Gene Transfer of Human Prostacyclin Synthase Prevents Neointimal Formation After Carotid Balloon Injury in Rats

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Background and Purpose—A disordered proliferative process in the vascular wall is thought to underlie the pathogenesis of restenosis after percutaneous transluminal angioplasty and carotid endarterectomy. A growth inhibitory property of overexpressed prostacyclin (PGI₂) synthase (PGIS) was recently implicated in the pathological proliferation of vascular smooth muscle cells (VSMC) in vitro. Here, we investigated the effects of increased PGI₂ synthesis on the pathological proliferation of VSMCs.

Methods—The cDNA encoding human PGIS was transfected into endothelium-denuded rat carotid arteries after arterial balloon injury with the use of hemagglutinating virus Japan (HVJ). HVJ liposome vector complex without PGIS cDNA was used for vehicle control. The level of 6-keto PGF₁α, a stable hydrolyzed metabolite of PGI₂, the histological distribution of the immunoreactivity for human PGIS and the ratio of neointimal/medial area were analyzed.

Results—In the analyses of 6-keto PGF₁α, the level in the carotid arteries was significantly elevated 3 days after PGIS expression-vector transfection compared with that in the arteries after vehicle transfection. Seven days after human PGIS expression-vector transfection, the PGIS cDNA–transfected neointimal cells were strongly positive for human PGIS immunoreactivity in 81% sections examined. Fourteen days after the injury, the ratio of neointimal/medial area was 1.2 ± 0.4 in the PGIS expression-vector transfected group, which was significantly smaller than that of the vehicle control group, 1.7 ± 0.5; *P* < 0.01.

Conclusions—It was thus demonstrated that the gene transfer of human PGIS expression-vector into rat carotid arteries resulted in the increased production of human PGI₂ in the vascular wall, the expression of human PGIS in the developing neointima and significantly inhibited the neointimal formation generated after balloon injury. (Stroke. 1999;30:419-426.)

Key Words: carotid arteries ■ genes ■ prostacyclins ■ stenosis ■ rats

The principal contributor to the pathogenesis of cerebral and myocardial infarction is the developing atherosclerosis. The process of atherosclerosis involves a chronic inflammatory response to the injury in the arterial wall, leading to a dysfunction of endothelial cells, the migration and activation of macrophages, and the proliferation of vascular smooth-muscle cells (VSMC).¹ The complex of inflammatory and fibroproliferative processes is manifested as intimal hyperplasia or the formation of fibrous plaques. Similar inflammatory and proliferative processes in the vascular wall are thought to underlie the pathogenesis of restenosis after percutaneous transluminal angioplasty (PTA) and carotid endarterectomy (CE).²–⁶

A neointima formation after balloon angioplasty involves a complex interaction between numerous growth-regulatory molecules that promote the migration and proliferation of VSMC.² Possible neointima-generating molecules include thrombin, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β).¹,⁷–⁹

Prostacyclin (PGI₂) is a short-lived endogenous inhibitor of platelet aggregation that can provide a nonthrombogenic condition for the endothelium.¹⁰–¹³ PGI₂ is also known as a potent vasodilator that contributes to the maintenance of homeostasis in vascular tone, which is generally balanced by the constitutive production of thromboxane A₂ (TXA₂), angiotensin II (A-II), or endothelin (ET).¹,¹⁴ In addition to these

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well-known functions of PGI₂, a growth regulatory property has been reported. 15–18

Prostacyclin synthase (PGIS) is known to catalyze the conversion of prostaglandin H₂ (PGH₂) to prostacyclin. 14 In 1994, 19,20 we determined the amino acid sequence of bovine and human endothelial PGIS by cDNA cloning. PGIS was found to be widely expressed in human and rat tissues. 20, 21 Importantly, smooth-muscle cells of arteries were shown to express abundant PGIS mRNA by in situ hybridization. 21 To study the growth-inhibitory effects of overexpressed PGIS on the proliferation of VSMC, Hara et al. 22 recently transfected human PGIS expression vector into rat cultured VSMC and demonstrated that overexpressed human PGIS resulted in increased PGI₂ synthesis. In addition, a concomitant suppression of serum-stimulated DNA synthesis was observed to occur in an autocrine and/or paracrine manner in the cultured VSMC.

