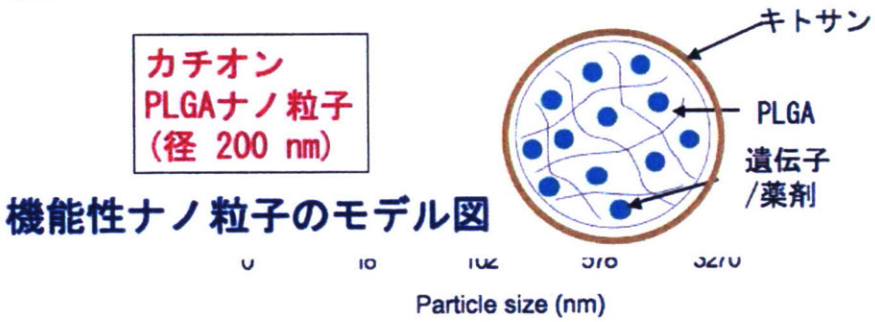
【参考データ】
我々が本研究で用いる PLGA ナノ粒子の特徴について、１）ナノ粒子の概要とモデル図、２）電子顕微鏡写真と粒径分布、３）加水分解のプロセス、４）ナノ粒子に封入された因子の徐放性、に関するデータを提示する。

１）

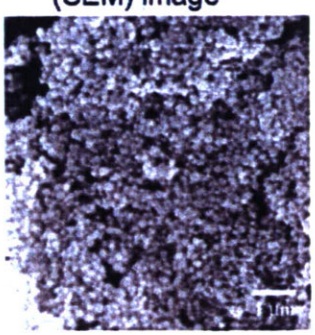
生体吸収性ナノ粒子がもたらす優れた細胞導入率、細胞内安定性と徐放性

- ◆**生体吸収性**高分子（PLGA）ナノ粒子：安全
- ◆**独自の封入技術**：水中エマルジョン溶媒法、水溶性・脂溶性の薬物／遺伝子を封入可能
- ◆**高い細胞内導入効率、安定性**：培養細胞では90％以上、細胞質内で安定して存在、加水分解により消失
- ◆**細胞選択性**：表面機能修飾による導入細胞選択性
- ◆**細胞内（細胞外）DDS**：導入後、封入因子の徐放性



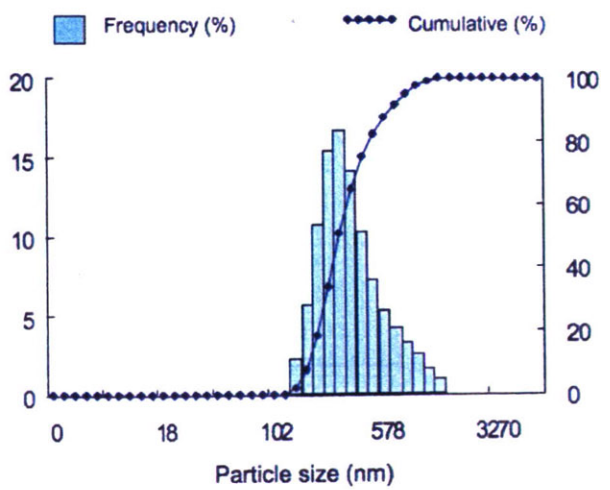
機能性ナノ粒子のモデル図

Scanning electron microscopy (SEM) image



1 μm

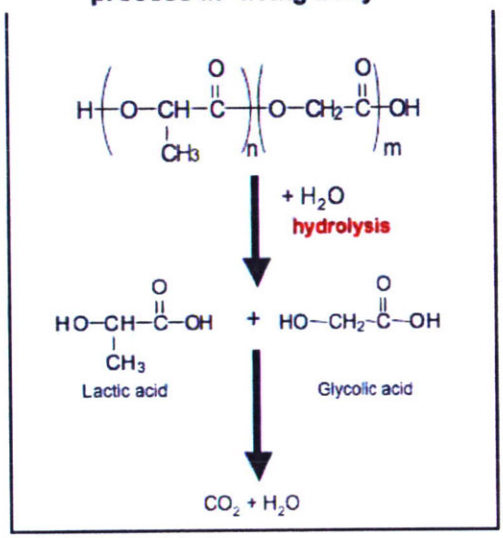
Particle size distribution of FITC-incorporated PLGA nanoparticles in water



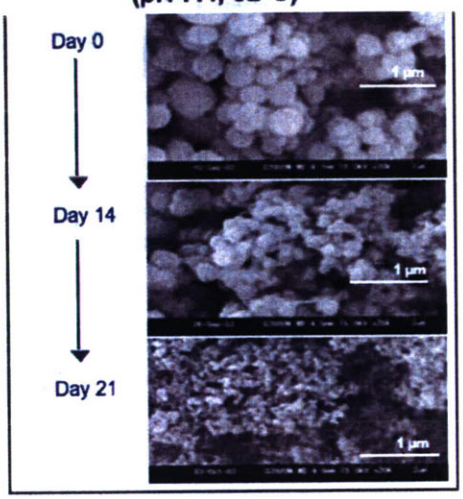
3）

Process of Bioabsorption (hydrolysis) of PLGA in Living Body

Chemical structure of PLGA and its hydrolysis process in living body



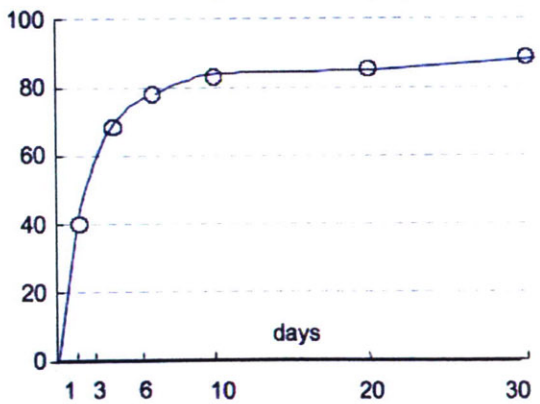
Biodegradation of PLGA nanoparticles (a mean diameter: 200 nm) in phosphate buffer solution (pH 7.4, 32°C)



