Inducible Nitric Oxide Synthase Expression in Smooth Muscle Cells and Macrophages of Human Transplant Coronary Artery Disease

Stefano Ravalli, MD; Arline Albala, MA; Ming Ming, PhD; Matthias Szabolcs, MD; Alessandro Barbone; Robert E. Michler, MD; Paul J. Cannon, MD

Background—The inducible isoform of the nitric oxide synthase (iNOS) produces large amounts of nitric oxide in response to cytokine stimulation. Previous investigations have demonstrated iNOS expression in the setting of acute and chronic rejection in experimental cardiac transplant models. The goal of this study was to investigate whether iNOS is upregulated in human transplant coronary artery disease (TCAD), a major cause of late mortality after cardiac transplantation.

Methods and Results—We studied 15 patients with TCAD and 10 with normal coronary arteries. In situ hybridization and immunohistochemistry were used in tissue sections to localize iNOS mRNA and protein, respectively. The presence of peroxynitrite was indirectly assessed by immunostaining with an anti-nitrotyrosine antibody. Normal coronary arteries had no evidence of iNOS expression. In contrast, 30 of 36 coronary artery segments with TCAD (83%) were immunostained by the iNOS antibody. The presence of iNOS mRNA was demonstrated in these vessels by in situ hybridization. Specific cell markers identified iNOS-positive cells as neointimal macrophages and smooth muscle cells. Nitrotyrosine immunoreactivity colocalized with iNOS expression in arteries with TCAD, distributed in macrophages and smooth muscle cells.

Conclusions—iNOS mRNA and protein are expressed in human arteries with TCAD, where they are associated with extensive nitration of protein tyrosines. These findings indicate that the high-output nitric oxide pathway and possibly the oxidant peroxynitrite might be involved in the process leading to the development of TCAD. (Circulation. 1998;97:2338-2345.)

Key Words: arteriosclerosis ■ endothelium-derived factors ■ transplantation

Long-term survival of cardiac transplant recipients has been limited by the development of a vasculopathy in the coronary arteries of the allograft.1 The pathogenesis of this vasculopathy, called TCAD, is not fully understood but is thought to result from the interaction of immunological and nonimmunological factors leading to the migration and proliferation of SMCs, T-lymphocytes, and macrophages in an expanded neointima.2–6 The inflammatory reaction leads to luminal narrowing, myocardial ischemia and infarction, and the development of heart failure and malignant arrhythmias.

The oxidation of the amino acid l-arginine by the family of enzymes known as NOS is important in a large number of physiological and pathological processes. Three isoforms of NOS have been identified and cloned: neuronal, cytokine-inducible (iNOS), and endothelial. Endothelial NOS, found predominantly in endothelial cells, is calcium-calmodulin dependent and produces small amounts of NO in response to shear stress or to agonists like bradykinin.7–9 iNOS, induced in macrophages, SMCs, endothelial cells, and other cells by cytokines, is not dependent on increased calcium and calmodulin and produces large amounts of NO for long periods of time.7–9 NO, by activating soluble guanylyl cyclases in target cells, can initiate responses such as vasodilation,10 inhibition of platelet aggregation and adhesion to the vessel wall,11 and inhibition of SMC proliferation.12 NO, when produced in higher concentrations by iNOS, has other effects, some of which are cytotoxic. It can cause nitration and nitrosylation of proteins, inhibit enzymes involved in oxidative metabolism and mitochondrial respiration, bind to FeS clusters in proteins, cause ADP ribosylation, and damage DNA.8,9 When NO combines with equimolar amounts of superoxide, peroxynitrate is formed, which (although an NO donor at low concentrations) can at high concentrations cause nitration of cell proteins and decompose to form cytotoxic hydroxyl radicals.13,14

In addition to its established function in the cardiovascular system, NO participates in immune responses15 and has been implicated in allograft rejection. Using a rat model of heterotopic cardiac transplantation, Yang et al16 reported that iNOS mRNA, protein, and enzyme activity were induced in

