Cardiac Allograft Vasculopathy and Dysregulation of the NO Synthase Pathway

Michael Weis, John P. Cooke

Abstract—Cardiac allograft vasculopathy is the most aggressive form of atherosclerosis in humans and is the leading cause of death after the first year of heart transplantation. Endothelial dysfunction is a major contributing factor to the acceleration of coronary vascular disease in these individuals. A reflection of this endothelial dysfunction is the severe impairment in endothelium-dependent vasodilation that occurs early after transplantation. The etiology of this allograft endothelial alteration is multifactorial and may include preexisting atherosclerosis of the graft vessels, reperfusion injury during transplantation, denervation, disruption of the lymphatic system, and acute and chronic immune injury, as well as traditional risk factors for coronary artery disease (hyperlipidemia, diabetes, hypertension, or hyperhomocysteinemia) and pathogens, such as cytomegalovirus. The alteration in endothelial function affects vasomotor tone of the coronary arteries. Evidence indicates that there may be an impairment of endothelial production and/or activity of NO. Because NO is a potent vasodilator, its deficiency would explain the abnormal vasomotor tone in these individuals. In addition, because NO inhibits key processes in vascular inflammation and atherosclerosis, its absence may contribute to the acceleration of transplant vascular disease. Recent studies from our group and others have shed light on the mechanisms of endothelial dysfunction and its importance in cardiac allograft vasculopathy. In addition, the alteration in endothelial function contributes to vascular inflammation and progression of the disease. (Arterioscler Thromb Vasc Biol. 2003; 23:567-575.)

Key Words: endothelium ■ coronary ■ transplantation ■ heart ■ inflammation

Accelerated graft atherosclerosis is a key feature of most chronic rejection syndromes. Coronary atherosclerosis frequently limits the long-term success of cardiac transplantation, and is characterized by intimal proliferation during the early phase of the disease, and ultimately manifests itself as luminal stenosis of epicardial branches, occlusion of smaller vessels, and myocardial infarction. The most salient difference between cardiac allograft vasculopathy (CAV) and typical atherosclerotic coronary artery disease is the diffuse involvement of the coronary vasculature. Whereas the structural changes of atherosclerosis largely affect the proximal epicardial coronary arteries, in CAV, the disease is much more extensive. Cardiac transplant recipients often manifest concentric intimal thickening that affects the distal epicardial vessels, as well as their branches. Intimal thickening and inflammation may extend into the donor aorta up to the suture lines and, in some instances, even into the great veins of the allograft. Histopathologic analysis reveals that morphologic manifestation of this vasculopathy may range from concentric, diffuse, intimal hyperplasia to fibrofatty plaques indistinguishable from spontaneously occurring atherosclerosis. The clinical detection of CAV is made more difficult by the frequent occurrence of typical symptoms of ischemia in the denervated allograft; by the insensitivity of coronary angiography, which frequently underestimates the extent and severity of the disease; by the common involvement of small intramyocardial vessels; and by the occurrence of functional coronary alterations independent of morphological changes.

Immunologic and nonimmunologic factors influence the evolution and progression of transplant vasculopathy. Allograft coronary endothelial cells serve as potent stimulators as well as targets of allogeic lymphocyte reactivity. The recipient’s dendritic cells are the first and major antigen-presenting cells that recognize foreign major histocompatibility complex molecules on the allograft endothelium. After circulating dendritic cells adhere to endothelial cells, they capture foreign antigens, transmigrate, and usually enter the lymphatic vessels and lymph nodes. After heart transplantation, lymphatic disruption may alter this pathway, favoring reentry (reverse transmigration) of dendritic cells into the blood circulation. Subsequently, dendritic cells stimulate T lymphocytes (1 dendritic cell may stimulate up to 1000 T lymphocytes). The activated lymphocytes adhere to graft endothelial cells, enter the vessel wall, and sustain the chronic immune injury. Alterations in endothelial adhesiveness in the
The prevalence of microvascular endothelial dysfunction is also time dependent. Early after transplantation, microvascular dysfunction is prominent in 20% of the patients and increases to ~30% of the patients during long-term follow-up. Intriguingly, there is no significant correlation between the degree of epicardial dysfunction and that of microvascular dysfunction. Microvascular endothelial dysfunction occurs even in patients with angiographically normal epicardial coronary arteries. Thus, there appear to be some independent determinants of endothelial dysfunction in the epicardial and resistance vessels of the cardiac allograft.

