

RESEARCH ARTICLE

Antimonocyte chemoattractant protein-1 gene therapy reduces experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys

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In-stent restenosis results exclusively from neointimal hyperplasia due to mechanical injury and a foreign body response to the prosthesis. Inflammation mediated by monocyte chemoattractant protein-1 (MCP-1) might therefore underlie in-stent restenosis. 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 experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys. Transfection of the mutant

MCP-1 gene suppressed monocyte infiltration/activation in the stented arterial wall and markedly reduced the development of neointimal hyperplasia. This strategy also suppressed local expression of MCP-1 and inflammatory cytokines. Therefore, inhibition of MCP-1-mediated inflammation is effective in reducing experimental in-stent restenosis. This strategy might be a useful form of gene therapy against human in-stent restenosis.

Gene Therapy (2004) 11, 1273–1282. doi:10.1038/sj.gt.3302288; Published online 3 June 2004

Keywords: monocyte; inflammation; restenosis; stent

Introduction

Each year, more than 1.5 million patients undergo percutaneous coronary intervention of atherothrombotic lesions worldwide. Stent implantation is now the major revascularization technique.¹ Although the stent technique reduces the restenosis rate in selected coronary artery lesions, restenosis continues to occur in high-risk lesions or patients, and thus still remains an unsolved clinical issue. Anatomically, in-stent restenosis results exclusively from neointimal hyperplasia, whereas restenosis after balloon angioplasty results from neointimal hyperplasia and negative remodeling of the arterial wall.² There is a two-fold greater incidence of neointimal hyperplasia after stent implantation than after balloon angioplasty.³ Recent evidence suggests that in addition to mechanical injury, an intense foreign body response to stent prosthesis induces acute and chronic inflammation in the arterial wall, ensuing production of cytokines and growth factors that subsequently induce proliferation and migration of vascular smooth muscle cells.^{3,4,5} Experimental and clinical data suggest that inhibition of cellular proliferation with sirolimus might be an effective strategy to suppress in-stent restenosis.^{6–9}

There is growing evidence from clinical and animal studies indicating that inflammation is a central mediator

in restenosis.^{10–12} Recruitment and activation of monocytes/macrophages are major early histopathologic findings after arterial injury. As monocyte chemoattractant protein-1 (MCP-1) is a potent and specific chemokine for monocytes,^{13,14} an anti-inflammatory strategy targeting MCP-1 and its receptor (CCR2) might be an appropriate and reasonable approach for the treatment of restenosis. We recently devised a new strategy for anti-MCP-1 gene therapy by transfecting plasmid cDNA encoding a mutant MCP-1 gene into skeletal muscle.¹⁵ This mutant MCP-1 lacks N-terminal amino acids 2–8, called 7ND, forms inactive heterodimers with wild-type MCP-1, and thus works as a dominant-negative inhibitor of MCP-1.¹⁶ This method (intramuscular (i.m.) transfection of the gene) is useful because direct gene transfer into the injured arterial wall is not necessary and the role of MCP-1 can be investigated under pathophysiologic conditions *in vivo*. We used this strategy to demonstrate that blockade of the MCP-1 signal reduces neointimal hyperplasia after injury^{17,18,19} and atherosclerosis.^{20,21} Roque *et al*²² reported reduced neointimal hyperplasia after intraluminal arterial injury in CCR2-deficient mice. With regard to the role of MCP-1 in in-stent restenosis, Horvath *et al*²³ demonstrated that blockade of the MCP-1 receptor (CCR2) with anti-CCR2 antibody reduced neointimal hyperplasia by 40% after stenting by inhibiting monocyte infiltration in normocholesterolemic monkeys. In the latter study, however, the efficacy of CCR2 blockade might have been limited due to possible antigenic actions from the use of murine antibody in monkeys. Furthermore, because of the use of

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Received 12 October 2003; accepted 19 March 2004; published online 3 June 2004

normocholesterolemic monkeys, the degree of stent-induced neointimal hyperplasia was markedly less than that reported in humans and in hypercholesterolemic animals.

Therefore, the primary aim of this study was to test the hypothesis that MCP-1-mediated arterial inflammation is the underlying mechanism in in-stent neointimal hyperplasia in hypercholesterolemic rabbits and monkeys, which might be more clinically relevant animal models than normocholesterolemic animals.²⁴

Results

Effects of 7ND gene transfer in rabbits

We measured gene expression of cytokines and chemokines 7 days after stenting (Figure 1a) (Table 1). The

mRNA levels of interleukin (IL)-6, tissue necrosis factor (TNF) α , IL-1 β , matrix metalloproteinases (MMP)-2, MMP-9, and vascular endothelial growth factor (VEGF) were higher in the stented artery than in the noninjured normal control artery. 7ND gene transfer did not affect the increase in mRNA levels of TNF α , MMP-2, and MMP-9, but reduced the increases in IL-6, IL-1 β , and VEGF levels. We immunohistochemically examined MCP-1 and VEGF expression and found that immunoreactive MCP-1 and VEGF increased 7 days after stenting (Figures 1b and c). Minor MCP-1 or VEGF immunostaining was detected in the noninjured control artery, whereas there was intense MCP-1 and VEGF immunoreactivity mainly in the media and intima 7 days after stenting. 7ND gene transfer markedly reduced the magnitude of MCP-1 and VEGF immunostaining (Figures 1b and c).