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rejecting allografts in association with reduced contractility and death of cardiac myocytes. Immune reactivity for iNOS was present in macrophages infiltrating the myocardium and in cardiac myocytes in the rejecting allografts. Using a similar model, Worrall et al\textsuperscript{17} confirmed these results and demonstrated that treatment with aminoguanidine, a relatively selective inhibitor of iNOS, prolonged allograft survival, improved contractility, and reduced the intensity of rejection. In studies of human endomyocardial biopsies, induction of iNOS mRNA was correlated with a reduced ejection fraction of the transplanted hearts.\textsuperscript{18} In rat cardiac allografts, Szabolcs and coworkers\textsuperscript{19} reported that apoptotic death of infiltrating macrophages and of cardiac myocytes in the rejecting hearts was correlated in time and intensity with the induction of iNOS mRNA, protein, and enzyme activity. iNOS is expressed in human atherosclerotic coronary arteries.\textsuperscript{20}Russel et al\textsuperscript{21} and Akyurek et al\textsuperscript{22} also reported recently that iNOS expression is upregulated in macrophages and SMCs in the intimal lesions of TCAD in rat transplantation models. The purpose of the present study was to investigate, with in situ hybridization and immunohistochemistry, the expression and cellular distribution of iNOS and nitrated proteins in the human lesions of TCAD.

**Methods**

**Patient Characteristics**

Patients demographics are summarized in Table 1. Written informed consent for the use of cardiac tissue samples was obtained from all subjects. Coronary arteries were obtained from the explanted allografts of 15 patients (12 men) undergoing retransplantation for severe TCAD. Allograft survival ranged from 13 to 126 months. Cyclosporine, corticosteroids, and azathioprine were used in combination for immunosuppression. Normal coronary arteries were obtained for comparison from the native hearts of 10 patients under- going transplantation for idiopathic cardiomyopathy or congenital heart disease.

**Tissue Preparation**

Coronary arteries were fixed in 10% buffered formalin, paraffin-embedded, sectioned at 4-µm intervals, and stained with hematoxylin and eosin. Parallel sections were applied to organosilane-coated slides and used for immunohistochemistry. For in situ hybridization, unfixed segments of coronary arteries were placed in OCT embedding compound (Miles Laboratory) and immediately snap-frozen. Cryosections were cut under RNase-free conditions, mounted on RNase-free glass slides (Fisher Scientific), air-dried, and fixed in 4% paraformaldehyde in PBS for 20 minutes in a humidified chamber. Sections were then dehydrated in a graded series of ethanol (2 minutes each), air-dried, and stored at −70°C until processing.

**Immunohistochemistry**

The source of each antibody used and the optimal working dilution are summarized in Table 2. The affinity-purified rabbit antibody to a synthetic peptide from mouse iNOS (amino acid residues 961 through 1144) was purchased from Transduction Laboratories. The presence of nitrated proteins was determined by a monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology). Antibodies directed against CD-68 (Dako) and α-smooth muscle actin (Bio Genex) were used to identify macrophages and SMCs, respectively.

Sections were deparaffinized, rehydrated in sequential alcohol baths, and then washed in PBS. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in ethanol and nonspecific antibody binding was suppressed with 20% nonimmune serum in PBS. Sections were incubated in a humidified chamber overnight at 4°C with the anti-iNOS antibody or for 1 hour at room temperature with the other antibodies. With intervening washes in PBS, sections were then incubated with a biotinylated secondary antibody. A goat anti-rabbit IgG (Vector Laboratories) was used for the anti-iNOS and von Willebrand antibodies, and a horse anti-mouse IgG (Vector) was used for the monoclonal anti-nitrotyrosine, CD-68, and α-smooth muscle actin antibodies. The avidin-biotin-immunoperoxidase complex (ABC Elite, Vector) was then applied. Peroxidase activity was visualized with diaminobenzidine (Sigma Chemical Co).