4）

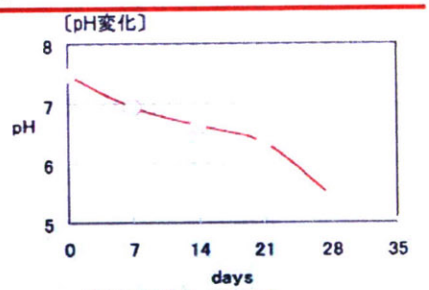
PLGAナノ粒子の溶解性と徐放性の *in vitro* 評価

Cumulative release of FITC from FITC-incorporated NP (%)

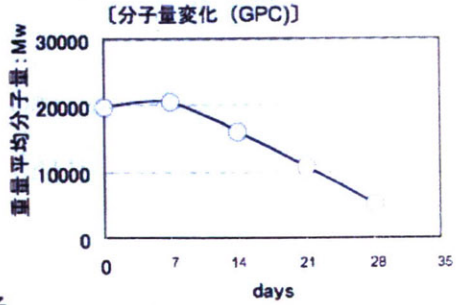


条件：ポリマー2%（酢酸トコフェロール封入PLGAナノ粒子100mg/水5ml)、H7.4、32℃、封入率15%

〔pH変化〕



〔分子量変化（GPC)〕



条件：空PLGAナノスフェア1%,リン酸緩衝液(pH7.4),32℃

8. 健康危険情報

なし

9. 研究発表

1）国内　口頭発表：　18件

原著論文による発表：　0件

それ以外（レビュー等）の発表：　12件

2）国外　口頭発表：　2件

原著論文による発表：　15件

それ以外（レビュー等）の発表：　0件

10. 知的財産権の出願・登録状況

出願2件　（うち国内2件、国外0件）

- 2007.4.27,「肺疾患治療薬」江頭健輔，小島順司，岩永恵，特願2007-119553
- 2007.12.13,「薬物含有ナノ粒子」江頭健輔，特願2007-322409

公開1件

- 2008.8.30,「薬物溶出型ステント及びその製造方法」辻本広行，原香織，塚田雄亮，江頭健輔，特開2007-215620

登録2件（いずれも北米）

- 2007.12. 4,「suppression of post-transplant arteriosclerosis」江頭健輔，竹下　彰，佐田政隆，特許登録US 7,304,040 B2
- 2008.3.18,「actin relrated cytoskeletal protein “LACS”」　江頭健輔，特許登録 US 7,345,158 B2

【研究成果の刊行に関する一覧表】

（1）学会誌発表

＜原著＞

1. Kumai Y, Ooboshi H, Ibayashi S, Ishikawa E, Sugimori H, Kamouchi M, Kitazono T, Egashira K, Iida M: Postischemic gene transfer of soluble Flt-1 protects against brain ischemia with marked attenuation of blood-brain barrier permeability. ***J Cerebr Blood F Met.*** 2007; 27(6):1152-1160.

2. Sasaki S, Inoguchi T, Muta K, Abe Y, Zhang M, Hiasa K, Egashira K, Sonoda N, Kobayashi K, Takayanagi R, Nawata H: Therapeutic Angiogenesis by Ex Vivo Expanded Erythroid Progenitor Cells. ***Am J Physiol Heart Circ Physiol.***2007; 292(1): H657-665.

3. Yamada M, Kuwano K, Maeyama T, Yoshimi M, Hamada N, Fukumoto J, Egashira K, Hiasa K, Takayama K, Nakanishi Y: Gene transfer of soluble transforming growth factor type II receptor by in vivo electroporation attenuates lung injury and fibrosis. ***J Clin Pathol.*** 2007; 60(8): 916-920.

4. Monden Y, Kubota T, Tsutsumi T, Inoue T, Kawano S, Kawamura N, Ide T, Egashira K, Tsutsui H, Sunagawa K: Soluble TNF receptors prevent apoptosis in infiltrating cells and promote ventricular rupture and remodeling after myocardial infarction. ***Cardiovasc Res.*** 2007; 73(4): 794-805.

5. Kusaba K, Kai H, Koga M, Takayama N, Ikeda A, Yasukawa H, Seki Y, Egashira K, Imaizumi T: Inhibition of Intrinsic Interferon-γ Function Prevents Neointima Formation After Balloon Injury. ***Hypertension*** 2007; 49(4):909-915.

6. Koga M, Kai H, Yasukawa H, Kato S, Yamamoto T, Kawai Y, Kusaba K, Seki Y, Kai M, Egashira K, Kataoka Y, Imaizumi T: Postnatal Blocking of Interferon-γ Function Prevented Atherosclerotic Plaque Formation in Apolipoprotein E-knockout Mice. ***Hypertension Res.*** 2007; 30(3):259-267.

7. Takewaki H, Egashira K, Kimura S, Nishida T, Morita S, Tominaga R: Blockade of Monocyte Chemoattractant Protein-1 by Adenoviral Gene Transfer Inhibits Experimental Vein Graft Neointimal Formation. ***J Vasc Surg.*** 2007; 45(6): 1236-1243.

8. Koga M, Kai H, Yasukawa H, Yamamoto T, Kawai Y, Kato S, Kusaba K, Kai M, Egashira K, Kataoka Y, Imaizumi T: Inhibition of progression and stabilization of plaques by postnatal interferon-gamma function blocking in ApoE-knockout mice. ***Circ Res.*** 2007; 101(4): 348-356.

