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Importance of Monocyte Chemoattractant Protein-1 Pathway in Neointimal Hyperplasia After Periarterial Injury in Mice and Monkeys

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***Abstract*—**Neointimal hyperplasia is a major cause of restenosis after coronary intervention. Because vascular injury is now recognized to involve an inflammatory response, monocyte chemoattractant protein-1 (MCP-1) might be involved in underlying mechanisms of restenosis. In the present study, we demonstrate the important role of MCP-1 in neointimal hyperplasia after cuff-induced arterial injury. In the first set of experiments, placement of a nonconstricting cuff around the femoral artery of intact mice and monkeys resulted in inflammation in the early stages and subsequent neointimal hyperplasia at the late stages. We transfected with an N-terminal deletion mutant of the human MCP-1 gene into skeletal muscles to block MCP-1 activity in vivo. This mutant MCP-1 works as a dominant-negative inhibitor of MCP-1. This strategy inhibited early vascular inflammation (monocyte infiltration, increased expression of MCP-1, and inflammatory cytokines) and late neointimal hyperplasia. In the second set of experiments, the cuff-induced neointimal hyperplasia was found to be less in CCR2-deficient mice than in control CCR2$^{+/+}$ mice. The MCP-1/CCR2 pathway plays a central role in the pathogenesis of neointimal hyperplasia in cuffed femoral artery of mice and monkeys. Therefore, the MCP-1/CCR2 pathway can be a therapeutic target for human restenosis after coronary intervention. **(*Circ Res*. 2002; 90:1167-1172.)**

Key Words: remodeling ■ growth substances ■ inflammation ■ monocytes ■ gene transfer

Neointimal hyperplasia is an essential stage in the development of restenosis after coronary intervention as well as atherosclerosis.[1] Therefore, studying the mechanism of neointimal hyperplasia in animals is indispensable to clarifying the underlying mechanisms and exploring the new treatment for vascular diseases. Recent evidence suggests that vascular injury may involve an inflammatory response that accelerates the recruitment and activation of monocytes through the activation of chemotactic factors including monocyte chemoattractant protein-1 (MCP-1).[2–4] MCP-1 is a potent chemotactic factor for monocytes.[5,6] Eliminating MCP-1 gene or blockade of MCP-1 signals has been shown to decrease atherogenesis in hypercholesterolemic mice.[7–9] However, no prior study addressed the definite role of monocytes or MCP-1–mediated signals in the development of neointimal hyperplasia after periarterial injury. Placement of nonconstricting cuff around the artery induces vascular inflammation at the early stages and subsequently causes intimal hyperplasia at the late stages.[10–12] Wu et al[12] recently reported that angiotensin II type 1 receptor blockade attenuated vascular inflammation (monocyte infiltration and activation, upregulation of MCP-1 and inflammatory cytokines) induced by perivascular cuff placement. The latter study suggests the importance of MCP-1–mediated inflammation in mediating the formation of neointimal hyperplasia. However, no prior study has addressed the role of MCP-1 in the pathogenesis of neointimal hyperplasia after cuff placement.

In the present study, we investigated the role of MCP-1 and its receptor pathway in neointimal hyperplasia after cuff-induced periarterial injury in mice and monkeys. We chose the cuff injury model, because it offers the advantage of inducing reproducible and site-controlled neointimal hyperplasia in mice and other larger animals. Vascular injury induced by intraluminal techniques in the mouse is methodologically difficult and the results are variable. In contrast, the cuff-induced injury stimulates vascular inflammation that seems to be a central mediator in the pathogenesis of restenosis and atherosclerosis. First, we determined if blockade of MCP-1 activity could decrease neointimal hyperplasia in normal mice and monkeys. To block MCP-1 activity in vivo, the animals were transfected with an N-terminal deletion mutant of the human MCP-1 gene, called 7ND (missing

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the N-terminal amino acids 2 to 8), into skeletal muscles.[9,13] 7ND has been shown to work as a dominant-negative inhibitor of MCP-1.[14] Second, the cuff-induced neointimal hyperplasia was examined in CCR2-deficient and wild-type CCR2$^{+/+}$ mice. Our present data show that the MCP-1/CCR2 pathway may play an essential role in the development of neointimal hyperplasia after cuff-induced neointimal hyperplasia.