Here, to further elucidate the effects of increased PGI₂ synthesis on the pathological proliferation of VSMC in vivo, the cDNA encoding human PGIS was transfected into endothelium-denuded rat carotid arteries after arterial balloon injury, using the gene transfer method with hemagglutinating virus Japan (HVJ). 23–27 PGIS cDNA was introduced into the injured arterial wall, and its effects on PGI₂ production, PGIS expression, and pathological thickening of the vascular wall were examined. Two weeks after balloon injury, the luminal narrowing was most pronounced, and total cell count in vessel wall was considered maximal in this model. 28

Materials and Methods

Construction of Plasmids

The expression vector for human PGIS was constructed as described previously. 20, 22 Briefly, the blunted Hind III/Bam HI fragment of the full-length human PGIS cDNA was ligated into the blunted Xho I site of the pUC-CAGGS expression plasmid. To verify that the pUC/PGIS construct encoded a biologically active PGIS protein, pUC/PGIS was transfected into 293 cells, and the PGIS activity of the PGIS protein was assayed in the transfected cells. The pUC-CAGGS vector lacking the PGIS insert served as the control vector.

Preparation of HVJ Liposomes

The preparation of HVJ liposomes has been described elsewhere. 23–27, 29–31 Briefly, pUC/PGIS or pUC-CAGGS vector was transfected into 293 cells, and the PGIS activity of the PGIS protein was assayed in the transfected cells. The pUC-CAGGS vector lacking the PGIS insert served as the control vector.

Carotid Artery Balloon Injury and In Vivo Gene Transfer Technique

Sixty-seven male Sprague-Dawley rats, weighing 350 to 400 g (SLC, Kyoto, Japan), were used. All rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg before surgery or 100 mg/kg before sacrifice). A balloon injury to the carotid artery was induced based on the experimental model described by Morishita et al. 23–27, 29–31 which is originated from the model described by Clowes et al. 24, 30 Under a surgical microscope, the left common, internal, and external carotid arteries (CCA, ICA, and ECA) were exposed with a midline linear skin incision in the neck. The left CCA 15 mm proximal to the carotid bifurcation and the left ICA at the orifice were temporary occluded by aneurysmal straight clips. The left ECA was ligated at the exposed distal end. A 2F balloon catheter (Fogarty, E-060-2F, Baxter) was used to induce the denudation and mechanical stretching injury of the left CCA. The catheter was introduced into the CCA through a small window opened in the ECA, which is proximal to the ligation site. After the clip of the CCA was removed, the deflated catheter was passed through the CCA into the aortic arch. An inflated balloon with 0.03 ml air in the aortic arch was slowly pulled back to the ECA to mechanically expand the left CCA. After 3 repetitions of the inflation-pull-deflation procedure, the catheter was removed. Soon after the removal of the catheter, the CCA was clipped at the location 15 mm proximal to the carotid bifurcation and prepared for the subsequent gene transfer procedure.

To achieve the in vivo gene transfer, the balloon-injured CCA segment was transiently isolated by temporary clips as shown in Figure 1. An infusion cannula was introduced into the segment of the CCA through the arteriotomy in the ECA. The HVJ liposome complex with PGIS cDNA and the infusion cannula were removed, and the CCA was ligated at the orifice. After the ligation of the ECA, the blood flow to the common and internal carotid arteries was restored by releasing the clips, and the wound was closed. No adverse neurological or vascular effects were observed in any animal undergoing this procedure.

The experimental protocols were approved by the animal research committee at the National Cardiovascular Center Research Institute. All efforts were made to minimize suffering and to minimize the number of animals used.