9. Nakano K, Egashira K, Ohtani K, Zhao G, Funakoshi K, Ihara Y, Sunagawa K:

Catheter-based adenovirus-mediated anti-monocyte chemoattractant gene therapy attenuates in-stent neointima formation in cynomolgus monkeys. ***Atherosclerosis.*** 2007; 194(2): 309-316.

10. Nakano K, Egashira K, Ohtani K, Iwata E, Miyagawa M, Sunagawa K: Azelnidipine Has Anti-atherosclerotic Effects Independent of its Blood Pressure- Lowering Actions in Monkeys and Mice. ***Atherosclerosis.*** 2008; 196: 172-179.

11. Ihara Y, Egashira K, Nakano K, Ohtani K, Kubo M, Koga J, Iwai M, Horiuchi M, Zhao G, Yamagishi S, Sunagawa K: Upregulation of the ligand-RAGE pathway via the angiotensin II type I receptor is essential in the pathogenesis of diabetic atherosclerosis. ***J Mol Cell Cardiol.*** 2007; 43(4): 455-464.

12. Egashira K, Nakano K, Ohtani K, Funakoshi K, Zhao G, Ihara Y, Koga J, Kimura S, Tominaga R, Sunagawa K: Local Delivery of anti-monocyte chemoattractant protein-1 by gene-eluting stents attenuates in-stent stenosis in rabbits and monkeys. ***Artemoscler Thromb Vasc Biol.*** 2007; 27(12): 2563-2568.

13. Koga M, Kai H, Egami K, Murohara T, Ikeda A, Yasuoka S, Egashira K, Matsuishi T, Kai M, Kataoka Y, Kuwano M, Imaizumi T: Mutant MCP-1 therapy inhibits tumor angiogenesis and growth of malignant melanoma in mice. ***Biochem Bioph Res Commun.*** 2008; 365(2): 279-284.

14. Ishibashi T, Zhao H, Kawabe K, Oono T, Egashira K, Suzuki K, Nawata H, Takayanagi R, Ito T: Blocking of monocyte chemoattractant protein-1 (MCP-1) activity attenuates the severity of acute pancreatitis in rats. ***J Gastroenterol.*** 2008; 43(1): 79-85.

15. Kimura S, Egashira K, Nakano K, Iwata E, Miyagawa M, Tsujimoto H, Hara K, Kawashima Y, TominagaR, Sunagawa K: Local Delivery of Imatinib Mesylate (STI571) Incorporated Nanoparticle Ex Vivo Suppresses Vein Graft Neointima Formation. ***Circulation.*** 2008 in press.

(2) 口頭発表

＜国内学会＞

1. 第 70 回日本循環器学会総会（平成 19 年 3 月 15 日、兵庫）江頭健輔： Sustained intracellular delivery of nanoparticles in porcine coronary arteries from a bioabsorbable polymeric nanoparticle-eluting stent（プレナリーセッション）

2. 第 13 回 Circulation Club（平成 19 年 6 月 16 日、兵庫）江頭健輔：血管内皮細胞標的化ナノ DDS ステントを用いたスタチン局所デリバリーによる低侵襲治療的血管新生療法の開発

3. 第32回九州MMC研究会（平成19年7月7日、福岡）江頭健輔：生体吸収性ナノDDS ステントの創製- 我が国発世界標準の次世代低侵襲血管内ナノ医療を目指して- （シンポジウム）

4. 第39回日本動脈硬化学会総会・学術集会（平成19年7月13日、大阪）江頭健輔：血管内皮細胞標的化ナノDDSステントを用いたスタチン局所デリバリーによる低侵襲治療的血管新生療法の開発（ジョイントシンポジウム）

5. 第55回日本心臓病学会学術集会（平成19年9月11日、東京）江頭健輔：薬剤溶出性ステントの問題点（遅発性血栓症、など）と新しい次世代アプローチ（分子細胞標的薬溶出・生体吸収性ナノDDSステント）（教育講演）

6. 第35回日本臨床免疫学会総会（平成19年10月19日、大阪）江頭健輔：心血管病態・臓器不全の病態におけるMCP-1の役割解明を基盤とするトランスレーショナルリサーチ（シンポジウム）

7. 第15回日本血管生物医学会学術大会（平成19年11月29日、福岡）江頭健輔：DESの陰から光を探る生体吸収性ナノDDSステントの創製（プレナリーセッション）

8. NEDO委託開発・橋渡し促進技術開発研究会（平成19年12月3日、岡山）江頭健輔：生体吸収性ナノDDS技術搭載ステント

9. 第1回低侵襲医療機器実用化フォーラム・第12回ナノメディシン研究会（平成19年12月20日、東京）江頭健輔：薬剤溶出ステントの問題点と新規対策（オーバービュー，コーディネーター講演）**資料添付**

10. CCT2008（平成20年1月31日、神戸）江頭健輔：DESの陰から光を探る薬剤溶出ステントの問題点と新しい次世代アプローチ（生体吸収性ナノDDSステントなど）（ランチョンセミナー）

11. 平成19年度　厚生労働科学研究費研究成果等普及啓発事業　医療機器開発推進研究　ナノメディシン研究成果発表会（平成20年2月27日、東京）江頭健輔：先端技術（医・工・薬・ナノ）融合のインテリジェントナノDDS制御技術開発に基づく低侵襲血管内医療システム（分子標的医薬溶出・生体吸収性ステントetc）の創製と臨床応用

12. 第81回日本薬理学会年会（平成20年3月18日、横浜）江頭健輔：血管内皮細胞選択的ナノDDSを基盤とするスタチン送達による低侵襲治療的血管新生療法の創製（シンポジウム）

