

Gene Transfer of Stromal Cell–Derived Factor-1 α 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-1 α (SDF-1 α) is implicated as a chemokine for endothelial progenitor cells (EPCs). We therefore hypothesized that SDF-1 α gene transfer would induce therapeutic neovascularization in vivo by functioning as a chemokine of EPC.

Methods and Results—To examine SDF-1 α –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-1 α or empty plasmids into the ischemic muscles. SDF-1 α 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-1 α induced vasculogenesis and angiogenesis. SDF-1 α 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-1 α –induced effects.

Conclusions—SDF-1 α 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-1 α Concentrations and White Blood Cell Count Before and After Intramuscular Transfection of SDF-1 α

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

Data are mean \pm 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-1 α (SDF-1 α), a family of CXC chemokines, and its receptor CXCR4.^{13,14} The SDF-1 α /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-1 α functions as a chemoattractant for EPCs in vitro.^{18–20} SDF-1 α also induces the expression of VEGF¹⁷ and induces angiogenesis in vivo.¹⁷ Recently, Yamaguchi et al²¹ reported that local delivery of SDF-1 α protein enhanced neovascularization of an ischemic hindlimb after administration of EPCs, suggesting that SDF-1 α augments EPC-induced vasculogenesis. No previous studies, however, have addressed whether local delivery of SDF-1 α alone has a therapeutic potential.

We examined whether local gene transfer of SDF-1 α 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-1 α 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-1 α 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-1 α cDNA (accession No. XM 005815), pcDNA3-SDF-1 α , at 100 μ g/50 μ L. The control BMT^{Wild \rightarrow Wild} mice (plasmid group,

n=15) received intramuscular injections of empty plasmid cDNA3 devoid of cDNA. Human SDF-1 α gene was cloned into the *EcoRI* (5') and *EcoRI* (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 *N* ω -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 (Pharmin-gen) 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 μ L 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-1 α , CXCR4, and VEGF

