Vascular Endothelial Growth Factor Is Necessary in the Development of Arteriosclerosis by Recruiting/Activating Monocytes in a Rat Model of Long-Term Inhibition of Nitric Oxide Synthesis

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Background—It remains unclear whether vascular endothelial growth factor (VEGF) is a proarteriosclerotic or an antiarteriosclerotic factor. We recently reported that long-term inhibition of nitric oxide by administering Nω-nitro-L-arginine methyl ester (L-NAME) induces coronary vascular inflammation and arteriosclerosis.

Methods and Results—We used this animal model to investigate the role of VEGF in arteriosclerosis. We blocked VEGF activity in vivo by transfecting with plasmid DNA encoding the murine soluble FLT-1 (sFLT-1) gene into thigh muscle. Soluble FLT-1 can suppress VEGF activity both by sequestering VEGF and by functioning as a dominant-negative inhibitor of VEGF receptors. We observed vascular inflammation associated with increased VEGF expression within 3 days of L-NAME administration, which was prevented by pretreatment with ACE inhibitor, angiotensin II receptor antagonist, or neutralizing monocyte chemoattractant protein-1 antibody. The sFLT-1 gene transfer attenuated the early vascular inflammation and prevented late arteriosclerosis. The sFLT-1 gene transfer also inhibited increased expression of monocyte chemoattractant protein-1 and transforming growth factor-β, indicating creation of a positive feedback loop to cause arteriosclerosis.

Conclusions—VEGF is necessary in the development of arteriosclerosis by mediating monocyte recruitment and activation in this model. (Circulation. 2002;105:1110-1115.)

Key Words: endothelium-derived factors • remodeling • inflammation • growth substances • gene therapy

vascular endothelial growth factor (VEGF) can induce endothelial cell migration and growth, differentiation/regeneration, and angiogenesis.1,2 These unique actions of VEGF have been thought to protect the artery from arteriosclerosis and atherosclerosis.3,4 However, there is a considerable debate over VEGF.5 It was reported that VEGF can induce migration and activation of monocytes through its receptor FLT-1,6 adhesion molecule,7 or monocyte chemoattractant protein-1 (MCP-1)8 and that administration of VEGF protein to hypercholesterolemic animals enhances atherogenesis.9 These recent reports suggest that VEGF may increase atherogenesis by inducing activation and migration of monocytes or by inducing intraplaque angiogenesis. Therefore, it remains unclear whether VEGF is a proarteriosclerotic or an antiarteriosclerotic factor.

The major reason for the inconsistent reports regarding proarteriosclerotic versus antiarteriosclerotic effects of VEGF may be due largely to the fact that effects of the selective inhibitor of VEGF have not been tested. Because the absence of VEGF results in defective vascularization and early embryonic lethality, it is practically impossible to investigate the pathogenic role of VEGF in postnatal life in VEGF-deficient animals.1,2 Although antibodies against VEGF contributed to the understanding for the role of VEGF, it remains unclear that the antibodies might attain sufficient concentration in sites of injury or might be neutralized by the host immune response. The currently known selective and specific inhibitor of VEGF is a soluble form of the FLT-1 VEGF receptor (sFLT-1).10 This isoform 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 receptors.10 Therefore, sFLT-1 can be used as a selective and potent inhibitor of VEGF in vivo.

VEGF has been shown to be regulated reciprocally against endothelium-derived nitric oxide (NO) to control endothelial
integrity after arterial injury. However, it remains unknown whether VEGF attenuates or promotes arteriosclerosis after inhibition of NO synthesis or endothelial injury. We and others have recently reported that long-term inhibition of NO by administering an inhibitor of NO synthase, N\textsuperscript{\text{-}} nitro-L-arginine methyl ester (L-NAME), induces coronary vascular inflammation (monocyte infiltration, MCP-1 expression, increased activity of ACE, and nuclear factor-\kappaB) and subsequent arteriosclerosis. Treatment with ACE inhibitor or angiotensin II receptor antagonist prevented the early vascular inflammation and subsequent arteriosclerosis. Therefore, this model may be useful to investigate the role of cytokines such as VEGF in the development of arteriosclerosis. We examined whether blockade of VEGF signals by sFLT-1 gene transfer into skeletal muscles can attenuate vascular inflammation and arteriosclerosis in a rat model of long-term inhibition of NO synthesis. This study demonstrates that transfection of sFLT-1 gene in skeletal muscles inhibits coronary vascular inflammation and subsequent arteriosclerosis.