Analyses of 6-Keto PGF₁α Contents in the Common Carotid Arteries

The level of the stable hydrolysis metabolite of PGI₂, 6-keto PGF₁α, was analyzed in the carotid wall after the balloon injury with a separate set of 25 rats to confirm the increased production of PGI₂ after PGIS expression-vector transfection. Three days after the rat carotid balloon injury treated with HVJ liposomes with PGIS expression vector (n=10) or control vector (vehicle control, n=7), all rats were killed and the left CCAs distal from the proximal ligation clip were harvested. For the normal control, 8 noninjured rats (without carotid injury or treatment with HVJ liposome complex) were sacrificed to analyze the baseline contents of 6-keto PGF₁α in the carotid arteries. The carotid segments were frozen, powdered in liquid nitrogen, and stored at −80°C until measurement. [1H]-6-keto PGF₁α (10 000 dpm, 6.55 TBq/mmol, Amersham) was added as a tracer for calculation of the recovery factor. 6-Keto PGF₁α was extracted in ice-cold ethanol, purified with a C-18 reverse phase cartridge (Sep-Pak Plus, Waters) and quantified by use of a 6-keto PGF₁α enzyme immunoassay kit (Cayman Chemical Co). 32 The protein content of the precipitate after ethanol extraction of each carotid artery was determined by the Lowry method. 33 The results are expressed as picograms of 6-keto PGF₁α per milligram of protein.

Preparation and Characterization of an Anti-Human PGIS Antibody

A synthetic peptide (PGEPPDLGSGPFLGYSALDC) containing a sequence from the human prostacyclin synthase (amino acids 27–45) 29 coupled with keyhole limpet hemocyanin, was prepared by Peptide Institute Inc. Approximately 1 milligram of the conjugated peptide mixed with complete Freund’s adjuvant was injected intradermally into Japanese White rabbits. These animals were subsequently boosted 3 times every other week with 1 mg of the conjugated peptide in incomplete Freund’s adjuvant. The serum was used as a human PGIS antibody; it cross-reacted specifically with human PGIS visualized as a single band and reacted weakly to rat and mouse enzymes in an immunoblot analysis (data not shown).
Histological and Morphometric Analyses

To examine the elevated expression of PGIS after cDNA or vehicle transfection, a separate group of rats was killed 7 (n=8) or 14 days (n=8) after the balloon injury for a determination of the intensity and regional and cellular distributions of PGIS. Cross sections of left carotid arteries transfected with HVJ liposomes harboring PGIS expression vector or with the control vector (vehicle control) was infused into the closed segment and incubated for 10 minutes at body temperature. The segment (indicated by asterisk) 5 mm distal to the occluded site in the CCA was used to measure the area and thickness of the intimal or medial layers in each rat. SCA indicates subclavian artery; CCA, common carotid artery; ICA, internal carotid artery; ECA, external carotid artery.

Results

In the analyses of hydrolyzed metabolite of unstable PGI₂, the level of 6-keto PGF₁α in the carotid arteries following PGIS expression-vector transfection, 2030±800 pg/mg protein, was significantly elevated compared with that in the arteries after PGIS vehicle transfection with the treatment of HVJ liposome complex (P<0.05; Figure 2). The baseline level of 6-keto PGF₁α contents in the normal arteries was calculated as 1230±280 pg/mg protein, the same level as that after PGIS vehicle transfection, 1240±510 pg/mg protein (Figure 2).