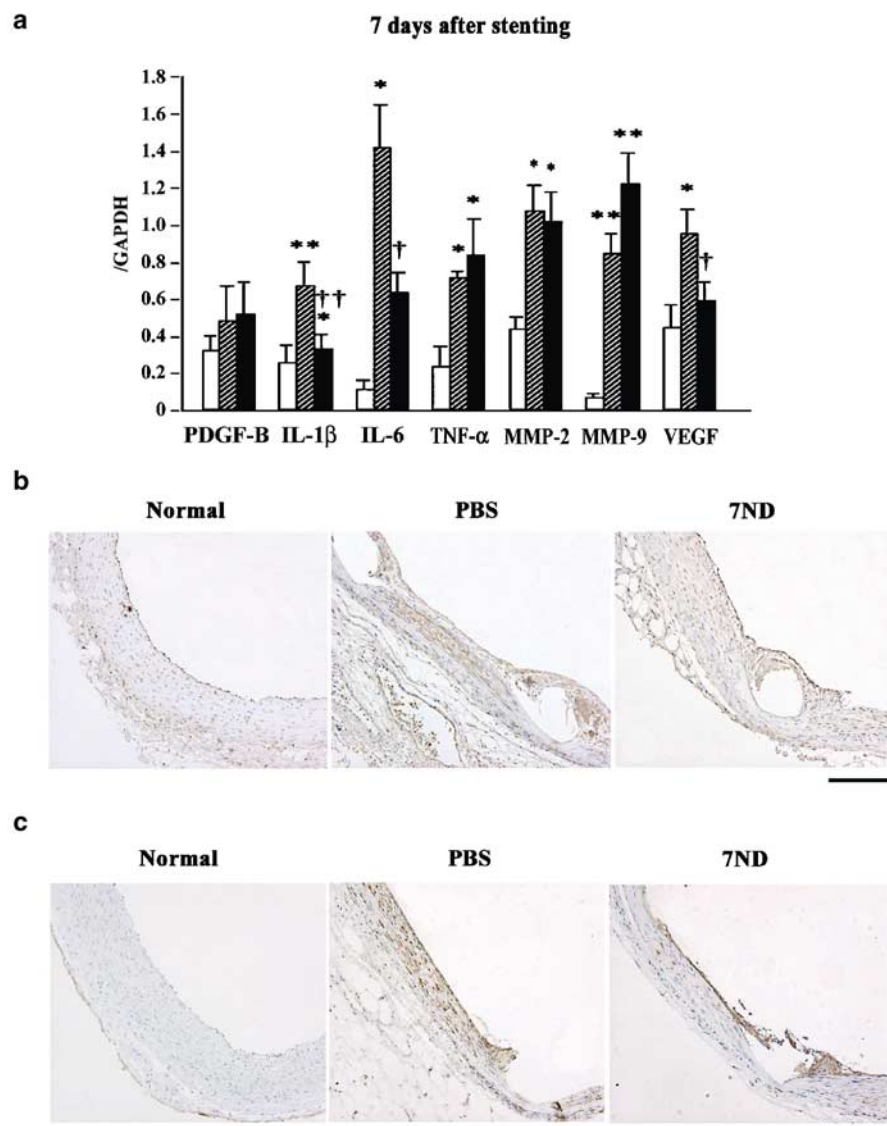


Figure 1 Gene expression and immunohistochemistry in the stented artery of rabbits. (a) Analysis of expression of various genes by real-time RT-PCR in noninjured controls (open bars) and stented arteries from PBS-treated rabbits (hatched bars) and stented arteries from rabbits transfected with 7ND gene (closed bars). $N=7-8$. * $P<0.05$, ** $P<0.01$ versus control (no injury); † $P<0.05$, **† $P<0.01$ versus the PBS group. (b) Arterial sections of control nonstented artery or stented arteries from the PBS-treated and 7ND-transfected group stained immunohistochemically with the antibody against MCP-1 7 days after stenting in rabbits. Bar = 200 μm . (c) Effects of 7ND gene transfer on protein expression of VEGF. Noninjured control artery section and stented artery sections from the PBS and 7ND group stained immunohistochemically with the antibody against VEGF. Bar = 200 μm .

