Essential Role of Monocyte Chemoattractant Protein-1 in Development of Restenotic Changes (Neointimal Hyperplasia and Constrictive Remodeling) After Balloon Angioplasty in Hypercholesterolemic Rabbits

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Background—Renarrowing of dilated arterial sites (restenosis) hampers the clinical benefits of coronary angioplasty. Infiltration and activation of monocytes in the arterial wall mediated by monocyte chemoattractant protein-1 (MCP-1) might be a major cause of restenosis after angioplasty. However, there is no direct evidence to support a definite role of MCP-1 in the development of restenosis.

Methods and Results—We recently devised a new strategy for anti–MCP-1 gene therapy by transfecting an N-terminal deletion mutant of the MCP-1 gene into skeletal muscles. We used this strategy to investigate the role of MCP-1 in the development of restenotic changes after balloon injury in the carotid artery in hypercholesterolemic rabbits. Intramuscular transfection of the mutant MCP-1 gene suppressed monocyte infiltration/activation in the injured arterial wall and thus attenuated the development of neointimal hyperplasia and negative remodeling.

Conclusions—MCP-1–mediated monocyte infiltration is necessary in the development of restenotic changes to balloon injury in hypercholesterolemic rabbits. This strategy may be a useful and practical form of gene therapy against human restenosis. (Circulation. 2002;105:2905-2910.)

Key Words: cells ■ proteins ■ gene therapy ■ restenosis ■ remodeling

Percutaneous interventions are now recognized as the treatment of choice for dilation of flow-limiting coronary and peripheral atherosclerotic stenosis.1,2 However, the overall benefits of this approach are hampered by the triggering of local arterial renarrowing (restenosis), which occurs in 20% to 50% of cases within 3 to 6 months and represents a major clinical and economic problem.1,2 Although several drugs have been shown to reduce restenosis rates in humans,3,4 their benefits are not definitive, and thus such drugs may not be used widely in patients receiving percutaneous interventions. Therefore, prevention of restenosis is a major challenge that highlights the need for new therapeutic options such as gene therapy.

Recently, inflammatory changes in the arterial wall have been recognized as playing a central role in the development of restenosis5 and atherosclerosis.6 Infiltration and activation of monocytes in the arterial wall are important early steps of vascular injury.6 Monocyte chemoattractant protein-1 (MCP-1) is the C-C chemokine and controls chemotaxis of monocytes/macrophages.7,8 Prior studies have indicated that MCP-1 expression is increased in atherosclerotic lesions in human and rabbit arteries9,10 and in balloon-injured arteries.11,12 Elimination of the MCP-1 gene decreases atheroma formation in hypercholesterolemic mice.13 We have shown that MCP-1 plays an important role in coronary arteriosclerosis in a rat model of nitric oxide synthesis inhibition.14–16 However, there is no direct, substantial evidence to support a role of MCP-1 in the restenotic reactions that occur after angioplasty.

We have devised a new strategy for anti–MCP-1 gene therapy by transfecting a mutant MCP-1 gene into skeletal muscle.17,18 This mutant MCP-1 lacks the N-terminal amino acid 2 to 8, called 7ND. 7ND usually forms a heterodimer with natural (wild type) MCP-1. This heterodimer has been shown to bind to the receptor for MCP-1 (CCR2) and to work as a dominant-negative inhibitor of MCP-1.7,8 We have shown that cells infected with 7ND secrete 7ND protein into circulating blood, and the 7ND protein binds to the MCP-1 receptor on monocytes or target cells in remote organs, thus blocking the signal of MCP-1. Such blockade of MCP-1 activity would suppress MCP-1–mediated inflammation and thereby improve the function of the target organs. Clinically,
negative arterial remodeling and neointimal formation are regarded as the major causes of human restenosis after balloon injury.\textsuperscript{19–21} In the present study, using our anti-MCP-1 gene therapy, we tested the hypothesis that blockade of MCP-1–induced monocyte chemotaxis can attenuate restenotic changes. We used a carotid artery balloon-injury model of hypercholesterolemic rabbits, which represents both neointimal hyperplasia and negative remodeling after balloon injury.

