Prostacyclin synthase gene transfer inhibits neointimal formation in rat balloon-injured arteries without bleeding complications

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Abstract

Objective: This study was designed to compare the effects of prostacyclin synthase (PCS) gene transfer with those of a systemic infusion of beraprost sodium (BPS), a prostacyclin analogue, on vascular smooth muscle cell (VSMC) proliferation and neointimal formation after arterial injury. Methods: PCS gene (3 or 30 µg) was transfected into rat balloon-injured carotid arteries by a non-viral lipotransfection method. BPS (100 or 300 µg/kg/day) was subcutaneously infused with osmotic pumps after the injury. LacZ gene (30 µg) was used as a control. VSMC proliferation was estimated by the bromodeoxyuridine (BrdU) index (BrdU-positive nuclei/total nuclei) at day 7. Neointimal formation was evaluated at day 14. Each treatment group had six rats. Results: PCS gene transfer prevented the increase in intimal/medial area ratio (3 µg: 46.6%, 30 µg: 61.1% reduction; P<0.05, P<0.01, respectively), as did BPS 300 µg/kg/day (49.8% reduction; P<0.05). BPS 100 µg/kg/day, however, had no effects on the ratio. PCS gene transfer and BPS 300 µg/kg/day significantly suppressed the BrdU index. BPS 300 µg/kg/day group had more frequent hematoma and longer bleeding time. There were no significant differences in blood pressure, heart rate, or urinary volume among all groups. Conclusion: Both PCS gene transfer and BPS 300 µg/kg/day reduced neointimal formation after arterial injury by inhibiting VSMC proliferation. PCS gene transfer may be a safer therapeutic modality against neointimal formation than a systemic infusion of BPS because the former method resulted in fewer bleeding complications. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a well-established strategy against severe and symptomatic coronary artery diseases. However, its efficacy is limited by a high incidence of chronic restenosis which has been reported to be as high as 20–50% within 3–6 months [1–3]. Several lines of evidence indicate that this restenosis might be multifactorial and caused mainly by vascular remodeling, the migration of vascular smooth muscle cells (VSMCs) from the media into the intima, and their unregulated proliferation [4–6]. Intravascular ultrasound examinations of human coronary arteries recently demonstrated that coronary restenosis resulted primarily from vascular remodeling and contraction rather than from intimal growth [7,8]. Although several randomized and placebo-controlled studies have revealed that coronary stents which generally prevented vascular remodeling reduced restenosis rates after angioplasty [9,10], the issue of restenosis mainly caused by intimal thickening after coronary intervention has not been resolved. Combined therapy against vascular remodeling and neointimal formation may be required to eliminate restenosis after coronary intervention. In animal models, several pharmacological [11–13] and genetic [14–16] approaches focusing on the inhibition of VSMC proliferation have proved effective in reducing restenosis rates after balloon injury. Prostacyclin (PGI2) is produced in vessel walls and...
protects vessels by dilating the vessels [17], suppressing platelet aggregation [18] and inhibiting VSMC proliferation [19]. Although several animal studies have shown that a PGI2 analogue suppressed experimental neointimal hyperplasia [20,21], restenosis occurred after coronary angioplasty with a PGI2 analogue in a clinical trial conducted by Gershlick et al. [22]. However, they employed a relatively small dose (4 ng/kg/min) of the analogue, which was infused for a relatively short period (36 h). This may have led to the failure of the analogue to prevent restenosis. Thus, the effects of PGI2 on VSMC migration and proliferation after balloon injury have not yet been clarified. We speculated that one of the reasons for the discrepancy between the experimental success and the clinical failure might be the difference in the amount of PGI2. Therefore, we hypothesized that a local overexpression of PGI2 at balloon-injured sites could reduce VSMC migration and proliferation after balloon injury without systemic complications such as hypotension and bleeding tendency. To test this hypothesis, we examined the effects of an overexpression of endogenous PGI2 as well as those of an exogenous PGI2 analogue in balloon-injured rat carotid arteries. For the former model, we developed a plasmid with cDNA for prostacyclin synthase (PCS) which catalyzes the conversion of prostaglandin H2 (PGH2) to PGI2, and performed a PCS gene transfer into balloon-injured arterial segments after we had confirmed the successful transfection of the gene into cultured VSMCs. For the latter model, beraprost sodium (BPS), a stable PGI2 analogue, was infused continuously after balloon injury.

