Expression of Endomyocardial Nitric Oxide Synthase and Coronary Endothelial Function in Human Cardiac Allografts

Stephen M. Wildhirt, MD; Michael Weis, MD; Costas Schulze, MD; Nicole Conrad, PhD; Sinan Pehlivanli, MD; Gabi Rieder, PhD; Georg Enders, MD; Wolfgang von Scheidt, MD; Bruno Reichart, MD

Background—Inducible nitric oxide synthase (iNOS) is expressed and is functionally active in the presence of transplant arteriosclerosis. However, the early involvement of iNOS in alterations of microvascular endothelial function in the absence of preexisting lesions remains unclear; this information would be of prognostic value. We studied the course of iNOS mRNA expression, transcardiac nitric oxide production, and their potential association with microvascular coronary endothelial dysfunction in human cardiac allografts.

Methods and Results—A total of 42 patients were studied at 1, 6, and 12 months after heart transplantation. Microvascular coronary flow velocity reserve (CFVR) was tested in an endothelium-dependent (acetylcholine) and -independent manner (adenosine) using a Doppler flow wire. Endomyocardial iNOS expression was determined by reverse transcription polymerase chain reaction. iNOS protein and nitrotyrosine levels were detected by immunohistochemistry. Transcardiac plasma nitrite/nitrate (NOx) levels were measured by the Griess reaction. CFVR was impaired in 26.1% of patients (n=11) at 1 month and in 31% of patients (n=13) at 12 months after heart transplantation. Patients who developed impaired CFVR in the first year showed a significant increase in iNOS gene expression. Patients with impairment of CFVR 1 month after heart transplantation had higher levels of iNOS mRNA than patients with a normal CFVR. Patients with an initial impairment of CFVR who did not improve over time presented with significantly higher iNOS mRNA levels. iNOS protein and nitrotyrosine were expressed in the endomyocardial vessels of patients with impaired CFVR. Transcardiac NOx release was higher in patients with impaired CFVR.

Conclusions—In human cardiac allografts, microvascular endothelial dysfunction is associated with an enhanced endomyocardial iNOS mRNA expression and higher transcardiac NOx production and is accompanied by the expression of nitrotyrosine protein, suggesting peroxynitrite plays a role in the disease process. The data from the present study suggest an important role for the iNOS/nitric oxide pathway in the regulation of microvascular function in the absence of preexisting atherosclerotic lesions. (Circulation. 2001;104[suppl I]:I-336-I-343.)

Key Words: transplantation ■ microcirculation ■ endothelium ■ nitric oxide synthase

The progressive development of transplant coronary artery disease (TxCAD) is the major limiting factor for long-term survival in patients after heart transplantation (HTx). The disease process develops in stages and involves the epicardial conduit and intramyocardial resistance vessels. An initial and potentially reversible phenomenon in the process of TxCAD is the development of coronary endothelial dysfunction, which precedes and predicts morphological lesion formation.1 It has been shown recently that a decrease in the microvascular response to the endothelium-dependent vasoconstritor acetylcholine predicts the development of allograft vasculopathy and death in human HTx recipients.2 Despite its multifactorial genesis, the enhanced production of nitric oxide (NO) by inducible NO synthase (iNOS) plays an important role in the development of TxCAD. In line with these findings, Russell and coworkers3 previously demonstrated upregulation of iNOS in rat cardiac allografts with chronic rejection and transplant arteriosclerosis. Moreover, Ravalli and colleagues4 demonstrated the presence of iNOS mRNA and protein in vascular smooth muscle cells and macrophages from human arteries with TxCAD. This was associated with extensive nitration of protein tyrosines, suggesting that the oxidant peroxynitrite might be involved in the process leading to the development of TxCAD. Others have demonstrated that iNOS mRNA and protein expression is involved in progression of preexisting experimental and human atherosclerosis.5-7

Despite a potential role for iNOS in the progression of preexisting morphological lesions, which are usually seen >1 year after HTx, it remains unclear whether iNOS is involved in early endothelial dysfunction (<1 year after HTx) in human cardiac allografts in the absence of morphological changes.