Table 1 Probes used for real-time PCR

Assay	Sequence	Acc. no.
PDGF-B		
Forward	5'-CCCATCTACATCATCACCGAGTAC-3'	AB020215
Reverse	5'-GAGTGCTGCTGCAGGAAGGT-3'	
TaqMan probe	5'-TGGACTACCTGCACCGCAACAAGC-3'	
IL-6		
Forward	5'-CATGGTGCTGAAGAACATCCAA-3'	AF169176
Reverse	5'-ACTGGTTTTCTGCTGCAGGT-3'	
TaqMan probe	5'-AATGAAGAAGCCACCCTCAAGCCAGC-3'	
IL-1 β		
Forward	5'-TCAGCACCTCTCAGACAGAGTACAT-3'	M26295
Reverse	5'-AAGACACGAATTCATGCTGAA-3'	
TaqMan probe	5'-AAACAACAGTGGCGGCCAAGACCTAA-3'	
TNF- α		
Forward	5'-CATGGTGCTGAAGAACATCCAA-3'	M12845
Reverse	5'-ACTGGTTTTCTGCTGCAGGT-3'	
TaqMan probe	5'-AATGAAGAAGCCACCCTCAAGCCAGC-3'	
MMP-2		
Forward	5'-GGAGAAGGCCGTGTTCTTTG-3'	D63579
Reverse	5'-CAGTTGAAGGCCGCTCTAC-3'	
TaqMan probe	5'-CCAAGCCTCTGACCAGCCTCGG-3'	
MMP-9		
Forward	5'-GCAGGATGTCAAAGCTCACGTA-3'	D26514
Reverse	5'-AACACACACGACGCTTCCAGTA-3'	
TaqMan probe	5'-TCACACGCCAGAAGAAGCGGTCC-3'	
VEGF		
Forward	5'-GGCTGCTGCAATGATGAAAG-3'	AB020216
Reverse	5'-TTGATCCGCATGATCTGCAT-3'	
TaqMan probe	5'-TGCCCACCGAGGAGTTCAACGTC-3'	
GAPDH		
Forward	5'-CTCTGGCAAAGTGGATGTTGTC-3'	L23961
Reverse	5'-GGGTGGAATCATACTGGAACATG-3'	
TaqMan probe	5'-CCATCAATGATCCATTTCATTGACCTCCA-3'	

Acc. no. indicates the accession number in GenBank.

Vascular inflammation, proliferation, and apoptotic cell death were examined 7 days after stent implantation. Infiltration of monocytes/macrophages (Figure 2a) and appearance of proliferating cells (Figure 2b) were observed in the intima and media. Apoptotic cells (Figure 2c) were observed in the intima. Increased inflammation, proliferation, and apoptotic cell death persisted mainly in the neointima at 28 days (data not shown). 7ND gene transfer attenuated the inflammation and proliferation, and enhanced cell death after stenting (Figure 2). There were equal numbers of endothelial cells, monitored by CD31 immunoreactivity in animals treated with phosphate-buffered saline (PBS) or transfected with 7ND gene (Figure 2d). Neointimal hyperplasia was examined by intravascular ultrasound (IVUS) and by histopathologic analysis 28 days after stenting (Figure 3). We detected significant neointimal hyperplasia 28 days after stenting in the PBS-treated rabbits. 7ND gene transfer markedly reduced the neointimal formation as assessed by ultrasonographic and histologic analyses (Figure 3).

We measured plasma and femoral muscle concentrations of 7ND after 7ND or empty plasmid transfection.

Plasma 7ND was detected in the plasma 3, 7, and 14 days after 7ND transfection (Table 2). On day 3 of transfection, 7ND could be detected only in 7ND-transfected muscle (Table 3). These data confirm that 7ND protein was released from transfected muscles to circulation.

Effects of 7ND gene transfer in monkeys

Histopathologic and immunohistochemical analyses were performed 28 days after stenting (Figure 4). MCP-1 immunoreactivity was not detected in the nonstented artery (data not shown), whereas intense MCP-1 and CCR2 immunoreactivity was evident mainly in the neointima around the stent prosthesis and weakly in the media (Figure 4a). As seen in rabbits, 7ND gene transfer reduced the magnitude of MCP-1 immunostaining (data not shown) and the neointimal formation (Figures 4b and c).

Effects of 7ND gene transfer in cholesterol levels

In rabbits, the total cholesterol levels before and 28 days after stenting were 922 ± 108 and 968 ± 166 mg/dl in the PBS-treated group, and 938 ± 74 and 950 ± 117 mg/dl in 7ND-transfected group. In monkeys, the total cholesterol