2. Methods

2.1. Preparation of PCS plasmid DNA

Based on the sequence of rat PCS [23], we designed the primers including an epitope tag of influenza hemagglutinin (forward primer: 5’-ccacc-atgtacccatacgctcagactacgct-agttctgggccgct-3’, reverse primer: 5’-ggaccaacaggaagtcaggag-3’). The forward primer contains the HA tag sequence to facilitate protein expression, shown above as the underlined region. The non-underlined sequences are based on the original sequence of rat PCS. Total RNA (5 μg) from rat aorta was reverse-transcribed using a first-strand cDNA synthesis kit (Pharmacia Biotech), containing Moloney Murine Leukemia Virus reverse transcriptase. In each tube, 0.5 μg of oligo(dT)12–18 primer (Invitrogen) was used as a primer for the first-strand cDNA synthesis. A polymerase chain reaction (PCR) was performed for 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min with a final 10-min extension period. The PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and extracted. The 1.6 kb rat PCS cDNA was cloned in the cytomegalovirus (CMV) enhancer/promoter-directed vector (pTargetT, Promega), which contains a neomycin resistance gene under the control of an SV 40 enhancer/early promoter with a polyadenylation signal sequence (pCMV-PCS). The plasmid carrying the LacZ gene substituted for the PCS gene was constructed as a control (pCMV-LacZ). The plasmid DNAs (pCMV-PCS and pCMV-LacZ) were purified with a plasmid purification kit (Qiagen) according to the manufacturer’s protocol. The sequence of plasmids was confirmed by a DNA sequence reader (4000L, LI-COR).

2.2. PCS gene transfer to cultured vascular cells

Rat VSMCs were explanted from rat aortas and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (GIBCO BRL), 100 U/ml penicillin and 100 μg/ml streptomycin. VSMCs were grown in 60 mm dishes to 60% confluency and transfected with 5 μg pCMV-PCS or pCMV-LacZ mixed with 50 μl of a liposomal transfection reagent, Lipofectamine Plus (GIBCO BRL). The VSMCs were incubated for 2 days after transfection. Untransfected VSMCs were also incubated with only 50 μl of the liposomal transfection reagent for 2 days. The medium was removed and replaced with 1 ml of fresh medium containing 10 μmol/l sodium arachidonate. After 60 min of incubation, the medium was extracted for the measurement of 6-keto-prostaglandin F1a (6-keto-PGF1a) and thromboxane B2 (TXB2) levels by chemiluminescence immunoassay kits (BioAssay Inc.). The remaining VSMCs were lysed with lysate buffer and stored at −80°C until assay by Western blotting.

2.3. Western blotting

The cell lysate was applied to a 10% sodium dodecylsulfate-polyacrylamide gel. The proteins obtained from the cells were transferred to polyvinyl difluoride membranes (Immobilon-P, Millipore Corp.). 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 anti-rat PCS antibody (1:500 in TBST, Cayman) 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.

2.4. Transgene efficiency in vitro

Transfected VSMCs were detected by LacZ gene expression using β-galactosidase staining [5 mmol/l
K₄Fe(CN)₆, 5 mmol/l K₄Fe(CN)₆, 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Invitrogen) for 2 h. Transduction efficiency was evaluated by the average percentage of positive cells in whole cultured VSMCs in 5–6 eyefields. We defined that β-galactosidase staining was positive if the nuclei had dark blue coloration. Therefore, cells with the blue cytosol but without dark blue nuclei were not counted as transduced cells.

2.5. Measurement of cyclic AMP levels

Cultured VSMCs were mixed with 6% trichloroacetate. The solution 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. Gene delivery in vivo and regimen of BPS infusion

This investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The in vivo gene transfer into rat carotid arteries was performed as previously described [24]. Male Sprague–Dawley rats (Japan SLC, Inc.) weighing 360–410 g were maintained under a 12-h light/dark cycle at 23°C and were fed standard laboratory chow (RC4, Oriental Yeast Co.) and water ad libitum. Rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally, i.p.). After a midcervical incision, the right common carotid artery and its bifurcation were exposed. To prevent acute thrombosis during the procedure, heparin sodium (200 IU/kg) was intravenously injected 5 min before the balloon injury according to the method of Zoldhelyi et al. [25]. The right common carotid artery was balloon-injured three times with a 2 F balloon catheter (2 F Fogarty, Baxter Healthcare) inserted through the external carotid artery. Two vascular clips were placed at the distal end and in the middle of the injured arterial segment. A 24-gauge cannula was introduced into the common carotid artery via the external carotid artery. The lumen of the injured segment between the two clips (10 mm long) was washed for 5 min with 9.57 mmol/l phosphate-buffered saline (PBS). pCMV-PCS or pCMV-LacZ with 100 µl Lipofectamine Plus (GIBCO BRL) or vehicle (9.57 mmol/l PBS) with 100 µl Lipofectamine Plus was instilled into the lumen for 30 min. Then the instillate was retrieved, the cannula removed, and the blood flow restored. After confirmation of the carotid arterial flow, the wound was sutured. All procedures were performed under sterile conditions. BPS was subcutaneously (s.c.) infused with an osmotic pump (Alzet 2ML2; ALZA Corp.) implanted in the back of rats, starting 30 min before the injury and continuing for 14 days. A pump filled with vehicle was implanted also in rats transfected with plasmids to equalize the surgical procedures among all groups. Rats were divided into five groups as follows: (1) pCMV-LacZ 30 µg plus the vehicle for BPS (control), (2) pCMV-PCS 3 µg plus the vehicle (pCMV-PCS-3), (3) pCMV-PCS 30 µg plus the vehicle (pCMV-PCS-30), (4) BPS 100 µg/kg/day without transfection (BPS-100), and (5) BPS 300 µg/kg/day without transfection (BPS-300) (n=6 each, Table 1).

2.7. Localization of plasmid in the vessel wall

Seven days after the transfection of pCMV-PCS and pCMV-LacZ, the carotid arteries were perfusion-fixed with 2.5% phosphate-buffered glutaraldehyde and harvested under anesthesia with sodium pentobarbital. The LacZ gene expression was determined by β-galactosidase staining for 24 h, and counterstaining with eosin. The β-galactosidase positive cells were defined as described above. The transduction efficiency was calculated by the following formula: (positive cells stained by β-galactosidase staining)/(total nuclei stained by eosin) (n=3).

2.8. PGI₂ and TXA₂ production in rat carotid artery

The injured rat carotid arteries treated with pCMV-PCS (30 µg) or pCMV-LacZ (30 µg) were resected 14 days after the balloon injury. The arteries were cut into 5-mm lengths, washed with 9.57 mmol/l PBS (pH 7.4), and incubated in 1 ml of 50 mmol/l Tris–HCl at 37°C for 45 min. Six-keto-PGF₁α and TXB₂ in the medium were measured to evaluate local PGI₂ and TXA₂ production, respectively, with the enzyme immunoassay kits described above.

Table 1

| Transfected gene | Systemic infusion |
|------------------|-------------------|
| Control          | pCMV-LacZ 30 µg   |
| pCMV-PCS-3       | pCMV-PCS 3 µg     |
| pCMV-PCS-30      | pCMV-PCS 30 µg    |
| BPS-100          | None              |
| BPS-300          | BPS 100 µg/kg/day |
|                  | BPS 300 µg/kg/day |

*CMV, cytomegalovirus; PCS, prostacyclin synthase; BPS, beraprost sodium.
2.10. Hemostatic parameters

The platelet count and template bleeding time were measured before and 14 days after balloon injury. The template bleeding time was measured after the measurement of hemodynamic parameters in vivo in each group. The abdomen was shaved carefully and an incision (10 mm in length and 3 mm in depth) was made with a scalpel. Bleeding from the incision site was wiped away with filter paper every 20 s, until it stopped. The template bleeding time was defined as the time elapsed from the initiation of the incision to hemostasis. The frequency of subcutaneous hematoma at the site of surgery was also determined on day 14. Hematoma was defined as a mass which was more than 10 mm in diameter, and was confirmed macroscopically.

2.11. Quantification of neointimal formation

Fourteen days after the vascular injury, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). After a midsternal incision was made, a blood sample was collected by heart puncture, and the common carotid arteries were perfusion-fixed with 2.5% phosphate-buffered glutaraldehyde and harvested. The arteries were stained with hematoxylin-eosin or elastica van Gieson stain. Cross-sectional areas of the lumen, intima, media and area surrounded by external elastic lamina were determined with computed planimetry on four sections from each artery that spanned the 1.0-cm region of arterial injury and were averaged.

