Attenuated Acute Cardiac Rejection in NOS2 −/− Recipients Correlates With Reduced Apoptosis

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Background—The mechanisms through which NOS2-mediated pathways regulate graft failure in acute cardiac rejection are ill defined. To determine whether apoptosis promoted by NOS2 may contribute, we used a heterotopic transplant model to study mouse cardiac allografts placed in recipients with targeted gene deletion of NOS2.

Methods and Results—Using 5 different indexes of apoptosis, we showed that mouse cardiac allografts placed in NOS2 −/− recipients (n = 7) had reduced apoptotic activity compared with those in NOS2 +/+ controls (n = 8). There were significantly fewer TUNEL-positive nuclei per high-powered field (P < 0.01), less DNA fragmentation (antinucleosome ELISA: P < 0.05), lower corrected transcript levels for caspase-1 and -3 (3P reverse transcriptase–polymerase chain reaction; P < 0.01), and reduced caspase-3 activity (cleavage of DEVD-pNA [P < 0.001] and poly [ADP-ribose] polymerase) in grafts from NOS2 −/− recipients. This concordant reduction in apoptotic indexes paralleled the improved histological outcome of grafts transplanted into NOS2 −/− recipients (assessed as rejection scores; P = 0.012). To identify pathways controlled by NOS2, we compared intragraft transcript levels of potential triggers and regulators. Whereas Fas ligand/Fas and tumor necrosis factor (TNF)-α/TNF receptor-1 levels were not altered by NOS2 deficiency, transcript levels for p53 were significantly lower in grafts from NOS2 −/− recipients, coinciding with a significant increase in the antiapoptotic Bcl-2/Bax balance and decrease in Bcl-X levels.

Conclusions—Using NOS2 knockout mice, we demonstrated that NOS2-mediated pathways can promote acute rejection, at least in part, by inducing apoptotic cell death. When NOS2 is present, p53 might control NOS2-mediated apoptosis by stimulating Bax and repressing Bcl-2 and Bcl-Xl expression, which may activate the cell death program in the rejecting heart. (Circulation. 1999;99:836-842.)

Key Words: transplantation • immune system • genes

NOS2 is an inducible isoform of a family of nitric oxide synthases (NOS), which catalyze the synthesis of NO.1 NO rapidly reacts with superoxide anion to form peroxynitrite (OONO−).1 In vitro, both NO and peroxynitrite have been identified as cytotoxic molecules capable of triggering programmed cell death (apoptosis).2–4 NO and peroxynitrite have been shown to directly induce DNA damage in different in vitro cell lines.3,5,6 NO-mediated DNA damage, in turn, can induce the tumor suppressor gene p53,3,6 leading to apoptosis by transcriptionally activating expression of proapoptotic genes like Bax while suppressing expression of antiapoptotic genes like Bcl-2 or Bcl-X. The balance between proapoptotic and antiapoptotic genes, in turn, has been shown to control activation of caspases required for induction of the cell death program.8 However, it is unclear whether this in vitro scheme is relevant in vivo.

Recently, NOS2-mediated pathways have been shown to contribute to graft failure in acute rejection.9,10 NOS2 expression is induced early and persistently within rejecting grafts in a variety of different cell types.11 Attenuation of rejection by immunosuppressive strategies is associated with reduced NOS2 expression.11,12 Most importantly, different measures to inhibit NOS activity have attenuated the course of acute rejection in animal models.9 Less is known about the mechanisms through which NOS2 contributes to graft failure.

Descriptive studies have identified apoptotic cells in tissues undergoing various forms of rejection.10,13 NO was linked to apoptosis in a recent study showing that apoptosis of cardiac myocytes correlated with expression of NOS2 in a rat cardiac transplant model of acute rejection.10 In another study, in vivo transfection of rat hearts with the endothelial isoform of NOS produced apoptotic cell death of transfected cardiomyocytes.14 From these findings, we hypothesized that apoptosis, promoted by NOS2, is one mechanism that contributes to graft failure during cardiac rejection.
To study this hypothesis, we compared cardiac allografts placed in mice with targeted deletion of the NOS2 gene with those placed in wild-type controls. We assessed allograft-specific apoptosis in both groups by comparison of TUNEL staining, DNA fragmentation, and caspase expression and activity (poly [ADP-ribose] polymerase [PARP] cleavage and DEVDase activity). We then sought to identify differences in intracellular pathways regulating the apoptotic activity in grafts from NOS2+/− and NOS2+/+ recipients by comparing allograft-specific transcript levels for potential triggers, mediators, and antiapoptotic and proapoptotic regulators.