＜国際学会＞

1. The 16th Asian Pacific Congress of Cardiology （December 13-16, 2007, Taipei）Egahira

K: Impact fo nanotechnology-Based Drug Delivery System (NanoDDS) for Treatment of Cardiovasculalr Disease（symposium）

2. Taiwan Society of Lipids & Atherosclerosis （December 15, 2007, Taipei）Egahira K: Impact of nanotechnology-Based Drug Delivery System (NanoDDS) for Treatment of Cardiovasculalr Disease（plenary session）

（3）出版物

＜総説＞

1. 船越公太、江頭健輔：ステント内再狭窄の分子機構と生体吸収性ナノ粒子電着による遺伝子溶出ステントがもたらす新たな治療戦略. 日本薬理学雑誌 社団法人日本薬理学会 2007；129(3)：171-176

2. 船越公太、江頭健輔：ナノ DDS 制御コーティング技術の創製による次世代ステント内再狭窄抑制対策の開発. Mebio メジカルビュー社 2007；24(4)：106-117

3. 竹本真生、江頭健輔:Sirolimus と Paclitaxel の薬理学的特徴と血管に与える「benefit」と「risk」. Coronary Intervention メディアルファ 2007；3(5)

4. 古賀純一郎、江頭健輔：循環器疾患における MCP-1 の重要性—動脈硬化性疾患を中心に. 医学のあゆみ 医歯薬出版株式会社 2007；221(13)：1190-1194

5. 江頭健輔：Over View 衝撃：DES は誰の心を痛くするのだろう？-患者，医師，それとも企業. 血管医学 メディカルレビュー社 2007；8(3)：77(301)-81(305)

6. 中野覚、月江教昭、増田征剛、江頭健輔：薬剤溶出ステントと遅発性血管症 ーDES の陰から光を探るー 血管生物医学の視点：DES 遅発性血管症の衝撃と分子機序. 血管医学 メディカルレビュー社 2007；8(3)：83(307)-88(312)

7. 増田征剛、江頭健輔：生体吸収性ナノ粒子電着コーティングステント. 炎症と免疫 先端医学社 2007；16(1)：9(9)-14(14)

8. 的場哲哉、古賀純一郎、江頭健輔：ケモカインと動脈硬化. 医学のあゆみ 医歯薬出版株式会社 2007；223(13) 1205-1210

9. 向井靖、江頭健輔：Nitric oxide. 高血圧ナビゲーター メディカルレビュー社 2008；1(1) 98-99

10. 向井靖、江頭健輔：特集 データブック 血栓症の大規模臨床試験 PART 2 4. 動脈硬化危険因子 7. 中等度および強化脂質低下療法が冠動脈硬化症の新興に与える影響- 無作為化比較対照試験. 血栓と循環 メディカルレビュー社 2007；15(4) 190(500)-191(501)

11. 中野覚、江頭健輔：4.新たなるテクノロジーとは？ ナノ医工学によるナノ DDS

技術開発に基づく生体吸収性ナノ粒子コーティングステントの創製. 循環器専門医　日本循環器学会　2008；16(1)　67-74

＜新聞報道＞

1. 平成20年3月28日掲載、日刊工業新聞「九大　次世代DESにめど　血管狭窄防止治療　副作用抑制を確認」

＜第15回日本血管生物医学会学術大会開催＞

1. 平成19年11月29、30日（九州大学百年講堂）**資料添付**

【研究成果の刊行物・別刷】

○をつけた論文の別刷あるいは資料を次のページ以降に添付します。

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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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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.

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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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

