Hence, the use of nonhuman primates may work for evaluation of the efficacy of ARB on in-stent neointima formation in clinically relevant conditions.

To obtain mechanistic insight into the beneficial effects of ARB, we first examined the time course of local expression of RAS components in rabbits (Figure 2). We found that expression of all of the components (ACE, angiotensin II, AT_1 receptor, and AT_2 receptor) increased, mainly in cells composed of neointima (monocytes and smooth muscle cells), at early stages (7 to 10 days after stenting), and persisted until 28 days after stenting. This RAS activation colocalized with increased NADPH oxidase–dependent DHE fluorescence (generation of superoxide anion) and was associated with increased levels of NADPH oxidase subunit mRNAs, consistent with previous reports showing that increased reactive oxygen species can be detected in activated smooth muscle cells after balloon injury.[21,22] These in vivo observations are also consistent with previously published in vitro data suggesting that proliferation and migration of smooth muscle cells are critically mediated by oxidative stress via AT_1-mediated activation of NADPH oxidases.[23–29] Interestingly, treatment with ARB not only attenuated the levels of oxidative stress markers but also reduced the level of immunoreactive AT_1. These data suggest the presence of a positive feedback loop in which activation of AT_1 further enhances expression and activity of the AT_1 receptor in vivo, as seen in the present study.

It is known that oxidative stress–induced inflammatory and proliferative processes are central to neointima formation after vascular injury.[24,25] We and others have demonstrated that[1] increased monocyte-mediated inflammation or MCP-1 expression is associated with greater neointima formation after stenting,[26,27] and[2] anti–MCP-1 gene therapy[15,28–30] or administration of blocking antibody against the MCP-1 receptor[31] markedly reduces neointima formation after vascular injury. However, no previous study examined whether or not those inflammatory and proliferative changes after stenting depends on the AT_1 receptor. In the present study, we, therefore, examined the effects of ARB on monocyte recruitment and MCP-1 expression after stenting and found that ARB reduced monocyte/macrophage recruitment, as well as MCP-1 immunoreactivity and gene expression. Furthermore, ARB inhibition increased the expression of growth-promoting factors, such as PDGF and IL-1β. These data suggest that the beneficial effects of ARB may be attributed to the inhibition of oxidative stress–induced inflammatory and proliferative changes.

Recent studies have shown that peripheral blood contains bone marrow-derived progenitor cells, which contribute to neointima formation after injury.[16,18,32] However, the role of RAS in the recruitment/differentiation of progenitor cells into the neointimal cells after stenting has not been addressed. Here we found that differentiation to SMPCs increased in MNCs from rabbits fed a high-cholesterol diet and was further enhanced in those rabbits that had also undergone stenting. Differentiation into EPCs was not affected by either the diet or stenting. In vivo treatment with ARB suppressed the increased differentiation into SMPCs induced by diet or stenting. In contrast, in vitro treatment with angiotensin II or ARB did not affect the capacity to differentiate into SMPCs or EPCs. Therefore, the capacity to recruit or form SMPCs from MNCs after stenting might be determined by an AT1 receptor–mediated pathway in vivo and, thus, contribute to in-stent neointima formation.

It must be mentioned that ARB did not significantly reduced arterial blood pressure in rabbits. Although arterial pressure was not measured in monkeys, the dose of ARB used in the present study is reported to show no effects on arterial blood pressure in monkeys.[33] Plasma ARB level was within or below the clinical range. Furthermore, ARB did not affect serum lipid levels. Therefore, the beneficial effect of ARB on in-stent neointimal formation is likely to be independent of its effects on arterial blood pressure or serum lipid.

Perspectives

This study provides experimental evidence suggesting that oral treatment with ARB at a clinical dose range attenuates in-stent neointima formation in rabbits and nonhuman primates. The beneficial effects were associated with reduced local oxidative stress, reduced expression of MCP-1 and other inflammation-promoting factors, and reduced recruitment/differentiation of SMPCs, suggesting that ARB is of potential clinical benefit in patients who have undergone vascular interventions.

Sources of Funding

This study was supported by Grants-in-Aid for Scientific Research (14657172 and 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.

Disclosures

None.

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Stent-Based Local Delivery of Nuclear Factor-κB Decoy Attenuates In-Stent Restenosis in Hypercholesterolemic Rabbits

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Background—Nuclear factor-κB (NF-κB) plays a critical role in the vascular response to injury. However, the role of NF-κB in the mechanism of in-stent restenosis remains unclear. We therefore tested the hypothesis that blockade of NF-κB by stent-based delivery of a cis-element "decoy" of NF-κB reduces in-stent neointimal formation.

Methods and Results—Stents were coated with a polymer containing or not containing NF-κB decoy, which represented a fast-release formulation (<7 days). Bare, polymer-coated, and NF-κB decoy–eluting stents were implanted in iliac arteries of hypercholesterolemic rabbits. Increased NF-κB activity was noted at early stages after stenting, which was suppressed by stent-based delivery of NF-κB decoy. NF-κB decoy–eluting stents also reduced monocyte infiltration and monocyte chemoattractant protein-1 expression and suppressed CD14 activation on circulating leukocytes. Importantly, NF-κB decoy–eluting stents attenuated neointimal formation on day 28. There was no evidence of an incomplete healing process (persistent inflammation, hemorrhage, fibrin deposition, impaired endothelial regeneration) at the site of NF-κB decoy–eluting stents. Transfection of NF-κB decoy suppressed proliferation of human coronary artery smooth muscle cells in vitro. No systemic adverse effects of NF-κB decoy were detected.

Conclusions—Stent-based local delivery of NF-κB decoy reduced in-stent neointimal formation with no evidence of incomplete healing. These data suggest that this strategy may be a practical and promising means for prevention of in-stent restenosis in humans. (Circulation. 2006;114:&NA;-.)

Key Words: inflammation ■ monocytes ■ restenosis ■ myocytes, smooth muscle

Each year, >1.5 million patients worldwide undergo percutaneous coronary intervention for atherothrombotic lesions. Local drug delivery by drug-eluting stents is now becoming a useful strategy for prevention of restenosis because of promising results in animal studies and clinical trials.[1,2] Currently marketed first-generation drug-eluting stents use antiproliferative drugs including rapamycin, its analogues, or paclitaxel. The current antiproliferative strategies are no longer a panacea, however, because this strategy involves potential problems such as impaired endothelial regeneration and an incomplete healing process (excessive inflammation and fibrin deposition) associated with increased risk of stent thrombosis.[3–5] Lack of long-term effects of sirolimus-eluting stents due to delayed inflammation and proliferation has been reported in a porcine coronary model.[6]

Clinical Perspective p ●●●

Recent experimental and clinical studies suggest that inhibition of stent-associated inflammation (monocyte recruitment and activation) can be a promising next-generation approach.[7–9] Nuclear factor-κB (NF-κB) is a redox-sensitive transcription factor that regulates inflammation and thus plays a critical role in the vascular response to injury.[10] Activated NF-κB is detected in human atherosclerotic and restenotic lesions of smooth muscle cells, monocytes, and endothelial cells.[11] In contrast, activated NF-κB is rarely detected in normal uninjured arteries. After vascular injury, rapid activation of NF-κB in smooth muscle cells correlates with proliferation of smooth muscle cells and induced expression of NF-κB–dependent genes.[12] Recently, blockade of NF-κB by transfection of adenoviral inhibitor-κB or NF-κB "decoy" oligodeoxynucleotides attenuated restenotic changes

Received August 11, 2005; revision received October 10, 2006; accepted October 13, 2006.
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The online-only Data Supplement, consisting of expanded Methods, tables, and a figure, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.582254/DC1.
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© 2006 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org DOI: 10.1161/CIRCULATIONAHA.105.582254

(neointimal formation) after balloon injury in animal models associated with reduced NF-κB–dependent genes like monocyte chemoattractant protein-1 (MCP-1).[13,14] As a clinically feasible technique, however, these gene transfer approaches are usually hampered by prolonged arterial occlusions. It has not yet been directly determined whether blockade of NF-κB inhibits neointima formation after stenting. This is important because the mechanisms underlying neointima formation differ between balloon injury and stent-induced injury, and stenting is the most frequently performed vascular interventional technique.

We therefore created the NF-κB decoy–eluting stent using polymer technology that facilitates local delivery of the NF-κB decoy oligodeoxynucleotide during stent expansion by balloon dilatation. This decoy-eluting stent strategy is a clinically feasible approach. Although local gene delivery from a polymeric plasmid DNA-coated stent has been reported, local vascular transfer of decoy oligodeoxynucleotide by polymeric-coated stent has not been reported thus far. We herein report inhibition of in-stent neointimal formation by stent-based local transfer of NF-κB decoy in vivo.

The aims of this study were (1) to create a NF-κB decoy–eluting metallic stent by the use of water-soluble polymer; (2) to evaluate the in vivo blockade of NF-κB activation by NF-κB decoy–eluting stent implantations; and (3) to determine whether the NF-κB decoy–eluting stent attenuates stent-associated inflammation and neointimal formation in vivo.

Methods

Stent Preparation

The NF-κB decoy sequences are 5′-CCTTGAAGGGATTTCCCTCC-3′ and 3′-GGAACTTCCCTAAAGGGAGG-5′. GGGATTTCCC is the consensus sequence for the NF-κB binding site. The decoy is directed against the NF-κB binding site in the promoter region that corresponds to NF-κB–responsive genes.[15,16] The decoy works to inhibit binding of this transcription factor to the promoter region.[15,16] The NF-κB decoy oligodeoxynucleotides have been shown to bind to free NF-κB, preventing NF-κB transactivation of the cytokine genes. Because NF-κB is activated immediately after stenting, we designed the NF-κB decoy–eluting stent as an early-release formulation. The 15-mm-long stainless steel balloon-expandable stent (Kawasumi Co, Osaka, Japan) was dip-coated with multiple thin layers of polyurethane containing or not containing NF-κB decoy (500 to 600 μg per stent) under sterile conditions. Another layer of decoy-free polyurethane was applied on top of the decoy-polyurethane matrix. We selected polyurethane as a polymer matrix material for stent coating because (1) it is water soluble and therefore stably absorbs NF-κB decoy oligodeoxynucleotides and (2) metallic stents coated with polyurethane containing DNA are reported to be useful for transgene delivery to the iliac arterial wall of rabbits.[17] In addition, of a number of polymer matrix materials evaluated for stent coating, polyurethane has been shown to prevent the thrombosis and inflammation that can occur with uncoated stents and some polymers used for stent coating.[18]

The coated stent was then mounted over a 3-mm balloon catheter. Uncoated bare stent mounted over the same balloon catheter was used as a control. Before implantation, all stents were sterilized with the use of ethylene oxide.

