Local Delivery of Anti-Monocyte Chemoattractant Protein-1 by Gene-Eluting Stents Attenuates In-Stent Stenosis in Rabbits and Monkeys

Kensuke Egashira, Kaku Nakano, Kisho Ohtani, Kouta Funakoshi, Gang Zhao, Yoshiko Ihara, Jun-ichiro Koga, Satoshi Kimura, Ryuji Tominaga, Kenji Sunagawa

Objective—we have previously shown that the intramuscular transfer of the anti–monocyte chemoattractant protein-1 (MCP-1) gene (called 7ND) is able to prevent experimental restenosis. The aim of this study was to determine the in vivo efficacy and safety of local delivery of 7ND gene via the gene-eluting stent in reducing in-stent neointima formation in rabbits and in cynomolgus monkeys.

Methods and Results—we here found that in vitro, 7ND effectively inhibited the chemotaxis of mononuclear leukocytes and also inhibited the proliferation/migration of vascular smooth muscle cells. We then coated stents with a biocompatible polymer containing a plasmid bearing the 7ND gene, and deployed these stents in the iliac arteries of rabbits and monkeys. 7ND gene-eluting stents attenuated stent-associated monocyte infiltration and neointima formation after one month in rabbits, and showed long-term inhibitory effects on neointima formation when assessments were carried out at 1, 3, and 6 months in monkeys.

Conclusions—strategy of inhibiting the action of MCP-1 with a 7ND gene-eluting stent reduced in-stent neointima formation with no evidence of adverse effects in rabbits and monkeys. The 7ND gene-eluting stent could be a promising therapy for treatment of restenosis in humans. (Arterioscler Thromb Vasc Biol. 2007;27:2563-2568.)

Key Words: restenosis ▪ inflammation ▪ leukocytes ▪ stents ▪ smooth muscle cells

The use of polymer-coated drug-eluting stents (DES) for local drug delivery has proved to be a useful strategy for the prevention of restenosis.1–3 However, recent clinical reports raise the possibility of a risk of stent thrombosis in DES compared with bare metal stent.4–6 Drugs released from first-generation DES (sirolimus or paclitaxel) exert distinct biological effects3,4: although primarily aimed to prevent vascular smooth muscle cell (VSMC) proliferation, which is one of central factors in the pathogenesis of restenosis, they also impair reendothelialization, which leads to delayed arterial healing and thrombogenesis. The use of sirolimus-eluting stents in a porcine model was associated with no apparent long-term effects and with the delayed inflammation and proliferation.7,8 In human pathologic study with 40 patients who died after the currently-approved DES implantation, it was suggested that the DES caused a persistent fibrin deposition and delayed reendothelialization compared with bare metal stent implantation.9 Therefore, the development of a novel DES system with less adverse effects is needed.

We have recently devised a new gene therapy strategy for the delivery of the anti–monocyte chemoattractant protein-1 (MCP-1) in which plasmid cDNA encoding a mutant MCP-1 gene is transfected into skeletal muscle.10 This mutant MCP-1 protein, called 7ND, lacks the N-terminal amino acids 2 through 8 and has been shown to function as a dominant-negative inhibitor of MCP-1. Using this systemic gene transfer strategy, we have demonstrated that blocking MCP-1-derived signals reduced neointima formation after balloon- and stent-induced injury11–14 and atherosclerosis15,16 in animals, including nonhuman primates. Overall, these data suggest that an antiinflammatory strategy targeting MCP-1 may be an appropriate and reasonable approach for the prevention of restenosis.

Local delivery of 7ND through a gene-eluting stent may have advantages beyond those of the current first-generation DES devices: 7ND does not affect endothelial regeneration and proliferation11 and may also inhibit proliferation of VSMC.17,18 Previous studies have reported that stents coated with a polymer emulsion containing plasmid DNA were able to effect successful transgene delivery and expression in arteries.19–21 In this study, we examined the possibility that a 7ND gene-eluting stent might reduce in-stent neointima...
formation. To assess its potential clinical utility, we used a nonhuman primate model of stent-associated neointima formation. The specific aims of this study were (1) to use biocompatible polymer technology to create a 7ND gene-eluting metallic stent; (2) to determine whether the 7ND gene-eluting stent was able to reduce in-stent inflammation and neointima formation, and to assess any potential adverse effects in vivo; and (3) to determine the effects of the 7ND protein on the chemotaxis of mononuclear leukocytes and on the proliferation of VSMCs in vitro.

Materials and Methods

Plasmid Expression Vectors
This section is available in the supplemental materials (available online at http://atvb.ahajournals.org).