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

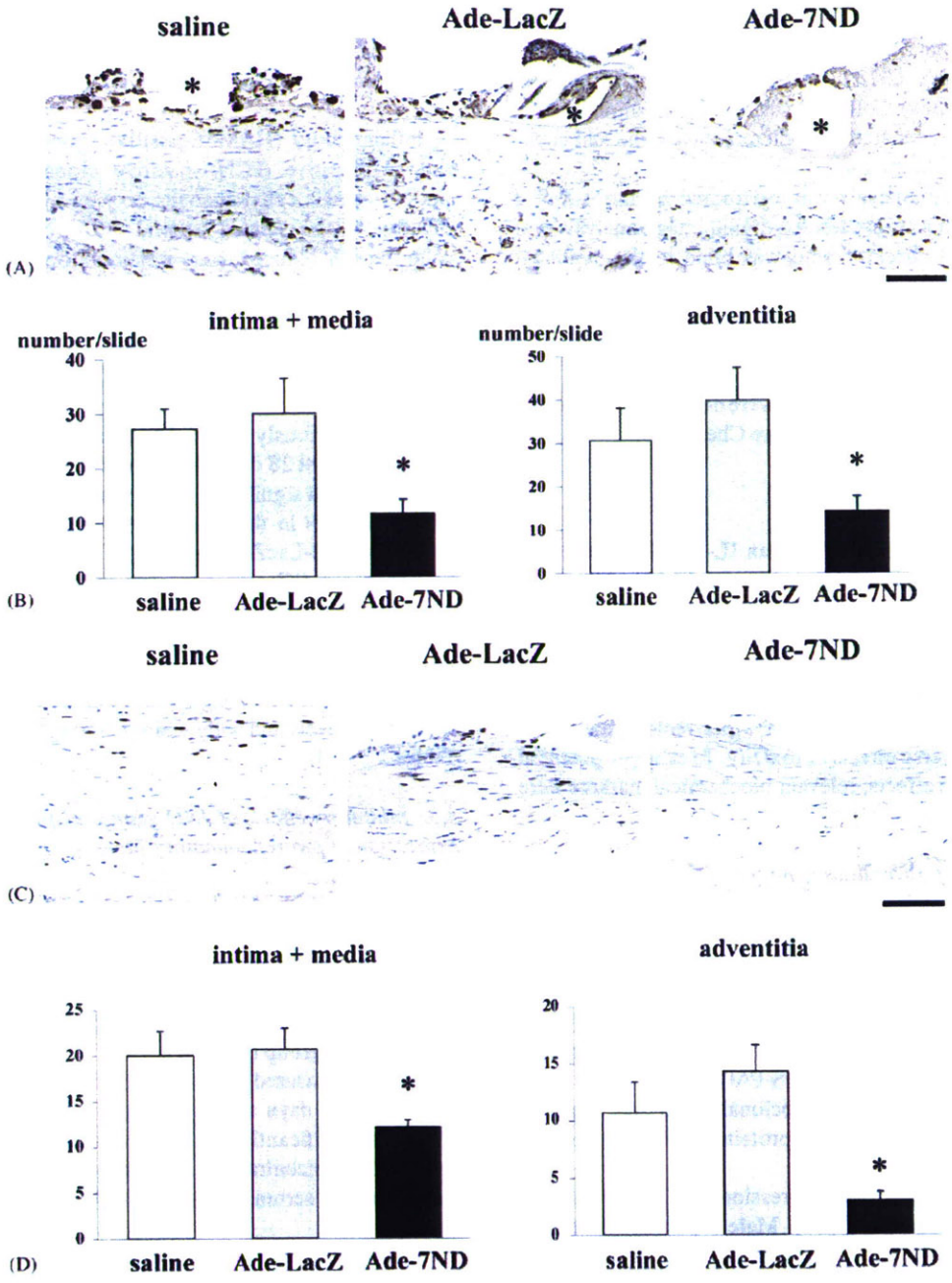

Fig. 1. Inhibitory effects of 7ND gene transfer on local inflammation and proliferation. (A) Effect of the 7ND gene transfer on local inflammation (HAM56-positive monocyte/macrophages) 7 days after stenting. Asterisk (*) indicates stent strut sites. Bar = 100 μm. (B) Summary of quantitative analysis ($n = 7$ 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

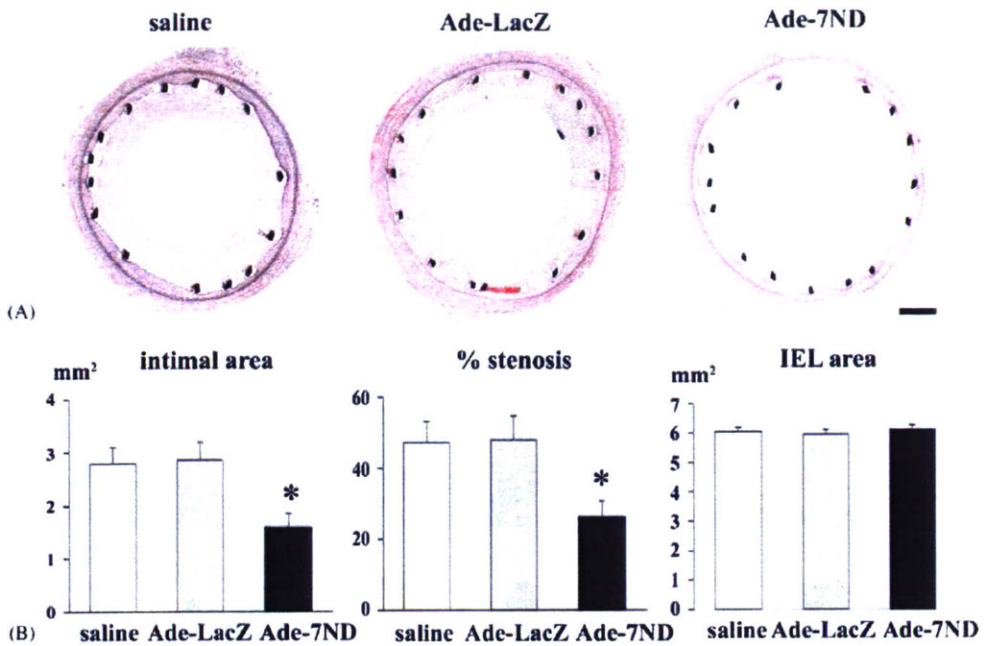

Fig. 2. Inhibitory effect of 7ND gene transfer on in-stent neointimal formation. (A) Iliac artery sections from the saline, the Ade-LacZ, and the Ade-7ND group 28 days after stenting, stained with elastic van Gieson. Bar = 500 μm. (B) Effect of 7ND gene transfer on intimal area, % stenosis, and IEL area 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 ± 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 ± 0.3 mm² and 1.4 ± 0.2 mm², 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 ± 0.2 mm² and 2.4 ± 0.2 mm², 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

