Cytomegalovirus Infection Impairs the Nitric Oxide Synthase Pathway
Role of Asymmetric Dimethylarginine in Transplant Arteriosclerosis

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Background—We hypothesized that cytomegalovirus (CMV) may contribute to the vasculopathy observed in cardiac allograft recipients by impairing the endothelial nitric oxide synthase pathway. We focused on asymmetric dimethylarginine (ADMA, the endogenous inhibitor of nitric oxide synthase) as a potential mediator of the adverse vascular effect of CMV.

Methods and Results—Heart transplant recipients manifested elevated plasma ADMA levels compared with healthy control subjects. Transplant patients with CMV DNA–positive leukocytes had higher plasma ADMA concentrations and more extensive transplant arteriopathy (TA). Human microvascular endothelial cells infected with the CMV isolates elaborated more ADMA. The increase in ADMA was temporally associated with a reduction in the activity of dimethylarginine dimethylaminohydrolase (DDAH, the enzyme that metabolizes ADMA). Infected cultures showed high levels of oxidative stress with enhanced endothelial production of superoxide anion.

Conclusions—CMV infection in human heart transplant recipients is associated with higher ADMA elevation and more severe TA. CMV infection in endothelial cells increases oxidative stress, impairs DDAH activity, and increases ADMA elaboration. CMV infection may contribute to endothelial dysfunction and TA by dysregulation of the endothelial nitric oxide synthase pathway. (Circulation. 2004;109:500-505.)

Key Words: endothelium □ transplantation □ viruses □ arteriosclerosis

Endothelial dysregulation in transplant arteriosclerosis (TA) may be due to hypertension, hyperlipidemia, hyperglycemia, reperfusion injury, alloimmune activation, and/or pathogens such as cytomegalovirus (CMV). Prior studies have documented that CMV infection induces an activated endothelial phenotype that promotes coagulation and inflammation. Furthermore, in patients with coronary arteriosclerosis, CMV seropositivity may be an independent predictor of death. In transplant recipients, CMV infection is associated with TA. Prophylactic treatment of cardiac transplant recipients with ganciclovir (an antibiotic effective in treating CMV infection) is associated with a reduced incidence of TA. These data suggest that CMV infection may play a role in the development of coronary vascular lesions by mechanisms that are not fully elucidated.

Endothelium-derived nitric oxide (NO) is a potent endogenous vasodilator. NO also prevents vascular inflammation and lesion formation in the vessel wall by inhibiting platelet and leukocyte adherence and by suppressing vascular smooth muscle cell proliferation. Thus, endothelium-derived NO may be thought of as an endogenous antiatherogenic factor. Genetic or pharmacological abrogation of NO synthesis accelerates atherosclerosis in animal models. Plasma asymmetric dimethylarginine (ADMA), the endogenous inhibitor of NO synthase, is elevated in patients with atherosclerosis or those with risk factors for atherosclerosis.

In the present study, we tested the hypothesis that CMV induces endothelial dysfunction and accelerates transplant arteriopathy in part by elevating plasma ADMA.

Methods

Subjects

Demographics
Thirty-six orthotopic heart transplant recipients without clinical evidence of acute rejection or infection were studied 67 ± 51 months after transplantation. The transplant recipients included 31 men and 5 women, 54 ± 11 years of age. The age of the donor heart was 28 ± 10 years. The patients tended to have elevated blood pressure...
Demographics of Study Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transplant Recipients</th>
<th>Control Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=36)</td>
<td>(n=16)</td>
</tr>
<tr>
<td>Age, y</td>
<td>54±11</td>
<td>50±10</td>
</tr>
<tr>
<td>Sex, male/female, %</td>
<td>86/14</td>
<td>56/44</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.8±0.7*</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>174±43</td>
<td>216±46</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>98±1.1</td>
<td>90±7</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>114±31</td>
<td>92±9</td>
</tr>
</tbody>
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*P<0.05 vs healthy control patients.

and fasting blood glucose, although these metabolic perturbations were fairly well controlled on medication (Table). We were receiving standard immunosuppressive regimens; 22 of 36 patients were taking cyclosporine, prednisone, and either azathioprine or MMF. The remaining 14 patients were using tacrolimus, prednisone, and either azathioprine or MMF. All patients were receiving statins and antihypertensive drugs. In addition, 16 healthy, age-matched individuals were studied (Table). The patients had significantly higher plasma creatinine, but age, gender, blood pressure, and blood levels of glucose and cholesterol were not significantly different. Peripheral venous blood was obtained after an overnight fast, and plasma was immediately isolated and stored at −70°C. The Stanford Human Subjects Committee approved the study protocol.