Stent Preparation and Measurement of In Vitro DNA Release Kinetics
A 15-mm-long stainless-steel balloon-expandable stent was dip-coated under sterile conditions with multiple thin layers of biocompatible polymer (polyvinyl alcohol [PVOH], GOHSENOL EG-05, Nippon Gohsei Inc) The polymer solution additionally contained either the 7ND cDNA plasmid, the GFP plasmid, or the β-galactosidase plasmid; polymer containing no added plasmid was also included as a control. The coated stent was then mounted over a 3-mm balloon catheter; a noncoated stent mounted over the same balloon catheter was used as a control. To measure DNA release kinetics in vitro, the 7ND plasmid-coated stents (n=8) were immersed in Tris-EDTA buffer, and the plasmid that was subsequently eluted into the buffer was measured using a thiazole fluorescence assay. Additional details are in the online data supplement.

Stent Implantation and Analysis in the Rabbit Model
The animal model 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 the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing 3.0 to 3.5 kg were fed a high-cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks before stent implantation. Animals were anesthetized and were randomly divided into 2 groups, which underwent deployment of either a noncoated bare metal stent (n=14) or a 7ND gene-eluting stent (n=14) in the right femoral artery as described previously. All animals received aspirin at 20 mg/d until euthanasia from 3 days before stent implantation procedure. After venous blood samples were taken, animals were euthanized with a lethal dose of anesthesia at days 10 (n=7 each) and 28 (n=7 each), and the stented arterial sites and contralateral nonstented sites were excised for biochemical, immunohistochemical, and morphometric analyses. In addition, the plasma levels of total cholesterol and low-density lipoprotein cholesterol were determined with commercially available kits (Wako Pure Chemicals).

The stented artery segments were processed as described previously. Additional details are in the online data supplement.

Stent Implantation and Analysis in the Monkey Model
This section is available online.

Purification of the 7ND Protein
This section is available online.

Protein Expression of the MCP-1 Receptor (CCR2)
This section is available online.

Leukocyte Chemotaxis Assay
This section is available online.

Proliferation Assay in Vascular Smooth Muscle Cells
This section is available online.

Angiogenic Activity of Endothelial Cells
This section is available online.

Agarose Gel Electrophoresis and Cell Transfection Studies
This section is available online.

Statistical Analysis
Data are expressed as means±SE. The statistical analysis of differences between 2 groups was assessed with the unpaired t test, and the statistical analysis of differences among 3 groups was assessed by using ANOVA and Bonferroni multiple comparison tests. Probability values <0.05 were considered to be statistically significant.

Results

Kinetics of DNA Release and Expression of Plasmid DNA
Scanning electron microscopy analysis revealed that polymer coating formed a uniform film over the outer surface of the stent (supplemental Figure 1A). After balloon expansion, the polymer stretched, but no fragmentation was observed. An analysis of the plasmid DNA release kinetics in vitro showed an early burst of release, such that ~80% of the total amount released was present 1 day after implantation, and maximal release occurred by 3 days after implantation (supplemental Figure 1B). Analysis of the DNA eluted from the stent by agarose gel electrophoresis showed that the DNA was structurally intact, and the functionality of the eluted DNA was confirmed by the ability of an eluted GFP plasmid to successfully be transfected and expressed in THP-1 cells and human coronary artery VSMC (hCASMC; supplemental Figure II).

Before examining the stent-based administration a plasmid encoding the 7ND protein, we first tested the stent-based delivery of the bacterial lacZ gene, which encodes the easily detectable protein β-galactosidase. Three days after stent implantation in the rabbit iliac artery, we saw expression of β-galactosidase at the gene-eluting stent site, but not at the site of implantation of a bare, non-coated metal stent, which was used as a negative control (Figure 1). X-gal staining of cross-sections was used to detect the expressed protein, and revealed that staining for β-galactosidase was localized mostly in the intima and on the luminal side of the media, and was present at a lesser extent in the adventitia. No induction of protein β-galactosidase was observed 7 days after stent implantation.

Effects of 7ND on Neointima Formation in Rabbit and Monkey Animal Models
The infiltration of RAM-11–positive macrophages around the stent strut for the non-coated control stent was observed at 10 days after stent implantation (Figure 2); this was consistent with our previous results. In contrast, the 7ND gene-eluting stents reduced the severity of macrophage-induced inflammation (Figure 2). Although an in-stent neointima formed similarly in the non-coated stent and 7ND gene-
eluting stent (histopathologic pictures in supplemental Figure IVA), quantitative analysis demonstrated a significant reduction in neointima formation in the 7ND gene-eluting stent site compared with the noncoated control stent sites (Figure 3A). However, there were no significant differences in stent area, IEL area, or medial area between rabbits receiving either the noncoated stent or the 7ND-eluting stent.