2.12. Proliferation index of VSMCs in vivo

Proliferating VSMCs were evaluated by the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) labeling technique [13]. The proliferation index (BrdU Index) was obtained in the control group (pCMV-LacZ 30 μg, n=6), the 30-μg pCMV-PCS-transfected group (n=6), and 300 μg/kg/day BPS-infused group (n=6). BrdU was injected (50 mg/kg, s.c.) 1, 8, 16, and 24 h before the removal of the carotid artery at day 7 after vascular injury. BrdU-positive cells were stained with a murine monoclonal antibody (Amersham), followed by goat anti-mouse Ig antibodies conjugated to peroxidase, and detected with diaminobenzidine (DAB). Adjacent sections were also stained with hematoxylin for the detection of nonproliferating cells. The positive nuclei were counted in the media and newly formed intima. The BrdU index was calculated by the following formula: (positive nuclei stained by DAB)/(total nuclei stained by hematoxylin).

2.13. Statistics

The results are expressed as mean±S.E.M. Differences among three or more groups were tested by a one-way ANOVA for repeated measures with a post hoc Scheffe’s F-test for multiple comparison. Serial changes in variables were evaluated by a two-way ANOVA for repeated measures followed by Scheffe’s F-test or a paired Student’s t-test where appropriate. A Kruskal–Wallis test followed by the Dunn procedure was performed to analyze non-parametric data. Differences in noncontinuous variables were tested with the chi-square test. A value of P<0.05 was considered significant.

3. Results

3.1. Prostacyclin production induced by pCMV-PCS transfer in cultured rat VSMCs

The baseline levels of both 6-keto-PGF₁α and TXB₂ produced by the pCMV-PCS- and pCMV-LacZ-transfected VSMCs did not differ significantly from those of the untransfected control VSMCs (Table 2). Treatment with 10 μmol/l sodium arachidonate for 60 min increased the production of 6-keto-PGF₁α by 2.5-fold in the untransfected VSMCs, by 3-fold in the pCMV-LacZ-transfected VSMCs, and by 6-fold in the pCMV-PCS-transfected VSMCs (Table 2). Thus, the pCMV-PCS-transfected VSMCs produced twice as much PGI₂ in the presence of exogenous arachidonic acid as in the pCMV-LacZ-transfected VSMCs and the untransfected VSMCs, which was a significant difference (P<0.05, each). In contrast, the TXB₂ production did not change even in the presence of exogenous arachidonic acid in each VSMC group.

The pCMV-PCS-transfected VSMCs expressed 2.1-fold more PCS protein than the pCMV-LacZ-transfected cells (n=5 each, P<0.01, Fig. 1). The cAMP level was 2.3 times higher in the VSMCs transfected with pCMV-PCS than in those transfected with pCMV-LacZ (17.3±0.5 vs. 7.5±0.4 pmol/mg protein, P<0.05, n=5 each). The

| Table 2 |
| --- |
| Prostanooid production after prostacyclin synthase gene transfer to cultured vascular smooth muscle cells* |
| Baseline | After treatment | AA(+) |
| --- | --- | --- |
| 6-keto-PGF₁α (pg/mg tissue) |
| Untransfected | 42±3 | 40±5 | 101±7a |
| pCMV-LacZ | 40±3 | 38±3 | 110±9b |
| pCMV-PCS | 42±4 | 37±4 | 225±12c |
| TXB₂ (pg/mg tissue) |
| Untransfected | 52±4 | 51±5 | 55±7 |
| pCMV-LacZ | 50±3 | 50±6 | 54±9 |
| pCMV-PCS | 49±4 | 50±4 | 55±6 |

* Data are mean±S.E.M. (n=6 each). AA, arachidonic acid; TXB₂, thromboxane B₂; CMV, cytomegalovirus; PCS, prostacyclin synthase.

a P<0.05 vs. AA(−).
b P<0.05 vs. untransfected and pCMV-LacZ.

were not significantly different among the five groups at both day 0 and day 14 (Table 3).

The baseline levels of urinary 6-keto-PGF$_{1a}$ were not significantly different among the five groups. Fourteen days after balloon injury, the urinary levels of 6-keto-PGF$_{1a}$ increased by 1.7-fold in BPS-100 group ($P<0.05$), and by 1.9-fold in BPS-300 group ($P<0.01$), while those of pCMV-PCS-3 and pCMV-PCS-30 groups did not significantly change (Fig. 5). Thus, pCMV-PCS locally delivered to the carotid artery did not affect the level of plasma 6-keto-PGF$_{1a}$.