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

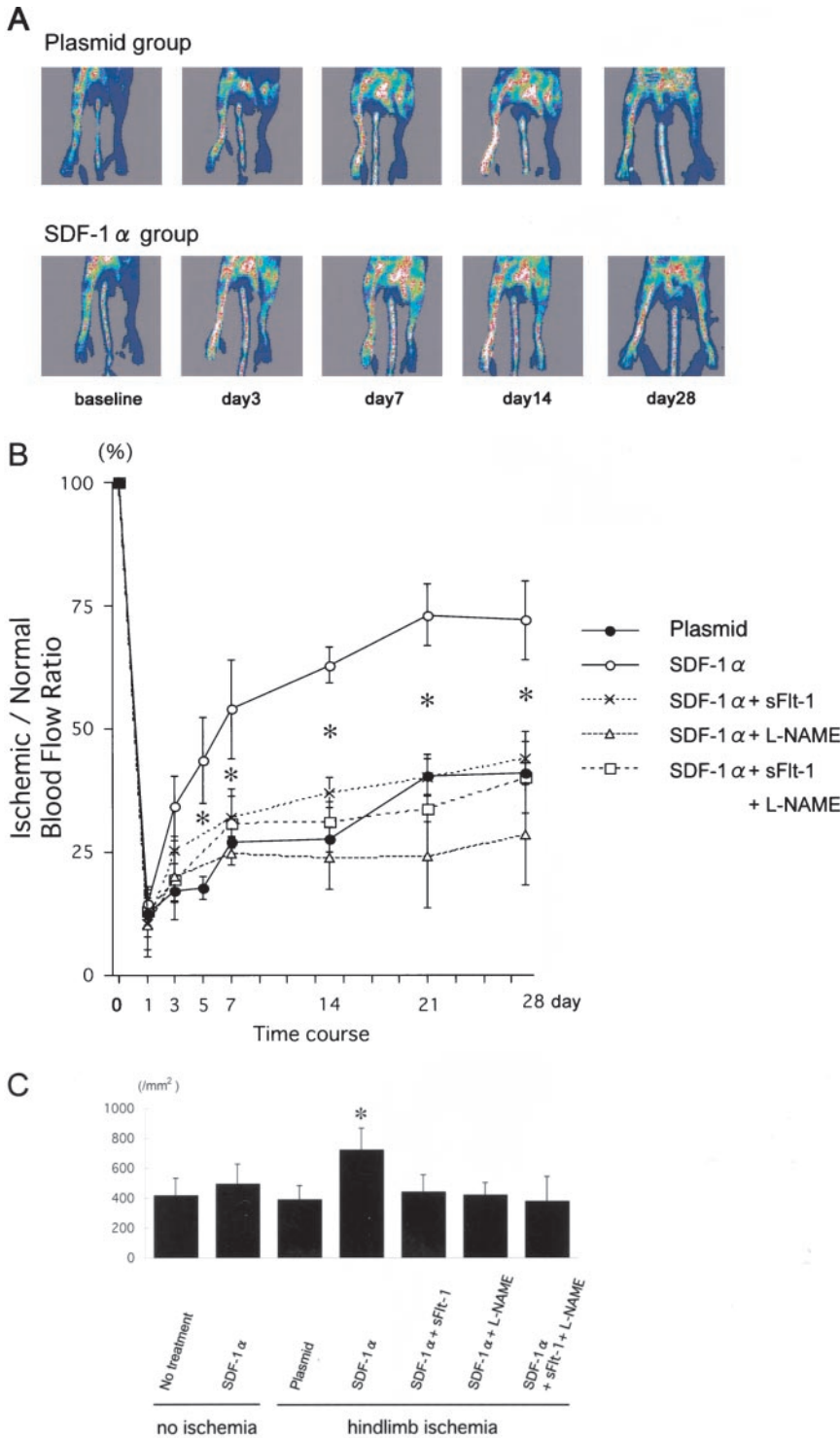


Figure 1. Effects of SDF-1 α gene transfer on ischemia-induced neovascularization in plasmid (n=18), SDF-1 α (n=18), SDF-1 α +sFit-1 (n=16), SDF-1 α +L-NAME (n=16), and SDF-1 α +L-NAME+sFit-1 (n=16) groups. A, Representative laser Doppler perfusion color imaging at indicated time points. In color-coded 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-1 α (n=18), SDF-1 α +sFit-1 (n=16), SDF-1 α +L-NAME (n=16), and SDF-1 α +L-NAME+sFit-1 (n=16) groups. Capillary density of nonischemic mice transfected with SDF-1 α or untransfected is also presented (n=7 to 8). *P<0.05 vs plasmid group.

μ m) were incubated with rabbit polyclonal antibody directed against SDF-1 α (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-horse-radish peroxidase visualization systems.

Plasma and Tissue Measurements of SDF-1

Plasma and tissue concentrations of SDF-1 α 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 μ g) were reverse transcribed, and the resultant cDNA was amplified by TaqMan real-time RT-PCR. The PCR primers for mouse SDF-1 α were: sense primer, 5'-CCCGATCCATGAACGCCAAGGTCGTG-3' and antisense primer, 5'-AGAGCTGGGCTCCTACTGTGCGGCCGCGGG-3'. The GADPH probe was obtained from Applied Biosystems.