Methods

Heterotopic Mouse Cardiac Transplantation

Model of Acute Rejection

We evaluated tissues from a series of vascularized, heterotopic abdominal cardiac transplants performed as described previously. Acutely rejecting allografts were produced by placing major histocompatibility complex class I and II mismatched donors in nonimmunosuppressed recipients. To study the impact of NOS2 deficiency on acute rejection, we compared male CBA/CaJ (H-2k) allografts placed in NOS2−/−, n=7 on a C57BL/6Jx129Sv (H-2b) background with those placed in C57BL/6Jx129Sv wild-type controls (NOS2+/+, n=8). Grafts were harvested at day 7 after transplantation when the characteristic histological features of acute rejection are maximally developed but before graft failure. This ensures adequate in vivo perfusion of the graft before tissue harvest. Six additional transplants (3 each from NOS2−/− and NOS2+/+ recipient groups) were performed exclusively to obtain sufficient tissue for caspase-3 activity assays. NOS2-deficient mice were generously supplied in collaboration with Dr. Carl Nathan (Cornell University, New York, NY). CBA/CaJ (stock number 000654), B6 (stock number 000664), and B6/129 mice (stock number 101045) were obtained from Jackson Laboratory (Bar Harbor, Me).

Histological Analysis

Allograft sections (4 μm) were stained with hematoxylin and eosin and Verhoeff’s elastin. Slides were examined by light microscopy, and severity of rejection was scored with a modified International Society for Heart and Lung Transplantation grading system (0=no rejection to 4=severe rejection). Grading was performed by 2 independent observers in a blinded fashion. Scores are reported as the mean value for all grafts in each recipient group.

TUNEL Procedure

For in-situ detection and localization of apoptosis at the level of single cells, we used terminal deoxynucleotidyl transferase (TdT) to incorporate fluorescein-labeled dUTP into DNA strand breaks (In Situ Cell Death Detection Kit, Boehringer Mannheim). TUNEL was performed in paraffin sections (4 μm) from 5 representative allografts per group according to the manufacturer’s recommendations. The number of apoptotic nuclei was determined in a sequence of high-powered fields (magnification ×400) covering the entire transverse section of each allograft and reported as apoptotic nuclei per high-powered field. Apoptotic cells were subclassified as inflammatory cells (macrophages, lymphocytes), vascular cells (vascular smooth muscle cells, endothelial cells), and cardiac myocytes.

Enzyme Immunoassay for Cytoplasmic Histone-Associated DNA Fragments

For semiquantitative determination of apoptotic activity, we measured cytoplasmic histone-associated DNA fragments (mononucleotides and oligonucleotides) using a photometric enzyme immunoassay (Cell Death Detection ELISA, Boehringer Mannheim). Homogenates (20%) were prepared from frozen ventricular sections in PBS–10 mmol/L EDTA from 4 allografts per group. Serial dilutions of the cytoplasmic fraction (supernatant after centrifugation at 15 000g, 4°C for 15 minutes) were then analyzed in triplicate by a quantitative sandwich enzyme immunoassay as recommended by the manufacturer. Negative controls included omission of (1) the coating antibody, (2) the sample, (3) the peroxidase-conjugated antibody, or (4) the substrate. The results are presented as corrected mean of the average absorbance at 405 nm (A405 nm) per group.

Semiquantitative 32P Reverse Transcripbase–Polymerase Chain Reaction

Relative gene transcript levels were measured with reverse transcriptase-polymerase chain reaction (RT-PCR) from cardiac allograft cDNA panels as published previously. In this model, in which the small size of allograft tissue is limiting, 32P RT-PCR allows study of a large number of factors in replicates in a semiquantitative manner. PCR primers were designed by use of MacVector 5.0 (Oxford Molecular Scientific), and specificity of the primer sequence was confirmed by BLAST analysis. For each individual primer pair, specific annealing temperature and cycle number were optimized by serial annealing studies, PCR cycle studies, and cDNA dilution studies. The logarithmic ranges of amplification were established as previously described to ensure that the amplified PCR product reflected the original mRNA level. Primer sequences, reverse anneal temperatures, cycle number, and cDNAs are listed in Table 1. Triplicate samples were amplified with 0.625 U AmpliTaq Gold DNA polymerase (Perkin-Elmer) in a total volume of 25 μL by use of the parameters described previously.

