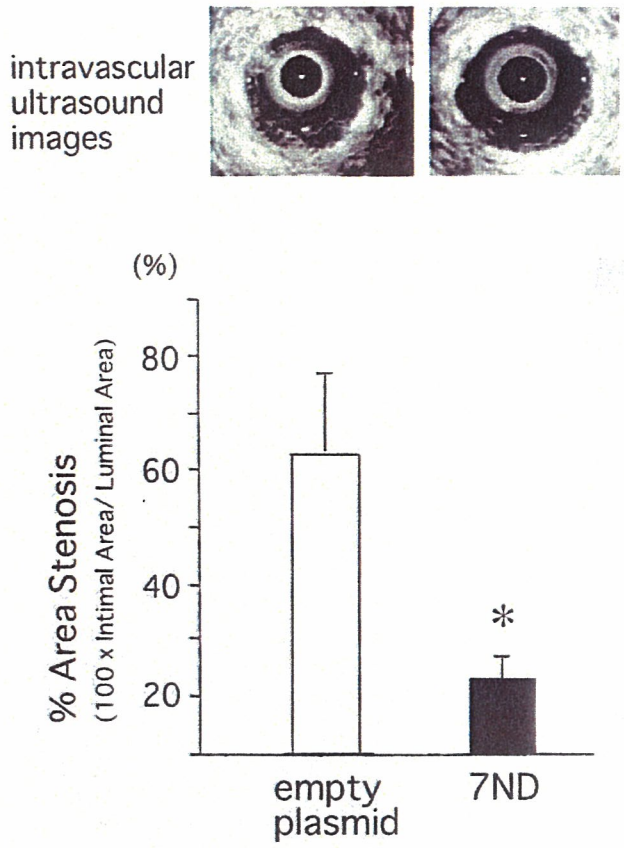

Figure 6. Effects of 7ND gene transfer on in-stent neointimal hyperplasia. Top, An intravascular ultrasound cross-section image in empty plasmid-transfected animal with large neointimal hyperplasia (left) and an intravascular ultrasound cross-sectional image in 7ND-transfected animal with small neointimal hyperplasia (right). Quantitative comparison of mean percent area stenosis within stent in the empty plasmid-transfected and 7ND-transfected rabbits as assessed by intravascular ultrasound. *$P<0.01$ vs empty plasmid.

progression of established preexisting atheroma but also limits transformation from destabilized plaques to stable plaques, suggesting that blockade of the MCP-1/CCR2 pathway might lead to reductions in atherosclerotic complications.

Effects of 7ND Gene Transfer on Experimental Restenosis

The benefits of percutaneous coronary interventions are hampered by triggering local arterial renarrowing (restenosis). As mentioned above, vascular injury owing to balloon dilatation or stent implantation induces inflammatory responses that accelerate the recruitment and activation of monocytes. Anatomically, in-stent restenosis results exclusively from neointimal hyperplasia, whereas restenosis after balloon angioplasty results from neointimal hyperplasia and negative remodeling of the arterial wall.[47] We hypothesized that MCP-1–mediated inflammation is essential in the development of restenotic changes after balloon injury or stent implantation in rats, rabbits, and monkeys. We demonstrated that blockade of MCP-1 by 7ND gene transfer suppressed monocyte infiltration/activation at the injured site and markedly inhibited restenotic changes (neointimal hyperplasia) after balloon injury of the carotid artery in rats and monkeys (Figure 5A and 5B).[48] This strategy also suppressed the local production of MCP-1 and inflammatory cytokines. In hypercholesterolemic rabbits in which neointimal formation and negative remodeling developed after balloon injury, 7ND gene transfer attenuated such changes (Figure 5C).[49] In hypercholesterolemic rabbits and monkeys, 7ND gene transfer inhibited monocyte infiltration/activation in the stented arterial wall and thus reduced the development of in-stent restenosis (Figure 6, K. Egashira, unpublished data, 2002).

Our data, therefore, indicate that locally produced MCP-1 not only induces the recruitment of monocytes but also activates lesional monocytes and vascular smooth muscle cells to produce the inflammatory cytokines, which might then cause experimental restenosis. Thus, MCP-1–mediated inflammation in the arterial wall is likely to create a positive-feedback mechanism to enhance inflammation and proliferation of the injured arterial wall (Figure 4). It is also possible that MCP-1 activated adventitial myofibroblasts, which may in turn contributed to the development of restenosis after injury. Our finding in nonhuman primates is meaningful because many therapeutic strategies that have proven effective in reducing restenosis in nonprimate animal models have failed to demonstrate substantial effect on human restenosis. Therefore, monocyte infiltration and activation mediated by MCP-1 are essential in the development of experimental restenosis.

Conclusion

In conclusion, the inflammatory changes mediated by MCP-1 are essential and important in mediating chronic inflammation in cardiovascular disease, especially in experimental restenosis as well as atherosclerosis and plaque destabilization. Future studies are needed to address the role of hematopoietic stem cells in the effect seen on atherosclerosis and restenosis. Our findings support the hypothesis that (1) MCP-1 is a novel therapeutic target against cardiovascular inflammation and related diseases, and (2) anti–MCP-1 gene therapy with mutant MCP-1 transfection might be a useful and practical form of therapy against human restenosis after coronary intervention. Because of the potential pathogenetic role of MCP-1 in other treatment-intractable inflammatory disorders, our strategy might have broader clinical applications.

Perspectives

From a clinical point of view, the potential side effects of anti-MCP-1 gene therapy merit mentioning. We assume that blockade of MCP-1 with our strategy does not cause serious local or systemic side effects, because (1) mice lacking MCP-1 or CCR2 display no serious health problems, (2) the delivery of plasmid DNA by intramuscular injection is now in clinical stages and is proven to be safe, and (3) intramuscular transfer of 7ND gene is nontoxic and safe in nonhuman primates. We have not yet investigated whether long-term inhibition of MCP-1 function affects the systemic immunoprotective ability in humans. Future studies will require careful observation over a long period of time to establish the

true risk/benefit ratio. We are planning to apply this strategy to clinical restenosis after percutaneous coronary intervention, and this clinical protocol is now under deliberation by the Gene Therapy Committee of Ministry of Health, Labor and Welfare of the Japanese government. Future clinical study would open a new therapeutic window for antirestenosis and antiatherosclerosis paradigms.

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Monocyte Chemoattractant Protein-1 Is an Essential Inflammatory Mediator in Angiotensin II-Induced Progression of Established Atherosclerosis in Hypercholesterolemic Mice

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Objective—Chronic inflammatory processes might be involved in the progression and destabilization of atherosclerotic plaques. Therefore, identification of the mechanism underlying arterial inflammatory function might lead to the development of novel therapeutic strategies. Angiotensin II (AngII) is implicated in atherogenesis by activating the vascular inflammation system, mainly through monocyte chemotaxis. Therefore, we hypothesized that AngII increases plaque size and promotes destabilization of established atheromas by activating the monocyte chemoattractant protein-1 (MCP-1) pathway.

Methods and Results—We report here that 4-week infusion of AngII not only increased plaque size but also induced a destabilization phenotype (ie, increased macrophages and lipids and decreased collagen and smooth muscle cells) of pre-existing atherosclerotic lesions of hypercholesterolemic mice. AngII also enhanced the gene expression of inflammatory cytokines (TNFα, IL-6, etc.) and chemokines (MCP-1, CCR2, etc). Blockade of MCP-1, by transfecting the deletion mutant of the human MCP-1 gene into the skeletal muscles, limited AngII-induced progression and destabilization of established atherosclerotic lesions and suppressed the induction of proinflammatory genes.

Conclusions—These data suggest that MCP-1 functions as a central inflammatory mediator in the AngII-induced progression and changes in plaque composition of established atheroma. (***Arterioscler Thromb Vasc Biol.* 2004; 24:534-539.**)

Key Words: atherosclerosis ■ hypercholesterolemia ■ cell adhesion molecules ■ inflammation ■ gene therapy

Atherosclerosis and its complications are the major cause of death in Western countries. Recent evidence suggests that chronic inflammatory processes have an important role in atherosclerotic plaque progression, destabilization, and subsequent rupture/thrombosis, resulting in acute coronary syndrome and stroke.[1] Therefore, identification of the critical inflammatory pathway involved in plaque progression and destabilization of pre-existing established atheromas might aid in the development of novel therapeutic strategies to reduce atherothrombotic complications. Angiotensin II (AngII) is implicated in atherogenesis beyond its hemodynamic effects.[2] Infusion of AngII into hypercholesterolemic mice dramatically accelerates the development and/or progression of atherosclerotic lesions and the effects of AngII occurred independent of changes in arterial pressure or plasma lipid concentration.[3,4] The mechanism of AngII-induced enhancement of atherogenesis is probably multifactorial, and includes hemodynamic effects, endothelial dysfunction and activation,[5] oxidative stress,[6] and inflammation.[7,8] AngII increases monocyte chemotaxis, activates nuclear factor-κB, and augments production of inflammatory cytokines and chemokines by arterial wall cells and monocytes.[9–12] AngII is very important in the pathogenesis of atherothrombotic complications, as evidenced by clinical benefits of angiotensin-converting enzyme inhibition[13] and AngII receptor blockers.[14] There are no reports, however, that address the mechanism of AngII-induced enhancement of atherogenesis and plaque destabilization under in vivo conditions.

Emerging evidence suggests that AngII activates cell inflammatory systems in arterial lesion.[1,2] Inflammatory changes in arterial lesions are characterized by the recruitment and activation of monocytes/macrophages, which are regulated by monocyte chemoattractant protein-1 (MCP-1).[15,16] Matrix metalloproteinases (MMP) and tissue factor,

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which are produced mainly from lesional macrophages, are believed to greatly contribute to destabilization of human atherosclerotic lesions.[1] Recently, our group and others have also reported that MCP-1 has a vital role in the initiation and progression of atherosclerotic or arteriosclerotic lesions in experimental animals.[17–24] Essential roles of MCP-1 and its interactions with its receptor (CCR2) in AngII-induced arteriosclerosis are shown in CCR2-deficient mice.[25] Therefore, we hypothesized that AngII increases plaque size and promotes destabilization of established atherosclerotic lesions by activating MCP-1. To block the MCP-1/CCR2 signal pathway, an N-terminal deletion mutant of the MCP-1 gene (7ND), which lacks the N-terminal amino acids 2 to 8, was transfected into the skeletal muscle.[17,18] This mutant MCP-1 binds to its receptor CCR2 and blocks MCP-1–mediated monocyte chemotaxis. In previous studies, we demonstrated that 7ND protein was secreted from the transfected skeletal muscle cells into the circulating blood and subsequently blocked MCP-1–induced chemotaxis in remote organs. Here, we report that AngII infusion into hypercholesterolemic apolipoprotein-E knockout (apoE-KO) mice not only increased plaque size but also promoted atherosclerotic plaque transformation to a more destabilized phenotype, which had more lipid and macrophages and less collagen and fewer smooth muscle cells.[1] In addition to the morphological changes, AngII markedly induced gene expression of several important cytokines and chemokines. Blockade of the MCP-1 pathway limited AngII-induced lesion progression and destabilization and suppressed gene expression of proinflammatory factors.

