American Journal of Pathology, Vol. 151, No. 4, October 1997

Short Communication

Inducible Nitric Oxide Synthase Expression in Coronary Arteries of Transplanted Human Hearts with Accelerated Graft Arteriosclerosis

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Inducible nitric oxide synthase (iNOS) is a high-output isoform of NOS that produces nitric oxide (NO), a nonspecific immune effector molecule. In some animal models of autoimmunity, the induction of iNOS has been shown to lead to inflammation and tissue damage, and it has been suggested that iNOS is an immune mediator in humans as well. Using *in situ* hybridization and immunohistochemical techniques, we demonstrate that iNOS mRNA and protein are present in the coronary arteries of transplanted human hearts with accelerated graft arteriosclerosis (AGA). iNOS is expressed in cells morphologically consistent with macrophages in the neointima of 7 of 10 of the transplanted vessels with AGA that were examined. In serial sections, these same cells express the macrophage marker CD68. In contrast, iNOS is absent from five native coronary arteries with atherosclerosis and absent from two normal coronary arteries. Although iNOS is expressed in macrophages in AGA, its role in the pathogenesis of AGA is unknown. ***(Am J Pathol 1997, 151:919–925)***

Nitric oxide (NO) is an unusual second messenger molecule; it is small, uncharged, and highly reactive.[1–6] NO is made by nitric oxide synthase (NOS), which converts arginine and oxygen into citrulline and NO. Three isoforms of NOS exist: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).[7–13] The constitutive isoforms eNOS and nNOS are normally expressed in certain cells but are inactive until calcium concentrations rise. In contrast, iNOS is normally absent from resting cells, but when it is induced, it continually synthesizes large amounts of NO. Cytokines or lipopolysaccharides can induce the expression of iNOS, and cytokines have been shown to induce iNOS expression in cardiac myocytes.[14–20]

During acute cardiac allograft rejection, cytokines are released that could induce iNOS. Cells that express iNOS during acute rejection of cardiac allografts in rats include cardiac myocytes, macrophages, and endothelial cells.[21–23] iNOS is also expressed during acute cardiac rejection in humans, and iNOS expression is associated with higher levels of NO metabolites in the serum of these patients.[24,25] Excess NO can impair cardiac contractility[26] and conceivably could damage or kill cells, leading to further inflammation. Although the precise role of iNOS in acute rejection is unclear, relatively specific iNOS inhibitors prolong graft survival in rats, whereas nonspecific NOS inhibitors do not.[27–31]

Coronary arteries of cardiac allografts are susceptible to a rapid form of arteriosclerosis called accelerated graft arteriosclerosis (AGA). AGA is the major long-term cause of death in cardiac transplant recipients. In contrast to native vessel atherosclerosis, AGA is diffuse and circumferential. AGA is characterized by a cellular infiltrate consisting predominantly of macrophages and lymphocytes and a neointima formed by smooth muscle cells.[21] Shi et al[32] have recently demonstrated that macrophages are necessary for the development of the neointima of AGA in a mouse model. However, it is unclear how these cells interact with other factors that may influence the development of AGA, such as cytomegalovirus (CMV) infec-

Supported by NIH grants P01 HL56092 (R. H. Hruban and C. J. Lowenstein), P50 HL52315 (A. Lafond-Walker and C. J. Lowenstein), and PSA K1102451 and R01 HL53615 (C. J. Lowenstein), grant MDSG2195 from the Maryland Affiliate of the American Heart Association (R. H. Hruban), and grants from the Bernard and Rebecca Bernard Foundation (C. J. Lowenstein), the John H. and Cora Davis Foundation (C. J. Lowenstein), and Novartis AG (R. H. Hruban and C. J. Lowenstein).

Accepted for publication July 22, 1997.