Methods

Plasmid Expression Vectors

Human 7ND cDNA was constructed by recombinant polymerase chain reaction (PCR) with a wild-type MCP-1 cDNA as template and inserted into the BamHI (5') and NotI (3') sites of the pcDNA3 (Invitrogen) expression vector plasmid. In this plasmid, gene expression is controlled by the cytomegalovirus immediate early enhancer/promoter.

Animal Model of Balloon Injury and Gene Transfer

The present experiments were reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and were performed according to the guidelines of American Physiological Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Male Japanese White rabbits (KBT Oriental, Tokyo, Japan) weighing 2.5 to 3.0 kg were fed a high-cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks. They were then anesthetized by intramuscular injection of xylazine (5 mg/kg)\textsuperscript{22} and ketamine (35 mg/kg).\textsuperscript{22} Their right common carotid arteries were anesthetized by intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg).\textsuperscript{22} Their right common carotid arteries were injured by 3 passages of an inflated 2F Fogarty catheter. We took care to keep the diameter of the balloon and the resistance during injury.

Electroporation In Vivo

Electroporation was performed as described previously.\textsuperscript{23} Immediately after either PBS, empty plasmid, or 7ND gene (500 µg/0.3 mL PBS) was injected into the femoral artery muscle with a 27-gauge needle, a pair of electrode needles (Tokiwa Science) spaced 5 mm apart were inserted into the muscle on either side of the injected sites, and three 25-V square wave pulses (spaced 1 second apart) were applied, followed by 3 pulses of opposite polarity for 10 ms with an electric pulse generator CUY201 (BTX), and the wound was closed. No inflammation was observed at the injection sites.

We selected this condition (500 µg plus electroporation) of plasmid injection because we preliminarily examined the transfection efficacy of several doses (100, 300, and 500 µg) of plasmid injection by the luciferase assay and found that the present condition showed the most effective and long-term transfection activity. Compared with PBS-injected muscle (260±32 relative light units/mg protein), luciferase activity of the muscles injected with luciferase gene plasmid was significantly (P<0.01) higher on days 2 (8.2±0.8×10^4), 7 (5.6±0.7×10^4), 14 (1.5±0.2×10^5), and 28 (1.0±0.2×10^5) relative light units/mg protein.

Histopathology and Immunohistochemistry

At various points in time, animals were killed with a lethal dose of anesthesia. The right and left common carotid arteries were perfused at 100 mm Hg and were excised for morphometric, immunohistochemical, and biochemical analysis. The common carotid arteries were fixed for 24 hours with 10% paraformaldehyde. Five arterial segments of ∼25 mm in length were obtained. The tissue was dehydrated, embedded in paraffin, and cut into 5-µm-thick slices.

Sections were either stained with elastin-van Gieson and Azan-Mallory stain or subjected to immunostaining with RAM11 and proliferating cell nuclear antigen (PCNA). For immunohistochemistry, slides were preincubated with 3% skim milk to decrease nonspecific binding. Sections were incubated overnight at 4°C with the mouse anti-rabbit monocytic/macrophage antibody RAM11 (DAKO Co), the PCNA antibody (DAKO Co), or nonimmune IgG (Zymed).

Morphometric Measurement

Morphometric analysis was performed with a microscope that had a computerized digital image-analysis system (Mac Scope) by a single observer who was blinded to the treatment protocol. Each sample of tissue (5 sections per artery) stained with RAM11 and PCNA was scanned. The total immunopositive cells and total cells in each section were counted. Then, the percentage of immunopositive cells per total number of cells in each section was calculated, and the average of the 5 sections was reported for each animal.

