Patterns of Myocardial Endothelin-1 Expression and Outcome After Cardiac Transplantation

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Background—Endothelin-1 (ET-1), a potent vasoconstrictor, is released in response to several inflammatory cytokines after heart transplantation. The present study correlated patterns of myocardial ET-1 expression in heart biopsies with acute rejection, post-transplantation ischemic injury, and subsequent development of coronary vasculopathy.

Methods and Results—Patterns of myocardial ET-1 expression were evaluated in 47 heart transplant recipients at 3 months after transplant. Transplant vasculopathy was documented by coronary angiography at 2 years after transplant. Expression of ET-1 was tabulated for both blood vessels and the interstitium. Vascular ET-1 expression was positive in 7/17 (41%) of patients with greater than grade 2 (International Society Heart Lung Transplant) rejection compared with 3/30 (10%) of patients with grade 0 and grade 1A rejection (P=0.02). Compared with patients with negative interstitial ET-1 expression (n=22), patients with positive interstitial ET-1 expression (n=25) had higher incidence of post-transplantation ischemic injury (52% versus 9%, P=0.002), lower mean episodes of acute rejection (≥ grade 2) during the first 3 months of transplant (1.09±0.66 versus 1.86±1.6, P=0.048), and more common vasculopathy at 2 years (50% versus 15%, P=0.02), and they tended to have worse survival (83.2% versus 100%, P=0.058).

Conclusions—Vascular ET-1 expression is likely to be associated with acute rejection. Interstitial ET-1 expression, however, is more likely to be associated with post-transplantation ischemic injury and subsequent development of coronary vasculopathy. (Circulation. 2002;105:1768-1771.)

Key Words: endothelin ■ rejection ■ transplantation ■ ischemia ■ vasculopathy

Endothelins are 21-amino-acid peptides that are produced by vascular endothelial cells, smooth muscle cells, and other cells in different organs. Endothelins are secreted as big-endothelins that are converted to active proteins by the endothelin-converting enzyme. These peptides are vasoconstrictors with mitogenic properties and are involved in both native atherosclerosis and transplant coronary vasculopathy. 

This study was undertaken to evaluate patterns of myocardial endothelin-1 (ET-1) expression in heart biopsies taken at 3 months after transplant and to correlate these patterns with acute rejection, post-transplantation ischemic injury, and subsequent development of vasculopathy.

Methods

Patient Population
A total of 47 heart transplant recipients (mean age, 57±8 years) underwent serial endomyocardial biopsies. All patients were on standard immunosuppressive therapy. The Ethics Review Committee of our institution approved the protocol.

Endothelin-1 Expression
The endomyocardial biopsy specimens were evaluated for the presence of post-transplantation ischemic injury (manifested histologically by myocyte necrosis and interstitial fibrosis on subsequent biopsies), vascular rejection (by immunofluorescence), and acute cellular rejection by use of International Society Heart Lung Transplant (ISHLT) criteria. The average number of rejection episodes (greater than grade 2) during the first 3 months of transplant was calculated for each patient. All episodes of rejection were biopsy proven. Biopsies that were read as “previous biopsy site” were not considered as post-transplantation ischemic injury.

Endothelin-1 Expression
Myocardial samples, evaluated for ET-1 expression at 3 months after transplant, were fixed by immersion in Zamboni’s solution at 4°C overnight and then washed in phosphate-buffered (0.015 mol/L) saline (PBS) containing 15% sucrose and 0.01% sodium-azide. Immunohistochemistry was performed on cryostat sections (10 μm) and collected onto poly-l-lysine–coated slides. Air-dried sections were immersed in PBS containing Triton-X-100 for 30 minutes at room temperature and processed according to indirect immunofluorescence methods, after counterstaining in a 0.05% PBS solution of
pontamine sky blue in PBS for 30 minutes to decrease background autofluorescence. Immunostaining was performed using a previously characterized polyclonal antisera for ET-1 (dilution, 1:200; Phoenix Pharmaceutical Inc). The specificity of this antibody has been demonstrated before. The sections were incubated with primary antisera for 16 to 20 hours at 4°C, rinsed in PBS (3 times for 5 minutes each), and incubated with fluorescein isothiocyanate–labeled goat anti-rabbit IgG (dilution, 1:80) for 1 hour at room temperature. Each preparation was mounted in glycerol/PBS (dilution, 1:1) and viewed under a fluorescence microscope. Immunohistochemistry was performed twice in different sections of the same specimen, and each time a positive control was included to check the binding of the first antibody and of the fluorescein-conjugated immunoglobulin. Controls included the omission of primary antisera and replacement with preimmune serum.

Coronary Angiography
Cardiac catheterization was performed on all patients at baseline (within 1 month of transplant) and on 42/47 patients at 2 years of transplant. Coronary vasculopathy was classified as mild (coronary obstruction <50%), moderate (coronary obstruction 50% to 70%), or severe (coronary obstruction >70%).

The endomyocardial biopsies and the coronary angiograms were evaluated in a blinded fashion without the knowledge of the immunofluorescence results.

Statistical Analysis
Categorical variables were compared by χ² test. Continuous variables were compared by Student's t test. Differences were considered significant at P<0.05. An estimation of the distribution of time-to-death was computed by the Kaplan-Meier method. A log-rank test was the primary efficacy analysis to compare the estimated time-to-death distribution. Data are expressed as mean±SD.