In Vitro Kinetics

In vitro kinetics studies were performed by placing a NF-κB decoy–coated stent in Tris-EDTA buffer at 37°C. The stent was periodically removed from the buffer, and the decoy eluted into the buffer was measured by a high-performance liquid chromatography system. The incremental quantities of the decoy released from the stent were plotted against time (n=8).

Stent Implantation in Animal Models

The experiments were reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and were performed according to the US National Institutes of Health 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 divided randomly into 3 groups, which underwent deployment of either an uncoated bare metal stent (n=22); decoy-free polyurethane-coated stent (n=22); or NF-κB decoy–coated stent (n=22) in the right femoral artery as described previously.[9] All animals received aspirin at 20 mg/d until euthanasia from 3 days before the stent implantation procedure. After venous blood samples were taken, animals were killed with a lethal dose of anesthesia at days 3 (n=6 each), 10 (n=8 each), and 28 (n=8 each). Stented arterial sites and contralateral unstented sites were excised for biochemical, immunohistochemical, and morphometric analyses.

Histopathological and Immunohistochemical Analysis

The stented artery segments were processed as described previously.[9] The segment was divided into 2 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. Serial sections were stained with elastica van Gieson and hematoxylin-eosin. Neointimal area, the area within the internal elastic lamina and external elastic lamina, and the lumen area were measured by computerized morphometry. A single observer who was blinded to the experiment protocol performed morphometry. All images were captured by an Olympus microscope equipped with a digital camera (HC-2500) and were analyzed with the use of 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 as previously described[19,20] (see Table I in the online-only Data Supplement for details).

The distal part was used for immunohistochemical analysis. After stent struts were removed gently with microforceps, the tissue was dehydrated, embedded in paraffin, and cut into 5-μm-thick slices. They were subjected to immunostaining with antibodies against rabbit monocytes/macrophages (RAM-11; Dako, Glostrup, Denmark), endothelial cells (CD31; Dako), an epitope (α-p65) on the p65 subunit of NF-κB (α-p65; Boehringer Mannheim, Roche Diagnostics, Basel, Switzerland), MCP-1 (a gift from Dr Matsukawa, Kumamoto University), or nonimmune mouse IgG (Dako). The α-p65 monoclonal antibody recognizes an epitope on the p65 subunit that is masked by bound I-κB.[11] Therefore, this antibody exclusively detects activated NF-κB. For quantification of immunohistochemical images, care was taken to select stented sites with minor injury in the neointima induced by detachment of stent strut. Because this process of selecting sections with the least injury may introduce bias, at least 5 representative images were selected, and the percentage of immunopositive cells per total cells in each image was calculated. The average of the 5 images was reported for each animal.

Electrophoretic Mobility Shift Assay

The electrophoretic mobility gel shift assay was performed on nuclear extracts prepared immediately from rabbit femoral arteries after stent implantation with the method described previously.[21] For competition studies, a 50-fold molar excess of unlabeled probe for NF-κB was added. For supershift assays, 1 μg anti-p50 or anti-p65 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) antibodies was added and incubated for 20 minutes. Nuclear extracts of HeLa cells were used as positive control.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Real-time polymerase chain reaction amplification was performed with the rabbit cDNA with the use of the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif) as described previously.[22] The respective polymerase chain reaction primers and TaqMan probes were designed from GenBank databases with a software program (Applied Biosystems; Table II in the online-only Data Supplement).

Fluorescence-Activated Cell Sorting

Peripheral blood was obtained at day 10 after stent implantation (n=7 each). Flow cytometry for $CD14^+$ cells was performed with the use of R-phycoerythrin–conjugated anti-CD14 (Dako). Data were analyzed by a flow cytometer and software (Becton, Dickinson and Co, Franklin Lakes, NJ).

Blood Cholesterol Measurements

Plasma total cholesterol levels were determined with commercially available kits (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Human Coronary Artery Smooth Muscle Cell Culture

This section is available in the online-only Data Supplement.

Potential Systemic Adverse Effects or Toxicity

To examine systemic adverse effects, biochemical markers were measured before and after implantation of the NF-κB decoy–eluting stent in rabbits (n=7). Five 5-year-old male cynomolgus monkeys weighing 4.2 to 5.0 kg were purchased and fed a normal diet (n=5). Biochemical markers were measured before and after intravenous injection of NF-κB decoy at 1 mg.

Statistical Analysis

Data are expressed as mean±SD. Statistical analysis of differences between the 2 groups was performed by unpaired t test. Statistical analysis of differences among the 3 groups was performed with the use of ANOVA and Bonferroni multiple comparison tests. A level of $P<0.05$ was considered statistically significant.

The authors had full access to the data and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

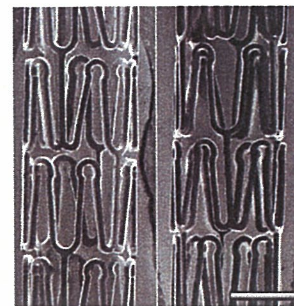
Eluting Stent and In Vitro Release Kinetics

Scanning electron microscopy analysis revealed that the polymer coating formed a uniform film over the outer surface of the stent (Figure 1A). After balloon expansion, stretching of the polymer with no fragmentation was observed (Figure 1A). In vitro release kinetics showed an early burst release of NF-κB decoy as designed (Figure 1B).

Early Activation of NF-κB After Stenting and Effects of NF-κB Decoy–Eluting Stents

Time course and localization of NF-κB activation were examined by immunohistochemical studies with the antibody against α-p65. This antibody recognizes the I-κB binding region on the p65 component of NF-κB.[11] In the unstented artery, no positive cells for α-p65 were noted in the media and adventitia, whereas there were some positive cells in the endothelial layer (Figure 2A). On day 3, activation of NF-κB was noted in the smooth muscle cells in the media. On day 10, activation of NF-κB decreased markedly in the media, but it was noted in neointimal cells of the luminal side. On day 28,

A



B

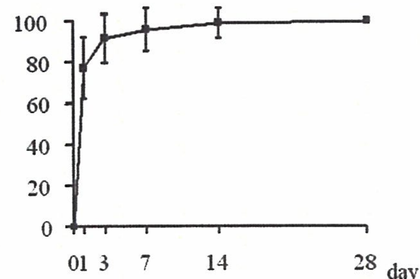


Figure 1. A, Scanning electron microscopic images of balloon-expanded uncoated stent (left) and NF-κB decoy–coated stent (right). Scale bar=1 mm. B, In vitro time course of cumulative NF-κB decoy release from the eluting stents (n=8). The percentage of incremental quantities of the decoy released from the stent was plotted against time.

NF-κB activation was rarely noted in the media and neointima, but it was noted in cells around the stent strut.

The effects of NF-κB decoy–eluting stent on NF-κB activation were examined on day 3 (Figure 2B and 2C). As expected, compared with the uncoated stent site, the number of α-p65–positive cells in the media was less ($P<0.01$) in the NF-κB decoy–eluting stent site.

To confirm immunohistochemical data, an electrophoretic mobility shift assay was performed (Figure 3). No DNA binding activity of NF-κB was noted in samples from unstented arteries. In contrast, the binding activity increased strikingly in samples from the uncoated stent site, which peaked on day 1 and gradually decreased on days 3 and 7. This NF-κB binding activity was attenuated in samples from the NF-κB–eluting stent site. Competition for increased binding of NF-κB was observed by an excess amount of NF-κB. A diminution of main band with supershifted band was observed in samples from uncoated stent sites treated with the p65 antibody but not with the p50 antibody.

Inhibitory Effects of NF-κB Decoy–Eluting Stent on Neointimal Formation

The in-stent neointima was formed equally in the uncoated stent and polyurethane-coated stent sites. Quantitative analysis demonstrated a significant reduction ($P<0.01$) of neointimal formation (neointimal area and thickness) and percent stenosis in the NF-κB decoy–eluting stent site compared with the other 2 sites (Figure 4). In contrast, there were no

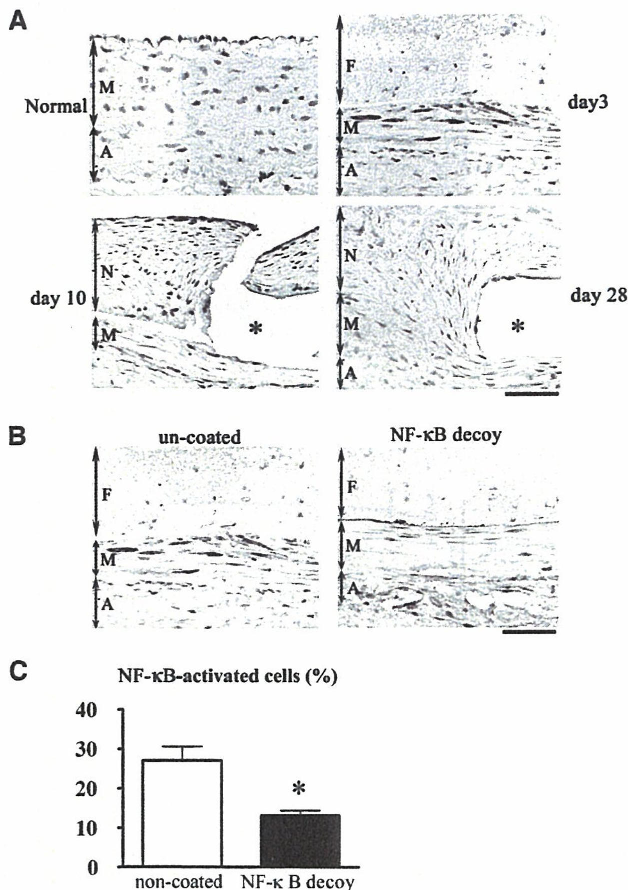


Figure 2. Immunohistochemical detection of activated NF-κB after stenting and inhibitory effects of NF-κB decoy–eluting stents on NF-κB activation. A, Immunohistochemistry of arterial cross sections stained with α-p65. *Stent strut. F indicates fibrin layer; N, neointima; M, media; and A, adventitia. Bar=50 μm. B, Artery sections from the uncoated stent site and NF-κB–eluting stent site stained immunohistochemically with the antibody against α-p65. *Stent strut. Bar=50 μm. C, Comparison of NF-κB activity between uncoated stent and NF-κB–eluting stent sites (n=6 each). Percentage of NF-κB–activated (α-p65–positive) cells is shown. *$P<0.01$ vs uncoated stents by unpaired t test.