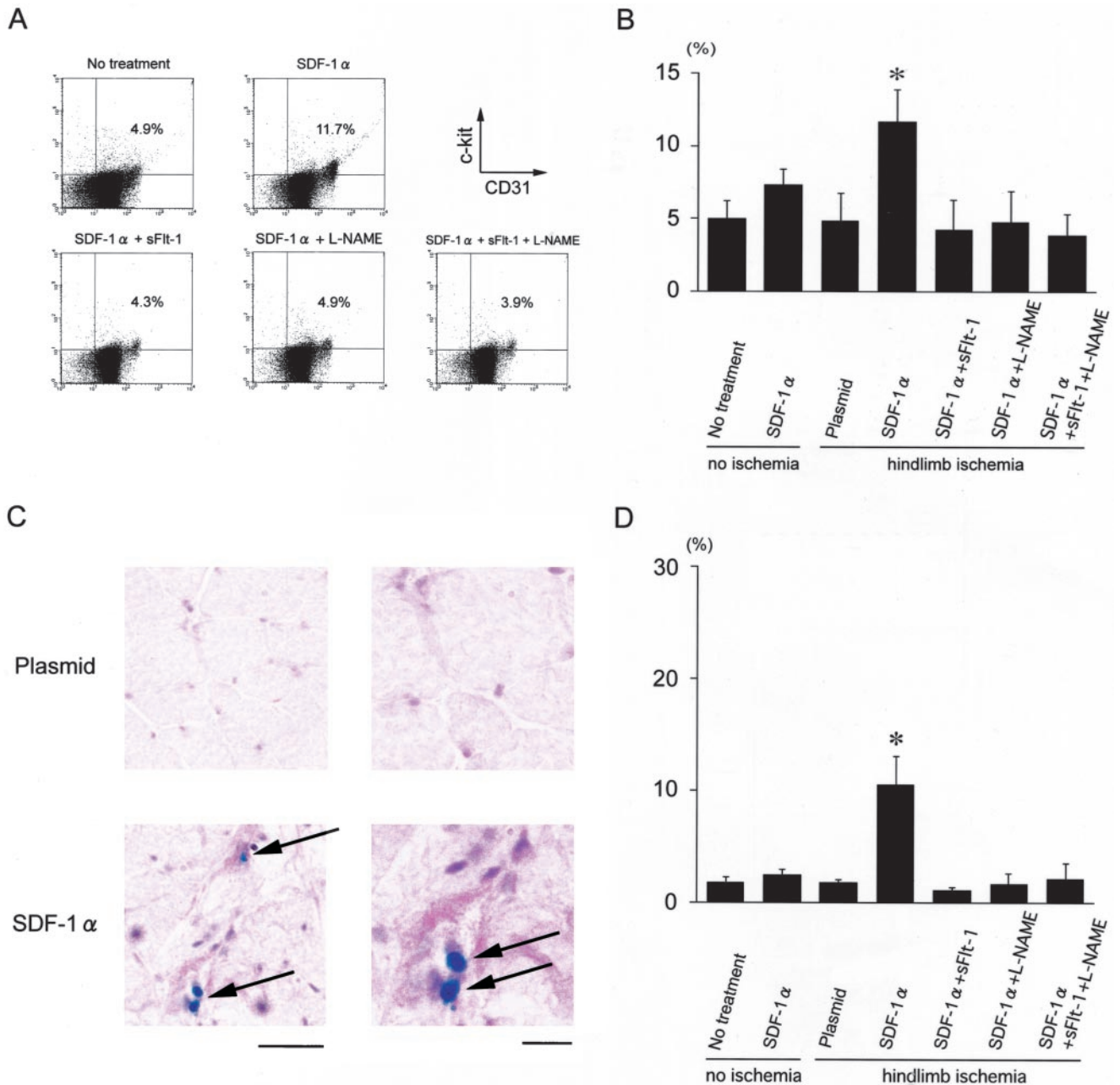


Figure 2. Effects of SDF-1 α gene transfer on EPC mobilization and incorporation. A and B, CD31 and c-kit double-positive cells were analyzed by flow cytometry. SDF-1 α -induced mobilization of double-positive cells in peripheral circulation are presented in plasmid (n=8), SDF-1 α (n=8), SDF-1 α +sFlt-1 (n=6), SDF-1 α +L-NAME (n=6), and SDF-1 α +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-1 α -induced incorporation of X-gal-positive cells into capillary vessels. Bars: left, 50 μ m; right, 20 μ m. 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 μ L in volume) with or without SDF-1 α 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 μ m) were stained with hematoxylin-eosin or with anti-CXCR4 antibody, anti-CD31 antibody, or c-kit antibody.

Statistical Analysis

Data were expressed as mean \pm 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-1 α Gene Transfer on Ischemia-Induced Neovascularization in BMT Mice

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

Hindlimb perfusion was measured serially with laser Doppler imaging (Figure 1, A and B). SDF-1 α gene transfer increased blood flow recovery on days 7, 14, 21, and 28 after ischemia. The SDF-1 α -induced enhancement of blood perfusion was suppressed by blockade of VEGF by sFlt-1 gene transfer or by inhibition of NO synthesis by treatment with L-NAME. Combined treatment with sFlt-1 and L-NAME did not have further inhibitory effects.

