Blockade of Vascular Endothelial Growth Factor Suppresses Experimental Restenosis After Intraluminal Injury by Inhibiting Recruitment of Monocyte Lineage Cells

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Background—Therapeutic angiogenesis by delivery of vascular endothelial growth factor (VEGF) has attracted attention. However, the role and function of VEGF in experimental restenosis (neointimal formation) after vascular intraluminal injury have not been addressed.

Methods and Results—We report herein that blockade of VEGF by soluble VEGF receptor 1 (sFlt-1) gene transfer attenuated neointimal formation after intraluminal injury in rabbits, rats, and mice. sFlt-1 gene transfer markedly attenuated the early vascular inflammation and proliferation and later neointimal formation. sFlt-1 gene transfer also inhibited increased expression of inflammatory factors such as monocyte chemoattractant protein-1 and VEGF. Intravascular VEGF gene transfer enhanced angiogenesis in the adventitia but did not reduce neointimal formation.

Conclusions—Increased expression and activity of VEGF are essential in the development of experimental restenosis after intraluminal injury by recruiting monocyte-lineage cells. (Circulation. 2004;110:2444-2452.)

Key Words: restenosis ■ remodeling ■ inflammation ■ endothelium-derived factors ■ gene therapy

Vascular endothelial growth factor (VEGF) has attracted attention for endothelial regeneration and angiogenesis.1-3 VEGF is one of the most potent vascular permeability factors known, is thought to function as an endogenous regulator of endothelial integrity after injury, and thus, protects the artery from disease progression.4 Previous animal studies have reported that local delivery of VEGF after endothelial injury promotes endothelial regeneration, accelerates the recovery of endothelium-dependent relaxation, and reduces neointima after balloon injury. Increased expression of VEGF and its 2 receptors (VEGFR-1, Flt-1; VEGFR-2, Flk-1) in atherosclerotic and restenotic lesions has been reported.5-7 However, there is still considerable debate over the vasculoprotective versus atherogenic effects of VEGF.8 Emerging evidence suggests that (1) VEGF induces migration and activation of monocytes;9 (2) VEGF induces adhesion molecules10 and monocyte chemoattractant protein-1 (MCP-1)11; and (3) VEGF enhances neointimal formation and atherosclerosis by stimulating intraplaque angiogenesis in hypercholesterolemic animals without balloon injury12,13 or by increasing monocyte infiltration into atherosclerotic lesions.14 Therefore, it remains unclear whether VEGF protects the artery from vascular disease or accelerates vascular disease.

Clinical and experimental studies involving arterial gene transfer of VEGF showed that it failed to reduce restenosis after balloon angioplasty.15-17 The role of VEGF in restenotic changes (neointimal formation and negative remodeling) after injury therefore remains a mystery. This is mainly because the inhibitor of VEGF has not been tested for experimental restenosis, although inhibitors of VEGF are currently being evaluated for tumor angiogenesis and other treatment-intractable inflammatory disorders.3 It is practically impossible to investigate the role of VEGF in postnatal life in mice lacking VEGF or its receptors, because the absence of VEGF function leads to embryonic lethality owing to vascular defects.4 A soluble form of the VEGF receptor-1 (sFlt-1) is expressed endogenously by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor against VEGF.18 We and others have demonstrated that intramuscular transfection of the sFlt-1 gene effectively and specifically

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blocks VEGF and thus, “quenches” the activity of VEGF in remote organs in vivo.21,22

The aim of this study was to decisively determine a role for VEGF in restenotic changes after intraluminal injury. We report herein that blockade of VEGF by systemic sFlt-1 gene transfer attenuates the development of neointimal formation after intraluminal injury by inhibiting inflammation, which suggests an essential role for VEGF in the pathogenesis of restenosis after injury. Our present data are clinically important because VEGF gene therapy for therapeutic angiogenesis and restenosis has been attempted in clinical studies.16,17,21

Methods

Expression Vector

The 3.3-kb mouse sFlt-1 gene, originally obtained from the mouse lung DNA library, was cloned into the BamHI (5’) and NotI (3’) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen). Plasmid cDNA3 encoding the luciferase gene was used to detect gene transfection.

