Upregulation of Bcl-2 Through Caspase-3 Inhibition Ameliorates Ischemia/Reperfusion Injury in Rat Cardiac Allografts

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Background—Oxidative stress after ischemia/reperfusion of cardiac allografts leads to cytokine production. Bcl-2, an inhibitor of apoptosis, also has strong antioxidant properties. Caspase-3 is known to cleave bcl-2. This study tests the hypothesis that bcl-2 is downregulated while tumor necrosis factor-α (TNF-α) levels increase after cardiac transplantation. Furthermore, the use of caspase-3 inhibition was investigated as a strategy for preserving myocardial bcl-2 and mitochondrial cytochrome c release after transplantation.

Methods and Results—PVG-to-ACI rat heterotopic cardiac transplantations were performed in 4 groups designed with 30 minutes’ ischemia and 4 or 8 hours of reperfusion (n=4 per group). Treatment consisted of DEVD-CHO 500 µg IP per animal to donor and recipient 2 hours before transplantation and 250 µg IC into allograft. Controls were treated with saline. Grafts were analyzed by reverse transcription–polymerase chain reaction for bcl-2 mRNA, by ELISA for TNF-α, for myeloperoxidase activity, and by Western blot for cytochrome c. In untreated groups, bcl-2 mRNA decreased significantly over time, whereas TNF-α increased significantly at 4 hours (P=0.003) and returned to baseline after 8 hours’ reperfusion (P=NS compared with normal hearts). Treatment with caspase-3 inhibitor showed significant upregulation of bcl-2 mRNA expression after 4 and 8 hours of reperfusion (P<0.001 versus control), with a concomitant decrease in TNF-α to baseline levels. Myeloperoxidase activity in all groups was no different from that of normal hearts. Mitochondrial cytochrome c release increased in both control and treatment groups.

Conclusions—Bcl-2 is actively downregulated and TNF-α is upregulated in this model of cardiac allograft ischemia/reperfusion. Furthermore, the caspase-3 pathway is linked to this process, and blockade of caspase-3 can ameliorate reperfusion injury by upregulating bcl-2 and inhibiting TNF-α without affecting cytochrome c release. (Circulation. 2001;104[suppl I]:I-202-I-206.)

Key Words: ischemia ■ transplantation ■ reperfusion ■ inhibitors

Reperfusion of ischemic tissue is accompanied by generation of oxygen free radicals that can overwhelm cellular defenses and induce tissue damage. These reactive oxygen intermediates cause direct damage to cellular DNA, protein, and lipids in addition to activating pathways of stress response. This nonspecific injury initiates a cytokine-mediated cascade, which results in the production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), leading to microcirculatory failure, followed by necrosis and cell death. In particular, TNF-α has been shown to be produced by myocytes in response to cellular stress. Several authors have reported that myocardial TNF-α production increases in the first several hours after ischemia and reperfusion or in response to lipopolysaccharide. In the transplant setting, such cytokine expression by graft tissue helps to recruit circulating recipient leukocytes to areas of inflammation, contributing to a cyclical cascade of injury.

The proto-oncogene bcl-2 and others of the bcl-2 family play key roles in the regulation of cell death and appear to govern the decision to die at multiple levels. Bcl-2 was originally identified as the proto-oncogene involved in the t(14;18) translocation in human low-grade B-cell lymphomas. Bcl-2 serves as a powerful antioxidant to cell death and may counteract the effect of both caspase-dependent and -independent modes of cell death.

Caspases are cysteine proteinases that have been shown to be specifically involved in the initiation and execution phases of apoptosis. Additional data, however, indicate that caspase activation plays an important proinflammatory role in the absence of cell death. Caspase-1 (IL-1β converting enzyme [ICE]) and caspase-3 (CPP32/Yama/apopain) are detected in cardiomyocytes, and in some systems, bcl-2 protein cleavage by activated caspase-3 promotes the release of cytochrome c, leading to further cellular damage. In addition, the transcription of bcl-2 is known to be regulated by the presence of cytokines, and recent evidence suggests that bcl-2 expression may be influenced by the presence of cytokines during reperfusion.
tional downregulation of bcl-2 may also depend on the inhibition of a tyrosine kinase that is regulated by caspase activation.9 Thus, the production and function of bcl-2 may be manipulated via alteration of caspase activity.