Materials and Methods

Expression Vector

The human 7ND gene was cloned into the *Bam*HI (5′) and *Not*I (3′) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen).[9] Human MCP-1 cDNA was a generous gift from Dr T. Yoshimura, National Cancer Institute, Frederick, Md. Plasmid cDNA3 encoding luciferase gene was used to detect gene transfection.

Animals

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

Mice with a genetic background of C57BL/6J were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice deficient in CCR2 and wild-type CCR2$^{+/+}$ mice with the same genetic background (hybrids of C57BL/6J and 129/svjae) were supplied from Dr Charo.[8] Male CCR2-deficient and wild-type CCR2$^{+/+}$ mice were age matched for all experiments. Male adult cynomolgus monkeys weighing 4 to 5 kg were purchased from Japan Charles-River, Shizuoka. The mice and monkeys were fed commercial laboratory diet.

Placement of Cuff

Mice were anesthetized with intraperitoneal pentobarbital, and the left femoral artery was dissected from its surroundings. A nonconstrictive polyethylene cuff (PE50, 0.58-mm inner diameter, 0.97-mm outer diameter, and 1.5-mm length) was placed loosely around the left femoral artery. Monkeys were anesthetized with ketamine hydrochloride (10 mg/kg IM) and sodium pentobarbital (30 mg/kg IV to effect) and a nonconstrictive polyethylene cuff (3.0-mm inner diameter, 4.0-mm outer diameter, and 10.0-mm length) was placed loosely around the left femoral artery.

Gene Transfer and Electroporation

Three days before the cuff placement, animals received an intramuscular injection of empty plasmid or plasmid with 7ND gene into the femoral muscles after light anesthesia. To enhance transgene expression, these animals received electroporation at the injected site as described.[15] Either empty plasmid or 7ND gene (300 μg/150 μL PBS in mice and 1000 μg/500 μL PBS in monkeys) was injected into the right femoral muscle using a 27-gauge needle. Then, a pair of electrode needles (Tokiwa Science) spaced 5 mm apart was inserted into the muscle on either side of the injected sites, and six 100-V square wave pulses (spaced 1 second apart) were applied, followed by 3 pulses in the opposite polarity for 50 ms in duration using an electric pulse generator CUY201 (BTX), and the wound was closed. No inflammation was observed at the injection sites.

We used in vivo matrigel plug assay to determine the effect of 7ND gene transfer on MCP-1 activity. Matrigel (9 mg/mL; 0.3 mL/mouse) alone or mixed with MCP-1 at a concentration of 100 ng/mL was injected subcutaneously into the flank of C57BL/6J mice.[15] We examined MCP-1–induced angiogenesis in the plugs by the use of histopathological analysis and found that 7ND gene transfer suppressed the angiogenesis induced by MCP-1 to a level similar to that observed in the control matrigel plugs lacking MCP-1 until 21 days after transfection.

Histopathological Assessment of Intimal Lesions

At euthanasia, mice were anesthetized with pentobarbital. The thorax was opened, and mild pressure-perfusion (100 mm Hg) with 3.7% formaldehyde in 0.9% NaCl (wt/vol) for 10 minutes was performed by cardiac puncture. After perfusion, the femoral artery was harvested, fixed overnight in 3.7% formaldehyde in PBS, and paraffin-embedded. Serial cross sections (5 μm thick) were used throughout the entire length of the cuffed femoral artery for histological analysis. Cryosections were made of 2 mice in each condition. All samples were routinely stained with hematoxylin-eosin (HE) or van Gieson. Smooth muscle cells were visualized with α-smooth muscle cell actin staining (Boehringer Mannheim), and Mac3 (Serotec) macrophage staining was used to detect monocytes/macrophages. PCNA (Santa Cruz Biotech) was detected to examine vascular proliferation. An antibody against von Wilbrand factor (Sigma) was used as endothelial cell marker. Some sections were also stained for MCP-1 with a rabbit anti-rat polyclonal antibody.[9]

Monkeys were euthanized with pentobarbital, and the femoral artery was harvested, fixed overnight in 3.7% formaldehyde in PBS, and paraffin-embedded without perfusion.