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tion, graft ischemia, complement, humoral antibodies, and lymphocytes.[33]

Although macrophages are essential for the development of AGA, the mechanisms of their recruitment into the vessel wall, and the factors that they elaborate once they are present in the vessel wall, are not well defined. Cytokines are produced in vessels with AGA, and these cytokines can activate macrophages, causing them to secrete substances that could promote the development of a neointima.[33–35] Recent reports show that iNOS is expressed in mononuclear cells and smooth muscle cells in the coronary arteries of rat cardiac allografts with AGA.[36,37] We hypothesized that a combination of inflammatory mediators activate macrophages to express iNOS in human vessels with AGA. Using *in situ* hybridization and immunohistochemical staining, we were able to demonstrate the presence of iNOS mRNA and protein in macrophages in human AGA.

Materials and Methods

Patients and Specimens

Between July 1983 and June 1985, 199 patients received a total of 211 heart transplants at The Johns Hopkins Hospital. Each transplant was assigned a unique number (H-1 to H-211). Twelve of the recipients were retransplanted because of severe AGA in their first grafts. Fresh-frozen tissue was available from 10 of these retransplants (H-13, H-36, H-38, H-44, H-61, H-116, H-126, H-149, H-167, and H-228). As controls, fresh-frozen sections of coronary arteries were obtained from hearts surgically explanted from five hearts of patients with native vessel coronary artery atherosclerosis, of whom two also had dilated cardiomyopathy, and from two patients with normal coronary arteries.

AGA was defined histologically as a diffuse luminal reduction by concentric fibro-intimal thickening.[21] This process often involved both the large epicardial vessels and the smaller perforating arteries. Grading of the degree of acute heart allograft rejection in the myocardium was based on the Working Formulation established by the International Society of Heart and Lung Transplantation.[38] Recipients were considered to be CMV-positive after transplantation if they had a positive serological titer of CMV, if they had histological evidence of CMV infection on biopsy, or if they had a positive culture for CMV.[39]

Immunohistochemistry

Immunohistochemical staining for iNOS was performed using two different commercial antibodies, a monoclonal and a polyclonal anti-iNOS antibody (both from Transduction Laboratories, Lexington, KY), and staining for the CD68 antigen was performed with a monoclonal anti-CD68 antigen (KP-1 clone, Dako Laboratories, Carpinteria, CA). Tissue was fresh-frozen and sectioned into 5-μm sections. Sections were processed as described previously.[21] In brief, sections were incubated with primary and then secondary antibodies, incubated with a tertiary reagent (an avidin-alkaline phosphatase complex for iNOS staining or an avidin-horseradish peroxidase complex for CD68 staining; Sigma Chemical Co., St. Louis, MO), incubated with a chromogen (3,3-diaminobenzidine or Fast Red substrate (Sigma)), counterstained with Mayer's modified hematoxylin (Polyscientific, New York, NY), and then coverslipped.

For controls, sections were stained either with the tertiary reagent alone or with the secondary antibody and the tertiary reagent. As an additional control, the sections were stained with a monoclonal antibody of the same isotype as the anti-iNOS monoclonal antibody against an irrelevant antigen *Coxiella*.

The number of cells staining was graded in a semiquantitative manner as has been previously described.[21]

In Situ *Hybridization*

Riboprobes

A recombinant plasmid containing a cDNA fragment of the human iNOS gene was constructed by isolating a 4.1-kb fragment containing an *Eco*RI linker at the *Eco*RV site and ligating it to the pBS vector that had been digested with *Eco*RI.[40] This pBS-hu-iNOS plasmid was linearized and transcribed *in vitro* with T7 or T3 RNA polymerase in the presence of digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN) to generate sense or antisense riboprobes. In those reactions using digoxigenin, 1% of the transcription products of 1.5 μg of the plasmid DNA and the digoxigenin-labeled RNA provided as a control by the manufacturer were compared by dot-blot hybridization. In each case, the signal associated with the plasmid's digoxigenin RNA was comparable to or exceeded that of the 0.2 ng of digoxigenin control RNA supplied by the manufacturer. As controls, an irrelevant anti-sense HPV69 riboprobe transcript was used.[41]