Caspase-3 Protease Activity Assays

Caspase-3 protease activity assays were performed in cell lysates from frozen allograft samples (200 mg/mL) as published previously. Briefly, fluorometric caspase-3 protease activity assays were performed in 96-well plates by incubating 50 μL of cell lysate (10 μg) with 100 μL of reaction buffer (1% NP-40, 20 mmol/L Tris, pH 7.5, 137 mmol/L NaCl, 10% glycerol) and 100 μmol/L of peptidic substrate Ac-DEVD-AMC (Calbiochem) at 37°C for 2 hours. The rate of caspase enzymatic hydrolysis was measured by release of AMC from the caspase substrate (emission of 460 nm on excitation at 380 nm).

PARP cleavage analysis was performed by immunoblotting with PARP (Santa Cruz Biotechnology Inc). After incubation with horseradish peroxidase-labeled anti–goat-IgG antibody (1:5000) and detection with an enhanced chemiluminescence detection system (Amersham), bands were visualized by autoradiography.

Statistical Analysis

Group data are expressed as mean±SEM. For comparison of 2 groups, an unpaired t test was used. A value of P<0.05 was considered significant.
Results

NOS2 in Acute Rejection

Mean histological rejection scores were significantly lower in allografts placed in NOS2 \(-/-\) recipients (1.6 \pm 0.2) than in those in NOS2 \(+/+\) recipients (2.7 \pm 0.3; \(P = 0.012\)). This reduction in rejection scores in grafts from NOS2 \(-/-\) recipients indicates that when present, NOS2 promotes parenchymal destruction in the acutely rejecting heart. The presence of the apoptotically active NO metabolite peroxynitrite was confirmed by immunostaining for nitrotyrosine (a nitration product of peroxynitrite) and localized predominantly within infiltrating mononuclear cells (Figure 1A).

Reduced In Situ Detection of Apoptosis in NOS2 \(-/-\) Recipients

Classic TUNEL positivity was characterized by focal nuclear staining. In apoptotic cells, nuclear and cell membrane integrity was intact (Figure 1B). TUNEL-positive nuclei were detected throughout the allograft sections. However, the overall frequency of apoptotic TUNEL positivity was significantly lower in sections from NOS2 \(-/-\) recipients (1.0 \pm 0.2 nuclei per high-powered field; \(P = 0.007\)) compared with those from NOS2 \(+/+\) recipients (3.2 \pm 0.5 nuclei per high-powered field). The distribution of TUNEL positivity between parenchymal myocytes, vascular cells (endothelial cells and smooth muscle cells), and infiltrating mononuclear cells was comparable in grafts from both recipient groups (Table 2). In addition, the TUNEL reaction produced a second staining pattern with diffuse nuclear and cytoplasmic positivity. As described previously,\(^{11}\) this pattern is consistent with cellular necrosis characterized by disruption of the cellular membrane integrity (early sign of necrotic cell death).

Decreased Apoptotic Activity in NOS2 \(-/-\) Recipients

The reduction in TUNEL-positive cells correlated with lower apoptotic activity from allografts placed in NOS2 \(-/-\) recipients than in those placed in NOS2 \(+/+\) recipients. Levels of histone-associated DNA fragments in grafts from NOS2-deficient recipients were significantly lower (NOS2 \(-/-\) 0.22 \pm 0.03 [absorbance A\(_{405} \text{ nm}\)] compared with NOS2 \(+/+\) 0.41 \pm 0.05 [absorbance A\(_{405} \text{ nm}\); \(P = 0.046\); Figure 2A). Corrected transcript levels for caspase-1 and -3 were significantly reduced in grafts placed in NOS2 \(-/-\) recipients (caspase-1, 0.32 \pm 0.09 relative units; caspase-3, 0.46 \pm 0.07 relative units) compared with NOS2 \(+/+\) recipients (caspase-1, 0.64 \pm 0.06 relative units; \(P = 0.008\); caspase-3, 0.74 \pm 0.03 relative units; \(P = 0.003\)). Hence, transplanted hearts placed in NOS2 \(-/-\) recipients showed a significant reduction in apoptotic activity and less rejection.

