Coronary Heart Disease

Dimethylarginine Dimethylaminohydrolase Overexpression Suppresses Graft Coronary Artery Disease

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Background—Graft coronary artery disease (GCAD) is the leading cause of death after the first year of heart transplantation. The reduced bioavailability of endothelium-derived nitric oxide (NO) may play a role in endothelial vasodilator dysfunction and the structural changes that are characteristic of GCAD. A potential contributor to endothelial pathobiology is asymmetric dimethylarginine (ADMA), an endogenous NO synthase inhibitor. We hypothesized that lowering ADMA concentrations by dimethylarginine dimethylaminohydrolase (DDAH) overexpression in the recipient might suppress GCAD and long-term immune responses in murine cardiac allografts.

Methods and Results—In one series, donor hearts of C-H-2<sup>bm12</sup>KhEg (H-2<sup>bm12</sup>) wild-type (WT) mice were heterotopically transplanted into C57BL/6 (H-2<sup>b</sup>) transgenic mice overexpressing human DDAH-I or WT littermates and procured after 4 hours of reperfusion (WT and DDAH-I recipients, n=6 each). In a second series, donor hearts were transplanted into DDAH-I–transgenic or WT mice and procured 30 days after transplantation (n=7 each). In DDAH-I recipients, plasma ADMA concentrations were lower, in association with reduced myocardial generation of superoxide anion (WT versus DDAH-I, 465.7±79.8 versus 173.4±32.3 μmol·L<sup>-1</sup>·mg<sup>-1</sup>·h<sup>-1</sup>; P=0.02), inflammatory cytokines, adhesion molecules, and chemokines. GCAD was markedly reduced in cardiac allografts of DDAH-I–transgenic recipients as assessed by luminal narrowing (WT versus DDAH-I, 79±2% versus 33±7%; P<0.01), intima-media ratio (WT versus DDAH-I, 1.1±0.1 versus 0.5±0.1; P<0.01), and the percentage of diseased vessels (WT versus DDAH-I, 100±0% versus 62±10%; P<0.01).

Conclusions—Overexpression of DDAH-I attenuated oxidative stress, inflammatory cytokines, and GCAD in murine cardiac allografts. The effect of DDAH overexpression may be mediated by its reduction of plasma and tissue ADMA concentrations. (Circulation. 2005;112:1549-1556.)

Key Words: nitric oxide synthase • coronary disease • reperfusion • arginine • transplantation

The development of graft coronary artery disease (GCAD) frequently limits the long-term success of cardiac transplantation. GCAD is a key feature of most chronic rejection syndromes and is the leading cause of death in patients surviving >1 year after transplantation. GCAD 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. Ischemia/reperfusion injury seems to be the strongest alloantigen-independent factor for the subsequent development of GCAD in a case-control study. This injury induces oxidative stress, leading to the elaboration of cytokines, chemokines, and adhesion molecules that participate in GCAD. No selective treatment for GCAD is currently available. The reduced bioavailability of endothelium-derived nitric oxide (NO) may play a role in the endothelial vasodilator dysfunction and structural changes that are characteristic of GCAD. NO inhibits platelet and leukocyte adherence to the vessel wall, suppresses the expression of adhesion molecules and chemokines regulating endothelial interactions with the circulating blood, and inhibits vascular smooth muscle cell proliferation. Notably, NO bioavailability is reduced in human allograft coronary arteries very early after transplantation and is predictive of later transplant arteriopathy.

Preclinical studies provide further support for the notion that a deficiency of NO bioavailability contributes to GCAD. In a murine chronic-rejection model, GCAD is accelerated in aortic allografts of endothelial NO synthase (eNOS)–deficient mice. Iwata et al demonstrated that intraoperative, liposome-mediated gene delivery of eNOS to rabbit donor hearts reduced ischemia/reperfusion injury by inhibiting nuclear factor-κB activation, adhesion molecule expression, and...
leukocyte infiltration. Thus, eNOS appears to protect cardiac grafts from ischemia/reperfusion injury and reduce the development of GCAD.