We also examined the effect of 7ND gene-eluting stents on inflammation and neointima in a monkey model. At sites in which a noncoated stent was implanted, an in-stent neointima was present at 1, 3, and 6 months after stenting (histopathologic pictures in supplemental Figure IVB). Quantitative analysis revealed that there was a significant reduction in neointima formation at sites in which the 7ND gene-eluting stent had been implanted compared with the noncoated control stent sites (Figure 3B). There were no significant differences in stent area, IEL area, or medial area between the 2 groups.

Histological and Biochemical Analysis
Biochemical analysis showed that after stenting, serum concentrations of MCP-1 increased transiently after deployment of bare metal and 7ND gene-eluting stents in monkeys. There was no significant differences in MCP-1 levels between the 2 groups (supplemental Figure V).

A histological analysis showed that there was no significant difference in the injury score or the inflammation score between the two groups of rabbits (supplemental Tables I and II) or monkeys (supplemental Table III). The endothelial cell linings, as monitored by CD31 immunoreactivity, were present at an approximately equal extent in the 2 groups (supplemental Tables II and III).

Delivery of 7ND gene-eluting stents did not have any significant effect on serum cholesterol levels, as serum cholesterol was similar in animals receiving the noncoated stent or the 7ND-coated stent; this was true both in rabbits (data not shown) and in monkeys (supplemental Table IV).
We additionally measured body weight, serum biochemical markers, and blood cell count in monkeys (supplemental Tables IV, V, and VI) and found no systemic adverse effects resulting from 7ND administration or significant treatment-associated differences in body weight between the 2 groups.

The Presence of CCR2 Protein on Human Coronary Arterial Smooth Muscle Cells

To validate our method for CCR2 detection, Western blot analysis was performed in peritoneal macrophages as control. Protein expression of CCR2 was actually detected in peritoneal macrophages isolated from wild-type mice. In contrast, no signal was detected in CCR2-knockout mice (supplemental Figure IIA). Immunoblot was then performed in hCASMC and human macrophages (THP-1) using the same antibody. The presence of CCR2 was detectable in hCASMCs as well as in human macrophages (supplemental Figure IIB).

Effect of the 7ND Protein in Cultured Vascular Cells

The 7ND protein inhibited the MCP-1-induced chemotaxis of mononuclear cells (Figure 4A). The dose of 7ND at which 50% of the observed chemotaxis was inhibited (IC$_{50}$), was at a ratio of 1:10 relative to the concentration of the MCP-1. This inhibition was specific for MCP-1, as 7ND had no effect on the interleukin (IL)-8–induced chemotaxis of polymorphonuclear leukocytes. 7ND inhibited the MCP-1–induced proliferation of hCASMCs (Figure 4B).

To examine the effects of 7ND on endothelial proliferation, we examined whether 7ND had any effect on the known capacity of VEGF to increase the capillary density of CD31-positive endothelial cells, and found that 7ND had no apparent effect on VEGF-induced angiogenic activity (Figure 4C).

Discussion

In this study we found that implantation of a 7ND gene-eluting stent reduced in-stent neointima formation with no evidence of adverse effects in rabbits or in nonhuman primates (cynomolgus monkeys). Although there is currently no clear consensus regarding which animal model (rabbit, dog, pig, monkey, etc.) is most appropriate for the evaluation of in-stent restenosis, nonhuman primate models may have advantages over nonprimate animal models, because the results of efficacy and safety tests performed in such nonhuman primates can be applied to humans. Therefore, the use of nonhuman primates may allow for the evaluation of the efficacy and safety of therapies under conditions that more closely approximate those of the human physiology. The results presented here support the notion that MCP-1 plays a central role in the pathogenesis of in-stent neointima formation (in-stent restenosis), and also provide evidence for feasibility of using the 7ND gene-eluting stent for prevention of in-stent restenosis in a human interventional setting.

Although DES reduces the rate of restenosis and target-vessel revascularization below 10%, increased risk of late in-stent thrombosis resulting in acute myocardial infarction and death after the use of the first-generation DES devices is becoming a big problem. Silorimus and paclitaxel have
been shown to impair reendothelialization and arterial healing process, resulting thrombogenesis attributable to increased expression of tissue factor. In addition, nonbiocompatible polymers used to load these drugs have been associated with DES thrombosis. However, no such adverse reactions were noted in this study especially in monkeys even after cessation of ticlopidine. In addition, 7ND showed no effect on proliferation of human endothelial cells in vitro. This suggests that the 7ND gene transfer does not appear to impair the healing process of endothelial cells in a stented arterial wall, so in these respects, this approach may have an advantage over the first-generation DES devices. We have shown that the biocompatible polymer and plasmid DNA coating material used in this study did not appear to cause any adverse reactions during a 1-month observation period in rabbits and during a 6-month observation period in monkeys. Therefore, we suggest that the blockade of MCP-1 via the 7ND gene-eluting stent may become a promising therapeutic strategy for treatment of restenosis, and that this strategy may have a low level of potential adverse effects.

