Catheter-based prostacyclin synthase gene transfer prevents in-stent restenosis in rabbit atheromatous arteries

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Received 11 February 2003; received in revised form 16 October 2003; accepted 20 October 2003

Abstract

Objective: Prostacyclin synthase (PGIS) gene transfer have been shown to accelerate re-endothelialization and prevent neointimal formation in balloon-injured arteries. The aim of this study is to evaluate how overexpression of endogenous prostacyclin exerts those beneficial effects in atheromatous arteries. Methods: New Zealand White Rabbits fed a 0.5% cholesterol diet underwent balloon injury and Palmaz-Schatz stent implantation in the iliac arteries followed PGIS gene (pCMV-PGIS, 200 μg) delivery by the lipotransfection method via Dispatch catheter (n = 6 each). Results: One week after transfeciton, arterial segments of pCMV-PGIS produced higher levels of 6-keto-PGF1α than those of control, pCMV-LacZ (p < 0.05). The levels of vascular endothelial growth factor (VEGF) expression was greater in the vessels of pCMV-PGIS than in those of pCMV-LacZ demonstrated by immunohistochemical analysis and quantitation of Western blotting (1.8-fold, p < 0.05). At 2 weeks, in-stent endothelialization was significantly greater in the vessels of pCMV-PGIS than in those of pCMV-LacZ (p < 0.01). The percentage of BrdU-positive nuclei in the injured arterial segments was lower in vessels of pCMV-PGIS than pCMV-LacZ (p < 0.01). At 4 weeks, PGIS gene transfer reduced the neointimal area by 38% (p < 0.05) and widened the lumen area by 71% (p < 0.01). Conclusion: PGIS gene transfer accelerated re-endothelialization, and attenuated neointimal formation after stent implantation in atheromatous rabbit arteries, at least in part, via increased production of VEGF protein.

Keywords: Atherosclerosis; Gene therapy; Prostaglandins; Restenosis; Stents

1. Introduction

Intra-coronary artery stent implantation was developed to overcome restenosis which is seen in 20% to 50% after balloon angioplasty [1-3]. Although stent implantation have become more widely applicable and succeeded to reduce restenosis rate by 10% to 20% after angioplasty, there remained the profound problem of thrombogenicity in acute and sub-acute phase and in-stent restenosis in late phase due to biological interaction between the foreign body of metallic stent and circulating blood cells like macrophages and platelets and vascular smooth muscle cells (VSMCs) [3-6]. The former problem of thrombus formation was overcome by systemic administration of antithrombotic and antiplatelet agents [4-6]. Thus, the scientific interests have been shifted to the mechanism of in-stent restenosis and methods to inhibit such unfavorable bioreactivity.

Recent reports have shown that antiproliferative drug-eluting stent implantation dramatically reduces in-stent restenosis in animal models and human clinical trials [7-10]. However, there might be potential problems such as, (1) little selectivity for the target of VSMCs with damage on endothelial cells, which might cause delay of re-endothelialization and following late stent thrombosis, (2) arterial wall necrosis due to chronic exposure of toxic substances, and (3) unfavorable effects on vulnerable plaques downstream of a stent by elution of drug [7-10]. As an alternative of drug elution, gene therapy would be a potential strategy to overcome those disadvantages.
Intensive studies on gene therapy, most focusing on the acceleration of reendothelialization and inhibition of VSMC proliferation, have demonstrated the superior efficacy of gene therapy against restenosis after balloon injury in animal models [11]. Thinking of the proper gene to deliver to injured arteries, secreted type molecules like prostacyclin (PGI₂) [12–15], C-type natriuretic peptide [16], and nitric oxide [17,18] may be favorable because those synthases are expected to exert in a paracrine manner and required less transduction rate if compared with cell cycle modulators like E2F decoy or antisenses undergoing in the study of venous graft occlusion after coronary bypass surgery [11].