Quantification of Intimal Lesions in Sections of Cuffed Femoral Artery

Ten equally spaced cross sections were used in all mice to quantify intimal lesions. Using image analysis software, total cross-sectional medial area was measured between the external and internal elastic

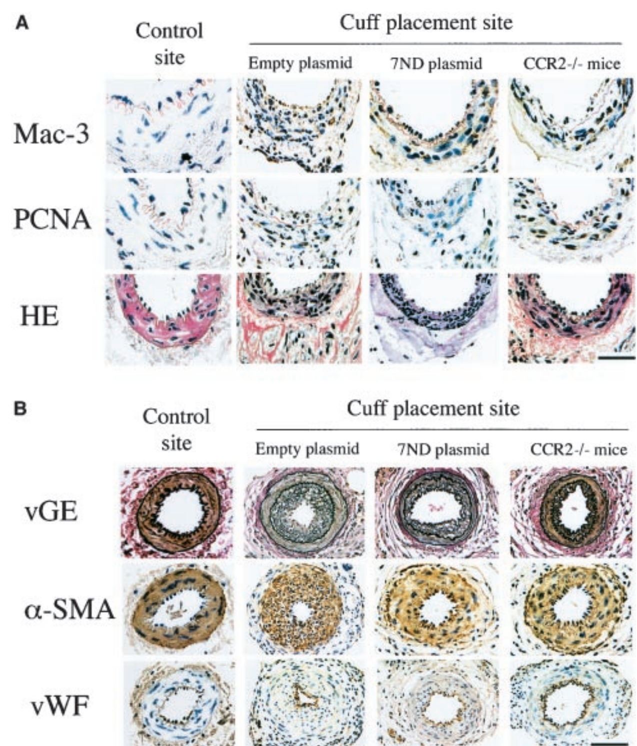

Figure 1. Histopathology and immunohistochemistry of the cuffed femoral arteries. A, Femoral artery sections 7 days after cuff placement from a mouse transfected with empty plasmid, a mouse transfected with 7ND plasmid, or CCR2-deficient mouse are stained with hematoxylin-eosin (HE) or immunohistochemically for monocyte/macrophage (Mac3), and proliferating cells (PCNA). Bar=25 μm. B, Femoral artery sections 21 days after cuff placement from control wild-type mice with and without 7ND gene transfer and CCR2-deficient mice are stained with van Gieson Elastica (vGE) or immunohistochemically for α-smooth muscle actin (α-SMA) and von Wilbrand factor (vWF). Bar=100 μm.

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lamina; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

RT-PCR and RNAse Protection Assays

RNA was prepared from the pooled samples (n=5 to 7 for each group) using TRIzol reagent (GIBCO-BRL). First-strand DNA was synthesized using reverse transcriptase with random hexamers from 1 μg total RNA in 20 μL reaction volume according to the manufacturer's protocol (GeneAmp RNA PCR Kit; Perkin-Elmer), then one-tenth of the resulting reverse transcription (RT) product was applied to each 25 μL PCR. PCR primers used for MCP-1, CCR2, and β-actin are 5′-AGAGAGCCAGACGGAGGAAG-3′ and 5′-GTCACACTGGTCACTCCTAC-3′ for MCP-1, 5′-GGTCATGAT-CCCTATGTGG-3′ and 5′-CTGGGCACCTGATTTAAAGG-3′ for CCR2, and 5′ATGGATGACGATATCGCT-3′ and 5′-ATGAGGT-AGTCTGCTAGGT-3′ for β-actin. PCR products were separated by 2% agarose gel electrophoresis and were detected as a single band of the expected size in each PCR.

RNAse protection assays (RPAs) 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 (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.

