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）

4. 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）

5. 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）

6. 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）

7. 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）

8. 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）

9. 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

10. 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）

11. 第 40 回日本動脈硬化学会総会・学術集会（平成 20 年 7 月 10 日、茨城）江頭健輔：生体吸収性ナノ DDS ステントによる革新的血管内ナノ医療の創製（Featured Session）

12. 第 31 回日本高血圧学会総会（平成 20 年 10 月 9 日、札幌）江頭健輔：Critical role of the CCL2/CCR2 pathway in the pathogenesis of angiotensin II-induced atherosclerosis and plaque rupture（Special Lecure）

13. 第 16 回日本血管生物医学会 The 6th Korea-Japan Joint Symposium on Vascular Biology 合同学術集会（平成 20 年 12 月 4 日、石川）江頭健輔：Therapeutic Neovascularization by Nanotechnology-Mediated Cell-Selective Delivery of Pitavastatin into the Vascular Endothelium（ランチョンセミナー）

14. 第 5 回ナノバイオ国際シンポジウム（平成 21 年 2 月 19 日、東京）江頭健輔：血管内皮細胞選択的ナノ DDS 技術開発を基盤とする革新的低侵襲治療的血管新生療法の実現（招待講演）

15. 平成 20 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 21 年 2 月 25 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

16. 次世代医療システム産業化フォーラム（平成 21 年 3 月 2 日、大阪）江頭健輔：生体吸収性ナノ粒子 DDS を基盤とする血管内ナノ医療（ステント、カテーテル）の研究開発

17. 第 7 回京都 Cardio カンファレンス（平成 21 年 4 月 7 日、京都）江頭健輔：ナノテクノロジーによる動脈硬化プラーク破綻の治療（特別講演）

18. 第 48 回日本生体医工学会大会（平成 21 年 4 月 25 日、東京）江頭健輔：生体吸収性ナノ粒子 DDS を基盤とする血管内医療（ステント、カテーテル）の研究開発（オーガナイズドセッション）

19. 遺伝子デリバリー研究会（平成 21 年 7 月 9 日、大阪）江頭健輔：生体吸収性ナノ粒子 DDS を基盤とする血管内医療（ステント、カテーテル）の研究開発（シンポジウム）

20. 新産業を創る先端科学技術フォーラム２００９（平成 21 年 10 月 22 日、大阪）江頭健輔：血管内皮細胞選択的ナノ DDS 技術を基盤とする革新的ナノ医療の創製：新しい治療的血管新生療法の実用化を目指して

21. CCT2010（平成 22 年 1 月 28 日、兵庫）江頭健輔：Beyond Restenosis –New Approaches to Optimal Medical Therapy-（シンポジウム）

22. 平成 21 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 22 年 2 月 24 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

＜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. The 2nd Oriental Congress of Cardiology (May 30, 2008, Shanghai) Egashira K: Drug-eluting stents: where are we and where we are going? （Special lecture）

4. The Annual Scientific Meeting of Taiwan Society of Lipids and Atherosclerosis 2008 and

The 8th Taipei International Vascular Molecular Biology Symposium(Sep 20, 2008, Taipei) Egashira K: Impact of Nanotechnology on Innovation of Cardiovascular Medicine (Special Lecture)

5. The Annual Scientific Meeting of Taiwan Society of Lipids and Atherosclerosis 2009 and The 9th Taipei International Vascular Molecular Biology Symposium(Sep 27, 2009, Taipei) Egashira K: Impact of Nanotechnology-based Drag Delivery on Treatment of Cardiovascular (Special Lecture)

6. Scientific Sessions 2009 of the American Heart Association （November 14-18, 2009, Orlando） Tsukie N, Egashira K, Nakano K, Matoba T, Sunagawa K: Pitavastatin incorporated nanoparticle-eluting stents attenuate in-stent stenosis without anti-healing effects induced by sirolimus-eluting stents (Cypher) in porcine coronary artery model

(3) 出版物

＜著書＞

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＜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」

2. 平成20年6月15日掲載、西日本新聞「ステント治療副作用少なく　九大病院、ナノテクで開発」

3. 平成20年10月22日掲載、日経産業新聞「2030年への挑戦　次世代産業技術」

4. 平成20年11月18日掲載、日本経済新聞「先端医療技術実用化促す　政府　スーパー特区24件決定」

5. 平成20年11月19日掲載、産経新聞「「先端医療開発特区」政府選定　スーパー特区24件　iPS応用、虫歯治療…」

6. 平成20年11月19日掲載、西日本新聞「初のスーパー特区に24件」

7. 平成20年11月28日掲載、科学新聞「先端医療開発特区の課題決定　iPSなど5分野24件　関係省庁一体で取り組み」

8. 平成20年12月17日掲載、西日本新聞「先進的医療　早期実現へ　九大など開発特区　「人工脳」実用化目指す」

9. 平成21年3月23日掲載、化学工業日報「九大-ホソカワ粉体技研　次世代DES、前臨床へ　ナノ粒子に薬剤封入　実用化でメーカーと交渉」

＜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）、大会長：江頭健輔

【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。

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

Original received December 24, 2006; final version accepted September 12, 2007.

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K.E., K.N., and K.O. contributed equally to this study.

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.107.154609

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-

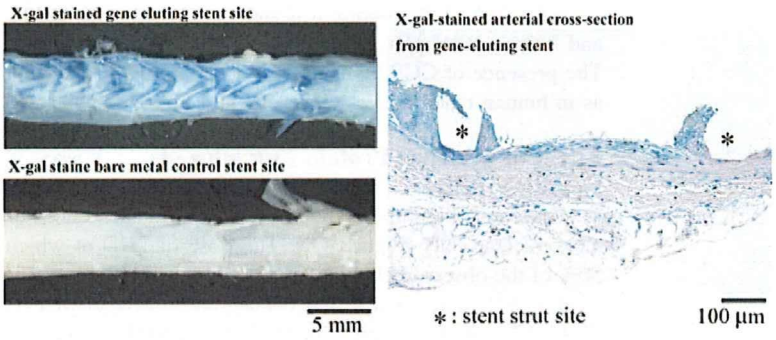

Figure 1. Gene transfer in the rabbit iliac stented artery 3 days after β-galactosidase gene-eluting stent. Upper and lower left: Macroscopic image of the luminal surface of the stented iliac artery. Stented arterial segments were excised, cut longitudinally, and stained with X-gal. Right: X-gal-stained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

