Identification and Characterization of Two Genes (MIP-1β, VE-CADHERIN) Implicated in Acute Rejection in Human Heart Transplantation

Use of Murine Models in Tandem With cDNA Arrays

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Background—Genes and mechanisms of action involved in human acute rejection after allogeneic heart transplantation remain to be elucidated. The use of a murine allograft model in tandem with cDNA arrays and quantitative real-time polymerase chain reaction (Q-PCR) can greatly help in identifying key genes implicated in human heart acute rejection.

Methods and Results—Hearts from Balb/c mice were either not transplanted or transplanted heterotopically in the abdomen of Balb/c (isografts) and C57BL/6 (allografts) mice. Histological analysis showed acute rejection only in allografts. Total RNA was extracted from isografts (n=3), allografts (n=4), and not transplanted hearts (n=4); reverse transcribed; and labeled with P32. Each probe was hybridized to cDNA macroarrays. Eight genes were overexpressed and 7 genes were underexpressed in allografts compared with isografts. Macrophage inflammatory protein-1β (MIP-1β), an overexpressed gene, and VE-cadherin, an underexpressed gene, were validated by immunohistochemistry and Q-PCR in the murine models. Genes of interest, validated in the 3 murine groups, were then investigated in human heart tissues. Immunohistochemistry and Q-PCR performed on endomyocardial biopsies after heart transplantation showing no rejection (n=10) or grade IB (n=10) or IIIA (n=10) rejection, according to International Society of Heart and Lung Transplantation criteria, confirmed the results obtained from the murine model.

Conclusions—We have demonstrated that the upregulation of MIP-1β and downregulation of VE-cadherin may strongly participate in human acute heart rejection. (Circulation. 2005;111:2636-2644.)

Key Words: genes ■ immunohistochemistry ■ polymerase chain reactions ■ rejection ■ transplantation

Acute rejection observed after heart transplantation is initiated by the recognition of the major histocompatibility complex class II antigen on transplanted organ by host CD4+ T cells.1 Major histocompatibility complex class II antigen activates CD4+ T lymphocytes, which subsequently release cytokines. Released cytokines target vascular endothelial cells (ECs), the first cells to be recognized by the host’s immune system, and induce the expression of adhesion molecules and chemokines implicated in T-lymphocyte adhesion and extravasation. Adhesion stage provides the necessary signal for full T-lymphocyte activation and transendothelial migration.2 Lymphocyte transendothelial migration to the donor organ is controlled by 3 cell junctions identified as tight, gap, and adherent junctions3 and by adhesion molecules such as CD31 and VE-cadherin.4,5

To date, the exact mechanisms involved in acute rejection are not completely understood. In this study, we used cDNA macroarray to investigate gene expression of heart tissue in acute allograft rejection in a murine model of heterotopic heart transplantation. Validation of the expression of 2 genes of interest (macrophage inflammatory protein-1β [MIP-1β] and VE-cadherin) was performed by immunohistochemistry and quantitative real-time polymerase chain reaction (Q-PCR) on murine and human heart tissues.

Methods

Animal Study

Heterotopic Heart Transplantation
Hearts from Balb/c (H-2d) mice (age, 7 to 10 weeks; IFFA CREDO, France) were either not transplanted (NT; n=4) or transplanted...
heterotopically in the abdomen of Balb/c (isografts; n=4) and C57BL/6 (H-2b) mice (allografts; n=4) as previously described.©

Hearts were evaluated by daily abdominal palpation. The day on which the heartbeat was reduced before stopping completely was considered the day of acute rejection, and the excision was triggered. Hearts were transversely cut and snap-frozen. The apex was used for RNA extraction, and the upper part was used for histological and immunohistochemical analyses.© Animals were treated according to INSERM guidelines.