The synthesis of NO is inhibited by the endogenous arginine analogues asymmetric dimethylarginine (ADMA) and N-monomethylarginine, the more abundant species being ADMA. Plasma levels of ADMA are dynamically regulated by the enzymes responsible for its synthesis (protein arginine N-methyltransferases) and more important, by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), responsible for its degradation. The plasma level of ADMA modulates NOS activity; specifically, DDAH-I–transgenic mice show a 2-fold reduction in plasma ADMA levels associated with a 2-fold increase in tissue NOS activity and urinary nitrogen oxide production. Elevated ADMA plasma concentrations may therefore contribute to the vascular pathophysiology observed in atherosclerosis. ADMA levels are elevated in patients with cardiovascular risk factors, eg, hypertension, hyperlipidemia, hyperhomocysteinemia, and diabetes mellitus. Moreover, ADMA appears to be an independent predictor of acute coronary events and cardiovascular and overall mortality.

Therefore, the aim of our study was to determine whether lowering ADMA concentrations by DDAH overexpression in the recipients would suppress GCAD and immune response in murine cardiac grafts.

**Methods**

**Animals**

Inbred female C-H-2\(^{mnt}\)KhEg (H-2\(^{m11}\)) mice, 6 to 10 weeks old, were purchased from the Jackson Laboratories (Bar Harbor, Me). DDAH-I–transgenic mice (DDAH) overexpressing the human isoform of DDAH-I were generated as previously described in detail. For maintenance of the colony, male transgenic mice were mated with C57BL/6 female mice (Jackson Laboratory, Bar Harbor, Me). Offspring were screened for transgene expression by polymerase chain reaction and Southern blot analysis with tail DNA, as described earlier. Female C-H-2\(^{mnt}\)KhEg (H-2\(^{m11}\)) mice were used as donors, and 7- to 8-month-old female heterozygous DDAH-transgenic mice and age- and weight-matched wild-type (WT) littermates were used as recipients for the study of GCAD. All mice were housed in the animal care facility at Stanford University Medical Center under standard temperature, humidity, and timed lighting conditions and were provided mouse chow and water ad libitum. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996). The study protocol was approved by the Administrative Panel on Laboratory Animal Care, Stanford University.

**Heterotopic Cardiac Transplantation**

Heterotopic abdominal cardiac transplantation was performed according to the method of Corry et al, with some modifications. In brief, the donor pulmonary veins, azygous vein, and inferior (IVC) and superior vena cavae were ligated. The donor ascending aorta was sutured end-to-side to the recipient lateral abdominal aorta below the right kidney, and the donor pulmonary artery was anastomosed to the recipient IVC. The heterotopically transplanted hearts behave functionally as aorto caval fistulas. Blood enters the donor ascending aorta from the recipient abdominal aorta and is diverted into the coronary arteries by the closed aortic valve. After the myocardium is perfused, venous blood drains into the right atrium through the coronary sinus and is pumped back into the recipient IVC by the right ventricle.

Anesthesia was induced with 5% inhaled isoflurane (Halocarbon Laboratories). During surgery, the animals were maintained on 2.5% inhaled isoflurane. Donor animals were systemically heparinized (50 IU/kg) before heart procurement. The donor heart was rapidly excised after retrograde coronary perfusion with ice-cold saline. The procured hearts were kept in ice-cold saline for 20 minutes. Because standard graft implantation averages ~30 minutes, the total ischemic time was 50 minutes.

In one series, donor hearts of C-H-2\(^{mnt}\)KhEg (H-2\(^{m11}\)) WT mice were heterotopically transplanted into C57BL/6 (H-2\(^{b}\)) transgenic mice overexpressing human DDAH or WT littermates and procured after 4 hours of reperfusion (6 WT recipients, 6 transgenic [DDAH] recipients) to investigate the impact of oxidative stress and chemokine activation on cardiac allograft vasculopathy in the early phase immediately after heterotopic heart transplantation.

In a second series, donor hearts of C-H-2\(^{mnt}\)KhEg (H-2\(^{m11}\)) mice were heterotopically transplanted into C57BL/6 (H-2\(^{b}\)) DDAH-transgenic mice or WT littermates and procured 30 days after transplantation (7 WT recipients, 7 DDAH recipients). No immuno-suppression was used. Graft viability was assessed daily by direct abdominal palpitation of the heterotopically transplanted hearts.