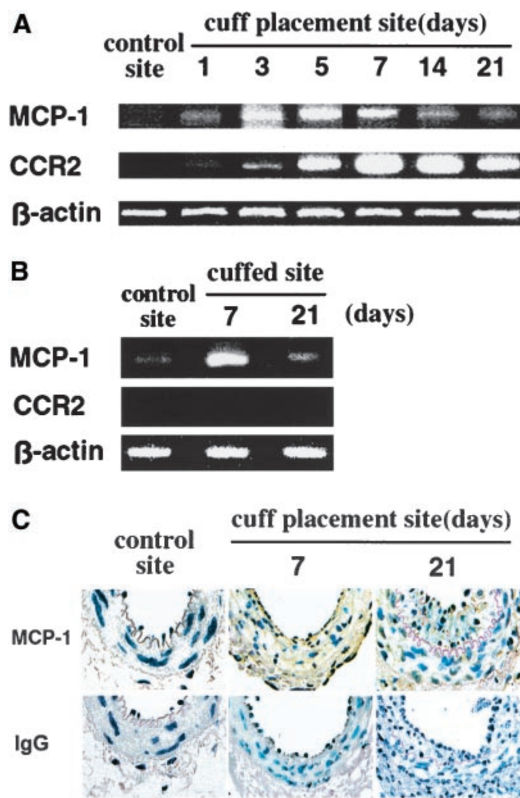

Figure 2. RT-PCR analysis for MCP-1 and CCR2. A, Cuffed arteries and control intact arteries were harvested and frozen at the indicated days. Pooled samples (n=5 for each time point) were used for RNA preparation. One representative result from 4 independent RT-PCR (each 2 from different pools of tissue samples) is shown. B, RT-PCR analysis for MCP-1 and CCR2 in the cuffed femoral artery in CCR2-deficient mice. One representative result of 3 independent RT-PCR (each 2 from different pools of tissue samples) is shown. C, Photomicrographs of femoral artery sections from control intact site as well as from 7 and 21 days after cuff placement are stained immunohistochemically for MCP-1. Bar=25 μm.

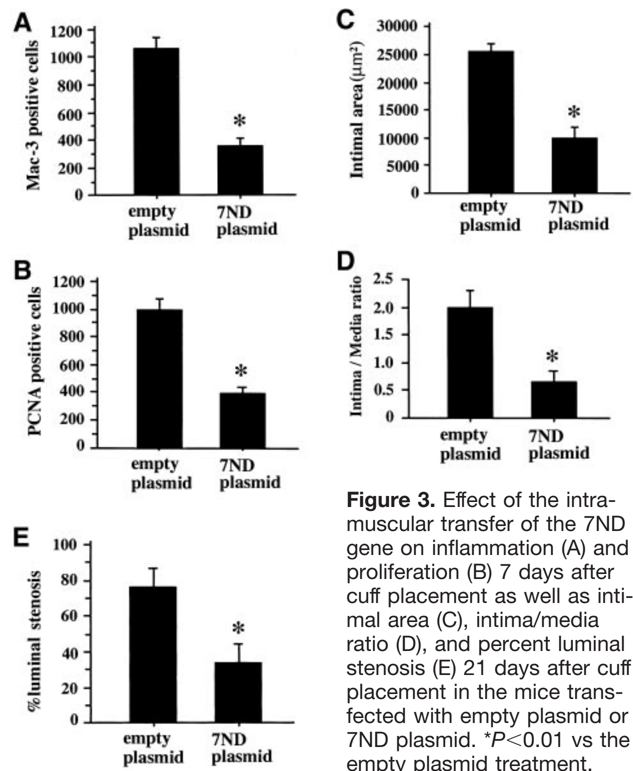

Figure 3. Effect of the intramuscular transfer of the 7ND gene on inflammation (A) and proliferation (B) 7 days after cuff placement as well as intimal area (C), intima/media ratio (D), and percent luminal stenosis (E) 21 days after cuff placement in the mice transfected with empty plasmid or 7ND plasmid. *$P<0.01$ vs the empty plasmid treatment.

ELISA

To measure 7ND released by the transfected skeletal muscle, plasma concentrations of 7ND 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 and recognize the C-terminus of human MCP-1, plasma 7ND concentrations are likely to be measured by the use of this human ELISA kit. Plasma concentrations of MCP-1 were also measured by the use of mouse MCP-1 ELISA kit (Biosource).