Detection of CMV in Leukocytes From Transplant Recipients

CMV DNA was detected in peripheral blood mononuclear and polymorphonuclear cells of patients by means of polynucleotide chain reaction (single round and nested PCR for CMV DNA in monocytes and granulocytes), but with primers IE1P1A and IE3P2B for the initial rounds and IE2P1A and IE2P2B for the nested rounds of PCR.10

Coronary Angiography in Transplant Recipients

All patients underwent coronary angiography with biplane cineangiography and standard angiographic techniques used. Coronary angiograms were reviewed in a blinded fashion by an experienced cardiologist who used qualitative features (ie, any distal pruning of vessels or any visually apparent stenosis, regardless of severity) to designate angiograms as positive or negative for transplant arteriopathy. This qualitative assessment was followed by a quantitative analysis performed by a second cardiologist, using standard methods of quantitative angiography. “Significant” transplant coronary artery disease was defined as the presence of any coronary stenosis involving >50% luminal obstruction and/or the presence of severe diffuse distal pruning of vessels, based on a consensus reached from the qualitative and quantitative analysis of the angiograms. This 50% level of stenosis was selected as the threshold for defining significant coronary artery disease because it was the point of greatest consensus between the qualitative and quantitative analysis and because of the known prognostic importance of this degree of coronary stenosis in heart transplant recipients.11 No attempt was made to classify patients according to the number and distribution of vessels involved because of the diffuse nature of transplant coronary artery disease that is known to already exist by the time stenosis is apparent angiographically.12

Cell Culture Studies

Endothelial Cell Culture

Primary human adult dermal microvascular endothelial cells (HMVEC) were cultured in endothelial cell basal medium supplemented with 10% fetal bovine serum, 0.1 mg/mL endothelial growth supplement, 5 µg/mL human epidermal growth factor, 0.5 mg/mL hydrocortisone, 50 U/mL penicillin, and 50 µg/mL streptomycin. The immortalized human dermal microvascular endothelial cell line HMEC-1 was provided by the Centers for Disease Control, Atlanta, Ga. HMEC-1 were cultured in DMEM supplemented with 10% fetal bovine serum, 10 ng/mL human epidermal growth factor, and 50 U/mL penicillin and 50 µg/mL streptomycin. Cells were maintained at 37°C and 5% CO₂.

Viral Infection of Cell Monolayers

CMV VHL/E11 and TB40/E,14 clinical CMV strains known to be endotheliotrophic, were used for EC infection. Virus stocks were generated by propagation in human fibroblasts and stored in aliquots at −80°C until use. HMVEC and HMEC-1 were seeded in 4-well chamber slides at 3×10⁶ cells/well (HMEC-1) or 2×10⁶ cells/well (HMVEC). Cells were washed in PBS and infected with CMV, diluted in regular medium on a subconfluent monolayer (70%) at a multiplicity of infection (MOI) of 1, 3, 5, or 10 for 1 hour at 37°C in 5% CO₂.

Determination of Viral Infection

To determine if exposure to the virus resulted in an infection, endothelial cells were fixed with paraformaldehyde and incubated for 1 hour at 37°C with a monoclonal mouse anti-CMV immediate early antigen (E1/E2) antibody (dilution 1:1000; MAB810). Alexa488 goat anti-mouse IgG or Alexa 594 rabbit anti-mouse IgG (dilution 1:1000; 3-hour incubation) was used as a secondary antibody. Nuclei were counterstained with Hoechst stain or propidium iodide. Inducible nitric oxide synthase (iNOS) was detected with the use of a monoclonal iNOS antibody (1:50) prelabeled with FITC. Imaging was performed with a Zeiss LSM 510 Confocal Laser Scanning Microscope.

Biochemical Measurements

Measurement of Dimethylarginines

ADMA and symmetric dimethylarginine (SDMA) were measured in conditioned medium or plasma by high-performance liquid chromatography (HPLC),15 with the following modification. A gradient mobile phase, consisting of potassium phosphate buffer (pH 6.5) and acetonitrile, was used. The column was run 1500 molecules each 20 minutes. This modification attenuates peak broadening, enhances separation, and decreases the measurement’s run time. The detection limit of the assay was 0.1 µmol/L.