**Graft Survival and Allograft Function Analysis**

Mice used in the second series of the study were monitored daily. Graft viability was assessed by direct abdominal palpation of the heterotopically transplanted hearts. Cardiac graft function was expressed as a beating score and assessed by the Stanford cardiac surgery laboratory graft scoring system (0 = no contraction; 1 = contraction barely palpable; 2 = obvious decrease in contraction strength, but still contracting in a coordinated manner, with rhythm disturbance; 3 = strong, coordinated beat but noticeable decrease in strength or rate, with distortion/stiffness; and 4 = strong contraction of both ventricles, regular rate, no enlargement or stiffness).

**Myocardial Generation of NO Ex Vivo**

DDAH-transgenic and WT mice (n = 6) were fasted overnight and on nitrate-low water. After an injection with ketamine/xylazine (0.5/0.1 mg per 10 g body weight IP), hearts of these animals were explanted. Each heart was placed into a glass scintillation vial containing 2.0 mL of Krebs-Ringer bicarbonate buffer supplemented with 6 mM/L glucose and 0.05% bovine serum albumin and oxygenated with a 95% O\(_2\)/5% CO\(_2\) gas mixture (2 L/min) in a shaking water bath (30°C). A sample of the conditioned medium was taken after 30 minutes and again 15 minutes after addition of the calcium ionophore (10\(^{-7}\) mol/L). Samples were stored at −20°C until nitrite/nitrate (NO\(_3\))/NO\(_2\)) analysis. At the end of the study, the hearts were collected from the vials and immediately frozen, and weight was measured.

**Measurement of NO**

NO concentration in the conditioned medium was measured with a colorimetric assay kit that measures total NO concentration in a 2-step process (Cayman Chemical). Absorbance was measured at 540 nm with a multimode microplate reader (Tecan GENios). Each sample was measured in duplicate. The NO concentration is expressed in micromoles per 100 mg heart tissue.

**Measurement of Plasma ADMA**

Plasma ADMA concentrations were measured with a newly developed, highly sensitive ELISA kit (DLD Diagnostika GmbH). The intensity of the color reaction is inversely proportional to the amount of ADMA in the sample and is measured by reading the optical density at 450 nm with a microtiter plate reader (Tecan GENios). In brief, blood samples were obtained from the IVCs, collected in 1.5-mL Eppendorf tubes, and immediately centrifuged at 4°C (10 minutes, 4000 rpm) to avoid possible degradation of ADMA through DDAH activity of blood cell components, and plasma was stored at −20°C. Each sample was measured in duplicate. The use of this
ELISA for murine plasma samples has been recently validated in our laboratory.23

Superoxide Production
Superoxide levels were measured in excised cardiac tissue by the spin-trap method after 4 hours of reperfusion in the acute group of animals. Superoxide accumulation was measured with conditioned medium supplemented with the spin-trapping agent 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (tempamine, Sigma-Aldrich), as previously described.24 Electron paramagnetic resonance (EPR) spectra were recorded at room temperature with a spectrometer (model 9400, Resonance Instruments). The EPR signal intensity was quantified by comparing the double integral of the recorded first-derivative EPR peaks of each sample with a standard tempamine spin solution. When tempamine reacts with other radical species such as superoxide, it loses its EPR signal. The reduction in peak height is directly proportional to the amount of superoxide produced. Measurements were normalized to the protein concentration of each sample, determined by the bicinchoninic acid method.

Protein Isolation and ELISA
Snap-frozen myocardial tissue specimens were homogenized in phosphate-buffered saline and centrifuged at 12 000g for 20 minutes at 4°C. The protein concentration of the supernatant was determined by the bicinchoninic acid method, and aliquots were stored at 4°C. The protein concentration of the supernatant was determined by calculating the number of arterial segments in a given heart section with intimal proliferation (model 9400, Resonance Instruments). The EPR signal intensity was quantified by comparing the double integral of the recorded first-derivative EPR peaks of each sample with a standard tempamine spin solution. When tempamine reacts with other radical species such as superoxide, it loses its EPR signal. The reduction in peak height is directly proportional to the amount of superoxide produced. Measurements were normalized to the protein concentration of each sample, determined by the bicinchoninic acid method.