Methods

Experimental Animals

C57BL/6J apoE-KO and wild-type mice, purchased from Jackson Laboratory (Bar Harbor, Me), were bred and maintained in the Laboratory of Animal Experiments at Kyushu University. The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments at Kyushu University Graduate School of Medical Sciences. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core at Kyushu University School of Medical Sciences.

Expression Vector

7ND was constructed by recombinant polymerase chain reaction using a wild-type human MCP-1 cDNA as the template and cloned into *Bam*HI (5′) and *Not*I (3′) sites of the pcDNA3 expression vector (Invitrogen).[18]

Treatment

Male apoE-KO mice were fed a normal chow diet (Oriental Yeast) during the experiment. At age 30 weeks, mice were randomly assigned to 1 of 5 groups. The first group (sham group, n=10) received physiological saline via a subcutaneously implanted osmotic minipump (model 2004; Alzet). The second group (AngII+plasmid group, n=10) received AngII dissolved in saline through the minipump for 4 weeks and intramuscular injections of pcDNA3 plasmid DNA at biweekly intervals. The third group (AngII+7ND group, n=10) received AngII dissolved in saline through the minipump for 4 weeks and intramuscular injections of pcDNA3–7ND plasmid DNA (100 μg) into the femoral muscle at biweekly intervals. The fourth group (ARB group, n=10) received AngII dissolved in saline through the minipump for 4 weeks and AngII receptor blocker (ARB) (olmesartan 7.5 μg/g) in chow. This dose of olmesartan has no effect on AngII-induced increase in arterial pressure.[26] The fifth group (sham+7ND group, n=10) received physiological saline and intramuscular injections of pcDNA3–7ND plasmid DNA (100 μg).

In the sham+7ND, AngII+plasmid, and AngII+7ND groups, transgene expression was enhanced by local intramuscular electroporation at the injection site immediately after the injection. Six 100-V, 50-ms electronic pulses were applied to each injection site using an Electroporator CUY21 (BTX).[27] The dose of AngII (0.75 $mg \cdot kg^{-1} \cdot d^{-1}$) provides a serum level of AngII similar to that reported in patients with renovascular hypertension.[28] After 4 weeks of saline or AngII infusion, mice were euthanized after collection of blood from the vena cava.

Tissue Preparation

Tissue preparation was performed essentially as previously described.[18] Briefly, after the mice were euthanized, the heart and total aortic segments were rapidly removed after perfusion with phosphate-buffered saline. The total aortic segments (ascending thoracic aorta, arch, descending thoracic aorta, and abdominal aorta) were rapidly frozen in liquid nitrogen for later extraction of RNA. The heart, including the aortic root, was snap-frozen in OCT compound (Tissue-Tek) for histology and immunohistochemistry.

Histology and Immunohistochemistry

Serial cryostat sections (6 μm) of the aortic root were prepared as described.[18] In brief, atherosclerotic lesions in the aortic root were examined at 5 locations, each separated by 120 μm, and 5 to 6 serial sections were prepared from each location. Some of these sections were conventionally stained with orcein (for elastic fiber staining) and oil red O (for lipid staining). Interstitial collagens were stained by Picrosirius red (Sigma Chemical, St. Louis, Mo) and photographed under polarization microscopy as described previously.[29] The remaining sections were used for immunohistochemical analysis. Air-dried cryostat sections were fixed in acetone and stained with the respective antibody: anti-mouse monocyte/macrophage monoclonal antibodies (MOMA-2; Serotec), anti-alpha-SM actin monoclonal antibodies (alkaline phosphatase conjugated; Sigma Chemical), anti-human MCP-1, CCR2, MMP-13 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse MMP-9 polyclonal antibodies (Santa Cruz Biotechnology), and anti-rabbit tissue factor monoclonal antibody (American Diagnostica), as described previously.[18] Respective nonimmune IgGs (Dako) were used as negative controls. After incubation with the appropriate biotinylated affinity-purified secondary antibodies (Nichirei, Tokyo), the sections were incubated with alkaline phosphatase-labeled streptavidin solution (Nichirei) and visualized using a fast red substrate kit (Nichirei). The sections were then counterstained with Mayer hematoxylin.

A single observer blinded to the experiment protocol performed quantitative analysis of atherosclerotic lesions. All images were captured with a Nikon microscope equipped with a video camera and analyzed using Adobe Photoshop 6.0 and National Institute of Health Image Software. Orcein staining was used to delineate the internal elastic lamina for determination of the intimal area. The lipid composition of the lesion was evaluated by calculating the percent of the oil red O positive area versus the total cross-sectional vessel wall area. Similarly, the percent area of macrophage accumulation (MOMA-2–positive area), alpha-SM actin–positive area, and collagen deposition were estimated. In each case, the average value for 4 or 5 locations for each animal was used for analysis.

RNA Extraction and RNAse Protection Assay

Total RNA was extracted from the aorta using the acid guanidinium thiocyanate-phenol-chloroform method (Isogen, Nippon Gene). RNAse protection assays were performed with 20 μg of total RNA using a RiboQuant kit with a custom template set according to the manufacturer's protocol (PharMingen; San Diego, Calif). After RNase digestion, protected probes were resolved on denaturing polyacrylamide gels and quantified using a BASS-3000 system (Fuji

Film, Tokyo). The value of each hybridized probe was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) included in each template set as an internal control.

Serum Analysis and Systolic Blood Pressure Determination

Serum total and high-density lipoprotein cholesterol and triacylglycerol concentrations were determined using commercially available kits (Wako). Systolic blood pressure was measured weekly using a tail-cuff system with the mice in a conscious state.

Commercially available ELISA kits (Biosource) were used to measure human MCP-1 and mouse MCP-1 according to manufacturer's instructions. Plasma 7ND concentrations were measured by the use of this human ELISA kit.

Statistical Analysis

Data were expressed as mean±SEM. Differences between groups were determined using 2-way analysis of variance and a multiple comparison test; $P<0.05$ was considered to be statistically significant.

Results

7ND Gene Transfer Limits AngII-Induced Progression and Destabilization of Atherosclerotic Lesions

After a 4-week treatment, cross-sections of the aortic sinus region were examined. The sham group had established lesions characteristic of early fibrous plaques containing necrotic cores and a few foam cells covered by a well-formed fibrous cap (Figure 1A), as previously reported.[17,30,31] There was a marked increase in the intimal area in the AngII+plasmid group, compared with that observed in the sham and sham+7ND group (Figures 1A and 2). 7ND gene transfer prevented the AngII-induced increase in the intimal area. Treatment with ARB also prevented the AngII-induced enhancement of the intimal area.

We next examined the effects of 7ND gene transfer on AngII-induced changes in plaque composition using immunohistochemical analysis. In particular, lipid deposition, macrophages, smooth muscle cells, and interstitial collagen content were evaluated (Figures 1A and 2). AngII infusion enhanced lipid staining and macrophage staining and attenuated α-SM–positive and collagen–positive areas (Figures 1A and 2). 7ND gene transfer reduced the AngII-induced increases in lipid deposition and macrophage infiltration, and AngII-induced decrease in α-SM actin and collagen (Figures 1A and 2). Treatment with ARB also prevented the AngII-induced changes in lesion composition.

7ND Gene Transfer Attenuates AngII-Induced Upregulation of Cytokine and Chemokine Expression

To explore the mechanisms by which 7ND gene transfer limited progression and destabilization of pre-existing atheromas, we examined gene expression of a number of chemokines, chemokine receptors, and cytokines (Figure 3). RNase protection assay revealed AngII-induced increases in proinflammatory genes. 7ND gene transfer significantly attenuated the increased gene expression of inflammatory cytokines and chemokines (Figure 3).

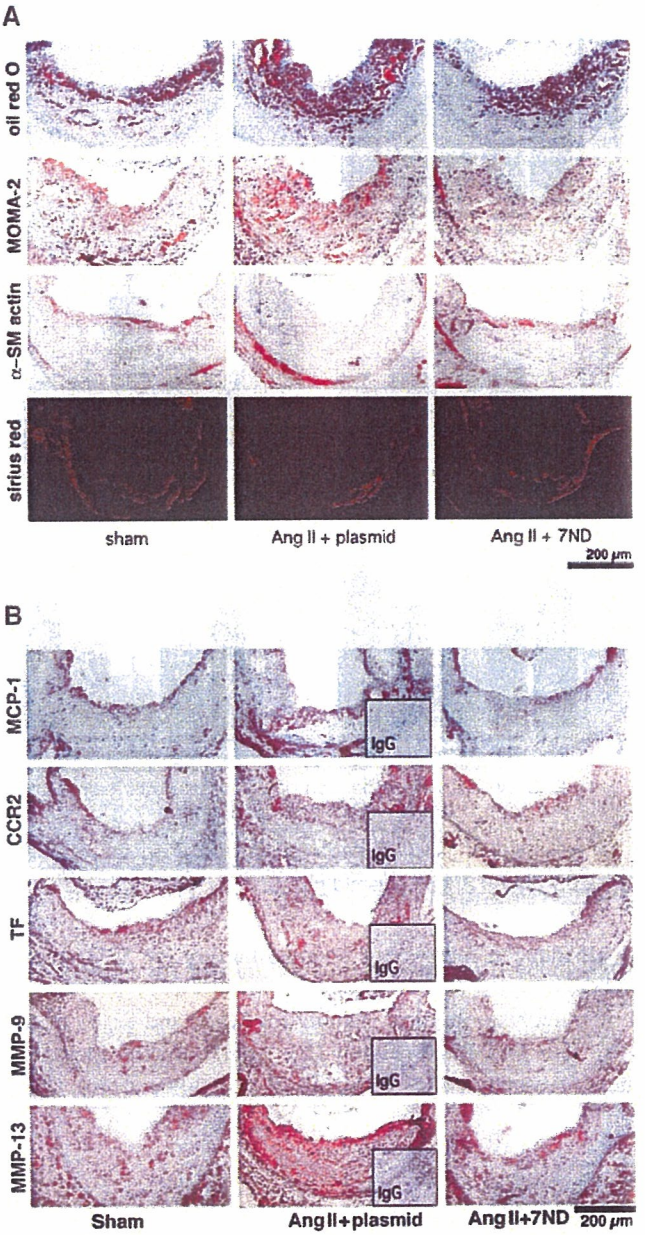

Figure 1. Histopathologic and immunohistochemical pictures of atherosclerotic lesions in the aortic root. A, 7ND gene transfer suppressed AngII-induced destabilization of the atheroma. From the top to the bottom panel, photomicrographs of atherosclerotic lesions stained with oil red O, immunostained with anti-murine macrophage antibody (MOMA-2) or anti-human α-SM actin antibody. Interstitial collagen was visualized using polarization microscopy after staining with Picrosirius red. Bar=200 μm. B, 7ND gene transfer suppressed the AngII-induced increase in immunoreactive MCP-1, CCR2, MMP-9, MMP-13, and tissue factor. Bar=200 μm.