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We hypothesized that iNOS is present in intramyocardial resistance vessels early after HTx and contributes to alterations in microvascular endothelial function in the absence of macroscopic evidence of TxCAD. This would have prognostic implications regarding the role of iNOS in the development and progression of morphological changes.

The purposes of the present study were to characterize iNOS mRNA and protein expression, to investigate its cellular source, and to determine its potential role in alterations of microvascular coronary endothelial function in the absence of preexisting atherosclerotic lesions in human cardiac allografts. Moreover, the presence and distribution of nitrotyrosine immunoreactivity as an indirect marker for peroxynitrite-mediated cell damage was investigated.

**Methods**

A total of 42 HTx recipients were included in this prospective, longitudinally designed study. The study group consisted of 37 men and 5 women (mean age, 50 years; range, 16 to 67 years) who received orthotopic HTx at our center. Triple drug immunosuppression (Table 1) was initiated immediately after organ implantation and was maintained throughout the study. Other medications, including ACE inhibitors, calcium antagonists, statins, and diuretics, were discontinued 24 to 48 hours before each examination. All patients agreed to participate in the study and gave written, informed consent. The study protocol was approved by the Ethics Committee of Ludwig-Maximilians University, Munich, Germany. No study-related complications and no deaths occurred during the 1-year follow-up of patients.

**Study Protocol**

Patients were examined 1 month (37±8 days), 6 months (205±12 days), and 12 months (370±17 days) after transplantation. Left heart catheterization was performed at 1 and 12 months after HTx and consisted of a measurement of hemodynamics and routine coronary angiography to exclude donor-transmitted CAD. In addition, assessment of endothelium-dependent and -independent epicardial (quantitative coronary angiography) and microvascular (Doppler flow wire) coronary vasomotor function was performed.

**TABLE 1. Donor and Recipient Criteria at Transplantation**

<table>
<thead>
<tr>
<th></th>
<th>Impaired CFVR (n=11)</th>
<th>Normal CFVR (n=31)</th>
<th>P</th>
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<tr>
<td><strong>Donors</strong></td>
<td></td>
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<tr>
<td>Age, y</td>
<td>36.2±15.5</td>
<td>31.6±11</td>
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</tr>
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<td>Weight, kg</td>
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<td>Height, cm</td>
<td>174.3±7.7</td>
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<tr>
<td>Sex, % male</td>
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<td>51.6</td>
<td>0.72</td>
</tr>
<tr>
<td>CMV-positive, %</td>
<td>45.4</td>
<td>50.1</td>
<td>0.41</td>
</tr>
<tr>
<td>Graft ischemic time, min</td>
<td>223±39</td>
<td>197±49</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Recipients</strong></td>
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<td>Height, cm</td>
<td>172±7</td>
<td>177±8.7</td>
<td>0.38</td>
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<tr>
<td>Sex, % male</td>
<td>82</td>
<td>93</td>
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<tr>
<td>CMV-positive, %</td>
<td>36</td>
<td>29</td>
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<tr>
<td>CMV mismatch, n (%)</td>
<td>5 (45.5)</td>
<td>15 (48.3)</td>
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<td>Pre-transplant Dx, % CAD</td>
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<td>Number of CAD risk factors per patient</td>
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<td>1.35</td>
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<td><strong>Native CAD risk factors, n (%)</strong></td>
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<tr>
<td>Diabetes</td>
<td>2 (18.1)</td>
<td>4 (12.9)</td>
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<tr>
<td>Hypertension</td>
<td>2 (18.1)</td>
<td>6 (19.3)</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>5 (45)</td>
<td>16 (51.6)</td>
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<tr>
<td>Tobacco</td>
<td>2 (18.1)</td>
<td>7 (22.5)</td>
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<tr>
<td>Hyperuremia</td>
<td>1 (9)</td>
<td>4 (12.9)</td>
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<tr>
<td>Alcohol</td>
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<td>2 (6.4)</td>
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<tr>
<td>Total bypass time, min</td>
<td>95.7±22</td>
<td>117±29.8</td>
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<td>Weaning time from bypass, min</td>
<td>33.5±8.5</td>
<td>36.9±14.3</td>
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<tr>
<td><strong>Immunosuppressive regimen, n (%)</strong></td>
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<tr>
<td>Tacrolimus, mycophenolate and prednisolone</td>
<td>8 (72)</td>
<td>21 (67.7)</td>
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<tr>
<td>Tacrolimus, azathioprine and prednisolone</td>
<td>1 (9)</td>
<td>4 (12.9)</td>
<td></td>
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<tr>
<td>Cyclosporine, azathioprine, prednisolone</td>
<td>2 (18.1)</td>
<td>6 (19.3)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM or n (%), unless otherwise indicated. Patients are grouped according to microvascular CFVR in response to acetylcholine as determined 1 month after HTx. CMV indicates cytomegalovirus; PVR, pulmonary vascular resistance; and Dx, diagnosis.
At each follow-up appointment, endomyocardial biopsies were obtained from the interventricular septum to determine iNOS and endothelial NOS (eNOS) mRNA and protein expression. To account for differences in sample quality, we always used homogenates from 2 biopsy samples for each reverse-transcription polymerase chain reaction (RT-PCR) analysis. Additional endomyocardial biopsy samples were used to detect iNOS and nitrotyrosine protein by immunohistochemistry.