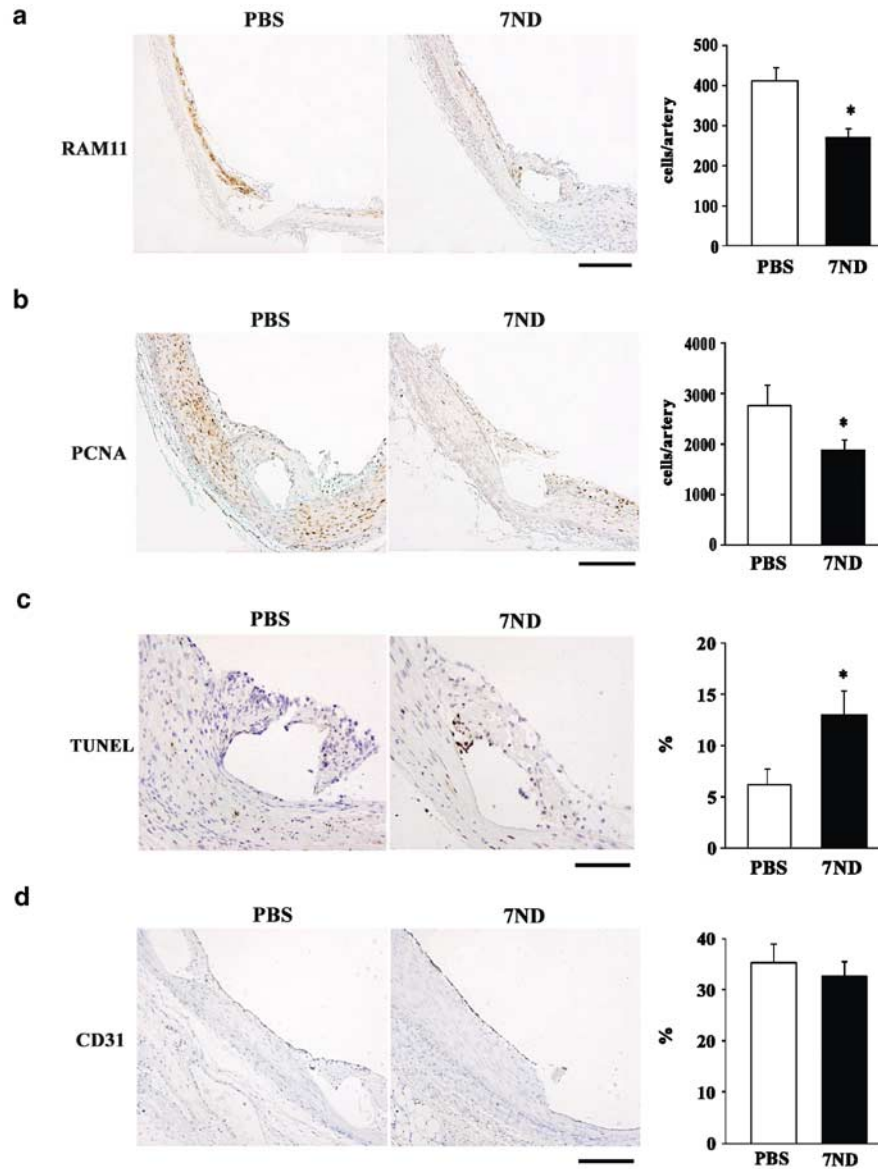


Figure 2 Inflammation, proliferation, and cell death in the stented artery of rabbits. (a) Effects of 7ND gene transfer on inflammatory changes. Artery sections 7 days after stenting from a rabbit transfected with PBS and a rabbit transfected with 7ND plasmid immunohistochemically stained for monocytes/macrophages (RAM11) and summary of quantitative analyses are presented ($n = 8$ each). $*P < 0.01$ versus PBS. Bar = 200 μm . (b) Effects of 7ND gene transfer on proliferative changes. Artery sections 7 days after stenting immunohistochemically stained for PCNA and summary of quantitative analyses are presented ($n = 8$ each). $*P < 0.01$ versus PBS. Bar = 200 μm . (c) Effects of 7ND gene transfer on cell death. TUNEL-stained artery sections 7 days after stenting and summary of quantitative analyses are presented ($n = 8$ each). $*P < 0.05$ versus PBS. Bar, 100 μm . (d) Effects of 7ND gene transfer on CD31-positive endothelial lining 7 days after stenting and summary of quantitative analyses are presented ($n = 8$ each). Bar, 100 μm .

levels before and 28 days after stenting were 444 ± 43 and 429 ± 37 mg/dl in the PBS-treated group, and 469 ± 30 and 488 ± 44 mg/dl in 7ND-transfected group. These data indicate that the observed effects of 7ND gene transfer were not due to changes in serum cholesterol levels.

Antibody production in 7ND-transfected monkeys

In ELISA assay, IgG and IgM antibodies against 7ND protein were not detected after 7ND transfection ($n = 6$ each, Figure 5).

Discussion

The present study demonstrated that blockade of MCP-1 by 7ND gene transfer attenuated monocyte infiltration, and thus suppressed the development of neointimal formation after stent placement in hypercholesterolemic rabbits and non-human primates (cynomolgus monkeys), indicating a role of MCP-1 in stent-induced experimental restenosis (neointimal formation).

Recent studies suggested that inflammation is an important determinant of in-stent neointimal hyperplasia. Inflammation associated with coronary stenting was