In Situ *Hybridization*

A modified RNA *in situ* hybridization method, which has been described previously, was used.[42] Briefly, 5-μm tissue sections were dewaxed and rehydrated, digested with proteinase K, and incubated with digoxigenin-labeled riboprobes for 6 hours at 50°C. The slides were then washed, incubated with RNAse A, dehydrated, and developed with a detection kit that includes anti-digoxigenin antibody (Boehringer Mannheim) according to manufacturer's instructions. Slides were counterstained with aqueous eosin solution. Normally, hybridization was performed without denaturation of the DNA in tissue sections. Negative controls included an unrelated sense probe to human papilloma virus.[41]

Results

Graft survival for the 10 hearts with AGA ranged from 36 months to 131 months (mean, 69 months; Table 1). The donor age ranged from 10 to 47 years (mean, 28 years), the recipient age ranged from 13 to 58 years (mean, 40

Table 1. Clinical Characteristics of Cardiac Transplant Patients with Accelerated Graft Arteriosclerosis

Transplant Number	Recipient age (years)	Donor age (years)	Graft ischemic time (minutes)	Graft survival time (months)	Rejection grade	CMV after transplant
13	17	18	195	131	1A	+
36	48	33	197	55	3B	
38	48	21	227	67	0	+
44	50	17	220	82	1A	
61	13	10	190	99	1A	
126	52	47	188	45	1A	
149	36	34	153	46	1A	
228	58	41	185	36	0	
116	25	29	154	55	3A	
167	48	27	140	72	3A	

years), and the graft ischemic time ranged from 140 to 227 minutes (mean, 185 minutes). Of the 10 transplanted patients, 2 developed a CMV infection after transplantation, 2 had no rejection (grade 0), 5 had grade 1A rejection, 2 had grade 3A rejection, and 1 had grade 3B rejection.

iNOS Protein Is Expressed in AGA

iNOS is expressed in vessels with AGA (Figure 1; Table 2). The cells expressing iNOS are large, vacuolated cells located throughout the neointima, including the subendothelial space (Figure 1). These cells are morphologically consistent with macrophages (Figure 1D). Positive staining slides show no staining when processed without the primary antibody (not shown). The slides did not stain when incubated with an isotype-matched irrelevant antibody (not shown).

These results were obtained using a monoclonal anti-iNOS antibody. We repeated the immunohistochemistry using a polyclonal antibody. Both antibodies stained similar cells, which histologically appear to be macrophages; these cells were located in the neointima and in the subendothelial area (not shown). The polyclonal anti-