Histopathological Study
Serial sections of formalin-fixed, paraffin-embedded heart tissue were stained with hematoxylin-eosin-safran (HES). Specimens were graded for acute rejection with the International Society of Heart and Lung Transplantation (ISHLT) criteria.©

RNA Extraction
Hearts were mechanically homogenized in TRIzol (Gibco BRL). Total RNA was extracted as described,© treated with MessageClean (GenHunter) to remove DNA contamination, and quality controlled.

cDNA Arrays
Total RNA was reverse transcribed to cDNA, 32P labeled, and hybridized to the Atlas mouse 1.2 array (Clontech) bearing 1176 genes. Each experiment had 1 allograft compared with 1 isograft sample and was analyzed in a paired fashion. cDNA hybridization intensity was analyzed by autoradiography and quantified with Atlasmage 1.01 software (Clontech). To allow calculation of ratios, a threshold of 10 U was assigned to any gene with a hybridization signal equal to 0. Genes were considered overexpressed or underexpressed if the signal obtained with the probe from the allograft group was more than 10 times that obtained from the isograft group (n=3).

Immunohistochemistry
Formalin-fixed, paraffin-embedded heart tissues were deparaffinized and treated with microwave in 10 mmol/L citric buffer, pH 6.0 (10 minutes). Alternatively, heart tissues were OCT embedded, snap-frozen in liquid nitrogen, and acetone fixed. Sections (4 µm) were incubated with primary (60 minutes) and secondary (30 minutes) antibodies and then revealed with streptavidin immunoperoxidase.

Antibodies used were as follows: rabbit anti-mouse CD31 polyclonal antibodies (1:100, Santa Cruz Biotechnology), goat anti-mouse MIP-1β polyclonal antibody (1:100, Research Diagnostics Inc), and rat anti-mouse VE-cadherin monoclonal antibody (1:10, BD Biosciences), followed by goat anti-rabbit-biotin (1:100, Bio-Rad), rabbit anti-goat-biotin (1:500, Dako), or rabbit anti-rat-biotin (1:400, Dako). MIP-1β-positive controls used heart, aorta, and spleen harvested from Balb/c mice injected intraperitoneally with lipopoly-saccharide 5 mg/kg and sacrificed 2 hours later.© VE-cadherin–positive controls used spleen tissues of NT Balb/c mice. Negative controls were performed with the use of irrelevant isotype-matched antibodies.

Q-PCR Procedure
CDNA was obtained by reverse transcription from 1 µg total RNA from 14 murine heart tissues, including 10 tested on the macroarrays and immunohistochemistry (NT, 4; isografts, 6; allografts, 4). Q-PCR primers, reagents, and TaqMan probes were from Applied Biosystems (ABI), and protocols are described in Assays-on-Demand (www.appliedbiosystems.com). The level of 18S rRNA was used as an endogenous control. Normalized values were obtained by subtracting the sample average 18S rRNA threshold cycle (Ct) value from the sample average target Ct value (ΔCt). Isografts and NT samples were used as calibrators for comparative results. Comparative threshold cycle (ΔΔCt) method was used as described.© Samples were done in duplicate and run on a prism 7000 (ABI).

Human Study
Patients and Endomyocardial Biopsies
All heart transplant recipients undergoing right ventricular endomyocardial biopsies in our hospital during 2001 as part of routine surveillance were eligible for this study. Endomyocardial biopsies were performed in accordance with institutional guidelines. All patients had a normal systolic ventricular function detected by echocardiography at the moment of the endomyocardial biopsy. Thirty paraffin-embedded biopsy specimens (from 20 patients) were selected for immunohistochemical staining. Fourteen patients had 1 biopsy specimen, and 8 patients had 2 specimens. For each patient, biopsy specimens were spread among categories: no acute rejection, grade IB acute rejection, and grade IIIA acute rejection. Biopsy specimens were selected on the basis of the number, size, and quality of myocardial pieces as recommended by the ISHLT.© Biopsy specimens presenting lesions as “quilty effect,” fibrosis, or infection were excluded. Patients with histopathological diagnosis of acute rejection according to the ISHLT criteria received high-dose methylprednisolone and/or antithymocyte globulin treatment after the biopsy specimen was obtained. In 4 patients, immunohistochemistry was performed before and after a rejection treatment.