Rat and Rabbit Models of Balloon Injury

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 American Physiological Society. A portion of this study was performed at the Kyushu University Station for Collaborative Research.

Twenty-week-old male, normal chow–fed Wistar-Kyoto rats were anesthetized, and their right common carotid arteries were injured by passage (3 times) of an inflated 2F Fogarty balloon catheter.24 Male Japanese white rabbits weighing 3.0 to 3.5 kg were fed a high-cholesterol diet for 2 weeks. Their right common carotid arteries were also injured by passage (3 times) of an inflated 2F Fogarty balloon catheter.24 After injury, all rabbits were fed the same high-cholesterol diet. Three days before balloon injury, the animals were randomly divided into 2 groups: the empty-plasmid group was injected with the empty plasmid, and the sFlt-1 gene was injected into femoral muscle (150 μg/50 μL TE buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0] in rats, 1500 μg/0.5 mL TE buffer in rabbits). To enhance transgene expression, all plasmid-injected animals received electroporation at the injection site immediately after injection with an electric pulse generator (CUY21, BTX) as previously described.19,23–25

Morphometric and Immunohistochemical Analyses

All animals were euthanized by intravenous injection of a lethal dose of sodium pentobarbital. Tissue sections from rabbits and rats were prepared as described and either (1) stained with Masson’s trichrome or elastica van Gieson’s stains or (2) subjected to immunostaining with antibodies against macrophages/monocytes (ED1, Serotec, for rats; RAM11, Dako, for rabbits), proliferating cells (proliferating cell nuclear antigen for rats from Dako, Ki-67 for rabbits from Dako), endothelial cells (CD31, Dako), VEGF (Santa Cruz), VEGFR-1 (Santa Cruz), VEGFR-2 (Santa Cruz), α-smooth muscle actin (Dako), MCP-1 (R&D Systems), interleukin-1β (IL-1β; R&D Systems), or nonimmune mouse IgG (Zymed). After avidin-biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin. Immunofluorescence double staining was performed to localize VEGF and its receptors by the use of fluorescence-conjugated antibodies in rats. Morphometric analysis was performed by microscopy with a computerized digital image-analysis system by a single observer who was blinded to the treatment protocol.

Real-Time Quantitative Reverse Transcription–PCR

Real-time polymerase chain reaction (PCR) amplification was performed with rabbit cDNA by 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 aided by a software program (Applied Biosystems; online Table I). Results were analyzed by sequence detection software (Applied Biosystems), expressed in arbitrary units, and adjusted for glyceraldehyde 3-phosphate dehydrogenase mRNA levels.

Mouse Femoral Wire-Injury Model With Bone Marrow Reconstitution

Intraluminal injury of the femoral artery of wild-type mice whose bone marrow had been replaced with that of ROSA26 mice, which expresses β-galactosidase (LacZ) ubiquitously, was performed.26 Four weeks after bone marrow transplantation, transluminal arterial injury was induced by inserting a straight spring wire (0.38 mm in diameter) into the femoral artery as described. The femoral artery was excised and stained with X-gal solution for 7 hours and then fixed in 4% paraformaldehyde. LacZ-positive cells were counted and expressed as a proportion of the total number of cells. The paraffin-embedded sections were stained with elastica van Gieson’s stain.

Peripheral blood was obtained from the retro-orbital venous plexus of the mice. Fluorescence-conjugated antibodies against CD31 (Pharmingen) and c-Kit (Pharmingen) were used as a circulating monocyte-lineage marker. A fluorescein isothiocyanate–conjugated antibody against Mac-1 (Pharmingen) was used as a circulating monocyte-lineage marker after gating for monocyte cell size with exclusion of granulocytes. Data were analyzed by flow cytometry and appropriate software (Becton Dickinson).

Blood Measurements

Plasma total cholesterol levels in rabbits were determined with commercially available kits (Wako Pure Chemicals). To measure sFlt-1 released by the transfected skeletal muscle, plasma concentrations of sFlt-1 were measured by use of an sFlt-1 ELISA kit (R&D Systems) in rabbits. Concentrations of VEGF in plasma and femoral arterial tissues were also measured in mice by use of an ELISA kit (R&D Systems).

