Anti-Monocyte Chemoattractant Protein-1 Gene Therapy Limits Progression and Destabilization of Established Atherosclerosis in Apolipoprotein E–Knockout Mice

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Background—Monocyte infiltration into the arterial wall and its activation is the central event in atherogenesis. Thus, monocyte chemoattractant protein-1 (MCP-1) might be a novel therapeutic target against atherogenesis. We and others recently reported that blockade or abrogation of the MCP-1 pathway attenuates the initiation of atheroma formation in hypercholesterolemic mice. It remains unclear, however, whether blockade of MCP-1 can limit progression or destabilization of established lesions.

Methods and Results—We report here that blockade of MCP-1 by transfecting an N-terminal deletion mutant of the MCP-1 gene limited progression of preexisting atherosclerotic lesions in the aortic root in hypercholesterolemic mice. In addition, blockade of MCP-1 changed the lesion composition into a more stable phenotype, ie, containing fewer macrophages and lymphocytes, less lipid, and more smooth muscle cells and collagen. This strategy decreased expression of CD40 and the CD40 ligand in the atherosclerotic plaque and normalized the increased chemokine (RANTES and MCP-1) and cytokine (tumor necrosis factor α, interleukin-6, interleukin-1β, and transforming growth factor β1) gene expression. These data suggest that MCP-1 is a central mediator in the progression and destabilization of established atheroma.

Conclusions—The results of the present study suggest that the inflammatory responses mediated by MCP-1 are important in atherosclerosis and its complications. (Circulation. 2002;106:2700-2706.)

Key Words: gene therapy ■ atherosclerosis ■ leukocytes ■ inflammation ■ lymphocytes

Atherosclerosis and its complications are the major cause of death in Western countries. Atherosclerosis is now recognized to involve chronic inflammatory and immune responses. A considerable body of evidence supports the notion that various mediators such as adhesion molecules, cytokines, and chemokines are involved in the early initiation of atherosclerotic lesions. The precise molecular mechanism underlying later complications of atherosclerosis, however, remains unclear. Investigation of this mechanism is clinically very important, because atherosclerotic complications such as acute myocardial infarctions and stroke develop during the later stages of atherosclerosis. Recently, several groups established that the immune mediator CD40 ligand (CD40L) and its receptor (CD40) are crucial not only in the initiation of atheroma formation but also in the progression and destabilization of established atheroma. CD40 receptor binding induces production of inflammatory cytokines, chemokines, matrix metalloproteinases (MMPs), and tissue factors in atheroma, which weakens the collagen frame of the plaque and renders it prone to rupture and thrombosis. There are likely to be other potential mediators, however, that might contribute to the progression and destabilization of established atheroma.

Monocyte chemoattractant protein-1 (MCP-1), a C-C chemokine, controls chemotaxis of mononuclear cells. MCP-1 and its receptor (CCR2) pathway recently attracted much attention, because the MCP-1/CCR2 pathway seems to be involved in the inflammatory aspect of atherogenesis. Atheroma-forming cells (endothelial cells, smooth muscle cells [SMCs], and macrophages) express MCP-1 and CCR2, and activity in this pathway is increased in atherosclerotic lesions. Furthermore, activation of the MCP-1/CCR2 pathway induces adhesion molecules, proinflammatory cytokines, chemokines, and MMPs and thus accelerates atherosclerosis in hypercholesterolemic animals. More importantly, MCP-1 induces tissue factor expression in human arterial SMCs. These findings suggest that MCP-1 contributes not only to vascular inflammation but also to plaque destabilization and thrombosis, which result in acute coronary syndrome. We and others have previously demonstrated that...
blockade or abrogation of the MCP-1/CCR2 pathway inhibits the early development of atherosclerotic lesions as well as neointimal hyperplasia after intraluminal injury in mice. No prior study, however, has addressed the effect of blockade of MCP-1 on established lesions. Therefore, the present study was designed to test the hypothesis that blockade of MCP-1 limits progression and destabilization of established lesions in apolipoprotein E–knockout (ApoE-KO) mice. ApoE-KO mice develop hypercholesterolemia and atherosclerotic lesions similar to those found in humans and are widely used to study the pathogenesis of atherosclerosis. To block the MCP-1/CCR2 signal pathway, an N-terminal deletion mutant of the MCP-1 gene (7ND), which lacks the N-terminal amino acids 2 through 8, was transfected into the skeletal muscle. This mutant MCP-1 binds to the MCP-1 receptor (CCR2) and blocks MCP-1–mediated monocyte chemotaxis. In a previous study, we demonstrated that 7ND protein was secreted from the transfected skeletal muscle cells into the circulating blood and subsequently blocked MCP-1–induced chemotaxis in remote organs.

