Antimonomocyte Chemoattractant Protein-1 Gene Therapy 
Attenuates Graft Vasculopathy

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Objective—Accelerated coronary arteriosclerosis remains a major problem in the long-term survival of cardiac transplant recipients. However, the pathogenesis of graft vasculopathy is poorly understood, and there is no effective therapy. Transplant arteriosclerosis is characterized by early mononuclear cell attachment on the transplanted vessel followed by development of concentric neointimal hyperplasia. Early and persistent expression of monocyte chemoattractant protein-1 (MCP-1) in cardiac allografts has been implicated for the pathogenesis of transplant arteriosclerosis.

Methods and Results—We investigated whether anti–MCP-1 gene therapy can inhibit the development of intima hyperplasia in a mouse model of cardiac transplantation. Either the dominant-negative form of MCP-1 (7ND) or control vector was transfected into the skeletal muscles of B10.D2 mice. Cardiac allografts from DBA/2 mice were transplanted heterotopically into B10.D2 mice. 7ND gene transfer was associated with a significant reduction of the number of mononuclear cells accumulating in the lumen of the graft coronary arteries at 1 week and an attenuation of the development of the lesion at 8 weeks (intima/media ratio 0.79 ± 0.05 versus 0.48 ± 0.04).

Conclusions—The MCP-1/CCR2 signaling pathway plays a critical role in the pathogenesis of graft vasculopathy. This new anti–MCP-1 gene therapy might be useful to treat graft vascular disease. (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words: cardiac transplantation ■ graft arteriosclerosis ■ MCP-1 ■ CCR2 ■ chemokine

Although recent advances in immunosuppressive therapy have contributed to a dramatic enhancement of the early survival of cardiac transplant recipients, transplant arteriosclerosis, an exuberant form of vessel narrowing in graft coronary arteries, has emerged as a leading cause of graft failure.1 The pathogenesis of graft vasculopathy is poorly understood, and there is no effective therapy. Histological examination of graft arteriosclerosis reveals diffuse concentric lesions with fewer lipids and more inflammatory cell accumulation, which is distinct from the usual coronary atherosclerotic lesions.1,2 In animal models, infiltrating macrophages concentrate early in the interstitium and vessels, and persistent cytokine and chemokine expression are evident.3,4 The lesion expands with infiltrates followed by a gradual increase in the presence of smooth muscle cells,5 which have been suggested to originate, at least in part, from the bone marrow–derived recipient cells.6–9 The temporal sequence of these events suggests that early attachment and homing of the recipient mononuclear cells onto the graft coronary arteries is an initiating event in the development of neointimal hyperplasia.10 Thus, the key molecule that regulates mononuclear cell recruitment to the graft arteries represents an ideal target to prevent graft vascular diseases.

Monocyte chemoattractant protein-1 (MCP-1) is a potent chemotactic factor for monocytes, activated CD4 and CD8 memory T lymphocytes, basophils, and hematopoietic progenitors.11 MCP-1 expression has been identified within the atherosclerotic lesions12 as well as within the proliferative intimal lesions associated with balloon injury.13 Early and persistent expression of MCP-1 in cardiac allografts has been implicated for the chronic inflammatory response and the pathogenesis of transplant arteriosclerosis.3,14

Here, we examined the hypothesis that anti–MCP-1 gene therapy inhibits the development of transplant arteriosclerosis in a murine model of cardiac transplantation. We blocked the MCP-1 signal by transfecting a dominant-negative form of MCP-1 gene (7ND) into the skeletal muscles of the recipient mice. 7ND gene therapy was associated with a reduced accumulation of the mononuclear cells on the luminal side of the graft coronary arteries and an attenuation of lesion formation. This new anti–MCP-1 gene therapy might hold a potential to treat graft vasculopathy.