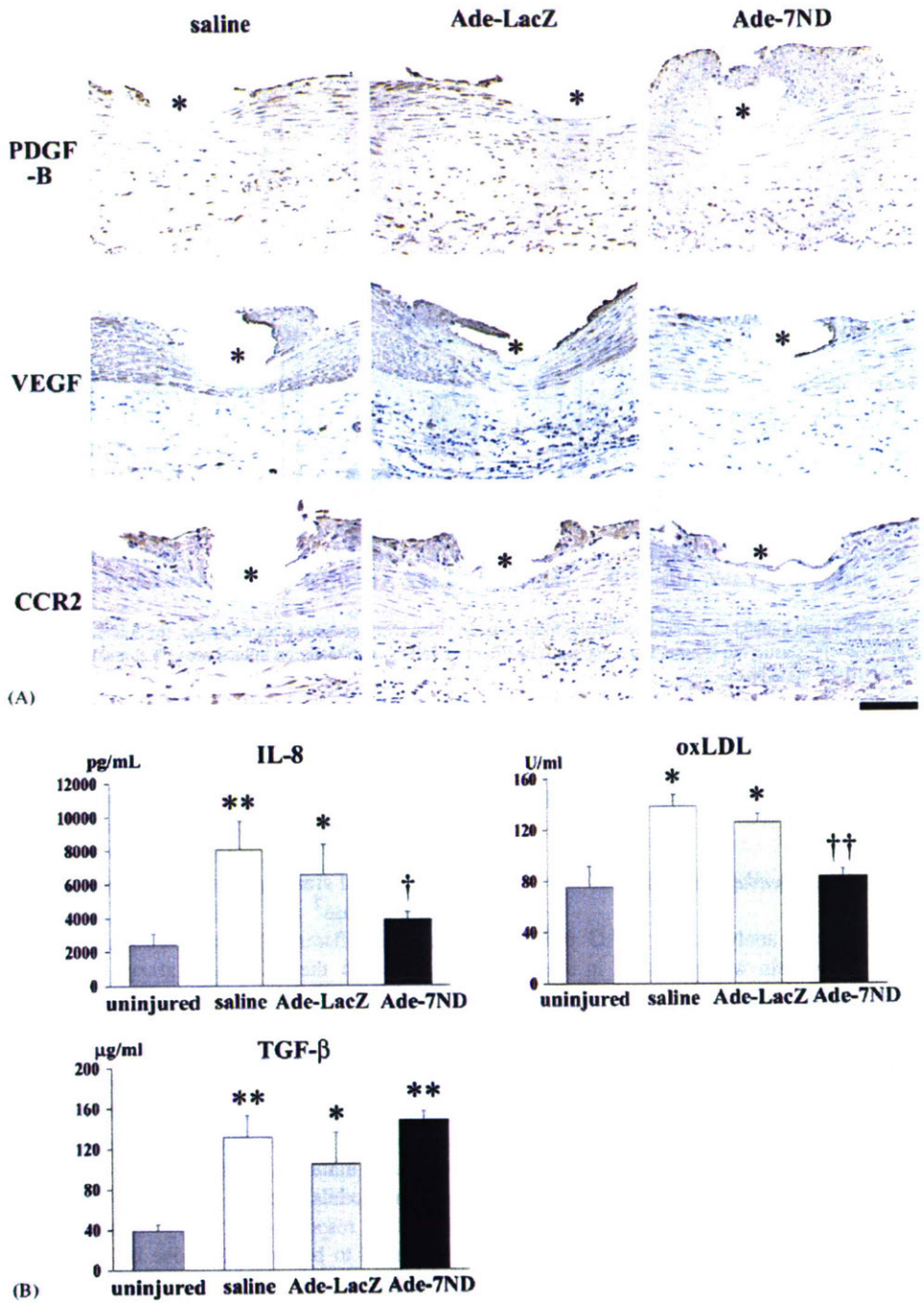

Fig. 3. Effects of 7ND gene transfer on immunohistochemical expression of PDGF-B, VEGF, and CCR2 and on plasma concentrations of IL-8, oxLDL, and TGF-β. (A) Iliac artery sections from uninjured normal arteries, and those from the Ade-LacZ and Ade-7ND groups 7 days after stenting. They are stained immunohistochemically 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

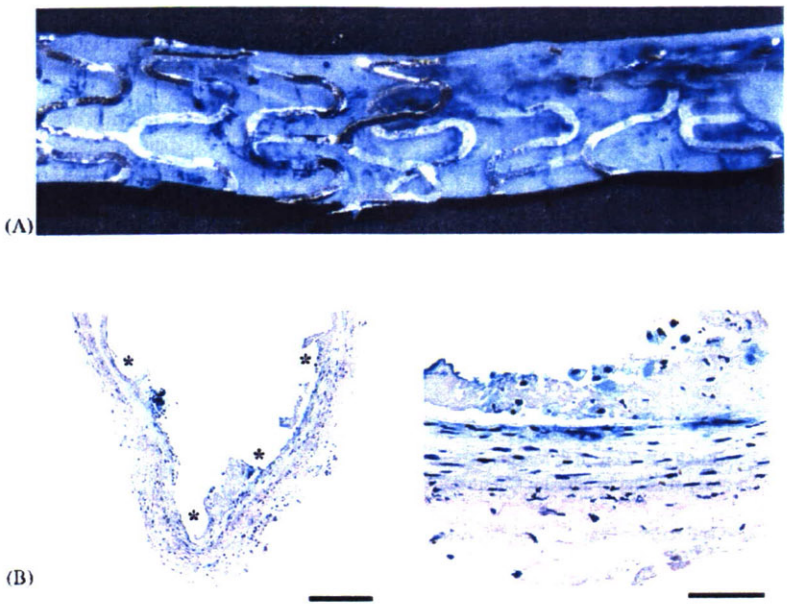

Fig. 4. Gene transfer into rabbit iliac stented artery after local infusion of de-LacZ via the Remedy delivery catheter. (A) Macroscopic picture of lumenal surface of the stented iliac artery staining with X-gal 4 days after transfection. Stented arterial segments were excised, cult longitudinally, and 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

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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.