From a perspective of clinical applicability, it is important to take into account any potential systemic toxicity associated with stent-based delivery of 7ND DNA plasmid. We demonstrated that 7ND gene-eluting stent, which elutes plasmid DNA at a dose of \( \approx 0.8 \text{ mg/g body weight} \) in rabbits (BW=about 3.5 kg) and \( \approx 0.16 \text{ mg/kg in monkeys} \) (BW=about 5 kg), did not induce any significant inflammatory or immune reactions. We have previously reported that the systemic intramuscular transfer of plasmid DNA encoding the 7ND gene at doses ranging from 0.5 to 10 mg/kg was nontoxic and safe in nonhuman primates, rabbits, and mice. In addition, knockout mice lacking MCP-1 or the MCP-1 receptor (C-C chemokine receptor 2: CCR2) displayed no serious health problems, suggesting that inhibition of MCP-1 is not physiologically toxic. From a toxicological point of view, because the dose of 7ND plasmid eluted from stents would be even lower in human subject (\( \approx 0.01 \text{ mg/kg for patients weighing 80 kg} \)), it would be unlikely that the 7ND gene-eluting stent would cause any toxicity in humans. In clinical trials of plasmid DNA-based gene therapy in which DNA was administered into the lower limb, myocardium, or coronary artery at 2 to 4 mg/body, no systemic adverse effects were reported. Overall, these safety and feasibility data support the notion that stent-based gene therapy could safely be applied to human subjects.

We have previously reported that 7ND gene transfer not only suppressed inflammation (monocyte infiltration), but also reduced the number of proliferating SMCs in the neointima after injury. Therefore, besides monocyte-mediated inflammation, we hypothesized that 7ND inhibits MCP-1–induced proliferation of SMCs. This notion is in line with several recent reports demonstrating that (1) mRNA and protein for the receptor for MCP-1, CCR2, are detectable in vascular SMCs; and (2) MCP-1 induces SMC proliferation in vitro. However, the effects of MCP-1 and CCR2 on SMC proliferation are controversial: several studies reported that MCP-1 either has no effect or inhibits proliferation. These conflicting conclusions are discussed to result from species specificity for MCP-1 activity in an article where human MCP-1 was used to proliferate human SMCs. Furthermore, MCP-1 induces tissue factor in murine SMCs from CCR2-/- mice, suggesting the possible presence of alternate MCP-1 receptor in murine SMCs. Therefore, we used human MCP-1 to stimulate hCASMCs in culture, and found that in addition to potent inhibitory actions on monocyte chemotaxis, 7ND inhibited proliferation of hCASMCs induced by human MCP-1. The presence of the receptor for MCP-1, CCR2, on the hCASMCs was also established. Therefore, our present data suggest that 7ND directly inhibits human SMC proliferation, in addition to its known effects on monocytes present in the in-stent vascular lesion.

In conclusion, strategy of inhibiting the action of MCP-1 with a 7ND gene-eluting stent reduced in-stent neointima formation with no evidence of either systemic or local adverse effects in rabbits and monkeys. These data suggest that anti–MCP-1 gene therapy via 7ND gene-eluting stents may be a clinically relevant and feasible therapeutic strategy for the treatment of in-stent restenosis. Further clinical trials are needed to examine this possibility.

**Sources of Funding**

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**Disclosures**

Dr Egashira holds a patent on the results reported in the present study.

**References**


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Table I. Histological scoring 1 month after stent implantation in rabbits

<table>
<thead>
<tr>
<th>Variable</th>
<th>BMS (n=8)</th>
<th>7NDES (n=8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neointima</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>1.86 ± 0.31</td>
<td>1.74 ± 0.19</td>
<td>0.28</td>
</tr>
<tr>
<td>Foreign reaction</td>
<td>0.61 ± 0.38</td>
<td>0.45 ± 0.57</td>
<td>0.48</td>
</tr>
<tr>
<td>Fibrinoid deposits</td>
<td>0.05 ± 0.16</td>
<td>0.14 ± 0.32</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Media</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction missing</td>
<td>0.40 ± 0.39</td>
<td>0.26 ± 0.41</td>
<td>0.44</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0.20 ± 0.38</td>
<td>0.33 ± 0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.00 ± 0.00</td>
<td>0.09 ± 0.20</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Adventitia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.44 ± 0.64</td>
<td>0.23 ± 0.41</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. BMS; Bare Metal Stent, 7NDES; 7ND Gene-Eluting Stent. Histopathological features were semiquantitative as defined grades for intimal, medial and adventititia. The appearance of the vasa vasorum and myocardium surrounding the artery were also systematically evaluated as described previously\(^1\). The degrees of extracellular matrix, foreign reaction, fibrinoid deposits, fraction missing, necrosis and fibrosis scored from 0 to 3, 3 was severest \(^1\).
Table II. Re-endothelialization, injury score, and inflammation score 1 month after stenting in rabbits