Capillary density was determined by immunohistochemical staining with anti-CD31 antibody on day 28 (Figure 1C). SDF-1 α gene transfer to the nonischemic limb did not affect capillary density; however, it enhanced capillary density of the ischemic hindlimb compared with the level observed in the nonischemic hindlimb or ischemic hindlimb. No SDF-1 α -induced increase in capillary density was seen in the sFlt-1, L-NAME, or L-NAME+sFlt-1 groups.

Effects of SDF-1 α Gene Transfer on EPC Mobilization and Incorporation in BMT Mice

Mobilization of EPCs to the peripheral circulation was determined by flow cytometry 14 days after ischemia. SDF-1 α gene transfer to nonischemic normal mice did not significantly affect the percentage of CD31- and c-kit-positive EPCs (Figure 2, A and B). In contrast, SDF-1 α gene transfer to ischemic mice induced mobilization of EPCs (Figure 2, A and B). This SDF-1 α -induced EPC mobilization was blunted in the sFlt-1, L-NAME, and L-NAME+sFlt-1 groups (Figure 2A).

To assess incorporation of bone marrow lineage cells to neovascularization, the percentage of capillaries positive for both LacZ and CD31 to total CD31 staining capillaries was calculated on day 28 (Figure 2, C and D). SDF-1 α gene transfer to nonischemic normal mice did not affect the percentage of LacZ-positive capillaries. In contrast, SDF-1 α gene transfer to the ischemic hindlimb significantly increased incorporation of LacZ-positive cells into the capillary vessels, which was blunted in the sFlt-1, L-NAME, and L-NAME+sFlt-1 groups (Figure 2, C and D). There was only a partial incorporation of LacZ-positive cells into the microvessels (only 12% of capillaries were LacZ-positive), suggesting that SDF-1 α gene transfer enhanced not only ischemia-induced vasculogenesis but also angiogenesis.

Effects of SDF-1 α on EPC Mobilization and Incorporation Into Neovessels

Because incorporation of LacZ-positive cells into capillary vessels might not prove incorporation of EPCs, an *in vivo* matrigel plug assay was performed to determine the SDF-1 α -induced incorporation of EPC lineage cells into neovessels in both wild-type and BMT mice (Figure 3). Matrigels containing SDF-1 α protein had significant neovessel formation in wild-type and BMT^{LacZ} \rightarrow Wild mice (Figure 3). Fluorescence microscopy revealed that most CD31-positive neovessels contained c-kit- and CXCR4-expressing cells, suggesting that CXCR4-positive EPCs might mobilize into the SDF-1 α -containing Matrigels, resulting in neovessel formation.

Regulation of VEGF, phospho-Akt, and phospho-eNOS Protein Levels in BMT Mice

VEGF protein content increased in the ischemic muscles compared with nonischemic controls at 14 days of treatment.

