Balanced expression of mitochondrial apoptosis regulatory proteins correlates with long-term survival of cardiac allografts

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Tung, Thomas C., Guanggen Cui, Kiyohiro Oshima, Hillel Laks, and Luyi Sen. Balanced expression of mitochondrial apoptosis regulatory proteins correlates with long-term survival of cardiac allografts. Am J Physiol Heart Circ Physiol 285: H2832–H2841, 2003. First published August 7, 2003; 10.1152/ajpheart.00054.2003.—Abnormal regulation of apoptosis is observed in ischemic injury and may contribute to the pathogenesis of atherosclerosis. However, its role in cardiac allograft vasculopathy (CAV), the fundamental lesion of chronic rejection (CR) in heart transplantation, remains uncertain. To clarify this issue, apoptosis was quantitated in myocardium and coronary arteries from 5 cardiac allograft donors (NL) and explanted hearts of 24 patients with ischemic cardiomyopathy (IsCM) and 15 patients with CR. Tissue samples were analyzed via end-labeling fragmented DNA [via deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)] and immunoblotting for activated caspase-3 and -9. Myocyte apoptosis assessed by TUNEL was similarly increased over NL (0.21%) in both the CR (0.88%; P < 0.01) and IsCM (0.88%; P < 0.01) groups. Activated caspase-9 levels were significantly higher in CR (14.7%) compared with IsCM (6.9%; P < 0.01) and NL (0%) groups, whereas activated caspase-3 levels were similarly elevated in both CR and IsCM (7.8 and 6.5% vs. 0% in NL; P < 0.01 and P < 0.05) groups. Expression of myocardial Bcl-2 and Bax was increased in CR compared with both NL (Box, 4.3-fold; P < 0.01; Bcl-2, 5.9-fold; P < 0.01) and IsCM (IsCM: Bax, 2.2-fold; P < 0.05; Bcl-2, 3.2-fold; P < 0.01) groups. The rate of apoptosis and the Bcl-2/Bax ratio independently correlated to graft survival in CR (activation of caspase-9: r = 0.87; P < 0.01; Bcl-2/Bax: r = 0.57; P = 0.05). Compared with native atherosclerosis, coronary arteries with CAV showed more medial apoptosis (7.8-fold; P < 0.01) and higher Bcl-2 levels (5.1-fold; P < 0.01) with lower Bax levels (threefold; P < 0.05) in the intima. These results indicate that abnormal Bcl-2 and Bax expression in myocardium and coronary arteries of cardiac allografts with CR is distinct from that in IsCM and suggest that balancing Bcl-2 to Bax in transplanted hearts promotes long-term graft survival.

CHRONIC REJECTION (CR) in the form of cardiac allograft vasculopathy (CAV) limits long-term survival among heart transplant recipients (12, 29, 31). Unlike the focal epicardial coronary lesions of native atherosclerosis (NA), CAV causes diffuse narrowing of the entire vascular tree of the allograft (12). This global vascular narrowing places transplant recipients at high risk for myocardial infarction, sudden death, and heart failure (29) and also causes ongoing subclinical graft injury (32). Because these events are often sudden and irreversible, alternative nonclinical measures of disease progression are required to allow timely diagnostic and therapeutic interventions for these patients.

The fundamental cellular process of apoptosis contributes to myocyte loss in many forms of ischemic injury including acute infarction (23), ischemia-reperfusion (7), and chronic coronary occlusion (19). Apoptosis is a mechanism of cell death that unlike necrosis requires activation of distinct cellular pathways (23). Two of its biochemical hallmarks are DNA fragmentation (23) and activation of a family of cysteine proteases called caspases (3, 27). In addition to its role in ischemic myocardial injury, apoptosis contributes to the pathogenesis of atherosclerotic plaques (16, 30). Vascular smooth muscle cells from atherosclerotic lesions are exceedingly susceptible to apoptosis, and their loss could destabilize plaques and cause rupture (15, 16, 30). Additionally, evidence exists for Fas-mediated apoptosis of endothelial cells, T lymphocytes, and macrophages within the coronary arteries of explanted hearts with CAV (6, 34). The presence of apoptosis in this setting could signal vascular remodeling or inflammation (30).