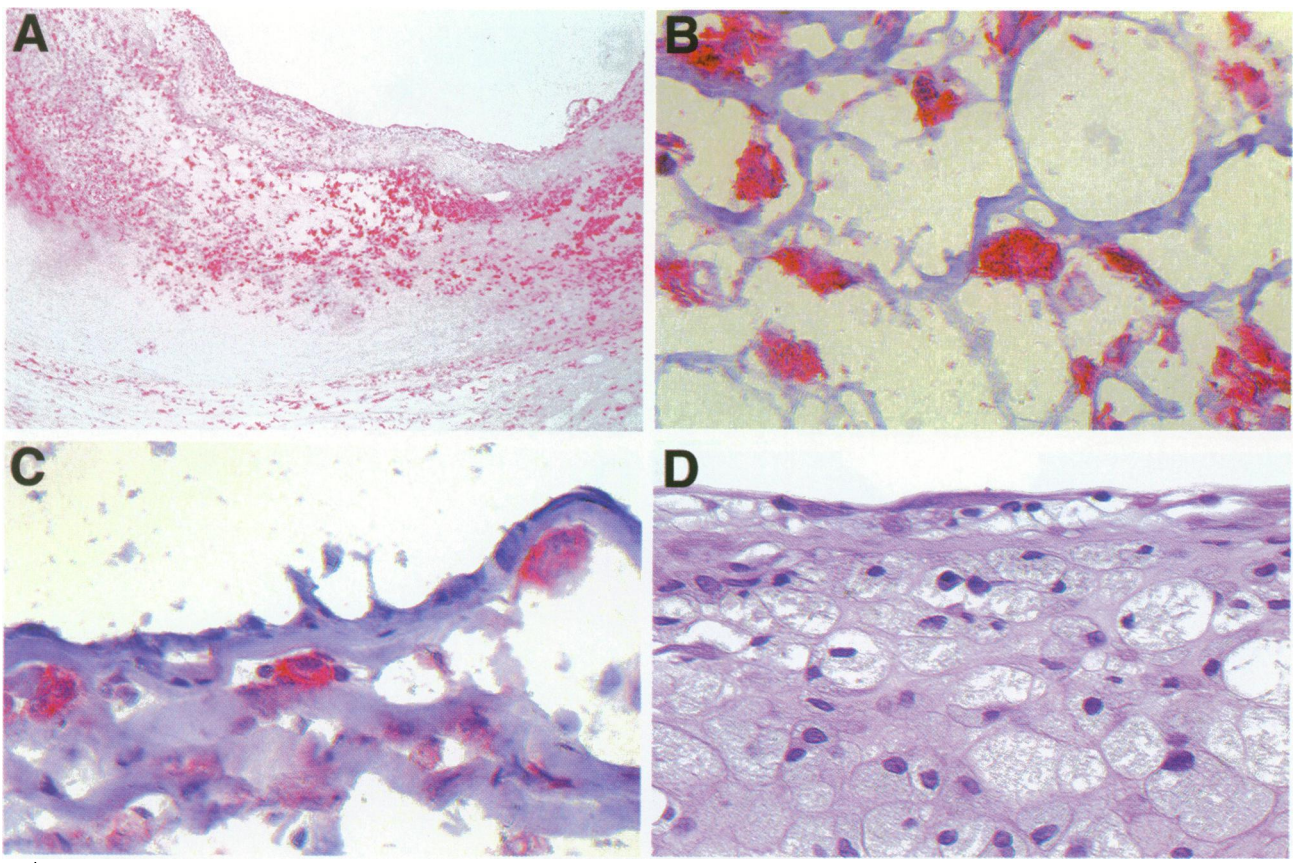

Figure 1. iNOS protein in accelerated graft arteriosclerosis. Immunohistochemical staining for iNOS (**A** to **C**) reveals numerous cells morphologically consistent with macrophages expressing iNOS. These cells are present in the neointima (**A** and **B**) as well as in subendothelial spaces (**C**). A hematoxylin and eosin (H&E)-stained section (**D**) reveals that many of the cells that express iNOS are morphologically consistent with macrophages.

Table 2. Cyclosporin Levels of Patients with AGA and Relative Staining for iNOS with Monoclonal and Polyclonal Antibodies

Transplant	Diagnosis	[Cy A]	iNOS (monoclonal)	iNOS (polyclonal)
13	Tx AGA	463	4	0
44	Tx AGA	202	4	ND
167	Tx AGA	ND	3	1.5
126	Tx AGA	189	2.5	1
149	Tx AGA	227	2.5	1.5
61	Tx AGA	183	2	0
228	Tx AGA	127	2	1.5
38	Tx AGA	115	1	1
36	Tx AGA	75	1	0
116	Tx AGA	276	2	1
	CAD, CMCAD	0	0	2
	CAD, CMCAD	0	0	0
	CAD, CM	0	ND	0
	CAD	0	0	0
	CAD	0	0	0
	nl	0	0	0

The polyclonal antibody stains fewer specimens and with less intensity than the monoclonal antibody. Tx AGA, transplant arteriosclerosis; CAD, coronary artery disease in native heart; nl, normal vessels in native heart ND, not done.