Dimethylarginine Dimethylaminohydrolase Activity

Activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that metabolizes ADMA, was assayed by measuring the amount of ADMA metabolized by the enzyme. In an ice bath, endothelial cell lysates (pooled from 3 independent experiments) were divided into two aliquots. To each aliquot, 500 µmol/L ADMA was added. After 1 hour, 30% SSA was added. The other aliquot of lysate was incubated at 37°C for 2 hours, then 30% SSA was added. The ADMA level in each aliquot was measured by HPLC as described above. The difference in ADMA concentration between the two aliquots reflects DDAH activity.

Quantitative Detection of NO Production

Fluorescence-based measurements of the stable end products of NO metabolism, nitrite and nitrate, were used for assessment of NO production. Total nitrite production released from endothelial cells was measured with a commercial fluorescent assay (detection limit, 0.2 µmol/L).

Immunossay of cGMP

To measure cGMP, a commercially available enzyme immunosassay kit was used. To reduce the degradation of cGMP, cells were treated with the phosphodiesterase V inhibitor zaprinast (30 µmol/L over 30 minutes). Thereafter, calcium ionophore (A23187, 1 µmol/L), which stimulates NO synthesis, was added to the media, and the incubation was continued for 1 hour. Subsequently, the media was aspirated; the cells were washed twice with ice-cold PBS and lysed. Immediately thereafter, intracellular cGMP was assayed by EIA.
Intracellular Oxidative Stress
Dichlorfluorescein-diacetate (2,7 DCFH-DA) was used as a marker for intracellular oxidative stress. Infected or noninfected endothelial cells were incubated with DCFH-DA over a period of 45 minutes. Then 2,3-dimethoxy-1,4-naphtoquinone (DMNQ, 100 μmol/L) was added for 10 to 60 minutes to generate intracellular O$_2^-$. DCFH fluorescence was measured at baseline and after treatment with DMNQ, using the GENIOS multireader at 485/530 nm.

Statistical Analysis
Statistical analysis of the results was performed with the Student’s t test for unpaired or paired data, respectively, or, if necessary, with ANOVA followed by the Student-Newman-Keuls post hoc test (StatView 5.0). Univariate correlations were performed with the use of the Pearson correlation coefficient for continuous variables and the Pearson χ² test for categorical variables. Data are presented as mean±SEM.

Results

Human Studies
Plasma Methylarginine Levels
Transplant recipients manifested a 200% increase in plasma ADMA concentration (Figure 1) in comparison to healthy control subjects. Notably, SDMA levels were comparable in the two different groups.

Correlation of Plasma ADMA Levels With Cardiovascular Risk Factors
There was a trend for positive correlations between plasma ADMA concentration and diabetes ($r=0.26$), hyperlipidemia ($r=0.31$), and hypertension ($r=0.19$) as well as creatinine concentration ($r=0.10$), which did not attain statistical significance.

Correlation of CMV Infection With ADMA Levels
The plasma concentration of ADMA in CMV-positive patients ($n=14, 39\%$) was $2.27±0.8 \mu$mol/L, compared with $1.96±0.7 \mu$mol/L in CMV negative recipients (an increase of 21%, $P<0.05$). A negative CMV test was associated with lower plasma ADMA levels ($\chi^2, 5.82; P=0.041$). Levels of SDMA and creatinine were not significantly different between CMV-negative and CMV-positive patients ($P=0.57$ for SDMA; $P=0.35$ for creatinine).

Correlation of Plasma ADMA Levels With Angiographically Documented Disease
Plasma ADMA concentration in patients with angiographically evident transplant arteriosclerosis was greater than those without TA (by 21±9%, $P<0.05$). Patients with high ADMA levels ($>2.0 \mu$mol/L) and presence of CMV DNA demonstrated the highest prevalence of TA ($P<0.05$ versus patients with ADMA $<2.0 \mu$mol/L and negative CMV).

Cell Culture Studies
Viral Infection
Both HMVEC and HMEC-1 were susceptible to CMV infection, as demonstrated by the presence of CMV immediate early antigen in the nuclei of CMV TB40/E–infected cells at day 3 after infection (Figure 2). Approximately half of the cells ($58±12\%$ of HMVEC and $47±5\%$ of HMEC-1) showed evidence of viral antigen after infection with CMV TB40/E.
Similar results were observed after infection with CMV VHL/E (MOI 3, day 3 after infection). The laboratory-adapted strains AD169 and Towne were ineffective at inducing infection (data not shown).