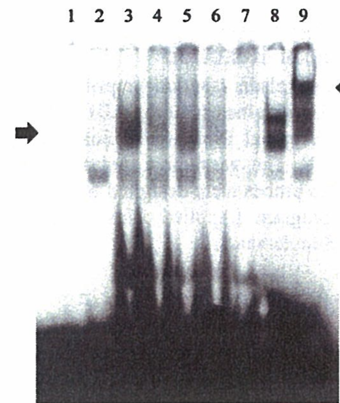


Figure 3. Assessment of activated NF-κB/DNA binding activity by electrophoretic mobility shift assay. NF-κB/DNA binding activity is determined with the use of nuclear extracts isolated from unstented and stented arteries. Lane 1, negative control (^{32}P-labeled NF-κB oligodeoxynucleotide without nuclear extract); lane 2, unstented artery; lane 3, 1-day artery after uncoated stent implantation; lane 4, 3-day artery after uncoated stent implantation; lane 5, 7-day artery after uncoated stent implantation; lane 6, 1-day artery after NF-κB decoy–eluting stent implantation; lane 7, 1-day artery after uncoated stent implantation incubated with extra amount of cold NF-κB oligodeoxynucleotide; lane 8, 1-day artery after uncoated stent implantation incubated with p50 antibody; lane 9, 1-day artery after uncoated stent implantation incubated with p65 antibody; arrowhead indicates supershift.

significant differences in internal elastic lamina area, external elastic lamina area, and medial area among the 3 groups.

A semiquantitative histological scoring system demonstrated that there was no significant difference in the injury score and inflammation score among the 3 groups (Table I in the online-only Data Supplement). Endothelial cell linings, monitored by CD31 immunoreactivity, were observed equally in the 3 groups (Table I in the online-only Data Supplement). There was no significant treatment effect on serum cholesterol levels and body weight among the groups (data not shown).

Inhibitory Effects of NF-κB Decoy–Eluting Stents on Local and Systemic Inflammatory Changes

As we previously reported,[9] infiltration of RAM-11–positive macrophages around stent strut was observed at 10 days after stent implantation (Figure 5A). NF-κB decoy–eluting stents reduced such inflammatory changes (Figure 5B).

CD14 expression on circulating monocytes was examined by flow cytometry for CD14$^+$ cells as a systemic inflammation marker. Maximum fluorescence intensity of CD14 on circulating monocytes increased ($P<0.01$) 10 days after uncoated stent implantation compared with unstented controls. No increase in CD14 expression on monocytes was observed in animals implanted with NF-κB decoy–eluting stent (Figure 5C).

Inhibitory Effects of NF-κB Decoy–Eluting Stents on Expression of Proinflammatory Factors

NF-κB decoy–eluting stents reduced the increased ($P<0.01$) gene expression of MCP-1, interleukin-6, tumor necrosis factor-α, and tissue factor (Figure 6A). NF-κB decoy–eluting stents did not affect increased gene expression of interleukin-1β and vascular cell adhesion molecule-1. Immunohistochemical staining performed 10 days after stenting revealed increased immunoreactive MCP-1 in cells in the neointima and smooth muscle cells in the media, which was attenuated ($P<0.01$) in the NF-κB decoy–eluting stent group (Figure 6B).

Blockade of NF-κB Inhibits Proliferation of Human Coronary Artery Smooth Muscle Cells

The serum-induced proliferation of human coronary artery smooth muscle cells was nearly prevented ($P<0.01$) by the adenovirus-mediated gene transfer of dominant-active I-κB or by transfection of NF-κB decoy (Figure in the online-only Data Supplement).

No Adverse Systemic Effects of NF-κB Decoy

Biochemical markers were measured as described in the online-only Data Supplement. These data show that no systemic adverse effects of NF-κB decoy were noted in rabbits or monkeys.

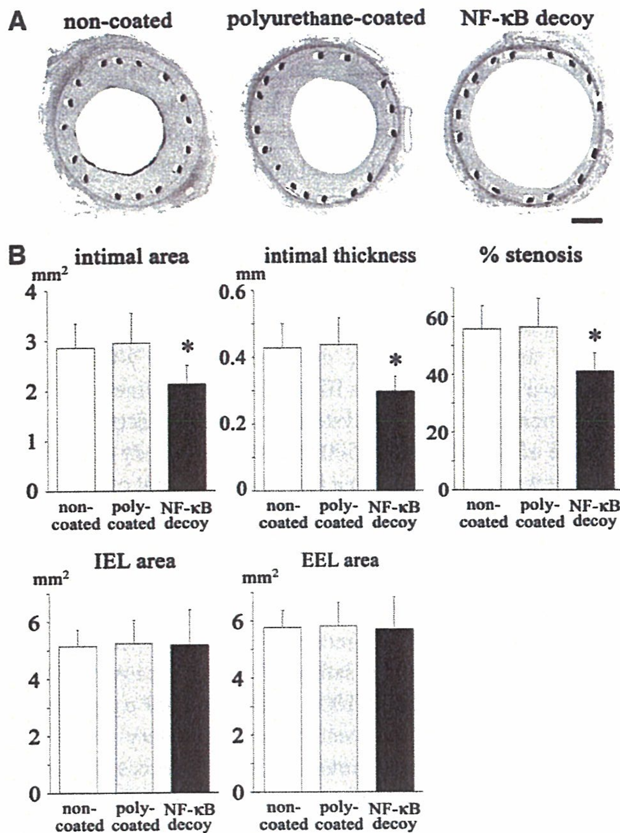


Figure 4. Inhibitory effect of NF-κB decoy–eluting stents on in-stent neointima formation. A, Iliac artery sections from the uncoated, polyurethane (poly)-coated, and NF-κB decoy–eluting stents 28 days after stenting stained with elastica van Gieson in rabbits. Bar=500 μm. B, Effect of the NF-κB decoy–eluting stents on intimal area, intimal thickness, internal elastic lamina (IEL) area, external elastic lamina (EEL) area, and percent stenosis 28 days after stenting in rabbits (n=8 each). *P<0.01 vs uncoated stents by ANOVA and Bonferroni multiple comparison tests.

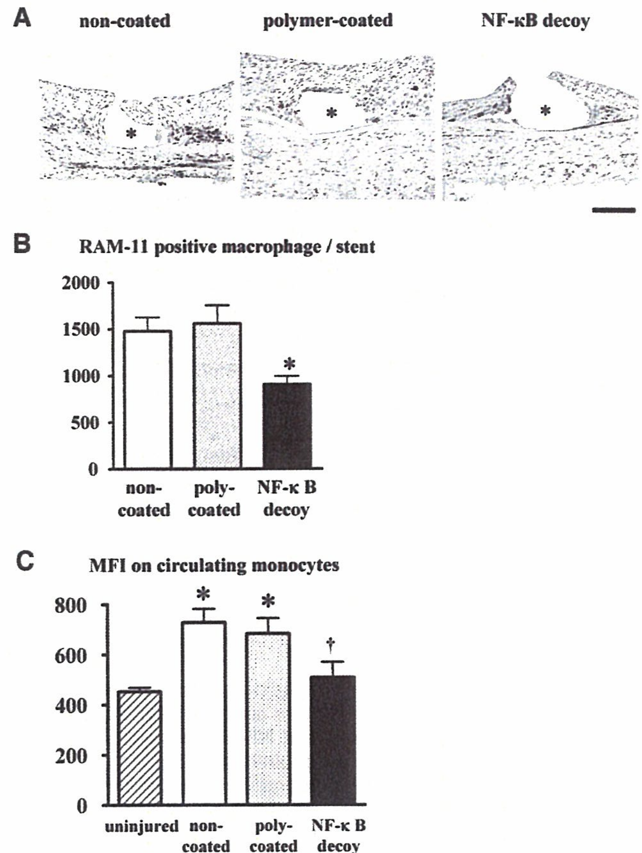


Figure 5. Effects of NF-κB decoy–eluting stents on local and systemic inflammation. A, Effect of NF-κB decoy–eluting stent on local inflammation (RAM-11–positive monocyte/macrophage) 10 days after stenting. *Stent strut. Bar=100 μm. B, Summary of quantitative analysis (n=7 each). Poly indicates polymer. *P<0.01 vs uncoated stents. C, Effects of NF-κB decoy–eluting stent on maximum fluorescence intensity (MFI) of CD14 on circulating monocytes 7 days after stenting (n=7 each). *P<0.01 vs unstented rabbits; †P<0.01 vs rabbits implanted with uncoated stents by ANOVA and Bonferroni multiple comparison tests.

Discussion

The present study reports, for the first time, the formulation of a stent-based delivery system of the NF-κB decoy oligodeoxynucleotide. A water-soluble polymer (polyurethane) was used to create a rapid-release type because NF-κB was found to be activated only at early stages but not at later stages after stenting. The present study clearly showed early activation of NF-κB after uncoated bare stenting and its inhibition by stent-based local delivery of NF-κB decoy in 2 approaches (immunostaining of a specific marker of NF-κB activation and DNA-finding assay). The inhibition of NF-κB activation was associated with reduced inflammatory changes such as reduced CD14 expression on circulating leukocytes as well as monocyte recruitment into stent sites. Although multiple factors are involved in the mechanism of decoy transfection, the mechanical force during stent expansion by the balloon dilatation procedure is likely to be a major contributing factor. The decoy might be transfected into medial and neointimal smooth muscle cells, which in turn reduced expression of various NF-κB–dependent inflammation-promoting factors. This polymeric technology-driven delivery system could be used for delivery of any other potential candidates of decoy oligodeoxynucleotides.

It has been reported that prolonged inflammatory changes were detected in arteries exposed to polymeric stent-coating materials in experimental animals[18,23] and humans.[3–5] However, no such adverse reaction was noted in this study. In addition, there was no evidence of an impaired healing process and endothelial regeneration at sites of stents coated with polyurethane alone and polyurethane plus decoy. These data suggest that the polymers used in this study may not cause an adverse reaction during a 4-week observation period.