Statistical Analysis

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

Results

Time Course of Development of Intimal Lesions in Mice

Control mice were killed at 3, 7, 10, 14, and 21 days after placement of the cuff. In these mice, marked infiltration of Mac3-positive monocytes and PCNA-positive cells into the adventitia, media, and intima was observed within 3 to 7 days after cuff placement (Figure 1A), as demonstrated by others.[12,16,17] These inflammatory and proliferative changes declined at 21 days. Modest neointimal formation was noted within 7 days and significant neointimal hyperplasia was observed 21 days after the placement of the cuff, as observed in previous studies by others.[10,11] This intimal formation consisted predominantly of α-smooth muscle cell actin–positive cells. No foam cells were detected in any of the sections taken from the cuffed arteries of mice. Endothelial cells, monitored by von

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Wilbrand Factor expression, were observed 7, 14, and 21 days after the cuff placement (Figure 1B).

We measured blood pressure and heart rate by the tail-cuff method in the mice of all groups before and after cuff placement. There was no statistical difference in blood pressure and heart rate among the groups and no interval changes in such parameters in each group (data not shown). No interval change in body weight was noted in each group (data not shown).

Expression of MCP-1 and CCR2 mRNA and Immunoreactivity in Mice

We examined the mRNA expression of MCP-1 and CCR2 in the cuffed artery RT-PCR (Figures 2A and 2B). The expression of MCP-1 and CCR2 was undetectable in the control intact artery, whereas the expression was induced markedly by cuff placement. The peak response was observed at 5 to 7 days after cuff placement.

Immunohistochemical staining for MCP-1 was performed at 3, 5, 7, and 21 days. MCP-1 immunoreactivity was undetectable in control intact arteries, but was intensely increased in endothelial layer at 5 to 7 days after cuff placement (Figure 2C). The increased immunoreactivity for MCP-1 declined at 21 days. Some intimal cells were stained for MCP-1 at 21 days.

7ND Gene Transfer Reduces Neointimal Hyperplasia in Mice and Monkeys

In the mice, both inflammation (Mac3-positive cells) and proliferation (the PCNA index) were markedly less in 7ND-transfected mice than in empty plasmid-transfected mice at 7 days (Figure 3). 7ND gene transfer significantly reduced the neointimal hyperplasia (increases in neointimal area, intima/media ratio, and luminal stenosis) 21 days after cuff placement.

The gene expressions of a battery of inflammatory cytokines, chemokines, and chemokine receptors were examined by RNAse protection assays 7 days after cuff placement in mice (Figure 4). The gene expressions of such genes all upregulated after cuff placement, and 7ND gene transfer prevented or inhibited the increased gene expression of various cytokines and chemokines.

In monkeys, significant neointimal hyperplasia was noted 21 days after cuff placement as well. 7ND gene transfer reduced the neointimal formation in monkeys (Figure 5).

Plasma Concentrations of 7ND and MCP-1

Plasma Concentrations of 7ND and MCP-1 were measured before and 3, 7, 14, and 21 days after 7ND transfection in mice. 7ND was detected in plasma 3, 7, 14, and 21 days after transfection, whereas plasma MCP-1 concentrations did not change during the course of experiments (Table).

Reduced Neointimal Formation in CCR2-Deficient Mice

CCR2 mRNA was not detected in the CCR2-deficient mice, whereas MCP-1 and CCR2 mRNA was comparably expressed in the $CCR2^{+/+}$ mice at 7 days after cuff placement (Figure 2B). Neointimal hyperplasia similar to that seen in C57BL/6J mice was observed at 21 days in $CCR2^{+/+}$ mice. Neointima formation was less in CCR2-deficient mice than in the $CCR2^{+/+}$ mouse (Figure 6).

Discussion

This study is the first to demonstrate that the MCP-1/CCR2 pathway is essential in the development of neointimal hyperplasia after cuff-induced perivascular injury in mice and monkeys. We found here that placement of cuff caused rapid and persistent activation of the MCP-1/CCR2 pathway, resulting in vascular inflammation (monocyte infiltration and the upregulation of various cytokines and chemokines) and proliferation (the appearance of PCNA-positive cells). Most importantly, blockade of MCP-1 by 7ND gene transfer reduced the early inflammatory and proliferative changes and thus attenuated the development of neointimal hyperplasia. Similar reduction in cuff-induced neointimal hyperplasia was noted in the absence of CCR2 in CCR2-deficient mice. Gene transfer of 7ND in CCR-deficient mice did not further reduce neointimal formation (the authors' unpublished data, 2002). Immunohistochemically, the endothelial layer was preserved after cuff placement during the course of these experiments. Most of cell types in the neointima was predominantly composed of α-smooth muscle actin–positive cells, indicating that invasion of medial smooth muscle cells into the intimal region may be a major cause of cuff-induced neointimal hyperplasia. Accordingly, it is concluded that vascular inflammation and proliferation mediated through increased expression and activity of the MCP-1/CCR2 pathway are essential in the pathogenesis of neointimal hyperplasia after