Because 7ND gene transfer reduced MCP-1 gene expression, immunohistochemical staining for MCP-1 and its receptor (CCR2) was performed (Figure 1B). As expected, AngII infusion increased immunoreactive MCP-1 and CCR2 in the intimal lesions. Incomplete colocalization of MCP-1 and CCR2 suggests that CCR2 expression might increase not only in lesional monocytes but also in activated cells such as vascular smooth muscle cells. 7ND gene transfer reduced the

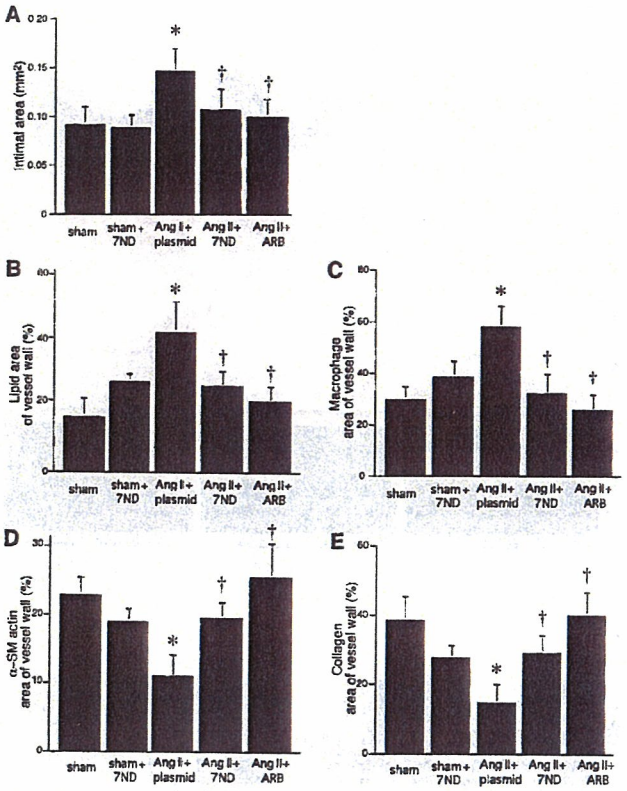

Figure 2. Quantitative comparison of atherosclerotic lesion size and lesion composition in sham-operation, sham+7ND, AngII+plasmid, AngII+7ND, and ARB groups. Data are reported as mean±SEM, n=8 to 10. *$P<0.05$ versus the sham group. †$P<0.05$ versus AngII+plasmid group.

AngII-induced increase in immunostaining for MCP-1 and CCR2.

Immunohistochemical staining for MMP and tissue factor were also performed (Figure 1B). AngII infusion increased immunoreactive MMP-9, MMP-13, and tissue factor. 7ND gene transfer reduced the AngII-induced increase in MMP and tissue factor.

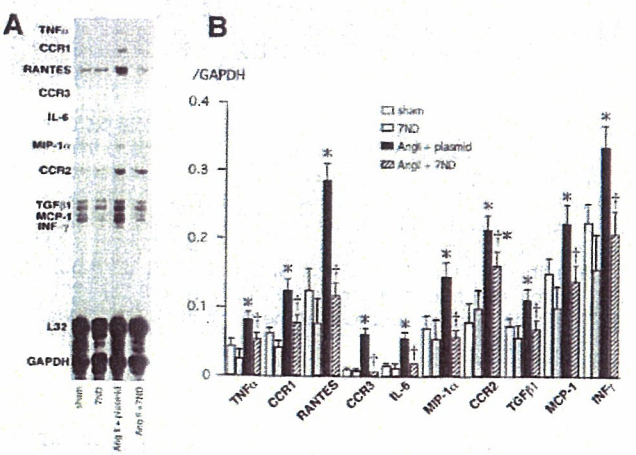

Figure 3. Effects of 7ND gene transfer on the AngII-induced gene expression of several chemokines (RANTES and MCP-1), chemokine receptors (CCR1, CCR2, and CCR3), and cytokine (TNF-α, IL-6, IL-1β, TGF-β1) in the aorta. A, Representative autoradiograph of RNase protection assay. B, Summary of densitometric analysis. Data are expressed as the ratio of each mRNA to the corresponding GAPDH mRNA. *$P<0.05$ versus the sham group. †$P<0.05$ versus AngII+plasmid group; n=6 to 7.

Beneficial Effects of 7ND Gene Transfection Had No Relation With Serum Lipid Levels or Systolic Blood Pressure

There were no statistically significant differences in serum total cholesterol levels among the groups (sham group, 480±21; AngII+plasmid group, 503±18; AngII +7ND, 488±19; ARB group, 478±22 mg/dL). 7ND gene transfer did not affect AngII-induced increases in systolic blood pressure (sham group, 102±3; AngII+plasmid group, 153±8; AngII +7ND, 157±6; ARB group, 110±8 mm Hg at 4 weeks of treatment).

7ND Gene Transfer Increases Plasma Concentrations of 7ND

Concentrations of 7ND and MCP-1 in plasma and transfected muscle were measured. In wild-type mice transfected with 7ND without AngII infusion, 7ND was detected in transfected muscle and plasma 3, 7, and 14 days after transfection (Table). Plasma MCP-1 concentrations did not change during the course of experiments.

Discussion

We demonstrated that blockade of MCP-1×7ND gene transfer limited AngII-induced progression of pre-existing advanced atherosclerotic lesions in hypercholesterolemic mice. Because 7ND gene transfer did not affect serum lipid or blood pressure levels, AngII-induced enhancement of atherogenesis must be a direct effect of AngII on cells in the atherosclerotic vascular wall. These data suggest that the essential role of MCP-1 in AngII increases plaque size of the pre-existing atheroma.

A clinically significant finding of the present study is that AngII increased markers of plaque instability. Qualitative changes in plaque are more likely than a decrease in plaque size or the degree of stenosis to contribute greatly to a reduction in cardiovascular events caused by atherothrombotic complications. Lesional macrophages might be a major source of cytokines, MMP, and tissue factor. Increased degradation of interstitial collagen by MMP and decreased cytokine synthesis have been demonstrated in human atherosclerotic lesions prone to rupture.[1] Resultant decreases in interstitial collagen might weaken the plaque's biomechanical strength, which in turn increases the likelihood of plaque rupture.[1] Tissue factor regulates plaque's thrombogenicity.[32] MCP-1 increases tissue factor expression and activity in human vascular smooth muscle cells.[33] We recently reported that 7ND gene transfer inhibited progression and destabilization of advanced atheroma by reducing inflammation in apoE-KO mice that were not received AngII.[24] We demonstrate the preservation of interstitial collagen in the atherosclerotic plaque associated with decreased expression of MMP and tissue factor by 7ND gene transfer. Therefore, MCP-1-induced inflammation appears to be an essential step not only in AngII-induced progression but also in AngII-induced changes in composition of established atheroma.

Plasma Concentrations of MCP-1 and 7ND After 7ND Transfection in Wild-Type Mice

		Days After 7ND Transfection			
	Baseline	3	7	14	28
Plasma MCP-1, pg/mL	88±5	92±6	94±6	90±6	86±5
Plasma 7ND, pg/mL	<20.0 (below detectable limit)	124±14	120±11	66±9	<20.0 (below detectable limit)
Muscular tissue 7ND, pg/mg protein	<20.0 (below detectable limit)	362±28	260±22	105±20	<20.0 (below detectable limit)

Values are mean±SE, n=6 to 8.

AngII causes endothelial dysfunction by decreasing nitric oxide synthase activity, mainly through oxidative stress, which in turn results in inflammation in the arterial wall.[1,2] We previously demonstrated an increase in tissue Ang-II activity after blockade of nitric oxide synthesis.[34] In the present study, 7ND gene transfer suppressed AngII-induced increase in MCP-1, CCR2, and other cytokine expression. Because recent data[1] suggest that lesional inflammatory cells release enzymes such as angiotensin-converting enzyme that generate AngII,[2,35,36] local AngII levels increase as macrophages becomes activated by oxidized LDL.[35] Our data strongly support the notion that an increased concentration and/or activity of AngII creates a positive-feedback mechanism for further increases in AngII generation, atherogenesis, and atherothrombotic events.

In conclusion, the present data demonstrate the essential role of MCP-1–mediated inflammation in AngII- induced progression of established atheroma in hypercholesterolemic mice. Because the activity and formation of AngII is enhanced at the inflamed human atherosclerotic sites that are prone to rupture, it is reasonable to propose that increased local action of AngII contributes greatly to the process of plaque rupture and subsequent cardiovascular ischemic complications by acting as an inflammatory mediator. Thus, AngII might augment inflammatory functions of atherosclerotic lesions in the presence of risk factors such as hypercholesterolemia. The clinical benefit of angiotensin-converting enzyme inhibitors or AngII receptor blockers might be caused by anti-inflammatory activity.

Acknowledgments

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RESEARCH ARTICLE

Inhibition of neointimal hyperplasia after balloon injury by cis-element 'decoy' of early growth response gene-1 in hypercholesterolemic rabbits

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Early growth response factor-1 (Egr-1) is a transcription factor that is rapidly activated after vascular injury and thus might contribute to vascular proliferation and inflammation. We hypothesized that Egr-1 might therefore be a therapeutic target against restenosis. Hypercholesterolemic rabbits were intraluminally administered synthetic DNA as a 'decoy' against Egr-1 immediately after carotid artery balloon injury. Efficient transfection was confirmed by the delivery of a fluorescence-labeled decoy. Gel mobility-shift assay showed increased Egr-1 activity after balloon injury and its prevention by Egr-1 decoy transfection in vivo. Egr-1 decoy transfection attenuated early inflammation and proliferation and later neointimal hyperplasia. In addition, Egr-1 decoy transfection reduced gene expression and protein production of Egr-1-dependent genes such as platelet-derived growth factor-B, transforming growth factor-β1, and monocyte chemoattractant protein-1. The Egr-1 pathway has an essential role in the pathogenesis of neointimal hyperplasia after balloon injury in hypercholesterolemic rabbits. This decoy strategy is a potential practical form of therapy for human restenosis.
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Keywords: *neointimal hyperplasia; inflammation; growth factor*

Introduction

Atherosclerosis and restenosis following percutaneous coronary intervention are now recognized to result from proliferative-inflammatory responses to injury in the arterial wall.[1–3] Both balloon angioplasty and stent implantation are useful treatments for dilating atherothrombotic stenosis.[4,5] The overall benefits of this intervention, however, are hampered by local arterial renarrowing (restenosis), which occurs in 30–50% of cases within 6 months.[4,5] No pharmacologic strategies have demonstrated definite benefits to reduce the rate of restenosis. Although silorimus-eluting stents might suppress restenosis,[6,7] its benefits have not yet been completely established.