The most important finding of the present study was inhibition of neointimal formation by stent-based delivery of NF-κB decoy. The beneficial effects of NF-κB decoy–eluting stents were associated with reduced gene expression of NF-κB–dependent genes (eg, MCP-1, interleukin-1β, interleukin-6) and with no change in NF-κB–independent genes (platelet-derived growth factor) (Figure 6). Immunoreactive MCP-1 expression was also reduced at sites of NF-κB decoy–eluting stent. These data indicate a specific function of the NF-κB decoy–eluting stent on local NF-κB activation. It is known that injury-induced inflammatory and proliferative

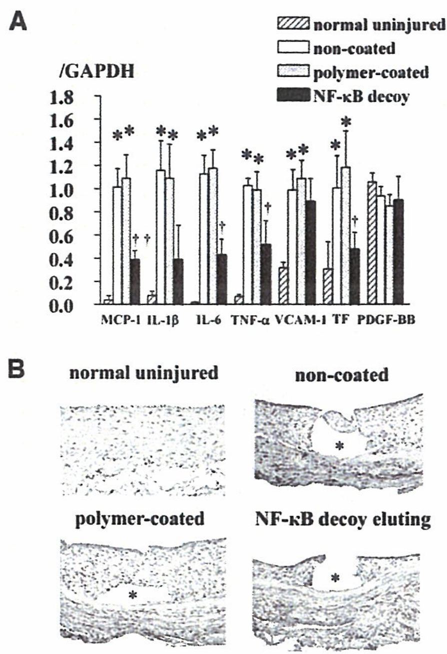


Figure 6. Effects of NF-κB decoy–eluting stents on gene expression of proinflammatory factors and immunohistochemical expression of MCP-1. A, Effect of NF-κB decoy–eluting stents on mRNA levels of various proinflammatory factors and tissue factor (TF) 10 days after stenting (n=7 to 8 each). *P<0.01 vs uninjured normal artery; †P<0.05, ††P<0.01 vs the control group by ANOVA and Bonferroni multiple comparison tests. B, Iliac artery sections from the uninjured normal rabbits and those from the uncoated, polyurethane-coated, and NF-κB decoy–eluting stents implanted in rabbits 10 days after stenting stained immunohistochemically with MCP-1. IL indicates interleukin; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule; and PDGF, platelet-derived growth factor. *Stent strut. Bar=100 μm.

changes are critical in restenotic changes after vascular injury.[8,24,25] We and others have reported that (1) increased monocyte-mediated inflammation correlates positively with in-stent neointimal formation[7,26] and (2) blockade of MCP-1 reduces neointimal formation after vascular injury.[9,27,28] Because the NF-κB–eluting stent reduced inflammation and MCP-1 expression in this study, the beneficial effects of NF-κB decoy–eluting stents can be attributable at least in part to inhibition of MCP-1–related inflammation resulting from reduced NF-κB activation. Otherwise, emerging evidence suggests that NF-κB regulates proliferation of vascular smooth muscle cells.[29,30] In this regard, we found that blockade of NF-κB activation by transfection of NF-κB decoy or dominant-active I-κB suppressed proliferation of human coronary artery smooth muscle cells in vitro. Therefore, it is also likely that NF-κB decoy–eluting stents might inhibit proliferation of vascular smooth muscle cells induced by NF-κB activation.

There are several caveats in our present findings in regard to potential clinical applicability. First, application of the present findings to treatment of restenosis in humans could be limited because the ideal animal model for drug-eluting stent evaluation is uncertain according to the recommendation from the consensus group.[31] They stated that the coronary arteries in pigs and iliac-femoral arteries of rabbits are suitable in that their size, access, and injury response are similar to those of human vessels and therefore they allow examination of devices that might be used in clinical evaluation. Thus, the rabbit peripheral artery model is considered an acceptable model of choice. Second, the observed efficacy and safety of NF-κB decoy and polymer at 28 days may be too short. Third, potential adverse effects or toxicity of NF-κB decoy may be important. In histopathological analysis, no adverse reactions such as incomplete healing or impaired endothelial regeneration were noted. Measurements of serum blood markers (glucose, aspartate aminotransferase, alanine aminotransferase, creatine kinase, γ-GTP, and C-reactive protein in Tables III and IV in the online-only Data Supplement) showed no systemic adverse effects. Because the dose of NF-κB decoy (500 to 600 μg per body) coated on the stent was very low from a toxicological point of view, our decoy-coated stent may not cause any toxicity in vivo. It has been reported that repeated bolus administration of high doses (eg, 10 mg/kg every other day for 28 days in monkeys, 20 mg/kg every other day for 28 days in mice) causes kidney damage.[32] In addition, we recently completed a clinical trial to test the feasibility and safety of NF-κB decoy in which NF-κB decoy at doses of 1000, 2000, or 4000 μg per body was transfected into the stented coronary artery sites via a channel balloon catheter immediately after successful percutaneous coronary intervention in 16 patients with flow-limiting coronary stenosis. The initial 2 cases have been reported,[33] and they showed no evidence of restenosis or systemic adverse effects during the 6-month observation period. Overall, these data support the notion that this NF-κB decoy–eluting stent system can be applied to the clinical setting.

In conclusion, the present study supports the experimental evidence that stent-based local delivery of NF-κB decoy reduces in-stent neointimal formation by inhibiting NF-κB–dependent gene expression and inflammation and perhaps by inhibiting proliferation of vascular smooth muscle cells. Inhibition of stent-associated inflammation by the NF-κB decoy–eluting stent may be a promising next-generation approach for the prevention of restenosis. Further preclinical studies and clinical trials are needed to prove this hypothesis.

Sources of Funding

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 (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.

Disclosures

Drs Egashira and Morishita hold a patent on the results reported in the present study. The remaining authors report no conflicts.

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CLINICAL PERSPECTIVE

Although first-generation drug-eluting stents are effective in reducing the rate of restenosis, the drug-eluting stent has no effect on the incidence of cardiovascular events compared with the bare-metal stent. In addition, recent clinical studies have demonstrated that drug-eluting stents increase the incidence of late stent thrombosis, leading to acute myocardial infarction and death after the discontinuation of clopidogrel. These serious late thrombotic events are thought to result from impaired endothelial regeneration and an incomplete healing process because of the drugs or polymers used in the construction of drug-eluting stents. Therefore, the formulation of a novel drug-eluting stent system with fewer adverse effects is warranted. In the present study, we formulate a nuclear factor-κB (NF-κB) decoy–eluting stent with biocompatible polymer technology and report inhibition of neointimal formation by stent-based delivery of NF-κB decoy. Importantly, no histopathological evidence of impaired endothelial regeneration and healing process was noted at sites of stents coated with polymer alone and polymer plus decoy. These data support the experimental evidence that the NF-κB decoy–eluting stent is effective in reducing in-stent neointimal formation and thrombosis. Our previous clinical trial testing the feasibility and safety of NF-κB decoy supports the notion that this NF-κB decoy–eluting stent system can be applied to the clinical setting. Ultimately, we propose that this system be used to treat vulnerable plaques leading to acute coronary syndrome and stroke.

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Atherosclerosis xxx (2006) xxx–xxx

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Catheter-based adenovirus-mediated anti-monocyte chemoattractant gene therapy attenuates in-stent neointima formation in cynomolgus monkeys

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Received 29 June 2006; received in revised form 27 September 2006; accepted 18 October 2006

Abstract

We have previously demonstrated great benefit from anti-monocyte chemoattractant protein-1 (MCP-1) gene therapy by "systemic" transfer of an N-terminal deletion mutant of human MCP-1 (called 7ND) gene into skeletal muscle for treatment of restenosis and atherosclerosis. However, recent evidence suggests that "local" gene transfer may be a clinically relevant approach. We therefore tested the hypothesis that catheter-based adenovirus-mediated anti-MCP-1 gene therapy attenuates stent-associated neointima formation.

Bare metal stents were implanted in iliac arteries of cynomolgus monkeys fed a high cholesterol diet. Immediately after the stenting procedure, normal saline or recombinant adenoviral vector containing LacZ or the 7ND gene was administered locally into the stenting site through a Remedy channel-delivery catheter. Compared to saline infusion or LacZ gene transfer, 7ND gene transfer markedly reduced inflammatory changes at an early stage and attenuated neointima formation after 4 weeks. This strategy also reduced the increased production of pro-inflammatory and growth-promoting factors such platelet-derived growth factor. No systemic adverse effects of 7ND gene transfer were detected. There were no significant differences in serum cholesterol levels among the three groups.

These data suggest that catheter-based adenovirus-mediated anti-MCP-1 gene therapy may be a clinically relevant and feasible strategy for treatment of in-stent restenosis.
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Keywords: Restenosis; Inflammation; Monocyte; Gene therapy; Catheter intervention

1. Introduction

There is ample clinical and experimental evidence suggesting that inflammation plays a central role in the pathogenesis of restenosis [1–3]. Recruitment and activation of monocyte/macrophages are a major histopathologic finding after arterial injury. Because monocyte chemoattractant protein-1 (MCP-1) is a potent and specific chemokine for monocytes [3–5], an anti-inflammatory strategy targeting MCP-1 and its receptor (CCR2) may be an appropriate and reasonable approach for restenosis. We have previously devised a new strategy for anti-MCP-1 gene therapy, in which plasmid cDNA encoding a mutant MCP-1 gene is transfected into skeletal muscle [6]. This mutant MCP-1, called 7ND, lacks N-terminal amino acids 2–8 and has been shown to work as a dominant-negative inhibitor of MCP-1. This systemic approach (intramuscular transfection of the gene) is useful, because direct gene transfer into the injured arterial wall is not necessary, and roles of MCP-1 can be investigated under pathophysiological conditions in vivo. With this strategy, we have demonstrated that blocking MCP-1 signals reduces neointima formation after balloon- and stent-induced injury [7–10] and atherosclerosis [11,12] in animals, including non-human primates. Roque et al. [13] reported that CCR2-deficient mice display reduced neointimal hyperpla-

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doi:10.1016/j.atherosclerosis.2006.10.029

Please cite this article in press as: Nakano K, et al., Catheter-based adenovirus-mediated anti-monocyte chemoattractant gene therapy attenuates in-stent neointima formation in cynomolgus monkeys, Atherosclerosis (2006), doi:10.1016/j.atherosclerosis.2006.10.029

sia after intraluminal arterial injury. Horvath et al. [14] have 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 monkeys.

It is becoming accepted that, rather than a systemic approach, a local delivery strategy should be a reasonable anti-restenotic therapy with minimal systemic adverse effects [15]. Indeed, the development of drug-eluting stents (DES) has had a major impact on in-stent restenosis. Their safety and extreme effectiveness have been proven in a majority of lesions, after more than a million DES implantations. However, currently marketed first-generation DES use sirolimus or taxol as the drug that elicits nonspecific anti-proliferative effects, not only on vascular smooth muscle cells, but also other cell types such as endothelial cells. It is suggested that impaired endothelial function and regeneration lead to acceleration of restenosis and atherosclerosis, and to cardiovascular events [16]. Thus, the first-generation DES still have a number of limitations that include significant restenosis rates in certain high-risk patients or lesions (bifurcation lesions, small vessels, diabetes, etc.), delayed healing (excessive late inflammation, proliferation, and fibrin deposition), and a small number of cases of late in-stent thrombosis [15,17–19]. In addition, it should be noted that 30–40% of coronary atherosclerotic lesions may not be appropriate for stenting, due to small arteries or branch sites. Therefore, catheter-based local gene transfer of relevant genes may represent a clinically relevant and alternative approach for treatment of restenosis beyond the first-generation DES strategy.

