Expression of matrix metalloproteinases in cardiac allograft vasculopathy and its attenuation by anti MMP-2 ribozyme gene transfection

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Received 12 December 2001; accepted 10 July 2002

Abstract

Objective: Proliferation and migration of vascular smooth muscle cells (SMCs) causes intimal thickening during cardiac allograft vasculopathy (CAV). This process requires the degradation or remodeling of extracellular matrix (ECM) surrounding the cells. Imbalance between degradation and accumulation of ECM also contributes to the development of CAV. In this study, we investigated the contribution of matrix metalloprotenases (MMPs), enzymes regulating ECM turnover, to the development of CAV. Methods: Donor hearts from male DBA mice were heterotopically transplanted to male B10.D2 recipient mice, and harvested at days 15 and 30 post transplantation. We examined expression MMP-2, -3, -9 and -13 of graft vessels using immunohistochemistry. To clarify the role of MMP-2 in CAV, anti MMP-2 ribozyme was delivered into donor hearts just before transplantation, mediated by a hemagglutinating virus of Japan–liposome complex to specifically suppress MMP-2 activity. Results: All MMPs were immunopositive in SMCs from the slightly thickened neointima at day 15. In the advanced stage of intimal thickening at day 30, in addition to increased number of SMCs, accumulation of collagenous fibers was observed; expression of MMP-3, -9 and -13 was decreased. In contrast, MMP-2 expression remained distinctly positive throughout the progression of the vascular remodeling. After the gene transfer of MMP-2 ribozyme, luminal occlusion was significantly decreased compared to non-treated allografts [25.0±6.5 vs. 55.1±7.0% (P<0.05)] at day 30 post transplantation. Conclusion: MMP-2 is a principle MMP throughout the progression of the vascular remodeling in CAV. Anti MMP-2 therapy could therefore be one of the candidates for a supplemental therapy for CAV.

Keywords: Extracellular matrix; Gene therapy; Remodeling; Smooth muscle; Transplantation

1. Introduction

While acute rejection of transplanted hearts can be well controlled with progressive immunosuppression, chronic rejection, which determines the long-term survival of transplant patients, remains a challenging problem. Chronic rejection, or cardiac allograft vasculopathy (CAV), is characterized by diffuse coronary arteriopathy formed by replication and migration of vascular smooth muscle cells (SMCs) inward to the lumen, which is stimulated by various growth factors. However, the replication and migration of SMCs also requires the degradation or remodeling of the extracellular matrix (ECM) surrounding the cells [1,2]. Matrix metalloproteinas (MMPs) belong to an endogenous family of enzymes responsible for ECM degradation and each MMP has a specific function. The interstitial collagenase (human MMP-1, mouse MMP-13) can cleave native fibrillar collagen; type IV collagenase or
gelatinase (MMP-2 and -9) degrade denatured interstitial collagen, native basement-membrane collagen and elastin. Stromelysins (MMP-3) have a wide specificity against matrix components, including gelatin, laminin, fibronectin, proteoglycan core proteins and elastin, and can activate the zymogen forms of other MMPs as well [3].

These MMPs contribute to the pathogeneses such as ECM turnover, cellular migration and proliferation in the neointimal formation seen after balloon injury; this pathogenesis quite resembles that of CAV. It has been shown that each MMP described above contributes differently to neointimal formation at various stages, and that MMP-2 contributes to the increase in neointimal volume throughout the progression of the intimal thickening [4]. MMP-2 is also revealed to be a principal MMP for SMC migration [5–8]. Furthermore, in our previous study, MMP-2 was shown crucially involved in the development of CAV in a primate model [9]. In this study, we investigated the expression of each MMP in the development of CAV in a murine model, and hypothesized that MMP-2 plays a crucial role, similar to that seen in neointimal formation of balloon injured arteries. We also delivered anti MMP-2 ribozyme to the donor hearts in order to specifically suppress MMP-2 activity, thus clarifying the role of MMP-2 in CAV.

2. Methods

2.1. Cell culture

DBA mouse aortic SMCs were harvested by modifying of the combined collagenase and elastase digestion method [10], and grown in Dulbecco’s modified Eagle’s medium (DMEM), pH 7.4, containing 50 μg/ml streptomycin and 50 IU/ml penicillin, and 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂. At confluence, approximately 4 days, the cells were passaged by rinsing in phosphate-buffered saline (PBS), followed by a 3-min incubation at 37 °C with 0.05% trypsin and 0.02% EDTA in PBS before resuspension in growth medium. All experiments were completed with cells between passages 3 and 8. Subconfluent SMCs were growth-arrested for 72 h in medium with 0.4% FBS in 100-mm plates. A 1-ml volume of each HVJ–cationic-liposome was added for experiments in 100-mm plates after removal of the medium. Cells were washed with PBS after a 2 h incubation at 37 °C, exposed to fresh medium containing 0.4% FBS and stimulated by 100 ng/ml 12-O-tetradecanoylphorbol 13-acetate (PMA) for 24 h.