**Functional Assessment of Coronary Vasomotor Function**

**Endothelium-Dependent and -Independent Epicardial Vasomotion**

Quantitative coronary angiography with a computerized, automatic analysis system (Hicor, Siemens) was used to assess the coronary vasomotor response (epicardial luminal diameter changes in percent). Endothelium-dependent changes were assessed with the intracoronary administration of acetylcholine (1.0 and 30.0 μg/min for 5 minutes each), and endothelium-independent changes were determined with an intracoronary infusion of adenosine (80.0 and 160.0 μg/min for 5 minutes each), as described elsewhere.6

**Endothelium-Dependent and -Independent Microvascular Vasomotion**

Microvascular vasomotor response was assessed by flow velocity measurements using an intracoronary Doppler flow wire (0.018 inches [0.04 cm]; Flo Wire, Cardiometrics Inc). The flow wire was introduced in a 6F Judkins catheter and positioned in the proximal part of the left anterior descending or circumflex coronary artery. After baseline flow velocity readings were obtained, hyperemic flow velocity data were determined using an intracoronary adenosine infusion (80.0 and 160.0 μg/min over 5 minutes). Endothelium-dependent changes in flow velocity were measured with intracoronary acetylcholine (1.0 and 30.0 μg/min over 5 minutes). Coronary flow velocity reserve (CFVR) was expressed as the ratio of peak to baseline blood flow velocity. Heart rate, mean arterial pressure, coronary flow velocity, and ECG were monitored continuously throughout the procedure. We ensured that measurements of the flow velocity reserve of the microvascular bed were not altered by epicardial vasoconstriction during adenosine infusion. An increase in flow velocity of factor <2.0 was considered pathological, as described previously.9

**Detection of iNOS and eNOS mRNA by RT-PCR**

Endomyocardial biopsy samples were immediately frozen in liquid nitrogen and stored at −80°C. For RNA extraction and cDNA preparation, a technique described previously was used.10 An aliquot (3 μL) of cDNA was amplified by PCR with a DNA thermal reactor (Perkin Elmer 480, Cetus Corp). The amplification reaction was performed as described previously.11 The nucleotide sequences of the chosen primers were as follows: GAPDH, 5′-TGAAAGGTCCGAGTCAACGGTATTGTTG-3′ for sense and 5′-CATGTGGGAGATGAGTGCCACC-3′ for antisense (product size, 983 base pairs(bp)); iNOS, 5′-GGCGCCCTGAAGGAGAAGAGC-3′ for sense and 5′-TTGGGTTGGAAGGCACACAGCT-3′ for antisense (product size, 506 bp); and eNOS, 5′-GAAGAGGAAGGGTCCAGTAAAC-3′ for sense and 5′-GTTGGGCTCTCGGACTCTGCTG-3′ for antisense (product size, 451 bp). Semiquantitative analysis was performed using a densitometric analysis system. We ensured that the amplification was within the linear range of the particular primer studied. Each signal was normalized for the DNA standard of the same gel to account for variances between gels. Normalization for the housekeeping gene GAPDH was performed to account for the variability of sample quality.

