Myocardial Ischemic Injury After Heart Transplantation Is Associated With Upregulation of Vitronectin Receptor (α,β3), Activation of the Matrix Metalloproteinase Induction System, and Subsequent Development of Coronary Vasculopathy

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Background—Myocardial ischemic injury after heart transplantation is associated with subsequent development of graft vasculopathy. Both vitronectin receptor (integrin α,β3) and tissue factor play key roles in vascular endothelial cell injury. Matrix metalloproteinases (MMPs) are activated in ischemic injury models.

Methods and Results—Thirteen patients developed myocardial ischemic injury within 2 weeks of cardiac transplantation (ischemia group). These were compared with 10 transplantation patients who had no evidence of ischemia (control group). Endomyocardial biopsies were evaluated within 2 weeks of transplantation for α,β3, tissue factor, and extracellular MMP inducer (EMMPRIN). At 1 year, MMPs were evaluated, and interstitial myocardial fibrosis was quantified. All patients underwent intravascular ultrasound at 1 month and 1 year after transplantation. Compared with control, the ischemia group demonstrated evidence of significant increased expression of α,β3 (3.2-fold, P<0.001), tissue factor (2.5-fold, P<0.001), and EMMPRIN (1.9-fold, P=0.01). At 1 year, the ischemia group had a significant increase in myocardial fibrosis (24±1.8% versus 14±1.1%, P<0.001) and zymographic activity of MMP-2 (1.4-fold, P<0.001), MMP-3 (1.2-fold, P<0.001), and MMP-9 (1.3-fold, P=0.01). Coronary vasculopathy progression was also more advanced in the ischemia group (change in coronary maximal intimal thickness over 1 year 0.54±0.1 versus 0.26±0.06 mm; P=0.031).

Conclusions—Myocardial ischemic injury after cardiac transplantation is associated with upregulation of α,β3, tissue factor, and activation of the MMP induction system, which may contribute to the subsequent development of allograft remodeling and vasculopathy. (Circulation. 2002;105:1955-1961.)

Key Words: glycoproteins | metalloproteinases | transplantation | ultrasonics

Myocardial ischemic injury after heart transplantation has been noted to be associated with subsequent development of coronary vasculopathy. Several factors contribute to myocardial ischemia, including graft ischemic time, hemodynamic status of the donor before transplantation, and graft preservation. However, the mechanism by which ischemia contributes to the development of accelerated atherosclerosis is not well defined.

Vitronectin receptor (integrin α,β3) is a cell surface adhesion receptor that mediates cell-cell and cell-extracellular matrix interaction with several ligands, including fibrinogen, fibronectin, thrombospondin, and prothrombin. α,β3 co-stimulates the release of tumor necrosis factor-α, which in turn induces expression of tissue factor, a major regulator of hemostasis, and regulates the expression of various matrix metalloproteinases (MMPs) (Figure 1). Both α,β3 and tissue factor have been shown to play key roles in smooth muscle cell migration and vascular endothelial cell injury. MMPs are an endogenous family of zinc-dependent enzymes, responsible for matrix remodeling in several disease states. MMPs also serve as ligands to α,β3. This complex interaction has been well demonstrated in tumor invasion. Recently, the MMP induction system has been shown to be upregulated in heart failure; however, its role in myocardial ischemic injury after transplantation has not been studied before.
Figure 1. Schematic diagram showing the relationship between $\alpha_v\beta_3$, MMP, and tissue factor. $\alpha_v\beta_3$ costimulates the release of tumor necrosis factor-$\alpha$, which in turn induces expression of tissue factor and stimulates the synthesis of MMP. Release of inflammatory cytokines such as interleukin-1 also induces expression of tissue factor. Both prothrombin and MMP serve as ligands to $\alpha_v\beta_3$. Activation of the coagulation cascade system results in fibrin formation.

We postulate that myocardial ischemic injury after transplantation is associated with upregulation of $\alpha_v\beta_3$ and tissue factor, which contributes to the subsequent development of coronary vasculopathy. We also postulate that in peritransplantation ischemia, the MMP induction system is activated, and this contributes to the subsequent development of myocardial remodeling after transplantation.