Decreased Caspase-3 Protease Activity in NOS2 \(-/-\) Recipients

As shown in Figure 3A, grafts from NOS2 \(-/-\) recipients showed a marked reduction in levels of the 25-kDa PARP cleavage product associated with more of the intact 116-kDa

| Table 1. Primer Sequences, Sequence Accession Numbers, Annealing Temperatures, and Cycle Numbers |
|-------------------------------------------------|------------------|-----------------|---------------------------------|
| **Primer** | **Annealing Temperature, °C** | **Number of Cycles** | **Primer Sequence** |
| Caspase-1 | 56 | 28 | Sense TTACTGCTATGGGCAAGGCCAGG |
| (L03799) | | | Antisense ATTAGGGGCAGCGGCTAGAG |
| Caspase-3 | 66 | 38 | Sense TCGTGAGACTGACACACATAC |
| (U19522) | | | Antisense TGTCGAGACTGACACACATAC |
| p53 | 59 | 27 | Sense AGAGTCACAGACATGACAGGA |
| (K01700) | | | Antisense AGGCAGACACAGAAAGTC |
| Bcl-2 | 59 | 28 | Sense TCCAGCCTGAGAGCAACCATA |
| (M16506) | | | Antisense TGGACCCACCGAATCCGAGG |
| Bax | 59 | 30 | Sense AGATGATTCGAGCGTTGAGAC |
| (L22472) | | | Antisense AGATGATTCGAGCGTTGAGAC |
| Bcl-x | 56 | 25 | Sense CGACTCACCACATCCTGATC |
| (U10101) | | | Antisense CCAGAAGAACCTGAAGCAAG |
| Fas ligand | 58 | 31 | Sense GCTTTGAGCTGAGCGTTGAGAC |
| (U06948) | | | Antisense TGGCCATTCCTGCTGAGAAC |
| Fas | 55 | 33 | Sense AGACAGACGTGCCACTCTTGAG |
| (M83649) | | | Antisense TGTCATGTCTCAGCAATTCTCGG |
| TNF-α | 58 | 30 | Sense AAAAAATGAGGGGCCTCCAGAATC |
| (M13049) | | | Antisense AGATACCATCGTGAGCTGAGG |
| TNF-R1 | 59 | 29 | Sense TGATGGTCTGTGAGCTGAGAAAC |
| (M59378) | | | Antisense GATCAAACACACCAAGCCCACAA |
| G3PDH | 50 | 20 | Sense TGAAGGCGTGTTGAAGAGTTG |
| (M32599) | | | Antisense CATGATTGAGGTCCTGACAC |

\(^{11}\) NOS2-Mediated Apoptosis Promotes Cardiac Rejection
Caspase-3–like (DEVDase) activity, as shown in Figure 3B, was significantly reduced in grafts from NOS2−/− recipients (n=3; 273±61 optical density [OD] at 460 nm) compared with those in NOS2 +/+ recipients (n=3; 2742±62 OD at 460 nm; P<0.001). Taken together, these results demonstrate that apoptotic activity is decreased when recipient NOS2 is absent.

NOS2 Regulates Intracellular Apoptotic Pathways

We identified alterations in potential apoptotic triggers (ligand/receptor), death agonists, and death antagonists by measuring corrected gene transcript levels in allograft cDNAs from both groups. As shown in Figure 4, transcript levels of p53 were significantly decreased in grafts from NOS2−/− recipients (0.62±0.09 relative units) compared with those from NOS2 +/+ recipients (0.30±0.04 relative units; P=0.027). Fas ligand transcript levels in grafts from NOS2−/− recipients (0.44±0.10 relative units) were comparable to those in NOS2 +/+ recipients (0.52±0.07 relative units). Fas transcripts were present at similar levels in grafts from NOS2−/− (0.69±0.09 relative units) and NOS2 +/+ recipients (0.67±0.05 relative units). Tumor necrosis factor (TNF)-α transcript levels were not different in grafts from NOS2−/− recipients (0.53±0.12 relative units) and NOS2 +/+ recipients (0.63±0.10 relative units). TNF receptor-1 (R1) was present in comparable levels in grafts from NOS2−/− recipients (0.49±0.09 relative units) and NOS2 +/+ recipients (0.58±0.04 relative units).

**Figure 1.** Localization of peroxynitrite and apoptotic activity. Representative photomicrographs from myocardial sections of acutely rejecting allografts placed in NOS2 +/+ recipient showing peroxynitrite-positive cells detected with anti-nitrotyrosine (A) and TdT-labeled DNA strand breaks (B). Cytoplasmic nitrotyrosine positivity is predominantly identified in infiltrating mononuclear cells (open arrowheads). Nuclear TUNEL positivity (red) is shown in infiltrating mononuclear cells (open arrowhead) and cardiac myocytes (solid arrowhead). Original magnification ×120.