**Methods**

**Experimental Animals**

C57BL/6J ApoE KO mice, purchased from Jackson Laboratory (Bar Harbor, Maine), were bred and maintained in the Laboratory of Animal Experiments at Kyushu University. The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core, Kyushu University School of Medical Sciences.

**Expression Vector Mutant**

MCP-1 (7ND) was constructed by recombinant polymerase chain reaction using a wild-type human MCP-1 cDNA (a generous gift from Dr T. Yoshimura, National Cancer Institute, Bethesda, Md) as the template and cloned into BamHI (5′) and NotI (3′) sites of the pcDNA3 expression vector (Invitrogen Corp). 

**Treatment**

ApoE-KO mice were fed a chow diet (Oriental Yeast) during the experiment. At 20 weeks of age, the baseline group of mice (n=10) was killed to determine the extent of baseline established lesions. Other mice were randomly assigned into two groups. The 7ND-transfected group (n=10) received intramuscular injections of pcDNA3-7ND plasmid DNA (100 μg) into the femoral muscle at biweekly intervals for up to 8 weeks. Transgene expression was quantified by intramuscular electroporation at the injection site immediately after injection. Six electronic pulses of 100 V, 50 ms each were applied to each injection site using an Electroporator CUY21 (BTX Corp). The second group was the plasmid group (n=10), which was transfected with an empty plasmid using the same method on the same time schedule. At the end of study, mice were killed after collection of blood from the vena cava.

**Tissue Preparation**

Tissue preparation was performed essentially as previously described. Briefly, after the mice were killed, the heart and aorta were rapidly removed after perfusion with PBS. The thoracic and abdominal aorta were rapidly frozen in liquid nitrogen for later extraction of RNA. The heart, including the aortic root, was snap-frozen in OCT compound (Tissue-Tek) for histology and immunohistochemistry. The remaining aortic arch was fixed in 10% buffered formalin for measurement of the surface area covered by lipid-staining lesions.

**Histology and Immunohistochemistry**

Serial cryostat sections (6 μm) of the aortic root were prepared as described. In brief, atherosclerotic lesions in the aortic root were examined at 5 locations, each separated by 120 μm. Ten serial sections were prepared from each location. Some of these sections were conventionally stained with orcein (for elastic fiber staining) and oil red O (for lipid staining). Interstitial collagens were stained by Picrosirius red (Sigma Chemical Co) and photographed using polarization microscopy as described previously. The remaining sections were used for immunohistochemical analysis. Air-dried cryostat sections were fixed in acetone and stained with the respective antibody: anti-mouse monocyte/macrophage monoclonal antibodies (MOMA-2, Serotec), anti-α-smooth muscle (SM) actin monoclonal antibodies (alkaline phosphatase conjugated, Sigma Chemical Co), anti-human MMP-3, MMP-13 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-mouse MMP-9 polyclonal antibodies (Santa Cruz Biotechnology), and anti-rabbit tissue factor monoclonal antibody (American Diagnostica Inc), as described previously. As negative controls, respective nonimmune IgGs (Dako) were used. After incubation with the appropriate biotinylated, affinity-purified secondary antibodies (Nichirei, Tokyo), the sections were incubated with alkaline phosphatase-labeled streptavidin solution (Nichirei) and visualized using a fast red substrate kit (Nichirei). The sections were then counterstained with Mayer’s hematoxylin.

Quantitative analysis of atherosclerotic lesions was performed by a single observer blinded to the experiment protocol. All images were captured by a Nikon microscope equipped with a video camera and analyzed using Adobe Photoshop 6.0 and National Institute of Health Image 1.62 Software. Two different methods were used to quantify the extent of the atherosclerotic lesions. First, adventitial tissue was removed from the aortic arch and the aortic arch was opened longitudinally, stained with oil red O, and pinned out on a black wax surface. The percentage of the endothelial surface area stained by oil red O was determined. The aortic arch region was defined as from the ostium to ~1 mm caudal from the left subclavian artery branch point. Alternatively, orcein staining, which stains the elastic fiber, was used to delineate the internal elastic lamina for determination of the intimal area. The lipid composition of the lesion was determined by calculating the percent of the oil red O–positive area to the total cross-sectional vessel wall area. Similarly, the percent area of macrophage accumulation (MOMA-2–positive area), α-SM actin–positive area, and collagen deposition were estimated. In each case, the average value for 5 locations for each animal was used for analysis.