Consequences of Allograft Vasomotor Dysfunction

The endothelium is the maestro of the circulation, a major determinant of vascular tone and blood flow. The healthy endothelium releases a panoply of vasodilator substances, such as NO, prostacyclin, atrial natriuretic peptide, endothelium-derived hyperpolarizing factor, and adrenomedullin. Thus, the healthy endothelium increases vessel diameter and reduces resistance to blood flow. When the endothelium becomes diseased, the synthesis and bioactivity of the vasodilators are reduced, and the balance tips in favor of endothelium-derived vasoconstrictors, such as endothelin and thromboxane. This derangement in endothelial function has clinical consequences. As a result of the impairment in endothelial vasodilator function, there is an increase in coronary vascular resistance, which can result in ischemia.

Hasdai et al have found that coronary endothelial dysfunction in humans is associated with reversible myocardial perfusion defects. In transplant recipients, an impaired coronary flow reserve has been associated with subsequent reduction in left ventricular ejection fraction during a 2-year follow-up, suggesting that repetitive subendocardial ischemia during myocardial stress can cause a deterioration of ventricular function. Consistently, heterogeneity of coronary vasodilator reserve is correlated with a significantly increased risk of cardiovascular events.

As discussed below, endothelial vasodilator dysfunction is associated with other vascular abnormalities (eg, expression of adhesion molecules, leukocyte adherence and infiltration, and smooth muscle cell proliferation). Several groups have reported changes in allograft endothelial functions (vasomotor independent) that are correlated with allograft failure. Thus, endothelial vasomotor dysfunction might be a marker of endothelial activation, increased adhesiveness and thrombogenicity, and the risk of atherogenesis. Indeed, early epicardial endothelial vasodilator dysfunction predicts the development of visible vasculopathy (as imaged by intravascular ultrasound) 1 year after transplantation. This is consistent with reports that coronary endothelial dysfunction in transplant and nontransplant patients is predictive of adverse cardiovascular events. It is also possible that the loss of the vasodilator contributes directly to the progression of transplant vasculopathy. As it turns out, most of the endothelium-derived vasodilators oppose key processes involved in atherogenesis, ie, cell adhesion, proliferation, and inflammation. Endothelium-derived NO is paradigmatic of an endothelium-derived antiatherogenic molecule.

Role of Endothelial NOS in Maintaining Vascular Homeostasis

Endothelium-derived NO is the most potent endogenous vasodilator known. NO induces vasodilation by stimulating soluble guanylate cyclase to produce cGMP. NO has a short half-life and avidly interacts with sulfhydryl-containing proteins, heme proteins, and oxygen-derived free radicals. By virtue of its ability to nitrosylate proteins, it may change their activity or behavior. The physiological importance of this
endothelium-derived vasodilator is reflected by the significant increase in vascular resistance that is induced in animals and humans exposed to pharmacological antagonists of NO synthase (NOS).51,52

Endothelium-derived NO also inhibits platelet and leukocyte adherence to the vessel wall.53,54 This effect of NO is mediated in part by the activation of cGMP and phosphorylation of intracellular signaling proteins, such as vasodilator-stimulated phosphoprotein.55 In addition, NO suppresses the expression of adhesion molecules and chemokines regulating endothelial interaction with circulating blood elements. Finally, endothelium-derived NO also inhibits vascular smooth muscle cell proliferation.56 This is in part mediated by an effect of NO, an increase in vascular smooth muscle cell apoptosis.57 In contrast, NO is a survival factor for endothelial cells.58 These observations are consistent with the view that NO is an endogenous antithromogenic molecule.

Impairment of endothelial NOS (eNOS) contributes to the pathological alterations in vascular reactivity and structure that are observed in atherosclerosis.59,60 Pharmacological inhibition or genetic deficiency of NOS inhibits endothelium-dependent vasodilation, impairs tissue blood flow, and raises the blood pressure.59 Furthermore, NO deficiency promotes the adherence and intimal accumulation of mononuclear cells and accelerates lesion formation in animal models of atherosclerosis.59,61 By contrast, enhancing NO production in the vessel wall slows or even reverses atherosclerosis.62–64 As discussed below, endothelial NO bioactivity is also a modulator of CAV.

