Donor Spontaneous Intracerebral Hemorrhage Is Associated With Systemic Activation of Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 and Subsequent Development of Coronary Vasculopathy in the Heart Transplant Recipient

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Background—Matrix metalloproteinase (MMP)-2 and MMP-9 have been shown to play a role in the progression of hemorrhagic stroke. We hypothesized that donor intracerebral hemorrhage (ICH) is associated with activation of the metalloproteinases before transplantation that play a key role in the subsequent development of transplant vasculopathy.

Methods and Results—We evaluated mRNA expressions of MMP-2 and MMP-9 in donor spleen lymphocytes (before transplantation) and in heart biopsies at 1 week after transplantation in 20 recipients from ICH donors and 20 recipients from trauma donors. Patients underwent serial coronary intravascular ultrasound, and interstitial myocardial fibrosis was quantified at 1 year. The baseline characteristics were similar except for increased donor age in the ICH group. Heart biopsies from the ICH group showed significant increased expression of MMP-2 (17-fold, \( P < 0.0001 \)) and MMP-9 (20-fold, \( P < 0.0001 \)) compared with the trauma group. Furthermore, the ICH group showed 1.8-fold \( (P=0.016) \) increased mRNA expression of MMP-2 and 1.7-fold \( (P=0.015) \) increased mRNA expression of MMP-9 in the donor spleen lymphocytes, suggesting the presence of systemic activation of metalloproteinases before transplantation. At 1 year, the ICH group showed increased myocardial fibrosis and accelerated coronary vasculopathy. Using multivariate regression analysis, MMP-9 was found to be associated with increased risk for vasculopathy independent of donor age \( (OR, 2.41; P=0.01; 95\% CI, 1.24 \text{ to } 4.69) \).

Conclusions—This is the first report to describe systemic activation of MMP-2 and MMP-9 in donors with intracerebral hemorrhage and subsequent development of allograft vasculopathy. \( (\text{Circulation. 2003;108:1724-1728}) \)

Key Words: metalloproteinases ■ hemorrhage ■ transplantation

Donors with spontaneous intracerebral hemorrhage (ICH) comprise 39% of the overall organ donor pool.\(^1\) Heart transplant recipients from donors with ICH are at increased risk of allograft vasculopathy.\(^1\) Activation of sympathetic nervous system activity and excessive catecholamine surge are among the proposed mechanisms underlying graft dysfunction and vasculopathy in donors with ICH.\(^2\)

Matrix metalloproteinases (MMPs) are an endogenous family of zinc-dependent enzymes, responsible for matrix remodeling in several disease states.\(^3\) MMP-2 and MMP-9 have been shown to play a key role in the degradation of basal lamina and disruption of blood-brain barrier in animal models of intracerebral hemorrhage,\(^4\) and inhibition of these MMPs has been shown to be helpful in reducing the vasogenic edema associated with intracerebral hemorrhage.\(^5\) Recently, baseline plasma MMP-9 level has been shown to serve as a biological marker for predicting intracerebral hemorrhage complications after thrombolytic therapy in human stroke.\(^6\)

We hypothesize that donor spontaneous intracerebral hemorrhage is associated with increased expression of MMP-2 and MMP-9 and subsequent development of coronary vasculopathy in the heart transplant recipient.

Methods

Study Population

Study subjects comprised 20 recipients of hearts from ICH donors (ICH group) and 20 recipients of hearts from trauma donors (trauma group). Brain injuries in the trauma group were related to falls \( (n=2) \), gunshot wounds \( (n=8) \), or motor vehicle crashes \( (n=10) \). There was no significant difference in the time elapsed from the ICH or trauma event to heart transplantation \( (59 \pm 57 \text{ versus } 68 \pm 58 \text{ hours}) \). All heart transplant recipients had endomyocardial biopsies at 1 week as part of the surveillance biopsy program and intravas-
cular ultrasound (IVUS) examination within 4 weeks and at 1 year after transplantation for the determination of the progression of transplant coronary vasculopathy. The study was approved by the institutional review board of our institution.

Endomyocardial Biopsy
Right ventricular endomyocardial biopsies were performed at 1 week after transplantation as part of the standard surveillance biopsy protocol. 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 −80°C) was evaluated for the purpose of the study.