**RNA Extraction and RNase Protection Assay**

Total RNA was extracted from the abdominal aorta using the acid guanidinium thiocyanate-phenol-chloroform method (Isogen, Nippon Gene), as described previously. RNA protection assays were performed with 5 μg of total RNA using a RiboQuant kit with a custom template set according to the manufacturer’s protocol (PharMingen). After RNase digestion, protected probes were resolved on denaturing polyacrylamide gels and quantified using a BASS-3000 system (FujiFilm, Fuji Photo Film Co). The value of each hybridized probe was normalized to that of glyceraldehyde-3-phosphate dehydrogenase included in each template set as an internal control.

**Measurement of Lipids, MCP-1, and 7ND**

Plasma total and high-density lipoprotein cholesterol and triglyceride concentrations were determined using commercially available kits (Wako Pure Chemicals).

Plasma and tissue concentrations of 7ND released by the transfected skeletal muscle were measured by the use of human MCP-1 ELISA kit (Biosource). Because this human MCP-1 ELISA kit does not react with the rat MCP-1, it is reasonable to consider that plasma 7ND concentrations can be measured by the use of this human ELISA kit.
Statistical Analysis
Data were expressed as mean±SEM. Differences between groups were determined using two-way ANOVA and a multiple comparison test. A probability value of 0.05 was considered to be statistically significant.

Results
7ND Gene Transfer Attenuates Progression of Established Atherosclerotic Lesions
At 20 weeks of age, the baseline group of ApoE-KO mice was killed to determine the extent of established lesions. Approximately 22±8% of the endothelial surface in the aortic arch was covered by lipid-staining atherosclerotic lesions (Figures 1A and 2A). From 20 weeks of age, the plasmid group and the 7ND group were transfected with empty plasmid and 7ND plasmid, respectively, at biweekly intervals. When the mice were killed at 28 weeks of age, the lipid-staining lesions in the plasmid group had progressed to more than twice that observed in the baseline group (Figures 1A and 2A). Transfection with 7ND significantly reduced lesion progression to a level similar to that observed in the baseline group (Figures 1A and 2A).

In addition, serial cross-sections of the aortic sinus region were examined using standard methods. The baseline group had established lesions characteristic of early fibrous plaques containing necrotic cores and a few foam cells covered by a well-formed fibrous cap (Figure 1B), as reported by other investigators. When mice were transfected with empty plasmid, there was a marked increase in the intimal area, compared with that observed in the baseline group (Figure 2B). 7ND gene transfection prevented additional increase in the intimal area, such that the size of the intimal area was similar to that observed in the baseline group (Figure 2B). There was no significant difference in the intimal area between the baseline group and the 7ND transfection group. The above data indicate that 7ND transfection did not regress established lesions but did prevent additional progression of the lesions.

7ND Gene Transfer Stabilizes Established Atheromatous Plaques
MCP-1 induces vascular SMC migration and dedifferentiation and mediates collagen synthesis. Therefore, we examined whether 7ND gene transfer affected the plaque composition using immunohistologic and immunohistochemical analysis. In particular, the lipid deposition, macrophages, SMCs, and interstitial collagen content were evaluated (Figure 1B). At the end of the study, empty plasmid-transfected mice had significantly elevated lipid deposition in plaques compared with that observed in the baseline group. In contrast, 7ND-transfected mice had only a moderate increase in lipid deposition, which was not significantly different from that in the baseline group (Figures 1B and 2C). Furthermore, the empty plasmid-transfected group had a dramatically increased macrophage area in the plaque compared with that of the baseline group (Figures 1B and 2D). The increased macrophage area was significantly inhibited in 7ND-transfected mice (Figures 1B and 2D).

Lesional SMCs and interstitial collagen contents are also important variables controlling plaque vulnerability and were analyzed using immunohistochrometry. The SMC area was significantly decreased in empty plasmid-transfected mice compared with that of the baseline group. Transfection with 7ND markedly preserved the plaque SMC content to a level similar to that in the baseline group (Figure 2E). Similarly, the interstitial collagen area was markedly decreased in the empty plasmid-transfected group after 8 weeks of study. Transfection with the 7ND gene also effectively preserved collagen deposition in the plaque (Figures 1B and 2F).