Experimentally, injury to the arterial wall by balloon or stents rapidly activates intracellular signals, such as mitogen-activated protein kinases, and various transcription factors, such as early growth response factor-1 (Egr-1).[8,9] Egr-1 is an immediate-early gene product and a zinc-finger transcription factor that regulates a number of pathophysiologically relevant genes.[8,10] Egr-1 is induced and activated after injury in vascular smooth muscle cells, endothelial cells, and leukocytes. A number of Egr-1-dependent genes are upregulated in human and experimental atherosclerotic lesions.[11,12] Among a variety of transcription factors that are activated after arterial injury, Egr-1 might be a practical and ideal target for therapeutic intervention to prevent restenosis, because (1) Egr-1 is expressed at low or undetectable levels in the normal artery, and is rapidly upregulated by mechanical injury; (2) Egr-1 is activated by multiple stimuli that might contribute to the development of restenotic and atherosclerotic lesions; and (3) Egr-1 controls the expression of various genes whose products are related to the development of vascular lesion – factors that induce proliferation/migration (platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), matrix metalloproteinases), inflammation (monocyte chemoattractant protein (MCP)-1), and thrombosis (tissue factor).[8,10] Khachigian *et al*[13] recently generated a DNAzyme that specifically cleaves Egr-1 mRNA and reported that adventitial delivery of the DNA enzyme with pluronic gel attenuated neointimal hyperplasia in balloon-injured rat carotid artery model, and that intraluminal delivery of the DNAzyme with the transport catheter inhibited in-stent neointimal hyperplasia of coronary arteries in pig model.[14] They did not, however, address the mechanism by which blockade of Egr-1 attenuated neointimal hyperplasia or causative factor(s) that might contribute to the inhibitory effects on neointimal hyperplasia under *in vivo* conditions. The delivery of anti-Egr-1 agents intraluminally might provide a reasonable starting point for clinical application of anti-Egr-1

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against restenosis. Transfection of the DNAzyme into the arterial wall *in vivo* is difficult compared to transfection of DNA oligodeoxynucleotides (ODNs).[15] We and others previously demonstrated that *cis*-element 'decoy' ODNs of the transcription factor-binding site can be successfully transfected using an intraluminal approach and is effective in blocking the activity of several transcription factors.[15–17] Although there is no ideal experimental model available, the hypercholesterolemic rabbit model might be more relevant and appropriate for studying the usefulness of novel approaches against human restenosis.

Therefore, the primary aim of the present study was to test the hypothesis that blockade of Egr-1 activity reduces neointimal formation after balloon injury by inhibiting Egr-1-dependent proliferative-inflammatory changes in hypercholesterolemic rabbits. To achieve effective blockade of Egr-1 *in vivo*, we utilized transfection of synthetic double-stranded Egr-1 decoy ODNs. This decoy strategy involves the intracellular delivery of 'decoy' ODNs, which are then bound by Egr-1. Occupation of the Egr-1 DNA-binding site by the decoy ODN renders the protein incapable of binding to the promoter regions of the target genes.

Results

Egr-1 expression and activity in a balloon-injured artery

Egr-1 mRNA was not detectable in normal noninjured artery. Egr-1 mRNA expression had biphasic changes; an early and rapid rise at 1 h, a spontaneous decline at 6 and 24 h, reinduction at 7 and 28 days after injury (Figure 1a). Immunohistochemical staining revealed increased Egr-1 protein levels in the medial cells at the early phase and in neointimal and medial cells at the later phase after balloon injury (Figure 1b). To identify which cell types upregulate Egr-1, we performed double immunostaining (Figure 1c). We found that immunoreactive Egr-1 located in monocytes/macrophages and smooth muscle cells in the media at the early phase of day 7, and in neointimal and medial vascular smooth muscle cells at later phase of day 28. Egr-1 was also present in the endoluminal endothelial cells on day 28.

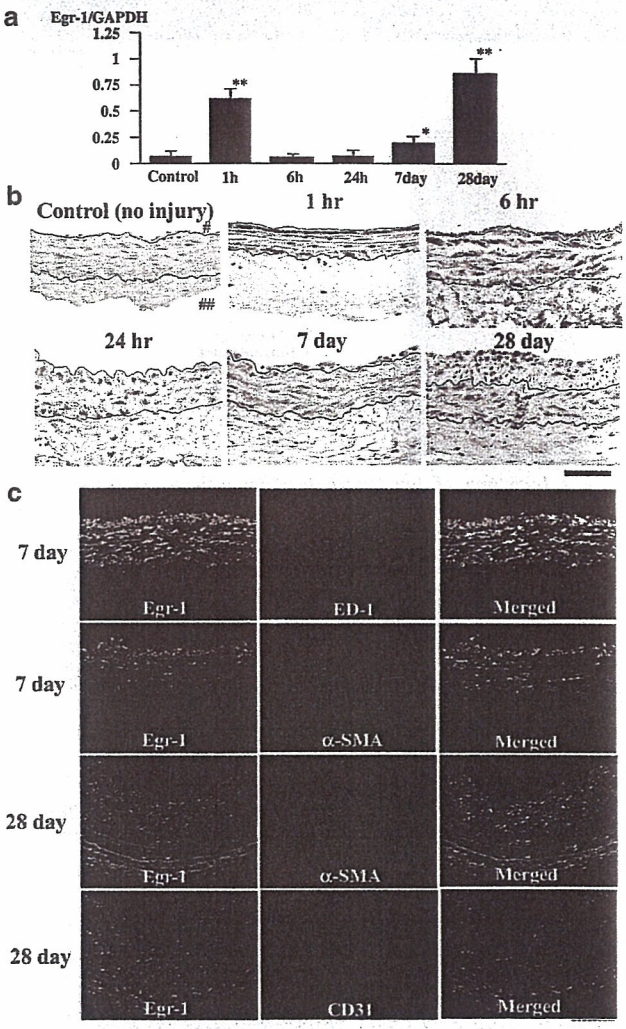

*Figure 1 Time course of Egr-1 gene and protein expression in the carotid artery after balloon injury. (a) Time course of Egr-1 gene expression by quantitative real-time PCR. *P<0.05, **P<0.01 versus noninjured normal control artery. (b) Immunohistochemical micrographs of rat carotid arteries after balloon injury stained for Egr-1. # and ## indicate the lumen and adventitia, respectively. Internal and external elastic layers are highlighted with blue and black lines, respectively. The bar indicates 50 μm. (c) Fluorescence double immunohistochemistry of rat carotid arteries after balloon injury. Micrographs of injured arteries are stained with Egr-1 in green. Micrographs of injured arteries are also stained with ED-1, α-SMA, or PECAM(CD31) in red. Single fluorescence-positive cells were stained in green or red, whereas double-positive cells were stained in yellow. The bar indicates 50 μm.*

Transfection of FITC-labeled Egr-1 decoy into the artery

Transfection of fluorescein-isothiocyanate (FITC)-ODNs resulted in widespread fluorescence in the medial layer of the injured arterial wall (Figure 2a). The transfection efficacy (percent of FITC-stained nuclei per total nuclei) was 6.2±1.6%.

Gel mobility-shift assay revealed that the Egr-1-binding activity was markedly increased compared with that of normal noninjured artery (Figure 2b). Complete competition of the increased Egr-1-binding activity by an excess Egr-1 decoy was observed. The increased Egr-1-binding activity was markedly diminished in the artery transfected with the Egr-1 decoy, but not in the artery transfected with the scrambled decoy ODN (Figure 2b).

Inhibitory effects of the Egr-1 decoy transfer on neointimal hyperplasia

On day 7, we detected RAM-11-positive monocytes and Ki-67-positive proliferating cells mainly in the media (Figure 3a). There was markedly less inflammation (RAM11-positive cells) and proliferation (Ki-67-positive cells) in Egr-1 decoy-transfected arteries than in untreated and scrambled decoy-transfected arteries (Figure 3b).

On day 28, thickened neointima was formed in the balloon-injured artery of all three groups (Figure 4a). Quantitative analysis demonstrated a significant reduction of the intima/media ratio in Egr-1 decoy-transfected arteries compared with the other two groups (Figure 4c). Endothelial cell linings, monitored by CD31

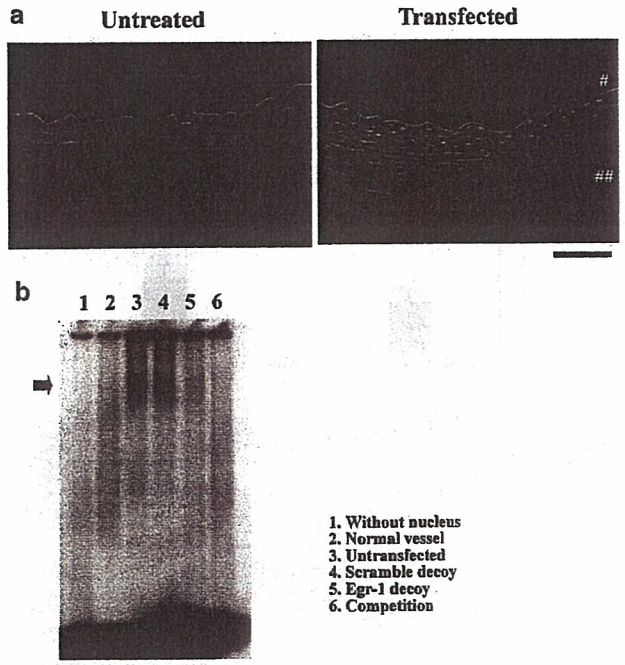

Figure 2 Inhibition of Egr-1 DNA-binding activity by Egr-1 decoy transfection in balloon-injured artery. (a) FITC-labeled decoy ODN transfection into the balloon-injured rabbit carotid artery. Weak background fluorescence is observed in elastic layers of untreated and transfected arteries. Spindle-shaped fluorescence staining, suggesting nuclear fluorescence in the transfected cells. The nuclear fluorescence-stained cells locate mainly in cells in the media. # and ## indicate the lumen and adventitia, respectively. The bar indicates 50 μm. (b) Gel mobility-shift assay for Egr-1-binding site. Lane 1, negative control (^{32}P-labeled Egr-1 decoy ODN without nuclear extract); lane 2, normal artery; lane 3, untreated balloon-injured artery; lane 4, balloon-injured artery transfected with scrambled decoy ODN; lane 5, balloon-injured artery transfected with Egr-1 decoy ODN; lane 6, balloon-injured artery transfected with Egr-1 decoy ODN incubated with extra amount of cold Egr-1 ODN. The arrow indicates migration of the band corresponding to Egr-1–DNA complex. These experiments were repeated five times, all with representative results.

