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Background—The role of vascular endothelial growth factors (VEGFs) in large arteries has been proposed to be either vasculoprotective or proatherogenic. Because VEGF family members are used for human therapy, it is important to know whether they could enhance atherogenesis. We tested the effects of the members of the VEGF gene family on atherogenesis in LDL-receptor/apolipoprotein (apo) B48 double-knockout (LDLR/apoB48) mice using systemic adenoviral gene transfer.

Methods and Results—Six groups of LDLR/apoB48-deficient mice (n = 110) were kept 3 months on a Western-type diet. After 6 weeks of diet, mice were injected via tail vein with recombinant adenoviruses expressing VEGF-A, -B, -C, or -D or LacZ (1 × 10^9 PFU) or rhVEGF-A protein (2 μg/kg) and euthanized 6 weeks later. Also, older mice (n = 36) were injected after 4 months on the diet and euthanized 6 weeks later (total time on the diet, 22 weeks) to evaluate the effects of gene transfers on the development of more mature lesions. Aortas were analyzed for the presence of macroscopic lesions, cross-sectional lesion areas, neovascularization, and cellular composition of the lesions. All groups had equivalent plasma cholesterol and triglyceride levels. Gene transfers with recombinant adenoviruses or administration of rhVEGF-A protein had no statistically significant effects on en face atherosclerotic lesions in the aorta, cross-sectional lesion area, neovascularization, or cellular composition of the lesions.

Conclusions—This study shows no proatherogenic effects of adenovirus-mediated gene transfers of VEGF-A, -B, -C, or -D in the LDLR/apoB48-deficient hypercholesterolemic mice, in which lipoprotein profile and atherosclerosis closely resemble those in human disease. (Circulation. 2005;112:1347-1352.)

Key Words: genes ■ atherosclerosis ■ gene transfer

Members of the vascular endothelial growth factor (VEGF)-gene family play important roles in vascular biology. It has been shown that several cell types express VEGF-A in atherosclerotic lesions and that expression of VEGF-A increases during atherogenesis. Also, VEGF-D is expressed in normal human arteries and atherosclerotic lesions. VEGF-A has been proposed to have several roles in the arterial wall: it can be protective by increasing the production of nitric oxide and prostacyclin and by lowering LDL toxicity. It has also been shown to mediate antiapoptotic effects in endothelium. Conversely, the capability of VEGF-A to stimulate monocyte/macrophage influx into the vessel wall suggests that it may contribute to atherogenesis. It has recently been shown that VEGF-A and angiogenesis are associated with atherosclerotic lesion formation in apolipoprotein (apo) E-knockout mice and cholesterol-fed rabbits.

See p 1248

bits. To gain a more comprehensive view about the issue, we decided to test the effects of VEGF-A, -B, -C, and -D on atherogenesis in LDL receptor (LDLR)/apoB48-deficient mice. Unlike in apoE-knockout mice, in these mice, most of the cholesterol is carried in apoB100-containing LDL, and the lipoprotein profile more closely resembles that in humans.
Also, macrophages in LDLR/apoB48-deficient mice can normally produce and secrete apoE. We chose adenoviral gene transfer as a tool to achieve effective transient systemic gene expression during a few weeks’ time, because this approach has already been applied in clinical trials. We also tested the effect of recombinant human (rh) VEGF-A protein to compare this model with the previous results obtained in apoE-knockout mice. It was found that adenovirus-mediated VEGF gene transfers had no effect on atherogenesis in LDLR/apoB48-deficient hypercholesterolemic mice.

Methods

Experimental Animals

LDLR/apoB48-deficient mice (n=110) were kept on a Western-type diet (Teklad Adjusted Calories, consisting of 21% [wt/wt] fat and 0.15% cholesterol without sodium cholate) for 3 months. Mice were put on the diet at the age of 2 months. After 6 weeks on the diet, the mice were injected via tail vein with recombinant E1–partial E3-deleted first-generation adenoviruses (1×10⁷ PFU): Ad-VEGF-A, Ad-VEGF-B, Ad-VEGF-C, and Ad-VEGF-D or control AdLacZ. Generation, production, and functionality of the adenoviruses containing cDNAs for human VEGF-A165, -B167, -C (full-length), and -D (proteolytically ΔNΔC-processed form) under the cytomegalovirus promoter have been described previously. One group of mice was treated with rhVEGF-A protein (2 µg/kg), and another control group of mice was injected with saline. Each group contained 15 to 20 mice, which were euthanized 6 weeks later. Another group of mice (n=36) was kept on the diet for 4 months, transduced similarly with adenoviruses or injected with rhVEGF-A protein or saline, and euthanized 6 weeks later (total time on diet, 22 weeks) to evaluate the effect of gene transfer on more established lesions. Diet and water were provided ad libitum. During the gene transfer, the animals were anesthetized with fentanyl-fluanisone (3.15 and 10 mg/kg)/midazolam (5 mg/kg) subcutaneously and euthanized with carbon dioxide.

