The Role of Vitronectin Receptor (αvβ3) and Tissue Factor in the Pathogenesis of Transplant Coronary Vasculopathy

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OBJECTIVES This study was undertaken to test the hypothesis that transplant coronary vasculopathy (CV) is associated with increased myocardial protein expression of both tissue factor (TF) and integrin αvβ3.

BACKGROUND The vitronectin receptor (integrin αvβ3) and TF have recently been found to play a key role in apoptotic cell death and vascular endothelial cell injury.

METHODS A total of 77 heart transplant recipients underwent simultaneous endomyocardial biopsy and intravascular ultrasound (IVUS) at one year of transplant. Patients with pre-existing donor coronary atherosclerosis (n = 35) or with acute rejection (grade >1A, n = 10) at the time of the IVUS were excluded from the analysis. The remaining 32 patients constitute the cohort of the present study. A computerized biopsy score was derived based on the duration and severity of cellular rejection. Both TF and αvβ3 expression in the heart biopsy specimens were evaluated by immunoperoxidase histochemistry and Western blot analysis.

RESULTS Patients with CV (n = 24) had increased expression of αvβ3 (2.7-fold, p = 0.003) and TF (7.9-fold, p = 0.04) compared with patients without evidence of vasculopathy (n = 8). In the absence of myocardial fibrosis, αvβ3 expression correlated significantly with the cellular rejection score (r = 0.58, p = 0.02).

CONCLUSIONS Transplant vasculopathy is associated with increased expression of both TF and αvβ3. The significant correlation of αvβ3 with cellular rejection suggests an important role for this integrin in serving as a mechanistic link between cellular rejection and vasculopathy. (J Am Coll Cardiol 2002;39:804–10) © 2002 by the American College of Cardiology Foundation

Allograft vasculopathy is the leading cause of death one year after heart transplantation. Following cardiac transplantation, release of cytokines may cause endothelial injury and stimulate proliferation of vascular smooth muscle cells (VSMCs), leading to graft atherosclerosis (1).

Integrins are a family of cell surface adhesion receptors that mediate cell–cell and cell–extracellular matrix interactions (2). Both the vitronectin receptor (integrin αvβ3) and tissue factor (TF), a major regulator of coagulation and hemostasis, have recently been shown to play a key role in apoptosis, vascular endothelial cell injury and the inflammatory process of the immune response (3–8). However, the roles of αvβ3 and TF in the transplant population have not been explored. We tested the hypothesis that coronary vasculopathy (CV) is associated with upregulation of αvβ3 and TF.

MATERIALS AND METHODS

Patient population. A total of 77 heart transplant recipients underwent serial endomyocardial biopsies and had serial coronary intravascular ultrasound (IVUS) at baseline (1 ± 0.01 months) and one year of transplant. Patients with pre-existing donor coronary atherosclerosis (defined as coronary maximal intimal thickness [CMIT] >0.3 mm at baseline, n = 35) or with acute cellular rejection (grade >1A, n = 10) at the annual IVUS were excluded from the analysis to eliminate any variation in results that could be influenced by these variables. The remaining 32 patients constitute the present study. Patients gave informed consent and the protocol was approved by the Ethics Review Committee of our Institution.

Endomyocardial biopsy. Serial endomyocardial biopsy specimens were evaluated histologically for acute cellular rejection (International Society Heart Lung Transplant criteria), acute vascular rejection, confirmed by immunofluorescence (9,10), acute ischemic injury (myocyte necrosis) and fibrosis. A computerized biopsy score was derived based on the duration (days) and severity of the cellular rejection component during the first year of transplant as recently described (11). The following measurements were derived: biopsy score = cellular rejection-1 score + cellular...
rejection-2 score; cellular rejection-1 score = cellular rejection score 0 to 6 months of transplant; and cellular rejection-2 score = cellular rejection score 6 to 12 months of transplant.