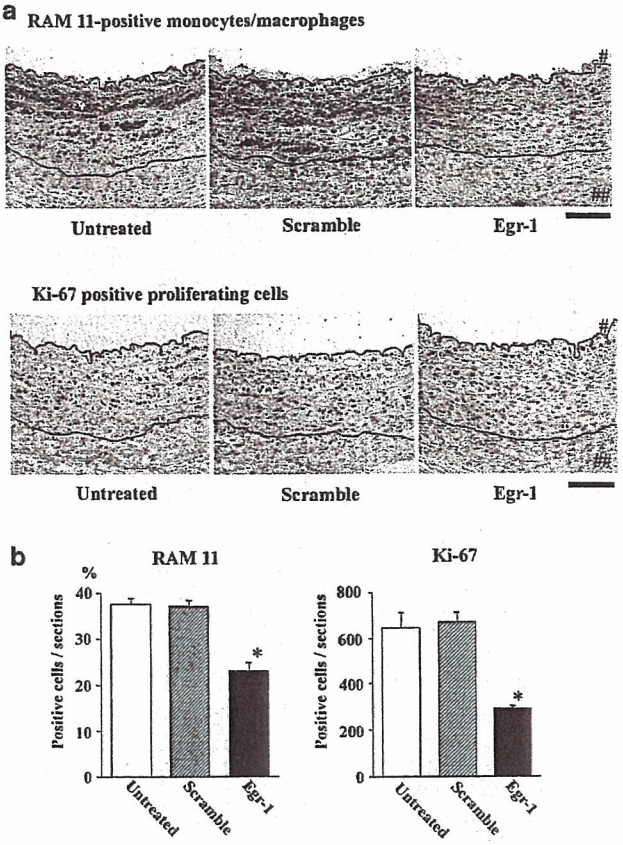

*Figure 3 Inhibitory effect of Egr-1 decoy transfection on early inflammatory and proliferative changes. (a) Carotid artery sections from the untreated group, scrambled group, and Egr-1 group 7 days after injury were stained immunohistochemically with the antibody against monocyte/macrophage (RAM11) or proliferating cells (Ki-67). # and ## indicate the lumen and adventitia, respectively. Bar=100 μm. (b) Effect of the Egr-1 decoy transfection on inflammation (RAM11-positive monocyte/macrophage) and proliferation (Ki-67 positive cells) 7 and after balloon-induced injury (n=8 each). *P<0.05 versus the untreated group.*

immunoreactivity, were observed equally in the three groups (Figure 4a).

Inhibitory effects of Egr-1 decoy transfer on expression of target genes

Egr-1 decoy transfection, but not scrambled decoy transfection, reduced the increased expression of PDGF-B, TGF-β1, and MCP-1 (Figure 5a). Egr-1 decoy transfection did not affect increased gene expression of interleukin (IL)-1β and TF. Immunohistochemical staining performed 7 days after balloon injury revealed increased immunoreactive PDGF-B, TGF-β1, and MCP-1 in cells in the neointima, and smooth muscle cells in the media (Figure 5b). Increases in PDGF-B, TGF-β1, and MCP-1 immunostaining were not observed in Egr-1 decoy-transfected arteries.

Plasma lipoprotein levels

There was no significant difference in the time course of plasma levels of low-density lipoprotein cholesterol among the three groups. The low-density lipoprotein cholesterol levels before and 28 days after injury were 452±47 and 471±63 mg/dl in the untreated group, 491±42 and 507±35 mg/dl in the scrambled decoy-transfected group, and 474±32 and 538±44 mg/dl in Egr-1 decoy-transfected group.

Discussion

Blockade of Egr-1 activation using the 'decoy' strategy attenuated early inflammation and proliferation and thus reduced later neointimal hyperplasia in balloon-injured arteries of hypercholesterolemic rabbits, suggesting that Egr-1 is an important target for restenosis. This study is the first to demonstrate the efficacy of Egr-1 decoy ODN transfection *in vivo* for restenosis therapy.

The most important finding in the present study is that blockade of Egr-1 activity by transfection of Egr-1 decoy ODN attenuated neointimal hyperplasia associated with the inhibition of inflammation (infiltration of RAM11-positive macrophages) and proliferation (appearance of proliferating cells). Also, Egr-1 decoy ODN transfection inhibited increased expression of Egr-1-dependent genes such as PDGF-B and TGF-β1 after balloon injury. Egr-1 decoy ODN transfection did not affect the expression of Egr-1-independent genes, such as IL-1β, suggesting specific inhibition of Egr-1-mediated gene transcription by Egr-1 decoy ODN transfection. Furthermore, there

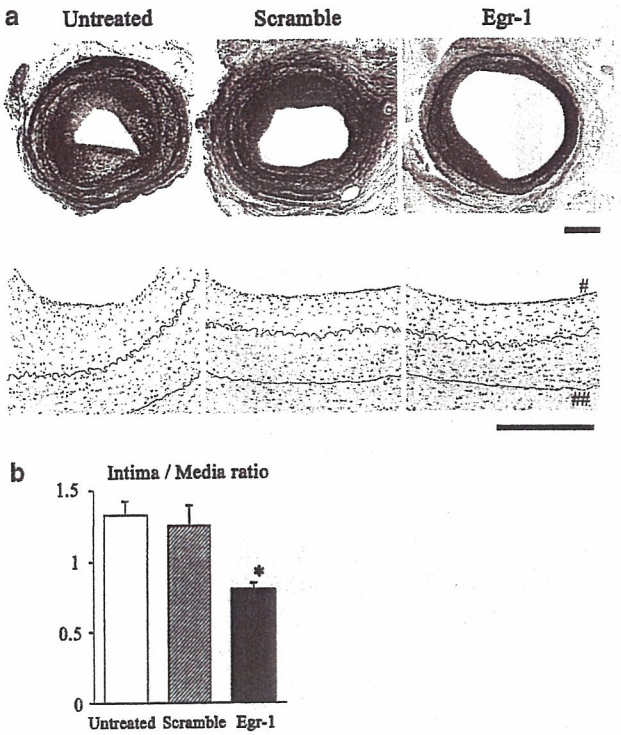

*Figure 4 Inhibitory effect of Egr-1 decoy transfection on neointimal hyperplasia. (a) Carotid artery sections 28 days after balloon-induced injury in untreated, scrambled decoy-transfected, and Egr-1 decoy-transfected arteries stained with elastic-van-Gieson (upper panels) or immunohistochemically for CD31 (lower panels). Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=200 μm. (b) Effect of the Egr-1 decoy transfection on the intima/media ratio 28 days after balloon injury (n=8 each). *P<0.05 versus the untreated and scrambled group.*

was increased immunoreactivity for PDGF-B, TGF-β1, and MCP-1 in balloon-injured arterial wall and increased inhibition by Egr-1 decoy ODN transfection. Neointimal hyperplasia is inhibited by blocking PDGF,[18,19] TGF-β1,[20,21] or MCP-1.[22–24] Therefore, it is reasonable to consider that the mechanisms by which the Egr-1 decoy ODN transfection attenuates neointimal hyperplasia after balloon injury is by decreased expression and activity of these target genes.

We demonstrated direct *in vivo* evidence for specific activation (increased DNA-binding activity) of Egr-1 in the balloon-injured artery and effective suppression of Egr-1 activation by transfection of synthetic double-stranded Egr-1 decoy ODN. As we could not stain Egr-1 immunohistochemically in rabbit tissues with available antibodies, we performed immunohistochemical staining of Egr-1 in a rat model. Egr-1 expression after balloon injury is biphasic and Egr-1-expressing cells locate in monocytes/macrophages and smooth muscle cells in the media at early phases and in the neointimal and vascular smooth muscle cells in the media at late phases of balloon injury. Egr-1 is activated by multiple external stimuli (ie, mechanical injury, cytokines, growth factors) that might contribute to the pathogenesis of neointimal hyperplasia after injury; therefore, the present data suggest that Egr-1 also participates in the chronic process of neointimal hyperplasia. A recent report by McCaffrey *et al*,[11] who demonstrated persistent expression of Egr-1 in activated smooth muscle cells and endothelial cells in chronic stages of both experimental and human atherosclerosis, supports this notion.

A caveat in the interpretation of the present data is the partial inhibition of neointimal hyperplasia. This might be due to the limited transfection efficiency of naked decoy ODN. Therefore, technologies that enhance transfection efficiency (ie, HVJ-liposome method, new catheters for decoy delivery) should be used for 'decoy' strategies in clinical gene therapy.

In conclusion, our present data suggest that Egr-1 has an important role in the pathogenesis of neointimal hyperplasia after balloon injury in hypercholesterolemic rabbits. The inhibitory effects of the Egr-1 decoy are explained by the inhibition of vascular inflammation and proliferation through decreased expression and activity of Egr-1-dependent genes. These results suggest that gene therapy using *in vivo* transfection of an Egr-1 decoy might be a new therapeutic option against restenosis. Recent studies reported that transfection of decoy ODN for other transcription factors (nuclear factor-κB,[25] activator protein-1[26]) also reduced neointimal hyperplasia after injury, possibly through inhibitory effects different from those of Egr-1 decoy ODN transfection. A double decoy strategy that facilitates simultaneous suppression of two transcription factors, therefore, would be another novel approach for restenosis therapy.

Materials and methods

Egr-1 'decoy' oligonucleotides

A double-stranded phosphorothioate Egr-1 decoy ODN was synthesized. The Egr-1 decoy ODN sequence was: 5′-CCGAGAGCGGGGGCGAGCGTG-3′ annealed to 5′-CACGCTCGCCCCGCTCTCGG-3′; the sequence of the scrambled ODN, which included unrelated oligonucleotides, was 5′-GAGGCCGGCAGGTCGCGAGGG-3′ annealed to 5′-CCCTCGCGACCTGCCGGCCTC-3′. Egr-1 ODN labeled with FITC at the 5′ end of one strand was used for fluorescent microscopic analysis of ODN distribution after transfection.