<table>
<thead>
<tr>
<th></th>
<th>BMS (n=8)</th>
<th>7NDES (n=8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-endothelialization (%)</td>
<td>92 ± 6</td>
<td>91 ± 5</td>
<td>0.88</td>
</tr>
<tr>
<td>Injury Score</td>
<td>1.55 ± 0.76</td>
<td>1.45 ± 0.60</td>
<td>0.68</td>
</tr>
<tr>
<td>Inflammation Score</td>
<td>0.35 ± 0.24</td>
<td>0.34 ± 0.19</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Data are Mean ± SEM (n=7 each). BMS; Bare Metal Stent, 7NDES; 7ND Gene-Eluting Stent.

The degrees of endothelial recovery (the length of CD31-positive layer/the length of IEL in cross-sections) in the three groups are also shown.

The injury score was determined at each strut site, and mean values were calculated for each stented segment. In brief, a numeric value from 0 (no injury) to 3 (most injury) was assigned: 0 = endothelial denudate, internal elastica lamina (IEL) intact; 1 = IEL lacerated, media compressed, not lacerated; 2 = IEL lacerated, media lacerated, external elastica lamina (EEL) compressed, not lacerated; and 3 = media severely lacerated, EEL lacerated, adventitial may contain stent strut. The average injury score for each segment was calculated by dividing the sum of injury scores by the total number of struts at the examined section.

The inflammation score took into consideration the extent and density of the inflammatory infiltrate in each individual strut. With regard to the inflammatory score for each individual strut, the grading is: 0 = no inflammatory cells surrounding the strut; 1 = light, noncircumferential inflammatory cells infiltrate surrounding the strut; 2 = localized, moderate to dense cellular aggregate surrounding the strut noncircumferentially; and 3 = circumferential dense inflammatory cells infiltration of the strut. The inflammatory score for each cross section was calculated in the same manner as for the injury score (sum of the individual inflammatory scores, divided by the number of struts in the examined section).
Table III. Re-endothelialization, injury score, and inflammation score 1, 3 and 6 months after stenting in non-human primate

<table>
<thead>
<tr>
<th>Re-endothelialization (%)</th>
<th>BMS</th>
<th>7NDES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 months</td>
<td>90 ± 4 (n= 10)</td>
<td>89 ± 5 (n= 12)</td>
</tr>
<tr>
<td>3 months</td>
<td>94 ± 3 (n=8)</td>
<td>95 ± 2 (n=8)</td>
</tr>
<tr>
<td>6 months</td>
<td>96 ± 2 (n=10)</td>
<td>95 ± 2 (n=8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Injury Score</th>
<th>BMS</th>
<th>7NDES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 months</td>
<td>1.1 ± 0.9 (n= 10)</td>
<td>0.9 ± 0.6 (n= 12)</td>
</tr>
<tr>
<td>3 months</td>
<td>0.9 ± 0.7 (n= 8)</td>
<td>1.1 ± 0.7 (n=8)</td>
</tr>
<tr>
<td>6 months</td>
<td>1.1 ± 0.5 (n=10)</td>
<td>1.0 ± 0.5 (n=8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inflammation Score</th>
<th>BMS</th>
<th>7NDES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 months</td>
<td>0.6 ± 0.5 (n= 10)</td>
<td>1.7 ± 0.2 (n= 12)</td>
</tr>
<tr>
<td>3 months</td>
<td>0.5 ± 0.6 (n=8)</td>
<td>0.7 ± 0.8 (n=8)</td>
</tr>
<tr>
<td>6 months</td>
<td>0.8 ± 0.5 (n=10)</td>
<td>1.4 ± 1.0 (n=8)</td>
</tr>
</tbody>
</table>

Data are Mean ± SEM. The degrees of endothelial recovery (the length of CD31-positive layer/the length of IEL in cross-sections) in the three groups are also shown. BMS; bare metal stent, GES; 7NDES; 7ND gene-eluting stent.