Morphometric Analysis of GCAD
Thirty days after transplantation, the cardiac grafts were harvested and embedded in paraffin. Elastica van Gieson’s staining was performed for morphometric analysis of arterial intimal proliferation, as recently described.25 We assessed the percentage of luminal narrowing, intima-media ratio, and percentage of affected vessels. Multiple transverse sections (10 μm) were analyzed. The lumen, neointima, and media were measured with SPOT Advanced version 3.4.2 software (Diagnostic Instruments, Inc.).

The percentage of intimal proliferation was determined by multiplying by 100% the ratio of intimal area (ie, the area bounded by the internal elastic lamina minus the luminal area) to the area bounded by the internal elastic lamina. In addition, the intima-media ratio was calculated by dividing the intimal area by the medial area (ie, the area bounded by the internal and external elastic laminas). In addition, the percentage of vessels affected by GCAD was determined by calculating the number of arterial segments in a given heart section with intimal proliferation >10% and dividing this number by the total number of arterial segments in that section (normal vessels of native mouse hearts may show intimal thickening of up to 10%).

Quantitative Immunohistochemistry for Myocardial Inflammation
The grafts were sectioned transversely and either embedded in OCT compound (Sakura Finetek USA, Inc), or fixed in 10% buffered formalin. Frozen sections of grafts were stained with anti-mouse CD3 (145-2C11) and B220 (RA3-6B2) (BD Pharmingen), followed by either biotinylated rabbit anti-rat or anti-hamster IgG and counterstained with hematoxylin. Stained tissues were examined with a Leica DMRB fluorescence microscope (Leica Microsystems). The number of positive cells in each cardiac allograft was counted by 2 investigators (M.T., F.G.) who were blinded to the experimental conditions. Cells were counted in 6 sections (4 fields each) at 4×200 magnification.

Figure 1. Effect of DDAH overexpression on NOx accumulation in explanted hearts. Hearts explanted from DDAH mice tended to exhibit higher NOx accumulation in the conditioned medium compared with WT mice, 30 minutes after incubation in a shaking water bath (WT vs DDAH, 1.2±0.1 vs 2.0±0.3 μmol·L⁻¹·100 mg⁻¹ heart tissue; P=NS). Furthermore, 15 minutes after stimulation with calcium ionophore [10⁻⁷ mol/L], NOx accumulation was significantly greater in the DDAH hearts compared with WT (WT vs DDAH, 1.2±0.3 vs 2.3±0.3 μmol·L⁻¹·100 mg⁻¹ heart tissue; P<0.05). Values are mean±SEM, n=6 per group.

Statistical Analysis
Values are expressed as mean±SEM. Differences in values were analyzed statistically by unpaired Student t test (StatView 5.0, SAS Institute). The χ² test was used to analyze differences in the percentage of diseased vessels between WT and DDAH-transgenic recipients. Significance was accepted at P<0.05.

Results
Acute Studies (Perioperative Period)
DDAH Overexpression Increases Myocardial NO, Formation
Native hearts from DDAH mice generated more NO, as manifested by greater NOx accumulation in the supernatant compared with WT mice 30 minutes after incubation in Krebs-Ringer buffer (Figure 1; WT versus DDAH, 1.2±0.1 versus 2.0±0.3 μmol·L⁻¹·100 mg⁻¹ heart tissue; P=NS). Furthermore, 15 minutes after stimulation with calcium ionophore, NOx production increased in the DDAH hearts (baseline versus stimulation, 2.0±0.3 versus 2.3±0.3 μmol·L⁻¹·100 mg⁻¹ heart tissue; P=NS), and NOx accumulation in the supernatant was significantly greater in the DDAH hearts (WT versus DDAH, 1.2±0.3 versus 2.3±0.3 μmol·L⁻¹·100 mg⁻¹ heart tissue; P<0.05).

DDAH Overexpression Reduces Plasma ADMA Concentrations
Plasma ADMA concentrations in the DDAH recipients were significantly lower postoperatively (WT versus DDAH, 0.92±0.12 versus 0.52±0.03 μmol/L; P<0.01). This difference in plasma ADMA concentrations was maintained for 30 days after heterotopic heart transplantation (WT versus DDAH, 0.70±0.04 versus 0.45±0.04 μmol/L; P<0.01).