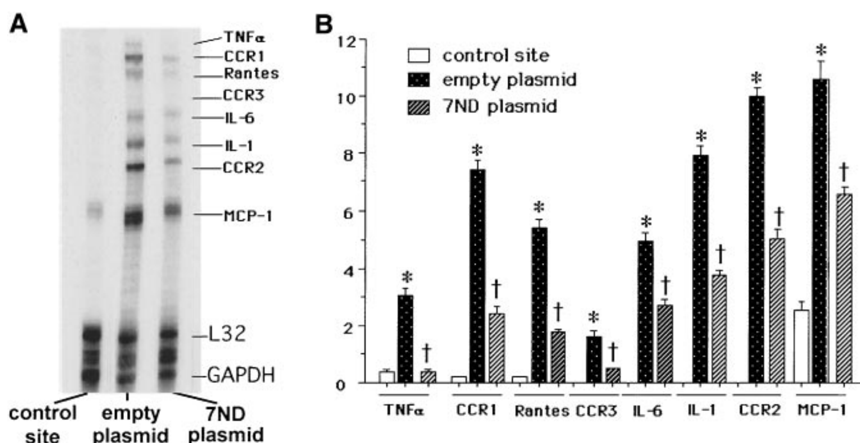

Figure 4. RNase protection assays for various inflammatory cytokines (TNFα, IL-6, IL-1β), chemokines (MCP-1 and RANTES), and chemokine receptors (CCR1, CCR3, and CCR2). A, Representative autoradiograph of RNase protection assay is shown. Arterial tissues from the noninjured control sites and cuff placement sites were harvested and frozen 7 days after transfection. Pooled samples (n=5 for one assay) were used for RNA preparation. Bands of L32 and GAPDH were used as internal controls. B, Summary of densitometric analysis. Data are expressed as the ratio of each mRNA to the corresponding GAPDH mRNA. *$P<0.01$ vs the control site; †$P<0.01$ vs empty plasmid group (n=6).

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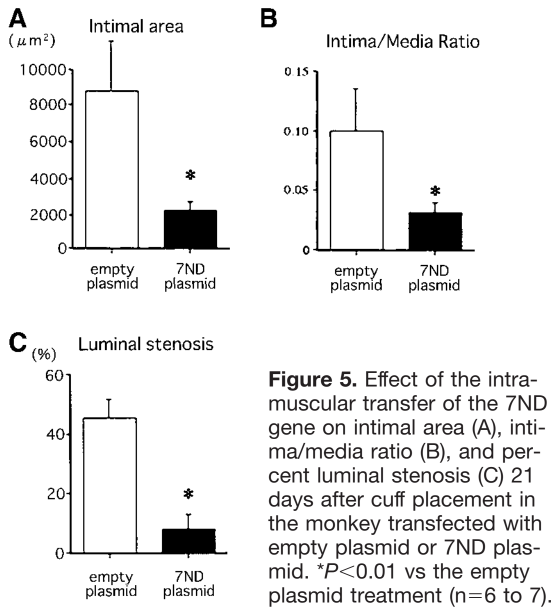

Figure 5. Effect of the intramuscular transfer of the 7ND gene on intimal area (A), intima/media ratio (B), and percent luminal stenosis (C) 21 days after cuff placement in the monkey transfected with empty plasmid or 7ND plasmid. *$P<0.01$ vs the empty plasmid treatment (n=6 to 7).