Isolation of Lymphocytes From Donor Spleen
Donor spleen was injected with HBSS, and the discharge containing cells was collected and centrifuged (700g for 20 minutes). Cells at interface were transferred to conical tubes and diluted with HBSS before additional centrifugation (700g for 15 minutes). Supernatant was discarded, the cell pellet was resuspended in 3 mL fresh HBSS, and concentrations were adjusted to 5×10^6 cells/mL. The cell suspension was centrifuged (1000g for 1 minute), and supernatant was discarded using a Hamilton syringe. Cells were then mixed with Lympho-Kwik T/B and incubated at 37°C for 20 minutes. HBSS 100 to 200 μL was added and then centrifuged (2000g for 20 minutes). Floating dead cells and supernatant were removed using a Pasteur pipette. Finally, the lymphocyte pellet was washed twice with HBSS (centrifugation 1000g for 1 minute) and resuspended in RPMI + 0.5% BSA.

Isolation of Total RNA
Donor Spleen Lymphocyte RNA
Ambion RNAqueous-4PCR kits were used to isolate total RNA from donor spleen lymphocytes. The lymphocytes were treated with 10 volumes of guanidinium thiocyanate lysis/binding solution, mixed thoroughly with an equal volume of 64% ethanol, and applied to RNAqueous silica-filter cartridges placed into nuclease-free collecting tubes. The filter cartridges were centrifuged at 10 000 rpm for 1 minute or until the lysate/ethanol mixture flowed through the filter. The flowthrough was discarded, and the filters were washed once with guanidinium–ethanol wash solution and twice with ethanol wash solution. The washed filter cartridges were placed into a fresh collection/elution tube, and 50 μL of preheated elution solution (nuclease-free water/EDTA at 80°C) was applied to the center of the filters. The cartridges were centrifuged at 14 000 rpm for 30 seconds to recover the eluate containing total RNA. RNA was quantified with a RiboGreen assay (Molecular Probes).

Cardiac Tissue RNA
Endomyocardial biopsy specimens were retrieved from frozen blocks (−80°C) and were rapidly processed to isolate total RNA by using the Ambion Totally RNA kit. Denaturation solution 200 μL was added, and tissue was homogenized in a 1.5-μL nuclease-free tube. An equal volume of phenol/chloroform/indoleacetic acid was added, vortexed for 1 minute, and then stored on ice (4°C) for 5 minutes. Then the mixture was centrifuged at 4°C (10 000g). The aqueous phase was transferred to new tube, and a 1:10 volume of sodium acetate solution was added and mixed by inversion. Acid:chloroform 200 μL was added, vortexed for 1 minute, and stored on ice (4°C) for 5 minutes before centrifugation (10 000g at 4°C). The upper aqueous phase was transferred to a new tube, precipitated with isopropanol, and stored at −20°C for 30 minutes. The precipitation mixture was centrifuged (10 000g) for 20 minutes, and supernatant was discarded. The pellet was washed with 70% ethanol and resuspended in 50 μL of DEPC water.

Assessing RNA Concentration and Purity
The concentration and purity of RNA were determined by its absorbency in a spectrophotometer at 260 nm. Total RNA was stored at −80°C until analysis.

### Reverse Transcription
RNA samples were reverse transcribed in a 40-μL volume by using the TaqMan reverse transcription kit (TaqMan Reverse Transcription Reagents, Applied Biosystems) containing 2× reverse transcription–polymerase chain reaction (RT-PCR) buffer, MgCl₂ (5.5 mmol/L), random hexamers (2.5 μL), RNA inhibitor (0.4 U/μL), and multiscribe reverse transcriptase (1.25 U/μL). The mix was aliquoted in individual tubes, and template RNA was added. After brief centrifugation, samples were incubated for 90 minutes at 25°C, 45 minutes at 48°C, and 5 minutes at 95°C. A tube with no reverse transcriptase was included to control for DNA contamination.

### TaqMan Quantitative RT-PCR
MMP-2 and MMP-9 primers and probes for quantitative RT-PCR were designed using PRIMER express program (Applied Biosys-tems). We conducted BLASTN searches against dbEST (GenBank, EMBL) to confirm the total gene specificity of the nucleotide sequences chosen for primers and probes and absence of DNA polymorphism. The oligonucleotide sequences of TaqMan probes and primers were as follows: for MMP-2, TaqMan probe FAM-1573, CAAGAACTCCGCTGTCCCGAGATGAC-TAMRA; forward primer 1514, CCTGATGGCAAACCTTACAC; reverse primer 1621, GAGCTCTTGAATGGCCTTG; for MMP-9, TaqMan probe FAM-498, ACAACCGGGACAGACATCG-TAMRA; forward primer 578, GCTCACCTTCTCGCGGT; reverse primer, 538, CGGCACACACCAACTGGATG. Both primers and probes were purchased from Perkin-Elmer, Applied Biosystems.