Lesional macrophages produce proteolytic enzymes that have an important role in weakening the fibrous cap and promoting plaque rupture. MMP is an important family of such proteolytic enzymes. The effect of 7ND gene transfer on the MMP expression was examined using immunohistochemistry. There was a marked increase of MMP-9 and MMP-13 immunoreactivity in the aorta of empty plasmid-transfected mice compared with that in the baseline group (Figure 3A). In contrast, in 7ND gene–transfected mice, MMP-9 and MMP-13 immunoreactivity did not obviously increase. Lesional macrophages also express tissue factor (TF), which is a strong activator of blood coagulation. Increased expression of such prothrombotic molecules is believed to contribute to thrombogenicity and subsequent acute coronary syndromes. The effect of 7ND gene transfer on the lesional expression of TF was examined. Compared with the baseline group, the empty plasmid-transfected group had a marked increase in TF protein levels, as assessed using immunohistochemistry (Figure 3A). Although TF expression also increased in the 7ND-transfected group compared with baseline, the increase was moderate and limited (Figure 3A).

7ND Gene Transfection Attenuates T-Lymphocyte Infiltration and Decreases CD40 and CD40L Expression
Empty plasmid-transfected mice exhibited a marked increase in CD4+ T-lymphocytes in the plaques compared with mice in the baseline group (Figure 3B). In contrast, in 7ND-transfected mice, CD4+ T-lymphocyte infiltration did not obviously increase compared with mice in the baseline group (Figure 3B). CD8+ T cells were also observed in the lesions but were sparse (data not shown). CD40 immunoreactivity in plaques was markedly increased in empty plasmid-transfected mice but was not markedly increased in 7ND-transfected mice compared with the baseline group (Figure 3B). The immunoreactivity of CD40L was similar to that of CD40 (Figure 3B). CD40 and CD40L immunoreactivity was observed in endothelial cells, inflammatory cells (mainly macrophages and T cells), and SMCs. These findings are consistent with previous reports.

7ND Gene Transfer Attenuates Upregulation of Chemokine and Cytokine Gene Expression
To explore the mechanisms underlying the limitation of atheroma progression and destabilization by 7ND gene transfer, we examined gene expression of a battery of chemokines and cytokines. RNase protection assay results revealed that 7ND transfection completely normalized the increased che-
Figure 1. A, Transfection with the 7ND gene inhibits progression of established atherosclerotic lesions in the aortic arch of ApoE-KO mice. Top, photomicrographs of the gross appearance of the aortic arch without staining. Bottom, photomicrographs of the intraluminal surface of the aortic arch stained with oil red O. B, 7ND transfection changes the composition of the atheroma such that it is more stable. From the top to bottom panel, photomicrographs of atherosclerotic lesions stained with orcein, oil red O, or immunostained with anti-murine macrophage antibody (MOMA-2) or anti-human α-SM actin antibody. Interstitial collagen was visualized using polarization microscopy after staining with Picro-sirius red. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=200 μm. C, Immunostaining of atherosclerotic lesions indicates increased immunoreactivity of MCP-1 and CCR2 in the empty plasmid-transferred group. Such increases in MCP-1 and CCR2 are not noted in the 7ND-transferred group. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=200 μm.
mokine and cytokine gene expression in the abdominal aorta compared with those of the empty plasmid–treated group (Figure 4).

Plasma Concentrations of MCP-1, 7ND, and Lipids

Plasma MCP-1 concentrations did not change during the course of experiments, whereas 7ND was detected in plasma 3, 7, and 14 days after transfection (Table 1). Tissue concentrations of 7ND were measured in various tissues 7 days after 7ND transfection in a separate set of animals (Table 2). 7ND could be detected only in transfected muscle, whereas 7ND levels were below detectable limits in other tissues such as the aorta, heart, liver, spleen, kidney, and thymus, suggesting that detectable amount of 7ND may not reach to the aortic and other tissues after secretion from the transfected muscle.

There were no statistically significant differences in serum total cholesterol and triacylglycerol levels among the 3 groups. Total cholesterol and triacylglycerol levels were 580±21 and 103±11 mg/dL in the baseline group, 588±19 and 118±12 mg/dL in the empty plasmid group, and 602±12 and 113±10 mg/dL in the 7ND group, respectively.

Discussion

We demonstrated for the first time that blockade of the MCP-1/CCR2 pathway by 7ND gene transfer markedly limited progression and destabilization of established atherosclerotic lesions. Clinically, lesion composition rather than size or degree of the stenosis of the lesion is believed to determine the likelihood of plaque rupture and subsequent

Figure 2. Quantitative comparison of atherosclerotic lesion size and lesion composition in baseline, empty plasmid, and 7ND-transfected ApoE-KO mice. Data are reported as mean±SEM, n=9 to 10.

Figure 3. A, Immunostaining of atherosclerotic lesions indicates increased immunoreactivity of MMP-9, MMP-13, and tissue factor in the empty plasmid-transferred group but not in the 7ND-transferred group. B, Immunostaining of atherosclerotic lesions exhibiting an increased infiltration of CD4-positive cells and increased immunoreactivity of CD40 and CD40L in the empty plasmid-transferred group but not in the 7ND-transferred group. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=200 μm.