PGI₂ protects vessels through cAMP by dilating the vessels [19] and by its multifunctional effects such as inhibition of platelet aggregation [20] and VSMC proliferation [21]. Furthermore, there practically remains significant restenosis in the very high-risk populations such as diabetics and those with chronic kidney disease or in unfavorable lesion characteristics such as long lesions or small vessels less than 2.5 mm in diameter. We have previously shown that PGI₂ synthase (PGIS) gene transfer accelerates re-endothelialization, suppresses VSMC proliferation, and inhibits neointimal formation in rat balloon-injured arteries [13–15]. Thus, gene therapy using PGIS would be a potential strategy against in-stent restenosis. However, its mechanism of accelerated re-endothelialization remains unclear and the application of PGIS gene transfer to atheromatous arteries has not been evaluated. In this study, we hypothesized that an overexpression of endogenous PGI₂ might augment the production of angiogenic growth factors that might accelerate endothelial recovery as well as directly inhibit VSMC proliferation after stent implantation, even in atheromatous arteries. To test our hypothesis, we examined the effects of catheter-based PGIS gene transfer on in-stent restenosis in atheromatous rabbits whose arteries had characteristics similar to those of the human coronary arteries [22].

2. Methods

2.1. Preparation of plasmid DNA

A plasmid DNA encoding rat PGIS cDNA driven by a cytomegalovirus (CMV) enhancer/promoter-directed vector (pCMV-PGIS) was generated based on previously described methods [13]. Briefly, the 1.6-kb rat PGIS cDNA was cloned in the CMV enhancer/promoter-directed vector driven under the control of a simian virus 40 enhancer/early promoter with a polyadenylation signal sequence. The functional character of these plasmids, such as protein expression level in vitro, has been confirmed in our previous reports [13–15]. A plasmid carrying the LacZ gene substituted for the PGIS gene was constructed as a control (pCMV-LacZ).

2.2. PGIS gene transfer in vivo

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were also performed in accordance with protocols approved by the Animal Ethics Committee of Nagoya University. Thirty-seven male New Zealand White Rabbits (Japan SLC, Hamamatsu, Japan) weighing 3.9 to 4.2 kg were fed a 0.5% cholesterol diet from 4 weeks before the transfection until euthanasia. To avoid acute and subacute complications due to in-stent thrombus formation, all animals received ticlopidine hydrochloride (Daiichi Pharmaceutical, Tokyo, Japan), 8 mg/kg/day, from 5 days before the intervention until euthanasia. The animals were anesthetized with ketamine (50 mg/kg intramuscularly) and sodium pentobarbital (30 mg/kg intravenously). A 5F introducer sheath (Terumo, Tokyo, Japan) was positioned in the right femoral artery under surgical exposure. Heparin sodium (200 IU/kg) was intraarterially injected. All catheters were subsequently introduced through this sheath and advanced to the external iliac artery through a 0.014-in. guide wire (Japan Lifeline, Tokyo, Japan). Baseline angiograms of the external iliac arteries were obtained for each animal. The vessel diameter was calculated by the caliper method. Arterial injury was performed using an angioplasty balloon catheter (Boston Scientific) at 506.5 kPa for three, 1-min periods at the same location. A 7-mm-long Palmaz–Schatz coronary stent (Johnson & Johnson) was delivered over the angioplasty balloon catheter; the stent was apposed to the vessel wall using an inflation pressure of 607.8 kPa to achieve a 1.1 to 1.2:1.0 stent/artery ratio. The in vivo gene transfer into stented iliac arteries was performed as previously described [23]. Briefly, 3.0-mm multichamber autopercision balloon catheter (Dispatch catheter; Boston Scientific) was introduced through the arterial sheath up to the stented site. The balloon was then inflated at 50.65 kPa. Two-hundred micrograms of pCMV-PGIS or pCMV-LacZ with 600-µL Lipofectamine Plus (Invitrogen/GIBCO BRL) were gradually injected and left standing in contact with the vessel wall for 60 min (n = 6 each). The solution was then withdrawn, the balloon deflated, and the catheter retrieved. The right femoral artery was ligated, the skin was sutured and the animals were allowed to recover. These procedures were performed under sterile conditions as well as in strict percutaneous fashion without surgical ligation or cross clamping of side branches. Two weeks after the transfection, the central ear arteries of conscious restrained rabbits were cannulated by percutaneous puncture with a 24G cannula for measurement of intra-arterial blood pressure. Blood pressure was measured through the arterial cannula after the animal was allowed to rest quietly for at least 15 min.

The total plasma cholesterol levels of the rabbits was increased after consuming a high-cholesterol diet for 2 weeks (702 ± 152 mg/dl, n = 10) if compared with normal diet controls (51 ± 7 mg/dl, n = 6, p < 0.0001). The typical
atherosclerotic lesion in the iliac arteries was confirmed by morphology and histochemical staining of oil red.