Accordingly, this study was designed to investigate whether blockade of MCP-1 by catheter-based adenovirus-mediated local 7ND gene transfer is effective in attenuating stent-associated neointima formation in non-human primates. To gain clinical significance for the results, we used a non-human primate model of stent-associated neointima formation [7]. The Remedy channel-delivery catheter was used for local delivery, because it is adopted for human use, and thus relevant to the human interventional setting.

2. Methods

2.1. Adenoviral constructs

Human 7ND cDNA was constructed by recombinant polymerase chain reaction (PCR) using a wild-type human MCP-1 cDNA (Dr. T. Yoshimura, National Cancer Institute) as a template, and inserted into the BamH1 (5′) and Not1 (3′) sites of the pcDNA3 (Invitrogen) expression vector plasmid [6]. Twenty four nucleotides encoding FLAG epitope (DYKDDDDK) were added directly at the 3′ terminus of MCP-1 sequence. Adenovirus vectors encoding the 7ND gene (Ade-7ND) or galactosidase gene (Ade-LacZ) were generated by use of the Adenovirus Expression Vector Kit (Takara) according to the manufacturer's instructions.

2.2. Stent implantation and arterial gene transfer

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 the American Physiologic Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Five-year-old adult male cynomolgus monkeys weighing 4–5 kg were fed laboratory diet containing 0.5% cholesterol, starting 2 months before stent implantation. Monkeys were anesthetized with ketamine hydrochloride (10 mg/kg IM) and sodium pentobarbital (30 mg/kg IV to effect), and underwent placement of a 3 mm × 15 mm stent in the external iliac artery, as described previously [20]. Immediately after stent implantation, the animals were randomly allocated to the normal saline, Ade-LacZ, or Ade-7ND group. Saline or adenovirus solution at amount of 2 mL (1.0×10^9 pfu) was locally infused through the Remedy channel balloon catheter (Boston Scientific Inc.) [21]. The titer of adenovirus used in this study was below the inflammatory threshold (1.6×10^9 pfu/body), as reported [22]. The Remedy is a triple-lumen local delivery device, and consists of a noncompliant angioplasty balloon surrounded by a set of 18–24 perforated channels (30 μm holes) [23]. These channels are directly connected to a separate infusion lumen, allowing independent low-pressure drug delivery. The balloon was inflated at 2 atm support pressure, and infusion of saline or virus solution was performed at 2 atm over 2 min. Efficient and relatively safe percutaneous gene transfer can reportedly be achieved without overt systemic toxicity using this approach [23], which is adopted for human use.

All monkeys were killed with a lethal dose of anesthesia 7 or 28 days after stenting for immunohistochemical and morphometric analysis. All animals received aspirin 81 mg/day and ticlopidine 100 mg/day until euthanasia.

2.3. Morphometric and immunohistochemical analysis

Stented arterial sections were excised and fixed for 24 h with 95% ethanol and 1% acetic acid. Each segment was divided into two parts at the center of the stent as described [20]. The proximal part was embedded in methyl methacrylate mixed with n-butyl methacrylate to allow for sectioning through metal stent struts. Serial sections were stained with elastica van Gieson and hematoxylin-eosin (HE). To evaluate the in-stent neointima formation, areas of internal elastic lamina, lumen, media, and neointima were measured.

The distal part was used for immunohistochemical analysis. After stent struts were gently removed with micro forceps, the tissue was dehydrated, embedded in paraffin, and cut into

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5-μm thick slices. They were subjected to immunostaining using antibodies against macrophage/monocytes (HAM56, Dako), endothelial cells (CD31, Dako), PDGF-B (Mochida), IL-1β (Endogen), CCR2 (Sigma), or with non-immune mouse IgG (Zymed). Following avidin–biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin.

Morphometric analysis was performed by the use of a microscope with a computerized digital image-analysis system by a single observer who was blind to the treatment protocol.

2.4. Blood cholesterol measurements

Plasma total cholesterol levels were determined with commercially available kits (Wako Pure Chemicals).

2.5. Biochemical measurements

Plasma concentrations of human IL-8, TGF-β, and oxidized LDL were measured using an ELISA kit (R & D). To detect MCP-1 transgene expression indirectly, tissue concentrations of human MCP-1 were also measured by the use of an ELISA kit (R & D). Although we avoided using high titers of adenovirus that may cause inflammatory reactions [22], adenovirus vectors may cause virus-related adverse effects or toxicity. To examine potential systemic adverse effects, relevant biochemical markers were measured.

2.6. Efficiency of adventitial gene transfer

The efficiency of arterial gene transfer was determined in monkeys and rabbits. In monkeys, the expression of FLAG protein in stented arteries after 7days of stenting were evaluated by western blot analysis, as described previously [6]. In brief, the same amount of extracted protein (25 μg for each experiment) was loaded for SDS-PAGE/immunoblot analysis using anti-FLAG M2 monoclonal antibodies (Sigma). The regions containing MCP-1 proteins were visualized by LAS-1000 (Fujifilm).

We also determined the expression of 7ND in rabbits, as we reported previously [21]. Male Japanese white rabbits weighing 3.0–3.5 kg were anesthetized and underwent stent placement, and LacZ gene transfer was performed as described above. The transfection efficiency was measured 4 days after LacZ transfection by X-gal staining of sections from the target artery and calculated as follows: 100× (X-gal positive cells/total number of cells in a section).

2.7. Statistical analysis

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

3. Results

3.1. Inhibitory effects of Ade-7ND gene transfer on inflammatory and proliferative changes

Inflammatory (HAM56-positive monocyte/macrophage) and proliferative (PCNA-positive monocytes and medial smooth muscle cells) changes became evident 7 days after stenting in the stented arterial wall (Fig. 1). The 7ND gene transfer reduced these inflammatory and proliferating changes (Fig. 1).

3.2. Inhibitory effects of 7ND gene transfer on neointima formation

As we previously reported [7], in-stent neointima formation was evident 28 days after stenting. Quantitative analysis demonstrated a significant reduction of neointima formation and % stenosis in the Ade-7ND group compared with the saline and Ade-LacZ groups (Fig. 2). In contrast, there were no significant differences in area of internal elastic lamina among the three groups (Fig. 2).

Endothelial cell linings, monitored by CD31 immunoreactivity, were observed equally in the three groups (online Table 1). There was no significant treatment effect on serum cholesterol levels and body weight among the groups (online Tables 2 and 3).

3.3. Inhibitory effects of 7ND gene transfer on tissue expression of pro-inflammatory factors

Immunohistochemical staining performed 7 days after stenting revealed increased immunoreactive PDGF-B, VEGF, and CCR2 in cells in the neointima, smooth muscle cells in the media, and in cells in the adventitia in the saline and Ade-LacZ groups. These changes were reduced in the Ade-7ND group (Fig. 3A).

We also measured serum concentrations of IL-8, TGF-β, and oxLDL 7 days after stenting. 7ND gene transfer partially but significantly reduced the increase in serum IL-8 and oxLDL concentrations. 7ND gene transfer did not affect the increase in serum TGF-β levels (Fig. 3B).

3.4. No adverse effects of 7ND gene transfer

White blood cell counts, inflammatory markers, and biochemical markers were measured (online Table 3). These data show no systemic adverse effects of 7ND gene transfer.

3.5. Serum and tissue concentrations of MCP-1 plus 7ND

We could not find an MCP-1 ELISA kit that differentiated between human and monkey MCP-1. Thus, this "human MCP-1" ELISA kit detects both 7ND and native monkey MCP-1. The tissue concentration of MCP-1 plus