DDAH Overexpression Decreases Superoxide and TNF-α Production in the Allograft
We measured myocardial superoxide production 4 hours after heart transplantation in a separate set of animals. Cardiac allografts of WT recipients manifested high levels of super-
oxide production. The generation of superoxide anions was significantly decreased in cardiac allografts of DDAH-transgenic recipients (Figure 2A; WT versus DDAH, 465.7±79.8 versus 1464.2±131.1 versus 1080.4±71.5 pg/mg; P<0.01). Furthermore, TNF-α production was significantly decreased in the cardiac grafts of the DDAH-transgenic group (B; WT vs DDAH, P<0.01). Values are mean±SEM, n=6 per group.

Chronic Studies (30 Days Postoperatively)

**DDAH Overexpression Reduces Myocardial Cytokines in the Allograft**

Donor hearts harvested 30 days after transplantation were studied for elaboration of inflammatory mediators. Notably, tissue levels of TNF-α were significantly lower in cardiac allografts in DDAH-transgenic recipients 30 days after transplantation (Figure 3A; WT versus DDAH, 655.1±72.0 versus 233.9±71.5 pg/mg; P<0.01). Furthermore, we found a significant reduction in the endothelial adhesion molecules ICAM-1 (Figure 3B; WT versus DDAH, 209.0±17.9 versus 124.6±7.9 ng/mg; P<0.01) and VCAM-1 (Figure 3C; WT versus DDAH, 97.3±7.1 versus 49.3±9.4 ng/mg; P<0.01) in hearts transplanted into DDAH-transgenic mice. Similarly, we found a diminished production of MCP-1/CCL2 in cardiac allografts in DDAH-transgenic recipients (Figure 3D; WT versus DDAH, 672.2±56.5 versus 359.1±82.5 pg/mg; P<0.01). Furthermore, the levels of IFN-γ (Figure 3E; WT versus DDAH, 1178.5±243.0 versus 428.6±66.6 pg/mg; P<0.01), IP-10/CXCL10 (Figure 3F; WT versus DDAH, 2382.9±296.4 versus 1084.3±172.6 pg/mg; P<0.01), and TGF-β (Figure 3G; WT versus DDAH, 4864.6±772.1 versus 1870.5±492.4 pg/mg; P<0.01) were markedly decreased. There was no significant difference in cytokine production between the native hearts of transgenic and WT recipients (data not shown).

**Overexpression of DDAH in Recipients Reduces GCAD**

Marked fibrointimal thickening and luminal narrowing, morphologically resembling typical human GCAD, were observed in donor hearts transplanted into WT recipients (Figure 4A-a). In contrast, less intimal thickening and a preserved vessel lumen were observed in donor hearts transplanted into DDAH-transgenic recipients (Figure 4A-b). GCAD, assessed by the mean percentage of luminal narrowing (WT versus DDAH, 79±2% versus 33±7%; P<0.01), the intima-media ratio (WT versus DDAH, 1.1±0.1 versus 0.5±0.1; P<0.01), and the percentage of diseased vessels (WT versus DDAH, 66.6 pg/mg;
Overexpression of DDAH Reduces Graft Inflammation

Immunohistochemical analyses of transplanted hearts were performed to determine T- and B-cell infiltration into the cardiac allograft at 30 days after transplantation. We found significantly decreased graft infiltration by CD3+ T cells in the cardiac allografts of DDAH recipients (Figure 5). No difference in infiltrating B cells (B220+) was observed between the 2 groups of recipients.

DDAH Overexpression Improves Cardiac Allograft Function

All animals survived the duration of the 30-day study. Graft beating scores were significantly better in the hearts transplanted into DDAH-transgenic recipients compared with WT littermate recipients at 30 days after transplantation (Figure 6; P<0.01).

Discussion

The salient findings of this investigation are that overexpression of DDAH-I in the recipient reduces plasma ADMA levels and increases myocardial generation of NO. These effects of DDAH overexpression in the host are associated with less superoxide anion generation, cytokine elaboration, and inflammation in the allograft. Moreover, in DDAH recipients, the allograft manifested less GCAD and improved function. These observations may be explained by the effect of DDAH to reduce tissue and plasma ADMA levels and thereby increase tissue generation of NO.