**Figure 2.** Apoptotic activity in grafts from NOS2 +/+ and NOS2−/− recipients. Acutely rejecting allografts from NOS2-deficient recipients have significantly reduced apoptotic activity as assessed by quantification of mononucleosomes and oligonucleosomes in cytoplasmic fraction of cell lysates (A) and corrected transcript levels for caspase-1 and −3 (B). 32P RT-PCR amplification was normalized against G3PDH and presented as corrected levels in relative units. Data are mean±SEM.

**TABLE 2. Parenchymal Cellular Rejection and Apoptosis in Grafts Placed in NOS2 +/+ and NOS2−/− Recipients**

<table>
<thead>
<tr>
<th>Degree of Apoptosis</th>
<th>Parenchymal Rejection (Rejection Score)</th>
<th>Apoptotic Nuclei per High-Powered Field</th>
<th>Inflammatory Cells, %</th>
<th>Myocytes, %</th>
<th>Vascular Cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS2 +/+ (n=8)</td>
<td>2.7±0.8</td>
<td>3.2±0.5</td>
<td>71±2</td>
<td>25±3</td>
<td>4±1</td>
</tr>
<tr>
<td>NOS2−/− (n=7)</td>
<td>1.6±0.9</td>
<td>1.0±0.2</td>
<td>60±8</td>
<td>32±6</td>
<td>8±2</td>
</tr>
<tr>
<td>P</td>
<td>0.012</td>
<td>0.007</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
Because the balance between proapoptotic and antiapoptotic factors can regulate apoptotic activity, we compared the ratio of Bcl-2/Bax transcript levels in grafts from both groups (Figure 5). With individual transcript levels of Bcl-2 (NOS2<sup>+/+</sup>, 0.54 ± 0.07 relative units; NOS2<sup>−/−</sup>, 0.38 ± 0.09 relative units) and Bax (NOS2<sup>+/+</sup>, 0.10 ± 0.01 relative units; NOS2<sup>−/−</sup>, 0.13 ± 0.03 relative units), the ratio of Bcl-2/Bax transcript was significantly increased in grafts from NOS2<sup>−/−</sup> recipients (0.38 ± 0.06) compared with those from NOS2<sup>+/+</sup> recipients (0.18 ± 0.02; *P* < 0.005). Similarly, transcript levels for the antiapoptotic factor Bcl-X<sub>L</sub> were significantly increased in grafts from NOS2<sup>−/−</sup> recipients (0.41 ± 0.04 relative units) compared with those in NOS2<sup>+/+</sup> controls (0.23 ± 0.02 relative units; *P* = 0.002).

Taken together, these findings show that reduced apoptotic activity in grafts placed in NOS2-deficient recipients is associated with reduced intragraft transcript levels of p53 but not Fas ligand/Fas or TNF-α/TNF-R1 mRNA expression. However, a potential mode of NOS2-induced regulation of apoptotic activity may involve Bcl-X<sub>L</sub>, Bcl-2, and Bax.

**Discussion**

This is the first study using mice with targeted gene deletion to show that recipient sources of NOS2 contribute to acute rejection and programmed cell death. Using a mouse cardiac transplant model, we demonstrated in vivo that NOS2-mediated pathways regulate apoptotic cell death in the...
acutely rejecting heart. In concert with reduced severity of parenchymal rejection, there were fewer TUNEL-positive nuclei, less DNA fragmentation, lower corrected intragraft transcript levels for caspase-1 and -3, and reduced caspase-3 protease activity (assessed by DEVDase activity and PARP cleavage) in grafts placed in NOS2-deficient recipients. Hence, our in vivo studies showing attenuated apoptosis when NOS2 is deleted confirm and extend previous in vitro studies indicating that NOS2-mediated pathways can promote apoptotic cell damage.

NOS2, NO, and the Regulation of Apoptosis

NO is capable of either inducing or suppressing the cell death program, depending on its local concentration and the micro-environmental redox milieu. In vitro studies with cultured macrophages and smooth muscle cells have shown that generation of NO and its reactive oxidant, peroxynitrite, can directly induce DNA strand breaks.3,20 Another transferrable repair cycle is initiated that ultimately causes cellular energy depletion and apoptotic cell breakdown.3,20 Another intracellular pathway has been described in a pancreatic B cell line that involves activation of cGMP-dependent protein kinases. Different inhibitors of guanylyl cyclase, cGMP-dependent protein kinase, and cGMP analogs were used to demonstrate that activation of apoptosis by NO donors may be secondary to an increase in cGMP.21