Results
Patterns of expression of ET-1 were tabulated for both blood vessels and the interstitium. Sixteen patients had both negative vascular and interstitial ET-1 expression (Figure 1a). These patients had no evidence of post-transplantation ischemic injury or acute rejection at the time of the biopsy. On the other hand, 4 patients with positive vascular and positive interstitial ET-1 expression (Figure 1d) had both post-transplantation ischemic injury/fibrosis and evidence of acute rejection (grade ≥2) at the time of the biopsy. The pattern of ET-1 expression in relation to post-transplantation ischemic injury/fibrosis and acute rejection was further examined.

Vascular ET-1 Expression
Vascular ET-1 expression was positive (Figure 1b) in 7/17 (41%) of patients with grade 2 and grade 3A rejection compared with 3/30 (10%) of patients with grade 0 and grade 1A rejection (P=0.02). In these 3 latter patients with grade 0 and grade 1A rejection, 1 patient had 2 prior consecutive episodes of grade 3A rejection and the other 2 patients had active cytomegalovirus (CMV) disease at the time of the biopsy.

Interstitial ET-1 Expression
Interstitial ET-1 expression was positive (Figure 1c) in 25/47 (53%) and negative in 22/47 patients (47%). These 2 groups were similar in baseline characteristics (Table 1). The cyclosporine trough blood level at 3 months was similar in the
ET-1–positive and ET-1–negative groups (342±41 versus 326±50 ng/mL, P=NS). Patients in the ET-1–positive group had higher incidence of post-transplantation ischemic injury/fibrosis, lower mean episodes of acute rejection (≥ grade 2) during the first 3 months of transplant, and more common vasculopathy by coronary angiography at 2 years (Table 2). Further, over a mean follow-up period of 28.5±3.9 months, the ET-1–positive group tended to have poorer survival (Figure 2).

### Discussion

Cytokines, released during acute cellular rejection, are known to induce ET-1 release by vascular endothelial and vascular smooth muscle cells. The mitogenic and pressor actions of ET-1 have been shown to play a key role in the pathogenesis of chronic renal allograft rejection. Increased vascular ET-1 expression in the presence of acute rejection (≥ grade 2) is one of the main findings of the present study. Upregulation of myocardial ET-1 expression has been demonstrated in animal models of cardiac allograft rejection. In the present study, only 41% of patients with greater than grade 2 rejection had evidence of vascular ET-1 expression. Further investigation will be warranted to better define the relationship between vascular ET-1 expression and cardiac allograft rejection. Three patients with no rejection demonstrated vascular ET-1 expression. One patient had 2 prior consecutive episodes of grade 3A rejection, suggesting that vascular ET-1 was still expressed despite histologic resolution of rejection, and the other 2 patients had active CMV disease at the time of heart biopsy. CMV induces the expression of adhesion molecules, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1, but ET-1 expression in relation to CMV disease has not been evaluated.

Another main finding of the present study is that interstitial ET-1 expression was more commonly associated with post-transplantation ischemic injury/fibrosis. Most transplant studies have focused on the role of vascular endothelium in the pathogenesis of allograft vasculopathy. The present study addresses the importance of the interstitium, where ET-1 expression may have a prognostic role as well. Animal models of myocardial ischemia have shown that endogenous ET release is involved in the pathogenesis of ischemia/reperfusion injury, and that ET-1 stimulates cardiac fibroblast proliferation. The negative interstitial ET-1 expression in 2 patients with post-transplantation ischemic injury might be related to sampling error, a problem inherent in biopsy studies. Interstitial ET-1 was associated with a lower cellular rejection score, suggesting a dissociation between rejection and fibrosis, as has been shown in pediatric liver transplants. The concept of fibrosis after transplantation has been thoroughly examined by Armstrong and his colleagues, who evaluated serial endomyocardial biopsies on 50 cardiac transplant patients followed over 5 years. The investigators showed that myocardial fibrosis develops early and remains modestly elevated 2 months after transplant, indicating that peri-transplant factors might be responsible for this fibrotic process. The role of ET-1 in the pathophysiology of native atherosclerosis and transplant vasculopathy has been well demonstrated. The present study demonstrates that interstitial ET-1 expression at 3 months of transplant is associated

![Figure 2. Kaplan-Meier survival curve of patients with positive and negative interstitial ET-1 expression.](image-url)

### TABLE 2. Comparison of ET-1 Interstitial Positive and Negative Groups

<table>
<thead>
<tr>
<th></th>
<th>Interstitial Positive Group (n=25)</th>
<th>Interstitial Negative Group (n=22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posttransplant ischemic injury</td>
<td>13 (52%)</td>
<td>2 (9%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Av. episodes rejection</td>
<td>1.09±0.66</td>
<td>1.86±1.60</td>
<td>0.048</td>
</tr>
<tr>
<td>Coronary vasculopathy</td>
<td>11/22 (50%)</td>
<td>3/20 (15%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Mild</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values are n (%) or mean±SD.

Av. Episodes Rejection indicates the average number of episodes grade≥2 rejection per patient during the first 3 months of transplant.
with subsequent development of coronary vasculopathy at 2 years and a trend for worse survival.

In conclusion, vascular ET-1 expression is more likely to be associated with acute rejection; however, interstitial ET-1 expression is more likely to be associated with post-transplantation ischemic injury/fibrosis and subsequent development of coronary vasculopathy. Therapeutic interventions targeted against ET-1 may be helpful in aborting the process of post-transplantation ischemic injury and warrant future investigations.

References
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