Methods

Heterotopic Cardiac Transplantation

DBA/2 (H-2d) and B10.D2 (H-2d) mice were purchased from Japan SLC, Inc. Adult male 7- to 9-week-old mice were used throughout the study. All procedures involving experimental animals were performed in accordance with protocols approved in the local
institutional guidelines for animal care of the University of Tokyo. Cardiac transplantation was performed according to the method of Corry et al.6,7,15

Gene Transfer Into Skeletal Muscles by In Vivo Electroporation
7ND-protamine complementary DNA 3 (pcDNA3) plasmid, which encodes 7ND, was constructed as described previously.16 Human 7ND cDNA with an epitope tag FLAG in the carboxyl-terminal was constructed by recombinant polymerase chain reaction using a wild-type MCP-1 cDNA as template and cloned into the BamH1 (5′) and NotI (3′) sites of the pCDNA3 expression (Invitrogen) vector plasmid. A total of 50 μg each of closed circular plasmid DNA (pcDNA3 or 7ND-pcDNA3) in 50 μL of PBS was injected into bilateral femoral muscles of the recipients using a 30-gauge needle 2 days before cardiac transplantation. A pair of electrode needles (Nihon) was inserted into the muscles to a depth of 5 mm to encompass the injection sites. We applied 3 square pulses of 100 V followed by 3 more pulses of the opposite polarity at each injection site using an electric pulse generator (SEN-7203; Nihon) as described previously.17

RNA Extraction and Ribonuclease Protection Assay
One week after cardiac transplantation, allografts were excised. Naïve hearts were excised from DBA/2 mice. Total RNA was prepared with the use of Isogen reagent (Nippon Gene) according to the protocol of the manufacturer.18 Ribonuclease (RNase) protection assay (RPA) was performed with 5 μg of total RNA using a RiboQuant kit with a custom template set according to the protocol of the manufacturer (PharMingen). After RNase digestion, protected probes were resolved on denaturing polyacrylamide gels and quantified using a BASS-3000 system (Fuji Film). The value of each hybridized probe was normalized to that of glyceraldehyde-3-phosphate dehydrogenase included in each template set as an internal control.

In Vivo Matrigel Plug Assay
Effectiveness of anti–MCP-1 gene therapy was evaluated by the matrigel plug assay as described.19 Matrigel plug (100 μL in volume) containing MCP-1 protein (100 ng/mL) was injected sub-
We hypothesized that in vivo blockade of MCP-1/CCR2 signaling may attenuate development of intima hyperplasia in a mouse model of cardiac transplantation. Either the 7ND or control vector (pcDNA3) was transfected into the skeletal muscles of B10.D2 mice. In vivo electroporation increased muscle and plasma 3, 7, and 14 days after transfection. 18 The 7ND-pcDNA3 or pcDNA3 plasmid as described above. Fourteen days after injection, the plugs were removed and snap-frozen in ornithine carbamoyltransferase (OCT) compound. Frozen sections were incubated with antibodies against CCR2 (goat polyclonal) or F4/80 (rat monoclonal), followed by staining with an fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG antibody (American Qualex) and a rhodamine-conjugated anti-rat IgG antibody (Santa Cruz Biotechnology). The sections were counterstained with Hoechst 33258 (Sigma), mounted using ProLong Antifade Kit (Molecular Probes), and examined under a confocal microscope (FLUOVIEW FV300; Olympus) as described previously. The mononuclear cells accumulating in the lumen of mid-sized coronary arteries (n=5 for each graft) were counted (n=3 for each group). For immunohistochemistry, distributions of first antibodies were visualized by the avidin–biotin complex followed by Vector Red (Vector Laboratories).

**Immunohistochemical Analysis**

Grafts were harvested at 1, 2, and 8 weeks and embedded in paraffin. Cross-sections were incubated with antibodies against CCR2 (goat polyclonal) or F4/80 (rat monoclonal), followed by staining with an fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG antibody (American Qualex) and a rhodamine-conjugated anti-rat IgG antibody (Santa Cruz Biotechnology). The sections were counterstained with Hoechst 33258 (Sigma), mounted using ProLong Antifade Kit (Molecular Probes), and examined under a confocal microscope (FLUOVIEW FV300; Olympus) as described previously. The mononuclear cells accumulating in the lumen of mid-sized coronary arteries (n=5 for each graft) were counted (n=3 for each group). For immunohistochemistry, distributions of first antibodies were visualized by the avidin–biotin complex followed by Vector Red (Vector Laboratories).