**Measurement of Transcardiac Nitrite/Nitrate**

Total transcardiac nitrite/nitrate (NOx) levels were measured by the Griess reaction in samples derived from the aorta and coronary sinus. In brief, plasma was deproteinized by ultrafiltration (Centrifree micropartition system, Amicon). The nitrate content of the sample was reduced to nitrite with a nitrate reductase. Samples were measured by spectrophotometric analysis at 540 nm. A standard curve was performed in each experiment. The NOx content of the samples was calculated from the standard curve, which was linear within this range.

**Immunohistochemistry**

We used immunohistochemistry to detect iNOS and nitrotyrosine over time. Cryosections measuring 3 μm were cut from endomyocardial biopsies taken 1, 6, and 12 months after HTx. At least 15 sections from 2 biopsies from each patient were analyzed at each time point. In brief, sections were fixed with chloroform (30 minutes) and blocked with 1.5% H2O2 (10 minutes). After washing (twice in 5 minutes), nonspecific binding was blocked with protein serum-free block (DAKO). To detect iNOS, a monoclonal antibody (dilution 1:100; Transduction Laboratory) was incubated with the section for 45 minutes. After washing (3 times in 5 minutes), a biotinylated antibody was applied for 30 minutes and visualized with peroxidase-conjugated streptavidin for 30 minutes and with a peroxidase substrate kit (AEC, Vector Laboratory) for 10 to 15 minutes. To detect nitrotyrosine protein, a polyclonal antibody (dilution 1:300; Upstate/Biozol) was incubated with the section for 45 minutes. After washing (3 times in 5 minutes), a secondary antibody (EnVision; DAKO) was applied for 30 minutes and visualized with a peroxidase substrate AEC kit (Vector Laboratory). After rinsing, sections were counterstained with Mayer’s hemalum solution and mounted. All steps were performed at room temperature. Negative controls were obtained by omitting the primary antibody. Isotype controls for iNOS was obtained using mouse IgG, MOPC21 (dilution 1:400; Sigma) instead of the first antibody.

**Data Analysis**

The CFVR to acetylcholine was considered normal when flow increased more than a factor of 2.0 from baseline.9 Nonparametric data were analyzed using the Mann-Whitney U-test, and correlations were determined by the Spearman rank test. Multiple comparisons were made by ANOVA followed by Bonferroni post hoc analysis. Data are expressed as mean±SEM. \( P<0.05 \) was considered statistically significant. For multiple comparisons, \( P<0.0018 \) was considered statistically significant.

**Results**

**Donor and Recipient Demographics**

None of the patients included in the study showed significant donor-transmitted disease visible during angiography at 1 month after HTx. Table 2 shows important clinical parameters evaluated at 1 and 12 months of follow-up. None of patients included in the investigation had clinical signs of infection or acute rejection episodes of International Society for Heart Lung Transplantation (ISHLT) grade 1b or greater during the time of sample collection and functional assessment of the coronary vasculature. Because rejection is a heterogeneous process, 4 to 5 different biopsy samples were assessed for acute rejection episodes in addition to each sample used for immunohistochemistry.