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

SMCs were lysed with distilled water, and then total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method. After treatment with DNase, mRNA was reverse transcribed in RT solution (10 mmol/l Tris·HCl, pH 8.3; 50 mmol/l KCl; 1.5 mmol/l MgCl₂), 2 mmol/l DTT, 50 U RNase inhibitor, and 200 U MuLV reverse transcriptase (Perkin-Elmer, NJ, USA) for 20 min at 42 °C. Primers for MMP-2, -3, -9 and -13 were synthesized by Sawada Technology (Table 1) [11]. cDNA was amplified using the following parameters: 94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min; 35 cycles. Products were analyzed by electrophoresis on 1.2% agarose gels followed by ethidium bromide staining. cDNA preparations were analyzed for GAPDH as a positive control.

2.3. Animal and heterotopic cardiac allografting and ex vivo gene transfer

Male DBA (H-2d) and B10.D2 (H-2d) mice (age 4 to 6 weeks; weight 20–25 g) were obtained from Japan SLC (Hamamatsu, Japan). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Donor hearts from male DBA mice were heterotopically transplanted to male B10.D2 recipient mice by microsurgical technique [12,13]. The donor hearts were harvested at days 15 and 30 post transplantation. Ex vivo gene transfer of anti MMP-2 ribozyme, DNA-ribozyme and mismatched ribozyme was performed by injecting 0.1 ml of HVJ–artificial viral envelope (AVE) liposome complex into the ascending aorta

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oligonucleotode primers</th>
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<tbody>
<tr>
<td>MMP</td>
<td>Primer sequence</td>
</tr>
<tr>
<td>MMP-2</td>
<td>F 5′-TGCAACACCTTAACGTTGCTT-3′</td>
</tr>
<tr>
<td></td>
<td>R 5′-GGAAATGCGTGGAGTGGAAA-3′</td>
</tr>
<tr>
<td>MMP-3</td>
<td>F 5′-GGCTGTTGTTGTTGTTGTTGCTC-3′</td>
</tr>
<tr>
<td></td>
<td>R 5′-GCTCCTCCAGACCTTCAAAGC-3′</td>
</tr>
<tr>
<td>MMP-9</td>
<td>F 5′-TCAGGGAGATGCCCATTTCG-3′</td>
</tr>
<tr>
<td></td>
<td>R 5′-GAACGGGAACACACAGGGTTTG-3′</td>
</tr>
<tr>
<td>MMP-13</td>
<td>F 5′-CCTCTCGTTCGTGCAACAG-3′</td>
</tr>
<tr>
<td></td>
<td>R 5′-GGGATAGGGCTGACAGTACAT-3′</td>
</tr>
</tbody>
</table>
of the donor hearts just after harvest; hearts were then incubated 10 min on ice. The donor hearts with gene transfer were harvested at day 30 post transplantation.

2.4. Histological study

The hearts were divided into two parts. One half was fixed in 10% formalin and embedded in paraffin for hematoxylin and eosin (HE), Elastica van Gieson (EvG) and Masson Trichrome staining. Degree of acute rejection was evaluated according to the working formation of the International Society for Heart and Lung Transplantation [14]. Rejection score was defined as follows: 0 = no cell infiltration, 1 = faint and limited cell infiltration, 2 = moderate cell infiltration, 3 = severe and diffuse cell infiltration. The sections were photographed, blindly video digitized and stored in an image analysis system (NIH). The area encompassed by the lumen and internal elastic lamina (IEL) was traced and the cross sectional area of luminal stenosis (luminal occlusion) was calculated by the formula: luminal occlusion (%) = (IEL area − luminal area)/IEL area [15].

2.5. Immunohistochemistry

The other half of the divided hearts were stored in OCT compound and rapidly frozen in liquid nitrogen to be used for immunohistochemical study. Serial transverse sections (6 to 8 μm) were cut, dipped in cold acetone for 10 min, and dehydrated in phosphate-buffered solution. After dehydration, sections were incubated for 12 h at 4°C with primary antibodies against MMP-2 (C-19, Santa Cruz Biotechnology, CA, USA), MMP-3 (C-19, Santa Cruz Biotechnology), MMP-9 (C-20, Santa Cruz Biotechnology) and MMP-13 (Chemicon International, CA, USA) diluted 100×, and collagen I (Southern Biotechnology Associates, AL, USA) diluted 20×. Biotin–second antibody conjugate was detected with a Vector ABC kit (Vector, Burlingame, CA, USA).