RNA Extraction
Biopsy specimens were cut into 10-µm-thick sections and treated with xylene for paraffin removal. Total RNA was extracted with the Total Quick RNA Cells and Tissues protocol (Talent) (www.spin.it/talent). Total RNA was quantified at 260 nm and checked for integrity.
**Immunohistochemistry**

Unless otherwise indicated, treatment and staining of paraffin sections were identical to the methods described in the Animal Study section. Antibodies used were as follows: goat anti-mouse MIP-1β polyclonal antibody (1:50; Research Diagnosis), mouse anti-human CD34 monoclonal antibody (1:50; Dako), and mouse anti-human VE-cadherin monoclonal antibody (1:50, Chemicon International), followed by rabbit anti-goat-biotin (1:500, Dako) or anti-mouse-biotin (prediluted, Dako). Epicardial vessels served as positive controls for VE-cadherin. Negative controls were performed with the use of irrelevant isotype-matched antibodies.

**Q-PCR Procedure**

Q-PCR was performed as described in the Animal Study section. cDNA was obtained by reverse transcription from 1 μg total RNA from biopsy specimens. No acute rejection and acute rejection grade IB samples were used as calibrators for comparative results.

**Scoring of Immunohistochemical Staining**

Sections were assessed by 2 independent observers (A.L.S.R., L.C.) and scored according to the number of stained ECs and intensity of staining: grade 0, no staining; grade 1, ≤50% weakly (+) stained cells; grade 2, 50% to 100% weakly (+) stained cells; grade 3, ≥50% moderately (+++) stained cells; grade 4, 50% to 100% moderately (+++) stained cells; grade 5, ≤50% strongly (++++) stained cells; and grade 6, ≥50% to 100% strongly (++++) stained cells. Sections (≥4 for each sample) were completely examined and scored in ×400 magnification. Images were generated on a microscope (Leica) at ×400 magnification. The intraobserver reproducibility and interobserver reproducibility were 97% and 92%, respectively.

**Statistical Analysis**

The Kruskal-Wallis test was used to compare the 3 groups. When this global test was significant, the Mann-Whitney U test was used to compare between 2 groups. In the human study, all biopsy specimens were considered independent measurements even when obtained from the same patient because they were classified in different groups. For the comparison between human biopsies before and after an acute rejection treatment, the Wilcoxon signed-rank test for paired data was used. Values of P≤0.05 were considered to indicate statistically significant differences.

**Results**

**Clinical Signs of Rejection**

Grafted heart function, evaluated by daily abdominal palpation, showed in the allograft group a reduced heart rate at day 6.75±0.5 bpm. This clinical sign of acute rejection was not observed in the isografts (hearts harvested at day 6, 6.50±0.57 bpm) or in the NT group.

**Histopathological Evaluation**

**Murine**

No evidence of acute rejection in isografts or the NT group was observed. In contrast, in allografts, histological examination demonstrated grade III to IV acute cellular rejection characterized by diffuse inflammatory infiltrate consisting of large lymphocytes, myocyte necrosis, and interstitial edema (Figure 1).

**Human**

All biopsies were constituted by ≥4 adequate fragments showing no acute rejection (n=10), grade IB acute rejection (n=10), or grade IIIA acute rejection (n=10).

**TABLE 1.** Overexpressed Genes: Expression Ratio of the Intensity of Genes Present in Allografts Compared With Isografts

<table>
<thead>
<tr>
<th>Clontech Gene Code</th>
<th>Gene Description</th>
<th>Allografts vs Isografts</th>
<th>Allografts vs Isografts</th>
<th>Allografts vs Isografts</th>
</tr>
</thead>
<tbody>
<tr>
<td>B04I</td>
<td>Interferon inducible protein-1</td>
<td>6.51</td>
<td>6.93</td>
<td>2195.3</td>
</tr>
<tr>
<td>B08I</td>
<td>Interferon regulatory factor-1</td>
<td>750.5</td>
<td>4.97</td>
<td>195.2</td>
</tr>
<tr>
<td>C14j</td>
<td>T-cell activation antigen CD27</td>
<td>137.6</td>
<td>59</td>
<td>151.7</td>
</tr>
<tr>
<td>D03c</td>
<td>Programmed cell death-1 protein precursor</td>
<td>1110.8</td>
<td>5.99</td>
<td>16.18</td>
</tr>
<tr>
<td>D04a</td>
<td>DNA-damage-inducible transcript 3 (DDIT3)</td>
<td>368.5</td>
<td>9.62</td>
<td>4.52</td>
</tr>
<tr>
<td>D06c</td>
<td>Interleukin-2 receptor γ subunit (IL2RG)</td>
<td>59.91</td>
<td>6.86</td>
<td>7.04</td>
</tr>
<tr>
<td>D13c</td>
<td>γ-Interferon–induced monokine precursor</td>
<td>50.8</td>
<td>16.44</td>
<td>3181.9</td>
</tr>
<tr>
<td>D14j</td>
<td>MIP-1β</td>
<td>3.24</td>
<td>10.49</td>
<td>386.8</td>
</tr>
</tbody>
</table>