Methods

Patient Population
Thirteen heart transplant recipients sustained myocardial ischemic injury (ischemia group), manifested histologically by myocyte necrosis, within 2 weeks of transplantation. These patients were compared with a group of 10 patients who had no evidence of hemodynamic compromise or ischemia after transplantation. Patients with acute cellular rejection (grade $\geq 2$) on their initial 2 biopsies or with acute vascular rejection were excluded from the analysis to eliminate any potential variation in results that might be influenced by the presence of rejection. The Ethics Review Committee of our institution approved the protocol.

Endomyocardial Biopsy
Serial endomyocardial biopsy specimens were evaluated histologically for the presence of acute cellular rejection by use of International Society Heart Lung Transplant criteria.

Immunohistochemistry Staining
Five endomyocardial biopsy specimens were obtained from each patient at the time of the biopsy. Four of these specimens were analyzed for histopathology, and 1 specimen (snap frozen in liquid nitrogen and stored at $-75^\circ$C) was evaluated for immunoblotting. The biopsies were obtained within 2 weeks of transplantation. Immunoperoxidase staining for $\alpha_v\beta_3$ and tissue factor was performed using rabbit anti-human integrin $\beta_3$ polyclonal antibody (dilution 1:50, Chemicon International Inc) and goat anti-human tissue factor polyclonal antibody (dilution 1:300, American Diagnostica Inc), respectively. Immunoperoxidase staining for the different MMPs was performed using mouse anti-human monoclonal antibody against the corresponding MMP (MMP-1, dilution 1:400; MMP-2, dilution 1:100; MMP-3, dilution 1:100; and MMP-9, dilution 1:200) (ICN Biomedicals Inc). Co-localization of MMP with $\alpha_v\beta_3$ was performed using serial 6-µm sections.

Immunoblotting
Protein expression of $\alpha_v\beta_3$, tissue factor, and extracellular MMP inducer (EMMPRIN) was determined by Western blot analysis as described previously. Briefly, cardiac tissue (3 to 5 mg wet weight/biopsy specimen) was homogenized in 230 µL of lysis buffer (10 mmol/L HEPES at pH 7.4, 2 mmol/L EDTA, 0.1% CHAPS, 5 mmol/L DTT, 10 µg/mL aprotinin, 1 mmol/L PMSF, and 10 µg/mL leupeptin). After homogenization, the samples were centrifuged at 14 000 g (16 000 ×g) at 4°C. Tissue homogenate or supernatant of the homogenate was separated on 10% polyacrylamide gels by Tris-glycine SDS-PAGE. Protein concentration was measured using the bichinchoninic assay method (Pierce Inc.). The amount of protein loaded in each well was 25 µg. After transfer to polyvinylidene fluoride membranes (0.2 µm, BioRad Inc), the membranes were blocked in 3% milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). After two 10-minute washes in TBST, the membranes were incubated overnight with rabbit anti-human integrin $\beta_3$-polycyonal antibody (Chemicon International Inc), goat anti-human tissue factor polyclonal antibody (American Diagnostica Inc), and anti-GAPDH monoclonal antibody (Chemicon International Inc). Because $\alpha_v\beta_3$ and $\alpha_\text{IIb}\beta_3$ share the same $\beta$-subunit, immunoblotting for $\alpha_\text{IIb}\beta_3$ was performed using rabbit anti-human $\alpha_\text{IIb}\beta_3$-monoclonal antibody to exclude any possible variation in results that could be related to $\alpha_\text{IIb}\beta_3$. Platelets were used as positive control for $\alpha_v\beta_3$ and $\alpha_\text{IIb}\beta_3$. Human cirrhotic liver and breast cancer tissues were used as positive controls for tissue factor and EMMPRIN, respectively. Protein expression was normalized to GAPDH and was quantified by arbitrary units by densitometric ratio and was expressed as fold increase.