Because apoptosis is relevant to both the cause and effect of myocardial ischemia, characterizing its extent and regulation during CR might provide a useful pathological measure of disease progression. In this study, we compared the extent of apoptosis in the myocardium and coronary arteries from explanted hearts of 15 retransplanted patients to hearts from 24 patients with ischemic cardiomyopathy (IsCM) and 5 normal (NL) controls. Because regulation of apoptosis is accomplished in part through the stoichiometric balance of proapoptotic to antiapoptotic Bcl-2 proteins (28), we also studied alterations in the levels of two antiapoptotic (Bcl-2 and Bcl-XL) and one proapoptotic (Bax) member of this protein family via immunoblotting and immunohistochemistry of these tissues.

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METHODS

Patients. Fifteen patients with initial graft survival in excess of 2 yr (range, 2.1–13.3 yr) underwent retransplantation at UCLA Medical Center at Los Angeles between September 1999 and June 2003. All patients received triple-drug immunosuppression that included cyclosporine, azathioprine, and prednisone with a 10-fold prednisone taper over the 6 mo after transplantation. Episodes of cardiac rejection were treated with oral prednisone boluses followed by subsequent taper, or OKT3 murine monoclonal antibody when episodes were severe. CAV was assessed by yearly planar coronary angiography and was confirmed by final pathology of explanted hearts.

Twenty-four patients with IsCM were randomly selected from recipients of a first allograft transplanted between November 1998 and June 2003. These patients were New York Heart Association (NYHA) class III or IV and were on maximal medical heart failure therapy. In all patients undergoing first or second transplantation, cardiac function was assessed by two-dimensional echocardiography and right heart catheterization in the 6 mo preceding transplantation. Coronary arteries taken as histological specimens from explanted hearts were removed from areas of palpable atherosclerotic disease. Myocardium was taken distal from visible infarct sites. Ventricular tissue from five cardiac allograft donors was used for NL controls. These nonfailing donor hearts were all trauma victims and were free of cardiovascular pathology. These control hearts came from potential donors was used for NL controls. These nonfailing donor hearts were all trauma victims and were free of cardiovascular pathology. These control hearts came from potential donors were rejected as heart transplant donors due to size mismatch, blood group incompatibility, or underlying systemic disease in one patient (hepatitis B positive). None of patients included in this study were on dobutamine or dopamine at the time of harvest in donors or explantation in recipients. This study was approved by the Committee for the Protection of Human Subjects at the UCLA School of Medicine.

DNA gel electrophoresis. Myocardial fragments were homogenized in 1 ml of DNA digestion buffer and shaken for 12 h at 50°C with 0.4 mg of proteinase K. The fragments were then extracted with an equal volume of phenol-chloroform-isooctyl alcohol, and 0.5 ml of 7.5 M ammonium acetate and 2 ml of absolute ethanol were added to the upper aqueous layer. After centrifugation at 16,000 g for 30 min, the pellet was washed with 70% ethanol, air-dried, and resuspended in 200 μl of Tris-EDTA buffer (10 mM Tris·HCl, pH 8.0, and 0.1 mM EDTA). DNA (3 μg) from each sample was loaded onto a 2% agarose gel for electrophoresis, which was followed by ethidium bromide staining (10 mg/ml).

DNA end-labeling of tissue sections. After fixation with 4% formaldehyde in PBS, frozen tissue sections (thickness, 4–6 μm) were permeabilized with proteinase K (20 μg/ml in 10 mM Tris·HCl, pH 8.0) for 10 min at room temperature. After sections were rinsed in Tris-buffered saline ([TBS] 20 mM Tris, pH 7.6, and 140 mM NaCl), endogenous peroxidases were inactivated with 3% H2O2 in methanol. Sections were then incubated at room temperature with deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) staining. For DNA end-labeling, a minimum of 1,000 total cells (2,069 ± 900) were counted for each tissue section. For immunohistochemistry, a minimum of 1,177 total cells (2,291 ± 717) were counted for each section. The percentage of positive cells per high-powered field was compared between patient groups using the unpaired Student’s t-test with unequal variance.

For deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) staining and immunohistochemistry, positive cells and total nuclei were counted for each high-powered field at ×400 magnification. For DNA end-labeling, a minimum of 1,000 total cells (2,069 ± 900) were counted for each tissue section. For immunohistochemistry, a minimum of 1,177 total cells (2,291 ± 717) were counted for each section. The percentage of positive cells per high-powered field was compared between patient groups using the unpaired Student’s t-test. The Spearman correlation coefficient was used to assess nonparametric relationships between the Bax/Bcl-2 ratio, rate of apoptosis, and graft survival. Numeric values are expressed as means ± SD. Pearson correlation coefficients were calculated for linear regressions, and the significance for each regression was estimated by ANOVA. A probability value <0.5 was considered to be statistically significant.

RESULTS

Patient population. All 15 retransplanted patients had CAV by final pathology, and 7 had evidence of prior infarction. One of these patients (patient no. 9; Table 1) received a heterotopic transplant for right
end-labeling of myocardium. TUNEL-positive myocyte nuclei were rare in normal myocardium and usually scattered among unlabeled myocytes in diseased tissue (Fig. 1A). However, one patient with CR had a 100-fold greater frequency of end-labeled nuclei than any other patient. This patient was the only one to exhibit an oligonucleosome DNA ladder by ethidium bromide staining (Fig. 1B). The rate of myocyte apoptosis (Fig. 1C) by TUNEL in patients with either CR or IsCM (8.8 and 2.1 positive nuclei per 1,000 cells) exceeds that of NL controls by 4.35-fold (P < 0.01 for both disease groups). Because the rate of TUNEL-positive nuclei (15%) in the patient with the visible nucleosome ladder introduced unrepresentative variability to the average from the CR group, this patient was excluded from comparative analyses.

**Activation of caspase-3.** The enzymatically inert 32-kDa form of caspase-3 is cleaved to active 11- and 17-kDa subunits during apoptosis (27). To assess activation of caspase-3, total myocardial cell lysates from all patients were assessed by immunoblotting with an antibody that recognizes both the full-length 32-kDa form and the active 11-kDa subunit. Caspase-3 is activated in the myocardium of patients with either CR or IsCM (Fig. 2A). No activated caspase-3 is seen in NL controls. The fraction of activated caspase-3 (Fig. 2B) is 7.8% among patients with CR and 6.5% among patients with IsCM (P > 0.05); both of these figures are significantly higher than NL controls (CR, P < 0.01; IsCM, P < 0.01). Caspase-3 activation correlates with the degree of DNA end-labeling among all 14 patients with CR or 15 with IsCM (Fig. 2C; r = 0.93; P < 0.01).

**Activation of caspase-9.** In mitochondrial-dependent apoptosis, cytochrome c is released from mitochondria...
into cytosol and then interacts with Apaf-1, thereby triggering the 46-kDa procaspase-9, which proteolytically cleaves itself to form the 35-kDa active caspase-9 \((33, 36)\). Caspase-9 can signal downstream and activate procaspase-3 and -7. Caspase-9 is activated in the myocardium of patients with either CR or IsCM (Fig. 2A). No activated caspase-9 is seen in NL controls. The fraction of activated caspase-9 (Fig. 2D) in patients with CR was 14.7%, which was significantly higher than in patients with IsCM (6.9%; \(P < 0.01\)). Caspase-9 activation significantly correlates with the degree of DNA end-labeling among all 14 patients with CR or 15 with IsCM (Fig. 2C; \(r = 0.87; P < 0.01\)).

Expression of apoptosis regulatory proteins. Immunoblotting of myocardial tissue homogenates from all patients in the study reveals increased expression of proapoptotic Bax (4.3-fold; \(P < 0.01\)) and antiapoptotic Bcl-2 (5.9-fold; \(P < 0.01\)) among patients with CR relative to NL controls (Fig. 3). Patients with CR also show a weak trend toward greater Bcl-xL expression (3.2-fold; \(P = 0.05\)). For Bcl-2, protein levels among patients with CR exceed those from IsCM patients (3.2-fold; \(P < 0.01\)). For Bax, there is a strong trend toward greater (2.2-fold) Bax expression in hearts with CR than in IsCM (\(P = 0.05\)).