Our results are consistent with earlier observations revealing an antiinflammatory and antithrombogenic effect of NO.26–28 The effects of DDAH overexpression on superoxide anion generation, vascular inflammation, and vascular structure are consistent with the effects of genetic or pharmacological modulation of NO availability in similar models of ischemia/reperfusion or transplant arteriopathy.10,11,28 We have previously shown that NO production can be enhanced by overexpression of DDAH, the enzyme that degrades the endogenous NOS inhibitor ADMA. In DDAH-transgenic mice, plasma levels of ADMA were reduced, urinary NOx levels were increased, and systemic resistance was reduced.14 With respect to the latter, it is possible that the reduction in blood pressure in the transgenic DDAH mice contributed to the attenuation of vascular lesions. In addition, we hypothesized that enhanced NO activity in the DDAH-transgenic mice would limit vascular inflammation and proliferation. Supporting this hypothesis, we found less intimal thickening and luminal narrowing and fewer diseased vessels in donor hearts transplanted into DDAH-transgenic recipients.

Our results are concordant with other studies indicating that NOS activity is a determinant of vascular lesion formation.15,29 Boger et al29 showed that ADMA plasma concentrations and aortic intima-media ratio were increased in cholesterol-fed rabbits. Furthermore, myointimal proliferation and intima-media ratio were significantly correlated to...
plasma ADMA levels. Dietary l-arginine reduced monocyte accumulation, myointimal cell proliferation, and intimal thickening. Miyazaki et al\textsuperscript{15} demonstrated that plasma ADMA levels were significantly correlated with carotid intima-media thickness in humans.

When endothelial cells undergo inflammatory activation, the increased expression of selectins, VCAM-1, and ICAM-1 promotes the adherence of monocytes.\textsuperscript{30} Uptregulation of adhesion molecules may be important for GCAD development, because treatment with anti–VCAM-1 antibody induces long-term acceptance of murine cardiac allografts.\textsuperscript{31} In addition, treatment with anti–ICAM-1 antibody has been shown to inhibit GCAD in rats.\textsuperscript{32} The diseased graft shows abundant ICAM-1, especially on the endothelial surface.\textsuperscript{30} These adhesion molecules may facilitate transmigration of inflammatory and immune cells to the graft and contribute to ischemia/reperfusion injury as well as GCAD. Reduced ICAM-1 and VCAM-1 production in the chronic phase may relate to decreased GCAD. Adhesion molecule expression is induced by the proinflammatory cytokine TNF-\(\alpha\). Interestingly, in cardiac allografts from DDAH-transgenic recipients, we observed less expression of TNF-\(\alpha\) immediately after transplantation and a blunted increase in the later expression of VCAM-1 and ICAM-1.

Once adherent, monocytes transmigrate into the tunica intima, the innermost layer of the arterial wall. This monocyte migration is directed along a concentration gradient of MCP-1, via interaction with the monocyte receptor CCR2.\textsuperscript{33} We found a significant decrease in MCP-1/CCL2 production, a potent chemokine secreted by activated endothelial and vascular smooth muscle cells as well as monocyte/macrophages in cardiac allografts.\textsuperscript{34} MCP-1/CCL2-mediated effects appear to be an important step in the development of GCAD. Therefore, decreased production of proinflammatory stimuli should not only result in inhibition of immune cell infiltration to the allograft but also inhibit processes directly leading to GCAD development.

A relation between elevated ADMA plasma concentrations and monocyte adhesion has been investigated in vitro and in vivo. Incubation of endothelial cells with ADMA stimulated MCP-1 formation and increased the adhesiveness of endothelial cells for human monocyted cells.\textsuperscript{35} Incubation of endothelial cells with oxidatively modified LDL enhanced adhesion of monocytes to endothelial cells, increased ADMA levels by impairing DDAH activity, and decreased the content of NO.\textsuperscript{36} Furthermore, TNF-\(\alpha\) and MCP-1 concentrations were elevated in this model. Goonasekera et al\textsuperscript{37} found increased plasma levels of ADMA and VCAM-1 in hypertensive humans compared with normotensives. In that study, higher VCAM-1 concentrations were significantly associated with higher ADMA levels in hypertensive humans. In patients with acute congestive heart failure, plasma ADMA and TNF-\(\alpha\) levels were higher than in those with chronic heart failure and control subjects.\textsuperscript{38} TNF-\(\alpha\) and ADMA levels were positively correlated in these patients. Furthermore, plasma ADMA levels and adhesiveness of mononuclear cells (specifically, monocytes and T lymphocytes) were elevated in hypercholesterolemic patients.\textsuperscript{39} Adhesiveness was inversely correlated with the plasma l-arginine–ADMA ratio, and oral administration of l-arginine attenuated monocyte and T-lymphocyte adhesiveness. Interestingly, in cardiac allografts from DDAH-transgenic recipients, we observed less T-cell activation compared with WT recipients.