Figure 4. Effect of 7ND transfection on chemokine (RANTES and MCP-1) and cytokine (tumor necrosis factor-α, interleukin-6, interleukin-1β, and transforming growth factor-β) gene expression in the abdominal aorta. Data are expressed as the ratio of each mRNA to the corresponding GAPDH mRNA. *P<0.05 vs the baseline and 7ND-transfected group. N=5 to 6.
thrombotic complications such as acute coronary syndrome. Therefore, anti-MCP-1 gene therapy might not only reduce plaque size but also reversed markers of plaque instability. Degradation of collagen by MMPs has been demonstrated in human atherosclerotic lesions prone to rupture. Lesional monocytes are believed to be a major source of MMP expression. We have demonstrated herein the preservation of interstitial collagen associated with decreased lesional expression of MMP-9 and MMP-13 by 7ND gene transfer. This finding warrants clinical attention, because interstitial collagen in the shoulder region is considered to be a critical determinant of fibrous cap integrity. Furthermore, suppression of MCP-1 and the other chemokine and cytokine expression by 7ND gene transfer suggests that MCP-1-mediated inflammation creates a positive feedback loop (a vicious cycle) to enhance vascular inflammation and atherogenesis possibly through activating lesional monocytes. A positive feedback effect of MCP-1 on lesion formation is supported by prior studies that demonstrated the acceleration of atherogenesis in hypercholesterolemic animals by overexpression of MCP-1. Therefore, the beneficial effects of 7ND gene transfer on established atherosclerotic lesions might be attributed mainly to suppression of monocyte recruitment and activation.

Based on recent evidence regarding the role of the CD40/CD40L system in the inflammatory aspect of atherogenesis, we hypothesized that the MCP-1/CCR2 pathway affects expression of the CD40/CD40L system. We found here that blockade of MCP-1 decreased immunohistochemically-detectable CD40 and CD40L expression within the atheroma. Although CCR2 is expressed on activated CD4-positive T lymphocytes, it is presumed that MCP-1 may not function as a significant chemoattractant for T cells in vivo. It is reasonable to assume that 7ND gene transfer decreased CD40 and CD40L expression secondarily by inhibiting a T cell chemoattractant such as RANTES. It is therefore plausible that the beneficial effects of 7ND gene transfer on established atheroma are attributable to the secondary decrease in expression and activity of the CD40/CD40L system in atheroma. The present study is the first to demonstrate a link between the MCP-1/CCR2 and CD40/CD40L systems in atherogenesis.

In conclusion, this study suggests that anti-MCP-1 gene therapy not only limits progression of established preexisting atheroma but also limits transformation from destabilized plaque to stable plaques. Thus, MCP-1 might be a promising therapeutic target against atherosclerosis. Blockade of the MCP-1/CCR2 pathway might lead to reductions in atherosclerotic complications.

**Acknowledgments**

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**References**


### TABLE 1. Plasma Concentrations of MCP-1 and 7ND After 7ND Transfection

<table>
<thead>
<tr>
<th>Days After 7ND Transfection</th>
<th>Baseline</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1, pg/mL (mouse MCP-1)</td>
<td>76±5</td>
<td>85±7</td>
<td>88±6</td>
<td>77±5</td>
<td>80±6</td>
</tr>
<tr>
<td>7ND, pg/mL (human MCP-1)</td>
<td>&lt;20.0 (below detectable limits)</td>
<td>226±21</td>
<td>220±20</td>
<td>140±12</td>
<td>&lt;20.0 (below detectable limits)</td>
</tr>
</tbody>
</table>

Values are mean±SEM, n=6 to 8.

### TABLE 2. Tissue Concentrations of 7ND 7 Days After 7ND Transfection

<table>
<thead>
<tr>
<th>Tissues</th>
<th>7ND, pg/mg Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle 7ND transfection (+)</td>
<td>260±32</td>
</tr>
<tr>
<td>Skeletal muscle 7ND transfection (−)</td>
<td>&lt;20.0</td>
</tr>
<tr>
<td>Heart</td>
<td>&lt;20.0</td>
</tr>
<tr>
<td>Liver</td>
<td>&lt;20.0</td>
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<tr>
<td>Aorta</td>
<td>&lt;20.0</td>
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<tr>
<td>Kidney</td>
<td>&lt;20.0</td>
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<tr>
<td>Thymus</td>
<td>&lt;20.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>&lt;20.0</td>
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Values are mean±SEM, n=5.


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