2.3. PGI2 production in stented arteries

The stented iliac arteries transfected with pCMV-PGIS or pCMV-LacZ were resected 7 days after the balloon injury (n = 6 each). The exact stented segments were retrieved, washed with 9.57 mmol/l PBS (pH 7.4), and incubated in 1 ml of 0.1 mmol/l Tris–HCl containing 10 μmol/l sodium arachidonate at 37 °C for 45 min. To evaluate local PGI2 production, the level of 6-keto-PGF1α in the medium was measured using an enzyme immunoassay kit (BioAssay).

2.4. Localization of plasmid in the vessel walls and remote organs

Seven days after the transfection of pCMV-LacZ, the animals were perfusion-fixed with 2.5% phosphate-buffered glutaraldehyde and the stented vessels were harvested under anesthesia with ketamine and sodium pentobarbital. For analysis of transgene expression at remote organs, samples of liver, lung, heart, kidney and skeletal muscle (n = 6 each) were taken for β-galactosidase staining (5 mmol/l K4Fe(CN)6, 5 mmol/l K3Fe(CN)6, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Invitrogen). Twenty sections were selected at random from each tissue sample and scanned for presence of β-galactosidase staining. The LacZ gene expression was determined by β-galactosidase staining for 24 h followed by counterstaining with eosin. Cells with nuclei appearing dark blue in color were considered to have stained positive. The transduction efficiency in the stented arteries was calculated by the following formula: (number of cells stained positive by β-galactosidase)/(total nuclei stained by eosin) (n = 3).

2.5. c-AMP levels in the vessel walls

Seven days after the transfection, the stented iliac arteries were immersed in liquid nitrogen. The tissues were homogenized in 6% trichloroacetate and centrifuged. The supernatant was washed with water-saturated diethyl ether three times and lyophilized. The cAMP levels in an appropriate dilution were measured by an enzyme immunoassay kit (Amersham) according to the manufacturer’s protocol.

2.6. Quantification of neointimal formation

Four weeks after the transfection, the animals were perfusion-fixed with 2.5% phosphate-buffered glutaraldehyde and the iliac arteries were harvested and embedded in epoxy-araldit resin. The stented arteries were serially sectioned into four slices (70 μm thick) with a rotating diamond-coated saw (Leica) and were stained with hematoxylin–eosin. Cross-sectional areas of the lumen, intima and media were measured and quantified with NIH Image software (by Wayne Rasband, National Institute of Health, USA).

2.7. Assessment of re-endothelialization

Two and four weeks after the transfection, animals were anesthetized with ketamine and sodium pentobarbital. One hour before the animals were killed, they received an intravenous injection of 6 ml of 0.5% Evans blue dye (Sigma) [24] to identify the remaining nonendothelialized area. After perfusion–fixation, the stented iliac arteries were retrieved and incised longitudinally. The arterial segments were then pinned to a corkboard and further fixed in 100% methanol. Planimetric analysis was performed using the photograph of the harvested arterial segment taken through the dissecting microscope (Olympus). The area of the intimal surface that was stained blue after the application of Evans blue dye was defined as the portion of the arterial segment that remained endothelium deficient. Surface endothelialization was quantified by outlining the Evans blue-positive and -negative areas using NIH Image software. This procedure was performed by a single technician who was blinded to the treatment protocol. Specifically, the extent of endothelialized area was calculated as a percentage of the intimal area encompassed within the stented segments.

2.8. Proliferation index of VSMCs in vivo

Proliferating VSMCs were evaluated by the thymidine analogue BrdU labeling technique [13,14]. The proliferation index was obtained in the pCMV-LacZ- and pCMV-PGIS-transfected groups (n = 3 each), which underwent balloon injury and stent implantation. BrdU was injected (50 mg/kg subcutaneously) 1, 8, 16, and 24 h before the removal of the iliac artery at 14 days after vascular injury. BrdU-positive cells were stained with a murine monoclonal antibody (Amersham), followed by goat anti-mouse IgG antibodies conjugated to peroxidase, and detected with diacylaminobenzidine (DAB). Adjacent sections were also stained with hematoxylin for the detection of non-proliferating cells. The positive nuclei were counted in the media and newly formed intima. The BrdU labeling index was calculated by the following formula: (number of nuclei stained positive by DAB)/(total nuclei stained by hematoxylin).