The present study tests the hypothesis that bcl-2 expression is reduced and TNF-α levels are increased after cardiac transplantation. Furthermore, the use of caspase-3 inhibition was investigated as a strategy for the preservation of myocardial bcl-2 after transplantation.

Methods

Animals

Adult, inbred male PVG (RT1b) and ACI (RT1a) rats weighing 250 to 350 g were purchased from Harlan Sprague-Dawley, Indianapolis, Ind. All rats were housed under conventional conditions and maintained on standard laboratory rat chow and water ad libitum. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Experimental Groups and Treatment Protocol

Animals were grouped according to treatment and, within each treatment group, time of reperfusion (n=4 per time point). Two hours before transplantation, donors and recipients were injected intraperitoneally with either (1) 300 μL of PBS solution containing 10% DMSO (vehicle only) or (2) 500 μg of cell-permeable caspase-3 inhibitor (DEVD-CHO, Calbiochem) dissolved in 300 μL of 10% DMSO in PBS. In addition, allografts were subjected to intracoronary administration of either (1) 150 μL of 10% DMSO in PBS (vehicle only) or (2) 250 μg of DEVD-CHO in 150 μL of 10% DMSO in PBS during procurement.

Heterotopic Heart Transplantation

PVG rats served as donors and ACI rats as recipients. Both donor and recipient rats were anesthetized with isoflurane (inhalational) and sodium pentobarbital (50 mg/kg IP). Native hearts were procured from donor animals after intravenous administration of 50 mg/kg heparin. Stanford cardioplegia solution (3 mL) was infused into the donor heart proximal to an aortic cross-clamp, followed by intracoronary administration of either vehicle alone or DEVD-CHO solution. End-to-side anastomoses were performed from the ascending aorta of the donor heart to the abdominal aorta of the recipient and from the donor pulmonary artery to the recipient vena cava according to the methods described by Ono and Lindsey.10 Allografts incurred 30 minutes of warm global ischemia, during which time the anastomoses were performed, and were procured for analysis after 4 or 8 hours of reperfusion. Tissue samples were snap-frozen in liquid nitrogen and then stored at −80°C for future processing.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

Total cellular RNA was isolated from snap-frozen tissue samples weighing ~100 mg with the total RNA isolation system (RNAgent, Promega). Reverse transcription–polymerase chain reaction (RT-PCR) was performed with Access RT-PCR (Promega) with the following sequences of intron-spanning bcl-2–specific primers: upper primer 5′-CGT TAC GGC CCC AGC ATG CG-3′, lower primer 5′-GCT TTG TTT CAT GGT ACA TC-3′ (cDNA length 231 bp), and for the housekeeping gene GAPDH, upper primer 5′-CGT CTT CAC CAC CAT GGA GA-3′, lower primer 5′-CGG CCA TCA CGC CAC AGT TT-3′ (cDNA length 395 bp). RT-PCR conditions for complete heart samples were as follows: RT 45 minutes at 48°C, RT inactivation and RNA/DNA/primer denaturation 5 minutes at 94°C; 35 cycles of PCR amplification: denaturation 1 minute at 95°C, annealing 1 minute at 55°C, extension 2 minutes at 72°C, and final extension 7 minutes at 72°C. Products were fractionated on a 1.5% agarose gel, visualized with ethidium bromide staining under ultraviolet light, and quantified by densitometry with a computerized digital imaging system. Densitometric analysis was used to determine a ratio between the amount of target mRNA to internal standard (GAPDH) mRNA (B/G ratio).

Caspase-3 Activity Assay

To verify inhibition of caspase-3 activity, pooled tissue samples for each of the treatment groups were homogenized in 1.5 mL of PBS, assayed with the CPP32/caspase-3 colorimetric protease assay kit (Chemicon International, Inc), and read at 405 nm in a microtiter plate reader. Activity was standardized to total protein concentration determined with a bichinchoninic acid (BCA) total protein detection kit (Pierce Chemical Co).