Animal model of balloon injury

The present experiments were reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and according to the Guidelines of the American Physiologic Society. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core.

Male Japanese white rabbits weighing 2.7–3.2 kg were fed a high cholesterol diet containing 1% cholesterol and 3% peanut oil for 2 weeks. After anesthesia, the right common carotid artery was injured by three passages of an inflated 2F Fogarty catheter.[22] The sham operation involved simple ligation of the left external carotid artery without balloon injury. After the operation, all rabbits were fed the same high cholesterol diet. After balloon injury, rabbits were randomly divided into three groups: the untreated group (n=31) received no treatment, the scrambled group (n=31) underwent transfection of the scrambled decoy ODN at the balloon injury site,

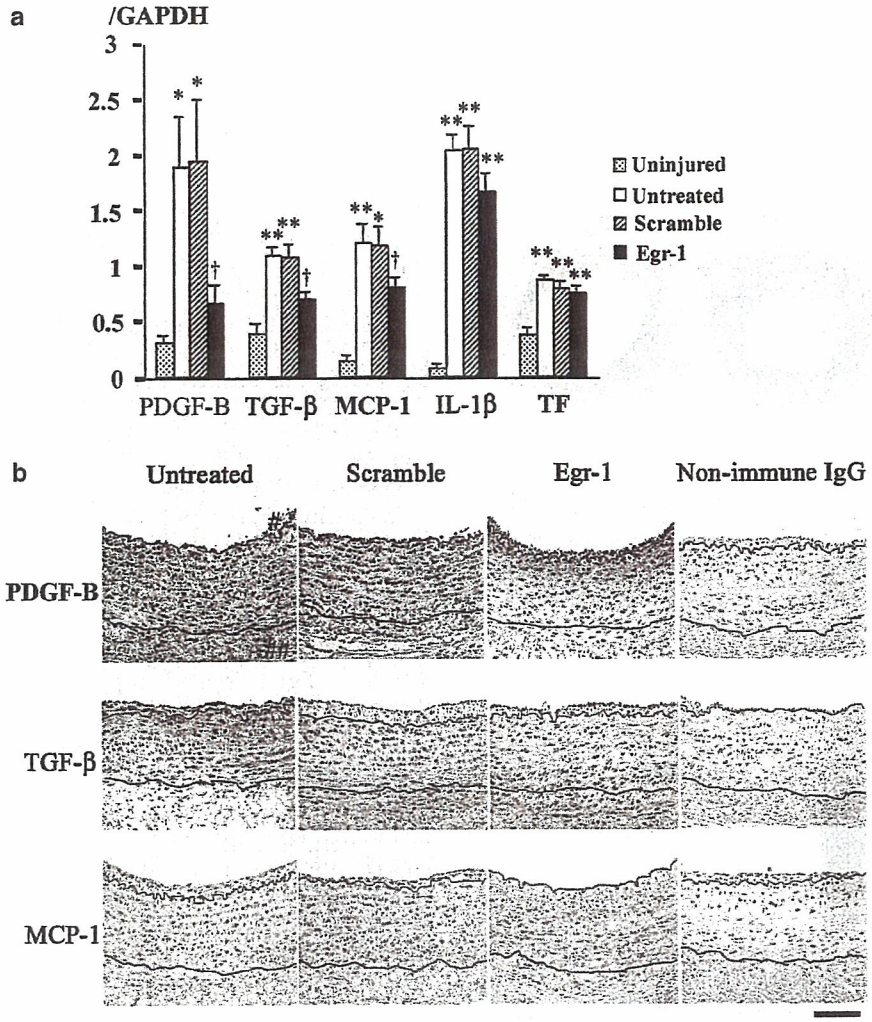

*Figure 5 Inhibitory effect of Egr-1 decoy transfection on the expression of target genes. (a) Effect of Egr-1 decoy transfection on mRNA levels of various genes. Quantitative real-time PCR was performed. *P<0.05, **P<0.01 versus uninjured control artery, †P<0.05 versus untreated and scrambled decoy-transfected artery. (b) Carotid artery sections from the untreated group, scrambled group, and Egr-1 group 7 days after balloon injury stained immunohistochemically with PDGF-B, TGF-β1 and MCP-1. Sections from the untreated group stained with nonimmune IgG are also presented. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=100 μm.*

the Egr-1 group (n=33) underwent transfection of the Egr-1 decoy at the balloon injury site. Before insertion of the catheter, heparin (100 IU) was intravenously injected. For transfection of the decoy ODN, the pulse infusion catheter (Aishin Human Systems Co, Japan) was introduced into the injured common carotid artery site via the right external carotid artery immediately after balloon injury. A measure of 1 ml of the ODN solution (1 mg/1 ml, 80 μM) was infused via the catheter under a 300 mmHg pressure for 15 min. The infusion catheter was then removed and blood flow of the common carotid artery was re-established. At various time points after the operation, rabbits of each group were anesthetized and the injured or transfected common carotid arteries as well as the contralateral untransfected, unmanipulated carotid arteries were collected.

To determine the time course of Egr-1 protein expression, immunohistochemical staining for Egr-1 was performed in a rat model of carotid artery balloon injury. A rat model was used because the appropriate (rabbit Egr-1) antibody was not available. Male Wistar–Kyoto rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and balloon injury of the right common carotid artery was performed using an inflated 2F Fogarty balloon catheter.[23]

Analysis of FITC-ODNs

FITC-labeled ODN were instilled into the injured carotid arteries as above and the arteries were harvested 24 h after transfection. The efficacy of FITC-labeled ODN was assessed using a fluorescence microscope. Serial 5 μm cryosections of carotid arteries transfected with Egr-1 with FITC-labeled ODNs were prepared and recorded. Transfected cells were identified by enhancement of the fluorescein signal.

Electrophoretic mobility shift assay (EMSA)

The EMSA was performed on nuclear extracts prepared immediately from rabbit carotid arteries 2 h after injury using the method described previously.[17,27] Two carotid arteries were pooled so as to obtain one sample for one EMSA. Double-stranded ODN probes for Egr-1 (Santa

Cruz Biotechnology, Santa Cruz, CA, USA) were 5′-end labeled with [γ-^{32}P]dATP by using T4 polynucleotide kinase and standard procedures. For competition studies, a 100-fold molar excess of unlabeled probe for Egr-1 was added.

Histopathology and immunohistochemistry

The rabbit common carotid arteries were harvested, fixed with 10% paraformaldehyde or methacarn solution (methanol 60%; 1,1,1-trichloroethane 30%; and glacial acetic acid 10%). Sections were either stained with elastic-van Gieson stain or subjected to immunostaining. For immunohistochemistry, the slides were heated to induce epitope retrieval. To reduce nonspecific reactivity, sections were preincubated with 0.3% hydrogen peroxide and normal bovine serum. The primary antibodies used in the present study were as follows: mouse anti-rabbit macrophage antibody RAM11 (DAKO Co), the mouse anti-human Ki-67 antibody (DAKO Co), goat anti-human MCP-1 (R & D systems), mouse anti-human PDGF-B antibody PGF007 (Mochida Pharmaceutical Co), chicken anti-human TGF-β antibody (R & D Systems), or nonimmune IgG (Zymed).

The rat common carotid arteries were harvested, fixed with methacarn solution, embedded in paraffin, and cut into 5-μm thick sections. Sections were first stained with primary antibodies, rabbit anti-rat Egr-1 IgG (Santa Cruz Biotechnology), sequentially treated with a biotinylated species-specific secondary antibody, and stained with streptavidin-conjugated peroxidase substrate (Nichirei, Tokyo, Japan). The slides were counterstained with hematoxylin. Immunofluorescence double staining was performed to localize Egr-1 by the use of fluorescence-conjugated antibodies. The primary antibodies used in this study were mouse anti-rat ED-1 (Serotec), mouse α-smooth muscle actin (Dako), goat anti-PECAM (Santa Cruz).

Morphometric analysis

Morphometry and cell counting were performed by a single observer who was blind to the treatment protocols. To evaluate the neointimal thickening of the rabbit carotid arteries, the neointima to media ratio (*I*/*M* ratio) was measured using a computer-assisted analyzer (NIH Image).

Each tissue (four sections per artery) stained with RAM11 and Ki-67 was scanned. The number of positive cells in each section was determined. The sum of the immunopositive cells and the number of total cells in each section were counted. Then the percentage of immunopositive cells per total cells in each section was calculated, and the average of the four sections was reported for each animal.

Table 1 Probes used for real-time PCR

Assay	*Sequence*	*Acc. no.*
Egr-1		
Forward	5′-GCTTCCAGGTCCCCATGAT-3′	AJ291320
Reverse	5′-CCTTGATGGTGGAGAGTGGAG-3′	
TaqMan probe	5′-CCGACTACCTGTTTCCGCAGCA-3′	
PDGF-B		
Forward	5′-CCCATCTACATCATCACCGAGTAC-3′	AB020215
Reverse	5′-GAGTGCTGCTGCAGGAAGGT-3′	
TaqMan probe	5′-TGGACTACCTGCACCGCAACAAGC-3′	
TGF-β1		
Forward	5′-TGCTTCAGCTCCACAGAGAAGA-3′	AB020217
Reverse	5′-GGCAGAAGTTGGCGTGGTA-3′	
TaqMan probe	5′-TGTGCGGCAGCTGTACATTGACTTCC-3′	
MCP-1		
Forward	5′-TGAGCACGTTTCAGTGAGCAT-3′	M28883
Reverse	5′-ACCACACCTGCCTTTACACCTAA-3′	
TaqMan probe	5′-ATGAAGTCGTAGACCAGCAGCCCCC-3′	
IL-1β		
Forward	5′-TCAGCACCTCTCAGACAGAGTACAT-3′	M26295
Reverse	5′-AAGACACGAATTCCATGCTGAA-3′	
TaqMan probe	5′-AAACAACAGTGGCGGCCAAGACCTAA-3′	
TF		
Forward	5′-CACGGTGGCTCAGACATCAG-3′	M55390
Reverse	5′-CGTGCAAAAGGCACCGTAGT-3′	
TaqMan probe	5′-CGGTACAGCCAATCCCAGCTTGTTTC-3′	
GAPDH		
Forward	5′-CTCTGGCAAAGTGGATGTTGTC-3′	L23961
Reverse	5′-GGGTGGAATCATACTGGAACATG-3′	
TaqMan probe	5′-CCATCAATGATCCATTCATTGACCTCCA-3′	

Acc. no. indicates the accession number in GenBank.

Real-time reverse quantitative transcription-polymerase chain reaction

Real-time polymerase chain reaction (PCR) amplification was performed with the rabbits' cDNA using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as described previously.[23] The respective PCR primers and TaqMan probes were designed from GenBank databases using a software program (Applied Biosystems; Table 1). The results were analyzed using the Sequence Detection Software (Applied Biosystems) and expressed in arbitrary units and adjusted for GAPDH mRNA levels.