2.9. Immunohistochemistry

Immunohistochemical analysis of VEGF was performed in the pCMV-PGIS- and pCMV-LacZ-transfected groups (n = 3 each), which underwent balloon injury and stent implantation. Transfected vessels were perfusion-fixed, harvested and paraffin-embedded at 7 days after transfection. To block endogenous peroxidase activity and nonspecific
binding, 5-μm sections were incubated in 0.3% hydrogen peroxidase and then pretreated with 1% bovine serum albumin for 30 min. Specimens were incubated with rabbit polyclonal anti VEGF antibody (1 μg/ml) (Lab Vision) for 30 min at room temperature. Thereafter, the specimens were stained by the ABC method (Vector Laboratories).

2.10. Western blotting

Seven days after transfection, the stented vessels were harvested and immersed in liquid nitrogen. The tissues were homogenized in a lysis buffer, followed by sonication and centrifugation. The supernatant was applied to a 10% sodium dodecylsulfate-polyacrylamide gel. The proteins obtained from the tissues were transferred to polyvinyl difluoride membranes (Immobilon-P; Millipore). The membranes were blocked in skim milk, washed three times with TBST (25 mmol/l Tris, 140 mmol/l NaCl buffer, pH 7.4, and 0.2% Tween-20), and incubated with the rabbit polyclonal anti-VEGF antibody described above (1:500 in TBST; Lab Vision) and β-actin antibody 1:1000 in TBST; DAKO) for 1 h at room temperature. The membranes were then washed three times with TBST, incubated with anti-rabbit immunoglobulin antibody (1:5000), washed again three times with TBST, developed with enhanced chemiluminescence reagent (ECL Western Blotting Analysis System; Amersham) and exposed to X-ray film.

2.11. Statistical analysis

The results are expressed as mean ± S.E.M. ANOVA followed by Scheffe’s post hoc test was used to determine significant differences in multiple comparison testing among
three groups. Unpaired Student’s t-test was used for comparisons between two groups. A value of \( p < 0.05 \) was considered statistically significant.

3. Results

3.1. Effects of gene transfer on the stented arterial walls and remote organs

The cells stained by \( \beta \)-galactosidase were recognized mainly in the media and to a lesser degree in the adventitia. The transduction rate was \( 4.8 \pm 0.6\% \) (\( n = 6 \)). Light microscopical analysis disclosed no evidence of gene expression at any organs remote from the pCMV-LacZ-transfected arterial segments.

The 6-keto-PGF\(_{1\alpha}\) production in pCMV-PGIS-transfected vessels was significantly higher than that in the PBS-instilled (control) and pCMV-LacZ-transfected vessels (Fig. 1).

The cAMP level was 1.6 times higher in the pCMV-PGIS-transfected vessels than in the pCMV-LacZ-transfected counterparts (\( 12.1 \pm 0.4 \text{ vs. } 7.3 \pm 0.5 \text{ pmol/mg protein, } n = 6 \text{ each, } p < 0.05 \)).

3.2. Effects of gene transfer on physiological parameters

Systolic blood pressure was not significantly different between the pCMV-PGIS and pCMV-LacZ groups at 2 weeks after the transfection (pCMV-PGIS: \( 136 \pm 4 \text{ mm Hg, } p = \text{NS} \)). There was also no significant difference in heart rate between the two groups (pCMV-PGIS: \( 326 \pm 14 \text{ vs. } \text{pCMV-LacZ: } 333 \pm 11 \text{ beats per minute, } p = \text{NS} \)).

3.3. Effects of PGIS gene transfer on VSMC proliferation and neointimal formation after stent implantation

Fig. 2 shows representative histological photomicrographs of the stented iliac arteries. Table 1 shows the morphometric analysis of these arteries. PGIS gene transfer markedly suppressed neointimal formation by \( 38\% \) (\( p < 0.05 \)) and widened the lumen area by \( 71\% \) (\( p < 0.01 \)), but did not affect the medial area, which provided larger lumen areas in the stented iliac arteries.