In addition, no significant differences with regard to cardiac hemodynamics were observed at either follow-up appointment (Table 2). Moreover, we found no correlation between NOS gene expression or NOx levels and cardiac hemodynamics at any time point. Subanalysis did not reveal significant differences between sex-matched and sex-mismatched transplanted hearts. In the present study, recipients with male or female donor hearts showed comparable
values with regard to values for iNOS mRNA expression, microvascular CFVR, and plasma NOx.

### Epicardial Vasomotor Function

The administration of acetylcholine resulted in an epicardial coronary diameter change of $-5.0 \pm 2.0$% in proximal and $-11.0 \pm 5.0$% in distal segments at 1 month and of $-7.0 \pm 3.0$% in proximal and $-12.0 \pm 4.0$% in distal segments at 12 months after HTx. The epicardial vasomotor response was not associated with the endomyocardial expression of either iNOS or eNOS mRNA.

### Microvascular Vasomotor Function

Eleven of the 42 patients (26.1%) showed an impaired CFVR at 1 month after HTx, which was significantly different from those patients with a normal increase in CFVR (3.0 $\pm 0.13$; $P<0.001$), as shown in Figure 1A. Thirteen patients (31%) presented with an impaired CFVR in response to acetylcholine at 12 months after transplant. These patients also showed a significantly reduced endothelium-independent microvascular vasomotor function in response to adenosine ($2.4 \pm 0.29$ versus $3.5 \pm 0.28$; $P=0.03$) 12 months after HTx (Figure 1A).

Importantly, 7 of 12 patients (58.3%) who showed an impaired CFVR in response to acetylcholine at 1 month after HTx significantly improved over time; they had a normal response of the coronary microvasculature at 12 months after HTx. However, 9 of 13 patients (69.2%) with an impaired CFVR in response to acetylcholine at 12 months after HTx demonstrated a normal CFVR at 1 month after HTx (Figure 1B).

### NOS mRNA Expression and Coronary Vasomotor Function

No significant differences in overall eNOS mRNA expression were noted between patients with and without an impairment of endothelium-dependent CFVR at 1 month (normal response, 2.97 $\pm 0.61$; dysfunctional response, 1.53 $\pm 0.34$; $P=NS$) and 12 months after HTx (normal response, 2.35 $\pm 0.44$; dysfunctional response, 1.81 $\pm 1.47$; $P=NS$).

The expression of endomyocardial eNOS isoforms was not associated with changes of epicardial vasomotor function or with the microvascular response to either acetylcholine or adenosine.

Patients who had a normal CFVR in response to acetylcholine over the first year had the lowest iNOS mRNA levels (Figure 2A). Patients who developed vasomotor dysfunction over the first year showed a significant increase in iNOS gene expression (Figure 2B). Those patients with initial impairment of CFVR at 1 month after HTx had higher levels when compared with the values obtained for patients with a normal CFVR (Figure 2C). These patients had higher levels than those of patients with a normal CFVR and those with an initial impairment of CFVR who improved over time. Patients with an initial impairment of CFVR who did not improve over time presented with significant higher iNOS mRNA levels when compared with patients who had a normal CFVR (Figure 2D).

### Transcardiac NOx Production

Plasma NOx levels derived from the aorta and coronary sinus revealed higher levels in the coronary sinus, resulting in a net release of NOx over the heart. Transcardiac NOx production was significantly higher in patients with impaired CFVR when compared with patients having a normal CFVR in response to acetylcholine at both 1 and 12 months after HTx (1 month: normal CFVR, 3.7 $\pm 1.2$ $\mu$mol/L versus impaired CFVR, 7.1 $\pm 2.1$ $\mu$mol/L; $P<0.05$; 12 months: normal CFVR, 3.5 $\pm 1.1$ $\mu$mol/L versus impaired CFVR, 6.4 $\pm 1.4$ $\mu$mol/L; $P<0.05$; Figure 3).