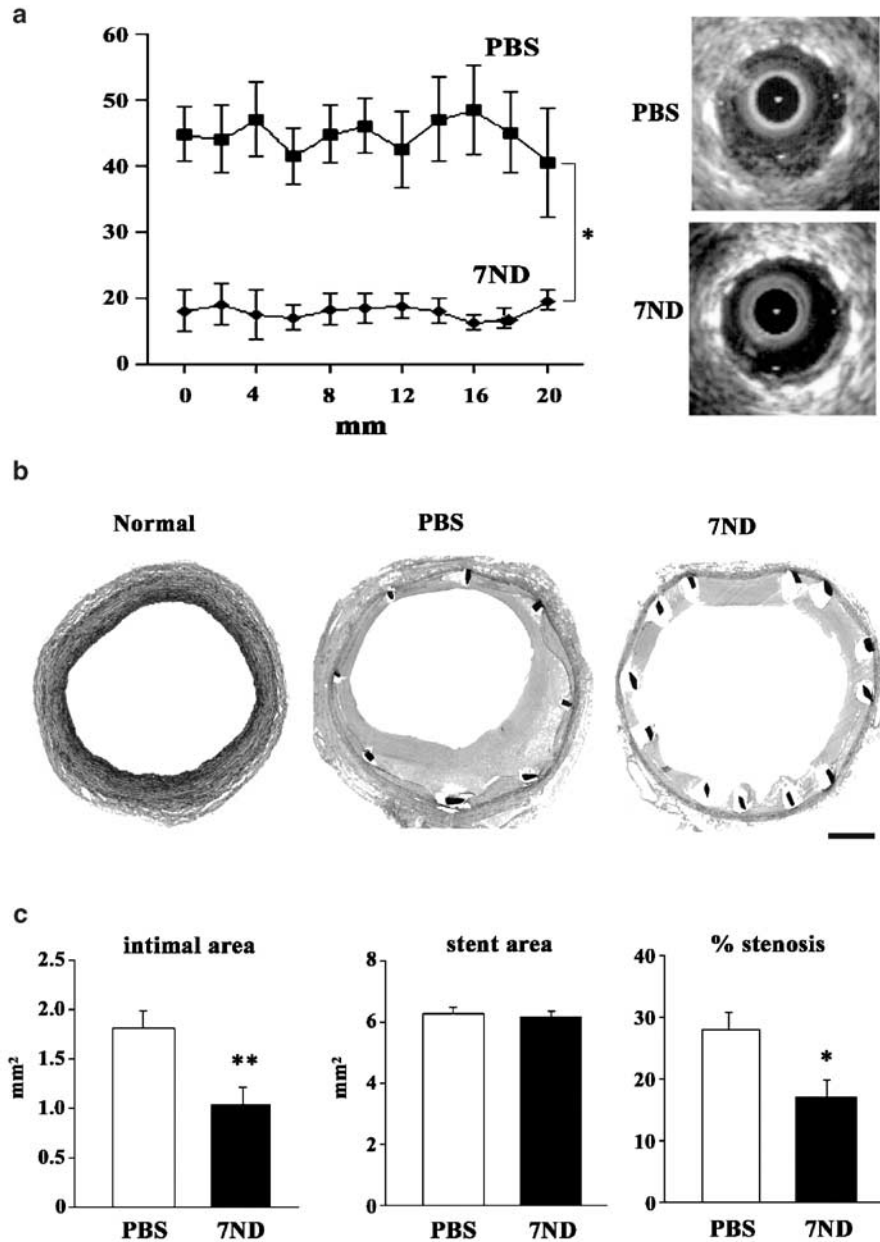


Figure 3 Effects of 7ND gene transfer on in-stent neointimal hyperplasia in rabbits. (a) Left: mean percent area stenosis within stent in the PBS-treated and 7ND-transfected rabbits as assessed by intravascular ultrasound. X-axis: distance from the distal to the proximal stent end; Y-axis: % cross-sectional stenosis. The upper right panel shows an intravascular ultrasound cross-section image in PBS-treated animal with large neointimal hyperplasia. The lower right panel displays an intravascular ultrasound cross-section image in 7ND-transfected animal with small neointimal hyperplasia. * $P < 0.01$ between PBS and 7ND. (b) Noninjured control artery section (left panel) and artery sections from the PBS-treated group (middle panel) and 7ND-transfected group (right panel) 28 days after stenting stained with elastic van-Gieson in rabbits. Bar = 600 μm . (c) Effect of 7ND gene transfer on intimal area, stented area, and % stenosis 28 days after stenting in rabbits ($n = 8$ each). * $P < 0.05$, ** $P < 0.01$ versus PBS.

described previously^{3,10}; neutrophils surrounding stent struts are observed only transiently within early stages, whereas chronic inflammatory cells such as monocytes are observed both in early (within 7 days) and late stages (6 months or later) after stenting. Increased monocyte inflammation is associated with greater neointimal formation after stenting in animals²⁵ and humans.¹⁰ Furthermore, a monoclonal antibody against the adhesion molecule Mac-1 reduced monocyte recruitment and neointimal formation after rabbit iliac artery stenting.²⁶ As inflammation is unavoidable during stent placement, therapies directed against stent-induced inflammation

are a reasonable approach to reduce stent-associated restenosis. Recent experimental studies indicate that a drastic reduction in neointimal formation with rapamycin-eluting stents is mediated by its antiproliferative and anti-inflammatory effects.⁶

There is a rapid increase in plasma MCP-1 within the first day of coronary intervention.^{27,28} Persistent increase in MCP-1 after angioplasty is a significant and independent predictor of restenosis.²⁷ We demonstrated increased immunostaining of MCP-1 after stenting. 7ND gene transfer markedly attenuated inflammatory and proliferative changes and increased apoptotic cell death,

Table 2 Plasma concentrations of 7ND after empty or 7ND plasmid transfection in rabbits

	Baseline	Days after transfection			
		3	7	14	28
No transfection (pg/ml)	<20.0 (below detectable limits)	<20.0	<20.0	<20.0	<20.0
Empty plasmid transfection (pg/ml)	<20.0	<20.0	<20.0	<20.0	<20.0
7ND transfection (pg/ml)	<20.0	96 ± 19	76 ± 15	63 ± 9	<20.0

Values are mean ± s.e.m., *n* = 6–8.

Table 3 Tissue concentrations of 7ND 3 days after empty or 7ND plasmid transfection in rabbits

	Tissue (pg/mg)
No transfection	<20.0
Empty plasmid transfection	<20.0
7ND plasmid transfection	306 ± 39

Values are mean ± s.e.m., *n* = 5–6.

and thus suppressed in-stent neointimal hyperplasia. Our present data, therefore, suggest that locally produced MCP-1 not only induced the recruitment of monocytes but also activated lesional monocytes and vascular smooth muscle cells to produce the inflammatory cytokines and growth factors (IL-6, IL-1 β , VEGF), which in turn might result in in-stent neointimal hyperplasia. We previously reported that 7ND gene transfer suppressed neointimal formation after balloon or cuff injury.^{17–19} The present study therefore extends our previous reports with regard to the beneficial effects of 7ND gene transfer on balloon or cuff injury-induced neointimal hyperplasia to in-stent neointimal hyperplasia.