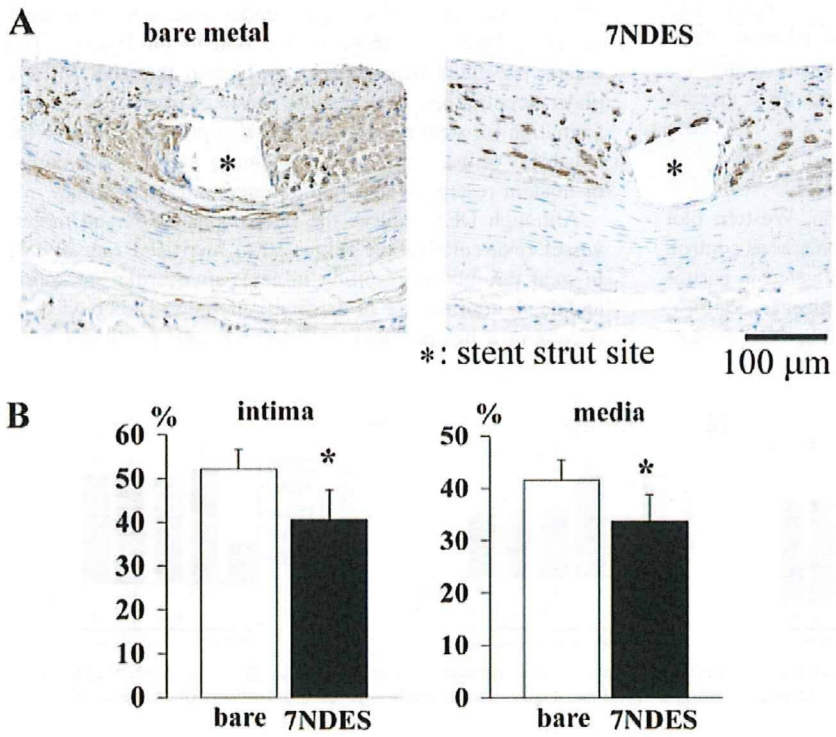

Figure 2. Effect of 7ND gene-eluting stents (7NDES) on local inflammation in rabbits. A, Inflammation (RAM-11–positive monocytes/macrophages) 10 days after stenting. B, Summary of quantitative analysis, as reported by the percentage of immunopositive cells per total cells; n=7 each. *$P<0.01$ vs the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

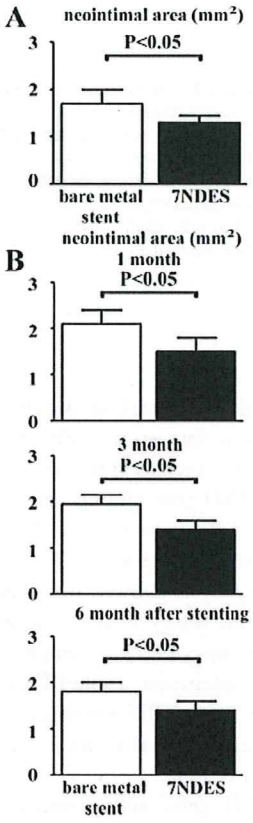

Figure 3. Inhibitory effect of 7ND gene-eluting stents (7NDES) on in-stent neointima formation in rabbits (A) and monkeys (B). A, Neointimal area 28 days after stenting (n=7 each). B, Neointima area at 1, 3, and 6 months (M) after stenting (n=6 each).

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

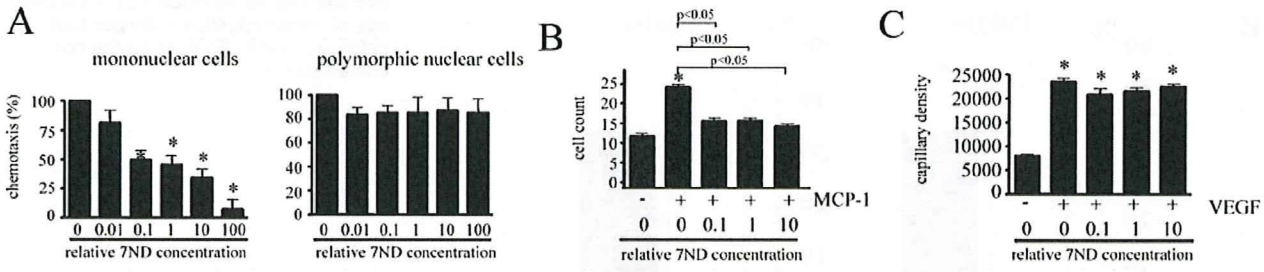

Figure 4. Effect of 7ND on chemotaxis of mononuclear leukocytes (A, n=8 each), proliferation of hCASMCs (B, n=8 each), and angiogenic activity of endothelial cells (C, n=8 each). Concentrations of 7ND are expressed in relation to concentrations of the agonist. *$P<0.05$ vs control.

been shown to impair reendothelialization and arterial healing process, resulting thrombogenesis attributable to increased expression of tissue factor.[4] In addition, nonbiocompatible polymers used to load these drugs have been associated with DES thrombosis.[25] However, no such adverse reactions were noted in this study especially in monkeys even after cessation of ticlopidine. In addition, 7ND showed no effect on proliferation of human endothelial cells in vitro. This suggests that the 7ND gene transfer does not appear to impair the healing process of endothelial cells in a stented arterial wall, so in these respects, this approach may have an advantage over the first-generation DES devices. We have shown that the biocompatible polymer and plasmid DNA coating material used in this study did not appear to cause any adverse reactions during a 1-month observation period in rabbits and during a 6-month observation period in monkeys. Therefore, we suggest that the blockade of MCP-1 via the 7ND gene-eluting stent may become a promising therapeutic strategy for treatment of restenosis, and that this strategy may have a low level of potential adverse effects.

From a perspective of clinical applicability, it is important to take into account any potential systemic toxicity associated with stent-based delivery of 7ND DNA plasmid. We demonstrated that 7ND gene-eluting stent, which elutes plasmid DNA at a dose of ≈0.8 mg/ body [≈0.23 mg/kg in rabbits (BW=about 3.5 kg) and ≈0.16 mg/kg in monkeys (BW=about 5 kg)], did not induce any significant inflammatory or immune reactions. We have previously reported that the systemic intramuscular transfer of plasmid cDNA encoding the 7ND gene at doses ranging from 0.5 to 10 mg/kg was nontoxic and safe in nonhuman primates,[12,14,26] rabbits,[11] rats,[14] and mice.[12] In addition, knockout mice lacking MCP-1[27] or the MCP-1 receptor (C-C chemokine receptor 2: CCR2)[28] displayed no serious health problems, suggesting that inhibition of MCP-1 is not physiologically toxic. From a toxicological point of view, because the dose of 7ND plasmid eluted from stents would be even lower in human subject (≈0.01 mg/kg for patients weighing 80 kg), it would be unlikely that the 7ND gene-eluting stent would cause any toxicity in humans. In clinical trials of plasmid DNA-based gene therapy in which DNA was administered into the lower limb,[29] myocardium,[30] or coronary artery[31,32] at 2 to 4 mg/ body, no systemic adverse effects were reported. Overall, these safety and feasibility data support the notion that stent-based gene therapy could safely be applied to human subjects.

