positive cells are actually cells undergoing oncosis in a rabbit model of 30 min of ischemia and 4 h of reperfusion. They suggested that apoptotic cell death during ischemia and reperfusion reported in other studies [1,5,19] may belong to the category of oncosis. In the present study, we cannot distinguish the difference between apoptosis and oncosis owing to the limitation of the methods used. Studies, however, have confirmed the ultrastructural evidence of apoptotic changes in dog myocardium after ischemia and reperfusion by TUNEL staining and electron microscopy [9,18,24].

Many genes have been reported to be linked with the regulation of programmed cell death under physiological and pathological conditions, but the Bcl-2 family has been suggested as a major, controlling point in the pathway to apoptotic cell death. Bcl-2 possesses a putative transmembrane domain including mitochondria, endoplasmic reticulum, and nuclear membrane. Its family members consist of Bcl-2, Bcl-x, Bcl-w (three main inhibitors of apoptosis), Bax, and Bak (two main accelerators of apoptosis). The ratio of Bcl-2 to Bax proteins has been suggested to determine survival or death after an apoptotic stimulus. Several mechanisms in which Bcl-2 is involved in regulation of the anti-apoptotic pathway have been proposed including a direct antioxidant effect, inhibition of release of proapoptotic proteins, and attenuation of cytotoxic effect of proapoptotic regulators such as Bax and Bak [7,8]. In the present study, ischemia/reperfusion significantly decreased the expression of Bcl-2 and increased the expression of Bax in necrotic myocardium, consistent with the suggested roles of these proteins in regulation of apoptotic cell death. In addition to an increase in Bax and a decrease in Bcl-2 after reperfusion, some other members of the Bcl-2 family such as Bcl-x and Bak, may also be involved in regulation of apoptosis. However, these proteins were not measured in the present study. The anti-apoptotic effect of Bcl-2 in salvaging ischemic myocyte and proapoptotic effect of Bax in mediating apoptotic cell death have been also reported in clinical and experimental studies [12,13]. It has been reported that infiltrated inflammatory cells such as neutrophils at the late stage of infarction may undergo apoptotic death pathway. Therefore, these cells could be experienced in the TUNEL-positive staining and may induce changes in expression of Bcl-2 and Bax [17]. However, the acute time course of the present study (6 h of reperfusion) and the absence of TUNEL-positive neutrophils in the necrotic myocardium suggested that neutrophils may contribute to the overall postischemic apoptosis in the later phase of reperfusion. Changes in Bcl-2 and Bax may be primarily induced by apoptotic myocytes during early reperfusion.

The role of neutrophils in ischemia/reperfusion-induced myocardial injury has been widely investigated in recent years. The major morphologically identified difference in the necrotic tissue induced by permanent ischemia and ischemia followed by reperfusion in the present study was neutrophil accumulation. Significantly increased neutrophil accumulation in ischemic/reperfused myocardium indicates that there may be different mechanisms involved in cell death pathway in permanent ischemia and ischemia followed by reperfusion. In this connection, Reimer et al. [26] have reported that the progression of cell death after permanent occlusion injury is primarily related to the transmural distribution of coronary collateral blood flow. However, comparable measurement of transmural myocardial blood flow in ischemic myocardium during coronary occlusion in both groups allowed us to exclude a role of blood flow change in determining the extent of cell death. Although the role of neutrophils in the development of reperfusion-induced necrotic cell death has been well investigated [22,27–30], the precise effect of neutrophil accumulation in ischemic myocardium on apoptosis still remains unclear at the present time. Controversial results have been reported. One study from a rabbit model of ischemia and reperfusion showed that granulocytopenia did not prevent reperfusion induced apoptosis [1], while another study in the rat has shown that increased neutrophil accumulation in ischemic myocardium is associated with augmentation of apoptotic cell death [5]. As shown in the present study, increased TUNEL-positive cells after reperfusion were in proportion to neutrophil accumulation, suggesting a role of neutrophils in modulating apoptotic cell death. Although the factors that may induce apoptosis in the ischemic/reperfused myocardium are not fully understood, studies have shown that proinflammatory mediators such as reactive oxygen species and various cytokines released from activated neutrophils may trigger the development of apoptosis [1,5,11]. However, our results did not allow us to conclude a role of neutrophils in

induction of apoptotic cell death. Direct experimental proof confirmed by anti-neutrophil therapy before reperfusion in vivo is required.

In conclusion, the present study demonstrates that both necrosis and apoptosis coexist in necrotic myocardium after ischemia/reperfusion. However, apoptosis was not observed in infarcted myocardium in the absence of reperfusion. Apoptosis, as a distinct form of cell death triggered during reperfusion, is associated with alterations primarily occurred during reperfusion. The downregulation of Bcl-2 and the upregulation of Bax principally in myocytes may represent the molecular triggers or modulators of apoptotic cell death in ischemic/reperfused heart.

However, more studies are needed to identify the precise mechanism responsible for reperfusion-induced apoptosis. Although apoptosis is now recognized as a new contributor of cell death in ischemia/reperfusion injury, there are no cardiac models to show how apoptosis is operative especially during the long-term of reperfusion. Because apoptosis represents a potentially preventable form of cell death owing to its active nature, understanding of this genetic cell death pathway may leads to potential clinical therapeutic strategies for altering reperfusion injury by inhibiting apoptosis.

Acknowledgements

The authors are grateful for the technical contributions of Sara Katzmark, Susan Schmarkey and Jill Robinson in performing this study, and for the assistance of Gail H. Nechtman in preparing the manuscript. The authors also thank Dr. Robert Rothlein of Boehringer-Ingelheim (Ridgefield, CT, USA) and Dr. C. Wayne Smith of Department of Pediatrics, Baylor College of Medicine (Houston, TX, USA) for the generous supply of the antibodies used in this study. This work was supported by grants from National American Heart Association (Scientist Development Award, Z.-Q. Zhao, and Grant-In-Aid, J. Vinten-Johansen) as well as the Carlyle Fraser Heart Center of Emory University.

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