Our finding in non-human primates might have clinical significance, because many therapeutic strategies that have proven effective in reducing restenosis in nonprimate animal model failed to demonstrate a substantial effect on human restenosis. Horvath *et al*²³ demonstrated that injection of an antibody against murine CCR2 attenuated neointimal hyperplasia after iliac arterial stenting in normocholesterolemic cynomolgus monkeys. The magnitude of inflammation and neointimal formation, however, was markedly less in the study by Horvath *et al*²³ (mean intimal thickness in animals with no treatment: 0.25 ± 0.03 mm) than in the present study (0.33 ± 0.02 mm) and those reported in humans^{3,10} (0.3–0.5 mm). Small-scale neointimal formation is also reported in normocholesterolemic rabbits (0.13 ± 0.03 mm).²⁹ This difference might be because hypercholesterolemic animals were used in the present study. Although the appropriate animal model for evaluation of experimental in-stent restenosis is uncertain, the hypercholesterolemic non-human primate model might have an advantage over normocholesterolemic nonprimate animal models, because (1) adequate

degrees of neointimal hyperplasia develop after stenting and (2) vascular inflammatory and proliferative responses to injury in non-human primates are presumed to be closer to those in humans than other nonprimate models. Therefore, the use of non-human primates might allow us to evaluate the efficacy of therapies such as 7ND gene transfer on in-stent neointimal hyperplasia under more reliable conditions.

Although this anti-MCP-1 therapy by 7ND gene transfer has yet to be tested for the prevention of restenosis in humans, the beneficial effects observed in rabbit and monkey models suggest a significant potential for this new mode of treatment. For translational research, we must determine as to what is the effective range of 7ND concentration in humans. Basal plasma levels of MCP-1 protein in patients before PCI are reported to be 500 pg/ml.²⁷ Other reports show plasma MCP-1 levels in patients to be in the range of 150–852 pg/ml.^{28,30,31} Compared to plasma MCP-1 levels in humans, 7ND levels achieved by i.m. transfection of 7ND gene in the present study seem to be less. Therefore, further studies are needed to determine the effective range of 7ND concentration in humans before this anti-MCP-1 strategy proceeds to clinical study. As no antibody against 7ND protein was detected after 7ND transfection, it is unlikely that the observed effects of 7ND were due to anti-7ND antibody formation. If this mode of treatment is effective and safe, it could be used as an independent therapy for high-risk lesions, small vessels (<2.5 mm in diameter), or recurrent restenosis. It could also be used as an adjunct therapy for restenosis after drug-eluting stents. 7ND-eluting stents might be associated with a drastic reduction in restenosis in humans.

In conclusion, this study provides evidence that MCP-1-mediated inflammation is an essential mediator in the development of experimental restenosis (neointimal formation) after stenting. Inhibition of stent-associated inflammation by 7ND gene transfer might be a promising next-generation gene therapy to reduce restenosis and to improve clinical outcome after stent placement.

Materials and methods

Plasmid expression vectors

Human 7ND cDNA was constructed by recombinant polymerase chain reaction (PCR) using a wild-type human MCP-1 cDNA (Dr T Yoshimura, National Cancer