Morphometric Analysis
Morphometric analysis was performed on myocardial biopsies taken at 1 year after transplantation at the time of coronary intravascular ultrasound. Color (RGB) images of the picrosirius-stained myocardial tissue sections were obtained using an Ektron Applied Imaging 1412 scanner attached to an Olympus BH-2 microscope; 2048×2048 images of the myocardial samples were obtained (pixel size 1.384 µm). A microscopic field of interest was selected, avoiding areas of artifacts and scar tissue from prior biopsy sites. The imaging software is programmed to recognize the red color as interstitial fibrosis, which is highlighted within the field of interest. The RGB images were converted to HSV (hue-saturation-value) for further processing. The tissue was segmented from the surrounding background in the images by Otsu’s automated thresholding algorithm and the saturation band of the color-converted image. This tissue mask was then applied to the hue band of the color-converted image to mask out the surrounding background region. The collagen was masked out the surrounding background region. The collagen was

Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control (n=10)</th>
<th>Ischemia (n=13)</th>
<th>P</th>
</tr>
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<td>Male sex, n</td>
<td>7</td>
<td>10</td>
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<tr>
<td>Donor age, y</td>
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<td>5</td>
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<td>8</td>
<td>NS</td>
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<td>172±8</td>
<td>0.85</td>
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<td>LVAD, n</td>
<td>2</td>
<td>2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are n or mean±SEM. LVAD indicates left ventricular assist device as a bridge to transplant.
segmented from the tissue within the masked area by applying Otsu’s automated thresholding algorithm to the masked hue band image. The percentage of collagen was calculated according to the following: [number of segmented collagen pixels/number tissue pixels]×100.

The ratio of the fibrotic area to the total myocardial tissue area (×100%) is calculated to provide a measure of the percentage of fibrosis.

Matrix Metalloproteinases
MMP-1, MMP-2, MMP-3, and MMP-9 were evaluated by ELISA and zymography in myocardial specimens obtained at 1 year after transplantation at the time of coronary intravascular ultrasound.

MMP zymography was performed as described previously. Briefly, frozen cardiac tissue (3 to 5 mg wet weight/biopsy specimen) was homogenized in 1 mL of extraction buffer (in mmol/L: cacodylic acid 10, NaCl 150, ZnCl2 20, and Na3 1.5 and Triton X-100 0.01%) and concentrated using Amicon Microcon (Ym-10). The protein concentration was then determined using Bio-Rad DC protein assay. MMPs were activated in a buffer solution (Brij-35 0.02%, CaCl2 1 mmol/L, Trypsin 0.5 μg/mL) at 37°C for 5 minutes. Activation of the enzymatic activity was terminated by PMSF 2× SDS buffer and kept at room temperature for 15 minutes. Equal amounts of protein (1 μg) were then loaded on SDS-PAGE (1% gelatin), and electrophoresis was performed at 15-mA constant current for stacking separation and at 20-mA constant current for protein separation. The gel was removed and incubated for 1 hour at room temperature in 100 mL of 2.5% Triton X-100 solution. The Triton X-100 solution was decanted and replaced with 100 mL of enzyme buffer (50 mmol/L Tris at pH 7.5, 200 mmol/L NaCl, 5 mmol/L CaCl2, 0.02% Brij-35). The gel was then incubated for 24 hours. Each gel was stained with 100 mL of 0.5% Coomassie blue G 250 for 3 hours and then was destained. The degree of digestion was quantified by the Adobe Photo Scanner system, and the integrated density of a particular band was reported in volume units of pixel intensity×millimeters2. Conditioned cell media from the HT1080 cell line was used a positive control. Western blot β-actin was used as an internal control (β-actin monoclonal antibody, Chemicon International Inc). Gel zymographic analysis of MMPs may not distinguish between active and latent forms, as these enzymes usually become activated during processing.

MMP ELISA was performed as described previously. Briefly, MMP standards and cardiac tissue extract were incubated at 25°C for 2 hours and washed. The wells were filled with a horseradish peroxidase–conjugated anti-rabbit sera and incubated for an additional hour. After a final series of washes, the reaction was initiated by the addition of 3,3’,5,5’-tetramethylbenzidine/hydrogen peroxide in dimethylformamide (30% vol/vol) and allowed to proceed for 30 minutes. The colorimetric reaction was then terminated and stabilized by the addition of 100 μL of 1 mol/L sulfuric acid. The plate

Figure 2. Myocardial biopsy specimens taken at 1 week of cardiac transplantation showing normal myocardium (top), focal area of ischemic necrosis (center), and an extensive myocyte necrosis (bottom). Magnification ×200.