2.6. Gene transfer complex

The sequences of ribozyme oligonucleotides (ONs) against MMP-2 mRNA are shown in Fig. 1. The target sequence of MMP-2 is identical among mouse and rat species [16,17]. In this study, we used DNA-based control ON as the negative control, because DNA ribozyme has no catalytic activity. Moreover, mismatched ribozyme from which catalytic activity was inactivated by replacing G* with A was used as an additional negative control.

2.7. Preparation of HVJ-liposome

HVJ-AVE liposomes were prepared as described previously [18]. Briefly, dried lipids containing phosphatidylethanolamine and cholesterol was hydrated in 200 μg of balanced salt solution (BSS) containing anti MMP-2 ribozyme, DNA-ribozyme and mismatched ribozyme ONs. Liposomes were prepared by shaking and sonication; in cationic liposome, DC-cholesterol was substituted for phosphatidylethanolamine and cholesterol. Purified HVJ (Z strain) was inactivated by UV irradiation (198 mJ/cm²) just before use. The liposome’s suspension (0.5 ml, containing 19 mg of lipid) was mixed with HVJ 15,000 hemagglutinating units in a total volume of 1 ml BSS. The mixture was incubated at 4°C for 10 min and at 37°C for 60 min with gentle shaking. Free HVJ was removed from HVJ–liposome by sucrose density gradient centrifugation. The liposomes were unilamellar, and the size of ON-containing HVJ–liposome complex was 400–500 nm in diameter [19].

2.8. Statistical analysis

Data were expressed as mean±S.D. Differences were compared using analysis of variance (ANOVA). A value of P<0.05 was considered statistically significant.

3. Results

3.1. Efficacy and specificity of anti MMP-2 ribozyme in cultured SMCs

RT-PCR confirmed that MMP-2, -3, -9 and -13 were expressed in cultured SMCs after stimulation by PMA. MMP-2 expression was suppressed in cultured SMCs with anti MMP-2 ribozyme after stimulation, while this suppression was not observed in control ribozyme or DNA ribozyme. Expression of the other MMPs was not sup-
Fig. 2. RT-PCR confirmed that MMP-2, -3, -9 and -13 were expressed in cultured SMCs after stimulation by PMA (B), but not in those without stimulation (A). MMP-2 expression was suppressed in cultured SMCs with anti MMP-2 ribozyme (C) after stimulation, while this suppression was not observed in control ribozyme (D) or DNA ribozyme (E). Expression of the other MMPs was not suppressed by anti MMP-2, control or DNA ribozyme. cDNA preparations were analyzed for GAPDH as a positive control.

pressed by anti MMP-2, control or DNA ribozyme (Fig. 2).

3.2. **MMP-2 is enhanced in the development of CAV**

Pathologically, neointimal formation was observed at days 15 and 30, and no significant difference was detected among luminal occlusion ratios of non-transferred, mismatch ribozyme transferred or DNA ribozyme transferred allografts. In the intramyocardium, signs of severe rejection (rejection score 2–3) were seen accompanied by diffuse interstitial edema, perivascular and interstitial mononuclear cell infiltration and focal interstitial hemorrhage; no significant difference was observed in the average of this score among non-treated cardiac allografts (Table 2). Masson Trichrom staining showed accumulation of collagenous fibers in the advanced stage in neointima and intracellular space of media, in addition to increased number of SMCs (Fig. 3). We performed immunostaining for collagen type I and ensured that collagen I was expressed in the severely thickened neointima of control allografts (Fig. 3).

Immunohistochemically, all MMPs were enhanced in slightly thickened neointima of the allografts harvested at day 15 (Fig. 4). At day 30, MMP-2 expression remained distinctly positive throughout the progression of the CAV, whereas expression of MMP-3, MMP-9 and MMP-13 was decreased in severely thickened neointima with increased cell number (Fig. 5A–D). In recipient hearts, neither MMP expression, cell infiltration or intimal thickening were observed.

3.3. **Anti MMP-2 ribozyme attenuates CAV development**

Treatment with MMP-2 ribozyme ON significantly suppressed intimal thickening of coronary arteries compared to non-treated allografts, mismatch ribozyme or DNA ribozyme treated allografts (Table 2). There was no MMP expression in arteries of anti MMP-2 treated allograft without intimal thickening, while unsuppressed MMP-2 expression was seen in neointima of allografts treated with control ONs (Fig. 5E, F). In the intramyocardium,