We have previously reported that 7ND gene transfer not only suppressed inflammation (monocyte infiltration), but also reduced the number of proliferating SMCs in the neointima after injury.[11,13,14] Therefore, besides monocyte-mediated inflammation, we hypothesized that 7ND inhibits MCP-1–induced proliferation of SMCs. This notion is in line with several recent reports[17,18,33] demonstrating that (1) mRNA and protein for the receptor for MCP-1, CCR2, are detectable in vascular SMCs; and (2) MCP-1 induces SMC proliferation in vitro. However, the effects of MCP-1 and CCR2 on SMC proliferation are controversial: several studies reported that MCP-1 either has no effect[34] or inhibits proliferation.[35] These conflicting conclusions are discussed to result from species specificity for MCP-1 activity in an article[17] where human MCP-1 was used to proliferate human SMCs. Furthermore, MCP-1 induces tissue factor in murine SMCs from $CCR^{-/-}$ mice,[36] suggesting the possible presence of alternate MCP-1 receptor in murine SMCs. Therefore, we used human MCP-1 to stimulate hCASMCs in culture, and found that in addition to potent inhibitory actions on monocyte chemotaxis, 7ND inhibited proliferation of hCASMCs induced by human MCP-1. The presence of the receptor for MCP-1, CCR2, on the hCASMCs was also established. Therefore, our present data suggest that 7ND directly inhibits human SMC proliferation, in addition to its known effects on monocytes present in the in-stent vascular lesion.

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

Sources of Funding

This study was supported by Grants-in-Aid for Scientific Research (14657172, 14207036, etc) from the Ministry of Education, Science, and Culture, Tokyo, Japan, by Health Science Research Grants (Research on Translational Research and Nanomedicine) 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

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

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Local Delivery of Imatinib Mesylate (STI571)–Incorporated Nanoparticle Ex Vivo Suppresses Vein Graft Neointima Formation

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Background—Clinical outcome of surgical revascularization using autologous vein graft is limited by vein graft failure attributable to neointima formation. Platelet-derived growth factor (PDGF) plays a central role in the pathogenesis of vein graft failure. Therefore, we hypothesized that nanoparticle (NP)-mediated drug delivery system of PDGF-receptor (PDGF-R) tyrosine kinase inhibitor (imatinib mesylate: STI571) could be an innovative therapeutic strategy.

Methods and Results—Uptake of STI571-NP normalized PDGF-induced cell proliferation and migration. Excised rabbit jugular vein was treated ex vivo with PBS, STI571 only, FITC-NP, or STI571-NP, then interposed back into the carotid artery position. NP was detected in many cells in the neointima and media at 7 and 28 days after grafting. Significant neointima was formed 28 days after grafting in the PBS group; this neointima formation was suppressed in the STI571-NP group. STI571-NP treatment inhibited cell proliferation and phosphorylation of the PDGF-R-β but did not affect inflammation and endothelial regeneration.

Conclusions—STI571-NP–induced suppression of vein graft neointima formation holds promise as a strategy for preventing vein graft failure. (***Circulation***. **2008;118[suppl 1]:S65–S70.**)

Key Words: nanotechnology ■ drug delivery system ■ grafting ■ platelet-derived factors ■ signal transduction

Clinical outcome of surgical revascularization using autologous vein graft is limited by vein graft failure resulting from accelerated neointima formation; 30% to 50% of vein grafts fail within 10 years.[1] Because platelet-derived growth factor (PDGF), expressed by proliferating vascular smooth muscle cells (VSMCs) and infiltrating monocytes, plays a central role in the pathogenesis of vein graft failure,[2] targeted molecular blockade of PDGF signaling is a potential strategy for preventing vein graft failure. Imatinib mesylate (STI571),[3] a potent inhibitor of the c-Abl tyrosine kinase (TK), the c-Kit receptor kinase, and the PDGF-R TK, is approved for the treatment of patients with chronic myeloid leukemia. It has been shown that c-Kit–positive progenitor cells can differentiate into α-actin–positive VSMCs and may contribute to neointima formation after vascular injury.[4] It has also been reported that c-Abl TK is involved in angiotensin II–induced VSMC hypertrophy.[5] In contrast, STI571 has been shown to have little antiproliferative effects on endothelial cells.[6] These data suggest that STI571 appropriately inhibits neointima formation without negative effects on endothelial regeneration/vascular healing, and thus provide a rationale for the use of STI571 as a VSMC-selective molecular targeting drug in the prevention of neointima formation associated with vein graft failure.

STI571 has been reported to inhibit balloon injury–induced neointima formation in rats[7] when dosages beyond the clinical norm were used (50 mg/kg per day). In contrast, STI571 had no effect on neointima formation in rabbits when administered in a clinically relevant dosage (10 mg/kg per day).[8] Recent clinical studies in humans have detected no beneficial effects of oral administration of STI571 (600 mg/d for 10 days)[9] on in-stent restenosis. These data suggest that systemic administration of STI571 at clinical dosages may not be sufficient to antagonize PDGF-induced vascular responses at the site of vascular injury. It has been suggested that STI571 administered at standard dosages (400≈800 mg/d) may not reach sufficient serum concentrations (maximum concentration: <10 μmol/L) to function as an inhibitor of PDGF-R signaling.[10] Furthermore, long-term administration of STI571 causes cardiac mitochondrial dysfunction that results in cardiotoxicity and ventricular dysfunction.[11]

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Presented at the American Heart Association Scientific Sessions, November 4–7, 2007, Orlando, Fla.

The online Data Supplement can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.740613/DCI.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.107.740613**

Therefore, preventing vein graft failure via STI571-mediated PDGF-R signaling blockade requires an efficient local drug delivery system. Ex vivo local delivery of drugs or genes to the vein has been used as a clinically relevant approach. We have recently developed bio-absorbable polymeric nanoparticles (NP) formulated from the polymer polyethylene-glycol (PEG)-modified poly(DL-lactide-*co*-glycolide) (PLGA).[12,13] PEG-PLGA NP offers the advantages of safety, efficient intracellular delivery of different classes of therapeutic agents, and the capacity for sustained intracytoplasmic release.[14,15] Therefore, we hypothesized that STI571-incorporated NP could be an innovative therapeutic strategy for preventing vein graft failure. We investigated whether our NP-based drug delivery system worked as an intracellular ex vivo delivery system to the excised vein, and whether blockade of PDGF-R TK by STI571-incorporated NP suppressed vein graft neointima formation in vivo.