**Methods**

**Expression Vector**

The 3.3-kb mouse sFLT-1 gene, originally obtained from the mouse lung DNA library, was cloned into the BamH1 (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.

**Animal Model of Long-Term Inhibition of NO Synthesis**

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 part of this study was performed at the Kyushu University Station for Collaborative Research.

Male Wistar-Kyoto rats, bred as an established colony at the Animal Research Institution of Kyushu University and housed singly in a pyrogen-free facility and fed untreated rat chow, were studied. The control group received untreated chow and drinking water. The L-NAME group received L-NAME in drinking water (1 mg/kg). The L-NAME+plasmid group received L-NAME plus an intramuscular injection of empty plasmid (500 μg/150 μL PBS) into the femoral muscles with a 27-gauge needle 3 days before L-NAME administration began and 2 weeks thereafter. The L-NAME+sFLT-1 group received L-NAME plus an intramuscular injection of sFLT-1 plasmid on the same time schedule. To enhance transgene expression, the third and fourth animal groups received electroporation at the injected site immediately after injection. Six electric pulses of 100 V for 50 ms were applied with the use of an electric pulse generator CUY21 (BTX). Other groups of rats received D-NAME, L-NAME plus imidapril (an ACE inhibitor from Tanabe Pharmaceutical Co), L-NAME plus candesartan (an angiotensin II receptor blocker from Takeda Pharmaceutical Co), L-NAME plus TAK-044 (an endothelin receptor antagonist from Tanabe Pharmaceutical Co, 1 mg/kg per day), or L-NAME plus monomoclonal antibody against rat MCP-1 (2 mg/kg per day).

At various time points, systolic blood pressure was measured by the tail-cuff method. Venous blood was collected and the rats were killed for morphometric, immunohistochemical, and biochemical analysis. The hearts were then isolated and either fixed in a methacarn solution or snap-frozen in liquid nitrogen and stored at −80°C.

**Histopathology and Immunohistochemistry**

Tissue sections were prepared as described and either stained with Masson’s trichrome or subjected to immunostaining with antibodies against macrophage/monocyte (ED1, Serotec), proliferating cell nuclear antigen (PCNA) (Dako), α-smooth muscle actin antibody (1 μg/mL, Dako), MCP-1, transforming growth factor (TGF)-β, (AHG0051, Biosource), or nonimmune mouse IgG (Zymed). The slides were washed and incubated with biotinylated, affinity-purified goat anti-rabbit IgG. After avidin-biotin amplification, the slides were incubated with diaminobenzidine and counterstained with hematoxylin.

**Morphometry and Cell Counting**

Morphometry and cell counting were performed by a single observer who was blind to the treatment protocols. Each section (5 per heart) stained with an antibody against ED1 or PCNA was scanned. The number of positive cells in each section was determined. The average number of positive cells per section was determined for each animal.

To evaluate the thickening of the coronary arterial wall and the extent of perivascular fibrosis, short-axis images of the coronary arteries were analyzed. The wall-to-lumen ratio (the ratio of the medial thickness to the internal diameter) and perivascular fibrosis were estimated.

**Northern Blot Analysis**

Total RNA was extracted from each sample, poly (A)+RNA was purified, and Northern blot hybridization was performed as we have described previously. The cDNA probes used were rat MCP-1, rat TGF-β, and mouse GAPDH (American Type Culture Collection).