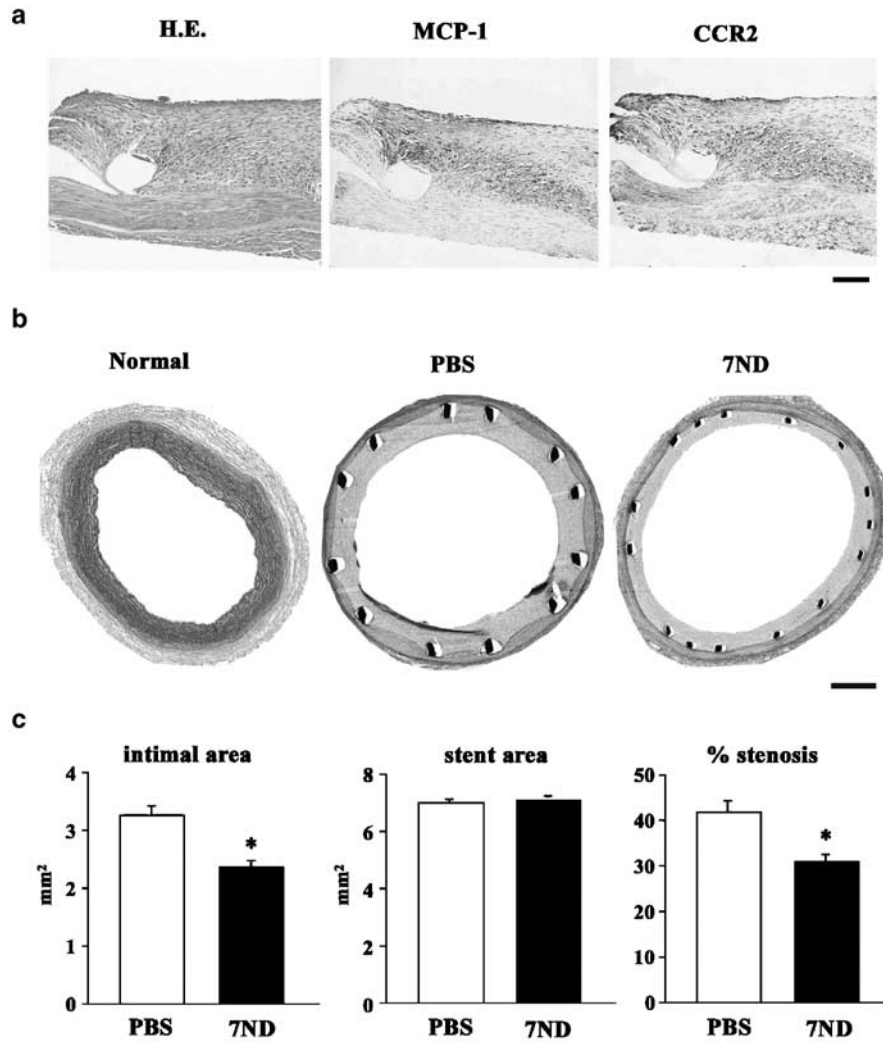


Figure 4 Effects of 7ND gene transfer on in-stent neointimal hyperplasia in cynomolgus monkeys. (a) Arterial sections of stented artery from the PBS group stained with HE or immunohistochemically with the antibodies against MCP-1 and CCR2 28 days after stenting in monkeys. Bar = 100 μ m. (b) Noninjured control artery section (left panel) and artery sections from the PBS-treated group (middle panel) and 7ND-transfected group (right panel) 28 days after stenting stained with elastic van-Gieson in monkeys. Bar = 500 μ m. (c) Effect of 7ND gene transfer on stented area, intimal area, and % stenosis 28 days after stenting ($n = 8$ each). * $P < 0.01$ versus PBS.

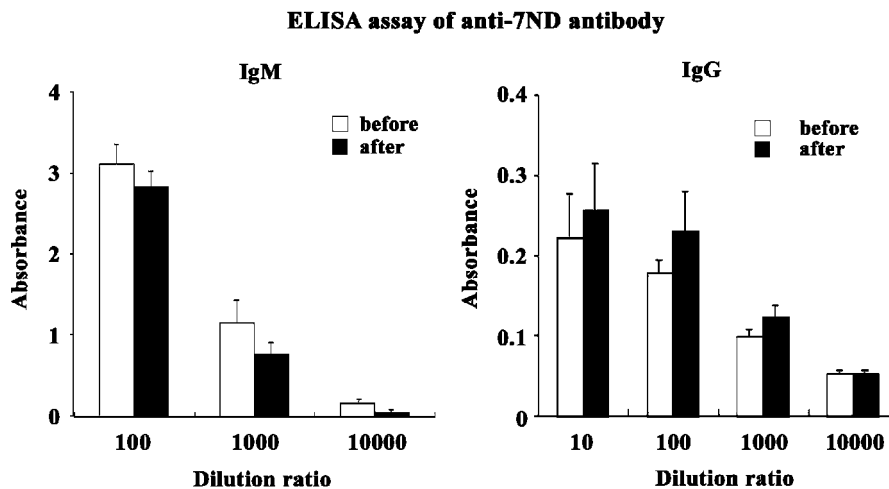


Figure 5 ELISA assay of anti-7ND antibody (IgM and IgG) in paired serum from 7ND-transfected monkeys.

Institute, Japan) as a template and inserted into the BamHI (5') and NotI (3') sites of the pcDNA3 (Invitrogen) expression vector plasmid.¹⁵

Animal model of in-stent restenosis and gene transfer

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

Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing 3.0–3.5 kg were fed a high cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks. They were then anesthetized by i.m. injection of xylazine (5 mg/kg) and ketamine (35 mg/kg). The right common carotid arteries were surgically exposed, and a 3F Fogarty balloon catheter was passed into one iliac artery under fluoroscopic guidance, and the balloon was then withdrawn and inflated over a 3-cm section of artery three times; then, a 15 mm-long Multilink stent (RX Multi-link plus, Guidant/ACS Inc.) mounted over the 3-mm balloon was implanted in the iliac artery (30-s inflation, 10 atm, stent-to-artery ratio of 1.2:1.0). When the stent was intended to be implanted bilaterally, the procedure was repeated in the contralateral iliac artery. The carotid artery was then ligated and the incision was closed. After the operation, all rabbits were fed the same high cholesterol diet.

At 3 days before stenting, rabbits were randomly divided into two groups: the PBS group (16 stents in 14 rabbits) was injected with PBS and the 7ND group (16 stents in 14 rabbits) was injected with the 7ND gene (500 µg/0.3 ml PBS) into their femoral muscle. To enhance transgene expression, all plasmid-injected animals received electroporation at the injection site immediately after injection with an electric pulse generator CUY21 (BTX, San Diego, CA, USA) as described previously.^{17,19} All rabbits were killed with a lethal dose of anesthesia 7 or 28 days after stenting for biochemical, immunohistochemical, and morphometric analyses. IVUS study was also performed 28 days after stenting.