Myeloperoxidase Activity Assay

Samples were then analyzed for myeloperoxidase (MPO) activity. In brief, the tissue was disrupted by homogenization in 10% (wt/vol) hexadecyltrimethyl-ammonium bromide in 50 mmol/L potassium phosphate buffer (pH 7.0) with a Polytron homogenizer (Fisher Scientific). The homogenate was sonicated on ice for 15 seconds, underwent 3 freeze-thaw cycles, and then centrifuged at 12 000g for 15 minutes. Aliquots (35 μL) of supernatant were added to 3 mL of assay buffer (100 mmol/L of guaiacol, 0.17% H2O2, and 50 mmol/L potassium phosphate buffer, pH 7.0). Absorbance at 470 nm was measured by spectrophotometry (Beckman Instruments). One unit of MPO is defined as the activity degrading 1 μmol of peroxide per minute at 25°C.

Measurements of TNF-α

An enzyme-linked immunosassay kit (BioSource International) was used to determine TNF-α concentrations in myocardium homogenates. In brief, as reported by the manufacturer, this kit is a solid-phase sandwich ELISA. A specific anti-TNF-α antibody was coated onto the wells of the microtiter strips. Standards of known TNF-α content, control specimens, and unknown samples were placed into the wells by pipette, followed by the addition of biotinylated secondary antibody. After a first incubation and the removal of excess secondary antibody, streptavidin peroxidase was added, which bound to the biotinylated antibody to complete the 4-member sandwich. After a second incubation and washing to remove all unbound enzyme, a substrate (tetramethyl benzidene) solution was then added to produce color. The intensity of this colored product is directly proportional to the concentration of TNF-α present in the sample. Absorbance was read with a microtiter plate reader at 450 nm.

Western Blot Analysis

Western blotting was used for detection of mitochondrial cytochrome c from myocytes fractionated into mitochondrial and cytoplasmic compartments as described previously.11 In brief, myocytes were harvested in buffer A containing 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, and 250 mmol/L sucrose, pH 7.5. Cells were allowed to swell on ice for 15 minutes, homogenized, and centrifuged at 750g at 4°C. The supernatant was aspirated and centrifuged at 10 000g at 4°C. The pellet containing the mitochondrial fraction was resuspended in buffer A. The supernatant was centrifuged an additional time at 100 000g at 4°C to remove any mitochondrial contamination. Protein concentration of the mitochondrial and cytoplasmic fractions was determined with the BCA total protein assay (Pierce Chemical Co), and aliquots were stored at −70°C until use. The mitochondrial and cytoplasmic fractions (50 μg) were denatured in Laemmli buffer at 100°C and separated by electrophoresis on a 12% Tris-glycine gel. Proteins were then transferred to a nitrocellulose membrane in the presence of transfer buffer (20 mmol/L Tris-base, 0.15 mol/L glycine, 0.1% SDS, 20% methanol). Nitrocellulose filters were blocked with 5%
nonfat milk in TBST buffer (0.1 mol/L Tris-HCl, 1.5 mol/L NaCl, 0.5% Triton X-100) overnight at 4°C. Membranes were then exposed to the appropriate antibody for target protein expression. Mouse antibody directed toward mitochondrial cytochrome c (Pharmingen) was used as the primary antibody. Bound antibodies were detected with horseradish peroxidase–conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) and visualized by chemiluminescence with enhanced chemiluminescence reagents (Amersham).

Statistical Analysis
Statistical analyses were performed with SPSS for Windows. All measurements were compared between the various groups and all time points by repeated-measures ANOVA with Bonferroni corrections. Statistical significance was assigned to probability values <0.05.

Results
mRNA Expression of Bcl-2 in Ischemia-Reperfusion Injury
First, we examined the effect of ischemia/reperfusion injury on the expression of bcl-2, which was determined by mRNA detection through RT-PCR. In saline-treated groups, bcl-2 mRNA decreased stepwise after reperfusion, becoming significantly decreased after 8 hours (B/G ratio 0.85±0.73 for 4-hour reperfusion and 0.23±0.13 for 8-hour reperfusion, P=0.044 and P<0.001, respectively, versus 1.16±0.11 for normal hearts). However, treatment of donor and recipient animals with caspase-3 inhibitors resulted in significant upregulation of bcl-2 mRNA expression after 4 and 8 hours of reperfusion (B/G ratio 2.06±0.62 and 1.95±0.52, respectively, P<0.001 versus normal hearts for both; Figure 1).