Materials and Methods

Preparation of PEG-PLGA NP

PEG-PLGA NP encapsulated with fluorescence marker or STI571 was prepared using an emulsion solvent diffusion method, as previously reported.[12,13] Additional details can be found in the online Data Supplement.

Cellular Uptake and Intracellular Distribution of NP In Vitro

We cultured rat aortic smooth muscle cells (SMCs) and evaluated the cellular uptake of PEG-PLGA NP by fluorescence microscopy. Additional details can be found in the online Data Supplement.

Measurement of In Vitro FITC Release Kinetics From NP

To measure FITC release kinetics, FITC-NP (n=8) was immersed in Tris-EDTA buffer, and the released FITC from NP was measured.

Cell Proliferation, Migration, Cytotoxicity, and TUNEL Assay

We cultured human coronary artery SMCs and evaluated proliferation, migration,[16] cytotoxicity, and apoptosis. Additional details can be found in the online Data Supplement.

Experimental Animal Models

Male Japanese white rabbits (KBT Oriental, Tokyo, Japan) weighing 2.5 to 3.0 kg were fed a high-cholesterol diet for 2 weeks before the operation. Animals were anesthetized, a midline neck incision was made, and an approximately 3-cm segment of the jugular vein was dissected free; all side branches were ligated. The vein segments were gently flushed, and placed in a buffer alone (n=11) or in a solution containing either FITC-encapsulated PEG-PLGA NP at 0.5 mg/mL (n=11), STI571- encapsulated PEG-PLGA NP at 0.5 mg/mL containing STI571 at 100 μmol/L (n=11), or STI571 alone at 100 μmol/L (n=11) for 30 minutes at room temperature. The treated vein segments were interposed into ipsilateral carotid arteries in an end-to-side fashion. The animals were maintained on the same high-cholesterol diet throughout experimental period.

All animals received aspirin at 20 mg/d from 3 days before the graft procedure until euthanasia. After venous blood samples were taken, animals were killed with a lethal dose of anesthesia on days 7 (n=5 each) and 28 (n=6 each). The vein grafts were harvested, flushed with saline, and used for histopathologic, immunohistochemical, and biochemical studies.

Ex Vivo NP Delivery in Human Vein

Segments of internal thoracic vein were obtained from patients undergoing coronary arterial bypass surgery. Additional details can be found in the online Data Supplement.

Histopathologic and Immunohistochemical Analysis

Tissue sections from the grafts were harvested and prepared for analysis. Additional details can be found in the online Data Supplement.

Western Blot Analysis

Protein was extracted from cultured VSMCs or frozen vein graft tissues. Western blot analysis was performed with antibodies against human PDGF-R-β, phospho-PDGF-R-β, phosphotyrosine, phospho-p44/42 MAPK (ERK 1/2), ERK 1/2, c-Abl TK, or actin (additional details can be found in the online Data Supplement).

Statistical Analysis

Data are expressed as the mean±standard error of the mean (SEM). Statistical analysis of differences was performed by ANOVA and Bonferroni's multiple comparison tests. Statistical significance was set at $P<0.05$.

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

Results

In Vitro Cell Uptake and Intracellular Distribution of NP and NP Release Kinetics In Vitro

When incubated with rat aortic and human coronary artery SMCs, the fluorescence-encapsulated NP showed excellent capacity of intracellular distribution (Figure 1A). In contrast, no fluorescence was detected when the SMCs were incubated with blank NP or fluorescent molecules only. A large fraction (>90%) of the NP rapidly entered into the cells, and this incorporation rate sustained until 24 hours (Figure 1B). An endocytosis inhibitor (chlorpromazine hydrochloride) attenuated the NP-mediated intracellular incorporation of fluorescence (supplemental Figure I). Fluorescence confocal microscopic study revealed the intracellular retention of NP (Figure 1C). Transmission electron microscopy of the cellular cross-sections revealed the intracellular localization of NP at 1 hour of incubation (Figure 1D).

An analysis of the in vitro FITC release kinetics from FITC-NP showed an early burst of FITC release such that approximately 40% of the total amount ultimately released was present on day 1, followed by sustained release of the remaining FITC over the next 28 days (Figure 1E).

In Vitro Effects of STI571-NP on PDGF-Induced Proliferation and Migration of VSMCs and on Receptor Phosphorylation

As reported by other investigators,[6,17] non–NP-STI571 attenuated PDGF-induced proliferation and migration in a dose-dependent manner (Figure 2A and B). STI571 at a concentration of 10 μmol/L prevented the PDGF-induced cell proliferation and migration. The PEG-PLGA NP-containing STI571 at 10 μmol/L also prevented the PDGF-induced responses of VSMCs (Figure 2A and B). Results of a TUNEL assay showed no detectable increase in STI571-induced increase in apoptotic cells (data not shown). Results of a

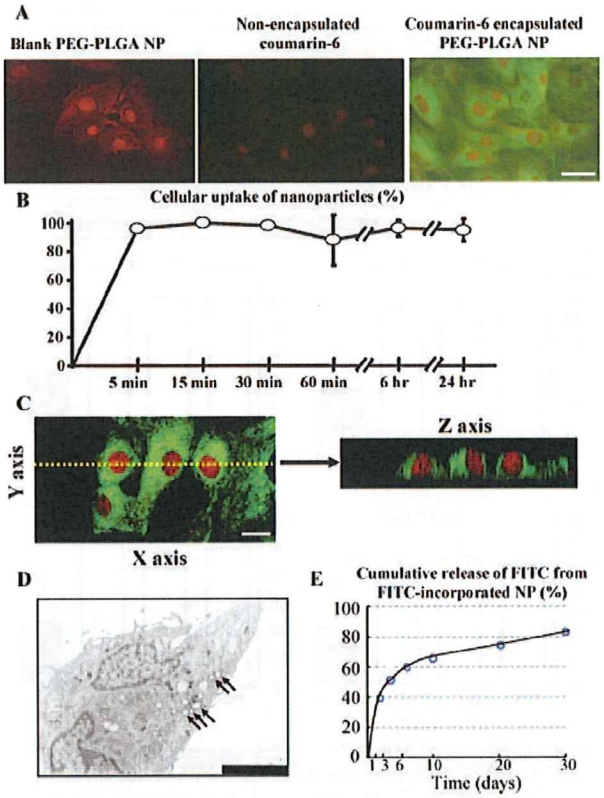

Figure 1. In vitro cellular uptake and intracellular distribution of NP. A, Fluorescence microscopic pictures of rat aortic SMCs incubated with blank PEG-PLGA NP, nonencapsulated coumarin-6, and coumarin-6–encapsulated PEG-PLGA NP (0.5 mg/mL) for 60 minutes. Nuclei were counterstained with propidium iodide (PI). Scale bar=200 μm. B, In vitro time course of the percentage of cellular uptake of NP (100× fluorescence-positive cells/total cells per field) are shown. Data are means±SEM (n=4). C, Confocal fluorescence microscopy photographs (left: an X–Y axis image, right: a Z axis image of cross-sections from yellowish dashed line displayed on an X–Y axis image) of rat aortic SMCs incubated with medium containing coumarin-6- encapsulated NP at 0.5 mg/mL. Coumarin-6 fluorescence is green. Nuclei are stained red. Scale bar=10 μm. D, Transmission electron microscopic picture of cross-section of human coronary artery SMCs incubated with NP for 60 minutes. Arrows indicate NP in the cytoplasm. Scale bar=500 nm. E, In vitro time course of cumulative FITC release from the FITC-incorporated NP (n=8 each). The percentage of incremental quantities of released FITC was plotted against time.