**In Vivo Matrigel Plug Assay**

VEGF-induced angiogenesis was examined by the matrigel plug assay. Matrigel (300 μL) alone or mixed with VEGF164 at a concentration of 100 ng/mL was injected subcutaneously into the flank of rats. The rats were injected with intramuscular plasmid with or without sFLT-1 gene as described above. On days 1 (n=3), 5 (n=3), 7 (n=5), and 14 (n=5), the rats were killed; plugs were removed and fixed, paraffin was embedded, and staining was done with hematoxylin and eosin. In a separate set of rats injected with empty plasmid or sFLT-1 gene (n=4 each), angiogenesis induced by MCP-1 (matrigel with MCP-1 at 100 ng/mL) was examined.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was prepared from the carotid arteries with the use of an RNA purification kit (QUIAGEN) and reverse-transcribed using murine leukemia virus reverse transcriptase. Quantity and quality of mRNA from all samples were certified by reverse transcription–polymerase chain reaction (RT-PCR) amplification of the GAPDH gene. Amplification was done with the Perkin-Elmer Gene Amp PCR system 9700. PCR reactions were carried out with the following primers used for VEGF: sense primer 5’-GAC CCT GGT GGA CAT TCT CCA GGA-3’ and antisense primer 5’-GTT GAG AGG TCT AGT TCC CGA-3’ with expected sizes of 514, 462, and 330 bp for amplification of VEGF188, VEGF 164, and VEGF120, respectively. Primers for the internal control, GAPDH, were included in each reaction: sense primer 5’-TCA CCA TTT ACC ACC TTC CTT TTG A-3’ and antisense primer 5’-CTG CTT ACC ACC TTC TGG A-3’. Primers for the internal control, GAPDH, were included in each reaction: sense primer 5’-TCA CCA TTT ACC ACC TTC CTT TTG A-3’ and antisense primer 5’-CTG CTT ACC ACC TTC TGG A-3’.

**Measurements of ACE Activity and Plasma NOx Concentration**

Five rats in each group were killed on the third day of treatment. Cardiac tissue was isolated and the ACE activity was measured by means of fluorometric assay as previously described. Tissue ACE activity was calculated as nanomoles of His-Leu generated per milligram of tissue weight per hour. Plasma NOx concentration was measured by a fluorometric assay with a commercially available NOx assay kit (NOx/NO2, Assay Kit-F, Wako).
Figure 1. Angiogenesis in matrigel plugs. A, Histological appearance of matrigel plug sections with or without VEGF from rat with no treatment and rat transfected with empty plasmid or sFLT-1 plasmid after 7 days. Similar suppression of VEGF-induced angiogenesis is noted after 14 days. S indicates stroma surrounding plugs. Arrows point to endothelial cells forming vessels. B, Histological appearance of matrigel plug sections with or without VEGF from rat with no treatment and rat transfected with empty plasmid or sFLT-1 plasmid 7 days after injection.

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

Results

In Vivo Matrigel Plug Assay
Histological sections of the matrigel plugs showed a significant angiogenic effect induced by VEGF or MCP-1 compared with matrigel alone (Figure 1A). Soluble FLT-1 gene transfer suppressed the angiogenesis induced by VEGF to a level similar to that observed in the control matrigel plugs lacking VEGF. Injection of empty plasmid did not show such suppression. Soluble FLT-1 gene transfer had no effect on the angiogenesis induced by MCP-1 (Figure 1B).

Transgene expression in the transfected muscle was examined by the luciferase assay. Compared with PBS-injected muscle (50±10 RLU/mg protein), the luciferase activity of the muscles injected with luciferase gene plasmid on days 1, 5, 7, and 14 was 2.6±0.1, 7.8±0.5, 9.8±0.9, and 8.8±0.6×10^7 RLU/mg protein (n=6 to 8 each, all P<0.01 versus PBS, respectively).

Systolic Arterial Pressure and ACE Activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>ACE Activity in the Left Ventricle, nmol · mg^-1 · h^-1</th>
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<tr>
<td>Control</td>
<td>Day 0 134±4</td>
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<tr>
<td></td>
<td>Day 3 135±2</td>
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<tr>
<td></td>
<td>Day 28 140±4</td>
<td>NM</td>
</tr>
<tr>
<td>L-NAME</td>
<td>Day 0 138±2</td>
<td>NM</td>
</tr>
<tr>
<td></td>
<td>Day 3 162±6†</td>
<td>1.5±0.2†</td>
</tr>
<tr>
<td></td>
<td>Day 28 162±5†</td>
<td>NM</td>
</tr>
<tr>
<td>L-NAME+empty plasmid</td>
<td>Day 0 130±4</td>
<td>NM</td>
</tr>
<tr>
<td></td>
<td>Day 3 166±6†</td>
<td>1.6±0.1†</td>
</tr>
<tr>
<td></td>
<td>Day 28 172±4†</td>
<td>NM</td>
</tr>
<tr>
<td>L-NAME+sFLT-1</td>
<td>Day 0 132±2</td>
<td>NM</td>
</tr>
<tr>
<td></td>
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<td>Day 28 168±3†</td>
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<td>L-NAME+hydralazine</td>
<td>Day 3 170±5†</td>
<td>1.6±0.3†</td>
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<tr>
<td>L-NAME+TAK-044</td>
<td>Day 3 172±6†</td>
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<td>L-NAME+MCP-1Ab</td>
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<tr>
<td>D-NAME</td>
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</table>