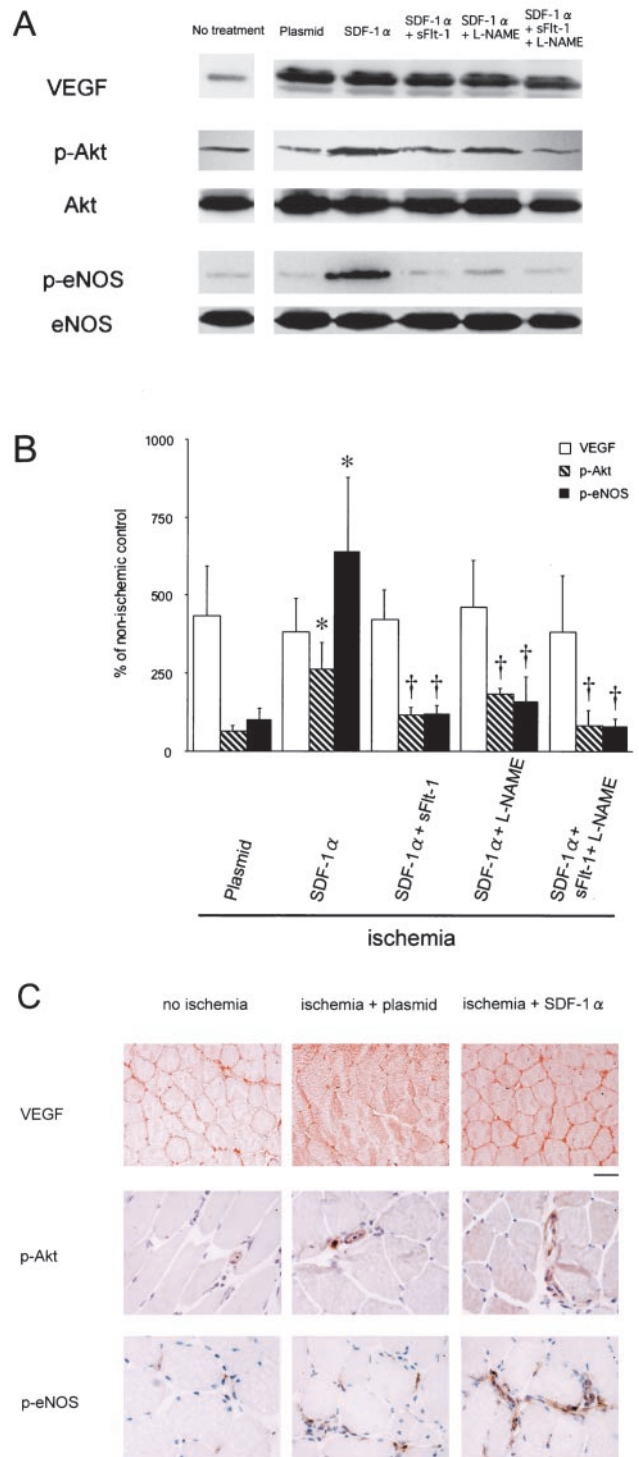


Figure 3. Regulation of VEGF, phospho-Akt, and phospho-eNOS protein in plasmid (n=5), SDF-1 α (n=5), SDF-1 α +sFlt-1 (n=5), SDF-1 α +L-NAME (n=5), and SDF-1 α +L-NAME+sFlt-1 (n=5) groups. A, Western blot of VEGF, phospho-Akt, and phospho-eNOS proteins in ischemic and non-ischemic muscle 7 days after ischemia. B, Quantitative evaluation of VEGF, phospho-Akt, and phospho-eNOS protein levels expressed as a percentage of non-ischemic control. * P <0.05 vs plasmid group; † P <0.05 vs ischemia+SDF-1 α . C, Pictures of ischemic muscle sections stained immunohistochemically with antibodies against VEGF, phospho-Akt, and phospho-eNOS protein. Scale bar: 50 μ m.