The association of IFN-\(\gamma\) with GCAD is supported by studies showing a lack of GCAD in IFN-\(\gamma\)-knockout recipients\textsuperscript{40} and the fact that IFN-\(\gamma\) directly causes vascular remodeling and intimal proliferation in the absence of immune cells.\textsuperscript{41} We found significantly decreased production of IFN-\(\gamma\) and the IFN-\(\gamma\)-related chemokine, IP-10/CXCL10, in cardiac allografts of DDAH-transgenic mice. Interstimulation of IFN-\(\gamma\) and IFN-\(\gamma\)-related chemokine is thought to promote inflammation and GCAD, owing to its strong chemotraction of antigen-primed T cells.\textsuperscript{42}

Furthermore, we found significantly decreased TGF-\(\beta\) production in the hearts transplanted into DDAH-transgenic recipients. TGF-\(\beta\) is a powerful and essential immune regulator in the vascular system and is capable of modulating inflammatory events in both leukocytes and vascular endothelial cells. TGF-\(\beta\) is also a potent regulator of the cell cycle in many cell types, including vascular smooth muscle and endothelial cells. Defects in the ability to respond to TGF-\(\beta\) have been strongly implicated in the dysregulated smooth muscle cell hypertrophy/hyperplasia that is known to be a hallmark of neointimal formation or restenosis after vascular injury.\textsuperscript{43}

ADMA competes with the naturally occurring substrate l-arginine for the NOS binding site. Therefore, by inhibiting NOS, ADMA changes the balance of NO and superoxide production, increasing oxidative stress within the vessel and myocardium.\textsuperscript{44} Therefore, overexpression of DDAH, by decreasing plasma ADMA, increases NO formation and decreases superoxide production by the allograft vasculature. Alternatively, the reduced myocardial generation of superoxide anion observed in the allografts of DDAH-transgenic mice could be secondary to a reduction in inflammatory infiltrate.

In conclusion, overexpression of DDAH-I in the recipient reduced plasma ADMA levels, myocardial oxidative stress, cytokine elaboration, and inflammation in the allograft. These effects were associated with less GCAD and improved function of the allograft. Lowering plasma levels of ADMA in human heart transplant recipients with angiotensin-converting enzyme inhibitors,\textsuperscript{45–47} angiotensin II receptor blockers,\textsuperscript{45,47} rosiglitazone,\textsuperscript{48} or metformin\textsuperscript{49} before and after heart transplantation might reduce GCAD and improve long-term results after transplantation.

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**Disclosure**

Stanford University owns patents on the therapeutic use of l-arginine and on assays for ADMA detection. Dr Cooke is an inventor on these patents and receives royalties from the licenses.
References


**CLINICAL PERSPECTIVE**

The development of graft coronary artery disease (GCAD) frequently limits the long-term success of cardiac transplantation. GCAD is a key feature of most chronic rejection syndromes and is the leading cause of death in patients surviving >1 year after transplantation. Endothelial dysfunction is thought to play a major role in the derangements of vascular reactivity and structure that are associated with GCAD. One important endothelial dysfunction is impairment of the nitric oxide synthase (NOS) pathway. NOS produces nitric oxide (NO). Endothelium-derived NO is vasoprotective. The ability of endothelium-derived NOS to produce NO is limited by asymmetric dimethylarginine (ADMA), the endogenous inhibitor of NOS. In patients, ADMA is elevated by cardiovascular risk factors, impairs endothelium-dependent vasodilation, and is associated with vascular disease. The present paper describes a transgenic mouse that has a greater capacity to metabolize ADMA. Consequently this mouse makes more NO and is protected from GCAD. This finding suggests that strategies to reduce levels of ADMA might be therapeutic in GCAD.
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