Data are mean±SEM (n=6 to 8). NM indicates not measured. *P<0.01 vs day 0; †P<0.01 vs control group; ‡P<0.01 vs L-NAME+empty plasmid group.

not differ among the L-NAME, L-NAME+plasmid, and L-NAME+sFLT-1 groups.

Rats that received L-NAME, L-NAME+imidapril, L-NAME+candesartan, or L-NAME+hydralazine showed no significant changes in systolic arterial pressure. In contrast, rats that received L-NAME+TAK-044 or L-NAME+MCP-1 antibody showed a significant rise in systolic blood pressure (Table).

Cardiac tissue ACE activity was significantly higher in the L-NAME group than in the control group (Table). As we reported,12,14,15 the increase in tissue ACE activity was not affected by hydralazine, TAK-044, or MCP-1 antibody but was reduced by candesartan and suppressed by imidapril.

Increased Expression of VEGF
A significant increase in the cardiac VEGF mRNA level was detected during the course of L-NAME administration, which peaked on day 3 and declined from day 3 to day 28 with further administration of L-NAME (Figure 2, A and B). At day 28, no significant increases in gene expression of three
isoforms of VEGF were detected in the L-NAME group (Figure 2, A and B).

To determine the mechanisms of increased VEGF expression after L-NAME administration, effects of various inhibitors were examined (Figure 2C). Treatment with imidapril or candesartan but not with hydralazine or TAK-044 prevented the L-NAME–induced increases in VEGF expression. Also, MCP-1 antibody prevented the L-NAME–induced increase in VEGF expression. Immunohistochemical staining studies showed that immunoreactive VEGF, Flt-1, and Flk-1 were faintly observed in the endothelial layer of control rats (Figure 3A). Such endothelial staining with VEGF and VEGF receptors was more intense in the L-NAME group than in the control group. Most of monocytes infiltrated into the inflammatory lesions of coronary artery seen in the L-NAME group were stained intensely with VEGF on day 3 (Figure 3A). Increased immunoreactivity of Flt-1 and Flk-1 was observed in the endothelial layer in the L-NAME group on day 3 (Figure 3A). The VEGF-positive and VEGF receptor–positive cells became less prominent from day 3 to days 7 and 28 (data not shown).

Marked increases in ED1-positive monocytes in the intima and perivascular area of coronary arteries was also observed in the L-NAME group on day 3 (Figure 3B). Nuclear staining with PCNA antibody was observed in arterial wall cells such as endothelial cells, smooth muscle cells, and myofibroblast (Figure 3B). These inflammatory and proliferative changes peaked on day 3 and declined from day 3 to day 28. No evidence of such inflammation was observed in the control group and in rats that received D-NAME.

**Inflammatory and Proliferative Changes on Day 3**

When ED1-positive monocytes or PCNA-positive cells were counted, the number of immunopositive cells per section was significantly greater in the L-NAME and L-NAME+plasmid groups than in the control group (Figures 3B, 4A, and 4B). The increases in ED1-positive cells and PCNA-positive cells were both markedly reduced by sFLT-1 gene transfer. The sFLT-1 gene transfer also reduced the appearance of α-smooth muscle actin–positive myofibroblasts (data not shown).

The L-NAME–induced increase in immunoreactive VEGF, Flt-1, and Flk-1 was reduced to the baseline level by sFLT-1 gene transfer but not by empty plasmid transfection (data not shown).