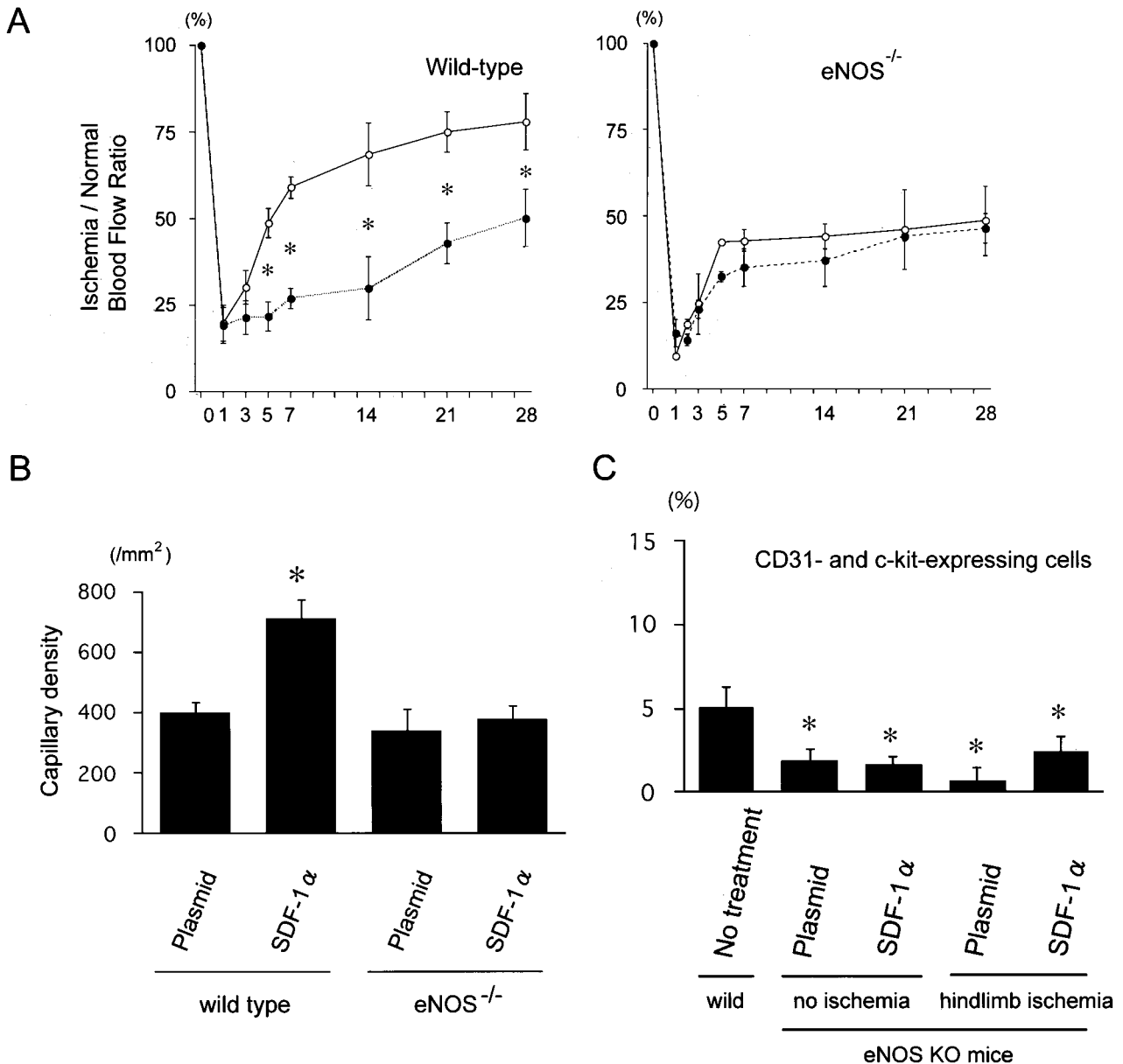


Figure 4. Effects of SDF-1 α gene transfer on ischemia-induced neovascularization in wild-type and eNOS^{-/-} mice. A, Summary of the blood flow ration of ischemic limb to that of non-ischemic limb. Open and closed circles indicate SDF-1 α gene transfer and empty plasmid groups, respectively. B, Effects of SDF-1 α gene transfer on capillary density. N=7 to 8. *P<0.05 vs plasmid group. C, Effects of SDF-1 α gene transfer on EPC mobilization to peripheral circulation CD31 and c-kit double-positive cells is presented in non-treated wild type mice and mice transfected with empty plasmid or SDF-1 α gene into ischemic muscles. *P<0.01 vs no treatment.

VEGF protein also increased in the SDF-1 α , sFlt-1, L-NAME, and L-NAME+sFlt-1 groups (Figure 4A). Immunohistochemistry revealed that increased VEGF was localized in ischemic myocytes (Figure 4B). In contrast, phospho-Akt and phospho-eNOS were localized in microvascular endothelial cells, and their intensity was enhanced by SDF-1 α transfection. Neither phospho-Akt nor phospho-eNOS protein levels changed in ischemic muscle of the plasmid group, but they were increased in SDF-1 α -transfected ischemic muscle. The SDF-1 α -induced increases in phospho-Akt and phospho-eNOS protein levels were blunted in the sFlt-1, L-NAME, and L-NAME+sFlt-1 groups (Figure 4B).

Effects of SDF-1 α Gene Transfer on Ischemia-Induced Neovascularization in eNOS^{-/-} Mice

SDF-1 α gene transfer augmented recovery of blood perfusion, increased capillary density, and EPC mobilization in wild-type (eNOS^{+/+}) mice as in BMT^{LacZ}→^{Wild} mice. In contrast, such SDF-1 α -induced effects were blunted in eNOS^{-/-}.