In the histological study, the neointimal cells were strongly positive for PGIS immunoreactivity in 30 of 37 sections (81%) examined 7 days after balloon carotid injury with PGIS cDNA transfection (Figure 3A). In contrast, no or only faint immunoreactivity was observed in the medial smooth muscle cell layer in both the PGIS cDNA-transfected and vehicle-transfected groups. Positive immunoreactivity for PGIS was also observed in the endothelial cells and adventitial cell layer, including fibroblasts in both groups (Figure 3A). The positive immunoreactivity for human PGIS in both groups is considered a cross-reaction with rat PGIS, because the synthetic peptide containing a sequence from the human PGIS (described in “Materials and Methods”) showed a 90% identity with that of rat PGIS.20,21 In a higher-magnification view in the PGIS transfected group (Figure 3B), the immunoreactivity for PGIS was seen primarily in the cytoplasm in the neointimal cells, which was in accord with the observation that PGIS could be detected in the microsomal fraction in endothelial cells.19 In contrast, 2 weeks after the balloon injury (with or without PGIS gene transfection), the rats were killed and the left carotid arteries were perfusion fixed with 10% (wt/vol) formaldehyde. Cross-sections at the middle segment of the left CCAs 5 mm distal from the proximal ligature clip (indicated in Figure 1) were stained with hematoxylin and cosin and Masson trichrome stain (smooth-muscle cells, red; collagen fibers, blue; neo-intimal layer, red and blue) analyzed by means of a computerized analysis system (SD-510C, WACOM) in a blinded manner by the analyzer. Cross-sectional areas of the medial smooth-muscle-cell layer and neointimal layer were calculated by tracing the exact border of each area under constant magnification with the use of a microscope and the computerized system. To calculate the average thickness of each layer, the analyzed area was divided by the mean of the outer and inner circumferences of the intimal layer.

Statistical Analysis

All values are expressed as mean±SD. ANOVA with subsequent Bonferroni test was used to determine significant differences in multiple comparisons. P<0.05 was considered significant.
Discussion

The frequency of restenosis after successful coronary angioplasty ranges from 30% to 50% of patients within 3 to 6 months.\textsuperscript{34-36} The main cause of restenosis is neointimal formation, which has been observed in 60% of necropsy cases after successful coronary angioplasty.\textsuperscript{37}

A derangement in vascular eicosanoid metabolism has been implicated in the development of thrombosis and the atherogenic process.\textsuperscript{17} The balance between the productions of TXA\textsubscript{2} from platelets and PGI\textsubscript{2} from vessel walls (particularly by endothelium and, to a lesser extent, by medial smooth-muscle cells) is an important maintenance factor of vascular integrity.\textsuperscript{14,17} The regulation of PGI\textsubscript{2} has been a target of research regarding the prevention and management of diseases such as cerebral and myocardial infarction/ischemia. In 1987, it was reported\textsuperscript{46} that the intravenous administration of PGI\textsubscript{2} for 5 days reduced the mitotic activity of smooth muscle cells studied using specimens from human femoral or popliteal arteries. It was later demonstrated that the administration of a stable analogue of PGI\textsubscript{2}, beraprost sodium, inhibited the insulin-stimulated and PDGF-stimulated proliferation of cultured smooth-muscle cells from rat aorta.\textsuperscript{38} These studies directly showed the growth inhibitory action of exogenous PGI\textsubscript{2} in vitro. Despite this beneficial growth inhibitory action, several studies have demonstrated that PGI\textsubscript{2} synthesis is decreased during the process of atherosclerosis.\textsuperscript{39-43} Moreover, in the chronic and systemic intra-vascular administration of PGI\textsubscript{2}, platelet desensitization and a "rebound phenomenon" have been reported.\textsuperscript{44,45}