Figure 3. Myocardial biopsy specimens taken from patients without (A, C, and E) and with (B, D, and F) ischemia. Marked positive staining of tissue factor (B), αβ3 (D), and MMP-9 (F) in the myocytes and intramyocardial blood vessels is noted in the presence of ischemia. Sections D and F are serial, 6 μm apart. Magnification ×200.
Coronary Intravascular Ultrasound

Coronary intravascular ultrasound (IVUS) was performed in 48 coronary vessels in 23 patients (2.1±0.1 arteries/patient). Coronary intimal thickness was measured at baseline (1.0±0.01 months) and 1 year (12.0±0.01 months) after transplantation. Coronary sites with minimum and maximum intimal thickness were identified. Paired analysis of matched sites (10 sites/patient) at 1 year measured the change in maximal intimal thickness. Intimal thickening of >0.3 mm was considered pathological, based on reported values of intimal thickness in the young adult population.18,19

Statistical Analysis

Data are expressed as mean±SEM. Categorical variables were compared by χ² test. Continuous variables were compared using Student’s t test. Differences were considered significant at P<0.05.

Results

All patients were on a standard triple immunosuppressive therapy of prednisone, cyclosporine, and mycophenolate mofetil. The baseline characteristics were similar, except for an increased donor age in the ischemia group (Table). The baseline characteristics were similar, except for an increased donor age in the ischemia group (Table). The baseline characteristics were similar, except for an increased donor age in the ischemia group (Table). The baseline characteristics were similar, except for an increased donor age in the ischemia group (Table). All patients were on a standard triple immunosuppressive therapy of prednisone, cyclosporine, and mycophenolate mofetil. The baseline characteristics were similar, except for an increased donor age in the ischemia group (Table).

Immunohistochemistry Staining

A marked positive staining of tissue factor, αβ3, and MMP is noted in the cardiomyocytes and intramyocardial blood vessels in the ischemic patients (Figure 3). αβ3 and MMP sections are serial, 6 μm apart.

Immunoblotting

Compared with controls, the ischemia group demonstrated evidence of increased expression of αβ3 (3.2-fold, P<0.001) (Figure 4A), tissue factor (2.5-fold, P<0.001) (Figure 4B), and EMMPRIN (1.9-fold, P=0.01) (Figure 4C) on endomyocardial biopsies evaluated within 2 weeks of transplantation. The negative immunoblotting of αβ3 (Figure 4D) indicates that the β3-protein expression findings are mainly related to αβ3 rather than to αβ3.

Compared with patients with focal myocardial necrosis (n=7), patients with extensive myocardial necrosis (n=6) had significant increased expression of αβ3 (1.6-fold, P=0.04). The increase in tissue factor (1.4-fold, P=0.14) and EMMPRIN (1.5-fold, P=0.19) in relation to the extent of necrosis, however, did not reach statistical significance.

MMP Zymography and ELISA

Compared with controls, the ischemia group showed increased zymographic activity (Figure 5) of MMP-2 (1.4-fold, P<0.001), MMP-3 (1.2-fold, P<0.001), and MMP-9 (1.3-fold, P<0.01). ELISA determination (Figure 6) also confirmed increased MMP-2 (1.8-fold, P=0.038), MMP-3 (1.7-fold, P=0.039), and MMP-9 (4.8-fold, P<0.001) in the ischemia group.

Morphometric and IVUS Analysis

The ischemia group had a significant increased mean percent of fibrosis of the biopsies taken at 1 year (24±1.8% versus 14±1.1%, P<0.001) and increased coronary vasculopathy.
progression on IVUS (change in coronary maximal intimal thickness 0.54 ± 0.1 versus 0.26 ± 0.06 mm, *P* = 0.031) compared with the values of controls (Figure 7).

**Discussion**

Increased expression of α,β3, and tissue factor in the presence of ischemia is one of the main findings of this study. Ischemia at the time of transplantation has been shown to cause significant endothelial injury,20 and with the release of cytokines, may result in the proliferation of vascular smooth muscle cells.21 Cytokines may also stimulate α,β3 expression, interleukin-4, interleukin-6, granulocyte-monocyte colony-stimulating factor, and tumor necrosis factor-α; all upregulate gene expression of the integrin β-subunit.22 The histologic degree of ischemic injury has been shown to be a strong predictor of subsequent development of coronary vasculopathy.1 Both, α,β3, and tissue factor have been shown to be implicated in the pathogenesis of atherosclerosis,5,9 and we have recently shown increased myocardial expression of both in the presence of transplant coronary vasculopathy (data not shown).