**ADMA Accumulation**

The level of ADMA in the cell culture medium was significantly increased after infection with CMV TB40/E. The concentration of ADMA in the conditioned media from HMEC-1 (Figure 3) or HMVEC was increased by day 3. SDMA concentrations were comparable in the conditioned media of control and CMV-infected endothelial cells (data not shown).

**Effect of Viral Infection on EC DDAH Activity**

Levels of DDAH activity were determined in endothelial cells by assessing the rate of degradation of exogenous ADMA added to the cell lysates. Lysates from uninfected endothelial cells metabolized 73% of the exogenous ADMA over a period of 2 hours. Infected endothelial cells metabolized ADMA much more slowly. On day 3 after infection (MOI=3), CMV TB40/E or VHL/E metabolized only 15% or 2%, respectively of the exogenous ADMA (Figure 4).

**Viral Infection and EC Nitrite Production**

Basal total nitrite production in HMVEC was 2.4 ± 0.7 nmol. Nitrite production in ECs infected with CMV TB40/E for 24 hours was increased compared with uninfected ECs (by 32 ± 11%, MOI 1, P=NS; by 49 ± 17%, MOI 3, P<0.05). However, nitrite production by day 3 after infection had returned to levels not significantly different from uninfected ECs (2.7 ± 1.1 versus 2.4 ± 0.9 nmol). iNOS was undetectable in noninfected endothelial cells. However, after infection of HMEC-1 with TB40/E (MOI 3), expression of iNOS was detectable in 47 ± 28% (range, 22% to 78%) of the CMV-positive endothelial cells (data not shown).

**Viral Infection and EC cGMP Production**

Intracellular cGMP levels reflect NO bioactivity. The stimulated production of cGMP was reduced in cells 3 and 7 days after infection. On the 7th day after exposure to the virus, the reduction in cGMP production was 59% (TB40/E) and 74% (VHL/E), respectively (Figure 5).

**Viral Infection and EC Oxidative Stress**

Basal O2− release from endothelial cells was enhanced after CMV TB40/E infection (65 ± 14% at MOI 1; 311 ± 86% at MOI 3; P<0.01), as was DMNQ-stimulated O2− release (Figure 6). O2− generation was negatively correlated to cGMP levels (r = −0.72; P<0.01). Addition of intracellular antioxidant polyethylene glycol–conjugated superoxide dismutase (22 U/mL) to the medium partially reversed the effect of CMV on oxidative stress (reduction in DMNQ-stimulated O2− release by 72 ± 13%; P<0.001) and significantly reduced...
endothelial ADMA elaboration (reduction in ADMA levels by 66±7%; P<0.01).

Discussion
The major findings of the present study are (1) plasma ADMA levels are elevated in human heart transplant recipients; (2) the presence of CMV DNA at levels consistent with active infection (in mononuclear or polymorphonuclear cells) of heart transplant recipients is associated with a greater increase in plasma ADMA; (3) transplant arteriosclerosis is more prevalent in transplant recipients with high ADMA concentrations and CMV positivity; and (4) CMV infection of cultured endothelial cells increases oxidative stress, reduces DDAH activity, and increases ADMA accumulation. These data indicate that CMV may contribute to endothelial dysfunction and arteriosclerosis by dysregulation of the NOS-pathway.

CMV and Vascular Disease
In line with recent in vitro and in vivo data, our study indicates that CMV infection of the endothelium promotes processes that favor atherogenesis and vascular lesion formation.2,17–21 In the human aorta, endothelial cells and smooth muscle cells appear to be the primary site of infection, suggesting that the vasculature may serve as a reservoir for CMV.22 CMV infection increases expression of endothelial surface adhesion molecules and promotes mononuclear adhesion, activation, and transendothelial migration within the vasculature.18,23,24 Intriguingly, in one study, 76% of patients with ischemic heart disease had evidence of CMV DNA in the affected arterial wall,25 suggesting that the biological effects of CMV on the vessel wall may have clinical implications. In this study, we elucidate a novel mechanism by which CMV causes endothelial dysfunction. We found that CMV is a stimulus of ADMA accumulation, acting in a similar fashion as known risk factors for vascular disease.14,26