Immunohistochemistry of left ventricular tissue sections from patients with CR and NL controls also shows that the myocardial expression of these three proteins is significantly elevated during CR (\(P < 0.01\); Fig. 4). Hearts from patients with IsCM also show significant increases in the percentage of myocytes that express these proteins compared with NL controls. Additionally, hearts from patients with CR exhibit a significantly greater percentage of positive myocytes compared with IsCM specimens for Bax (2.2-fold; \(P < 0.01\)), Bcl-2 (1.4-fold; \(P < 0.01\)), and Bcl-xL (1.8-fold; \(P < 0.01\)). These findings are similar to those seen from immunoblot analysis.

Apoptosis and graft survival. The rate of apoptosis measured through activation of caspase-3 has a weak linear correlation with graft survival (\(r = 0.39; P = 0.08\); Fig. 5A). In contrast, as shown in Fig. 5B, the percentage of activated caspase-9 in procaspase-9 is significantly correlated with graft survival (\(r = 0.87; P < 0.01\)). The activation of caspase-9 is significantly correlated with the ratio of Bcl-2/Bax expression (\(r = 0.59; P < 0.05\); Fig. 5C). The Bcl-2/Bax ratio by densitometry correlates with the graft survival (\(r = 0.57; P < 0.05\); Fig. 5D).

Apoptosis in atherosclerotic coronary arteries. End-labeling of fragmented DNA in atherosclerotic epicardial arteries from 12 patients with CR (22 vessels) and 11 patients with IsCM (21 vessels) demonstrated diff-

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**Fig. 1.** A: DNA end-labeling of fragmented nuclei (arrows) from the myocardium of a normal control (NL), a heart that exhibited chronic rejection (CR) that does not have a visible oligonucleosome ladder, and a heart with CR that shows positive laddering. Magnification is \(\times 400\) for all fields. B: oligonucleosome DNA ladder revealed by ethidium bromide staining in left ventricular myocardium of one patient. C: frequency of end-labeling in NL, CR, and ischemic cardiomyopathy (IsCM) groups.
ferences in the location of apoptosis between these two diseases. Although the overall prevalence of apoptosis is identical, the intimal apoptosis was more prominent in IsCM than in CAV (Fig. 6). In contrast, the number of end-labeled nuclei within the media was 7.8-fold greater ($P < 0.01$) in CAV compared to NA (Fig. 6B) vessels. Markedly more apoptosis was seen in regions near necrotic cores of NA plaques, which accounts for a widely variable rate of nuclei end-labeling in the intima of these samples (Fig. 6B).

Fig. 2. A: activated caspase-3 and -9 increases revealed by immunoblotting during CR and IsCM compared with NL controls. B, D: percentages of activated caspase-3 and -9 were significantly increased during both CR and IsCM. C: percentages of activation of caspase-3 and -9 show a linear correlation to the frequency of end-labeled nuclei.

Fig. 3. A: immunoblotting shows that in hearts with CR and IsCM, levels of proapoptotic Bax and antiapoptotic Bcl-2 and Bcl-xL were increased. B, C, D: densitometric analyses show that expression in hearts with CR was significantly greater than NL hearts for Bax and Bcl-2. Increases in Bcl-2 in hearts with CR were also greater than in hearts with IsCM. (Average optical density units are arbitrary and are not comparable between different proteins.)
Immunohistochemical staining for Bcl-2, Bcl-xL, and Bax reveals that within the intima of NA lesions, Bax expression (Fig. 7, A and C) is increased 3.1-fold ($P < 0.05$), whereas Bcl-2 expression (Fig. 7, B and C) is 5.1 times lower ($P < 0.01$) compared with CAV. In contrast, a greater Bcl-xL expression with a wide variable rate was found in NA (threefold; $P < 0.05$). Although apoptosis is increased within the media of patients with CAV compared with those with NA, no significant difference in the expression of these three Bcl-2 family proteins occurred within this layer of the vessel wall (data not shown).