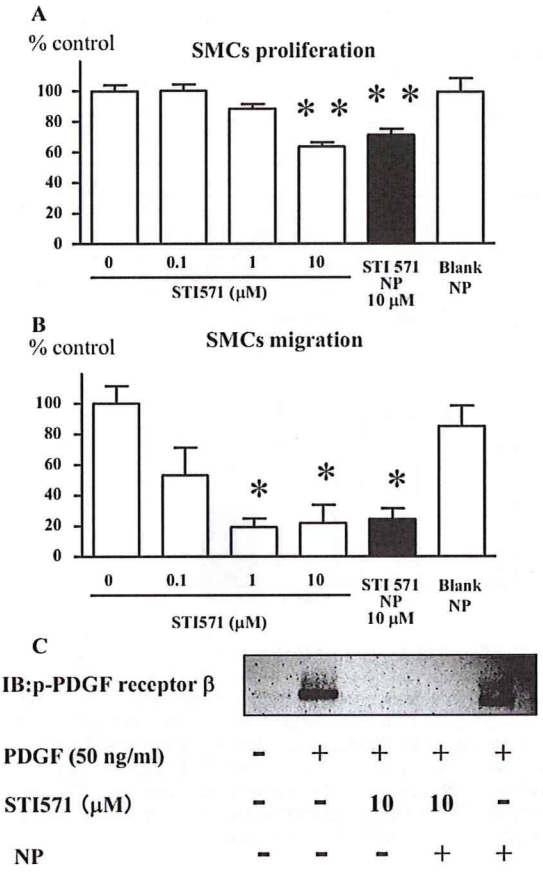

Figure 2. In vitro effects of STI571-encapsulated PEG-PLGA NP on PDGF-induced proliferation and migration of SMCs and on PDGF-R-β phosphorylation. A, Effects of STI571 and STI571-NP on PDGF-stimulated proliferation of human coronary artery SMCs. Data are mean±SEM (n=6 each). $^{**}P<0.001$ vs PDGF-induced response. Data are percentage changes from PDGF-induced responses (100%). B, Effects of STI571 and STI571-NP on PDGF-stimulated migration of rat aortic SMCs. Data are mean±SEM (n=6 each). $^{*}P<0.01$ vs PDGF-induced response. C, Effects of STI571 and STI571-NP on PDGF-induced PDGF-R-β phosphorylation and in human coronary artery SMCs. These Western blot experiments were repeated 3 times; results from all 3 trials were similar and representative results are shown.

cytotoxicity assay showed that human coronary artery SMCs incubated with blank PEG-PLGA NP at a concentration of 1 mg/mL remained 100% viable relative to control (data not shown).

Western blot analysis showed that in human coronary artery SMCs, PDGF-induced phosphorylation of PDGF-R-β was suppressed by STI571 at 10 μmol/L as well as by STI571-NP (Figure 2C).

Efficacy of NP-Mediated Drug Delivery System to Vein Grafts

Ex vivo incubation of NP with excised rabbit jugular vein or human internal thoracic vein for 30 minutes resulted in high SMC uptake in the media and in some cells in the adventitia (supplemental Figure IIA and IIB). After 7 days of grafting in rabbits when a thin neointima was formed, FITC-positive cells were detected in the neointima and media (% positive area: 51±9%). After 28 days, many FITC-positive cells were still noted in the neointima and media (% positive area: 12±5%). In contrast, no FITC immunoreactivity was noted in veins incubated with PBS.

Effects of STI571-NP on Vein Graft Failure in Rabbits

As we previously reported,[18] significant neointima developed 28 days after grafting in animals interposed with control PBS-treated vein grafts. Ex vivo treatment with STI571-NP, but not with STI571 only or FITC-NP, markedly attenuated neointima formation at day 28 (Figure 3).

Increased monocyte infiltration and PCNA-positive proliferating cells were observed in the intima-media and adventi-

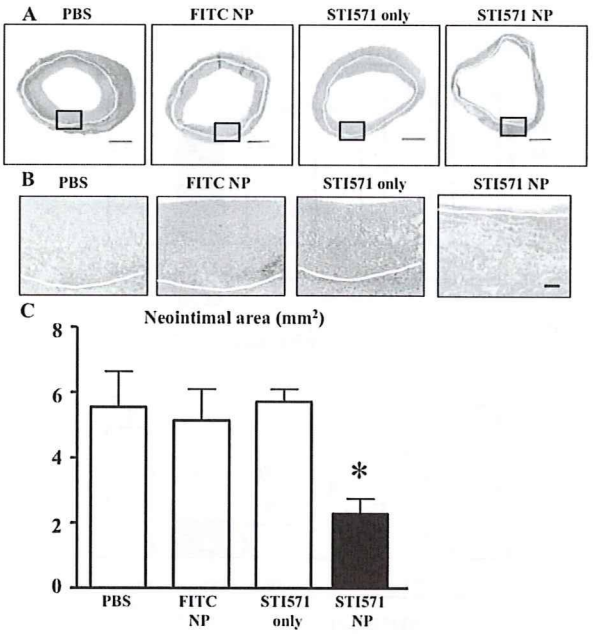

Figure 3. Effects of ex vivo treatment with STI571-NP on vein graft neointima formation in rabbits. A, Low-powered micrographs of whole vessel cross-sections of vein grafts from PBS-, FITC-NP-, STI571 only-, and STI571-NP–treated vein grafts after 28 days, stained with Elastica Van Gieson. White line shows internal elastic lamina. Scale bar=1 mm. B, Expanded high-powered microphotos from boxed area in A. Scale bar=100 μm. C, Effects of STI571-NP on neointima area 28 days after grafting (n=6 each). *P<0.05 vs PBS-treated group.