n=3 for allografts and isografts. Each experiment had 1 allograft sample compared with 1 isograft.

**TABLE 2.** Underexpressed Genes: Expression Ratio of the Intensity of Genes Present in Isografts Compared With Allografts

<table>
<thead>
<tr>
<th>Clontech Gene Code</th>
<th>Gene Description</th>
<th>Isografts vs Allografts</th>
<th>Isografts vs Allografts</th>
<th>Isografts vs Allografts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A05d</td>
<td>Btb and cnc homolog 2 (BACH2)</td>
<td>88.8</td>
<td>44</td>
<td>4.66</td>
</tr>
<tr>
<td>B14b</td>
<td>VE-cadherin</td>
<td>10.66</td>
<td>4.62</td>
<td>3.86</td>
</tr>
<tr>
<td>C01b</td>
<td>Dystroglycan 1</td>
<td>20.10</td>
<td>4.40</td>
<td>3.56</td>
</tr>
<tr>
<td>C03e</td>
<td>RAB19, member of RAS oncogene family</td>
<td>7.70</td>
<td>4.99</td>
<td>7.93</td>
</tr>
<tr>
<td>E14j</td>
<td>Phosphatidylinositol 3-kinase regulatory α subunit</td>
<td>3.01</td>
<td>8.85</td>
<td>4.17</td>
</tr>
<tr>
<td>F06e</td>
<td>Syndecan 2 (SYND2)</td>
<td>66.4</td>
<td>7.98</td>
<td>58.8</td>
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<tr>
<td>F08h</td>
<td>Collagen 6 α1 subunit precursor (col6A1)</td>
<td>507.2</td>
<td>7.87</td>
<td>4.49</td>
</tr>
</tbody>
</table>

n=3 for allografts and isografts. Each experiment had 1 isograft sample compared with 1 allograft.
cDNA Expression

Transcription profile was obtained by comparing the 3 murine groups. Eight genes were overexpressed and 7 genes were underexpressed in allograft hearts compared with isografts (Tables 1 and 2). Two genes, MIP-1β and VE-cadherin, were selected from the transcriptional modulated pool of genes (Figure 1) because of their presence on ECs, the first graft cells to be in contact with the host cells.

Immunohistochemistry

MIP-1β staining and VE-cadherin staining were differentially expressed between the 3 groups in the murine (P=0.02 and P=0.01, respectively) and human (P=0.0052 and P=0.0007, respectively) studies. Figure 2 shows the strong expression of MIP-1β on ECs in allografts compared with isografts (P=0.03) and NT hearts (P=0.03). VE-cadherin was detected as a strong, thin, linear staining on ECs in the NT and isografts compared with allografts (P=0.03) as shown in

<table>
<thead>
<tr>
<th>Groups</th>
<th>MIP-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not Transplanted (n=4)</td>
<td>2, 2, 2, 4</td>
</tr>
<tr>
<td>Isografts (n=4)</td>
<td>2, 3, 3, 2</td>
</tr>
<tr>
<td>Allografts (n=4)</td>
<td>6, 6, 6, 6 * , †</td>
</tr>
</tbody>
</table>