Statistical Analysis

Data are expressed as mean±SE. Statistical analysis of differences was compared by ANOVA and Bonferroni’s multiple-comparison tests. A level of P<0.05 was considered statistically significant.

Results

Increased Expression of VEGF and Its Receptors in Rabbits and Rats

Significant increases in VEGF mRNA levels were detected after balloon injury in rabbits, which peaked on day 7 and persisted until day 28 (Figure 2A). Immunohistochemical staining revealed that VEGF and VEGFR-1 increased in vascular smooth muscle cells in the media and regenerated endothelial layer during the early phase (day 7) and in cells in the neointima, media, and adventitia during the later phase (day 28) after balloon injury in rabbits (Figure 1B). VEGFR-2 did not increase on day 7 but did increase in the injured artery on day 28. sFlt-1 gene transfer reduced the increased immunoreactivities of VEGF, VEGFR-1, and VEGFR-2 on day 28 (Figure 1B).

The localization of VEGF, VEGFR-1, and VEGFR-2 was studied in rats by immunohistochemistry. As observed in rabbits, immunoreactive VEGF and VEGFR-1 increased in the media on days 3 and 7 and in the neointima, media, and adventitia on day 28 (Figure 2A). The increase in immunoreactive VEGFR-2 was less prominent during the early phase but became apparent on day 28 (Figure 2A).
Fluorescence double immunohistochemistry revealed that VEGF and VEGFR-1 were expressed predominantly in α-smooth muscle actin–positive cells in the media and neointima on day 28 (Figure 2B). During the early phase, ED1-positive monocytes recruited into the intima and adventitia expressed VEGFR-1 but not VEGFR-2 (Figure 2C). sFlt-1 gene transfer reduced the increased immunoreactivities of VEGF, VEGFR-1, and VEGFR-2 on day 28 (data not shown).

**Inhibitory Effects of sFlt-1 Transfer on Inflammatory and Proliferative Changes in Rabbits**

As we reported,23,24 inflammatory and proliferative changes became evident by 3, 7, and 28 days after balloon injury in rabbits (Figure 1C and 1D). sFlt-1 gene transfer reduced these inflammatory and proliferating changes.

**Inhibitory Effects of sFlt-1 Transfer on Neointimal Formation and/or Negative Remodeling in Rabbits and Rats**

The carotid arteries in the control and empty-plasmid groups developed significant neointimal formation and negative remodeling (smaller lumen size, internal elastic lamina, and external elastic lamina) in rabbits by day 28 (Figure 3A and 3B). The arteries from the sFlt-1 group showed less neointimal formation, negative remodeling, perivascular fibrosis, and adventitial vasa vasorum; an adventitial VEGFR-2–positive vasa vasorum; and a larger lumen area. There was no significant difference in plasma levels of total cholesterol between the 2 groups (online Table II), indicating that the observed effects of sFlt-1 gene transfer were not caused by changes in serum cholesterol levels. In rats, neointimal
formation was also less in the sFlt-1 group than in the empty-plasmid group on day 28 (Figure 2D).

To assess transfection efficacy of sFlt-1, plasma sFlt-1 concentration was measured in rabbits. The plasma sFlt-1 levels were 96 ± 14, 377 ± 25 (P < 0.01 versus before), 413 ± 20 (P < 0.01), 284 ± 15 (P < 0.05), and 113 ± 16 (P > 0.1) pg/mL before and at 3, 7, 14, and 28 days after sFlt-1 transfection, respectively, indicating that sFlt-1 was released from the transfected muscle to the circulation.

No Significant Effects of sFlt-1 Gene Transfer on Endothelial Regeneration in Rabbits and Mice

In rabbits, there were no significant differences between the empty-plasmid and sFlt-1–transfected groups in the ratio of luminal surface area covered with endothelium (Figure 4A) and that of the CD31-positive endothelial layer 7 days after injury (Figure 4B). In mice, endothelial recovery was scarcely observed on day 7 (data not shown) but was noted equally in the 2 groups on day 14 (Figure 4C).