Male adult cynomolgus monkeys (5 years old) weighing 4–5 kg were purchased from Primate Ltd., Kumamoto, Japan and were fed laboratory diet containing 0.5% cholesterol starting 2 months before stent implantation. Monkeys were anesthetized with ketamine hydrochloride (10 mg/kg i.m.) and sodium pentobarbital (30 mg/kg i.v. to effect), and underwent placement of a 3 × 15 mm² stent as described above. Monkeys were also injected with PBS (eight stents in six monkeys) or 7ND plasmid (2.5 mg/500 µl PBS, eight stents in six monkeys) into their femoral muscle immediately after stenting. To enhance transgene expression, all monkeys were pretreated with i.m. injection of bupivacaine (0.25 mg/kg) at the injection site.¹⁷ We reported the biologic efficacy of 7ND gene transfer using *in vivo* matrigel plug assay in monkeys.¹⁷ In brief, MCP-1-induced inflammatory angiogenesis in the plugs was suppressed until 14 days after 7ND gene transfer. All monkeys were killed with a lethal dose of anesthesia 28 days after stenting for morphometric analysis. All animals received aspirin 81 mg/day and ticlopidine 100 mg/day until euthanasia.

Histopathology and immunohistochemistry

Stented arterial segments were excised and fixed for 24 h with 6–10% paraformaldehyde or methacarn solution for several days. Each segment was divided into two parts at the center of the stent. The proximal part was embedded in methyl methacrylate mixed with *n*-butyl methacrylate to allow for sectioning through metal stent struts. Sections were stained with elastica van Gieson and hematoxylin–eosin (HE). To evaluate the thickening of the neointima, the areas encroached by the external elastic lamina, the internal elastic lamina, and the lumen area were measured. The distal part was used for immunohistochemical analysis. After stent struts were gently removed with microforceps, the tissue was dehydrated, embedded in paraffin, and cut into 5-µm thick slices. In rabbits, the sections were subjected to immunostaining using antibodies against rabbit monocytes/macrophages (RAM11, Dako), proliferating cell nuclear antigen (PCNA) (Dako), MCP-1 (R & D), endothelial cell (CD31, Dako), VEGF (Santa Cruz), or non-immune mouse IgG (Dako). The cells that underwent apoptosis were detected by using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (*in situ* apoptosis detection kit, Takara). The number of apoptotic nuclei in arterial wall was counted, and the data were calculated and expressed as the percentage of apoptotic cells nuclei/total nuclei. In monkeys, the sections were immunohistochemically stained with antibodies against MCP-1 (R & D), CCR2 (Sigma Chemical), or non-immune mouse IgG (Dako). The slides were washed and incubated with biotinylated, affinity-purified goat IgG. Following avidin–biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin. The percentage of immunopositive cells per total cells in each section was calculated, and the average of five sections was reported for each animal.

Intravascular ultrasound procedure and analysis

High-resolution IVUS analysis was performed 28 days after stenting with an electron-scan IVUS system (Terumo TU-C100, Tokyo, Japan). A contour detection program was used for two-dimensional analysis. Quantitative IVUS measurements were performed at 10 cross-sections in each stent segment at 2-mm intervals from the distal stent end. Cross-sectional narrowing was calculated as the neointimal area divided by stent area. All the measurements were performed by a single sonographer who was unaware of the experimental protocol.

Plasma and tissue measurements

Plasma total cholesterol and LDL cholesterol were measured using a commercially available kit. To measure 7ND released by the transfected skeletal muscle, plasma and femoral skeletal muscle concentrations of 7ND were measured using the human MCP-1 enzyme-linked immunosorbent assay kit (Biosource) in rabbits.¹⁹ As this human MCP-1 ELISA kit does not react with the rabbit MCP-1, we can measure plasma 7ND in rabbits.

Real-time reverse quantitative transcription-PCR

The normal and stented arteries were excised and washed with cold normal saline 7 days after stent placement. Stent was removed gently. The samples were

cleaned of excessive perivascular tissue, and frozen in liquid nitrogen. Total RNA was reverse transcribed, and the resultant cDNA was amplified by TaqMan Real-Time Reverse Transcription-PCR on the ABI Prism 7000 Sequence Detection as described previously.¹⁸ The respective PCR primers and TaqMan probes were designed from GenBank databases using a software program (Applied Biosystems; Table 1). The results were analyzed using the Sequence Detection Software (Applied Biosystems) and expressed in arbitrary units and adjusted for GAPDH mRNA levels.

Measurement of antibody productions in 7ND-transfected animals

We examined whether anti-7ND IgG and IgM antibodies were produced in 7ND-transfected monkeys. In all, 96-well plates (ELISA PLATE HTYPE, SUMITOMO BAKELITE Co., Ltd.) were coated with 7ND protein (0.1 µg/ml). Paired serum before and 7 or 28 days after 7ND transfection was incubated on each coated well for 90 min at 37°C followed by incubation with HRP-conjugated goat antibodies against monkey IgG or IgM (Kirkegaard & Perry Laboratories) for 1–2 h at 37°C. TMB one solution (Promega) was used, and the absorbencies of each well were detected by using ELISA plate reader.

Statistical analysis

Data are expressed as the mean ± s.e. Statistical analysis of differences was compared by analysis of variance and Bonferroni's multiple comparison tests. A *P*-value of less than 0.05 was considered to be statistically significant.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (14657172, 14207036) from the Ministry of Education, Science, and Culture, Tokyo, Japan, by Health Science Research Grants (Comprehensive Research on Aging and Health, and Research on Translational Research) from the Ministry of Health Labor and Welfare, Tokyo, Japan, and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Tokyo, Japan.

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