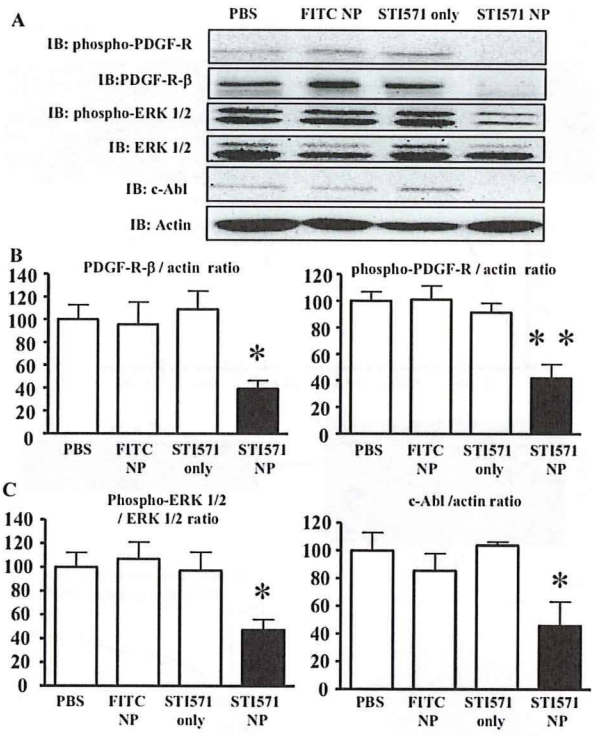

Figure 4. Effects of STI571-NP on the expression of phospho-PDGF-R, PDGF-R-β, phospho-ERK 1/2, MAP kinase (ERK 1/2), c-Abl TK, and actin, 7 days after grafting. A, Immunoblots are representative of 5 individual vein grafts from each group, showing identical results. B, Densitometric analysis of PDGF-R-β and phosphorylation expression (n=5 each). Data are expressed as percent change from PBS group (100%). *P<0.05 vs PBS-treated group. **P<0.01 vs PBS-treated group. C, Densitometric analysis of phospho-ERK 1/2 and c-Abl TK expression (n=5 each). Data are expressed as percent change from PBS group (100%). *P<0.05 vs PBS-treated group.

tia at 7 and 28 days after grafting (supplemental Figure IIIA). No effects on inflammatory changes were noted in STI571-NP–treated vein graft (supplemental Figure IIIB). In contrast, treatment with STI571-NP, but not with FITC-NP or STI571 only, markedly attenuated the number of PCNA-positive cells observed on day 7 (supplemental Figure IIIC). There were no significant differences in endothelial cell linings among the 4 groups at 7 and 28 days after grafting (supplemental Figure IIID). There were no significant differences in serum cholesterol levels after 4 weeks among the 4 groups (data not shown).

Effects of STI571-NP on PDGF, PDGF-R, PDGF-R Phosphorylation, and MAPK Pathway

Immunohistochemical studies showed that no PDGF was detected in normal veins. In contrast, intense immunohistochemical staining for PDGF was noted in vein graft tissues 7 days after grafting. There were no significant differences in the degrees of the positive staining area among the 4 groups (supplemental Figure IV). Western blot analysis showed that ex vivo treatment with STI571-NP, but not with STI571 only or FITC-NP, markedly attenuated expression of PDGF-R protein and phosphorylation of PDGF-R kinase, phosphorylation of ERK1/2, and c-Abl TK, at 7 days after grafting (Figure 4A through 4C).

Discussion

We demonstrated for the first time that PEG-PLGA NP is an excellent system for intracellular delivery of molecular targeting drugs in excised veins. This NP system is bioabsorbable polymer with a long history of safe use in medical applications. Therefore, this system may represent a novel NP-mediated drug delivery system to prevent vein graft failure.

We showed that the NP was endocytosed rapidly by VSMCs and was retained stably in the intracellular space. Impressive ex vivo delivery of NP into human veins suggests that this NP-mediated drug delivery system can be applied to clinical settings for humans. An important finding was that long-term retention of NP in neointima and medial cells of vein grafts was detected until day 28. After cellular uptake of NP, NP slowly releases encapsulated drugs or genes into the cytoplasm as PLGA is hydrolyzed, resulting in an intracellular drug delivery. The bio-absorption time of PLGA in the body can be controlled by changing material make-up of PLGA, thus the function of the intracellular drug delivery system can be modified. Therefore, (1) this NP-mediated drug delivery system works as an excellent ex vivo delivery system for the excised vein; and (2) this system provides an effective means of delivering drugs or genes that target intracellular proteins involved in the pathogenesis of vein graft neointima formation.

We selected STI571 because this compound is known to target the PDGF-R TK (see Introduction). NP-incorporated with STI571 attenuated the proliferation of human VSMCs in vitro and the formation of vein graft neointima formation in vivo, both of which are known to be associated with the inhibition of the target molecules of STI571 (PDGF-R TK, c-Abl TK) and downstream signal of PDGF-R (ERK). NP-incorporated with STI571 did not affect endothelial regeneration process after vein grafting. In preliminary experiments, tissue concentrations of STI571 were measured immediately after and 6 hours after incubation of excised veins with STI571-NP by HPLC system, which showed under the limit of detection (1 ng/mL). Although the precise intracellular concentration and distribution of STI571 is unclear, our present data (Figures 2 and 4) provide evidence that (1) STI571-incorporated NP may block PDGF-R signaling possibly via slow release of STI-571 into the cytoplasm as NP is hydrolyzed; and (2) PDGF-R signaling blockade by NP-incorporated with STI571 is a means for treating vein graft neointima formation in vivo.

Inflammatory-proliferative changes have been shown to play a central role in the pathogenesis of vein graft neointima formation. In early stages, the neointima lesion has an inflammatory nature characterized by mononuclear cell infiltration, followed by VSMC proliferation.[19] We recently reported that blockade of monocyte chemoattractant protein-1 (MCP-1) by adenovirus-mediated ex vivo transfer of 7ND gene to autologous vein grafts suppressed neointima formation in dogs.[18] We also have demonstrated that MCP-1 plays a central role in neointima formation following arterial mechanical injury.[20,21,22] In a previous study,[18] we showed that blockade of MCP-1 attenuated both inflammation (monocyte infiltration) and proliferation (appearance of proliferating VSMC) in vein grafts. In contrast, data from this study show that NP-mediated delivery of STI571 reduced PDGF-induced proliferation but not inflammation, suggesting that (1) PDGF-mediated proliferative changes might be located downstream of inflammatory changes, or (2) the mechanism of action of STI571-mediated inhibition of proliferation might be distinct from that of anti-MCP-1-mediated attenuation of proliferation and inflammation. If STI571 and anti-MCP-1 treatment exert their effects through different pathways, it would be interesting to examine whether combined blockade of PDGF and MCP-1 would have additive inhibitory effects on vein graft failure.