Table IV. Body weight among the groups in non-human primates

<table>
<thead>
<tr>
<th>body weight (kg)</th>
<th>baseline</th>
<th>1 months</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMS (n=6)</td>
<td>5.2 □ 0.2</td>
<td>5.0 □ 0.2</td>
<td>5.9 □ 0.2</td>
<td>6.0 □ 0.2</td>
</tr>
<tr>
<td>7NDES (n=5)</td>
<td>5.2 □ 0.1</td>
<td>5.7 □ 0.2</td>
<td>6.0 □ 0.5</td>
<td>5.6 □ 0.3</td>
</tr>
</tbody>
</table>

Data are Mean ± SEM. BMS; bare metal stent, 7NDES; 7ND gene-eluting stent.
Table V. Complete blood cell counts 6 months after stenting in non-human primates

<table>
<thead>
<tr>
<th>Variable</th>
<th>BMS (n=6)</th>
<th>7NDES (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (10^6/μL)</td>
<td>5.2 ± 0.4</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>35.2 ± 2.9</td>
<td>40.1 ± 1.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.8 ± 3.0</td>
<td>13.3 ± 1.0</td>
</tr>
<tr>
<td>Platelets (10^3/μL)</td>
<td>335 ± 23</td>
<td>292 ± 21</td>
</tr>
<tr>
<td>White blood cells (10^3/μL)</td>
<td>14.9 ± 1.9</td>
<td>11.7 ± 1.6</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. BMS; bare metal stent, 7NDES; 7ND gene-eluting stent.

Table VI. Time course of biochemical parameters in non-human primates

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Base line</th>
<th>1 months</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>BMS</td>
<td>116 ± 6</td>
<td>212 ± 36</td>
<td>417 ± 34</td>
<td>646 ± 55</td>
</tr>
<tr>
<td></td>
<td>7NDES</td>
<td>133 ± 21</td>
<td>231 ± 44</td>
<td>499 ± 24</td>
<td>599 ± 51</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>BMS</td>
<td>16 ± 3</td>
<td>67 ± 17</td>
<td>13 ± 1</td>
<td>18 ± 2</td>
</tr>
<tr>
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<td>7NDES</td>
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<td>18 ± 3</td>
<td>15 ± 1</td>
<td>11 ± 0</td>
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<td>Glucose (mg/dL)</td>
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<td>55 ± 3</td>
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<td>74 ± 5</td>
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<tr>
<td>GOT (Unit/L)</td>
<td>BMS</td>
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<td>86 ± 11</td>
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<td>50 ± 12</td>
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<tr>
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<td>21 ± 1</td>
<td>30 ± 5</td>
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<td>Creatinine (mg/dL)</td>
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<td>0.88 ± 0.03</td>
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<td>7NDES</td>
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<td>0.81 ± 0.08</td>
<td>0.81 ± 0.05</td>
<td>0.73 ± 0.04</td>
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Data are mean ± SEM. BMS; bare metal stent (n=6), 7NDES; 7ND gene-eluting stent (n=5), GOT; glutamate oxaloacetate transaminases, GPT; glutamate pyruvate transaminases, CPK; creatinine phosphokinase, ALP; Alkaline Phosphatase.

References
2. Schwartz RS, Huber KC, Murphy JG, Edwards WD, Camrud AR, Vlietstra RE, Holmes DR. Restenosis and the proportional neointimal response to coronary
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Materials and Methods

Purification of the 7ND protein  Recombinant 7ND was purified from serum-free conditioned medium from stably transfected CHO-cells. Medium diluted 1:1 with 0.02 mol/L Phosphate-buffer (pH 7.4) was circulated over a SP Sephadex column (Pharmacia) overnight, followed by elution using a NaCl gradient in 0.02 mol/L Phosphate-buffer (pH 7.4). Protein content was determined by the Bradford method using bovine serum albumin as a standard. Recombinant 7ND were stored as a final concentration of 100 µg/mL.

Analysis of the expression of CCR2 protein  To determine the CCR2 protein expression of human coronary arterial vascular smooth muscle cells, Western blot analysis were performed. Protein was extracted from cultured human coronary artery vascular smooth muscle cells (Cambrex Bio Science Walkersville, Inc), peritoneal macrophages from wild type mice or CCR2 knockout mice, and cultured human macrophages (THP-1). Samples were homogenized in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 50 mM NaCl, 30 mM sodium phosphate, 50 mM NaF, 1 % aprotinin, 0.5 % pepstatin A, 2 mM phenylmethylsulfonyl fluoride, and 5 mM leupeptin. Cell lysates (20 µg) were separated on 15 % polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA). Protein expression was analyzed using an antibody against human CCR2 (CKR2-B, C-20, 1:500, Santa Cruz). According to the manufacturer’s information, this antibody can detect both human and mouse CCR2 protein. Immune complexes were visualized with horseradish peroxidase-conjugated secondary antibodies. Bounded antibodies were detected by chemiluminescence with the use of an ECL detection system (Amersham Biosciences).
Leukocyte chemotaxis assay using human mononuclear and polymorphonuclear leukocytes