Discussion

The novel finding of this study is that intramuscular gene transfer of SDF-1 α (a strong EPC chemoattractant) into

ischemic limbs enhanced ischemia-induced neovascularization in mice associated with mobilization and partial incorporation of EPC into neovessels. Salcedo et al¹⁷ reported that serial intradermal injections of SDF-1 α protein into mouse skin induced significant microvessel formation associated with recruitment of leukocytes, which was equipotent to neovessel formation induced by VEGF protein injections. These previous studies, however, did not address whether SDF-1 α induces therapeutic angiogenesis, vasculogenesis, or both in vivo. Using BMT^{LacZ \rightarrow Wild} mice, we demonstrated that SDF-1 α gene transfer enhanced both ischemia-induced vasculogenesis and angiogenesis in vivo.

To investigate the mechanisms underlying the SDF-1 α -induced effects, we examined the local expression of VEGF, Akt, and eNOS. Previous studies established a role of NO in EPC podokinesis²⁸ (detachment of EPCs from bone marrow stromal cells), mobilization, and differentiation into endothelial cells in ischemic tissues.²⁹ Both Akt and eNOS are located downstream of VEGF-induced neovascularization.^{30,31} The present results demonstrated that SDF-1 α gene transfer did not affect ischemia-induced enhancement of VEGF expression but did enhance Akt and eNOS activity. VEGF upregulation was localized mainly in ischemic muscles, whereas activation of Akt and eNOS occurred in endothelial cells, implying that interaction of endogenous VEGF from ischemic muscles with endothelial Akt and eNOS is required for SDF-1 α -induced enhancement of ischemic neovascularization. We also demonstrated that the enhanced expression of Akt and eNOS was suppressed by treatment with a VEGF inhibitor, an NOS inhibitor, and their combination. Furthermore, SDF-1-induced neovascularization and EPC mobilization were not observed in eNOS^{-/-} mice. Upregulation of Akt and eNOS might result partly from increased EPC mobilization and neovascularization. Nevertheless, these data suggest that the increased expression and function of VEGF with subsequent activation of Akt and eNOS were involved, at least in part, in the mechanisms by which SDF-1 α enhanced ischemia-induced neovascularization.

Because EPCs express functional CXCR4 and VEGF receptors,^{17,21} an interaction between the SDF-1 α /CXCR4 pathway and VEGF might form a positive-feedback loop to further enhance therapeutic neovascularization in vivo. The present study might have significant clinical implications in that SDF-1 α gene transfer has therapeutic potential for enhancing ischemia-induced neovascularization, at least in part, by mobilizing EPCs to ischemic tissues. More research is required, however, on the effect of SDF-1 α on atherogenesis before translational clinical research begins. SDF-1 α is expressed in human atherosclerotic plaques³² and elicits proinflammatory and platelet-activating actions of SDF-1 α in vitro, suggesting SDF-1 α /CXCR4 inhibition as a therapeutic means for atherosclerosis and acute coronary syndrome.^{33,34} In contrast, anti-inflammatory and plaque-stabilizing effects of SDF-1 α are reported in vitro. Damas et al³⁵ reported that SDF-1 α (500 ng/mL) reduced the release of monocyte chemoattractant protein-1, interleukin-8, matrix metalloproteinases, and tissue factor in peripheral circulating mononuclear cells from patients with unstable angina. More recently,

Schober et al³⁶ reported that SDF-1 α is essential in neointimal formation after vascular injury associated with increased neointimal smooth muscle content. These studies support the concept that therapeutic interventions that enhance SDF-1 α activity could be beneficial in atherosclerotic vascular disease. Therefore, further studies are needed to establish the role of SDF-1 α in the process of atherosclerosis in vivo.

In conclusion, SDF-1 α gene transfer enhanced ischemia-induced vasculogenesis and angiogenesis in vivo through the VEGF/eNOS-related pathway, suggesting that this strategy might be used for the development of a next-generation chemokine therapy for therapeutic neovascularization. This mode of gene therapy might be a novel stand-alone or adjunctive approach for therapeutic neovascularization.

Acknowledgments

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