Expression of PDGF is known to be low in normal blood vessels, but mechanical forces stimulate SMC expression and release of PDGF, and induce PDGF-R phosphorylation (activation).[23] We show here that PDGF and the phosphorylation levels of its receptor were up-regulated in vein grafts. STI571-incorporated NP did not affect increased PDGF expression, but it did suppress the protein expression of PDGF-R, PDGF-R kinase, and c-Abl TK in vivo. This could suggest the presence of a positive-feedback loop that, in the absence of STI571, potentiates PDGF-mediated proliferation in vein grafts. It is also possible that reduced PDGF-producing cells (PCNA-positive cells) in the vein graft or blockade of multiple intracellular kinases might have contributed to the beneficial effects of STI571-incorporated NP on vein graft neointima formation in vivo.

One limitation in the present study is that only single dose of STI571-NP was examined. It is practically difficult to obtain the dose–response relationship of this NP system in small animals, because the dose–response relation of STI571 and polymer needs to be examined. For translation of our present findings into clinical medicine, further studies are therefore needed to define a dose-response relation in large animal models.

In conclusion, blockade of PDGF signaling by STI571-incorporated NP-inhibited proliferation of VSMCs in vitro and suppressed vein graft neointima formation in vivo. This NP-mediated drug delivery system provides an innovative and clinically feasible therapeutic strategy for preventing vein graft failure.

Sources of Funding

This study was supported by Grants-in-Aid for Scientific Research (19390216, 19650134) from the Ministry of Education, Science, and Culture, Tokyo, Japan and by Health Science Research Grants (Research on Translational Research and Nano-medicine) from the Ministry of Health Labor and Welfare, Tokyo, Japan.

Disclosures

Dr Egashira holds a patent on the results reported in the present study. The other authors report no conflicts.

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Nanoparticle-Mediated Delivery of Nuclear Factor κB Decoy Into Lungs Ameliorates Monocrotaline-Induced Pulmonary Arterial Hypertension

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Abstract—Pulmonary arterial hypertension (PAH) is an intractable disease of the small pulmonary artery that involves multiple inflammatory factors. We hypothesized that a redox-sensitive transcription factor, nuclear factor κB (NF-κB), which regulates important inflammatory cytokines, plays a pivotal role in PAH. We investigated the activity of NF-κB in explanted lungs from patients with PAH and in a rat model of PAH. We also examined a nanotechnology-based therapeutic intervention in the rat model. Immunohistochemistry results indicated that the activity of NF-κB increased in small pulmonary arterial lesions and alveolar macrophages in lungs from patients with PAH compared with lungs from control patients. In a rat model of monocrotaline-induced PAH, single intratracheal instillation of polymeric nanoparticles (NPs) resulted in delivery of NPs into lungs for ≤14 days postinstillation. The NP-mediated NF-κB decoy delivery into lungs prevented monocrotaline-induced NF-κB activation. Blockade of NF-κB by NP-mediated delivery of the NF-κB decoy attenuated inflammation and proliferation and, thus, attenuated the development of PAH and pulmonary arterial remodeling induced by monocrotaline. Treatment with the NF-κB decoy NP 3 weeks after monocrotaline injection improved the survival rate as compared with vehicle administration. In conclusion, these data suggest that NF-κB plays a primary role in the pathogenesis of PAH and, thus, represent a new target for therapeutic intervention in PAH. This nanotechnology platform may be developed as a novel molecular approach for treatment of PAH in the future. (***Hypertension.* 2009;53:877-883.**)

Key Words: pulmonary hypertension ■ lung ■ inflammation ■ leukocytes

Pulmonary arterial hypertension (PAH) is an intractable disease of the small pulmonary arteries that results in a progressive increase in pulmonary vascular resistance, right ventricular failure, and, ultimately, premature death.[1–3] Because its mortality remains high even after the introduction of prostacyclin infusion therapy (which has raised the 5-year survival rate to ≈50%), the development of a more effective and less invasive therapy for PAH is urgently needed.

Recent evidence suggests an important role of monocyte chemoattractant protein (MCP) 1–mediated inflammation in the mechanism of PAH.[4–8] However, the therapeutic benefits of MCP-1 blockade were not optimal for clinical application.[5,6] During the inflammatory process of PAH, several inflammatory factors (eg, MCP-1, interleukin [IL] 1, IL-6, and tumor necrosis factor [TNF] α) are overproduced, leading to a vicious circle.[1–3] A redox-sensitive transcription factor, nuclear factor κB (NF-κB), is known to regulate expression of chemokines such as MCP-1 and multiple inflammatory cytokines such as IL-6 and TNF-α. Blockade of NF-κB by transfection of NF-κB "decoy" oligodeoxynucleotides may attenuate the vascular pathology associated with reduced expression of NF-κB–dependent genes.[9–12] However, no previous study has addressed the specific role of the NF-κB pathway in the pathogenesis of PAH. Therefore, we hypothesized that controlled local delivery of NF-κB decoy into lungs, targeting a battery of multiple important inflammatory cytokines, would be a favorable therapeutic approach for PAH. To this end, we have recently developed bioabsorbable polymeric nanoparticles (NPs) formulated from a poly-(ethylene glycol)-*block*-lactide/glycolide copolymer (PEG-PLGA).[13–15]

The primary aim of this study was to investigate the role of the NF-κB pathway in the pathogenesis of PAH. We first examined the activity of NF-κB in patients with PAH. We then used a rat model of monocrotaline (MCT)-induced PAH to examine whether NP-mediated delivery of the NF-κB decoy can attenuate the development of PAH.

Methods

Histopathologic and Immunohistochemical Examination of Human Lungs

Human lung tissue was obtained from autopsy specimens from 4 patients whose deaths were attributed to idiopathic PAH and 2

Received August 10, 2008; first decision August 26, 2008; revision accepted March 2, 2009.

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Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.108.121418

patients whose deaths were attributed to nonlung disease (Figure S1, available in the online data supplement at http://hyper.ahajournals.org). Additional details are provided in the online data supplement.

Preparation of NPs

The NF-κB decoy oligodeoxynucleotides labeled with or without fluorescein-isothiocyanate (FITC) were prepared as described previously.[10,11] The decoy is directed against the NF-κB binding site in the promoter region that corresponds with NF-κB–responsive genes and works to inhibit binding of this transcription factor to the promoter region.[10,11] PEG-PLGA NPs encapsulated with FITC, NF-κB decoy, or FITC-labeled NF-κB decoy were prepared using an emulsion solvent diffusion method.[13,14] The average diameter of PEG-PLGA NPs was 44 nm. To measure FITC release kinetics, FITC-NP was immersed in Tris-EDTA buffer, and the released FITC was measured. Additional details are provided in the online data supplement.