**Does NO Deficiency Play a Role in the Progression of Transplant Vasculopathy?**

In organ transplantation, allograft eNOS expression and activity can be impaired by a number of mechanisms, including preexisting arteriosclerotic disease in the graft, graft ischemia before transplantation, immunosuppressive agents such as cyclosporin A and tacrolimus, classic risk factors (hyperlipidemia, hypertension, diabetes, and hyperhomocysteinemia), and, possibly, infectious diseases such as CMV.

In preclinical models of transplant vasculopathy, NO deficiency accelerates the disorder. The inducible form of NO (iNOS) is expressed in the vessel wall of the aortic allograft. Inhibition of iNOS activity in the aortic allograft significantly increases intimal hyperplasia at 4 weeks.65 Furthermore, early overexpression of iNOS by the use of ex vivo gene transfer completely prevents the development of structural changes in rejecting grafts.65 Furthermore, structural changes are accelerated in iNOS-knockout mice.66 The protective effects of iNOS in these studies may be due to an effect of NO, ie, the inhibition of SMC proliferation and suppression of the adhesion of platelets and leukocytes to the endothelium.66,67 In the context of these observations, it should be noted that there has been some controversy regarding what role iNOS may have in the development of vascular lesions. We hold the view that under the right conditions, iNOS suppresses inflammation and atherogenesis. Specifically, under circumstances in which L-arginine is not rate limiting, the product of iNOS is NO. NO is a survival factor for endothelial cells, but it induces apoptosis of macrophages and proliferating vascular smooth muscle cells.68 Indeed, by increasing vascular NO generation with supplemental L-arginine, apoptosis of macrophages and regression of preexisting lesions is observed in the fat-fed New Zealand White rabbit.69 In this case, the major source of NO in the vessel wall is iNOS. However, under circumstances in which L-arginine becomes rate limiting, the product of iNOS is the superoxide anion (O2·−), which can increase local oxidative stress and exacerbate the inflammatory process. In human cardiac allografts, microvascular endothelial dysfunction is associated with an enhanced endomyocardial iNOS mRNA expression and is accompanied by the expression of nitrotyrosine protein, suggesting peroxynitrite-mediated vessel damage.70 Importantly, dietary L-arginine has been shown to attenuate the structural changes of transplant vasculopathy in vivo associated with downregulation of insulin-like growth factor-I and interleukin-6.71

The literature supports a protective role for eNOS. In a murine chronic-rejection model, transplant atherosclerosis is accelerated in aortic allografts of eNOS-deficient mice.72 Iwata et al73 have demonstrated that intraoperative liposome-mediated gene delivery of eNOS to rabbit donor hearts results in early gene expression sufficient to reduce ischemia/reperfusion injury by inhibiting nuclear factor-κB activation, adhesion molecule expression (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1), and leukocyte infiltration.73 Enhanced eNOS expression extends graft survival without immunosuppression. Most important, eNOS immunoreactivity is gradually lost after human heart transplantation,74 and a reduced myocardial eNOS gene expression has been associated with coronary endothelial dysfunction.75 Thus, eNOS in the endothelium appears to protect allografts from endothelial activation and structural changes.

**Is Impairment of the NOS Pathway a Risk Factor for Vascular Disease in Humans?**

Do alterations in the NOS pathway contribute to the initiation and/or progression of atherosclerosis in humans? Several lines of evidence indicate that impairment of the NOS pathway does cause human coronary artery disease and vascular events. Genomic studies of the NOS pathway indicate that genetic alterations in NO or O2·− generation contribute to human vascular disease. Certain eNOS gene polymorphisms are predictive of coronary artery disease.76,77 The eNOS gene Glu298Asp polymorphism is more prevalent in patients with variant angina, essential hypertension, and acute myocardial infarction.78–80 Similarly, a quadruple repeat of a 27-bp sequence in intron 4 of the eNOS gene (allele a) is associated with the risk of coronary artery disease and acute myocardial infarction.81 There is an interaction with smoking, with homozygotes for the eNOSa allele at risk for greater severity of coronary artery disease. Of note, it has been reported that the eNOSa allele is associated with a decrease in the level of plasma nitrogen oxides.82 These studies suggest that genetically determined alterations in the NOS pathway could predispose an individual to atherosclerosis. Recently, several groups have independently offered
evidence that endothelial vasodilator dysfunction is an independent predictor of vascular events.83–85 These data provide a compelling rationale for understanding the mechanisms of NO deficiency, with a view toward developing new therapeutic avenues to prevent coronary artery disease and its progression.