**Double Immunofluorescence**

Deparaffinized, rehydrated sections were simultaneously incubated with either anti-iNOS antibody and anti–smooth muscle actin or anti-iNOS and anti-CD68 antibody. After rinsing with PBS, both secondary antibodies, tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse, were applied for 30 minutes, and death of cardiac myocytes. Immune reactivity for iNOS

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ICMP indicates idiopathic cardiomyopathy; P, prednisone; A, azathioprine; C, cyclosporine; IHD, ischemic heart disease; and CHD, congenital heart disease.

Selected Abbreviations and Acronyms

IFN = interferon
IL = interleukin
iNOS = inducible isoform of NOS
NO = nitric oxide
NOS = nitric oxide synthase
SMC = smooth muscle cell
SSC = standard saline citrate
TCAD = transplant-associated coronary artery disease
TNF = tumor necrosis factor

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...and demonstrating the presence of iNOS by red immunofluorescence labeling and the presence of smooth muscle actin or CD-68 by green immunofluorescence labeling. Sections were analyzed with a fluorescence microscope (Olympus BX 40) equipped with a blue filter for FITC (green immunofluorescence) and a green filter for TRITC (red immunofluorescence). Double-labeled sections in which one of the primary antibodies was replaced with an irrelevant isotype-specific antibody (Sigma) served as controls.

**Histological/Immunohistochemical Analysis**

Sections were independently examined by two observers for the presence of iNOS immunoreactivity and its distribution in the vessel wall. The intensity of staining was graded numerically on a scale from 1 to 3 as follows: 1=weak, 2=moderate, and 3=intense. Coronary arteries with TCAD were classified as atheromatous if they had at least three of the following histological features: (1) eccentric plaque, (2) disrupted internal elastic lamina, (3) large lipid deposits (“cholesterol clefts”), or (4) calcifications. Conversely, lesions were defined as proliferative if they consisted of a concentric accumulation of myointimal cells, with a mostly intact internal elastic lamina, without lipid deposits and calcifications.

**In Situ Hybridization**

A fragment of the mouse iNOS cDNA corresponding to the nucleotides 524 through 869 (85% homology with the human iNOS) was amplified by polymerase chain reaction from 1 μg of mouse iNOS cDNA (generous gift of Dr Carl Nathan, Cornell University Medical College) and cloned into the polymerase chain reaction II vector (TA Cloning Kit, Intron). Sense and antisense DNA templates were synthesized from this gene construct by polymerase chain reaction. Digoxigenin-labeled sense and antisense RNA probes were generated from the corresponding DNA templates by use of the polymerase chain reaction II.

Sections were rehydrated in PBS containing 5mmol/L MgCl₂ and then digested in 5 μg/mL proteinase K (Boehringer-Mannheim) for 15 minutes at 37°C. The tissue was postfixed in 4% paraformaldehyde in PBS for 10 minutes, washed in diethylpyrocarbonate (DEPC)-treated distilled water, and acetylated in triethanolamine-acetic anhydride (TEA) buffer (100 mmol/L triethanolamine with 0.25% acetic anhydride, pH 8.0). After being rinsed in DEPC-treated distilled water, sections were prehybridized in a humid chamber for 1 hour at 53°C in hybridization buffer consisting of 50% formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim), and 0.1% N-lauroylsarcosine. Hybridization was then performed overnight in a humid chamber at 56°C with digoxigenin-labeled sense and antisense probes in hybridization buffer. Subsequently, the sections were washed in 2× SSC at 65°C and 1× SSC and 0.1× SSC at 72°C for 30 minutes each; then they were incubated in maleic acid buffer (100 mmol/L maleic acid, 150 mmol/L NaCl, pH 7.5) plus 1.5% wt/vol blocking reagent (Boehringer Mannheim) for 30 minutes with slow agitation. To detect the hybridization signal, the sections were incubated with an anti-digoxigenin antibody (Boehringer Mannheim), diluted 1:500, followed by rabbit anti-mouse immunoglobulin and standard immunooalkaline phosphatase reaction, with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) as a substrate. After development, the slides were washed in TE buffer (10 mmol/L TRIS-HCl, pH 8.0), containing 1 mmol/L EDTA) for 5 minutes and mounted with coverslips by use of aqueous mounting medium (Innovex Biosciences).