### Detection of iNOS and Nitrotyrosine Protein

In patients with normal microvascular CFVR, no specific staining for iNOS was detected. In contrast, patients with impaired CFVR showed specific iNOS protein expression staining. iNOS was detected in intramyocardial vessels but...
As shown in Figure 4, in patients who had an impaired CFVR at 1 month after HTx but who improved over time, iNOS staining gradually declined (Figures 4A through 4C). In contrast, patients whose CFVR was normal at 1 month after HTx but was impaired at 12 months after HTx showed an increase in specific iNOS expression over time (Figures 4D through 4F).

Similarly, nitrotyrosine staining was absent whenever microvascular CFVR was normal. Patients with a normal CFVR who developed an impaired microvascular response to endothelium-dependent stimulation after 12 months showed specific staining for nitrotyrosine (Figures 5A through 5C).

Nitrotyrosine was found in the intramyocardial vessels and infiltrating cells. In contrast, in patients in whom CFVR improved over time, the specific staining for nitrotyrosine gradually declined (Figures 5D through 5F).

Discussion

It has been demonstrated that endomyocardial iNOS is expressed and is functionally active during the progression of
preexisting native and allograft atherosclerosis. However, its role in alterations of coronary microvascular endothelial function over time in the absence of morphological changes in human cardiac allografts remains unknown. The major findings of this prospective, longitudinal study, are as follows. First, there is persistent expression and an increase of endomyocardial iNOS mRNA expression over time in patients with impaired CFVR in response to acetylcholine (Figure 2). Second, impaired endothelium-dependent CFVR of the coronary microvasculature is associated with higher transcardiac NOx production at both 1 and 12 months after HTx (Figure 3). These data suggests that under immunosuppressive therapy sufficient to prevent acute rejection episodes, a considerable inflammatory process is active within cardiac allografts. Third, only patients with an impaired endothelium-dependent CFVR show specific immunoreactivity for iNOS protein in intramyocardial vessels (Figure 4); the protein colocalizes with the expression of nitrotyrosine protein (Figures 5 and 6), suggesting peroxynitrite-mediated nitrosylation of proteins in the coronary vasculature.

Although a causal relationship cannot be drawn from the present data, it may be that an impaired CFVR of the microvasculature reflects an inflammation-mediated relative decrease in vascular tone. Higher transcardiac NO production (as determined by plasma NOx) and enhanced nitrotyrosine protein expression in patients with impaired CFVR at both time points support this contention.

It is important to note that in the present study, cardiac hemodynamics were not associated with iNOS mRNA or protein expression. This is in line with data from Birks et al., who prospectively studied the potential role of NO (as measured by plasma NOx) on myocardial function after human HTx.

**Chronic iNOS Expression: Vascular Dysfunction Versus Protection**

Recent experimental and clinical studies provide intriguing data regarding the role of iNOS in allograft atherosclerosis. The question arises: under what circumstances does endomyocardial iNOS-derived NO production contribute to or prevent the progression of endothelial dysfunction and coronary morphological changes? There is experimental evidence from iNOS-deficient mice suggesting that the development of TxCAD is augmented in the absence of iNOS. In addition, it has been demonstrated by Shears and colleagues that transfection of the iNOS gene protects against the development of TxCAD by inhibiting intimal and medial thickening. Indeed, as shown in the present study, 9 patients with an initial impairment of CFVR at 1 month after HTx improved within the 12-month follow-up period. This was accompanied by a significant reduction of iNOS mRNA (Figure 2), a reduction in transcardiac NOx release (Figure 3), and decreased immunoreactivity of nitrotyrosine protein (Figure 5). On the basis of the experimental data, one could argue that iNOS-derived NO may have improved CFVR by inhibiting smooth muscle and fibroblast proliferation and neointima formation. It may be that iNOS is expressed secondary to chronic inflammation at sites of vascular lesions and may protect from the smooth muscle and fibroblast proliferation that subsequently results in reduced neointima formation. In this regard, Yogo and colleagues showed that eNOS knockout mice develop significantly more neointima formation, whereas in iNOS.
knockout mice, constrictive remodeling was more pronounced.