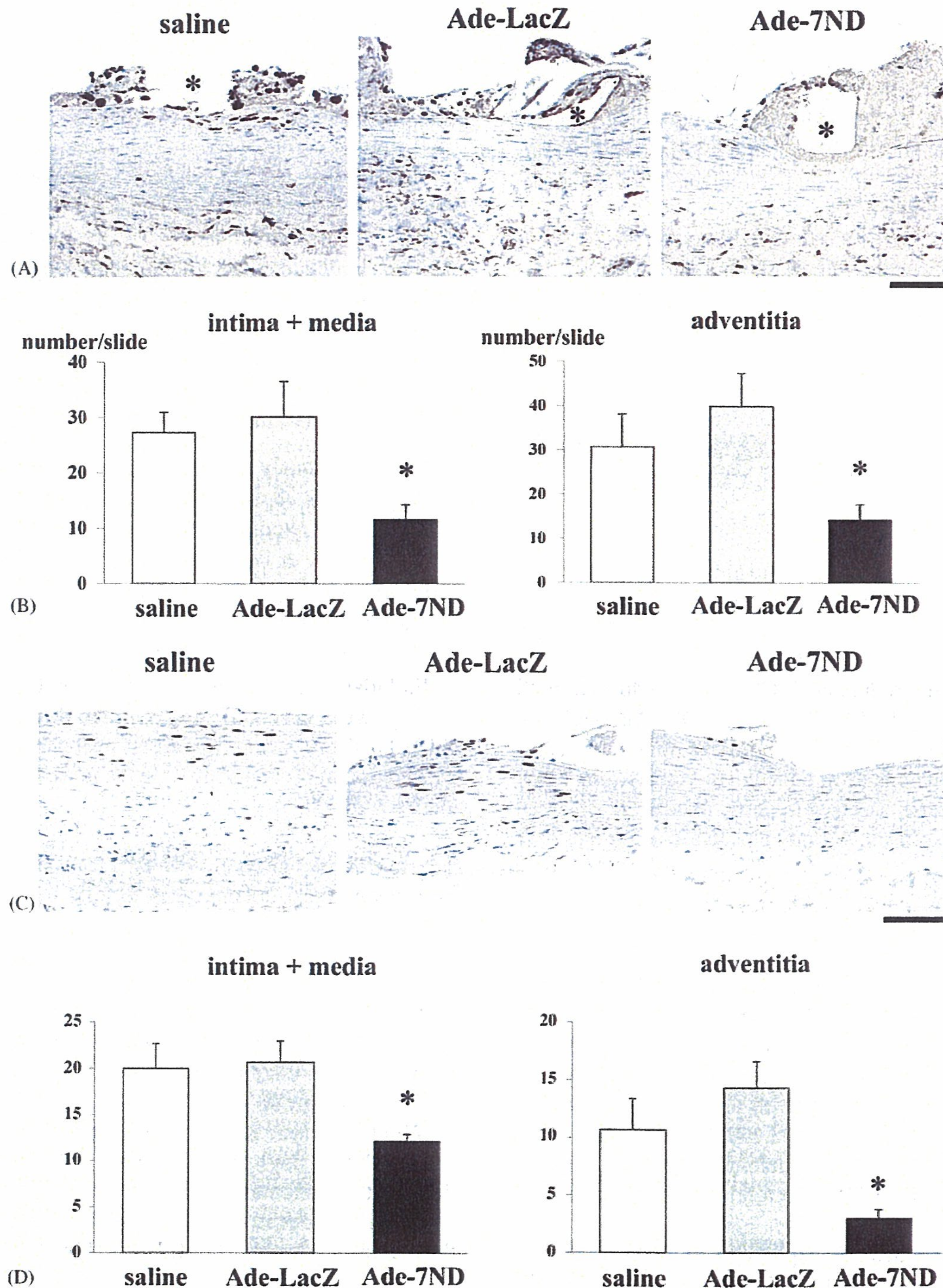

Fig. 1. Inhibitory effects of 7ND gene transfer on local inflammation and proliferation. (A) Effect of the 7ND gene transfer on local inflammation (HAM56-positive monocyte/macrophages) 7 days after stenting. Asterisk (*) indicates stent strut sites. Bar = 100 μm. (B) Summary of quantitative analysis (n = 7 each). Positive cell counts per section in the intima plus media (left) and in the adventitia (right) are shown. *P < 0.01 vs. the saline or Ade-LacZ group. (C) Effect of the 7ND gene transfer on local proliferation (PCNA-positive monocytes and medial smooth muscle cells) 7 days after stenting. (D) Summary of quantitative analysis (n = 7 each). Positive cell counts per section in the intima plus media (left) and in the adventitia (right) are shown. *P < 0.01 vs. the saline or de-LacZ group.

7ND at 7 days after stenting was higher in stented artery sites from the saline (120 ± 42 pg/mg protein) and Ade-LacZ (132 ± 46 pg/mg protein) groups than in unstented normal artery (24 ± 12 pg/mg protein), indicating increased production of MCP-1 from stented artery sites. Because, there was no significant difference in values between the saline and Ade-LacZ groups, we suggest that transfer of the LacZ gene did not affect the degree of MCP-1 production. Importantly, the tissue MCP-1 + 7ND levels were 2- to 3-fold higher in the stented artery sites from the Ade-7ND group

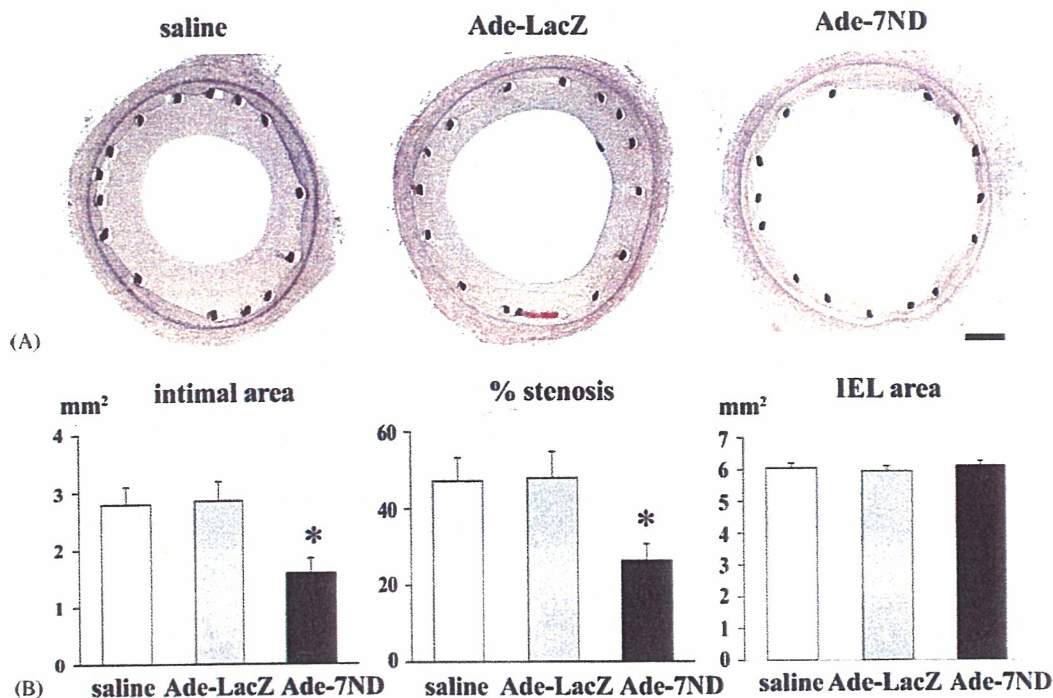

Fig. 2. Inhibitory effect of 7ND gene transfer on in-stent neointimal formation. (A) Iliac artery sections from the saline, the Ade-LacZ, and the Ade-7ND group 28 days after stenting, stained with elastic van Gieson. Bar = 500 μm. (B) Effect of 7ND gene transfer on intimal area, % stenosis, and IEL area 28 days after stenting (n = 7 each). *P < 0.01 vs. the saline groups.

(564 ± 128 pg/mg protein) than in those from the saline and Ade-LacZ groups, indicating possible production of 7ND from the transfected arterial sites as the result of transgene expression.

3.6. Transgene expression in monkeys and rabbits

In monkeys, western blot analysis for FLAG protein showed that FLAG/7ND protein was produced in stented arteries (online Figure I). We further examined, 4 days after stent implantation and gene transfection in the rabbit iliac artery, the expression of β-galactosidase was noted at the Ade-LacZ-transfected site (Fig. 4). Nuclear staining for LacZ was localized mostly in the intima and the lumenal side of the media, and to a lesser extent in the adventitia. As reported by other investigators [23,24], the transfection efficiency was $9.2 \pm 0.6\%$ (n = 4).

4. Discussion

This study reports, for the first time, that blockade of MCP-1 by catheter-based adenovirus-mediated local gene transfer of 7ND markedly reduced in-stent neointima formation in non-human primates (cynomolgus monkeys). Transgene expression was confirmed directly by FLAG/7ND protein expression after Ade-7ND transfer and X-gal staining of stenting sites after Ade-LacZ transfer, and indirectly by measuring arterial tissue concentrations of MCP-1 plus 7ND after Ade-7ND transfer. Another important point is that the magnitudes of inhibitory effects on in-stent neointima formation afforded by this local gene transfer strategy (neointimal area in control and Ade-7ND groups: $2.8 \pm 0.3\ mm^2$ and $1.4 \pm 0.2\ mm^2$, respectively) are greater than those afforded by "systemic" gene transfer strategy in our previous study [7] (neointimal areas in control and 7ND gene transfer groups: $3.2 \pm 0.2\ mm^2$ and $2.4 \pm 0.2\ mm^2$, respectively). No non-specific inflammatory effects on in-stent neointima were detected in the Ade-LacZ group. Furthermore, our findings in non-human primates may have clinical significance, because many therapeutic strategies that have proven effective in reducing restenosis in nonprimate animal models have failed to demonstrate substantial effect on human restenosis. Although it is uncertain which animal model is most appropriate for the evaluation of in-stent restenosis, non-human primate models may have advantages over nonprimate animal models, because, (1) vascular inflammatory and proliferative responses to injury in non-human primates are presumed to be closer to those in humans than other, non-primate models and (2) the results of safety tests can be applied to humans. Therefore, the use of non-human primates may allow us to evaluate the efficacy and safety of therapies such as 7ND gene transfer on in-stent neointimal formation in more reliable conditions. These findings suggest that catheter-based adenovirus-mediated gene transfer of 7ND is a feasible approach for treatment of restenosis, with minimal potential systemic adverse effects.

It is well known that inflammatory changes (monocyte recruitment and activation) induced by stent-induced injury are critical in the pathogenesis of in-stent restenosis

Please cite this article in press as: Nakano K, et al., Catheter-based adenovirus-mediated anti-monocyte chemoattractant gene therapy attenuates in-stent neointima formation in cynomolgus monkeys, Atherosclerosis (2006), doi:10.1016/j.atherosclerosis.2006.10.029

