Myocardial Ischemic Injury After Heart Transplantation Is Associated With Upregulation of Vitronectin Receptor (αvβ3), Activation of the Matrix Metalloproteinase Induction System, and Subsequent Development of Coronary Vasculopathy

Mohamad H. Yamani, MD; E. Murat Tuzcu, MD; Randall C. Starling, MD, MPH; Norman B. Ratliff, MD; Yang Yu, BS; D. Geoffrey Vince, PhD; Kimery Powell, PhD; Daniel Cook, PhD; Patrick McCarthy, MD; James B. Young, MD

Background—Myocardial ischemic injury after heart transplantation is associated with subsequent development of graft vasculopathy. Both vitronectin receptor (integrin αvβ3) 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 αvβ3, 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 αvβ3 (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 αvβ3, 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.1 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 αvβ3) is a cell surface adhesion receptor that mediates cell-cell and cell-extracellular matrix interaction with several ligands, including fibronogen, fibronectin, thrombospondin, and prothrombin.2,3 αvβ3 co-stimulates the release of tumor necrosis factor-α,4 which in turn induces expression of tissue factor, a major regulator of hemostasis,5 and regulates the expression of various matrix metalloproteinases (MMPs)6,7 (Figure 1). Both αvβ3 and tissue factor have been shown to play key roles in smooth muscle cell migration and vascular endothelial cell injury.8,9 MMPs are an endogenous family of zinc-dependent enzymes, responsible for matrix remodeling in several disease states,10 MMPs also serve as ligands to αvβ3.11 This complex interaction has been well demonstrated in tumor invasion.12 Recently, the MMP induction system has been shown to be upregulated in heart failure13; however, its role in myocardial ischemic injury after transplantation has not been studied before.

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From the Departments of Cardiovascular Medicine (M.H.Y., E.M.T., R.C.S., Y.Y., J.B.Y.), Anatomic Pathology (N.B.R.), Biomedical Engineering (D.G.V., K.P.), and Cardiothoracic Surgery (P.M.), and the Allegen Laboratory (D.C.) and Kaufman Center for Heart Failure (M.H.Y., E.M.T., R.C.S., Y.Y., J.B.Y., P.M.), Cleveland Clinic Foundation, Ohio.

Correspondence to Mohamad H. Yamani, MD, Cleveland Clinic Foundation, Cardiology, F25, 9500 Euclid Ave, Cleveland, OH 44195. E-mail yamanim@ccf.org

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We postulate that myocardial ischemic injury after transplantation is associated with upregulation of \( \alpha_\beta_3 \) 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 \( \alpha_\beta_3 \) 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 \( \alpha_\beta_3 \) was performed using serial 6-\( \mu m \) sections.

**Immunoblotting**

Protein expression of \( \alpha_\beta_3 \), 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 \( \mu L \) of lysis buffer (10 mMol/L HEPES at pH 7.4, 2 mMol/L EDTA, 0.1% CHAPS, 5 mMol/L DTT, 10 \( \mu g/mL \) aprotinin, 1 mMol/L PMSF, and 10 \( \mu g/mL \) leupeptin). After homogenization, the samples were centrifuged at 14,000 \( \times \)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 bichinonic acid assay method (Pierce Inc.).

**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 sections were obtained using an Eckelon Applied Imaging 1412 scanner attached to an Olympus BH-2 microscope; 2048×2048 images of the myocardial samples were obtained (pixel size 1.384 \( \mu m \)). 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 collage was

**Baseline Characteristics**

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Control (n=10)</th>
<th>Ischemia (n=13)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, n</td>
<td>7</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>Donor age, y</td>
<td>27±1.8</td>
<td>31±1.1</td>
<td>0.045</td>
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<tr>
<td>Recipient age, y</td>
<td>54±4.1</td>
<td>53±3.9</td>
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</tr>
<tr>
<td>Cause, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>4</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Ischemic cardiomyopathy</td>
<td>6</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Ischemia time, min</td>
<td>169±7</td>
<td>172±28</td>
<td>NS</td>
</tr>
<tr>
<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: \[
\text{percentage of collagen} = \frac{\text{number of segmented collagen pixels}}{\text{number of tissue pixels}} \times 100
\]

The ratio of the fibrotic area to the total myocardial tissue area (\(\times 100\%\)) 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, ZnCl 20, and Na3 1.5 and Triton X-100 0.01%) and concentrated using Amicon Microconn (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 \(\times\) millimeters\(^2\). Conditioned cell media from the HT1080 cell line was used as a positive control. Western blot α-actin was used as an internal control (α-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′-tetrathymethylbenzidine/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 \(\times 200\).

**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), α3β3 (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 \(\times 200\).
was read at 450 nm (Vmax Kinetic Microplate Reader, Molecular Devices), and optical density values were determined.

**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 histological changes of myocyte necrosis in the ischemia group were seen on the first biopsy (1 week after transplantation) in 9 patients and on the second biopsy (2 weeks after transplantation) in another 4 patients. Myocyte necrosis was focal in 7 of 13 patients (Figure 2, middle) and extensive in 6 of 13 patients (Figure 2, bottom). There was no relation between early myocyte necrosis and the extent of necrosis; at 1 week, necrosis was focal in 4 patients and extensive in 5 patients. None of these patients had evidence of acute vascular rejection on immunofluorescence. Two patients with extensive necrosis had evidence of graft dysfunction, with posttransplantation left ventricular ejection fraction of 30% to 35%, which recovered gradually to normal within 7 to 10 days after transplantation.

**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 αmβ3 (Figure 4D) indicates that the β3-protein expression findings are mainly related to αβ3, rather than to αmβ3.

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 \( \alpha,\beta_i \) 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, and with the release of cytokines, may result in the proliferation of vascular smooth muscle cells. Cytokines may also stimulate \( \alpha,\beta_i \) expression, interleukin-4, interleukin-6, granulocyte-monocyte colony-stimulating factor, and tumor necrosis factor-\( \alpha \); all upregulate gene expression of the integrin \( \beta_i \)-subunit. The histologic degree of ischemic injury has been shown to be a strong predictor of subsequent development of coronary vasculopathy. Both, \( \alpha,\beta_i \), and tissue factor have been shown to be implicated in the pathogenesis of atherosclerosis, and we have recently shown increased myocardial expression of both in the presence of transplant coronary vasculopathy (data not shown).

\( \alpha,\beta_i \) has also been shown to play a key role in the activation of metalloproteinases. The complex interaction between \( \alpha,\beta_i \), tissue factor, and the extracellular matrix is depicted in Figure 1. \( \alpha,\beta_i \) 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 lung 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 \( \alpha,\beta_i \)-integrin, however, its relation to \( \alpha,\beta_i \) 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 transplantation and was linked to peritransplantation ischemia. 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. Myocardial fibrin deposits noted soon after transplantation were found to predict subsequent development of vasculopathy and graft failure. 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 \( \alpha,\beta_i \), 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 \( \alpha,\beta_i \) 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 \( \alpha \beta \), 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

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