In vitro studies with rat hepatocytes and human umbilical venous endothelial cells have shown that NO can also inhibit apoptosis. Preexposure of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) to rat hepatocytes induced the expression of heat shock protein 70 associated with protection of hepatocytes from TNF-α–induced toxicity and apoptosis.22 In human umbilical venous endothelial cells, NO interfered with the TNF-α–induced cell death signal by inhibition of cysteine protease activation by blocking S-nitrosylation of a cysteine group.23

The functional role of NO (inducer or suppressor of apoptosis) may depend on its redox biochemistry within specific microenvironments. For example, pH and redox potential could regulate the transport, lifetime, and targeting properties of the various forms of NO. This would produce redox pools of NO that evoke specific biological responses.24 Perhaps the inflammatory milieu within the acutely rejecting graft produces a biochemical microenvironment favoring conversion of NO into activated forms that promote apoptosis. The next step will be to determine the exact cellular biological effects downstream of NO generation that control the induction of the cell death program within the graft microenvironment (eg, peroxynitrite formation or guanylyl cyclase activation).

Pathways to Control NO-Mediated Apoptosis

Given the differences in apoptotic activity in cardiac allografts from NOS2 −/− and NOS +/+ recipients, we sought to identify potential regulatory pathways controlled by NOS2. We found that attenuated apoptosis in mice with targeted gene deletion of NOS2 was associated with reduced intragraft transcripts for p53, coinciding with an increase in antiapoptotic Bcl-2/Bax ratios and Bcl-Xi transcripts. The tumor suppressor gene p53 can induce apoptosis by both transcriptionally dependent and independent mechanisms, depending on cell type.25 NO causes accumulation of p53 in various cell lines.5,6 p53 accumulation promotes transcription of Bax expression and repression of Bcl-2 expression.7 Shifts toward lower Bcl-2 or Bcl-Xi levels and higher Bax levels have been reported to promote apoptosis. In a mouse macrophage cell line, Bcl-2 transfectants showed substantial protection from cell death mediated by NOS2 activation or exposure to NO donors.26 NOS2-mediated apoptosis could be prevented by coexpression with Bcl-2 in a human cervix carcinoma cell line.27 Both Bcl-2 and Bcl-XI have been shown to prevent downstream activation of the cell death program like caspase-3–mediated cleavage of PARP.19,27,28 Hence, the balance of proapoptotic and antiapoptotic members of the Bcl-2 family appears to control activation of the caspase family of cysteine proteinases and cleavage of different enzymes that are involved in DNA repair and genomic maintenance.8,19

Roles of Apoptosis in Allograft Rejection

In the present model, the correlation between apoptotic activity and degree of allograft rejection suggests that the net effect of NO-mediated forms of apoptosis in the rejecting heart contributes to parenchymal damage of the transplanted heart.

Although apoptotic cells have been identified in the rejecting heart,10,13 the functional roles of apoptosis need elaboration. Depending on the target of programmed cell death, 3 different roles have been suggested for apoptosis in response to alloimmune stimulation. First, apoptosis has been detected in cardiac myocytes in the course of cardiac allograft rejection.10 Hence, apoptotic mechanisms could mediate increased myocyte damage, thereby contributing to reduced contractile capabilities and a loss of graft function. Second, apoptosis has been detected in endothelial cells of the allograft vasculature.13 Hence, apoptosis may mediate endothelial injury and possibly contribute to accelerated transplant arteriosclerosis as histological hallmarks of late cardiac rejection. Finally, deletion of donor-specific T cells through apoptosis has been one mechanism proposed to mediate allograft acceptance.29 Support for this comes from ex vivo studies showing that isolated graft-infiltrating cells from liver allografts showed an inverse relationship between prominent apoptotic activity and decreasing cytototoxic T lymphocyte activity.30

In the cytokine-activated milieu of the transplanted heart, it is possible that other coexisting pathways contribute to regulation of the apoptotic program. In the present study, we elaborated on NOS2-mediated apoptosis as 1 of the other effector mechanisms that promote tissue damage in response to alloimmune stimulation. By studying intracellular pathways when the NOS2 gene has been deleted, we found that a p53-dependent pathway involving transcriptional regulation of Bcl-2/Bax and Bcl-Xi is likely to control NOS2-mediated apoptosis through caspase-3. Hence, manipulation of NOS2 (mediator) or apoptosis (mechanisms) may serve as a useful measure to attenuate the acute alloimmune response in transplanted organs.
Acknowledgments
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References
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