Determination of Caspase-3 Activity
To verify the ability of DEVD-CHO to inhibit caspase-3 activity, we assayed the pooled samples of the DEVD-CHO–treated and saline-treated animals after 4 and 8 hours of reperfusion with a CPP32/caspase-3 colorimetric protease assay kit. Caspase activity increased 2-fold in saline-treated animals compared with normal hearts. In the DEVD-CHO group, caspase-3 activity was clearly suppressed, showing levels similar to those of normal hearts (Figure 2).

Effect of Bcl-2 Upregulation on TNF-α Levels and MPO
To further elucidate any protective effect of bcl-2 upregulation in this ischemia/reperfusion model, we assayed TNF-α in cardiac allografts with a commercially available ELISA kit. We showed that TNF-α levels in saline-treated animals were significantly higher than normal heart controls after 4 hours of reperfusion (669.99±127.09 versus 276.84±73.65 pg/mg total protein; P=0.003) and were returning to baseline after 8 hours of reperfusion (352.8±70.0 pg/mg total protein; P=NS versus normal heart controls). Interestingly, animals that were treated with caspase-3 inhibitors did not demonstrate this elevation in TNF-α levels at either reperfusion time point (338.42±124.81 pg/mg total protein at 4 hours and 262.88±58.43 pg/mg total protein at 8 hours, P=NS versus normal heart for both; Figure 3). Allograft MPO levels in saline-treated and in DEVD-CHO–treated animals after 4 and 8 hours of reperfusion showed no significant differences compared with normal heart controls (data not shown).

Figure 1. Bcl-2 mRNA expression by RT-PCR. A, Compared with normal hearts, bcl-2 mRNA expression decreased over time with ischemia and reperfusion in saline-treated hearts, becoming nearly undetectable after 8 hours of reperfusion. B, Treatment with caspase-3 inhibitor DEVD-CHO resulted in increased bcl-2 mRNA expression after 4 and 8 hours of reperfusion (B/G ratio 2.06±0.62 and 1.95±0.52, respectively, P<0.001 versus normal hearts for both; Figure 1).

Figure 2. Caspase activity assay. Caspase activity was determined in pooled samples of normal hearts, saline-treated hearts, and DEVD-CHO–treated hearts after 4 hours of reperfusion. Background reading was subtracted from readings of all samples before fold increase in caspase activity was calculated.

Cytochrome c Detection
To test the postulated role of caspase-3 as a trigger and bcl-2 as an inhibitor of mitochondrial cytochrome c release, we determined by Western blot whether caspase-3 inhibition...
Discussion

In the present study, we demonstrate that myocardial oxidative stress after transplantation causes a significant stepwise downregulation of the antioxidant gene bcl-2 over time. This pattern of expression is supported by the results reported by Maulik et al.\(^{11}\) in native heart ischemia and reperfusion. These findings are also in agreement with previous publications that demonstrated decreased bcl-2 and elevated bax levels in myocardial infarction\(^{14}\) and cerebral ischemia.\(^{15}\)

The present study also demonstrates that administration of a cell-permeable caspase-3 inhibitor, DEVD-CHO, protects cardiac allografts from ischemia/reperfusion injury via upregulation of bcl-2 and inhibition of TNF-\(\alpha\). In the event of oxidative stress in the presence of DEVD-CHO, stress-induced downregulation of bcl-2 mRNA is completely inhibited, which suggests that DEVD-CHO may be involved in the transcriptional regulation of bcl-2. It has been shown that tyrosine kinase activity, which is known to result in transcriptional overexpression of bcl-2, is inhibited by activation of caspase-3.\(^{3}\) In addition, bcl-2 cleavage is caspase mediated, and of all caspases tested, caspase-3 is most efficient in inducing bcl-2 cleavage.\(^{16}\) Therefore, in our model, DEVD-CHO blockade of caspase-3 may not only preserve native activity of bcl-2 by preventing direct bcl-2 cleavage but may also mediate tyrosine kinase activity, allowing for bcl-2 overexpression as a stress response to ischemia and reperfusion.