To measure gene expression, a reaction mix was prepared on ice with 10× TaqMan buffer; 55 mmol/L MgCl₂; 200 μmol/L dATP, 200 μmol/L dCTP, 200 μmol/L dGTP, 200 μmol/L dUTP; Amplerase UNG (0.01 U/μL); AmpliTaq Gold DNA polymerase (0.025 U/μL); 18S ribosomal forward and reverse primers and probe (all at 50 mmol/L); and forward and reverse primers for MMPs and probes (at 100-nmol concentrations). After mixing, the reaction mix was aliquoted into separate tubes of a 96-well plate (Applied Biosystems) and cDNA was added. Wells were sealed with optical caps, and the PCR reaction was carried out using ABI Prism 7700 (Applied Biosystems). Experiments were performed in a final volume of 50 μL with duplicate for each data point. Each PCR run included a no-template control, the calibrator, and unknown patient cDNAs. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 65°C for 1 minute. The data were analyzed and processed using the Sequence Detector Version 1.5 (Applied Biosystems) in accordance with the manufacturer’s instructions. Briefly, the software calculates the reaction cycle number at which fluorescence reaches a determined level for both 18S control and AT receptors. Ribosomal RNA was used as an internal control gene and validated for each experiment. Lymphoid cell–derived total human RNA (Applied Biosystems) and total human heart RNA (Applied Biosystems) were used as calibrators for the assessment of lymphocyte and myocardial mRNA expression of MMPs, respectively. Using the 2ΔΔCt method of relative quantification, we reported the fold change in gene expression normalized to ribosomal RNA and relative to the calibrator.

### Intravascular Ultrasound
The technique of intracoronary ultrasound was described before in detail.10 Briefly, using a standard technique for intracoronary catheter delivery, a catheter that incorporated a miniaturized high-resolution transducer (30 MHz) was passed over an angioplasty guidewire. The operator advanced the imaging catheter to the most distal position that could be safely reached and then retracted the catheter using an automatic pullback device (0.5 mm/s). Anatomical landmarks were identified to facilitate selection of identical segments for sequential examinations. This technique allows accurate volumetric assessment of changes in vessel dimensions and lesions. After excluding donor lesions, de novo lesions were identified as maximal disease based on intimal thickness. Matched sites were analyzed at 1 year to determine the change in maximal intimal thickness (CMIT).
TABLE 1. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Trauma (n=20)</th>
<th>ICH (n=20)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age, y</td>
<td>31±11</td>
<td>40±10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Recipient age, y</td>
<td>57±11</td>
<td>59±10</td>
<td>NS</td>
</tr>
<tr>
<td>Gender, male</td>
<td>14</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>Etiology of heart failure, ICM/DCM</td>
<td>12/8</td>
<td>11/9</td>
<td>NS</td>
</tr>
<tr>
<td>Ischemia time, min</td>
<td>159±43</td>
<td>162±35</td>
<td>NS</td>
</tr>
<tr>
<td>LVAD</td>
<td>3</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>12</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>13</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>11</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>CMV disease</td>
<td>3</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>Mean episodes of acute cellular rejection grade ≥3A</td>
<td>1.3±1.2</td>
<td>1.3±1.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

ICM indicates ischemic cardiomyopathy; DCM, dilated cardiomyopathy; LVAD, left ventricular assist device use as a bridge to transplant; ACE, angiotensin-converting enzyme; and CMV, cytomegalovirus.

and change in plaque volume at the worst affected sites. We defined coronary vasculopathy progression in this study as an increase in coronary intimal thickness of >0.3 mm from baseline.

Morphometric Analysis

Morphometric analysis was performed on myocardial biopsies taken at 1 year of transplant at the time of coronary intravascular ultrasound. Color (RGB) images of the Picosirisus-stained myocardial tissue sections were obtained using an Ektron Applied Imaging 1412 scanner attached to an Olympus BH-2 microscope. Images of 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 hue saturation value for additional processing. The tissue was segmented from the surrounding background in the images using Otsu’s automated thresholding algorithm and the saturation band of the color-converted image.11 This tissue mask was then applied to the hue band of the color-converted image to mask out surrounding background region. The collagen was segmented from the tissue within the masked area by applying Otsu’s automated thresholding algorithm to the masked hue band image. The percent 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 percent fibrosis.

Statistical Analysis

Data are presented as mean±SD. Categorical variables were compared by χ² or Fisher exact test as appropriate. Continuous variables were compared using the Student t test. The Kruskal-Wallis test was used for variables with nonnormal distribution. Multivariate regression models were used to identify the relative predictive power of gene expression. Differences were considered significant at P<0.05.