**Intravascular ultrasound.** Intravascular ultrasound was performed in 68 coronary vessels in 32 patients (2.1 ± 0.1 arteries/patient). Coronary intimal thickness was measured at baseline (1.0 ± 0.01 months) and one year (12.0 ± 0.01 months) of transplant. Coronary sites with minimum and maximum intimal thickness were identified. Paired analysis of matched sites (10 sites/patient) at one year measured the change in CMIT. Coronary vasculopathy was defined as an increase in CMIT >0.3 mm at any site at one year. A CMIT of 0.3 to 0.5 mm was considered mild and >0.5 mm as advanced vasculopathy. Intimal thickening of >0.3 mm was considered pathological on the basis of reported values of intimal thickness in the young adult population (12,13).

**Immunohistochemistry staining.** Five endomyocardial biopsy specimens were obtained from each patient at the biopsy. Four were analyzed for histopathology, and one (snap frozen in liquid nitrogen and stored at −75°C) for immunoblotting. Immunoperoxidase staining for αβ3 and TF was performed using rabbit antihuman integrin β3 polyclonal antibody (Chemicon International Inc., Temecula, California) and goat antihuman TF polyclonal antibody (American Diagnostica Inc., Greenwich, Connecticut), respectively. The staining was described as negative, weakly positive and strongly positive.

Myocardial fibrosis was evaluated by Trichome Masson staining, and intramyocardial blood vessels by Movat pentachrome staining. The number of blood vessels per high power field were quantified in a blinded fashion in a representative subpopulation of 15 patients (CMIT <0.3 mm, n = 5; CMIT = 0.3 to 0.5 mm, n = 5 and CMIT >0.5 mm with evidence of fibrosis, n = 5). Five sections were examined for each specimen.

**Immunoblotting.** Protein expression of αβ3 and TF was determined by Western blot analysis. Immunoblotting was performed as described previously (14). Briefly, cardiac tissue (3 to 5 mg wet weight per biopsy specimen) was homogenized in 230 μl of lysis buffer (10 mM N-(2-

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**Table 1.** Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients Without CV</th>
<th>Patients With CV</th>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>7 (87%)</td>
<td>21 (87%)</td>
</tr>
<tr>
<td>Recipient age (yrs)</td>
<td>52 ± 1.7</td>
<td>53 ± 1.5</td>
</tr>
<tr>
<td>Donor age (yrs)</td>
<td>26 ± 1.8</td>
<td>25 ± 1.7</td>
</tr>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic cardiomyopathy</td>
<td>6 (75%)</td>
<td>15 (63%)</td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>2 (25%)</td>
<td>9 (37%)</td>
</tr>
<tr>
<td>LVAD</td>
<td>2 (25%)</td>
<td>5 (21%)</td>
</tr>
<tr>
<td>Ischemia time (min)</td>
<td>174 ± 8</td>
<td>171 ± 7</td>
</tr>
</tbody>
</table>

**Abbreviations and Acronyms**

- **CMIT** = coronary maximal intimal thickness
- **CV** = coronary vasculopathy
- **GAPDH** = glyceraldehyde-3-phosphate dehydrogenase
- **IVUS** = intravascular ultrasound
- **TF** = tissue factor
- **VSMC** = vascular smooth muscle cell