Inflamed human tonsils, rich in activated macrophages, were used as positive controls for both in situ hybridization and immunohistochemistry. Labeling the tissue sections with the sense probe and replacing the primary antibody with normal rabbit serum provided negative controls for in situ hybridization and immunohistochemistry, respectively.

**Statistical Analysis**

All data are reported as mean±SEM. The rank-sum test was used for statistical comparison of the intensity of immunostaining. A value of P<0.05 was considered statistically significant.

**Figure 1.** Sections of a normal coronary artery (A) and of a coronary artery with TCAD (B) immunostained with the anti-iNOS antibody. Diaminobenzidine was used as a chromogen in the immunoperoxidase reaction, yielding a brown reaction product in positive cells. The normal artery has no immunoreactivity. In contrast, the artery with graft arteriosclerosis has intense immunoreactivity localized to spindle and foam cells in the thickened neointima. Hematoxylin counterstain. Magnification ×400.

**Results**

**Immunohistochemistry**

A total of 36 epicardial coronary artery segments from 15 patients with TCAD (2.4 segments per patient) and 22 segments from 10 patients with normal coronary arteries (2.2 segments per patient) were analyzed for iNOS and nitrotyrosine immunoreactivity.

**iNOS**

Immunostaining for iNOS was not present in any of the normal coronary arteries studied. In contrast, 30 of 36 coronary artery segments with TCAD (83%) were labeled by the iNOS antibody. Representative sections of a normal coronary artery and of an artery with advanced TCAD labeled with the iNOS antibody are shown in Figure 1. Immunoreactivity for iNOS was found in the neointimal layer of epicardial coronary arteries, localized to spindle-shaped mesenchymal cells, mononuclear cells, and foam cells, morphologically consistent with SMCs and macrophages, respectively (Figure 1). The intensity of iNOS immunostaining, assessed by a semiquantitative microscopic analysis, varied considerably not only from patient to patient but also in different vessels of the same patient. Labeling was confined to the cytoplasm of individual cells in the expanded neointima and was not observed in the extracellular space. To identify with certainty...
the cell types immunoreactive for iNOS, we used two different immunohistochemical techniques. In one, parallel sections were immunostained with the antibodies CD-68 and α-smooth muscle actin to recognize macrophages and SMCs, respectively. In the second, the same primary antibodies were incubated with TRITC- and FITC-labeled secondary antibodies, yielding red and green immunofluorescence, respectively, when viewed by fluorescence microscopy. The results are shown in Figure 2 (Figure 2A through 2E for immunohistochemistry; Figure 2F through 2I for double immunofluorescence). It is evident from the representative sections that iNOS expression was present in both neointimal SMCs and macrophages. Vasa vasorum, small blood vessels located in the thickness of the adventitia, consistently showed intense immunoreactivity for iNOS (Figure 3); intramyocardial arterioles, on the other hand, were consistently negative, even in cases with advanced occlusive disease (Figure 3). In all cases, the immunoreactivity was abolished when the primary antibody was replaced with nonimmune serum, confirming the specificity of the reaction (Figure 2).

Coronary arteries with TCAD were classified as atheromatous in 8 of 15 patients (53%) and proliferative in 7 of 15 (47%) on the basis of preestablished criteria that included lesion focality (ie, eccentric versus diffuse), the presence or absence of extracellular lipid deposits in the expanded intima, the integrity or disruption of the internal elastic lamina, and the presence or absence of calcifications. Foam cells and an intense neointimal lymphocytic infiltrate were present in both types of lesion. iNOS immunoreactivity was equally distributed among the two histological types of TCAD. The mean semiquantitative grade, ie, the intensity of iNOS immunostaining, was 1.9 for blood vessels with atheromatous features and 1.75 for those with proliferative ones (P=NS).