body, however, stained these cells with less intensity (Table 2). Furthermore, three of the sections with AGA that stained with the monoclonal antibody did not stain with the polyclonal antibody (Table 2).

iNOS mRNA Is Expressed in AGA

To confirm the localization of iNOS in mononuclear cells in coronary arteries with AGA, we used *in situ* hybridization. Positive hybridization for iNOS mRNA is present in large, vacuolated cells within the neointima of coronary arteries with AGA (Figure 2). As was true for the cells expressing iNOS protein by immunohistochemical staining, the cells expressing iNOS mRNA appear in two patterns: individual vacuolated cells lying directly under the endothelium and groups of foamy cells located throughout the neointima (Figure 2). The negative control using an unrelated sense probe did not hybridize to the sections (Figure 2D).

iNOS Protein and CD68 Co-Localize in AGA

Serial sections of vessels with AGA were immunohistochemically stained for CD68 and iNOS. These serial sections demonstrate that iNOS and CD68 are expressed in similar patterns and in similar cells (Figure 3).

Cyclosporin Levels Do Not Correlate with iNOS Expression

As cyclosporin has been reported to repress iNOS expression,[43–46] we compared the patients' serum cyclo-

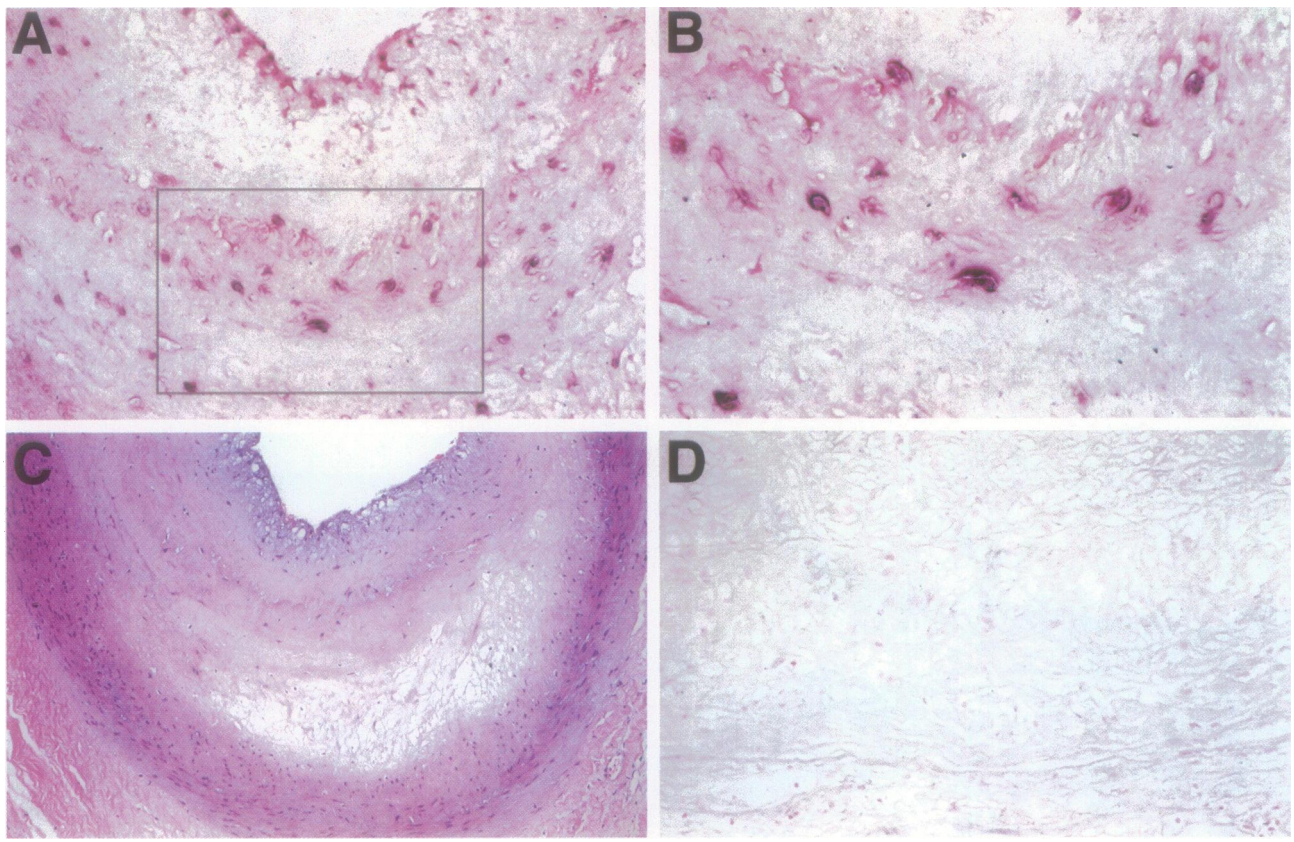

Figure 2. iNOS mRNA in accelerated graft arteriosclerosis. *In situ* hybridization for iNOS reveals that the iNOS message is expressed in cells morphologically consistent with macrophages in the neointima of a vessel with accelerated graft arteriosclerosis (**A** and **B**). The highlighted box in **A** is shown at higher magnification in **B**. **C**: H&E-stained serial section from this vessel. **D**: Control hybridization of the same vessel against HPV.

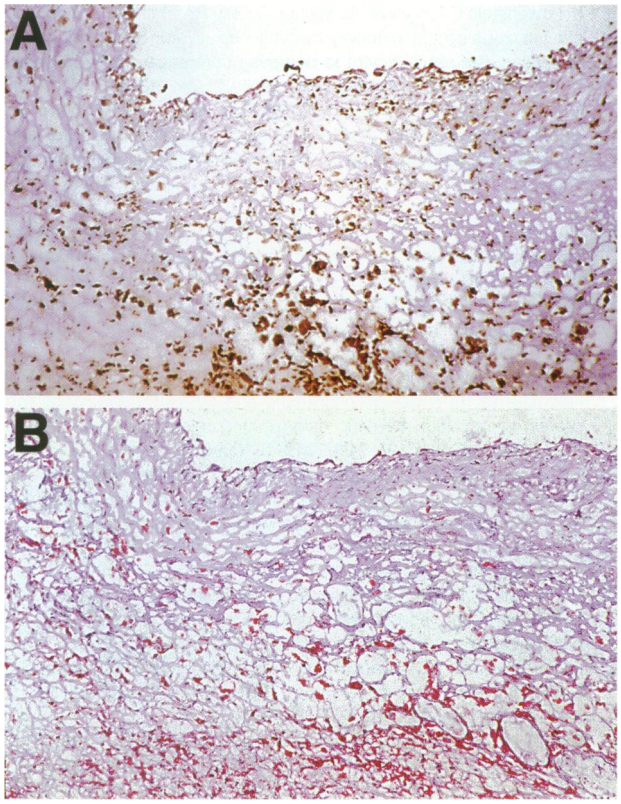

Figure 3. Co-localization of iNOS and CD68 in accelerated graft arteriosclerosis. Immunohistochemical staining of serial sections reveals numerous cells expressing CD68 (**A**), which in a serial section also express iNOS (**B**) in the neointima of a vessel with accelerated graft arteriosclerosis.