Inhibitory Effects of sFlt-1 Transfer on Expression of Proinflammatory Factors

sFlt-1 transfection reduced the increased gene expression of MCP-1, platelet-derived growth factor, transforming growth factor-β, IL-1β, IL-6, tumor necrosis factor-α, matrix metalloproteinase-9, and VEGF (Figure 5A). sFlt-1 transfer did not affect the increased gene expression of matrix metalloproteinase-1 and tissue factor. Immunohistochemical staining performed 7 days after balloon injury revealed increased immunoreactive MCP-1 and IL-1β in cells in the neointima and smooth muscle cells in the media, which were attenuated by sFlt-1 gene transfer (Figure 5B).

Contribution of Bone Marrow Cells to the Effect of sFlt-1 Gene Transfer in Mice

As reported,26 a considerable proportion of neointimal and medial cells were LacZ-positive 28 days after injury in mice whose bone marrow expressed LacZ ubiquitously. Intimal area, intima-media ratio, and LacZ-positive cells were decreased in sFlt-1–transfected mice than in empty plasmid–
transfected mice (Figure 6A and 6B). Wire injury also increased recruitment of bone marrow–derived monocytes (CD31-positive and c-Kit–positive) and circulating monocytes (Mac-1–positive) into peripheral blood (Figure 6C). sFlt-1 gene transfer attenuated such changes in cell distribution, suggesting that wire injury increased such cell lineages in peripheral blood through VEGF. Plasma and femoral arterial concentrations of VEGF increased after wire injury, which was partly attenuated by sFlt-1 transfection (online Tables III and IV).

Effects of VEGF₁₆₅ Gene Transfer on Neointimal Formation and Adventitial Angiogenesis in Rabbits
A recombinant adenoviral vector containing human VEGF₁₆₅ or the LacZ gene was produced. Gene transfer was performed by administering adenovirus solution (1 mL, 2×10⁹ plaque-forming units) by a channel balloon catheter (Remedy, Boston Scientific Inc) immediately after balloon injury of rabbit carotid arteries (online Data Supplement and Figure). There were no significant differences between the empty-plasmid and VEGF-transfected groups in terms of neointimal formation, perivascular fibrosis, and negative remodeling (smaller lumen size, internal elastic lamina, and external elastic lamina) on day 28. In contrast, the number of adventitial vasa vasorum (the degrees of adventitial angiogenesis) was markedly increased in the VEGF-transfected group.

Discussion
VEGF has conventionally been thought to be an endothelial cell–specific growth factor and that it inhibits vascular pathological processes by accelerating endothelial proliferation and regeneration through endothelial VEGFR-2. If so, blockade of VEGF would suppress endothelial regeneration...
and enhance neointimal formation after injury. In contrast to the conventional assumption, we here demonstrated that blockade of VEGF by sFlt-1 gene transfer attenuated neointimal formation in rabbits, rats, and mice, indicating the essential role of VEGF in experimental restenosis.

As previously reported by others, we demonstrated persistent increases in VEGF in arterial wall cells after injury. Emerging evidence suggests expression of functional VEGFR-1 and VEGFR-2 in cell types other than endothelial cells. We showed herein an increased expression of VEGFR-1 in lesional monocytes and medial smooth muscle cells during the early stage and in smooth muscle cells in the neointima and media during later stages. Increased VEGFR-2 expression was noted only at later stages. sFlt-1 gene transfer attenuated the increased expression of inflammatory and growth factors such as VEGF, MCP-1, IL-1β, and so forth at early stages. Expression of VEGFR-1 in monocytes mediates chemotaxis, and VEGFR-1 in smooth muscle cells mediates migration. VEGFR-1 has been shown to act as an important mediator of VEGF-induced inflammation. More recently, Yamada et al showed that VEGF-mediated angiogenesis and inflammation are mediated by MCP-1. We also demonstrated the central role of MCP-1 in the mechanism of neointimal formation after vascular injury. It is likely, therefore, that VEGF might cause vascular inflammation and migration of vascular smooth muscle cells and thus, cause neointimal formation after injury. Further studies are needed to elucidate the relative roles of VEGFR-1 and VEGFR-2 in the mechanisms of neointimal formation.