**Arteriosclerosis on Day 28**

Arteriosclerotic changes (increases in the wall-to-lumen ratio and perivascular fibrosis) of coronary arteries seen in the L-NAME group were significantly inhibited by sFLT-1 gene transfer but not by empty plasmid injection (Figures 3C, 4C, and 4D).

**Expression of MCP-1 and TGF-β1**

Cardiac MCP-1 and TGF-β1 mRNA levels were significantly greater in the L-NAME group (Figure 5). The increased expression of MCP-1 and TGF-β1 mRNA was prevented by sFLT-1 gene transfer but not by empty plasmid injection. In addition, the increased immunoreactive MCP-1 and TGF-β1 was prevented by sFLT-1 gene transfer but not by empty plasmid injection (Figure 3D).

**Plasma NOx Concentration**

Plasma NOx concentration was significantly decreased in the L-NAME group (0.8±0.1 μmol/L, P<0.01 versus control) compared with the control group (4.7±0.4 μmol/L). Transfection of empty plasmid or sFLT-1 plasmid did not affect the L-NAME–induced decrease in plasma NOx levels (0.7±0.1 μmol/L in L-NAME+plasmid group, 0.7±0.1 μmol/L in L-NAME+sFLT-1 group).

**Discussion**

The novel findings of the present study are that blockade of VEGF by intramuscular transfection of the sFLT-1 gene attenuated early inflammatory and proliferative changes of the coronary artery and late arteriosclerosis caused by long-term inhibition of NO synthesis. We have shown rapid and transient increase in VEGF genes and immunoreactive VEGF and its receptors after L-NAME administration. We also...
found that increased local activity of angiotensin II mediated by type I receptors may be responsible for the increase in VEGF activity. Angiotensin II has been shown to induce redox-sensitive transcription of MCP-1 or VEGF in vascular wall cells. Our data with the neutralizing antibody against MCP-1 and the matrigel assays indicate that lesional monocytes may be responsible mainly for increased VEGF expression after L-NAME administration.

The present study is the first to address the role of VEGF in coronary vascular inflammation and arteriosclerosis in the rat model of long-term inhibition of NO synthesis. We showed that intramuscular transfection of sFLT-1 gene plasmid suppressed VEGF-induced angiogenesis in matrigel plugs, indicating that sFLT-1 gene transfer blocked VEGF activity in remote organs in vivo. Therefore, our present data suggest that VEGF induced the migration and activation of monocytes at least in part through its effect on the monocyte receptor. Our present data regarding prevention of MCP-1 gene expression and activity by sFLT-1 gene transfer also suggest that VEGF induced monocyte chemotaxis through increasing MCP-1 activity. The latter finding is in agreement with a recent report demonstrating that VEGF increases MCP-1 gene expression and activity in endothelial cells in vitro. Because sFLT-1 gene transfer reduced proliferative changes (appearance of PCNA-positive cells) in the arterial wall, it is likely that lesional monocytes activated vascular smooth muscle cells and fibroblasts through monocyte-derived growth factors. Because VEGF is relatively specific for endothelial cells and monocytes, it is unlikely that VEGF acted directly to vascular smooth muscle cells or fibroblasts. Our present data also suggest that once VEGF or MCP-1 is induced and causes monocyte chemotaxis, such pathological
conditions may create a positive feedback mechanism to further enhance inflammatory and proliferative changes in the arterial wall.

In conclusion, this study provided direct in vivo evidence suggesting that VEGF may participate in the development of coronary arteriosclerosis by recruiting and activating monocytes through the FLT-1 VEGF receptor in monocytes or through inhibition of MCP-1 activity in a rat model of long-term inhibition of NO synthesis. Our current observation is in agreement with recent reports demonstrating that VEGF gene transfer into rabbit carotid arteries induces neointimal formation. Therefore, locally produced VEGF appears to exhibit proarteriosclerotic actions. A caveat for interpreting our present finding is that neither atherosclerotic plaque or intraplaque angiogenesis was observed in our model. It might be of clinical interest to know whether administration of VEGF protein or VEGF gene transfer increases or decreases atherogenesis in a certain subset of patients with advanced atherosclerosis because such patients are candidates of therapeutic angiogenesis with VEGF.

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References

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