To evaluate thickening of the neointima, the areas encroached by the external elastic lamina (EEL area), the internal elastic lamina (IEL area), and the lumen area were measured. Other areas were calculated as follows: medial area=EEL area–IEL area; neointimal area=IEL area–lumen area; neointima-to-media ratio (I/M)=neointimal area/medial area. The circumferential length of the EEL and IEL was also measured to determine negative remodeling.

Carotid Ultrasonography

High-resolution B-mode carotid ultrasonography was performed by use of a linear-array 10-MHz transducer (SSD-5500). After anesthesia, rabbits were allowed to lie in the supine position in a dark room. The right and left common carotid arteries were examined with the head tilted slightly upward in the midline position. A central region of the common carotid artery was identified, and lumen diameter and wall thickness were evaluated. These parameters were measured on the frozen frame of a suitable cross-sectional image with the image magnified to achieve a higher resolution of detail. The I/M interface could not be detected, and thus, intimal thickness was not reported. All measurements were performed by a single sonographer (C.K.) who was unaware of the experimental protocol.

Plasma Measurements

Blood samples were collected before (2 weeks on the high-cholesterol diet) and 4 weeks after balloon injury. Plasma total cholesterol and LDL cholesterol were measured with a commercially available kit.

To measure 7ND released by the transfected skeletal muscle, plasma concentrations of 7ND were measured with a human MCP-1 ELISA kit (Biosource). Because this human MCP-1 ELISA kit does not react with rat MCP-1, it is reasonable to consider that plasma 7ND concentrations can be measured by this human ELISA kit.

Reverse Transcription–PCR

Total RNA was prepared from carotid arteries with an RNA purification kit (QUIAGEN) and reverse transcribed with murine leukemia virus reverse transcriptase. Quantity and quality of mRNA from all samples were certified by reverse transcription–PCR amplification of the GAPDH gene. Amplification of MCP-1 was done with the Perkin-Elmer Gene Amp PCR system 9700. The sense and antisense primers for each gene were as follows: MCP-1, forward: 5’-GCAGCCATGGCTGCCGCTG-3’, reverse: 5’-CAATGAAATGTAGTAGAGGAGTG-3’. Thermal conditions of the system were as follows: 1 cycle at 94°C for 2 minutes; 36 cycles (GAPDH and MCP-1) or 26 cycles (collagen type I) at 94°C for 30
seconds, 60°C for 30 seconds, 72°C for 90 seconds, and 1 cycle at 72°C for 5 minutes.

Statistical Analysis
Data are expressed as mean±SEM. Statistical analysis of differences was compared by ANOVA and Bonferroni multiple comparison tests. A level of P<0.05 was considered statistically significant.

Results
We examined the time course of MCP-1 mRNA expression and macrophage infiltration after balloon injury. The MCP-1 mRNA level was undetectable in noninjured control artery where no fatty lesion formation began. We found that after balloon injury, the MCP-1 mRNA level significantly increased as early as day 1 but declined with time; at all points in time, however, gene expression of MCP-1 in the injured artery was significantly higher on days 3, 7, and 28 than that in the noninjured control artery (Figure 1, A and B). Appearance of RAM11-positive macrophages was not distinct on day 1 but became evident on days 3, 7, and 28 (Figure 1C). RAM11-positive cells were noted on all 3 layers of the arterial wall (Figure 2). No such macrophage infiltration was observed in sham-operated carotid artery (data not shown).

We then examined the effects of 7ND gene transfection and found that 7ND gene transfer markedly suppressed macrophage infiltration into the intima, media, and adventitia after balloon injury (Figure 2). Such suppression was not noted in the empty plasmid group (Figure 2). We further examined the appearance of PCNA-positive cells and found that PCNA-positive cells were noted in smooth muscle cells and macrophages in the media and in cells in the adventitia mainly on days 3 and 7 after balloon injury. On day 28, PCNA-positive cells were noted exclusively in the neointima. At all time points, 7ND gene transfection decreased the number of PCNA-positive cells. The number of positive cells per section in the PBS, empty plasmid, and 7ND groups were, respectively, 523±36, 579±131, and 107±39 on day 3 (P<0.01); 772±34, 694±18, and 451±31 on day 7 (P<0.01); and 236±30, 284±39, and 95±17 on day 28 (P<0.01).