The platelet counts measured at both day 0 and day 14 were not significantly different among the five groups (Table 4). The template bleeding times at day 0 were not different among the five groups. The bleeding time in the BPS-300 group was significantly prolonged at day 14 (day 14: 185±9 s, day 0: 145±7 s, $P<0.05$), while those of the other groups were not (Table 4). In addition, the BPS-300 group had significantly more frequent hematoma at the operated neck or the back where the osmotic pumps had been implanted (control: 0/6 vs. BPS-300: 3/6, $P<0.05$). In contrast, the pCMV-PCS groups showed neither bleeding tendency nor hemorrhagic complications.

### 3.4. The effects of PCS gene transfer and BPS infusion on VSMC proliferation and neointimal formation

Fig. 6 shows representative histological photomicrography of common carotid arteries 14 days after the injury. Table 5 shows the morphometric analyses of these arteries.
Fig. 3. Cross-section of pCMV-LacZ-transfected artery. The transgene efficiency in vivo was confirmed by β-galactosidase staining. β-Galactosidase-positive cells (arrowheads) were detected mainly in the media (M) and partly in the adventitia (A). E, endothelium. Magnification ×100.

Fig. 4. Effects of PCS gene transfer on the productions of PGI\(_2\) and TXA\(_2\) by the balloon-injured segments of rat common carotid arteries. (A) The level of 6-keto-PGF\(_{1\alpha}\). The production of 6-keto-PGF\(_{1\alpha}\) was significantly increased in the pCMV-PCS-transfected arteries. (B) The level of TXB\(_2\). There was no significant difference between the two groups. PCS gene transfer did not affect TXA\(_2\) production. Data are mean±S.E.M. (n=6 each). * P<0.0001 vs. control (n=6 each).
Table 3  
Hemodynamic parameters and urinary volume*  

|                | SBP (mmHg) | HR (bpm) | UV (ml/day) |
|----------------|------------|----------|-------------|
|                | Day 0      | Day 14   | Day 0       | Day 14   | Day 0       | Day 14   |
| Control        | 122±6      | 128±6    | 377±18      | 380±19   | 8.1±0.1     | 8.3±0.3  |
| pCMV-PCS-3     | 118±7      | 126±5    | 380±12      | 388±8    | 7.6±0.3     | 8.2±0.2  |
| pCMV-PCS-30    | 120±6      | 123±1    | 366±14      | 372±14   | 7.8±0.2     | 8.2±0.4  |
| BPS-100        | 115±2      | 117±3    | 390±14      | 388±13   | 8.2±0.4     | 7.9±0.4  |
| BPS-300        | 106±3      | 112±6    | 411±11      | 390±13   | 8.3±0.5     | 8.1±0.1  |

* Data are mean±S.E.M. (n=6 each). SBP, systolic blood pressure; HR, heart rate; UV, urinary volume; CMV, cytomegalovirus; PCS, prostacyclin synthase; BPS, beraprost sodium.

Fig. 5. Urinary excretion of 6-keto-PGF_{1a} before and 14 days after balloon injury. The baseline levels of urinary 6-keto-PGF_{1a} were not significantly different among the five groups. Fourteen days after balloon injury, the urinary levels of 6-keto-PGF_{1a} significantly increased both in BPS-100 group (P<0.05) and in BPS-300 group (P<0.01). At day 14, the urinary 6-keto-PGF_{1a} levels in BPS 300 mg/kg/day rats were significantly higher than those in control rats, while there was no significant difference among the pCMV-PCS 3 mg, 30 mg and control rats. Open bars: day 0; hatched bars: day 14. Data are mean±S.E.M. (n=6 each). * P<0.05, ** P<0.01, *** P<0.001 vs. control.

Both the local expression of PCS and the high dose of BPS (300 μg/kg/day) markedly suppressed neointimal formation and the intimal/medial area ratios, but did not affect the medial area and the vessel size expressed by the area surrounded by the external elastic lamina. Larger lumen areas were obtained in the pCMV-PCS-30 and the BPS-300 groups. A lower dose of BPS (100 μg/kg/day), however, had no inhibitory effect on neointimal formation.