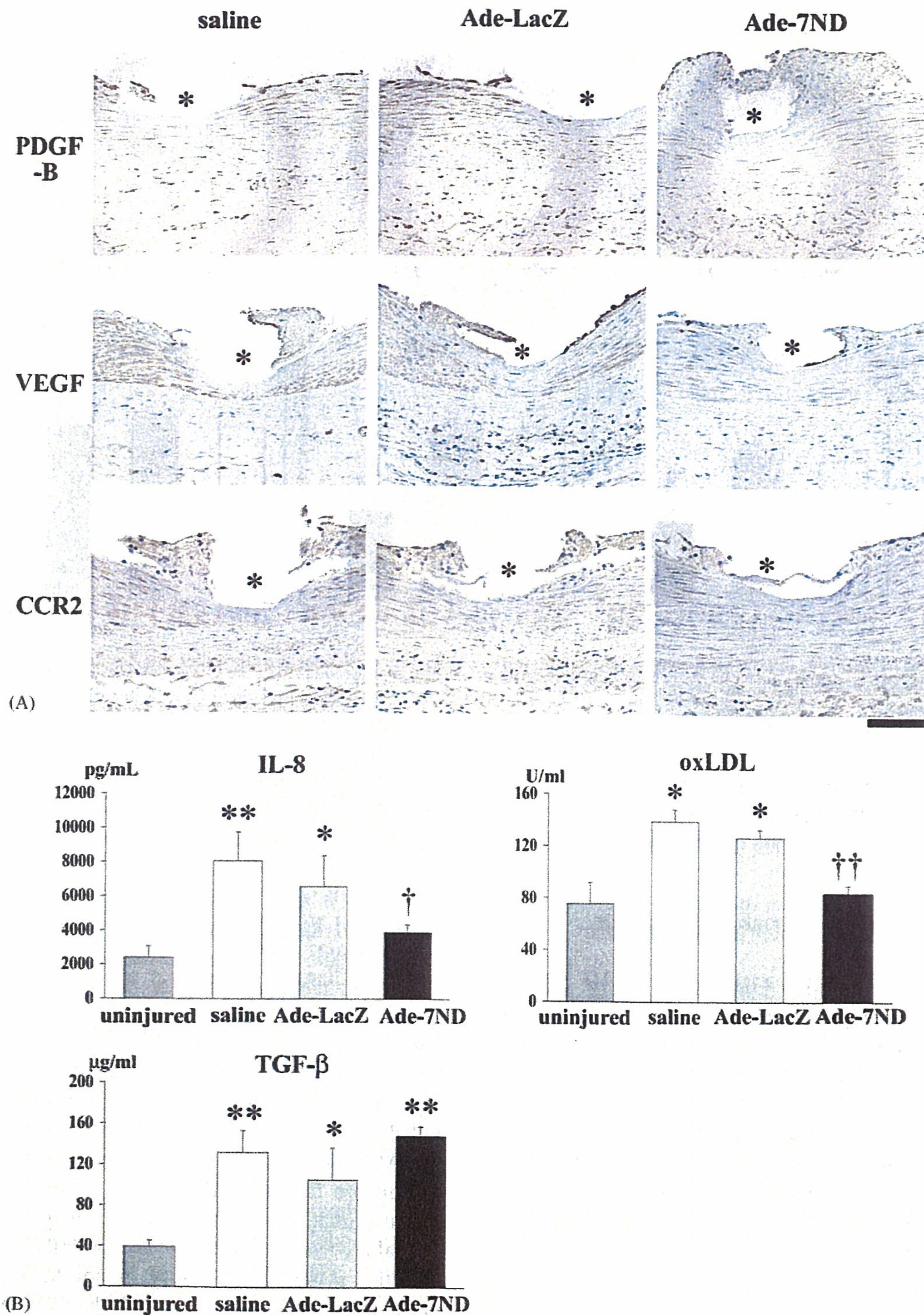

Fig. 3. Effects of 7ND gene transfer on immunohistochemical expression of PDGF-B, VEGF, and CCR2 and on plasma concentrations of IL-8, oxLDL, and TGF-β. (A) Iliac artery sections from uninjured normal arteries, and those from the Ade-LacZ and Ade-7ND groups 7 days after stenting. They are stained immunohistochemically for PDGF-B, VEGF, and CCR2. Asterisk (*) indicates stent strut sites. Bar = 100 μm. (B) Plasma concentrations of IL-8, oxLDL, and TGF-β 7 days after stenting $^{*}P < 0.05$, $^{**}P < 0.01$ vs. uninjured normal control, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, vs. the saline group.

[3,25,26]. We and others have reported that (1) increased monocyte-mediated inflammation correlates positively with in-stent neointima formation [1,2] and (2) blockade of MCP-1 reduces neointima formation after vascular injury [7–10,13,14]. Because, we demonstrated here that catheter-based Ade-7ND transfer reduced inflammation, the beneficial effects of Ade-7ND transfer can be attributed to inhibition of MCP-1-mediated inflammation. It is also possible that Ade-7ND transfer reduced in-stent neointima formation by inhibiting the proliferation of vascular smooth muscle

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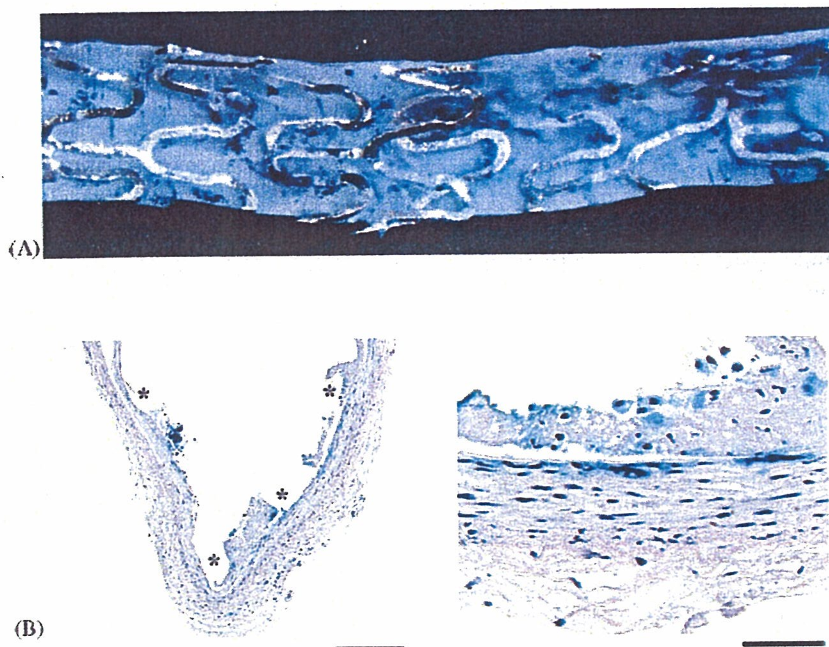

Fig. 4. Gene transfer into rabbit iliac stented artery after local infusion of de-LacZ via the Remedy delivery catheter. (A) Macroscopic picture of lumenal surface of the stented iliac artery staining with X-gal 4 days after transfection. Stented arterial segments were excised, cult longitudinally, and stained with X-gal. (B) X-gal-stained arterial cross-sections at low (right) and high (left) power of magnification. Asterisk (*) indicates stent strut sites. The bars indicate 400 μm (left) and 50 μm (right).

cells. Recent evidence suggests that vascular smooth muscle cells have a functional MCP-1 receptor (CCR2) [27]. In this study, local 7ND gene transfer attenuated (1) the appearance of proliferating smooth muscle cells and (2) the increased expression of mitogens of vascular smooth muscle cells (PDGF, VEGF, oxLDL) after stenting. Interestingly, local 7ND transfer reduced increased expression of CCR2 and IL-8, suggesting the presence of a positive-feedback loop to enhance inflammation and proliferation once vascular response to injury began. Therefore, it is possible that local 7ND gene transfer might reduce in-stent neointima formation by inhibiting inflammation, proliferation of vascular smooth muscle cells, or both.

In contrast, 7ND gene transfer did not affect endothelial regeneration, suggesting that the 7ND gene transfer may not impair the healing process of endothelial cells in stented arterial wall. This point may be an advantage of our approach over currently marketed first-generation DES. The Cypher (sirolimus) and Taxus (paclitaxel) stents impair regeneration of endothelial cells after stenting [28], and this may lead to impaired healing of the stented arterial wall. Hence, we presume that specific blockade of MCP-1-mediated signals in monocytes and smooth muscle cells, as described here using catheter-based local transfer of the 7ND gene, may provide a promising therapeutic strategy for restenosis with low potential toxicity.

In regard to clinical applicability, the potential systemic toxicity of catheter based adenovirus-mediated gene transfer deserves discussion. We demonstrated here that catheter-based adenovirus-mediated 7ND gene transfer induced no detectable inflammatory or immune reactions. We have previously reported that intramuscular systemic transfer of plasmid cDNA encoding the 7ND gene is nontoxic and safe in non-human primates [8,10,29], rabbits [7], rats [10], and mice [8]. In addition, mice lacking MCP-1 or CCR2 display no serious health problems. Furthermore, several protocols for adenovirus-mediated gene therapy have been in clinical trials, which reported no systemic adverse effects when the clinical trial protocol was performed properly. Overall, these toxicity data support the notion that this mode of gene therapy can be applied to human patients.

In conclusion, these data suggest that catheter-based adenovirus-mediated anti-MCP-1 gene therapy may be a clinically relevant and feasible therapeutic strategy for treatment of in-stent restenosis. If this mode of treatment is proven to be effective and safe, it could be used as an independent therapy for high-risk lesions, small vessels, or recurrent restenosis resistant to currently marketed DES. It could also be used as an alternative therapy beyond the first-generation DES strategy.

Acknowledgments

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; by the

Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Tokyo, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2006.10.029.

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Azelnidipine has anti-atherosclerotic effects independent of its blood pressure-lowering actions in monkeys and mice

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Received 17 October 2006; received in revised form 19 March 2007; accepted 22 March 2007

Abstract

Calcium channel blockers (CCBs) have been shown to improve clinical outcomes in atherosclerotic vascular disease. The mechanisms underlying the vasculoprotective effects of a third-generation calcium channel blocker, azelnidipine, are incompletely understood. We asked whether azelnidipine attenuates atherosclerosis in monkeys and mice beyond its blood pressure-lowering effects. Cynomolgus monkeys were randomized to three groups after 4 weeks of a high cholesterol diet: control group (no treatment) and 3 and 10 mg/kg daily azelnidipine; these doses have no effect on systemic arterial pressure or heart rate. Atherosclerosis was induced in the aorta by balloon injury, and the diet and treatment were continued for an additional 24 weeks. Azelnidipine did not affect blood lipid profiles, but reduced the development of atherosclerosis as detected by the elimination of local oxidative stress and reduced expression of monocyte chemoattractant protein-1 and platelet-derived growth factor. Azelnidipine also reduced the proliferation and migration of vascular smooth muscle cells in vitro. In atherosclerotic ApoE-knockout (ApoE-KO) mice fed a high cholesterol diet, azelnidipine but not amlodipine reduced the development of atherosclerosis. Neither drug changed the lipid profiles or systolic blood pressure of the mice. Thus, azelnidipine at clinically relevant doses exhibited anti-atherosclerotic effects in monkeys and mice independent of its blood pressure-lowering effects, suggesting that azelnidipine might be as a "vasculoprotective calcium channel blocker".
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Keywords: Atherosclerosis; Calcium channel blockers; Cytokines; Smooth muscle cells; Azelnidipine

1. Introduction

Calcium channel blockers (CCBs) have been used worldwide to treat patients with hypertension and angina pectoris. Recent clinical trials with CCBs, such as PREVENT, ALLHAT, VALUE, CAPARES, ACTION, and CAMELOT, have provided evidence that reducing arterial blood pressure to close to normal ranges is of great importance for reducing cardiovascular events [1–6]. These clinical trials suggest that CCBs may have pleiotropic actions beyond simply lowering blood pressure. The pleiotropic actions of CCBs may be distinct from its pharmacologic actions related to blocking L-type calcium channels, but may be attributable to their lipophilic character, which gives them a high affinity for the membrane phospholipids of arterial wall cells, such as vascular smooth muscle cells (see review by Mason et al. [7]). CCBs' vasculoprotective actions include improvement of endothelial function, anti-inflammation effects, anti-oxidant effects, and anti-proliferation effects on vascular smooth muscle cells (VSMCs).