**Flowcytometry and Cytokine Measurement**

Peripheral blood was obtained from the retro-orbital venous plexus of the recipient mice. At 1 week after transplantation, leukocytes were stained with macrophage antigen-1 (Mac-1)–FITC antibody (Immunotech) and analyzed by flowcytometry (EPICS XL; Beckman Coulter). At 3 and 14 days, serum concentrations of tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) were measured by use of murine ELISA kit (Quantikine Immunoassay R&D Systems).

**Morphometric Analysis and Statistical Analysis**

At 8 weeks, cardiac allografts were harvested and embedded in paraffin. Serial sections (5 µm) were deparaffinized and stained with hematoxylin and eosin. Morphometric analysis was performed as described previously (n=10 arteries for each graft). All data were expressed as the mean ± SEM. Means were statistically compared by ANOVA followed by Student t test. A value of \( P<0.05 \) was considered significant.

**Results**

**Persistent MCP-1 Expression and Accumulation of CCR2-Positive Cells in the Developing Atherosclerotic Lesions**

DBA/2 and B10.D2 mice share major histocompatibility antigens but differ in minor antigens. DBA/2 cardiac allografts transplanted into B10.D2 recipient mice develop severe vasculopathy, which mimics those observed in human cardiac allografts. To obtain insights into the pathogenesis of graft vasculopathy, we investigated cardiac allografts from DBA/2 mice to B10.D2 mice harvested at 14 days. Consistent with previous reports, immunohistochemistry revealed that MCP-1 was highly expressed in the arterioles of cardiac allografts (Figure 1). Mononuclear cells bearing CCR2, a receptor of MCP-1, were found on the luminal side and adventitia of the small vessels. These findings suggest that the persistent expression of MCP-1 in association with increased CCR2-positive mononuclear cell localization may play a role in the pathogenesis of transplant arteriosclerosis.

**Efficacy of Anti–MCP-1 Gene Therapy**

We hypothesized that in vivo blockade of MCP-1/CCR2 signaling may attenuate development of intima hyperplasia in a mouse model of cardiac transplantation. Either the 7ND or control vector (pcDNA3) was transfected into the skeletal muscles of B10.D2 mice. In vivo electroporation increased transfection efficiency of naked DNA by \( \approx 1000 \)-fold (M. Sata, unpublished data). 7ND was detected in the transfected muscle and plasma 3, 7, and 14 days after transfection.
efficacy of gene transfer of 7ND-pcDNA3 in blocking MCP-1 signaling was investigated by implanting MCP-1 containing matrigel in the flank of B10.D2 mice on the day of in vivo electroporation. The number of infiltrating cells at 2 weeks significantly decreased (205±6 cells per high-power field [HPF] versus 145±12 cells per HPF) in the mice treated with 7ND-pcDNA3 (Figure 2). These results suggest that anti-MCP therapy can attenuate the responsiveness of circulating CCR2-bearing inflammatory cells toward MCP-1.

Effects of Anti–MCP-1 Gene Therapy on Mononuclear Cell Recruitment to Cardiac Allografts

Next, we examined the effects of anti–MCP-1 gene therapy on the development of graft vasculopathy. Either 7ND-pcDNA3 or empty pcDNA3 vector was transfected into the skeletal muscles of B10.D2 mice. After 2 days, cardiac allografts from DBA/2 mice were transplanted into the B10.D2 mice heterotopically. One week after transplantation, many mononuclear cells accumulated on the luminal side of the coronary arteries. An immunofluorescent study revealed that most of the cells expressed F4/80 antigen, a marker for macrophages, as well as CCR2, the receptor for MCP-1 (Figure 3A). The number of CCR2-positive cells accumulating in the coronary artery was significantly lower in the 7ND-treated recipient mice than that in the control recipient mice (16.3±2.0 versus 9.0±0.5 cells per lumen). Interestingly, the proportion of Mac-1-positive cells in the recipient peripheral blood was significantly increased in the 7ND gene transfer group (control 6.9±1.4%; 7ND 20.3±5.8%; Figure 3B).