**DISCUSSION**

This study demonstrates that myocytes in transplanted hearts with chronic graft rejection undergo apoptosis at a rate similar to that seen with IsCM, which suggests a shared proapoptotic stress in these pathologies. However, a relatively high rate of caspase-9 activation in the myocardium from the hearts with CR suggests the importance of mitochondrial regulation in the mechanism of apoptosis. The expression of activated caspase-9, proapoptotic Bax, and antiapoptotic Bcl-2 in myocardium from hearts with CR was much more prominent than in hearts with IsCM as is shown by both immunohistochemical staining and immunoblotting. Of greater importance for each individual patient undergoing CR is that the ratio of myocardial Bcl-2 to Bax correlates with overall graft survival. The proportion of activated caspase-3 only has a logarithmic, not linear, relationship to graft survival, whereas activated caspase-9 has a significant linear relationship with graft survival; this further indicates the importance of the mitochondrial mechanism in triggering the caspase cascade in CR. Despite the independent predictive values of apoptosis rates and Bcl-2/Bax ratios for graft survival, these parameters show poor overall correlation with one another largely due to cases with high apoptosis rates in earlier graft failures. Alternative mechanisms of apoptosis independent of Bcl-2 and Bax could account for the additional apoptosis in these cases including cell surface-mediated pathways such as Fas or tumor necrosis factor (4). Rather than simply representing an alternative measure for the rate of apoptosis, the Bcl-2/Bax ratios among these patients reflect chronic apoptotic stresses with other stimuli potentially supervening in cases of early graft failure.
Ischemia and apoptosis in CR and IsCM. The rate of myocyte apoptosis found in this study for hearts with either IsCM or CR (8.7 per 1,000 cells) is within the range reported by others for IsCM. We are unaware of any other reports on the rate of myocardial apoptosis within the hearts of patients with CR. Narula et al. (20) found rates of apoptosis as high as 17% among three patients with IsCM. Although some authors report apoptotic indices of similar magnitude (25), others have found exponentially lower rates of myocyte apoptosis during IsCM (3, 18). End-labeling of nuclei with fragmented DNA is the most common method of describing apoptosis in these studies. However, this technique has well-described shortcomings including the labeling of cells undergoing necrosis or DNA repair rather than apoptosis (14, 21). Given the interobserver variability and the known deficiencies of DNA end-labeling, we corroborated our findings with a second specific measure of apoptosis: activation of caspase-3. Among our patients with either CR or IsCM, both activated caspase-3 and myocardial DNA fragmentation were increased compared with NL controls, and the values correlate well with one another.

The similarities in the rates of apoptosis from hearts with IsCM and CR indicate that the principal trigger for cell loss is likely myocardial ischemia. Ischemia causes apoptosis of ventricular myocytes in cell culture and animal models during acute injury with reperfusion or during periods of chronic ischemia (9, 11, 13). Inhibition of apoptosis via either transfection of the antiapoptotic Bcl-2 gene (13) or administration of caspase inhibitors (9) protects against this injury. Although the major reason for retransplantation in 2 patients was graft failure, all 15 patients in this study had pathological evidence of CAV with 14 of 15 cases graded as severe. Additionally, seven of these patients had scarring consistent with prior healed infarcts. Myocardial infarction is associated with increased myocyte apoptosis, even distant from the injured tissue (23). Although the vascular lesions of CAV are more diffuse with greater small-vessel involvement than those of IsCM, they have a similar physiological impact through the reduction of tissue perfusion and oxygenation.