The loss of bcl-2 expression on cardiac allograft reperfusion may have important implications. In the heart, ischemia/reperfusion injury results in cardiac myocyte cell death by both necrotic and apoptotic mechanisms.\(^{17}\) Although it is recognized that the expression of bcl-2 family proteins and mitochondrial dysfunction are key components of the apoptotic process,\(^{18}\) the degree of the importance of this process in ischemia/reperfusion remains to be elucidated. Investigators disagree as to the significant presence or absence\(^{19,20}\) and benefit or harm\(^{21}\) of apoptosis in myocardial ischemia and reperfusion. Our laboratory has demonstrated that inhibition of nuclear factor-\(\kappa B\) results in decreased reperfusion injury but increased apoptosis in transplanted hearts.\(^{22}\) Clearly, the relevance of apoptosis in myocardial ischemia and reperfusion is subject to debate. Therefore, the loss of bcl-2 expression in our model may be interpreted from a different perspective.

In addition to the well-documented role of bcl-2 in suppressing apoptosis, recent data indicate its importance in proinflammatory pathways. Specifically, Lee et al.\(^{6}\) demonstrated that overexpression of bcl-2 can inhibit (nonapoptotic) JNK activation induced by IL-1\(\beta\) and \(H_2O_2\). These authors introduce the concept of an expanded function of bcl-2, including nonapoptotic signal transduction.\(^{6}\) The duration of reperfusion of myocardial tissue in the present study correlated with a significant gradual downregulation of bcl-2 and an increase in TNF-\(\alpha\) expression after 30 minutes of ischemia. It has been generally believed that TNF-\(\alpha\) is produced by systemic leukocytes in response to ischemia/reperfusion, which in turn causes myocardial leukocyte infiltration.\(^{23}\) and this infiltration is considered to play a pivotal role in the phenomenon of myocardial reperfusion injury.\(^{3}\) Current findings, however, reveal that local synthesis from cardiomyocytes and cardiac resident macrophages is the main source of TNF-\(\alpha\) in the heart.\(^{3,4}\) Our MPO data, which suggest no significant leukocyte infiltration, are in agreement with this hypothesis and support the concept of TNF-\(\alpha\) as a sensitive marker for myocardial ischemia and reperfusion injuries. In our model, then, the early rise in TNF-\(\alpha\) levels mirrored by the decline in bcl-2 mRNA levels may represent an immediate byproduct of oxidative stress followed by an inability of the cellular machinery to adequately respond to that stress.

One of the reported mechanisms of protection by bcl-2 is that bcl-2 prevents the release of cytochrome c from the mitochondria and the subsequent activation of caspase-9.\(^{12}\) We were not able to confirm the postulated role of caspases as a trigger of mitochondrial cytochrome c release, because cytochrome c was detected mainly in the cytoplasm of...
DEVD-CHO–treated animals overexpressing bcl-2. This finding confirms the results of de Moissac et al.\(^4\) that suggest that the release of cytochrome c likely occurs proximal to the activation of caspase-3 and that myocytes survive with a significant amount of cytochrome c in the cytoplasm. Another study has shown that cells overexpressing bcl-2 fail to prevent bax-induced cytochrome c release, although bcl-2 colocalizes with bax to mitochondria. This indicates that bcl-2 can interfere with bax killing downstream and independently of cytochrome c release.\(^{24}\) Little has been reported regarding the mechanism of caspase-3 inhibition in the prevention of ischemia/reperfusion injuries, as well as a possible reduction of myocardial infarct size, and the available information is controversial.\(^{25,26}\)

In the present study, we have shown that bcl-2 upregulation can ameliorate ischemia/reperfusion injury independently of cytochrome c release in a heterotopic cardiac transplantation setting. In summary, the present study demonstrates that in vivo inhibition of caspase-3 is able to upregulate bcl-2 and reduce rat cardiac allograft damage from ischemia/reperfusion injury via restoration of TNF-\(\alpha\) levels to concentrations comparable to those in normal hearts. This myocardial preservation occurs regardless of cytochrome c release and may indicate a greater role for the direct antioxidant effects of bcl-2. This treatment strategy may prove useful in clinical transplantation and cardiac surgical procedures associated with ischemia and reperfusion.

**References**

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_Circulation_. 2001;104:I-202-I-206
doi: 10.1161/hc37t1.094833
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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