References

[1] Farb A, Weber DK, Kolodgie FD, Burke AP, Virmani R. Morphological predictors of restenosis after coronary stenting in humans. Circulation 2002;105:2974–80.
[2] Welt FG, Rogers C. Inflammation and restenosis in the stent era. Arterioscler Thromb Vasc Biol 2002;22:1769–76.
[3] Egashira K. Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. Hypertension 2003;41:834–41.
[4] Gerard C, Rollins BJ. Chemokines and disease. Nat Immunol 2001;2:108–15.
[5] Mukaida N, Harada A, Matsushima K. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev 1998;9:9–23.
[6] Egashira K, Koyanagi M, Kitamoto S, et al. Anti-monocyte chemoattractant protein-1 gene therapy inhibits vascular remodeling in rats: blockade of MCP-1 activity after intramuscular transfer of a mutant gene inhibits vascular remodeling induced by chronic blockade of NO systhesis. FASEB J 2000;14:1974–8.
[7] Ohtani K, Usui M, Nakano K, et al. Antimonocyte chemoattractant protein-1 gene therapy reduces experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys. Gene Ther 2004;11:1273–82.
[8] Egashira K, Zhao Q, Kataoka C, et al. Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. Circ Res 2002;90:1167–72.
[9] Mori E, Komori K, Yamaoka T, et al. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. Circulation 2002;105:2905–10.
[10] Usui M, Egashira K, Ohtani K, et al. Anti-monocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. FASEB J 2002;16:1838–40.
[11] Ni W, Egashira K, Kitamoto S, et al. New anti-monocyte chemoattractant protein-1 gene therapy attenuates atherosclerosis in apolipoprotein E-knockout mice. Circulation 2001;103:2096–101.
[12] Inoue S, Egashira K, Ni W, et al. Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. Circulation 2002;106:2700–6.
[13] Roque M, Kim WJ, Gazdoin M, et al. CCR2 deficiency decreases intimal hyperplasia after arterial injury. Arterioscler Thromb Vasc Biol 2002;22:554–9.
[14] Horvath C, Welt FG, Nedelman M, Rao P, Rogers C. Targeting CCR2 or CD18 inhibits experimental in-stent restenosis in primates: inhibitory potential depends on type of injury and leukocytes targeted. Circ Res 2002;90:488–94.
[15] Costa MA, Simon DI. Molecular basis of restenosis and drug-eluting stents. Circulation 2005;111:2257–73.
[16] Schmidt-Lucke C, Rossig L, Fichtlscherer S, et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 2005;111:2981–7.
[17] Virmani R, Farb A, Guagliumi G, Kolodgie FD. Drug-eluting stents: caution and concerns for long-term outcome. Coron Artery Dis 2004;15:313–8.
[18] Virmani R, Guagliumi G, Farb A, et al. Localized hypersensitivity and late coronary thrombosis secondary to a sirolimus-eluting stent: should we be cautious? Circulation 2004;109:701–5.
[19] McFadden EP, Stabile E, Regar E, et al. Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy. Lancet 2004;364:1519–21.
[20] Hiasa K, Ishibashi M, Ohtani K, et al. Gene transfer of stromal cell-derived factor-1alpha enhances ischemic vasculogenesis and angiogenesis via vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway: next-generation chemokine therapy for therapeutic neovascularization. Circulation 2004;109:2454–61.
[21] Ohtani K, Egashira K, Hiasa K, et al. Blockade of vascular endothelial growth factor suppresses experimental restenosis after intraluminal injury by inhibiting recruitment of monocyte lineage cells. Circulation 2004;110:2444–52.
[22] Channon KM, Qian HS, Youngblood SA, et al. Acute host-mediated endothelial injury after adenoviral gene transfer in normal rabbit arteries: impact on transgene expression and endothelial function. Circ Res 1998;82:1253–62.
[23] Varenne O, Gerard RD, Sinnaeve P, Gillijns H, Collen D, Janssens S. Percutaneous adenoviral gene transfer into porcine coronary arteries: is catheter-based gene delivery adapted to coronary circulation? Hum Gene Ther 1999;10:1105–15.
[24] Hiltunen MO, Laitinen M, Turunen MP, et al. Intravascular adenovirus-mediated VEGF-C gene transfer reduces neointima formation in balloon-denuded rabbit aorta. Circulation 2000;102:2262–8.
[25] Egashira K. Clinical importance of endothelial function in arteriosclerosis and ischemic heart disease. Circ J 2002;66:529–33.
[26] Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury. Part II. Animal and human studies. Circulation 2003;108:2034–40.
[27] Selzman CH, Miller SA, Zimmerman MA, Gamboni-Robertson F, Harken AH, Banerjee A. Monocyte chemotactic protein-1 directly induces human vascular smooth muscle proliferation. Am J Physiol Heart Circ Physiol 2002;283:H1455–61.
[28] Fukuda D, Sata M, Tanaka K, Nagai R. Potent inhibitory effect of sirolimus on circulating vascular progenitor cells. Circulation 2005;111:926–31.
[29] Kitamoto S, Nakano K, Hirouchi Y, et al. Cholesterol-lowering independent regression and stabilization of atherosclerotic lesions by pravastatin and by antimonocyte chemoattractant protein-1 therapy in nonhuman primates. Arterioscler Thromb Vasc Biol 2004;24:1522–8.

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 effects[3,4]: 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 injury[11–14] and atherosclerosis[15,16] 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 proliferation[11] 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

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formation. To assess its potential clinical utility, we used a nonhuman primate model of stent-associated neointima formation.[11] 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.[11] 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 levels were determined with commercially available kits (Wako Pure Chemicals).

The stented artery segments were processed as described previously.[11] 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 IA). 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 IB). 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.[11,22] 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-

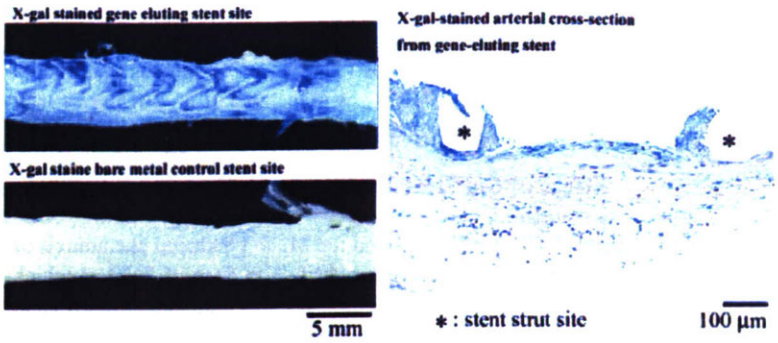

Figure 1. Gene transfer in the rabbit iliac stented artery 3 days after β-galactosidase gene-eluting stent. Upper and lower left: Macroscopic image of the luminal surface of the stented iliac artery. Stented arterial segments were excised, cut longitudinally, and stained with X-gal. Right: X-gal-stained arterial cross-sections.