Results

Both groups had similar baseline characteristics except for an increased donor age in the ICH group (Table 1). All heart transplant recipients were taking triple immunosuppressive drugs, including prednisone, cyclosporine, and mycophenolate mofetil. Both groups had similar comorbidities, such as diabetes, hypertension, and hyperlipidemia, and similar use of ACE inhibitors. Furthermore, there were no significant differences in incidence of cytomegalovirus disease or mean episodes of acute cellular rejection (grade ≥3A) between the 2 groups.

mRNA Expression Analysis of MMP-2 and MMP-9

At 1 week of transplant, heart biopsies from the ICH group showed significant increased expression of MMP-2 (17-fold, P<0.0001) and MMP-9 (20-fold, P<0.0001) compared with the trauma group (Table 2). Furthermore, the ICH group showed 1.8-fold (P=0.016) increased mRNA expression of MMP-2 and 1.7-fold (P=0.015) increased mRNA expression of MMP-9 in the corresponding donor spleen lymphocytes, suggesting the presence of systemic activation of metalloproteinases before transplantation. Using multivariate regression analysis, MMP9 was found to be associated with increased risk of vasculopathy independent of donor age (OR, 2.41; P=0.01; 95% CI, 1.24 to 4.69).

Morphometric and IVUS Analysis

At 1 year, the ICH group showed increased amount of myocardial fibrosis (29±10% versus 19±6%, P=0.003) and accelerated progression of coronary vasculopathy measured as CMIT (0.69±0.37 versus 0.46±0.35 mm; P=0.01) and change in plaque volume (6.3±2.6 versus 4.2±2.5 mm³; P=0.02) compared with the trauma group (Figure).

Discussion

The major findings of this study are the following: (1) significant increased mRNA expression of MMP-2 and MMP-9 in heart biopsies of heart transplant recipients from donors with spontaneous intracerebral hemorrhage; (2) increased mRNA expression of MMP-2 and MMP-9 was also present in the corresponding donor lymphocytes, suggesting that the index insult has occurred in the donor before transplantation; and 3) the ICH group showed increased amount of myocardial fibrosis and coronary vasculopathy compared with the trauma group.

Donor cause of death has been shown to influence the outcome in heart transplant recipients.1,12 We have observed that the donor cause of death was more likely to be brain injury–related in patients who develop myocardial ischemic injury/interstitial fibrosis after transplantation.13 We have also shown that myocardial ischemic injury after cardiac transplantation is associated with activation of the MMP induction system.14 These observations prompted us to study...
the mRNA expression of MMPs in relation to donor cause of death. MMP induction has been shown to play a significant role in several vascular disorders, including heart failure, myocardial infarction, aneurysm formation, rupture of atherosclerotic plaques, and, recently, hemorrhagic stroke. The extracellular matrix molecules, such as type IV collagen, laminin, and fibronectin, constitute the basement membrane underlying the vasculature and play a critical role in maintaining the integrity of the blood-brain barrier. At least 2 MMPs may be directly involved in the progression of stroke and hemorrhage, specifically MMP-2 and MMP-9, which degrade the extracellular matrix components of the basement membrane. A graded response was observed between the plasma MMP-9 level and the degree of intracranial hemorrhage. Our findings of significant increased mRNA expression of MMP-2 and MMP-9 in heart biopsies at 1 week of transplantation in recipients of hearts from donors with intracerebral hemorrhage highlight the impact of donor cause of death on cardiac remodeling. Furthermore, lymphocytes are among the potential sources of MMP biosynthesis. Our findings of increased mRNA expression of MMP-2 and MMP-9 in the corresponding donor lymphocytes in the presence of intracerebral hemorrhage suggest the presence of systemic activation of the metalloproteinase system and, therefore, indicate that the index insult has occurred before transplantation. We postulate that cerebral hemorrhage is associated with MMP release, with subsequent disruption of the blood-brain barrier resulting in a systemic activation process, as evidenced by the splenic upregulation of MMP expression. This, in turn, results in cardiac involvement, in which, on transplantation and in response to vascular injury, smooth muscle cells migrate from the media into the intima, where they contribute to the development of neointimal lesions. Increased MMP expression contributes to the migratory response of smooth muscle cells by releasing them from their surrounding extracellular matrix. This injury has been translated in the ICH group into increased vasculopathy and myocardial fibrosis. We have shown recently that myocardial fibrosis is associated with advanced vasculopathy and portends a poor survival. Heart transplant animal experiments have recently unmasked the key role of MMPs in the pathogenesis of coronary vasculopathy, and our intravascular data clearly show the increased vasculopathy in the ICH group.

In conclusion, MMP-2 and MMP-9 are upregulated in recipients of hearts from donors with intracerebral hemorrhage. This upregulation is associated with cardiac remodeling and subsequent development of coronary vasculopathy.

Acknowledgments
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References
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