Samples from the five nonfailing donor hearts that were used as NL controls were all trauma victims and free of cardiovascular pathology. Although the absence of pathophysiology was confirmed for all donor hearts, underlying systemic alterations such as activation of brain damage-induced stress hormones might be able to induce apoptosis in cardiac myocytes. Previous studies suggest that chronically high catecholamine exposure could induce cardiac myocyte apoptosis (34). In the present study, cardiac myocyte apoptosis was rarely found in all five NL donors with acute brain damage. This suggests that if acute brain injury did induce elevated catecholamine levels, their impact on cardiac myocyte apoptosis was very small. Cardiac myocyte apoptosis was increased four- to fivefold in the IsCM and CR groups compared with the NL controls. Nevertheless, the slight increase in cardiac myocyte apoptosis in the control group could only result in slight quantitative underestimation of the apoptosis in the IsCM and CR groups.

Role of mitochondria in regulating apoptosis. Abundant evidence supports a role for mitochondria in reg-
Fig. 6. A: end-labeling of endothelial cell nuclei in cardiac allograft vasculopathy (CAV), cells surrounding a necrotic core in the intima of native atherosclerosis (NA), and cells within the CAV media. End-labeled cells are shown (arrows). B: although overall vascular apoptosis did not differ between CAV and NA groups, medial apoptosis was significantly elevated in CAV. Magnification is ×400 for all fields.

Fig. 7. A: immunohistochemical staining for Bax in NA. B: staining for Bcl-2 in CAV. Stained cells are indicated (arrows). C: percentages of cells within the intima that expressed Bax, Bcl-2, and Bcl-xL are compared in NA and CAV groups. Magnification is ×400 for all fields.
ulating apoptosis. Mitochondria-mediated apoptosis is initiated by a number of death triggers that stimulate the release of several proteins including cytochrome c. Once released into the cytosol, cytochrome c binds to its adapter molecule, apoptotic protease activation factor-1, which oligomerizes and then activates procaspase-9. Caspase-9 can signal downstream of the tor-1, which oligomerizes and then activates pro-

The release of cytochrome c is regulated by different Bcl-2 family proteins: Bax and Bid potentiate cytochrome c release, whereas Bcl-2 and Bcl-xL antagonize this event. In the present study, we found a distinct difference in the importance of mitochondria-regulated apoptosis in two etiologically different subtypes of coronary artery disease. Although it is involved in both cases, mitochondria-mediated apoptosis plays a more significant role in CR than IsCM that is manifested by higher caspase-9 activation and better correlation between caspase activation and graft survival. Insufficient myocardium perfusion was present in all CR patients, including those without infarct, due to diffuse microvacularopathy. Based on the present results, we are not able to draw a conclusion as to whether alloimmune response plays a role in the upregulation of mitochondria-mediated apoptosis, although it is most likely.

Importance of myocardial Bcl-2 and Bax expression. Proteins of the Bcl-2 family collectively regulate the predisposition of a cell to undergo apoptosis through sequestration of critical activation factors or alterations of mitochondrial permeability (28). Like other investigators, we found that these proteins are increased in myocardium with IsCM (18, 22). The even higher myocardial Bax and Bcl-2 expression levels among patients with CR suggest that although ischemia plays a predominant role in triggering apoptosis, nonischemic stressors could also regulate this gradual cell loss in failed cardiac allografts. Although deficiencies in cardiac function associated with neurohormonal dysregulation can affect hearts with either CR or IsCM, the apoptotic stimuli from cell-mediated immunity (17), antidonor antibodies (24), and inflammatory cytokines (10) are unique to the host vs. graft response. Alternatively, the differences in protein expression among patients with CR and IsCM might suggest that subtle pathological differences exist that are due to the presence of either low-grade alloreactivity or diffuse microvacularopathy. The importance of mitochondria-mediated apoptosis through the Bcl-2/Bax pathway is emphasized by the poorer graft-survival rates among patients with CR whose hearts had a low Bcl-2/Bax ratio. Taken together, these findings support an important role for apoptosis during the pathogenesis of arterial lesions and the subsequent injury to heart muscle beyond the usual manifestations of atherosclerosis and myocardial ischemia. Additionally, proteins of the Bcl-2 family critically regulate apoptosis in this setting by collectively mediating the response of myocardial cells to apoptotic stresses. Regulating the balance of the Bcl-2/Bax ratio might be a new therapeutic strategy for prolonging allograft survival.

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DISCLOSURE

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