Values expressed as individual staining grade.
There was no difference in MIP-1β/H9252 and VE-cadherin staining between isografts and NT hearts (Figure 2 and 3). In humans MIP-1β/H9252 was strongly expressed on ECs in heart tissues showing a grade IIIA acute rejection compared with grade IB acute rejection (P<0.04) or cardiac tissues with no rejection (P=0.005) (Figure 4). There was no statistically significant difference in MIP-1β staining between grade IB acute rejection and no rejection tissues. Figure 5 shows VE-cadherin staining as a strong, thin, linear staining on ECs in cardiac tissues showing no rejection compared with acute rejection grade IB (P=0.004) and IIIA (P=0.008) tissue. Moreover, a successful treatment for acute rejection tended to decrease MIP-1β and to increase VE-cadherin immunostaining in each patient (P=0.06) (Figure 6).

**Q-PCR**

MIP-1β and VE-cadherin are differentially expressed between the 3 groups in the murine (P=0.01 and P=0.009) and...
human \((P=0.023\) and \(P=0.022\)) studies. There was no statistically significant difference in 18S rRNA Ct values between groups. Relative amounts of MIP-1\(\beta\) and VE-cadherin were increased and decreased, respectively, in allografts compared with isografts \((P=0.01\) and \(P=0.05\), respectively) and NT \((P=0.02)\) (Figures 2 and 3). The relative amounts of MIP-1\(\beta\) and VE-cadherin were increased and decreased, respectively, in grade IIIA acute rejection compared with no acute rejection \((P=0.048\) and \(P=0.008\), respectively) and grade IB acute rejection \((P=0.036\) and \(P=0.05\), respectively) (Figures 4 and 5). No statistically significant difference in the expression of MIP-1\(\beta\) and VE-cadherin was found when the grade IB acute rejection group was compared with no acute rejection group.
Discussion

The working hypothesis in this study is that the endothelium lining the vessel wall of the donor heart is first to be in contact with host cells and is subjected to immunological interactions. Such interactions result in the overexpression and underexpression of some molecules that facilitate the adhesion and transmigration of lymphocytes in the myocardial tissue, leading to myocyte damage and ultimately graft failure. In the present investigation, we find evidence for the first time that 2 molecules, VE-cadherin and MIP-1β, are implicated in acute rejection after heart transplantation. Indeed, we have demonstrated that (1) macroarray results showed an overexpression of MIP-1β and an underexpression of VE-cadherin in murine allografts compared with the isografts and the NT group; (2) immunohistochemistry showed in murine cardiac tissue that the protein expression of

![Figure 5](image-url)
MIP-1β and VE-cadherin were overexpressed and underexpressed, respectively, in allografts compared with isografts or NT hearts; (3) Q-PCR validated these results; (4) immunohistochemistry and Q-PCR in the human study confirmed the results obtained in the murine model that MIP-1β and VE-cadherin were observed to be overexpressed and underexpressed, respectively, on transplanted hearts showing grade IIIA acute rejection compared with no acute rejection; and (5) successful acute rejection treatment showed a tendency to decrease MIP-1β and to increase VE-cadherin immunohistochemical staining in ECs of human heart tissues.

The sequence homology between human and murine MIP-1β is 76% at the protein level and 75% between human and murine VE-cadherin (www.ncbi.nlm.nih.gov/blast). MIP-1β, a chemokine that belongs to a novel family of cytokines, can induce chemotaxis and adhesion of T cells.12,13 The ability of MIP-1β to regulate the adhesion of T cells to the vascular endothelium is important for the migration of T cells into tissues. Adams et al.,13 studying the hepatic expression of MIP-1β after liver transplantation, showed by immunohistochemistry that MIP-1β was strongly expressed on infiltrating leukocytes and on ECs during episodes of acute rejection. Segerer et al.14 also showed increased expression of MIP-1β in infiltrating cells, tubular epithelial cells, and ECs during acute renal transplant rejection. In our study, we observed a very strong expression of MIP-1β in murine and human ECs but not in inflammatory cells during acute rejection. This finding strongly suggests production of MIP-1β by ECs as previously described.12