Fresh venous blood was collected from the ante-cubital vein of human healthy volunteers using a 19 gauge butterfly needle, and mononuclear and polymorphonuclear leukocytes were isolated by density centrifugation. Leukocyte chemotaxis assays were performed essentially as previously described in 96-well modified Boyden chambers device fitted with a 5-µm polycarbonate filter (Neuroprobe). For the chemotaxis assay of mononuclear cells, human MCP-1 at 6.25 mg/mL (Sigma, Tokyo, Japan) was added to the lower chamber and mononuclear cells (1 × 10^7 cells/mL in 25 µl) were added to the upper chamber. To assay for chemotaxis in polymorphonuclear cells, human interleukin-8 (IL-8) at 10 ng/mL was added to the lower chamber and polymorphonuclear cells were added to the upper chamber. The number of cells that migrated to the lower surface of the membrane was counted in several high-power (200 x) fields of view.

Proliferation assay in human vascular smooth muscle cells

Human coronary artery VSMCs (Cambrex Bio Science Walkersville Inc., Tennessee, USA) were cultured, and placed into 48-well culture plates. Proliferation was stimulated by the addition of either human MCP-1 at 10 ng/mL (Sigma). Either 7ND at varying concentrations or vehicle alone was added to the wells, and four days later, the cells were fixed with methanol and a single observer (who was blinded to the experimental protocol) counted the number of cells/plate.

Angiogenic activity of human umbilical vein endothelial cells

To assess angiogenic activity, we used a kit (Kurabo Industries) consisting of a 2-D coculture system of human umbilical vein endothelial cells (HUVECs)/fibroblasts. Recombinant human VEGF165 (10 ng/mL, Oncogene) was added to the basal medium, and cells were
incubated in the presence or absence of 7ND or vehicle for 11 days, with a change of medium every 3 days. Tube formation was assessed by staining with an anti-CD31 antibody according to the manufacturer’s instructions. Capillary density was measured with NIH Image software.

**Agarose gel electrophoresis and cell transfection studies**

GFP plasmid-coated stents (n=8) were immersed in Tris-EDTA as above. Aliquots of the DNA eluted into the Tris-EDTA solution were subjected to agarose gel electrophoresis and the DNA was purified from excised gel slices. The resultant DNA was digested with Hind III and electrophoresed; ethidium bromide was used to detect the DNA, which was then gel-purified. The resultant purified DNA was used to transfect THP-1 cells and hCASMC (human coronary artery smooth muscle cells) using lipofectamine. After 72 hours, the transfected cell cultures were assessed for GFP expression using fluorescence microscopy.

**Stent implantation and analysis in the rabbit model**

The animal-model 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 the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing 3.0 to 3.5 kg were fed a high cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks before stent implantation. Animals were anesthetized and were randomly divided into two groups, which underwent deployment of either a non-coated bare metal stent (n=14) or a 7ND gene-eluting stent (n=14) in the right femoral artery as described previously.4 All animals received aspirin at 20 mg/day until euthanasia from 3 days before stent
implantation procedure. After venous blood samples were taken, animals were euthanized with a lethal dose of anesthesia at days 10 (n=7 each) and 28 (n=7 each), and the stented arterial sites and contralateral non-stented sites were excised for biochemical, immunohistochemical, and morphometric analyses. In addition, the plasma levels of total cholesterol levels were determined with commercially available kits (Wako Pure Chemicals).

The stented artery segments were processed as described previously: The segment was divided into two parts at the center of the stent, and the proximal part was embedded in methyl methacrylate mixed with n-butyl methacrylate to allow for sectioning through the metal stent struts. Serial sections were stained with elastica van Gieson and with hematoxylin-eosin (HE). The neointimal area, the area within the internal elastic lamina (IEL), and the lumen area were measured by computerized morphometry, which was carried out by a single observer who was blinded to the experimental protocol. All images were captured by an Olympus microscope equipped with a digital camera (HC-2500) and were analyzed using Adobe Photoshop 6.0 and Scion Image 1.62 Software. The injury and inflammatory scores were determined at each strut site, and mean values were calculated for each stented segment (see Supplementary Table 2 online). The distal part of the stent was used for immunohistochemical analysis. After the stent struts were gently removed with micro-forceps, the tissue was dehydrated, embedded in paraffin, and cut into 5-μm thick slices. The slices were then immunostained with either non-immune mouse IgG (Dako) as a control, or with antibodies against rabbit monocytes/macrophages (RAM11, Dako) or endothelial cells (CD31, Dako). To collect and quantify images of immunohistochemical stains, stented sites were chosen in which detachment of the stent strut induced only minor injury in the neointima. At least five representative images were
collected, and the percentage of immunopositive cells per total cells in each picture was calculated; the average of the five pictures was reported for each animal.