In Vivo Experiments With a Rat Model of MCT-Induced PAH

Rats were SC injected with 60 mg/kg of MCT, which induces severe PAH within 3 weeks.[5,16,17] In the prevention protocol, animals were assigned to either an untreated control group or a group that received a single intratracheal instillation of NF-κB decoy alone (50 μg), FITC-NP (1000 μg of PEG-PLGA), or NF-κB decoy NPs (50 μg of NF-κB decoy per 1000 μg of PEG-PLGA) immediately after MCT (n=6 each). For intratracheal instillation, a volume of 0.1 mL of phosphate buffer suspension of NP or NF-κB decoy was injected gently into the trachea of animals accompanied by an equal volume of air. The biodistribution of FITC in the lung was also examined 3, 7, and 14 days after intratracheal instillation of FITC only, FITC-NPs, or FITC-labeled NF-κB decoy NPs in rats injected with MCT. In the treatment protocol, rats were divided into 2 groups (rats treated with a single intratracheal instillation of phosphate buffer and rats treated with NF-κB decoy NPs; n=33 each) 21 days after MCT injection, when severe PAH had been established.

Hemodynamic Measurements

Three weeks after MCT administration, the animals were anesthetized with sodium pentobarbital, and then polyethylene catheters were inserted into the right ventricle (RV) through the jugular vein and the carotid artery for hemodynamic measurements. RV systolic pressure and systemic blood pressure were measured with a polygraph system (AP-601G, Nihon Kohden).[5]

Assessment of Right Heart Hypertrophy and Pulmonary Arterial Remodeling

After systemic arterial and RV pressure had been recorded, the animals were euthanized, and the lungs and heart were isolated. The RV wall was dissected from the left ventricle (LV) and ventricular septum (S). The wet weight of the RV and LV+S was determined, and RV hypertrophy was expressed as follows: RV/(LV+S).[5]

The lungs were perfused with a solution of 10% phosphate buffered formalin (pH 7.4). At the same time, 10% phosphate buffered formalin (pH 7.4) was administered into the lungs via the tracheal tube at a pressure of 20 cm H_2O. These specimens were processed for light microscopy by routine paraffin embedding. The degree of remodeling (muscularization) of the small peripheral pulmonary arteries was assessed by double immunohistochemical staining of the 3-μm sections with an anti–α-smooth muscle actin antibody (dilution 1:500, clone 1A4, Dako) and anti–platelet endothelial cell adhesion molecule 1 (M-20) antibody (dilution 1:100, Santa Cruz Biotechnology) modified from a protocol described elsewhere.[18]

To assess the type of remodeling in the muscular pulmonary arteries, microscopic images were analyzed. In each rat, 30 to 40 intra-acinar arteries were categorized as muscular (ie, with a complete medial coat of muscle), partially muscular (ie, with only a crescent of muscle), or nonmuscular (ie, with no apparent muscle). The arteries were counted and averaged within a range of diameters from 25 to 50 μm.

Histopathologic and Immunohistochemical Analysis

The degrees of monocyte infiltration were evaluated by immunostaining with the ED-1 (analogue of human CD68) antibody against monocytes. For quantification, a blind observer counted the number of ED-1–positive cells in 10 fields.[4] Monocytes were also subjected to immunostaining with antibodies against FITC, an epitope (α-p65) on the p65 subunit of NF-κB, or nonimmune mouse IgG. The α-p65 monoclonal antibody recognizes an epitope on the p65 subunit that is masked by bound inhibitor of κB (I-κB).[9] Therefore, this antibody exclusively detects activated NF-κB.[12]

Electrophoretic Mobility-Shift Assays

Nuclear extracts were prepared from the whole-lung homogenates using a nuclear extract kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Science) according to the manufacturer's instructions. The protein was measured using a BCA Protein Assay kit (Thermo Science). For NF-κB activation, a nonradioactive electrophoresis mobility-shift assay kit (AY1030, Panomics) was used according to the manufacturer's instructions. Five μg of nuclear protein were incubated for 30 minutes at room temperature with a biotinylated oligonucleotide containing the NF-κB binding site, and then the samples were separated on a nondenaturing polyacrylamide gel and blotted onto a positively charged nylon membrane. After blotting, the oligos on the membrane were fixed using a UV cross-linker oven. Then, the membrane was incubated with streptavidin-horseradish-peroxidase solution at room temperature for 15 minutes and with detection reagents for 5 minutes. Nuclear proteins that were bound to the NF-κB binding site were detected by chemiluminescence with the use of the LAS-1000 detection system (Fujifilm).

Real-Time Quantitative RT-PCR

Real-time PCR amplification was performed with the rat cDNA with the use of the ABI PRI8:21 PM 7000 Sequence Detection System (Applied Biosystems), as described previously.[12] TaqMan primer/probes for MCP-1, TNF-α, IL-1, IL-6, intercellular adhesion molecule 1, and GAPDH, which served as the endogenous reference, were purchased from Applied Biosystems (Assay-on-Demand gene expression products Rn00580555, Rn99999017, Rn00580432, Rn00561420, and Rn00564227 and TaqMan Rodent GAPDH Control Reagents, respectively).

Intracellular Delivery of NPs Incorporated With an FITC-Labeled NF-κB Decoy to Human Monocytes and Pulmonary Arterial Smooth Muscle Cells

The human monocyte cell line THP-1 was obtained from the German Collection of Micro-organisms and Cell Cultures and was used between passages 4 and 8. Cells were cultured in RPMI 1640 with 10% FBS in a humidified atmosphere of 5% CO_2 in air. The cell density was adjusted to 10^6 cells per milliliter in 1 mL of serum-free medium in 35-mm-diameter dishes. The cells were serum deprived 24 hours before the experiment. The growth medium was replaced with FITC-conjugated NF-κB decoy encapsulated PEG-PLGA NP suspension medium (0.5 mg/mL) and then further incubated for 1 hour. At the end of the experiment, the cells were washed 3 times with PBS to eliminate excess NPs that were not incorporated into the cells. Then, the cells were fixed with 10% cold methanol, and nuclei were counterstained with propidium iodide. Cellular uptake of FITC-conjugated NF-κB decoy-encapsulated PEG-PLGA NPs was evaluated by fluorescence microscopy.

Human pulmonary artery smooth muscle cells (PASMCs) were obtained from Cambrex Bio Science, Inc, and cultured as described previously. Cells were used between passages 4 and 8. Human PASMCs were seeded on chambered cover glasses and incubated at 37°C/5% CO_2 until the cells were subconfluent. The following treatments were performed in the same manner.