The carotid artery in the PBS and empty plasmid groups developed a significant neointimal formation (Figure 3A) associated with fibrosis (stained blue in the Azan-Mallory–stained sections in Figure 3B) in the media and adventitia. Arteries from the 7ND group showed less neointimal formation, less fibrosis, and a larger lumen area.

We found that intimal area and I/M ratio were less in the 7ND group than in the PBS or empty plasmid groups (Figure 4) and that negative remodeling (smaller lumen size, IEL, and EEL) was also less in the 7ND group (Figure 5). As a result of the inhibition of neointimal formation and negative remodeling, luminal narrowing was prevented. Carotid ultrasonography showed a decrease in lumen diameter in the PBS group before and 1, 3, 7, and 28 days after 7ND transfection are shown. B, MCP-1/GAPDH ratios in PBS (solid bars) and 7ND (open bars) groups are shown at indicated time (n=5 each). *P<0.01 vs without injury. C, Carotid artery sections from PBS group and 7ND group were stained immunohistochemically with antibody against monocyte/macrophage (RAM11) or non-immune IgG (negative control). Bar=20 μm.

We examined mRNA expression of type I collagen 3 days after balloon injury. Type I collagen mRNA expression was increased in the PBS group (2.1±0.4) 1 day after balloon injury compared with noninjured artery (1.0±0.1) and was significantly decreased in the 7ND group (1.1±0.2).

There was no significant difference in time course of plasma levels of LDL cholesterol among the 3 groups. LDL cholesterol levels before and 28 days after injury were 195±26 and 548±64 mg/dL in the PBS group, 198±19 and 631±46 mg/dL in the empty plasmid group, and 193±9 and 600±50 mg/dL in the 7ND group.

7ND was measured before and 3, 7, 14, and 28 days after 7ND transfection. 7ND could not be measured in plasma before and 28 days after transfection but was detected 3 (20±6 pg/mL), 7 (56±12 pg/mL), and 14 (44±8 pg/mL) days after transfection, which indicates that 7ND was actually released from the transfected skeletal muscles.

Discussion
We have demonstrated in the present study novel observations that blockade of MCP-1 by 7ND gene transfer attenuated neointimal formation and negative remodeling after
balloon injury, which supports the essential role of inflammatory changes mediated by MCP-1 in the development of restenotic changes after balloon injury. Prior studies have shown that balloon injury of the artery induces rapid and transient increases in MCP-1 activity in intact rat and rabbit models. In contrast, we have demonstrated rapid and prolonged increase in MCP-1 activity after balloon injury. This difference in time course of MCP-1 gene expression may be because hypercholesterolemic animals were used in the present study. Interestingly, a rapid and prolonged production of MCP-1 is reported in patients with restenosis after coronary angioplasty; Cipollone et al demonstrated that patients with restenosis displayed a rapid and prolonged increase in plasma MCP-1, whereas nonrestenotic patients showed only transient increases in plasma MCP-1. Plasma MCP-1 levels 15 days after angioplasty proved to be statistically significant and an independent predictor of restenosis. Thus, human arteries with underlying hypercholesterolemia or atherosclerosis are likely to represent prolonged production of MCP-1 after arterial injury.

The present study is the first to demonstrate the distinct role of MCP-1 in the development of neointimal formation after balloon injury. Previously, repeated injection of polyclonal antibody against rat MCP-1 modestly reduced neointimal formation in a rat model of carotid artery balloon injury. In that study, the MCP-1 antibody neither reduced monocyte infiltration and PCNA-positive cells in vivo nor inhibited migration of vascular smooth muscle cells in vitro.

Figure 2. Effect of intramuscular transfer of 7ND gene on macrophage infiltration in intima (A), media (B), and adventitia (C). Percent of RAM-11-positive cells ([positive cell number/total cell number]×100) is presented. Total cell numbers (mean±SEM) are shown below each bar. *P<0.05, **P<0.01 vs PBS or empty plasmid, n=8 to 9.