<table>
<thead>
<tr>
<th>Treatment of allografts</th>
<th>No. of grafts</th>
<th>No. of arteries</th>
<th>Luminal occlusion (%)</th>
<th>Myocardial rejection score (0–3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti MMP-2 ON</td>
<td>10</td>
<td>41</td>
<td>25.0±6.5*</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>Mismatch ON</td>
<td>8</td>
<td>46</td>
<td>5.3±6.0</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>DNA ON</td>
<td>8</td>
<td>37</td>
<td>48.9±6.9</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>No ON</td>
<td>6</td>
<td>41</td>
<td>55.1±7.0</td>
<td>2.7±0.3</td>
</tr>
</tbody>
</table>

*P<0.05 vs. other groups. Rejection score was as follows: 0=no cell infiltration, 1=faint and limited cell infiltration, 2=moderate cell infiltration, 3=severe and diffuse cell infiltration.
Fig. 4. Immunohistochemically, all MMPs were enhanced in slightly thickened neointima of the allografts harvested at day 15: (A) MMP-2, (C) MMP-3, (E) MMP-9, (G) MMP-13. In recipient hearts, neither MMPs expression nor cell infiltration or intimal thickening were observed: (B) MMP-2, (D) MMP-3, (F) MMP-9, (H) MMP-13. Original magnification ×400.

Fig. 5. (A) MMP-2 expression remained distinctly positive throughout the progression of the CAV at day 30, whereas expression of (B) MMP-3, (C) MMP-9 and (D) MMP-13 was decreased in severely thickened neointima with increased cell number. There was no MMP-2 expression in arteries of anti MMP-2 treated allografts without intimal thickening (E), while unsuppressed MMP-2 expression was seen in neointima of allografts treated with control ribozyme (F). Original magnification ×400.

Signs of severe rejection (rejection score 2–3) were observed and there was no significant difference in average of this score between treated and non-treated cardiac allografts.

4. Discussion

Proliferation of vascular SMCs is known to be involved in the pathogenesis of intimal thickening in the coronary arteriosclerosis seen in chronically rejected hearts. This has been confirmed by studies suppressing luminal occlusion of the arteries by blocking cell-cycle regulating genes such as E2F [20], PCNA [21] and cdk2 kinase [22]. The results of these studies suggest new therapeutic methods utilizing gene therapy for treatment of chronic rejection of transplanted hearts, for which there is neither conventional nor radical therapy at present.

It has been shown that collagen turnover plays an important role throughout the progression of neointimal formation after balloon injury, which pathologically resembles CAV. In addition to cell proliferation, we observed collagen accumulation in the severely thickened neointima, indicating that collagen turnover also contributes to the increase in the neointimal volume. In remodeling of the vascular wall during neointimal formation after balloon injury, vascular SMCs require extracellular proteases which degrade basement membrane and ECM in the interstitium, allowing cell migration and proliferation [1,2]. MMPs are primarily involved in tumor metastasis, as well as in many physiological processes that are associated with ECM turnover and remodeling [23,24]. Though other studies have shown that MMPs contribute to SMC migration, but not replication [8,25,26], emerging data on the role of MMPs in vascular SMC migration have suggested...
that these enzymes could also be important in regulating collagen turnover and net collagen accumulation [5,7]. Aoyagi et al. have reported a balloon injury study in which SMCs immunopositive for MMP-1, -2, -3 and -9 appeared throughout the slightly thickened intima at 1 week after intimal denudation. They added that, as the neointimal formation proceeded, the expression of MMP-1, -3 and -9 decreased, while that of MMP-2 remained stable [4]. We observed a similar phenomenon in the neointima of rejected allografts in this study. Jenkins et al. demonstrated that persisting MMP-2 expression might be involved in the control of collagen accumulation in developing neointima after balloon injury [27], suggesting that MMP-2 contributes to collagen accumulation in the chronically rejected cardiac allograft neointima as well.

The anti MMP-2 ribozyme treatment to suppress local expression of MMP-2 in allografts resulted in significantly, but not completely, decreased luminal occlusion. As demonstrated in balloon injury neointimal formation, MMPs may not prevent SMC replication in CAV, or MMPs not suppressed by anti MMP-2 could also contribute to the incomplete suppression of CAV. Holleran et al. demonstrated that topical administration of GM 6001 suppressed the type IV collagen turnover could play an important role during the [15] Eich DM, Nestler JE, Johnson DE, Dworkin GH et al. Inhibition of matrix metalloproteinases in rabbit carotid arteries after balloon denudation. Histochem Cell Biol 1998;109:97–102.


We would like to thank Rie Siohara and Akiko Motohara for excellent technical assistance. This study was supported by grants from Grant-in-Aid from the Ministry of Education, Science, and Culture, Research Grant for Immunology, Allergy and Organ Transplant, the Ministry of Health and Welfare, and the Japan Heart Foundation Dr. Hiroshi Irisawa Commemorative Research Grant.

Acknowledgements

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