The cDNA for bovine PGI\textsubscript{2} has been cloned from aorta endothelial cells and contains a 1500-bp open reading frame coding for a 500-amino acid polypeptide with an Mr of 56,628.\textsuperscript{19} Miyata et al\textsuperscript{20} accomplished the cDNA cloning of human PGI\textsubscript{2} from aortic endothelial cells, which encoded a 500-amino acid polypeptide. Successively, cDNA for rat and mouse PGIS was cloned sharing 84% and 78% identity with that of human PGIS, respectively.\textsuperscript{21,46} Several cytokines (IL-1\textalpha, IL-1\beta, IL-6, and TNF-\alpha) were shown to induce PGIS gene expression, and TNF-\alpha was the most potent.\textsuperscript{19} A study of the cellular localization of rat PGIS mRNA revealed the existence of significant signals in smooth-muscle cells and fibroblasts.\textsuperscript{21} With use of an adenovirus-mediated cyclooxygenase-1 (COX-1) gene transfer method in balloon-injured porcine carotid artery, a 4-fold increase in PGI\textsubscript{2} synthesis was demonstrated, and subsequent intraarterial thrombosis was significantly inhibited. There was a tendency of reduced intimal hyperplasia in the increased PGI\textsubscript{2} level; however, neointimal formation was not analyzed quantitatively.\textsuperscript{47} Although there is no evidence whether the substrate of PGIS, PGH\textsubscript{2}, via COX-1 or -2, was available to the overexpressed PGIS in the present study, we had demonstrated that the elevated expression of PGIS in cultured VSMC resulted in increased PGI\textsubscript{2} synthesis with a concomitant suppression of the growth of VSMC.\textsuperscript{22} These lines of evidence indicated that a sustained production of a high level of PGIS suppresses VSMC proliferation via PGI\textsubscript{2} production in vivo.

In addition to PGI\textsubscript{2}, the direct smooth muscle relaxants nitric oxide (NO) and atrial natriuretic peptides (ANP) have also been implicated to play a role in inhibiting the migration and proliferation of VSMC.\textsuperscript{26,27,48} The administration of ANP (10\textsuperscript{-7} M) to serum-stimulated cultured rat aortic smooth muscle cells significantly suppressed this proliferation.\textsuperscript{48} Morishita et al transfected an ANP expression vector into cultured endothelial cells and observed high levels of ANP secretion from the transfected endothelial cells. It was demonstrated that these transfected cells showed significantly lower rates of DNA synthesis under bFGF-stimulated conditions.\textsuperscript{26} On the other hand, Leyen et al\textsuperscript{27} transferred cDNA
encoding endothelial cell NO synthase (ec-NOS), which inhibited neointima formation by 70% at 14 days after carotid balloon injury in rats. These results demonstrated that the muscle relaxants ANP and NO are also important endogenous inhibitory factors of injury-induced proliferative vascular lesion formation. Furthermore, exogenously administered C-type natriuretic peptide (CNP) inhibited the proliferation of VSMC in vivo via a stimulation of cGMP production.49

Newby et al reviewed the inhibitory effects of endothelium-dependent vasodilators on VSMC proliferation, and suggested that a cAMP-elevating agent such as PGI2 is more potent for the inhibition of VSMC proliferation than cGMP-elevating agents such as NO.18 However, it is likely that the endothelium-denuded arteries after balloon injury or progressing atherosclerotic lesions are lacking or accumulating lesser amounts of these constitutively produced endothelium-derived growth inhibitory factors that could be expected to act as suppressors of pathological neointimal formation. Furthermore, it was demonstrated that PGI2 production in the carotid wall was not increased 3 days after balloon injury as seen in the vehicle transfected group.

Recent developments in gene transfer techniques have emerged as therapeutic options in treating vascular diseases, especially restenosis after balloon angioplasty. In this study, we used the Sendai virus (HVJ) liposome method for the transfection of cDNA encoding PGIS into injured rat carotid arteries. In this method, the virus is incapable of replication and does not integrate into the genome.50,51 The reliability and high efficiency of this method used with arterial walls after balloon injury have been well established.23–26,29,30,48,52 This HVJ liposome method is nontoxic and produces a 10-fold higher efficiency of gene transfection compared with lipofection or passive uptake methods.30,50 In addition, this method, using a short incubation period, produced effectively high levels of particular endogenous and functional enzymes, antisense, or decoy in injured rat carotid arteries.23,27,29,30,50 To induce a sustained and site-specific overproduction of a short-lived agent such as PGI2 on the proliferating site in the arterial wall, the gene transfer method using the HVJ liposome technique (gene delivery system) is considered an adequate treatment for restenosis rather than that using an intravascular systemic administration of stable analogues,16,44,45,53