In contrast, Kessler et al. showed a direct impairment of the endothelial function of rabbit carotid arteries by iNOS-derived NO. They reported that iNOS protein is located in vascular smooth muscle cells and acts by inhibiting eNOS. In addition, there is evidence to suggest that iNOS contributes to endothelial dysfunction in the presence of superoxide, suggesting an important role for peroxynitrite. Skarsgard and colleagues reported direct vasodilation and inhibition of the myogenic tone of vascular smooth muscle cells by both eNOS and iNOS-based NO production in allograft resistance vessels. As shown in the present study, patients with an impaired microvascular CFVR developed vasomotor dysfunction in response to adenosine, suggesting an alteration of vasomotor function downstream of endothelium-mediated signaling.

Akyurek and colleagues found iNOS protein expression in infiltrating cells and vascular smooth muscle cells in neointima and media during the development of experimental transplant arteriosclerosis in rats. Supporting evidence that iNOS is involved in the development of TxCAD in humans has been reported by Ravalli and colleagues. They studied tissue from 15 patients with TxCAD and compared these findings with those from 10 patients with normal coronary arteries. They found a significantly higher expression of both iNOS and nitrotyrosine in the TxCAD group. iNOS was expressed in macrophages and smooth muscle cells and colocalized with nitrotyrosine formation, suggesting that iNOS contributes to the progression of TxCAD in humans.

Potential Mechanisms Underlying iNOS-Mediated Microvascular Endothelial Dysfunction

One potential mechanism leading to microvascular endothelial dysfunction may be that iNOS-derived NO production forms peroxynitrite by rapid binding to superoxide, thereby triggering cellular activation and dysfunction.

In this regard, Bauersachs and colleagues demonstrated that increased superoxide production by a NADH-dependent oxidase plays an important role in the development of experimental

Figure 5. Nitrotyrosine protein was detected by immunohistochemistry in endomyocardial biopsy specimens. At least 10 sections from 2 biopsies from each patient at each time point were analyzed using a specific polyclonal antibody. Two representative patients are shown. The left column (A through C) shows a patient with a normal endothelium-dependent CFVR at 1 month after HTx that declined over time (impaired CFVR at 12 months after HTx). A gradual increase in nitrotyrosine immunoreactivity was noted. The right column (D through F) shows a patient with an initial impairment of endothelium-dependent CFVR who significantly improved within the 12-month follow-up period. Nitrotyrosine protein expression decreased over time in this patient. Magnification ×200.

Figure 6. High-power magnification (×400) of 2 representative biopsy sections stained for iNOS (A) and nitrotyrosine (B) are shown. INOS protein is localized in intramyocardial vessels (closed arrows) and in inflammatory cells (open arrows). A similar staining pattern is noted for nitrotyrosine, with specific immunoreactivity in intramyocardial vessels (closed arrows) and inflammatory cells (open arrows). This was consistent for all endomyocardial sections from patients with impaired CFVR at any time point.
endothelial dysfunction in chronic myocardial infarction. This may occur by the cytotoxic effects of formed peroxynitrite and/or by a reduction of relative NO bioactivity due to its inactivation by binding to superoxide.\textsuperscript{17,23} Moreover, it has been shown that peroxynitrite inactivates manganese superoxide dismutase, thereby promoting irreversible oxidative injury in chronic rejection of human allografts.\textsuperscript{24} The finding that endomyocardial nitrotyrosine is associated with reduced coronary vasomotor function supports these observations and is indicative of peroxynitrite formation in this group of patients.

In conclusion, the present study demonstrates for the first time that in human cardiac transplant recipients, endothelial dysfunction of the coronary microcirculation within the first year after HTx is associated with enhanced endomyocardial iNOS mRNA expression and higher transcardiac NOX production. These results are accompanied by the expression of nitrotyrosine protein, suggesting a role of peroxynitrite in the disease process. Although a causal relationship between iNOS and CFVR cannot be ascertained, data from the present study suggest an important role for the iNOS/NO pathway in the regulation of microvascular function in the absence of preexisting atherosclerotic lesions.

Acknowledgments
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References
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