Mechanisms of NO Deficiency in CAV
Hypercholesterolemia, hypertriglyceridemia, hyperhomocysteinemia, hypertension, and hyperglycemia are all conditions that are associated with the posttransplant state, mainly triggered by the use of cyclosporine or tacrolimus and steroids for immunosuppression.13,15,86 Each of these conditions is associated with endothelial vasodilator dysfunction.

Of note, different immunosuppressive strategies have been shown to elicit different expression patterns of vasoactive mediators in the allograft.79 Specifically, the combination of tacrolimus and azathioprine is associated with decreased myocardial eNOS gene expression and endothelial dysfunction early after transplantation.75 In addition, coronary endothelial vasomotor dysfunction is associated with an increased myocardial expression and elaboration of the vasoconstrictor endothelin.87 Significantly, endothelin immunostaining is increased in coronary arteries affected by CAV.88 The early administration of an endothelin antagonist was associated with lower prevalence of functional and morphological abnormalities in a rat transplant model.89 Moreover, inhibition of the endothelin-1-converting enzyme significantly improved survival.90 Inasmuch as endothelial dysfunction appears to be a predictor of morphological changes,25 one might conclude that an imbalance between NO and endothelin bioactivity in the allograft may contribute to the development of CAV.

Cardiac cytokine release, a hallmark of allograft inflammatory activity and a common phenomenon early after heart transplantation, is related, at least in part, to endothelial vasomotor dysfunction of the epicardial and microvascular compartment.12 We found an association between an early elevation of coronary sinus levels of inflammatory cytokines and endothelial vasomotor dysfunction during a 1-year follow-up (M. Weis, unpublished data, 2002).

With respect to inflammation and vascular disease, increasing attention has been focused on the role of human CMV, a member of the herpesviruses.91–95 CMV can infect human vascular endothelial cells and induce changes relevant to atherogenesis.94 CMV infection increases the expression of endothelial surface adhesion molecules, which upregulate the recruitment of granulocytes.94 Furthermore, CMV infection promotes mononuclear cell adhesion, activation, and transendothelial migration within the allograft vasculature.96 CMV infection also shifts the balance between endothelial factors mediating blood fluidity so that a procoagulant state is favored.96 It appears that CMV infection of the endothelium promotes processes that favor atherosclerosis and vascular lesion formation. Indeed, human CMV is associated with transplant vasculopathy.96–98

The most direct evidence of a link between CMV and transplant atherosclerosis was recently produced by Valantine et al.99 In their study, prophylactic treatment of cardiac transplant recipients with ganciclovir reduced the incidence of vasculopathy.99 Thus, a therapy directed toward CMV infection dramatically improves the outcome of patients after transplantation.

These data suggest that CMV may contribute to the initiation and/or progression of transplant vasculopathy. However, the mechanisms by which CMV may trigger atherosclerosis are incompletely defined. The immediate-early gene of human CMV can code for a protein that has sequence homology and immunologic cross-reactivity with a domain of human leukocyte antigen-DR.97 Additionally, CMV interferes with the action of p53, a protein that inhibits proliferation and induces apoptosis of vascular smooth muscle cells.98 One of the major mechanisms by which CMV could initiate and/or accelerate transplant vasculopathy is by impairing the NOS pathway. Inflammation impairs endothelium-dependent vasodilation in humans,12,100 and the virus-induced impairment of the eNOS pathway might accelerate atherosclerosis.101 Indeed, in the hypercholesterolemic mouse, infection with murine forms of chlamydia accelerates plaque growth.102