CMV Impairs DDAH Activity
The CMV-induced accumulation of ADMA appears to be due to reduced activity of DDAH, the enzyme that degrades ADMA.9,27,28 We have previously shown that DDAH is a key regulator of plasma ADMA levels. The elevation of ADMA that is observed in hypercholesterolemia, hyperhomocysteinemia, or hyperglycemia is due to impaired DDAH activity in the setting of metabolically induced oxidative stress.9,14,29 A sulfhydryl group in the catalytic region of the active site confers on DDAH its exquisite sensitivity to oxidative stress. We have shown that homocysteine oxidatively attacks a sulfhydryl group in DDAH to form a mixed disulfide, inactivating the enzyme.14 In addition, the activity of DDAH can be diminished by S-nitrosylation of the sulfhydryl moiety in the setting of iNOS activation.30 Notably, S-nitrosylation of different enzymes by NO is more likely when the reducing potential of the cells is impaired.31 In the current study, we found that CMV infection imposes an intracellular oxidative stress. The oxidative stress is associated with a severe reduction of DDAH activity. The effect of CMV to inhibit ADMA metabolism is partially reversed by the intracellular antioxidant peg-SOD.

This same pathophysiological mechanism appears to be responsible for the elevation of plasma ADMA in the transplant recipients. An increase in ADMA, in the absence of an increase in plasma SDMA levels, strongly supports an impairment of DDAH activity. Other possible mechanisms for an increase in plasma ADMA elevation (ie, increased methylation of protein arginine levels, their increased catabolism, or reduced renal clearance) would be associated with an elevation of plasma symmetric dimethylarginine (SDMA, a methylarginine that does not block NOS).

CMV Attenuates Bioactive NO in Vascular Cells
The impairment by CMV in endothelial cGMP level reflects a reduction in bioactive NO. We also observed an increase in the degradation products of NO. The mechanisms by which CMV reduce bioactive NO are complex. We show that CMV induces iNOS and increases oxidative stress. This is not surprising, as CMV infection is known to induce the elaboration of TNF-α,32 and TNF-α is known to induce vascular expression of iNOS.33 It is well known that iNOS is capable of generating superoxide anion, particularly in the setting of l-arginine deficiency.34 We have previously reported that TNF-α downregulates endothelial DDAH activity and subsequently increases ADMA release.29 Furthermore, in the presence of ADMA, endothelial cells generate superoxide anion.35 Accordingly, it seems reasonable to propose that CMV has multiple effects to impair endothelial NO bioactivity, for example, to induce expression of iNOS, to increase superoxide anion generation, to reduce DDAH activity, and thereby to increase ADMA elaboration.

CMV, ADMA, and Transplant Arteriosclerosis
In a previous study, we have observed that in cardiac transplant recipients an intravenous infusion of l-arginine restores endothelium-dependent, NO-mediated increases in coronary blood flow with intracoronary acetylcholine infusion.36 This observation is consistent with the hypothesis that endothelial vasodilator dysfunction in cardiac allograft recipients is mediated by ADMA. An impairment of NO synthesis and bioactivity would be predicted to have profound effects on vascular structure as well as function.37 NO inhibits platelet aggregation, leukocyte adherence and infiltration into the vessel wall, and proliferation of vascular smooth muscle cells. In experimental models, pharmacological or genetic inhibition of the NOS pathway accelerates atherosclerosis.38,39 Similarly, genetic or nutritional enhancement of NO synthesis reduces vascular lesion formation.40,41 It is therefore intriguing that our patients with angiographically evident transplant arteriosclerosis had higher levels of plasma ADMA. This observation provides further support for the accumulating evidence that ADMA may be a risk factor for coronary vascular disease.

Study Limitations
Although we have found an association between CMV infection, circulating ADMA levels, and transplant vasculopathy in humans, association does not imply causation. Several large prospective clinical studies of an observational and/or interventional nature will be required to determine if CMV-induced increases in ADMA have a causal role in transplant arteriopathy.

Conclusions
To summarize, we found that plasma ADMA is elevated in cardiac transplant recipients.
actively infected with CMV have higher plasma ADMA levels and are at greater risk of transplant arteriosclerosis. Endothelial cells infected by CMV elaborate more ADMA and manifest an impairment of NO activity. The CMV-induced increase in oxidative stress is associated with impairment of DDAH activity and ADMA accumulation. We have elucidated a novel mechanism for CMV-induced endothelial dysfunction that may play an important role in the altered vascular function and structure observed in the coronary arteries of cardiac transplant recipients.

Acknowledgments

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References

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