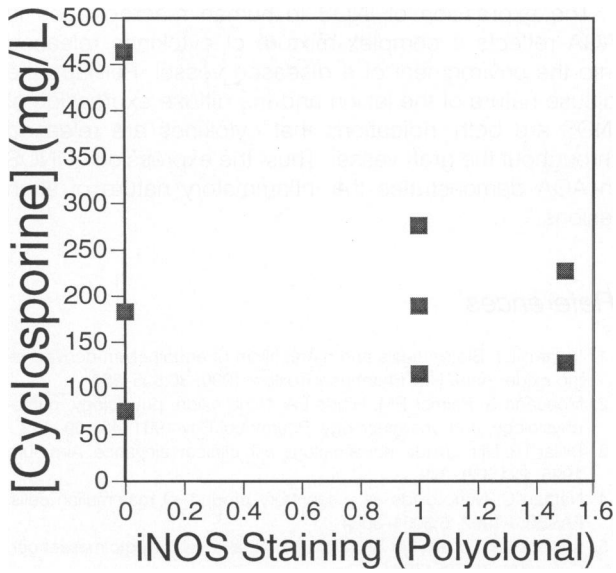

Figure 4. Lack of correlation between cyclosporin levels and iNOS staining. The polyclonal iNOS antibody was used to stain coronary arteries from eight patients with AGA. The intensity of staining was graded and compared with cyclosporin levels on the day of graft harvest.

sporin level on the day of tissue harvesting to the level of iNOS staining (Table 2). Although the number of patients is too small to draw statistically significant conclusions, the concentration of cyclosporin does not appear to be correlated with the intensity of iNOS staining with the polyclonal antibody (Figure 4) or monoclonal antibody.

Discussion

Accelerated graft arteriosclerosis is the major long-term cause of death for cardiac transplant recipients. AGA occurs in both pediatric and adult transplant patients. As the transplanted heart is denervated, typical symptoms of ischemia are frequently absent, and thus AGA can present as sudden death.[21] AGA is characterized by a diffuse and concentric narrowing of the lumen of the arteries of the transplanted heart. The lesions are composed of proliferating smooth muscle cells that form a neointima and by a cellular infiltrate of lymphocytes and macrophages.[21]

The pathogenesis of AGA is not precisely defined.[33] The initial injury may be influenced by factors such as graft ischemic injury, complement fixation, and CMV infection.[47,48] By unknown mechanisms, the initial injury recruits effector cells into the vessel wall. A recent report showed that, in a murine model of AGA, macrophages are critical to the development of AGA.[32] However, the mechanism by which macrophages influence AGA is unknown. Macrophages elaborate a variety of growth factors and cytokines that could affect AGA. For example, macrophages secrete transforming growth factor-β, interleukin-1, and tumor necrosis factor-α, which can stimulate smooth muscle cell proliferation, leading to the development of a neointima.

Macrophages can also produce NO by expressing iNOS. We found iNOS mRNA and protein expressed in the neointima of human coronary arteries with AGA but not in native vessel atherosclerosis or in normal coronary arteries. Others have found iNOS expressed in rodent and human hearts during acute graft rejection, and others have recently demonstrated iNOS in macrophages in arteries in rat cardiac allografts during AGA as well.[36,37]

The role of NO in the pathogenesis of AGA is not known. Moderate amounts of NO can affect cells in a variety of ways, including activating transcription factors such as the iron response binding element, inactivating phosphatases and thereby potentiating tyrosine kinase pathways, and inhibiting enzymes in the glycolytic and respiratory pathways.[49] For example, NO can protect hepatocytes from ischemic damage.[50] Furthermore, NO can inhibit the migration of cells[51] and perhaps could reduce the size of the neointima in AGA. However, large amounts of NO can damage cells, mutating DNA or inducing necrosis or apoptosis.[52–54] The cells in the vessel wall that are activated by moderate amounts of NO or necrotic cells killed by excess NO could induce further inflammation, contributing to the development of AGA. Although NO may damage cells and induce further inflammation, it is also possible that NO has no effect on AGA, merely serving as a marker for inflammation and the presence of cytokines. Selective inhibition of iNOS in animal models of AGA may reveal the role of iNOS and NO in the development of AGA.

The expression of iNOS in human macrophages in AGA reflects a complex mixture of cytokines released into the environment of a diseased vessel. Perhaps the diffuse nature of the lesion and the diffuse expression of iNOS are both indications that cytokines are released throughout the graft vessel. Thus, the expression of iNOS in AGA demonstrates the inflammatory nature of such lesions.

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