This study also demonstrated in rabbits the role of VEGF in the development of negative remodeling, another major cause of human restenosis after balloon angioplasty.
sis and vasa vasorum in the adventitia have been implicated to be the central pathological processes leading to constrictive negative remodeling after balloon injury. Therefore, our present data suggest that \textit{sFlt-1} gene transfer inhibited the development of negative constrictive remodeling by limiting adventitial fibrosis and angiogenesis.

VEGFR-1 has been shown to be an important mediator of stem cell recruitment and differentiation.

Sata and colleagues have shown that a considerable proportion of neointimal and medial cells were bone marrow–derived progenitor cells. However, the role of VEGF in the recruitment and differentiation of progenitor cells into neointimal cells after vascular injury has not been addressed. We showed here that \textit{sFlt-1} gene transfer inhibited recruitment of bone marrow–lineage cells into the peripheral circulation and injured arterial wall and thus, reduced neointimal formation after injury. These data suggest that VEGF might contribute to neointimal formation by recruiting bone marrow–derived and circulating monocytes.

Surprisingly, \textit{sFlt-1} gene transfer did not affect endothelial regeneration after endothelial injury, suggesting a minor role of endogenous VEGF in endothelial regeneration after endothelial injury. It remains to be determined whether inhibition of VEGFR-2–mediated activity of endothelial regeneration by \textit{sFlt-1} gene transfer might have been overshadowed by other stimuli (eg, basic fibroblast growth factor, angiopoietins, etc). On the contrary, adenovirus-mediated gene transfer of VEGF enhanced adventitial angiogenesis but did not reduce neointimal formation after balloon injury in rabbits. The latter observation is consistent with previous reports demonstrating that exogenous VEGF does not reduce neointimal formation in animals and humans. Taken together, the role or mechanisms of action of VEGF may differ between exogenous and endogenous VEGF and between angiogenesis and neointimal formation.

This study may have significant clinical implications. First, \textit{sFlt-1} gene transfer might be an attractive anti-VEGF therapy for inflammatory vascular disease and other inflammatory disorders. However, local delivery of \textit{sFlt-1} must be preferable for clinical use to avoid potential side effects by systemic delivery. Second, our finding indicates that more research is required, especially on the safety of VEGF, before translational clinical research proceeds. Deleterious effects associated with overexpression of VEGF have recently been reported: (1) injection of VEGF-expressing skeletal muscle myoblasts into the murine heart caused the formation of hemangiomas and lethagic heart failure and (2) VEGF gene transfer into rabbit carotid arteries induced neointimal

Figure 5. Inhibitory effect of \textit{sFlt-1} gene transfer on expression of various inflammatory factors in rabbits. A, Effect of \textit{sFlt-1} gene transfer on mRNA levels of various proinflammatory factors 1 day after injury. Quantitative real-time PCR was performed. * \(P<0.01\) vs uninjured control (uninjured) artery; † \(P<0.05\), †† \(P<0.01\) vs empty-plasmid group. B, Carotid artery sections from control uninjured animals and those from empty-plasmid and \textit{sFlt-1} groups 7 days after balloon injury stained immunohistochemically with MCP-1 and IL-1β. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=100 \(\mu\text{m}\). Immunohistochemical experiments were repeated 5 times, all with representative results.
formation with angiomatoid proliferation of endothelial cells. These studies highlight the potential side effects or toxicity that would against the clinical use of VEGF for therapeutic angiogenesis and restenosis.

In conclusion, this study has provided direct in vivo evidence suggesting that increased expression and activity of VEGF are essential for the development of experimental restenosis after intraluminal injury by recruiting monocyte-lineage cells. Although there is no clinical evidence suggesting enhancement of atherosclerosis or neointimal formation by VEGF therapy, our present data raise the question of whether VEGF therapy is useful without serious risks in patients with advanced atherosclerosis.

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