Nitrotyrosine
Representative examples of two coronary arteries with severe TCAD immunostained with a monoclonal anti-nitrotyrosine antibody are shown in Figure 4. Extensive immunoreactivity is shown in both cases. A large number of nitrotyrosine-positive cells were seen throughout the markedly thickened neointima. The labeling was found in cells morphologically consistent with macrophages and vascular SMCs in a distribution similar to that observed for iNOS. No labeling was present when the sections were incubated with control mouse IgG.
In Situ Hybridization

iNOS mRNA expression in coronary arteries with TCAD was investigated with antisense riboprobes labeled with digoxigenin and detected with an immunoalkaline phosphatase method. Inflamed human tonsils, rich in activated macrophages, were used as positive controls (Figure 5). Hybridization of the antisense iNOS probe was found in all arteries that immunostained for iNOS protein and was localized to the superficial and deep layers of the vessels neointima (Figure 6A through 6C) and occasionally to the SMCs of the tunica media (not shown). Comparison with parallel sections immunostained with the cell-specific antibodies CD-68 and α-smooth muscle actin indicated that iNOS mRNA-positive cells were macrophages and SMCs. Tissue sections from the same coronary arteries incubated with the sense probe, used as negative controls, did not show any hybridization signal (Figure 6D).

Discussion

The present study demonstrates that iNOS is expressed in coronary arteries of patients with TCAD. iNOS immunoreactivity was found in 30 of 36 arterial segments with TCAD (83%) and was localized to neointimal foam cells, SMCs, and macrophages. The presence of iNOS mRNA was demonstrated in the same vessels by in situ hybridization. We also showed that iNOS expression is associated with the presence of nitrated cellular proteins in the thickened neointima of coronary arteries with TCAD.

Russell and coworkers\textsuperscript{21} have previously demonstrated upregulation of iNOS in rat cardiac allografts with chronic rejection and transplant arteriosclerosis. In their study, iNOS mRNA was found to be markedly increased in coronary arteries of the transplanted hearts compared with paired host hearts and syngeneic grafts. iNOS protein immunoreactivity was demonstrated to be localized to medial and neointimal SMCs and macrophages in blood vessels with graft arteriosclerosis. Akyurek et al\textsuperscript{22} reported that iNOS mRNA and protein were expressed in rat aortic allografts. iNOS-positive SMCs and macrophages were found in the thickened neointima and in the media of the transplanted aortas. Recently, Lafond-Walker and coworkers\textsuperscript{23} have demonstrated the presence of iNOS in coronary arteries of human hearts with accelerated graft arteriosclerosis. By immunostaining of serial sections, these authors found iNOS in CD68-positive macrophages but not in neointimal SMCs. Our study therefore confirms previous findings in the experimental transplant model and demon-
A variety of growth factors and cytokines, including IL-1, IL-2, and IL-6; IFN-γ; and TNF-α. The presence of a cytokine-rich milieu in the vessel wall might therefore account for the expression of iNOS in this setting. Another immunologically mediated pathway might play an important role in the induction of iNOS in the cardiac allograft, namely the interaction between CD40, present on the surface of antigen presenting cells, endothelial cells, and SMCs, and the CD40 ligand (gp39) expressed by activated T-lymphocytes. Recent animal studies have shown that treatment of allograft recipients with an antibody directed against gp39 prolonged survival and markedly reduced the intragraft expression of iNOS. Preliminary data from our laboratory indicate that CD40 and CD40 ligand-positive cells are present in human lesions of atherosclerosis and TCAD.

In contrast to the marked iNOS upregulation we found in the epicardial coronary arteries of patients with TCAD, no expression was noted in intramyocardial arterioles. In this respect, our findings in humans differ from those of Russell and coworkers, who reported iNOS immunostaining in very small vessels, including intramyocardial arterioles, in a rat model of transplant arteriosclerosis. It is currently unknown whether the factors that have been demonstrated to regulate iNOS expression in large arteries such as the aorta or the epicardial coronary arteries also control iNOS production in small resistance vessels such as the intramyocardial arterioles. Clausell and coworkers have shown that histological abnormalities of small arterioles observed in endomyocardial biopsies specimens, albeit frequent, do not correlate with intravascular ultrasound findings and with endothelial dysfunction of large epicardial arteries. Taken together, these observations suggest that TCAD might be a more heterogeneous disease than usually thought in which different pathogenic factors contribute to disease development in different segments of the coronary artery tree.