It is possible that CMV infection could downregulate eNOS expression as well as activity. Tumor necrosis factor (TNF-α) has been reported to destabilize mRNA message for eNOS, possibly by inducing the expression of a binding protein for the 3′ untranslated region of eNOS mRNA.103 CMV infection of cells stimulates the expression of TNF-α104,105 as well as transcription factors (such as nuclear factor-κB) that stimulate the expression of TNF-α. Parenthetically, NO may inhibit viral replication in the THP-1 monocytic cell line.106 In addition to the effects of TNF-α on eNOS message stability, we and others have found that exposure of endothelial cells to TNF-α reduces NO synthesis and bioactivity.107 These effects appear to be due to the increased elaboration of asymmetric dimethylarginine (ADMA) and O2−, respectively (see discussion below). Furthermore, TNF-α may induce the endothelial expression of iNOS. The induction of iNOS does not necessarily increase endothelial NO production. This is because in the setting of inflammation and/or metabolic disturbances associated with vascular disease (eg, hypercholesterolemia), there are deficiencies of the cofactor tetrahydrobiopterin as well as the precursor, l-arginine.108,109 In the absence of tetrahydrobiopterin, NOS is no longer capable of transferring electrons to l-arginine to produce NO; instead, the preferred electron acceptor becomes oxygen, to form O2−, a phenomenon known as eNOS uncoupling.110 These data support the hypothesis that human CMV infection, one of the most common infectious complications in allograft recipients, may contribute to the development of CAV via interaction with the NOS system.

A Role for ADMA?

The impairment in endothelium-dependent vasodilation occurs early in the course of vascular disease and affects both conduit and resistance vessels.111–113 The impairment in endothelium-dependent vasodilation is multifactorial and dependent on the vessel and species studied, the stage of atherosclerosis, and the associated metabolic disorders. The mechanism of impairment may include endothelial generation of O2− and increased degradation of NO, elaboration of...
vasoconstrictor prostanoids and endothelin, reduced elaboration of prostacyclin, and/or impaired biosynthesis of NO.⁶⁰,¹¹⁴

Impaired biosynthesis of NO may be due to alterations in NOS affinity for L-arginine, to lipid-induced impairment of the high-affinity cationic amino acid transporter, to reduced availability of the cofactor tetrahydrobiopterin, or to increased levels of ADMA, the competitive inhibitor of NOS.¹¹⁵,¹¹⁶ Our group and others have accumulated extensive data to indicate that O₂⁻ and ADMA are major determinants of endothelial vasodilator dysfunction in humans at risk for atherosclerosis.¹⁰⁷,¹¹⁶–¹¹⁸ Whereas O₂⁻ degrades NO to reduce its bioactivity, ADMA inhibits NO synthesis. It is also possible that ADMA may “uncouple” eNOS, so that eNOS generates O₂⁻.¹¹⁹

Intriguingly, plasma levels of ADMA are elevated in a number of conditions associated with endothelial vasodilator dysfunction, including renal failure, hypercholesterolemia, hyperhomocysteinemia, hypertension, diabetes mellitus, and heart failure.¹²⁰ When ADMA is added to the medium at levels observed in renal failure, it induces vasoconstriction of vascular rings, which is reversible by addition of L-arginine to the medium.¹²¹ Dialysis normalizes plasma ADMA levels and improves endothelium-dependent relaxation of peripheral vessels in patients with renal failure.¹²¹ It appears that the levels of ADMA observed in patients with hypercholesterolemia and/or atherosclerosis are sufficient to explain the impairment of endothelial function and to explain the observations made repeatedly by multiple investigators that the endothelial dysfunction is reversible by administration of exogenous L-arginine.¹²²–¹²⁴ It is very likely that the L-arginine/ADMA ratio is a regulator of NOS activity that becomes disordered in atherosclerosis and with risk factors for atherosclerosis. Indeed, in hypercholesterolemic individuals, the plasma L-arginine/ADMA ratio is a better predictor of endothelial vasodilator dysfunction than is LDL cholesterol.¹¹⁷ Importantly, several investigations indicate that there is an L-arginine–reversible impairment of the NOS pathway in atherosclerosis that is at least in part mediated by ADMA.¹²⁵–¹²⁷ Recent studies from our laboratory indicate that the major mechanism responsible for elevated plasma levels of ADMA is a reduction in its degradation by the oxidant-sensitive enzyme dimethylarginine dimethylaminohydrolase (DDAH).¹⁰⁷,¹²⁸,¹²⁹