In the present study, it was demonstrated that the gene transfer of human PGIS cDNA into injured rat carotid arteries by the HVJ liposome gene delivery system resulted in the expression of human PGIS in the neointima. The elevated PGI2 synthesis in the carotid arterial wall was confirmed, and the neointimal formation was effectively inhibited. In a clinical study, it was reported that most of the atheromatous plaque excised by atherectomy consisted of dense connective tissue with abundant amounts of elastic fibers and lamellae.
This meshwork contained numerous cells, which were primarily proliferating smooth-muscle cells. Our present data demonstrated that a site-specific PGIS gene transfer was achieved, especially in the neointimal layer rather than in the normal medial smooth-muscle cell layer. The apparent failure of the HVJ liposome system to transfect the nonproliferating cells (medial smooth-muscle cells) may be expressing the suitability of this technique as an eventual gene therapy targeting a suppression of cell proliferation.

We conclude that the PGIS gene transfer method using the HVJ liposome gene delivery system may be useful for targeting the prevention of a disordered proliferation of smooth muscle cells, such as restenosis or lesions of developing atherosclerosis. However, this model is known to have limitations regarding the evaluation of the pathogenesis of human arterial diseases. The development of thickening of

Figure 4. Top, Cross section of the left CCA of a control rat at the site indicated in Figure 1 (hematoxylin and eosin, original magnification ×40). The intimal layer consists of a monolayer of endothelial cells that is hardly seen at this magnification. Middle, Cross section of the left CCA 14 days after the transfection of HVJ liposomes with control vector (without PGIS cDNA) after balloon injury. Neointimal hyperplasia consisting of migrated smooth-muscle cells is seen inside the normal medial smooth-muscle cell layer (Masson’s trichrome stain, original magnification ×40). Bottom, Cross section of the left CCA 14 days after the transfection of HVJ liposomes with PGIS expression vector after balloon injury. The growth of the neointimal formation is suppressed (Masson’s trichrome stain, original magnification ×40).

Figure 5. A, Average cross-sectional area of the neointimal layer of the left CCA in groups of rats transfected with HVJ liposomes harboring PGIS expression vector (PGIS, n=14) or control vector (Vehicle-Control, n=12). There was a significant difference between the groups (P<0.05). B, Average ratio of intimal area/medial area of the left CCA in groups transfected with HVJ-liposome harboring PGIS expression vector (PGIS) or control vector (Vehicle-Control). There was a significant difference between the groups (P<0.01).

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the human arterial wall takes a longer time than that seen in the present rat model, and the thickening develops on pathological atherosclerotic arteries, not on injured normal vessels.\textsuperscript{1,8,34} The adenosine-mediated arterial gene transfer method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall method, reportedly having been effective for endothelium-denuded arteries, was less effective in transfecting to the wall.

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Editorial Comment

Local gene transfer offers a potentially beneficial strategy to treat vascular disease at the molecular level. Already, a diverse group of genes have been studied by use of gene transfer technology in acute models of vascular growth—restenosis following vascular injury. For example, endogenous nitric oxide is known to be an important regulator of vascular growth, and gene transfer of endothelial nitric oxide synthase or soluble guanylate cyclase (a key molecular target for nitric oxide) reduces intimal growth after vascular injury.

The preceding study examined effects of viral-mediated gene transfer of another endothelial cell gene product, prostacyclin synthase, on vascular growth after acute injury of the carotid artery. Overexpression of human prostacyclin synthase increased local levels of prostacyclin and inhibited growth after balloon injury of the carotid artery. Although there are clearly differences between the acute balloon injury and carotid artery disease, which normally takes many years to develop, the findings nonetheless support the concept that genetic alterations of the vessel wall is an attractive approach to alter vascular function as well as vascular growth. An important unanswered question is whether chronic overexpression of prostacyclin synthase would reduce development or progression of atherosclerosis.

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