Statistical analysis

Data are expressed as the mean ± s.e. Statistical analysis of differences was compared by the analysis of variance and Bonferroni's multiple comparison tests. A *P*-value of less than 0.05 was considered to be statistically significant.

Acknowledgements

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Gene Transfer of Stromal Cell–Derived Factor-1α Enhances Ischemic Vasculogenesis and Angiogenesis via Vascular Endothelial Growth Factor/Endothelial Nitric Oxide Synthase–Related Pathway

Next-Generation Chemokine Therapy for Therapeutic Neovascularization

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Background—Stromal cell–derived factor-1α (SDF-1α) is implicated as a chemokine for endothelial progenitor cells (EPCs). We therefore hypothesized that SDF-1α gene transfer would induce therapeutic neovascularization in vivo by functioning as a chemokine of EPC.

Methods and Results—To examine SDF-1α–induced mobilization of EPC, we used bone marrow–transplanted mice whose blood cells ubiquitously express β-galactosidase (LacZ). We produced unilateral hindlimb ischemia in the mice and transfected them with plasmid DNA encoding SDF-1α or empty plasmids into the ischemic muscles. SDF-1α gene transfer mobilized EPCs into the peripheral blood, augmented recovery of blood perfusion to the ischemic limb, and increased capillary density associated with partial incorporation of LacZ-positive cells into the capillaries of the ischemic limb, suggesting that SDF-1α induced vasculogenesis and angiogenesis. SDF-1α gene transfer did not affect ischemia-induced expression of vascular endothelial growth factor (VEGF) but did enhance Akt and endothelial nitric oxide synthase (eNOS) activity. Blockade of VEGF or NOS prevented all such SDF-1α–induced effects.

Conclusions—SDF-1α gene transfer enhanced ischemia-induced vasculogenesis and angiogenesis in vivo through a VEGF/eNOS-related pathway. This strategy might become a novel chemokine therapy for next generation therapeutic neovascularization. (***Circulation.* 2004;109:2454-2461.**)

Key Words: angiogenesis ■ gene therapy ■ nitric oxide synthase ■ ischemia

Neovascularization in adults results not only from angiogenesis but also from vasculogenesis in which endothelial progenitor cells (EPCs) are mobilized from the bone marrow to the site of neovascularization and subsequently differentiate into endothelial cells.[1–3] Recent reports suggest that local or systemic administration of cultured or fresh EPCs enhances ischemic neovascularization and improves function of ischemic tissues in animals with hindlimb or myocardial ischemia.[4–6] More recently, the therapeutic benefits of EPC therapy were demonstrated in patients with severe ischemia in the lower limb and with acute myocardial infarction.[7–9]

There are at least 2 major problems that need to be overcome before EPC therapy may be applied for therapeutic neovascularization as a standard treatment. First, general anesthesia is necessary to harvest EPCs from the bone marrow. In addition, more than 5 to 6 L of blood must be harvested to obtain an adequate number of mononuclear cells that are rich in EPCs. Patients with severe ischemic disease, which is frequently associated with vital organ damage, might not be able to endure such invasive procedures. Second, evidence suggests that patients at risk for atherosclerosis (advanced age, hypertension, hypercholesterolemia, diabetes mellitus, etc) have a decreased number of EPCs and impaired EPC activity.[10,11] Because almost all patients targeted for EPC therapy have several risk factors, the therapeutic benefits of the cell therapy might be hampered by the presence of risk factors. Several potential approaches have been proposed to overcome these obstacles and include the improvement of EPC activity by transfecting genes of angiogenic factors.[12]

We hypothesized that local delivery of a chemokine that attracts EPCs to ischemic tissues and then differentiates them to matured endothelial cells might be another potentially

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Tissue and Plasma SDF-1α Concentrations and White Blood Cell Count Before and After Intramuscular Transfection of SDF-1α

	Time (days) After Transfection				
	0	3	7	14	28
SDF-1α in muscle, ng/g protein	Not detected	268±137	550±108	244±58	Not detected
SDF-1α in plasma, pg/mL	248±22	447±60*	680±188*	407±107*	198±64
White blood cell count, /μL	3720±1030	7537±1135*	11675±1144†	8525±695†	4120±618

Data are mean±SEM (n=5 each).
*$P<0.05$, †$P<0.01$ vs control.

useful approach for therapeutic neovascularization. This mode of "chemokine therapy" could solve the above-mentioned problems. To this end, we examined stromal cell–derived factor-1α (SDF-1α), a family of CXC chemokines, and its receptor CXCR4.[13,14] The SDF-1α/CXCR4 pathway is critical during embryogenesis for hematopoiesis, vascular development, and cardiac development. Recently, it was reported that (1) cells expressing markers of hematopoietic stem cells or EPCs express CXCR4,[15,16] (2) vascular endothelial cell growth factor (VEGF) induces CXCR4 in endothelial cells,[17] and (3) SDF-1α functions as a chemoattractant for EPCs in vitro.[18–20] SDF-1α also induces the expression of VEGF[17] and induces angiogenesis in vivo.[17] Recently, Yamaguchi et al[21] reported that local delivery of SDF-1α protein enhanced neovascularization of an ischemic hindlimb after administration of EPCs, suggesting that SDF-1α augments EPC-induced vasculogenesis. No previous studies, however, have addressed whether local delivery of SDF-1α alone has a therapeutic potential.

We examined whether local gene transfer of SDF-1α to an ischemic hindlimb enhances recruitment of EPCs from the bone marrow to the ischemic site, leading to enhanced neovascularization. To determine the contribution of EPCs, we investigated the effect of SDF-1α gene transfer in bone marrow–transferred mice with selective expression of β-galactosidase in the bone marrow cells.

Methods

Animals and Experimental Protocol

The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines of the American Physiological Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Male C57BL/6J wild-type mice and endothelial nitric oxide synthase (eNOS)-negative mice (Jackson Laboratory, Bar Harbor, Me) were bred and maintained in the Laboratory of Animal Experiments at Kyushu University. To determine the role of EPCs, bone marrow–transferred wild-type mice with ubiquitous expression of β-galactosidase (LacZ) in the bone marrow ($BMT^{LacZ\rightarrow Wild}$ mice) were used. Lethally irradiated (9.5 Gy) wild-type mice received 1×10^6 bone marrow mononuclear cells from ROSA26 mice.

After anesthesia with pentobarbital, animals underwent surgical ligation and resection of the left femoral artery to produce unilateral hindlimb ischemia as previously described.[22] To examine the role of SDF-1α in ischemia-induced angiogenesis, a group of $BMT^{LacZ\rightarrow Wild}$ mice (SDF-1 group, n=15) received intramuscular injections of the expression plasmid containing human SDF-1α cDNA (accession No. XM 005815), pcDNA3-SDF-1α, at 100 μg/50 μL. The control $BMT^{Wild\rightarrow Wild}$ mice (plasmid group, n=15) received intramuscular injections of empty plasmid cDNA3 devoid of cDNA. Human SDF-1α gene was cloned into the *Eco*RI (5′) and *Eco*RI (3′) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen). Mice were injected with plasmid into the left femoral and tibial muscles with a 27-gauge needle immediately after induction of hindlimb ischemia. Transgene expression was enhanced by local intramuscular electroporation at the injection site immediately after the injection.[23] Three other groups of $BMT^{LacZ\rightarrow Wild}$ mice received the NO synthesis inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) in drinking water (2 mg/kg; L-NAME group, n=15), intramuscular transfection of a soluble form of the VEGF receptor-1, Flt-1 (sFlt-1; the sFlt-1 group, n=15), or both L-NAME and sFlt-1 (L-NAME+sFlt-1 group, n=15). We and others previously demonstrated that intramuscular transfection of the sFlt-1 gene effectively and specifically blocks VEGF signaling and thus quenches VEGF activity in vivo.[24,25]

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging experiments (LDPI, Moor Instruments) were performed in mice as previously described.[22]

Capillary Density

Capillary density was determined by immunohistochemical staining with anti-CD31 antibody 28 days after hindlimb ischemia as previously described.[22]

Flow Cytometric Analyses of EPC Mobilization

Peripheral blood was obtained from the retro-orbital venous plexus of the BMT mice 2 weeks after hindlimb ischemia. EPCs are thought to derive from mononuclear leukocytes that are positive for both c-kit and CD31.[26] The percentage of mononuclear cells that were positive for both the c-kit-PE and CD31-FITC antibodies (Pharmingen) was then analyzed with a FACS Caliber flow cytometer (Becton Dickinson).

Determination of VEGF, Phosphorylated Akt, Akt, and eNOS Protein Expression

Tissue samples (gastrocnemius muscle from ischemic and nonischemic hindlimbs), obtained at days 3, 7, 14, or 28, were thawed and homogenized in 300 μL of buffer containing protease inhibitors. Proteins were separated in denaturing SDS/12% polyacrylamide gels and then blotted onto a nitrocellulose sheet (Hybond ECL, Amersham). Antibodies against VEGF (1:2000, Santa Cruz Biotechnology), phosphorylated (phospho-) Akt (1:500, Cell Signaling, Biolabs), Akt (1:1000, Cell Signaling), and eNOS (1:2000, Santa-Cruz-Biotechnology) were then used. The proteins were then stained with Ponceau red (Sigma Chemical Co) for 10 minutes. Results are expressed as the ratio of quantification of the specific band to quantification of the transferred total protein bands stained with Ponceau red (Sigma Chemical Co).