Table 4  
Hemostatic parameters*  

|                | Platelet (×10³/mm³) | Bleeding (s) | Hematoma |
|----------------|---------------------|--------------|----------|
|                | Day 0   | Day 14  | Day 0   | Day 14  |                |
| Control        | 84±10   | 87±9    | 135±5   | 144±9   | 0/6            |
| pCMV-PCS-3     | 79±4    | 77±4    | 138±13  | 148±8   | 0/6            |
| pCMV-PCS-30    | 97±8    | 98±11   | 153±19  | 164±12  | 0/6            |
| BPS-100        | 99±9    | 102±5   | 144±19  | 177±18  | 1/6            |
| BPS-300        | 102±5   | 109±7   | 145±7   | 185±9   | 3/6            |

* Data are mean±S.E.M. (n=6 each). CMV, cytomegalovirus; PCS, prostacyclin synthase; BPS, beraprost sodium.

b P<0.05 vs. control.

c P<0.05 vs. day 0.
Fig. 6. Representative histological sections of common carotid arteries. No injury (A), balloon injury plus pCMV-LacZ (B), the injury plus pCMV-PCS 30 μg (C) or the injury plus exogenous BPS 300 μg/kg/day (D). Arteries were stained with hematoxylin-and-eosin. Note that the PCS gene transfer as well as exogenous BPS markedly reduced neointimal formation. Magnification ×20.

Table 5
Morphometric analysis

|          | LA (mm²) | IA (mm²) | MA (mm²) | EEL (mm²) | IA/MA |
|----------|----------|----------|----------|-----------|-------|
| Control  | 0.20±0.03| 0.21±0.02| 0.16±0.02| 0.58±0.04 | 1.31±0.10 |
| pCMV-PCS-3 | 0.28±0.03| 0.08±0.02| 0.12±0.01| 0.49±0.04 | 0.70±0.15 |
| pCMV-PCS-30 | 0.52±0.05| 0.07±0.01| 0.15±0.01| 0.73±0.05 | 0.51±0.12 |
| BPS-100  | 0.31±0.03| 0.13±0.01| 0.14±0.02| 0.59±0.04 | 0.95±0.06 |
| BPS-300  | 0.39±0.04| 0.09±0.01| 0.14±0.01| 0.62±0.05 | 0.66±0.06 |

* Data are mean±S.E.M. (n=6 each). LA, lumen area; IA, intimal area; MA, medial area; EEL, area surrounded by external elastic lamina; IA/MA, intimal/medial area ratio; CMV, cytomegalovirus; PCS, prostacyclin synthase; BPS, beraprost sodium.

b P<0.05.
c P<0.01 vs. control.

Table 6
BrdU index

|          | Neointima (%) | Media (%) | Neointima + media (%) |
|----------|----------------|-----------|------------------------|
| Control  | 52.2±8.7       | 12.2±2.2  | 23.8±1.6               |
| BPS-300  | 18.6±3.6³      | 4.4±0.7³  | 9.6±1.3³               |
| pCMV-PCS-30 | 10.5±1.0³    | 2.2±0.5³  | 5.8±0.6³               |

* Data are mean±S.E.M. (n=6 each). BPS, beraprost sodium; CMV, cytomegalovirus; PCS, prostacyclin synthase.

b P<0.05.
c P<0.01 vs. control.
4. Discussion

In the present study, we demonstrated that a local overexpression of PGI₂ derived from PCS gene transfer and a systemic infusion of BPS prevent VSMC migration and proliferation (neointimal formation) in balloon-injured rat carotid arteries. We also found that a high dose of BPS causes more frequent hemorrhagic complications.