α,β3 has also been shown to play a key role in the activation of metalloproteinases.11 The complex interaction between α,β3, tissue factor, and the extracellular matrix is depicted in Figure 1. α,β3 expression was significantly increased in the presence of extensive myocardial necrosis. Two patients in this group developed graft dysfunction, with gradual recovery of function over 7 to 10 days.

MMPs are zinc- and calcium-dependent endopeptidases capable of degrading most of the extracellular matrix components, and their expression has been shown to be enhanced in animal models of lung23 and myocardial24 ischemia-reperfusion injuries. Our study confirms the upregulation of the MMP induction system in the presence of ischemia as reflected by the increased expression of EMMPRIN, which is known to induce MMP expression. It has been demonstrated that EMMPRIN forms a complex with α,β3-integrin,25 however, its relation to α,β3 was not studied before. At 1 year, MMP-2, MMP-3, and MMP-9 were significantly increased in the ischemia group. Further, the ischemia group had increased coronary vasculopathy progression and increased amounts of myocardial interstitial fibrosis, suggesting the presence of an early remodeling process. None of these patients had evidence of graft dysfunction at 1 year.

Interstitial myocardial fibrosis has been described as early as 1 to 2 months after transplantation26 and was linked to peritransplantation ischemia.27 The mechanism of fibrosis, however, has not been completely elucidated. It has been recently suggested that ischemia after transplantation is associated with subsequent microvascular damage and microvascular deposition of fibrin, linked to depletion of tissue plasminogen activator.28 Myocardial fibrin deposits noted soon after transplantation were found to predict subsequent development of vasculopathy and graft failure.29 Recently, we have shown that patients with ischemic-fibrotic injury after transplantation have increased progression of coronary vasculopathy and poor outcome over a 7-year follow-up period (data not shown). The finding of increased expression of α,β3, tissue factor, and activation of the MMP induction system in the present study add further insight to the pathophysiology of remodeling and allograft vasculopathy in patients with ischemia after transplantation. Targeting α,β3 may represent a novel therapeutic strategy for this disease process that warrants future investigations.

**Limitations**

The immunohistochemistry staining results may appear more reactive than the Western blot analysis results. These results could be related to the fact that ischemia may not be homogenous, especially if it involves a focal area, whereas immunoblotting analyzes the specimen as a whole. Therefore, the protein expression will represent a combined protein...
expression of areas that are ischemic and nonischemic. In addition, variability in results could also be related to variation in regional sampling from the same patient, because the specimens for immunohistochemistry and Western blot analysis represent different biopsy specimens. Despite these qualitative variations, we still observed significant increased expression of αβ3, tissue factor, and EMMPRIN in the ischemic group. Sampling and regional myocardial heterogeneity may also explain the small differences (although significant) in the MMP zymographic activities between ischemic and nonischemic groups. Finally, the fact that these patients were evaluated at an early phase (1 year after transplantation), when they have not yet developed left ventricular dysfunction, may also explain such small differences. We anticipate that these differences might widen when these patients develop graft dysfunction. A serial zymographic follow-up study may be a worthwhile future investigation.

Acknowledgments
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References
2. Byzova TV, Rabbani R, D’Souza SE, et al. Role of integrin αβ3, tissue factor, and EMMPRIN in the ischemic group. Sampling and regional myocardial heterogeneity may also explain the small differences (although significant) in the MMP zymographic activities between ischemic and nonischemic groups. Finally, the fact that these patients were evaluated at an early phase (1 year after transplantation), when they have not yet developed left ventricular dysfunction, may also explain such small differences. We anticipate that these differences might widen when these patients develop graft dysfunction. A serial zymographic follow-up study may be a worthwhile future investigation.

Figure 7. Representative intravascular ultrasound and myocardial biopsy specimens taken at 1 year after transplantation with picrosirius staining (stained in red) for morphometric analysis. An increased amount of interstitial fibrosis (C) and coronary intimal thickness (D; intimal thickness 0.2 mm; arrows) is noted in a patient who had ischemic injury after transplantation compared with a control patient (A and B; intimal thickness 0.83 mm; arrows). Both patients had no evidence of coronary vasculopathy at baseline.


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