To assess the efficacy of gene transfer, rabbits underwent γ-galactosidase plasmid-coated stent placement as described above, and lacZ gene transfer was measured 3 and 7 days after transfection by X-gal staining of sections from the target artery (n=3 each time point).

**Stent implantation and analysis in the monkey model**  Five-year-old adult male cynomologous monkeys weighing 4 to 5 kg were fed a laboratory diet containing 0.5% cholesterol starting at two months before stent implantation. Monkeys were anesthetized with ketamine hydrochloride (10 mg/kg IM) and sodium pentobarbital (30 mg/kg IV to effect), and were randomly divided into two groups, which underwent deployment of either a non-coated bare metal stent or a 7ND gene-eluting stent placement in one side or bilateral side of the iliac artery, as described previously.\(^5\) All monkeys were euthanized with a lethal dose of anesthesia prior to post-implantation morphometric analysis; the timing of euthanasia and the composition of the different groups follows: The group euthanized at 1 month after implantation consisted of 6 animals with 10 bare metal stents and 6 animals with 12 7ND gene-eluting stents. The group euthanized at 3 months after implantation consisted of 4 animals with 8 bare metal stents and 4 animals with 8 7ND gene-eluting stents. The group euthanized at 6 months after implantation consisted of 6 animals with 10 bare metal stents and 5 animals with 8 7ND gene-eluting stents. All animals received aspirin at 81 mg/day and ticlopidine at 100 mg/day starting 7 days before stent procedure. Aspirin was continued until euthanasia, and ticlopidine was stopped 28 days after stent procedure. After euthanasia, histopathologic and morphometric analyses of the target arterial sites were performed as described above and
the plasma concentrations of human MCP-1 were measured using an ELISA kit (R & D). To examine any potential adverse systemic effects resulting from 7ND gene elution from the stent in the 6-month group, blood cell count and the relevant biochemical markers and blood cell count were monitored over the entire course of the 6 months. In addition, total cholesterol plasma levels were determined with commercially available kits (Wako Pure Chemicals).

References
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Figure 1. A, Scanning electron microscopic images of the balloon expanded non-coated stent (left) and the plasmid-coated stent (right). Polymer coating formed a uniform film over the outer surface of the stent and after balloon expansion, the polymer stretched, but no fragmentation and crack were observed. B, In vitro time course of cumulative plasmid release from the eluting stents (n= 6 at each time point).
Figure 11. The structurally and functionally intact plasmid DNA eluted from the gene-eluting stents.

Left) Agarose gel electrophoresis showing intact plasmid DNA released from the gene-eluting stent: Lane 1 is GFP plasmid DNA eluted from stent surface and lane 3 is original GFP plasmid DNA. Lane 1 and lane 3 represent same band pattern, indicating that GFP plasmid eluted from stent surface were structurally intact. Lanes 2 and 4 is GFP plasmid DNA after Hind III restriction enzyme digestion. Right) The eluted GFP plasmid DNA from stent surface was subsequently transfected into THP-1 cells and hCASMC cells and expressed GFP protein in the presence of lipofectamine.
Figure III. Protein expression of the MCP-1 receptor, CCR2, in human coronary arterial vascular smooth muscle cells in vitro.

A, Representative Western blot analysis of protein extracts from peritoneal macrophages from wild type mice (C57BL/6J) and from CCR2-knockout mice (n=3 per condition).

B, Representative Western blot analysis of protein extracts from human coronary arterial vascular smooth muscle cells (hCASMCs) and from human macrophages (THP-1) (n=3 per condition).
Figure IV. Inhibitory effect of 7ND gene-eluting stents on in-stent neointima formation.

A, Iliac artery cross-sections of the bare metal and the 7ND gene-eluting stents (7NDES) 28 days after stenting in rabbits. Tissue was stained with elastic van Gieson. Bar = 500 µm.

B, Iliac artery cross-sections from the bare metal and the 7ND gene-eluting stents (7NDES) at 1, 3, and 6 months after stenting in monkeys. Tissue was stained with elastic van Gieson. Bar = 500 µm.
Figure V. Serum concentrations of MCP-1 in non-coated bare metal stent (n=6) and 7ND gene-eluting stent (n=5) groups in monkeys.