Many early responses to balloon injury, including platelet adhesion and aggregation, thrombus formation, and adhesion and invasion of macrophages and lymphocytes to arterial walls may take place, leading to the synthesis and/or release of various kinds of growth factors such as platelet-derived growth factor-α, transforming growth factor-β and basic fibroblast growth factor [26]. These growth factors in turn stimulate VSMCs to migrate from the media into the intima, where they start to proliferate and secrete extracellular matrix components [27,28]. PGI₂ has been reported to inhibit the chemotaxis and adhesion of monocytes [29], cytokines derived from activated macrophages, and VSMC migration and proliferation [30]. Moreover, it is likely that PGI₂ suppresses some of these reactions to balloon injury. Although each protective effect of PGI₂ may not be strong enough to inhibit VSMC proliferation, these effects may be combined and result in a marked suppression of proliferative changes after balloon injury. Thus, PGI₂ is a probable candidate for a preventive drug against restenosis after coronary intervention. However, in a clinical study, a relatively low dose of PGI₂ analogue (5.8 μg/kg/day) failed to prevent restenosis [22]. In an animal study reported by Isogaya et al., a high dose (200 μg/kg/day) of BPS had inhibitory effects on restenosis in atherosclerotic rabbits, whereas a low dose (100 μg/kg/day) had no inhibitory effects [21]. However, BPS at doses of 5.8–14.4 μg/kg/day, well below the low dose (100 μg/kg/day) used in animal studies such as ours and that of Isogaya et al., tends to cause intolerable systemic side effects in humans, as Pickles et al. reported [31]. Our present study also demonstrated that the high dose (300 μg/kg/day) of BPS caused more frequent hemorrhagic complications associated with increased levels of plasma and urinary 6-keto-PGF₁α, although it did prevent neointimal formation after balloon injury. In contrast, local PCS gene transfer to the injured arterial segment prevented neointimal formation without causing any hemorrhagic complications, and without increasing the plasma and urinary levels of 6-keto-PGF₁α. Hence, local PCS gene transfer may be more suitable for clinical application than a systemic administration of BPS in light of attenuating the systemic side effects of PGI₂.

In the present study, we used cationic liposomes to enhance the entry of plasmid DNA into cells. It has been reported that the transduction efficiency of liposomal vectors into vascular cells (4–5%) is lower than that of the other vectors [32]. Adenoviral vectors have been most frequently used for vascular gene transfer [14,25,33,34]. Although the adenovirus-mediated gene transfer method achieves high transgene expression in vivo (10–30%), it has several limitations. For example, adenovirus vectors retain the majority of the parent virus genome, which is associated with undesired gene expression that results in both immune and vascular inflammatory responses [35,36]. Gene expression from adenoviral vectors in vivo peaks within 1 week and is limited to 2–4 weeks due to these immune responses. In contrast, the liposomal vector method has several advantages. It is not antigenic, is feasible to prepare, and can be used repeatedly. Our liposomal vector had an acceptable transgene efficiency of 8.5% in vivo.

Our present findings showed that PCS gene transfer with a liposomal vector markedly suppressed neointimal formation after balloon injury. This desirable result may have been due to the following possible mechanisms. First, PGI₂ was overexpressed at the balloon-injured arterial segments on which it should act in order to prevent intimal thickening. The PGI₂ thus produced may be advantageous because it prevents the proliferation of untransfected cells as well as transducted cells through an autocrine/paracrine loop [24]. Second, PGI₂ has many protective effects on vessels as well as a direct inhibitory effect on cellular growth [30], similar to the other vasodilative molecules such as C-type natriuretic peptide [24] and nitric oxide [16]. Third, PCS gene transfer may effectively increase PGI₂ synthesis by diverting PGH₂ toward the production of PGI₂, resulting in less synthesis of TXA₂. Because TXA₂ opposes the protective effects of PGI₂ on vessels, and facilitates platelet aggregation and VSMC proliferation [19,37], a selective production of PGI₂ caused by PCS gene transfer is preferable. Zoldhelyi et al. showed that an adenovirus-mediated transfer of cyclooxygenase (COX)-1 gene into balloon-injured arteries significantly inhibited thrombosis by increasing PGH₂ synthesis by augmenting PGI₂ synthesis [25]. They speculated that the COX-1 gene transfer enhanced the production of PGI₂ with only a limited increase in TXA₂ production; however, they did not measure TXA₂ production. An overproduction of PGH₂ may cause vascular contraction and platelet aggregation [38–40], and may also result in an overproduction of TXA₂. Pritchard et al. [41] reported that PGI₂ production increased after balloon injury via the induction of COX-2 in the vessel wall, although the injury disturbed constitutive COX-1 activity. We speculated that a sufficient supply of PGH₂ led to an increased PGI₂ production catalyzed by overexpressed PCS in the injured arteries. In this study, the balloon-injured arteries transfected with PCS gene produced higher levels of PGI₂ without...
affecting TXA₂ production. The protective effects of PCS gene transfer on neointimal formation after balloon injury may thus be the result of the selective increase of PGI₂.

In conclusion, our study showed that a local overexpression of PGI₂ induced by PCS gene transfer as well as that induced by a systemic infusion of a PGI₂ analogue protected against neointimal formation in balloon-injured rat carotid arteries. Local PCS gene transfer may be a safer therapeutic modality against proliferative arterial diseases than exogenous PGI₂, because the former resulted in fewer hemorrhagic complications.

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