Cellular Localization of SDF-1α, CXCR4, and VEGF

To detect the localization of SDF-1α, CXCR4, and VEGF-expressing cells, frozen tissue sections from ischemic muscles (5

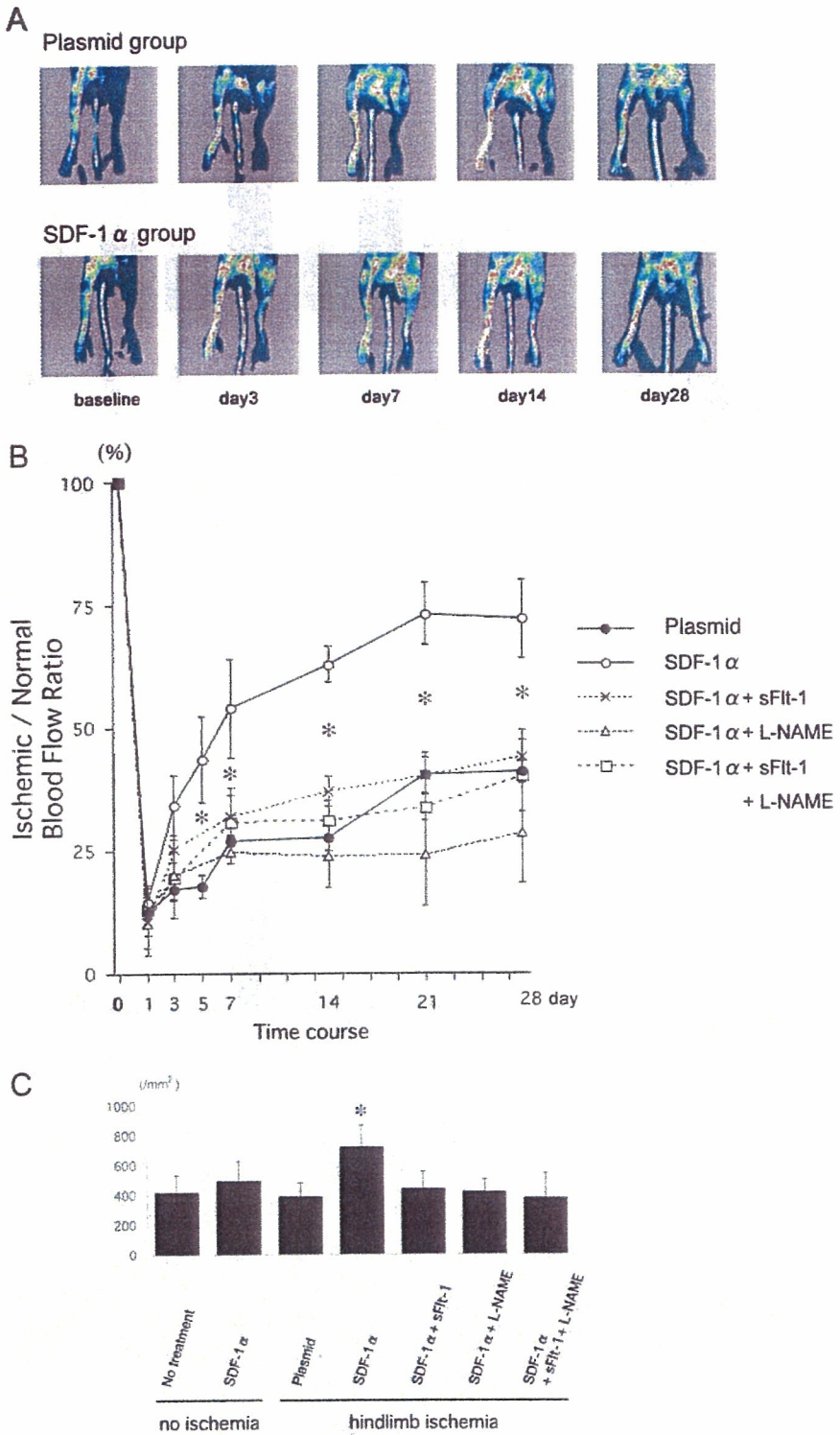

Figure 1. Effects of SDF-1α gene transfer on ischemia-induced neovascularization in plasmid (n=18), SDF-1α (n=18), SDF-1α+sFlt-1 (n=16), SDF-1α+L-NAME (n=16), and SDF-1α+L-NAME+sFlt-1 (n=16) groups. A, Representative laser Doppler perfusion color imaging at indicated time points. In color-coded images, normal perfusion is depicted in red and a marked reduction in blood flow of ischemic hindlimb in blue. B, Summary of blood flow ratio of ischemic limb to that of nonischemic limb. C, Capillary density in plasmid (n=18), SDF-1α (n=18), SDF-1α+sFlt-1 (n=16), SDF-1α+L-NAME (n=16), and SDF-1α+L-NAME+sFlt-1 (n=16) groups. Capillary density of nonischemic mice transfected with SDF-1α or untransfected is also presented (n=7 to 8). *$P<0.05$ vs plasmid group.

μm) were incubated with rabbit polyclonal antibody directed against SDF-1α (1:100, Santa Cruz Biotechnology) or VEGF (1:100, Santa Cruz Biotechnology). After incubation with biotinylated anti-rabbit IgG, immunostains were visualized by use of avidin-biotin–horseradish peroxidase visualization systems.

Plasma and Tissue Measurements of SDF-1

Plasma and tissue concentrations of SDF-1α released by the transfected skeletal muscle were measured by use of a human SDF-1 ELISA kit (R&D Systems).

TaqMan Real-Time Reverse Transcription–Polymerase Chain Reaction Analysis

TaqMan real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described.[27] Transcripts from total RNA (1 μg) were reverse transcribed, and the resultant cDNA was amplified by TaqMan real-time RT-PCR. The PCR primers for mouse SDF-1α were: sense primer, 5′-CCCGGATCCATGAACGCCAAGGTCGTG-3′ and antisense primer, 5′-AGAGCTGGGCTCCTACTGTGCGGCCGCGGG-3′. The GADPH probe was obtained from Applied Biosystems.

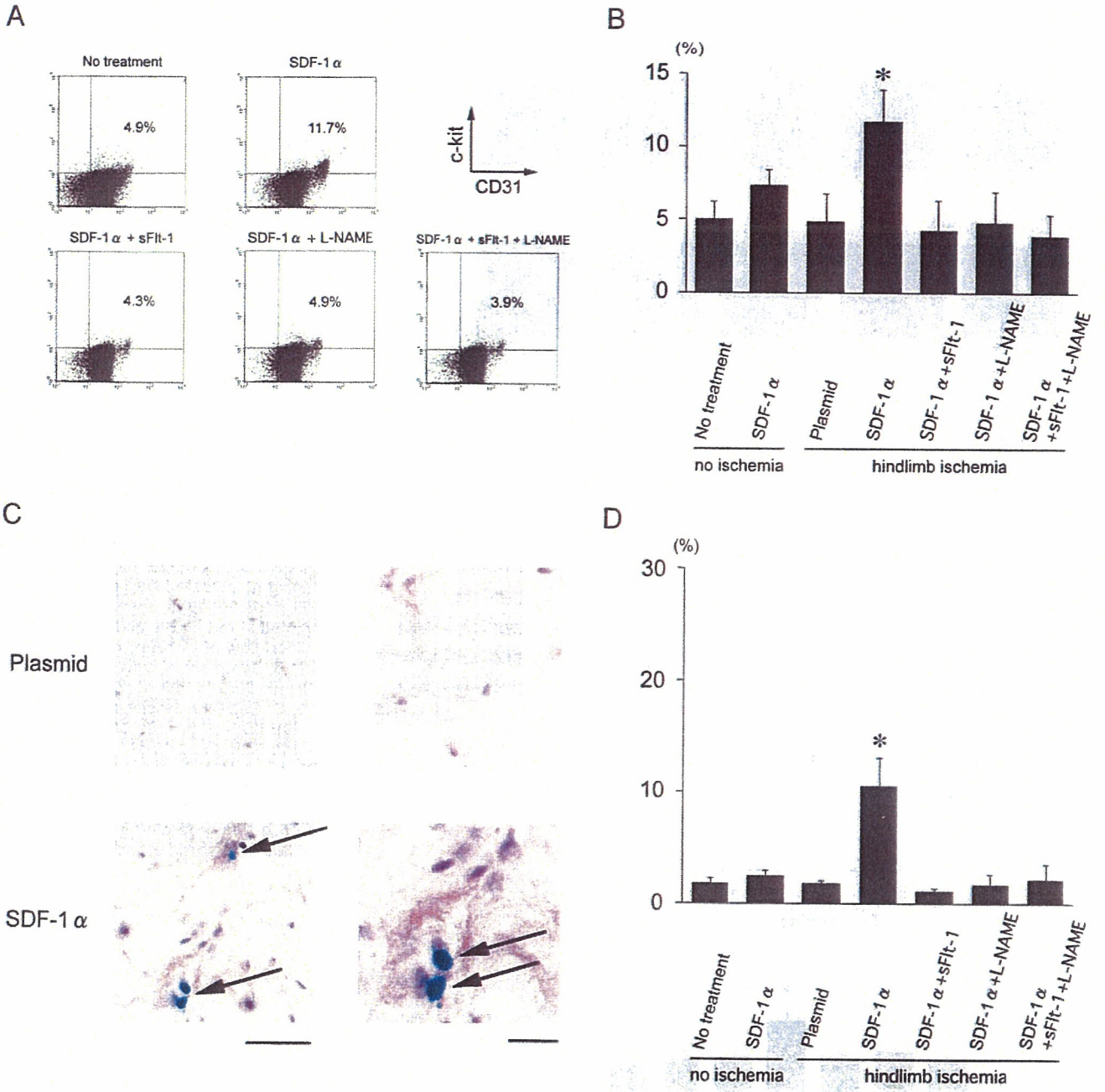

Figure 2. Effects of SDF-1α gene transfer on EPC mobilization and incorporation. A and B, CD31 and c-kit double-positive cells were analyzed by flow cytometry. SDF-1α–induced mobilization of double-positive cells in peripheral circulation are presented in plasmid (n=8), SDF-1α (n=8), SDF-1α+sFlt-1 (n=6), SDF-1α+L-NAME (n=6), and SDF-1α+L-NAME+sFlt-1 (n=6) groups. *$P<0.01$ vs plasmid group. C, Pictures of ischemic muscle sections stained with X-gal on day 28, showing SDF-1α–induced incorporation of X-gal–positive cells into capillary vessels. Bars: left, 50 μm; right, 20 μm. D, Summary of data in C. Percentages of capillary vessels double-positive with LacZ and CD31 to total CD31-stained capillaries are presented. n=6 to 8. *$P<0.01$ vs plasmid group.

In Vivo Matrigel Plug Assay

Matrigel plug (100 μL in volume) with or without SDF-1α protein (200 ng/mL) was injected subcutaneously into the flank of wild-type mice. Fourteen days after injection, the plugs were removed and snap-frozen in OCT compound as previously described.[23] Frozen sections (5 μm) were stained with hematoxylin-eosin or with anti-CXCR4 antibody, anti-CD31 antibody, or c-kit antibody.

Statistical Analysis

Data were expressed as mean±SEM. Statistical analysis of differences was compared by ANOVA with Bonferroni's correction for multiple comparisons. A probability value of $P<0.05$ was considered statistically significant.

Results

Effects of SDF-1α Gene Transfer on Ischemia-Induced Neovascularization in BMT Mice

Neither SDF-1α mRNA (real-time PCR, data not shown) nor protein (ELISA, Table) was detected in nonischemic or ischemic muscles. In contrast, tissue and plasma SDF-1α concentrations rose transiently after SDF-1α gene transfer into ischemic muscles (Table).