DDAH is the enzyme most responsible for the degradation of ADMA. We find that its activity is impaired by hypercholesterolemia, hyperglycemia, and hyperhomocysteinemia.¹⁰⁷ The reduction in DDAH activity is responsible for accumulation of ADMA, which then inhibits NO synthesis. To the extent that ADMA is responsible for the impairment of endothelial vasodilator dysfunction, it may be a predictor for vascular events. Indeed, it has been demonstrated in a study of 120 adults with varying levels of risk that ADMA and age were the only independent predictors of intimal-medial thickness of the carotid artery, as measured by ultrasound.¹³⁰ This finding has recently been confirmed and extended by Zoccali et al.¹³¹ They studied >200 individuals with end-stage renal disease. Most intriguingly, in a 5-year follow-up of these patients, the elevation in plasma level of ADMA was the strongest predictor of vascular events, with those in the upper quintile of plasma ADMA level having an odds ratio >10.¹³¹ Independently, another group of investigators found that ADMA is an independent predictor of cardiovascular events in patients with coronary artery disease.¹³² These data provide strong evidence for the crucial role of the NOS pathway in different stages of vascular disease. Of note, preliminary data from our laboratory indicate that ADMA is elevated in cardiac transplant recipients. Intriguingly, the elevation is greater in CMV-positive patients and is correlated with the extent of transplant coronary artery disease. The central role of the disrupted NO pathway after transplantation in the development of cardiovascular morbidity (based on the described studies) is outlined in the Figure.

**Therapeutic Options**

These recent insights indicate a significant contribution of endothelial dysfunction to the initiation and progression of atherosclerosis and CAV. Strategies to preserve normal endothelial function are likely to be useful therapies after heart transplantation.¹³³ For coronary atherosclerosis, endothelial protective agents such as the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) and ACE inhibitors have already been proven to reduce cardiac...
mortality in patients with coronary atherosclerosis. Statin therapy may be associated with a lower prevalence of graft endothelial dysfunction in humans, independent of lipid-lowering effects.\textsuperscript{134} Enhancement of myocardial eNOS expression, as well as anti-inflammatory actions of statins, may be particularly beneficial in the transplant setting.\textsuperscript{133} ACE inhibitors have been shown to reverse coronary endothelial dysfunction in nontransplant atherosclerosis\textsuperscript{135} and partially improve allograft microvascular endothelial dysfunction.\textsuperscript{136} Calcium channel blockers improve endothelial function in preclinical models but have not been convincingly shown to reduce the progression of coronary artery disease in humans. However, the situation is different in cardiac transplant recipients, inasmuch as the calcium channel blocker diltiazem appears to slow the progression of transplant coronary artery disease.\textsuperscript{137} Moreover, enhanced nifedipine-induced coronary vasorelaxation occurs in transplant recipients with coronary endothelial dysfunction.\textsuperscript{138} Other drugs that might have the potential to restore endothelial vasodilator dysfunction under circumstances relevant to CAV include antioxidants such as probucol and vitamins C and E,\textsuperscript{139} insulin sensitizers such as rosiglitazone, phosphodiesterase inhibitors such as cilostazol, and modifiers of NO synthesis, including L-arginine and tetrahydrobiopterin.

Another potential strategy is to inhibit the proliferation of infectious particles, such as CMV, that may affect endothelial function. Indeed, ganciclovir treatment appears to reduce the infective particles, such as CMV, that may affect endothelial function. Indeed, ganciclovir treatment appears to reduce the infective particles, such as CMV, that may affect endothelial function. Indeed, ganciclovir treatment appears to reduce the infective particles, such as CMV, that may affect endothelial function. Indeed, ganciclovir treatment appears to reduce the infective particles, such as CMV, that may affect endothelial function.

**Summary**

CAV remains the most troublesome long-term complication of heart transplantation. Allograft coronary endothelial cells, the allogeneic barrier between a recipient’s circulating immunoreactive cells and the transplanted organ, can serve as stimulators as well as targets of inflammatory reactivity. Immunologic and nonimmunologic factors likely influence the evolution and progression of transplant vasculopathy, mediated in part by dysregulation of the eNOS pathway. Activation and dysfunction of the arterial endothelium predict the development of CAV and may increase the risk of graft failure.

**References**


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