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0021-9150/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2007.03.036

In animals, CCBs have been shown to prevent or attenuate atherosclerosis [8], hypertension-induced vascular remodeling, neointimal formation after vascular injury [9]. Azelnidipine is a newly developed third-generation CCB that has anti-hypertensive effects comparable to amlodipine [10]. Azelnidipine is strongly lipophilic and has a high affinity for membrane of vascular wall cells, such as vascular smooth muscle cells [11]. We recently reported that azelnidipine attenuated stent-associated neointimal formation associated with reduced expression of monocyte chemoattractant protein-1 (MCP-1) in non-human primates (cynomolgus monkeys) [12]. In that study, we found that azelnidipine's attenuation of neointimal formation was independent of its blood pressure-lowering actions in vivo, and that azelnidipine directly reduced MCP-1-induced proliferation of VSMC, suggesting that azelnidipine's beneficial effects on in-stent restenosis were mediated in part by direct inhibition of VSMC proliferation. However, the mechanisms of the anti-atherosclerotic action and the clinical significance of azelnidipine were not fully addressed.

The aim of this study is to investigate whether azelnidipine at clinically relevant doses attenuates atherosclerosis in non-human primates (cynomolgus monkeys) in ways that are independent of its blood pressure-lowering effects. To confirm the clinical significance of the findings, we used a non-human primate model of atherosclerosis [13]. Although it is difficult to choose an appropriate animal model for studying atherosclerosis, a non-human primate model may have an advantage over non-primate animal models, such as rabbits and mice: vascular inflammatory and proliferative responses to injury in non-human primates are presumed to be more similar to those in humans than are those of other non-primate animals. Therefore, the use of non-human primates may allow us to evaluate the efficacy of any therapies on atherosclerosis in clinically relevant conditions. Furthermore, to examine whether the observed anti-atherosclerotic effects are unique to azelnidipine or are a class-effect of CCBs, we also compared the effects of azelnidipine and amlodipine in ApoE-knockout (ApoE-KO) mice.

2. Methods

An enhanced Methods and Results section is available online at doi:10.1016/J.atherosclerosis.2007.03.036.

2.1. Cynomolgus monkeys: animals and study protocol

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. Thirty-six 5-year-old male cynomolgus monkeys weighing 4.0 – 6.0 kg were purchased from Primate Ltd. (Gaoyao, Guang Dong, China). The monkeys were fed a high cholesterol diet (0.5% cholesterol and 6% corn oil) for 4 weeks prior to the balloon injury operation. Ticlopidine (100 mg) and aspirin (81 mg) were administered each day starting 7 days before the balloon procedure; ticlopidine was administered for 28 days, and aspirin was continued until euthanization at 6 months. The animals were randomized to 3 groups (n = 12 monkeys per group) as follows: (1) no treatment/vehicle control group (0.5% carboxymethyl cellulose sodium salt); (2) low-dose azelnidipine group (3 mg/kg per day; donated by Sankyo Pharmaceutical Co., Tokyo, Japan); and (3) high-dose azelnidipine group (10 mg/kg per day). These doses of azelnidipine were selected because we previously reported that first, the 3 and 10 mg/kg doses do not affect systemic arterial pressure and heart rate in conscious monkeys by telemetric measurements; and second, the low dose of azelnidipine used in the present study for monkeys was within clinical range, because the maximum drug concentration (C_{max}) of azelnidipine at 3 and 10 mg/kg per day was 36 ± 17 and 107 ± 17 ng/mL, respectively, in monkeys [12], while the C_{max} after oral administration of azelnidipine at 16 mg in hypertensive human subjects is reported to be 48 ± 19 ng/mL. Azelnidipine was administered to the monkeys once a day by gavage for 24 weeks. One week after starting azelnidipine treatment in the azelnidipine groups, all monkeys were anesthetized with ketamine hydrochloride (10 mg/kg IM) and sodium pentobarbital (30 mg/kg IV). The left femoral artery was surgically exposed, a 4 Fr sheath catheter was passed into the femoral artery, and monkeys received a balloon injury of the thoracic and abdominal aorta, as previously described [13]. The right femoral artery was then ligated and the incision was closed. After the operation, all monkeys were fed the same high cholesterol diet. Animal care before and after the operation took place in Gaoyao Kangda Laboratory Animal Science and Technology in China.

2.2. Comparison of azelnidipine and amlodipine in ApoE-KO mice

Male ApoE-KO mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). ApoE-KO mice were fed a Western-type diet (Oriental Yeast, Tokyo, Japan) during the experiment. At 8 weeks of age, mice were randomly assigned into the following groups: (1) no treatment/vehicle control group (n = 10); (2) low-dose azelnidipine group (3 mg/kg per day; n = 6); (3) high-dose azelnidipine group (10 mg/kg per day; n = 7); and (4) amlodipine group (same class CCB; 10 mg/kg per day, donated by Pfizer Japan Inc., Tokyo, Japan; n = 6). Azelnidipine and amlodipine treatment was carried out for 8 weeks by mixing the drug with food for the mice. After 8 weeks of treatment, all mice were sacrificed and tissue was prepared for analysis. Tissue preparation was performed as previously described [14]. Briefly, after the mouse was killed, the aorta was rapidly removed from the left ventricle after perfusion with phosphate-buffered saline. The aorta from the arch to the bifurcation of the iliac artery was fixed in 10% buffered formalin for measurement of the surface area covered by lipid-staining lesions. To quantify the extent of the atherosclerotic lesions, adventitial tissue was removed from

the aortic arch and the aortic arch was opened longitudinally, stained with oil red O, and pinned out on a black wax surface. The percentage of the endothelial surface area stained by oil red O was determined [14].

Plasma total cholesterol, high-density lipoprotein cholesterol and triglycerides concentrations were determined using commercially available kits (Wako Pure Chemicals, Tokyo, Japan). Systolic blood pressure and heart rate were measured every other week (by the tail-cuff method [14]). Moreover, to evaluate the safety of azelnidipine, a multiplex immunoassay was performed using the Luminex Lab MAP instrument by Charles River Inc. (see online Supplementary data).

3. Results

3.1. Histopathological and immunohistochemical measurements in monkeys

In the thoracic aorta, treatment with low and high doses of azelnidipine significantly reduced the neointimal area (Fig. 1A and B; vehicle group: $1.86 \pm 0.23\,mm^2$, low-dose group: $0.96 \pm 0.16\,mm^2$, high-dose group: $1.02 \pm 0.19\,mm^2$). These doses also significantly reduced the intima-media ratio (Fig. 1B: vehicle group: 0.45 ± 0.26, low-dose group: 0.23 ± 0.04, high-dose group: $0.28 \pm$

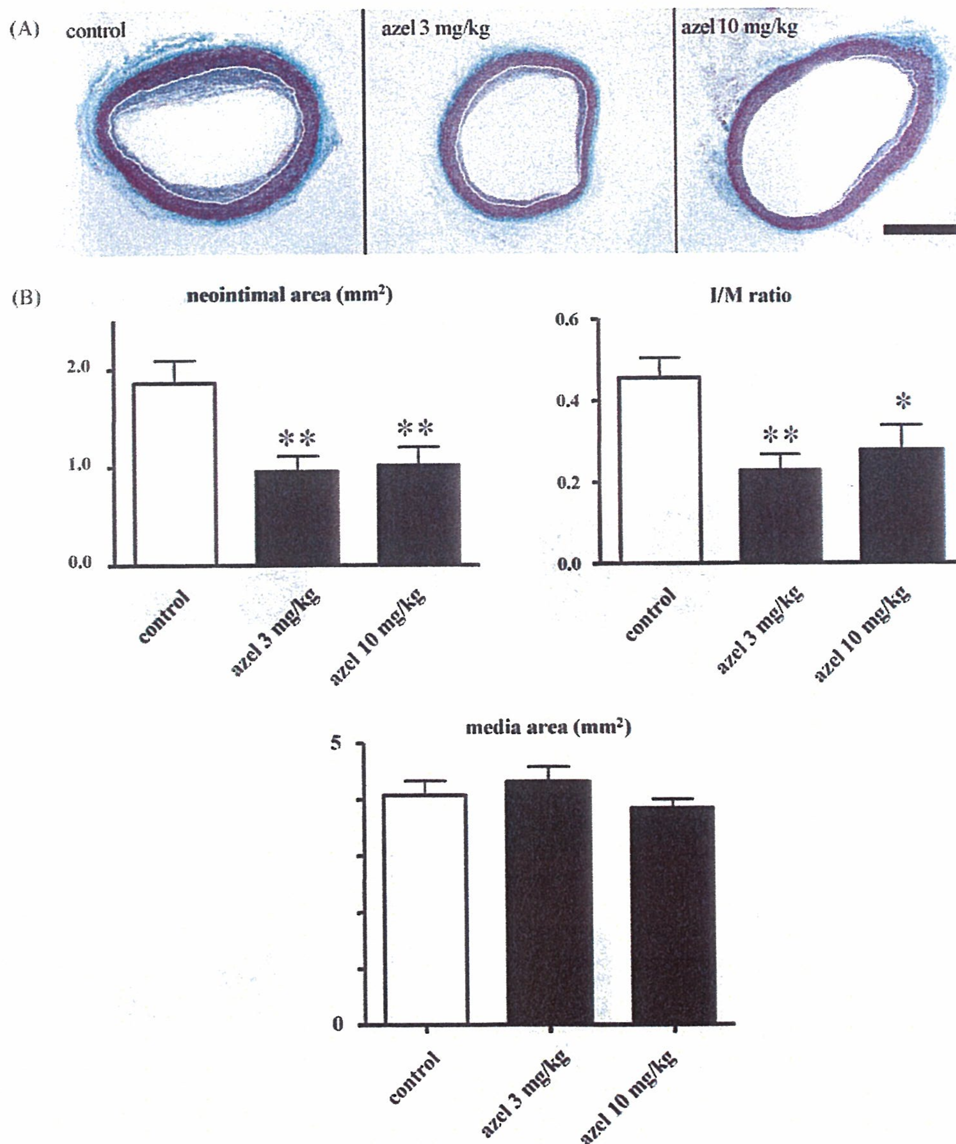

Fig. 1. Effects of azelnidipine on atherosclerosis in monkey thoracic aorta. (A) Representative photomicrographs are shown of Masson's trichrome-stained cross-sections of the injured thoracic aortas from the vehicle control group (control, left panel), low-dose azelnidipine group (azel 3 mg/kg, middle panel) and high-dose azelnidipine group (azel 10 mg/kg, right panel). Internal elastica lamina are outlined with white. Bar = 10 mm. (B) Measurements of the neointimal areas, intima/media (I/M) ratios, and media areas of the thoracic aortas from monkeys treated with or without azelnidipine as indicated (n = 12 each). $^*P < 0.05$, $^{**}P < 0.01$ versus vehicle control group. Each value represents mean ± S.E.M.

Please cite this article in press as: Nakano K, et al., Azelnidipine has anti-atherosclerotic effects independent of its blood pressure-lowering actions in monkeys and mice, Atherosclerosis (2007), doi:10.1016/j.atherosclerosis.2007.03.036