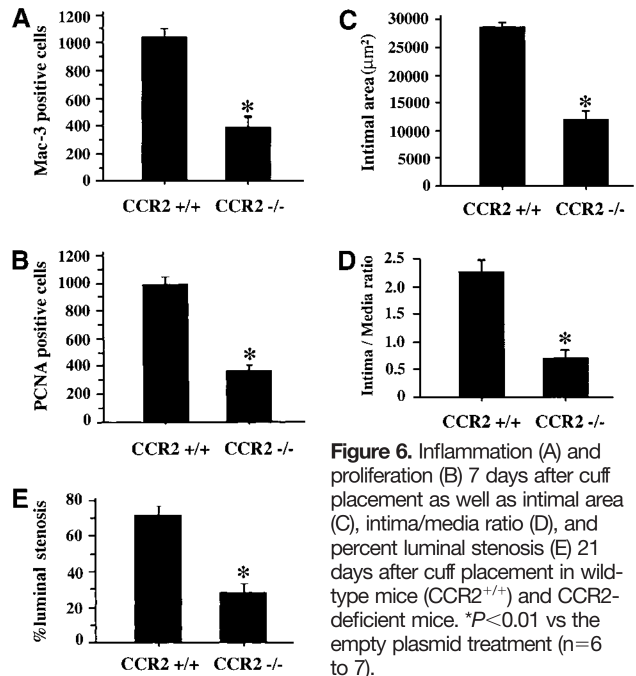

Figure 6. Inflammation (A) and proliferation (B) 7 days after cuff placement as well as intimal area (C), intima/media ratio (D), and percent luminal stenosis (E) 21 days after cuff placement in wild-type mice ($CCR2^{+/+}$) and CCR2-deficient mice. *$P<0.01$ vs the empty plasmid treatment (n=6 to 7).

cuff-induced perivascular injury. Our present data suggest an important role of increased expression of inflammatory cytokines and chemokines, resulting mainly from activation of lesional monocytes, in the pathophysiological process.

Our present data strongly suggest that the MCP-1/CCR2 pathway is important in mediating neointimal formation as well as vascular inflammation after perivascular injury. The beneficial effects of 7ND gene transfer in monkeys imply that this gene transfer strategy can be a novel therapeutic tool against human restenosis and atherosclerosis. It was reported that repeated injection of polyclonal antibody against rat MCP-1 modestly reduced neointimal formation in a rat model of carotid artery balloon injury.[18] In the latter study,[18] the mechanism by which the MCP-1 antibody attenuated neointimal formation remains unclear because the MCP-1 antibody did not reduce monocyte infiltration. It is possible, therefore, that the antibody might not attain a sufficient concentration at the injury sites or that it might be neutralized by host immune response. A recent clinical report,[19] demonstrating that patients with restenosis display rapid and prolonged increases in plasma MCP-1 levels compared with nonrestenotic patients, may support our current experimental observation. Thus, cuff-induced perivascular injury may cause neointimal formation through recruitment and activation of monocytes mediated by the MCP-1/CCR2 pathway. Although we did not determine the mechanism of the activation of the MCP-1/CCR2 pathway in cuffed femoral artery, it is plausible to speculate that increased oxidative stress and redox-sensitive transcription factors such as nuclear factor-κB might have contributed to the increased MCP-1 expression as we had reported in a rat model of long-term inhibition of nitric oxide synthesis.[20–23]

A caveat of interpreting our present data is that clinical significance regarding the role of MCP-1 in the cuff injury model is obscure. This is because the cuff injury may not represent any pathophysiological conditions clinically in humans. Therefore, the clinical relevance of our findings in humans remains to be defined.

In conclusion, our present data suggest that the MCP-1/CCR2 pathway is required for the development of fibromuscular neointimal hyperplasia by cuff-induced perivascular injury. Therefore, the MCP-1/CCR2 pathway can be a promising therapeutic target for restenosis after coronary interventions. Further studies are needed to verify that this form of anti–MCP-1 gene therapy may be useful for the treatment of acute coronary syndromes. This gene therapy strategy (the delivery of plasmid DNA by intramuscular injection) is simple and nontoxic and may have broader application for other inflammatory disorders.

Acknowledgments

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Plasma Concentrations of 7ND and MCP-1 After 7ND Transfection

		Days After 7ND Transfection			
	Baseline	3	7	14	21
MCP-1, pg/mL (mouse MCP-1)	66±4	72±2	71±2	70±3	68±5
7ND, pg/mL (human MCP-1)	0.0 (not detected)	186±12	220±10	150±7	124±12

Values are mean±SE (n=6 to 7).

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