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**Figure 1.** αβ3 and tissue factor immunohistochemistry staining in relation to coronary vasculopathy. Positive staining (brown) of both αβ3 (E, arrow, vascular endothelium) and tissue factor (F, myocardial interstitium) in a patient with coronary vasculopathy demonstrated on intravascular ultrasound (D) compared with a patient without evidence of coronary vasculopathy (A, B, C). Bar = 50 μm.
Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, pH = 7.4, 2 mM ethylenediamine-tetraacetic acid, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM dithiothreitol, 10 μg/ml aprotinin, 1 mM phenylmethyl sulfonyl fluoride and 10 μg/ml leupeptin. The samples were then centrifuged at 14,000 rpm (16,000 g) at 4°C. Tissue homogenate or supernatant of the homogenate was separated on 10% polyacrylamide gels by Tris-Glycine SDS-PAGE (Invitrogen Inc., Carlsbad, California). Protein concentration was measured using the bicinchoninic acid method (PIERCE, Inc., Rockford, Illinois). The amount of protein loaded in each well was either 25 or 35 μg. After transfer to polyvinylidene fluoride membranes (0.2 μm, Bio-Rad Laboratories, Hercules, California), the membranes were blocked in 3% milk in tris-buffered saline containing 0.1% tween-20. After two 10-min washes in this solution, the membranes were incubated overnight with rabbit antihuman integrin β3 polyclonal antibody (Chemicon International Inc.), goat antihuman TF polyclonal antibody (American Diagnostica Inc.) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Chemicon International Inc.). Protein expression was normalized to GAPDH and was quantified by arbitrary units using densitometric ratio. Immunoblotting results were correlated with the IVUS findings.

**Statistical analysis.** Data are expressed as mean ± SEM. Categorical variables were compared by chi-square test and continuous variables by Student's t test, and Pearson correlation coefficients were calculated. Intramyocardial blood vessel quantification analysis was evaluated using mixed modeling for repeated measurements for the overall group comparison, with Tukey's adjustment for pairwise comparison. All analyses were done using SAS software, version 6 (SAS Institute, Cary, North Carolina). Differences were considered significant at p < 0.05.

**RESULTS**

The baseline characteristics of patients with and without CV were comparable (Table 1). Eight patients had no evidence of vasculopathy (Fig. 1A) and 24 patients developed CV (CMIT >0.3 mm, Fig. 1D) at one year of transplant.

**Immunohistochemistry staining.** Compared with patients having no CV who showed negative staining for β3 integrin (Fig. 1B) and TF (Fig. 1C), patients with CV demonstrated strong positive staining of the β3 integrin in the vascular endothelium of the intramyocardial blood vessels (Fig. 1E, arrow). Tissue factor stained strongly positive in the interstitium of the myocardium (Fig. 1F).

**Immunoblotting.** Both αβ3 (2.7-fold, p = 0.003) and TF (7.9-fold, p = 0.04) protein expression were significantly increased in patients with CV (Fig. 2). When patients with CV were analyzed according to the severity of change in CMIT (Fig. 3), there was a trend toward a reduced αβ3 protein expression in patients with advanced CV (CMIT >0.5 mm). These patients had higher incidence of post-transplant ischemic injury/fibrosis (8/12 vs. 2/12, p = 0.04) than patients with CV 0.31 to 0.50 mm. There was no significant difference in TF expression between these groups of patients. In the absence of post-transplant ischemia/fibrosis, αβ3 protein expression correlated significantly with cellular rejection score mainly during the first six months of transplant (r = 0.58, p = 0.02, Fig. 4). However, this correlation was absent in patients whose post-transplant course was complicated by the development of fibrosis. Interestingly, a significant negative correlation was noted between αβ3 protein expression and change in
CMIT in the presence of post-transplant ischemia/fibrosis ($r = -0.60, p = 0.01$, Fig. 5).

Further subgroup analysis of patients with advanced CV progression (CMIT $>0.5$ mm) revealed significant reduced myocardial $\alpha\beta3$ protein expression in patients with post-transplant ischemia/fibrosis (Fig. 6). Careful analysis of the cellular rejection component reveals that patients with post-transplant ischemia/fibrosis had a significant reduced cellular rejection score in the latter half of the year (Fig. 7).

Quantifying the number of intramyocardial blood vessels per high power field showed that patients with CMIT 0.3 to 0.5 mm had increased number of blood vessels ($10.4 \pm 1.1$) than patients without vasculopathy ($4.7 \pm 0.2$, $p < 0.001$) or patients with advanced CV complicated by post-transplant ischemic injury/fibrosis ($5.5 \pm 0.3$, $p < 0.001$).