The proliferation index of VSMCs was detected by measuring the percentage of BrdU incorporation to nuclei in the injured vascular segments. The value was lower in the vessels transfected with pCMV-PGIS than in those with pCMV-LacZ (\( 0.4 \pm 0.1\% \text{ vs. } 3.3 \pm 0.1\%, \text{ } p < 0.01 \)).

Table 1

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<tr>
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<th>Lumen area (mm(^2))</th>
<th>Intimal area (mm(^2))</th>
<th>Medial area (mm(^2))</th>
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<td>pCMV-LacZ</td>
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<tr>
<td>pCMV-PGIS</td>
<td>( 4.1 \pm 0.4 )</td>
<td>( 0.8 \pm 0.1^* )</td>
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Data are shown as mean ± S.E.M. (\( n = 6 \) each). *\( p < 0.05 \), †\( p < 0.01 \) vs. pCMV-LacZ.

Fig. 4. Re-endothelialization after stent implantation. (A) At 2 weeks after transfection, the extent of re-endothelialization in the pCMV-PGIS group was significantly higher than that in the pCMV-LacZ group (\( p < 0.01 \)). (B) At 4 weeks after transfection, the extent of re-endothelialization in the pCMV-LacZ group increased up to 75%, whereas the re-endothelialization in the pCMV-PGIS group was almost completed and a significant difference was also observed (\( ^* p < 0.05 \)). Data are mean ± S.E.M.
3.4. Stent re-endothelialization

At 2 weeks after transfection, the coverage of stent by endothelium was significantly greater in the pCMV-PGIS-transfected vessels than in the pCMV-LacZ-transfected counterparts as assessed by Evans blue dye staining (89.4 ± 5.2% vs. 41.6 ± 6.1%, p < 0.01, n = 6 each). Even 4 weeks after transfection, a significant difference was observed (96.5 ± 4.2% vs. 75.2 ± 8.1%, p < 0.05, n = 6 each) (Figs. 3 and 4).

3.5. VEGF protein expression in the vessel wall

Immunostaining of the stented arterial segments with polyclonal anti-VEGF antibody revealed a marked increase of VEGF protein in the stent-implanted arteries at 1 week after the transfection of pCMV-LacZ, whereas the positive staining was hardly detected in the pCMV-LacZ-transfected counterpart (Fig. 5).

Western blot analysis showed that the vessels transfected with pCMV-PGIS produced 1.8-fold more VEGF protein than those transfected with pCMV-LacZ (p < 0.05, n = 6 each) (Fig. 6).

4. Discussion

This study has shown that (1) the percutaneous delivery of plasmid to the arterial wall after deployment of a metallic stent transfected 4.8% of cells in the segment with nonviral lipotransfection via Dispatch catheter, (2) PGIS gene transfer accelerated endothelialization on stent surface, (3) VEGF protein expression was enhanced in the stented arterial segments transfected with PGIS gene, and (4) PGIS gene transfer prevented in-stent restenosis in the atheromatous arteries without significant alteration of serum lipid levels.

4.1. Lipotransfection with Dispatch catheter

The gene transfer strategy used in the current study consisted of a percutaneous, catheter-based gene transfer with nonviral lipotransfection via Dispatch catheter. Long incubation times are required to achieve effective transduction, especially with lipotransfection in the arterial system. In the coronary vasculature, protracted exposure times are likely to result in severe ischemia. The Dispatch catheter can be inflated in the coronary arteries for periods as long as 16 h and effectively permit local drug
delivery without inducing myocardial ischemia due to its inside lumen flow [24]. Tahil et al. [23] reported that arterial gene transfer of an adenoviral vector carrying LacZ gene transduced 16 ± 8% of the endothelial cells and 0.7 ± 0.4% of the medial VSMCs in the iliac arteries of normal rabbits through Dispatch catheter. In this study, our liposomal vector had lower transgene efficiency (4.8%) via Dispatch catheter than did the adenovirus vector. However, the liposomal vector has several advantages, such as lower immunogenicity and more convenient handling than viral vectors [25]. The major limitation of the Dispatch catheter is the risk of gene dissemination at sites remote from the delivery site. The gene dissemination has most likely been the result of suboptimal isolation of transfection chambers between the catheter coils. However, lipotransfection with Dispatch catheter is not supposed to cause systemic gene dissemination even when there is leakage of gene solution, because the plasmid DNA mixed with liposomes that escapes into the circulation during the process of gene transfer is rapidly degraded by circulating nuclease as is the case with naked DNA. In fact, we observed no systemic dissemination of pCMV-LacZ in the present study.