Figure 3. Histopathology of carotid arteries. Arteries from 7ND group show less neointimal formation, negative remodeling (constriction), and fibrosis than those from PBS group. A, Carotid artery sections from PBS and 7ND groups on 28th day after injury stained with elastic-van Gieson. Bar=200 μm. B, Carotid artery sections from PBS and 7ND groups on 28th day after injury stained with Azan-Mallory stain. Bar=40 μm.

Figure 4. Effect of intramuscular transfer of 7ND gene on neointimal formation on day 28 after balloon injury. A, Neointimal area. B, I/M ratio. *P<0.05 vs PBS or empty plasmid, n=8 to 9.
It is possible that the antibody might not attain a sufficient concentration at the injury sites or that it might be neutralized by the host immune response. We show here that 7ND gene transfer also attenuated proliferative changes (the number of PCNA-positive cells) in the injured artery. Macrophages recruited into the arterial wall may serve as a source of growth factors, cytokines, and reactive oxygen species, which in turn activate vascular wall cells. Therefore, we conclude that locally produced MCP-1 induced the recruitment of monocytes during the early phase and activated vascular smooth muscle cells and monocytes, which in turn caused neointimal hyperplasia.

No prior study investigated the role of MCP-1 in the development of negative remodeling, another major cause of human restenosis after balloon angioplasty. Adventitial inflammatory responses and subsequent constrictive fibrosis have been proposed to be the major cause of constrictive negative remodeling after balloon injury.25,26 Our present data suggest that inhibition of the inflammatory response in the adventitia limited the development of arterial constrictive remodeling by reducing adventitial fibrosis.

In conclusion, inflammatory changes caused by increased activity of MCP-1 are essential in the development of restenotic changes (neointimal formation and negative remodeling) after balloon injury in hypercholesterolemic rabbits. This study may support the hypothesis that inflammation plays a central role in the pathogenesis of restenosis. From a clinical point of view, this strategy (the delivery of plasmid DNA by intramuscular injection) is simple and has been shown to be nontoxic. Moreover, mice lacking MCP-1 or CCR2 display no serious health problems. This strategy might be a useful and practical form of gene therapy against human restenosis. We are now planning to perform this gene therapy program for treatment of human restenosis, which would open a new therapeutic process not only for restenosis but also for other intractable inflammatory disorders.

Addendum

During the review process for this article, 2 relevant articles were published. Roque et al27 demonstrated that mice lacking CCR2 displayed diminished neointimal hyperplasia formation after femoral arterial injury. Horvath et al28 showed that blockade of CCR2 by an anti-CCR2 antibody was not effective in reducing neointimal hyperplasia after iliac arterial balloon injury in monkeys. The magnitudes of inflammatory response to injury appeared to be less in these 2 studies than in the present study. This is probably because of the use of normocholesterolemic versus hypercholesterolemic animals as discussed above. Failure to inhibit neointimal hyperplasia by CCR2 antibody in the latter study might have been caused by the use of a murine antibody in monkeys.

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**Carotid Ultrasonography**

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<th>PBS Group (n=9)</th>
<th>7ND Group (n=8)</th>
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<tr>
<td></td>
<td>Noninjured Left</td>
<td>Injured Right</td>
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<tr>
<td>Vascular wall thickness, mm</td>
<td>0.45±0.09</td>
<td>0.45±0.07</td>
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<tr>
<td>Luminal diameter, mm</td>
<td>2.07±0.15</td>
<td>1.66±0.11*</td>
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<tr>
<td>Luminal area ratio‡</td>
<td>0.80±0.07</td>
<td>1.04±0.10†</td>
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Values are mean±SEM.

*P<0.05 vs noninjured carotid artery; †P<0.05 vs PBS group.
‡Injured artery area/noninjured artery area.
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