VE-cadherin is an endothelium-specific membrane protein present in adherent junctions of ECs that is responsible for EC-cell adhesion. In 1992, Lampugnani et al.4 described VE-cadherin as an endothelium-specific membrane protein with a thin, sharp continuous line highlighting the margins of each cell. They observed that if EC permeability was increased, its distribution was punctuated. In our study, we found the same kind of staining described as a thin, continuous line in the margins of murine and human ECs. Previous studies15–17 have demonstrated that through their adhesion, leukocytes could transfer intracellular signals to ECs in different ways and affect gene transcription. VE-cadherin disappeared from endothelial adherent junctions after the adhesion of polymorphonuclear leukocyte to EC monolayers. Moreover, other authors18,19 have reported changes in cytosolic Ca²⁺ level in ECs during the adhesion of polymorphonuclear leukocytes. The polymorphonuclear adherence could conceivably induce a series of EC intracellular responses, leading to detachment of catenins from VE-cadherin. It is conceivable that, in our murine heart acute rejection model, activated lymphocytes adhering to vascular ECs could affect

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**Figure 6.** MIP-1β, VE-cadherin, and CD34 immunohistochemical staining of serial human heart tissues before and after acute rejection treatment. A–D, Grade IIIA acute rejection; E–H, no acute rejection (×400). A, E, HES staining; B, F, CD34 EC staining; C, G, MIP-1β staining; D, H, VE-cadherin staining; M, immunohistochemical staining grade of MIP-1β and VE-cadherin on ECs before and after acute rejection treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MIP-1β</th>
<th>VE-Cadherin</th>
</tr>
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<tbody>
<tr>
<td>Acute Rejection</td>
<td>5.0 ± 2.0</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>After Acute Rejection Treatment</td>
<td>2.0 ± 0</td>
<td>5.0 ± 2.5</td>
</tr>
</tbody>
</table>
the latter cells by inducing the disappearance of VE-cadherin from endothelial adherent junctions. Such an action could result in a significant increase in endothelial permeability because of the disassembly of endothelial adherent junctions.

These 2 markers were also investigated in settings other than acute rejection after heart transplantation. Indeed, we have assayed the expression of MIP-1β and VE-cadherin by Q-PCR in myocardial tissues from a small number of patients with ischemic cardiomyopathy (ICM) or dilated cardiomyopathy (DCM) who were scheduled for heart transplantation. The control group was formed by donors whose hearts could not be transplanted because of surgical or other reasons. Statistical analysis was performed with the Kruskal-Wallis and Mann-Whitney tests. No significant difference for MIP-1β was observed in ICM (P = 0.29) and DCM (P = 0.11) compared with the normal group. Using a ribonuclease protection assay, Damas et al20 studied the expression of MIP-1β in isolated mononuclear blood cells from chronic heart failure patients compared with healthy blood donors. Chronic heart failure patients included those with ICM and DCM. Damas et al observed MIP-1β overexpression in the chronic heart failure group but did not compare ICM and DCM patients. The relative amount of VE-cadherin in our study was significantly increased in ICM (P = 0.03) and DCM (P = 0.001) compared with the normal group. These results differ from those published by Shafer et al21 who observed a downregulation of VE-cadherin in DCM patients only. Differences between this study and the study by Shafer et al concerning the ICM group may be accounted for by the use of heart samples showing myocardial infarction and necrosis in our study and the exclusion of such patients in the Shafer et al study. The much larger number of patients in the Shafer et al study could explain differences obtained for DCM patients.

In this study, other genes were also found to be overexpressed or underexpressed in the acute rejection group but were not validated (Tables 1 and 2). Cytokines and cell adhesion molecules, previously shown to be overexpressed in acute rejection after heart transplantation,22,23 were also observed in this study but not systematically in all experiments.

In summary, we have demonstrated for the first time a correlation between 2 genes (MIP-1β, VE-cadherin) present in ECs lining the vessel walls and acute allograft rejection after human heart transplantation. These 2 validated genes could be used in the diagnosis of acute rejection in human heart transplantation. However, one should note that the statistical analysis of the human data failed to account for potential correlation within patients and that consequently the reported probability values from those analyses may not be accurate.

Acknowledgments

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References

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