### 4.2. Accelerated in-stent endothelialization

We could demonstrate that overexpression of PGI2 induced by PGIS gene transfer might activate VEGF protein production in balloon-injured and stented arteries via cAMP elevation. VEGF is induced by hypoxia [26,27], transforming growth factor-β [28], interleukin-1β [29], prostaglandin PGE2 and PGE1 [30], etc. Höper et al. [31] reported that PGI2 and PGE2 stimulated VEGF gene expression in monocytic cells and in isolated rat lungs via cAMP. They suggested that PGI2 activated several adenylate cyclases to form cAMP, which in turn activated cAMP-dependent protein kinase, leading to VEGF gene expression. We speculated that PGI2 gene transfer might activate VEGF expression via the same signaling pathway as proposed by Höper et al. Recently, van Belle et al. [32] indicated the feasibility of angiogenic gene transfer with VEGF gene against in-stent restenosis in rabbits. Antiproliferative gene transfer inhibits VSMC proliferation directly, whereas angiogenic gene transfer works more indirectly by reducing intimal thickening through the barrier function of the endothelium. In contrast, many reports suggest that atheromatous plaque is rich in neovascularization [33,34] and that the capillary in neointimal layer, vasa vasorum which is extended from the adventitia, controls VSMC’s ability to differentiate its phenotype from contractile-form to synthetic-form as well as infiltration of inflammatory cells after angioplasty and stent implantation [35,36]. Based on these findings, Moulton et al. demonstrated that anti-angiogenesis inhibitors, endostatin [37] and TNP-470 [38] could reduce plaque growth in genetically hypercholesterolemic mice [39]. In the other report, PGI2 is an angiogenic factor and administration of exogenous PGI2 induces capillary growth in vitro [40]. These results may be contradictory to our strategy of conveying endogenous PGI2. We speculate that to prevent in-stent restenosis, it is important to maintain the balance between regeneration of endothelium and suppression of abnormal capillary growth from the adventitia as well as inhibition of VSMC proliferation. In this study, our strategy of endogenous PGI2 overproduction would be beneficial for the injured arteries to maintain this balance. In future, it should be elucidated that how and to what vascular cells therapeutic angiogenesis [39] should be applied in atheromatous arteries.

### 4.3. Reduced neointimal formation

Our present findings showed that PGIS gene transfer with a liposomal vector markedly suppressed neointimal formation after stent implantation despite the low transduction efficiency. This desirable result may have been due to the following possible mechanisms. First, overexpressed PGI2 is secreted from transfected cells and may prevent proliferation of both the untransfected and the transfected cells through the bystander effect [41]. Second, PGI2 has many protective effects on vessels as well as a direct inhibitory effect on cellular growth [21], and these effects may act in multiple steps and result in marked inhibition of proliferative changes after stent implantation. Third, PGIS gene transfer effectively increases PGI2 production and decreases PGE2 production without affecting thromboxane A2 production after prostaglandin H synthase 2, or cyclooxygenase-2, induction, as previously described in our studies [13–15]. Finally, PGIS gene transfer might increase VEGF protein expression and accelerate re-endothelialization that might, in turn, restore the barrier function of the endothelium against thrombus formation and intimal hyperplasia in the balloon-injured and stented arterial segments.

In anticipation of a clinical application, we propose that PGIS gene transfer using gene-coated stents may further reduce in-stent restenosis in human atherosclerotic arteries. Further investigation is required for this gene delivery system.

### 5. Conclusions

In conclusion, our study showed that an overexpression of PGI2 induced by PGIS gene transfer inhibited VSMC proliferation and accelerated re-endothelialization with increase of VEGF protein expression resulting in prevention of neointimal formation after balloon injury and stent implantation in atheromatous rabbits. PGIS gene transfer may be a potential therapeutic strategy against in-stent restenosis in atheromatous human coronary arteries.
Acknowledgements

A part of this work was presented at the 2000 and 2001 Annual Congress of the European Society of Cardiology at Amsterdam and Stockholm. This work was in part supported by grants from the Ministry of Education, Science, and Culture of Japan (No. 12670658). The animal experiments were mainly carried out at the Institute for Laboratory Animal Research, Nagoya University.

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