Effects of Anti–MCP-1 Gene Therapy on Systemic Inflammation

We investigated whether an increase in the number of circulating mononuclear cells leads to an increase in systemic inflammatory reactions. We analyzed the expression of chemokine receptors and their proinflammatory chemokine ligands in the allografts at 1 week (Figure 4). Multiprobe RPA revealed that all cytokines, chemokines, and chemokine receptors were upregulated in the allografts (DBA/2 to B10.D2 mice) compared with the native hearts from DBA/2 mice. Particularly, upregulation of Rantes, MCP-1, and interferon-γ was noted. Anti–MCP-1 gene therapy to the recipient mice had no significant effect on mRNA expression for the cytokines, chemokines, and chemokine receptors in the whole cardiac allografts. Moreover, there was no significant change in serum levels of TNFα and IL-1β at 3 days and 14 days between the recipient mice treated with pcDNA3 and 7ND-pcDNA3 (Table). These results suggest that anti-MCP1 gene therapy has little effect on systemic inflammation of the recipient mice.

Inhibition of Neointimal Hyperplasia by Anti–MCP-1 Gene Therapy

At 8 weeks, grafts were harvested for histological examination. The coronary arteries of the allografts showed severe arteriosclerosis (Figure 5A). Immunohistochemical analysis revealed that the neointima was composed exclusively of α-smooth muscle actin-positive cells with occasional macrophage infiltration (data not shown). Morphometric analysis revealed that anti–MCP-1 gene therapy was associated with significant attenuation of the development of neointimal hyperplasia in the grafts (intima/media ratio 0.84±0.07 versus 0.54±0.09; Figure 5B).

Effects of 7ND-Gene Transfer on Serum Levels of Cytokines in Recipient Mice

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<th>Naive</th>
<th>pcDNA3</th>
<th>7ND</th>
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<tr>
<td>TNFα (pg/ml)</td>
<td>26.9±2.8</td>
<td>32.1±3.5</td>
<td>16.6±0.8</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>6.3±0.1</td>
<td>34.1±4.8</td>
<td>16.1±2.5</td>
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Peripheral blood was obtained at 3 days and 14 days from the retro-orbital venous plexus of the recipient mice treated with pcDNA3 (Control) or 7ND-pcDNA3 (7ND). Serum concentrations of TNFα and IL-1β were measured by the use of murine ELISA kit (Quantikine M, R&D Systems).
was persistently expressed in cardiac allografts in association with accumulation of CCR2-positive cells. It was reported that early and late chemokine production correlates with cellular recruitment in cardiac allograft vasculopathy.14 Hence, MCP-1 may play a role in the pathogenesis of transplant arteriosclerosis. Consistent with this notion, we found that in vivo blocking of MCP-1/CCR2 signaling resulted in the attenuation of mononuclear cell recruitment and development of arteriosclerosis.

In this study, the number of Mac-1–positive cells among peripheral mononuclear cells increased after 7ND gene transfer, whereas local accumulation of CCR2-positive cells was attenuated. It is likely that 7ND protein secreted from remote skeletal muscles successfully inhibited the function of circulating monocytes/macrophages to home to the coronary arteries because 7ND gene delivery was associated with the reduction of monocyte recruitment into the dermis induced by recombinant MCP-1.

In conclusion, our findings suggest that anti–MCP-1 therapy using intramuscular 7ND gene transfer is feasible and that the MCP-1/CCR2 signaling pathway plays a role, at least in part, in the pathogenesis of graft vasculopathy. Our strategy may serve as a prophylactic treatment of transplant-associated arteriosclerosis.

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References


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