**DISCUSSION**

The major findings of the present study are that both $\alpha\beta3$ and TF are upregulated in the presence of vasculopathy. Integrin $\alpha\beta3$ has been shown to play a significant role in endothelial cell migration and is essential for growth factor and cytokine-induced VSMC proliferation (15). The binding of $\alpha\beta3$ with its corresponding ligands, such as vitronectin, von Willebrand factor, fibronectin and thrombospondin, promotes a process of adhesive interactions between endothelial cells and matrix proteins (16). This integrin-mediated cell attachment regulates cell survival and proliferation and function in signal transduction processes (17). The activated endothelial cells in turn express TF (18,19), a major regulator of coagulation. The relation between $\alpha\beta3$ and TF is depicted in Figure 8. $\alpha\beta3$ has been shown to co-stimulate the release of tumor necrosis factor-alpha in macrophages (3). Tumor necrosis factor-alpha in turn induces TF expression (20,21). Tissue factor is also known to induce smooth muscle cell migration (22), and its activity has been demonstrated within the coronary intima in rat cardiac allografts (23). It may be a stimulus for fibrin deposition observed in the allograft vessels (24).

![Graph showing correlation between $\alpha\beta3$ and cellular rejection score](image)

Figure 4. Correlation of myocardial $\alpha\beta3$ protein expression with cellular rejection score during the first six months of transplant in the absence of post-transplant ischemic injury/fibrosis. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.
Figure 5. Correlation of myocardial αβ3 protein expression with severity of coronary vasculopathy in patients with post-transplant ischemia/fibrosis. CMIT = coronary maximal intimal thickness; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Figure 6. Myocardial αβ3 and tissue factor (TF) protein expression in patients with coronary vasculopathy in relation to the presence or absence of post-transplant ischemic injury/fibrosis. A significant decrease of αβ3 expression is noted in patients with post-transplant ischemic injury/fibrosis. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Figure 7. Biopsy score in patients with coronary vasculopathy in relation to the presence or absence of post-transplant ischemic injury/fibrosis. Patients with fibrosis have reduced cellular rejection score at one year (Y), mainly during 6 to 12 months of transplant (Y₂). *p = 0.05; Y, fibrosis versus no fibrosis. **p = 0.006; Y₂, fibrosis versus no fibrosis. Y₁ = cellular rejection score during the first 6 months of transplant.
Expression of TF by fibroblasts is a likely explanation for our findings of increased TF in the interstitium of the myocardium in the presence of allograft vasculopathy (18). Our study was limited to endomyocardial biopsies, so only small vessels were studied. That may explain why we did not note expression of TF on endothelial cells. Another possible explanation may be that vasculopathy in our patients was not severe enough to induce endothelial expression of TF.

The association of cellular rejection and transplant CV has been debated in the literature (25). It has been proposed that transplant vasculopathy is caused by an immune-inflammatory component. It has been re-indicating less in fact that these patients had a reduced cellular rejection score and macrophages. αvβ3 has been shown to co-stimulate the release of tumor necrosis factor-alpha (TNF-α) in macrophages. Tumor necrosis factor-alpha in turn induces tissue factor expression. Tissue factor expression is also induced by interleukin-1 (IL-1) and oxidized low density lipoprotein (LDL). Tissue factor binds factor VIIa to form a complex that converts factor X to its active form, Xa, which in turn accelerates the conversion of prothrombin to thrombin. Prothrombin serves as a ligand to αvβ3 and its binding to αvβ3 also accelerates the conversion of prothrombin to thrombin.

In conclusion, our results indicate that both αvβ3 and TF are upregulated in the presence of allograft vasculopathy. Therapeutic interventions targeted against αvβ3 may be helpful in aborting the process of vascular endothelial injury.

REFERENCES