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).

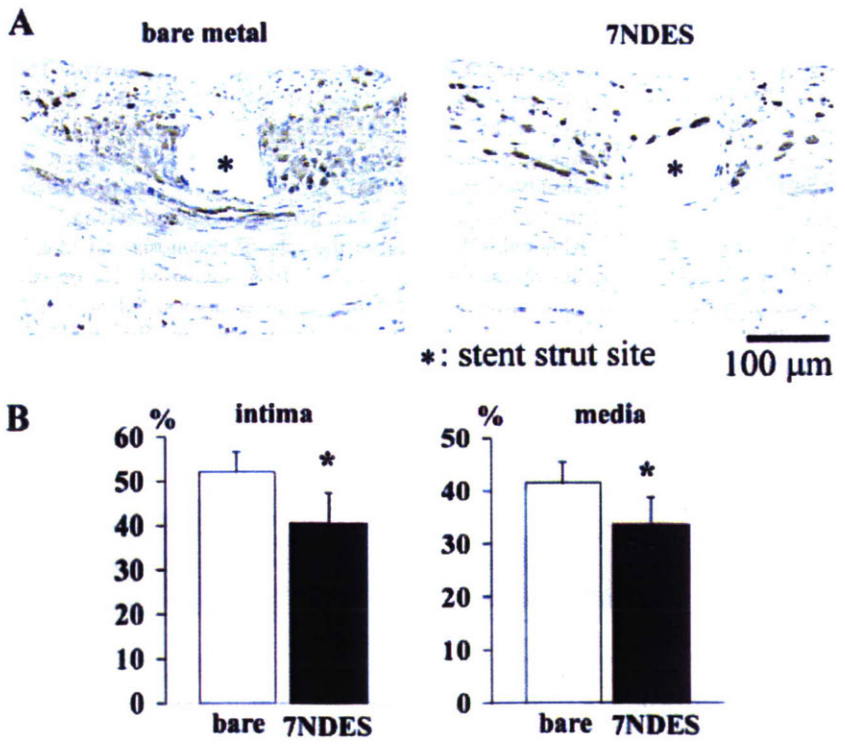

Figure 2. Effect of 7ND gene-eluting stents (7NDES) on local inflammation in rabbits. A, Inflammation (RAM-11–positive monocytes/macrophages) 10 days after stenting. B, Summary of quantitative analysis, as reported by the percentage of immunopositive cells per total cells; n=7 each. *$P<0.01$ vs the noncoated stents.

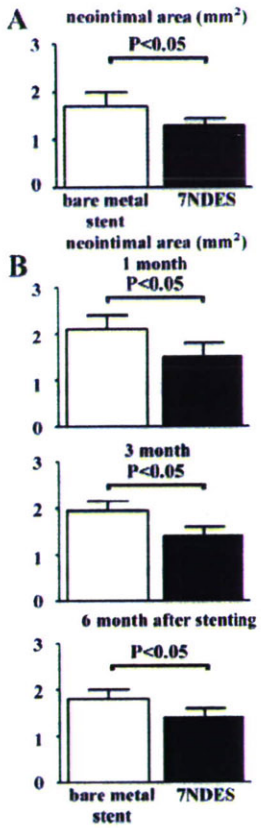

Figure 3. Inhibitory effect of 7ND gene-eluting stents (7NDES) on in-stent neointima formation in rabbits (A) and monkeys (B). A, Neointimal area 28 days after stenting (n=7 each). B, Neointima area at 1, 3, and 6 months (M) after stenting (n=6 each).

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 polymorphic nuclear 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,[23] 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,[24] 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 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.[4–6] Silorimus and paclitaxel have

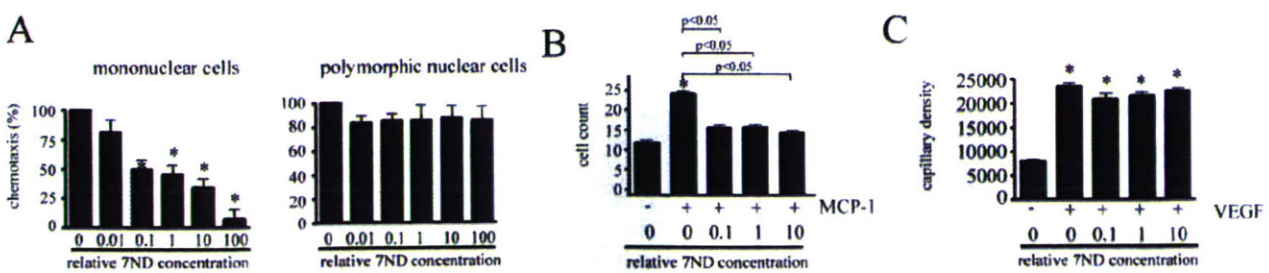

Figure 4. Effect of 7ND on chemotaxis of mononuclear leukocytes (A, n=8 each), proliferation of hCASMCs (B, n=8 each), and angiogenic activity of endothelial cells (C, n=8 each). Concentrations of 7ND are expressed in relation to concentrations of the agonist. *$P<0.05$ vs control.