The role that iNOS activation might play in the atherogenic process, in both native vessels and transplanted hearts, has yet to be elucidated. NO clearly has numerous potential antiatherogenic properties: it inhibits the proliferation of vascular SMCs in vitro, decreases platelet adhesion and aggregation, and reduces the endothelial expression of adhesion molecules and chemotactic factors. Inhibition of NOS with N-nitro-L-arginine methyl ester increases the extent of atherosclerotic lesions in the experimental animal, whereas administration of the NO precursor L-arginine has clear antiatherogenic effects in both native atherosclerosis and TCAD. NO has also been shown to induce apoptosis in vascular SMCs and macrophages in vitro through cGMP-dependent and independent mechanisms. Thus, iNOS- and NO-mediated apoptosis of macrophages and SMCs might decrease the cellularity of the atherosclerotic plaque and participate in the process of vascular remodeling. In a recent report, TCAD was exacerbated in iNOS-deficient mice, a finding consistent with a protective role for iNOS in this situation. Some of the effects deriving from the production of large amounts of NO by iNOS may nonetheless be proatherogenic. NO reacts with the free radical superoxide to form peroxynitrite, a strong oxidant that damages cellular proteins by nitration of tyrosine residues to form nitrotyrosine. In the present

Figure 5. In situ hybridization for iNOS mRNA in inflamed human tonsils. Staining with the antisense probe showed intense labeling for iNOS mRNA in activated macrophages (A). Sections incubated with the sense probe, used as negative controls, did not show any hybridization signal (B). No counterstaining. Magnification ×400.

strates, for the first time in humans, that iNOS is also produced by neointimal SMCs in arteries with TCAD.

The expression of iNOS has also been established in numerous other human tissues. Nicholson et al demonstrated that iNOS mRNA and protein are expressed by alveolar macrophages in patients with tuberculosis. iNOS enzyme activity, protein, and mRNA have been found in myocardial cells of patients with cardiomyopathy and cardiac allograft rejection and in human megakaryocytes. The demonstration of iNOS in human cells in vivo contrasts with the difficulties previously encountered by many investigators in inducing its expression in human cells in vitro. Recently, however, several laboratories have reported the induction of iNOS in human cells in vitro by appropriate cytokine combinations. Kolb et al have demonstrated that IFN-γ alone was not able to induce iNOS in human monocytes, unlike its effect in murine macrophages, but that its activation was triggered by the combination of IFN-γ and IL-4. Human epithelioid cells of the A549 cell line transfected with the human iNOS promoter were recently found to produce NO only when stimulated with a combination of IL-1β and IFN-γ but not with either cytokine alone. Additional stimulation was obtained by adding TNF-α. Thus, despite the marked similarity between the human and murine iNOS genes, differences exist in the control of their activation.

In animal models of TCAD, activated lymphocytes interact with macrophages and vascular endothelial cells to produce a
study, using a highly specific antibody developed by Beckman et al., we demonstrated extensive protein nitration in coronary arteries with TCAD, in concordance with previous observations in native atherosclerosis. Although little is known about the effects of tyrosine nitration on protein function in vivo, it has been shown that peroxynitrite or its decomposition products induce membrane lipid peroxidation and can initiate lipid peroxidation in human LDL. By altering the cellular redox state, it can also induce the expression of redox-sensitive genes (such as vascular cell adhesion molecule-1) that participate in the recruitment of inflammatory cells to the endothelial surface. The generation of large amounts of peroxynitrite consequent to the increased levels of NO and superoxide in the atherosclerotic lesion might therefore be a factor contributing to the progression of the disease.

In summary, the present study provides evidence that iNOS is expressed in macrophages and SMCs in the lesions of TCAD, where it is associated with extensive nitration of cellular proteins. Whether an increased local synthesis of NO is beneficial or detrimental in this process remains unclear and requires further investigation.

Acknowledgments
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References


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