All animal experiments were approved by the Experimental Animal Committee of Kuopio University. Blood samples were taken after 12 hours of fasting, and plasma lipid levels were measured with enzymatic kits: cholesterol (Ecoline 25, Merck Diagnostica, CHOD-PAP method) and triglycerides (Ecoline 25, Merck Diagnostica, GPO-PAP method). Circulating hVEGF-A, -B, -C, and -D levels were analyzed from the tail-vein blood samples at various time points after the gene transfer with ELISA (R&D; Quantikine, human VEGF-A and VEGF-D and Zymed ELISA kit for VEGF-C). To measure rhVEGF-B167, a sandwich ELISA was developed. Essentially, microtiter plates were coated with 3 µg/mL of anti–human VEGF-B monoclonal antibody (MAB3372, R&D Systems) in 50 µL of 100 mmol/L NaHCO₃ overnight at 4°C. Residual binding capacity of the plates was blocked by incubating with 50 µL PBS containing 3% BSA for 30 minutes at room temperature. The wells were then incubated overnight at 4°C with 50 µL of the diluted plasma samples.

Generally, the samples were analyzed by use of at least 2 different dilutions in duplicate. rhVEGF-B167 protein was used as a standard. After extensive washing with PBST, 50 µL of affinity-purified rabbit anti–VEGF-B immunoglobulin at a concentration of 1 µg/mL in PBS containing 1% BSA was added to detect antibody-bound VEGF-B167 and incubated for 2 hours at room temperature. After extensive washings as above, bound rabbit immunoglobulin was detected by incubating with 50 µL of anti–rabbit-IgG conjugated with alkaline phosphatase for 1.5 hours at room temperature using a 1:3000 dilution in 1% BSA-PBS. After extensive washing with PBST, bound enzyme conjugates were quantified and visualized with NPP substrate (Sigma-Aldrich). After the reaction had been stopped with 25 µL of 0.5 mol/L NaOH, the absorbance was measured at 405 and 650 nm with an ELISA plate reader. Proteins from the aortas were extracted with T-PER Tissue Protein extraction reagent including Halt protease inhibitors and analyzed with ELISA (R&D; Quantikine, human VEGF-165 and mouse VEGF-164). Total protein contents were assayed with a BCA Protein Assay kit (Pierce).

Quantification of Atherosclerotic Lesions

The arterial tree was perfused with PBS with buffered formalin (10%) and postfixed with 10% formalin for 2 hours. The whole aorta from the arch to the bifurcation was dissected from 8 to 12 mice in each group as described previously. These aortas were opened longitudinally and analyzed for the presence of en face macroscopic lesions with an MCID/M4-image analyzer (Imaging Research). Aortas from the rest of the mice were embedded in paraffin to evaluate cross-sectional lesion areas and cellular composition of the lesions. Complicated lesions were defined by the presence of atheroma and/or cholesterol crystals covered by several layers of cells and/or connective tissues. Cross-sectional lesion areas were quantified as whole lesion areas surrounding the vascular ring from the aortic sinus level, which is recognized by the presence of 3 valve cusps. The percentage of macrophages of the total lesion areas was also determined from the same sinus level (Analysis program, Media Cybernetics). All analyses were performed blindly without knowledge of the origin of the samples.
Ad-VEGF-A gene transfer from aortic extracts with ELISA and found that human VEGF-A was present at 4 to 10 times higher concentration than mouse endogenous VEGF-A (451±290 pg/mg hVEGF-A165, compared with 59±20 pg/mg mVEGF-A164).

No statistically significant differences were found between the study groups in serum total cholesterol or triglyceride levels, en face atherosclerosis, or atherosclerotic lesion areas measured from histological sections taken from the aortic sinus level (Table and Figure 2). Atherosclerotic lesions were rich in macrophage-derived foam cells, and staining patterns were similar in all groups (Figure 3). No differences were found in the percentage of lesion macrophages, which varied between 40% and 70% of the lesion areas (Table). We did not find any differences in the prevalence of complicated lesions. Even if there were more newly formed capillaries in the livers, especially in the VEGF-A and -D–transduced groups, no increased neovascularization was found in the atherosclerotic lesions or in the aortic adventitia (Figure 4).

Discussion

On the basis of recent animal studies, concerns have been raised about the potential proatherogenic effects of the VEGF gene or protein therapies. Celletti et al9 reported increased atherogenesis in apoE-deficient mice and cholesterol-fed rabbits, and Moulton et al10 showed that angiogenesis inhibitors can reduce atherogenesis in apoE-deficient mice. Because several members of the VEGF gene family are currently in clinical trials, it is important to clarify potential proatherogenic effects of the members of the VEGF family. We decided to use systemic delivery of adeno viruses encoding VEGF-A, -B, -C, and -D in LDLR/apoB48-deficient mice to gain further insights into the issue. Even though most human trials have used local intracoronary or intramyocardial delivery of VEGF genes or recombinant proteins, systemic delivery is the only feasible nontraumatic approach for gene or recombinant protein delivery in mice. However, we recognize that our results do not exclude the possibility of VEGF-derived harmful effects potentially caused by higher doses or alternative delivery routes.

In this study, we found no evidence that transient expression of the members of the VEGF gene family would cause any proatherogenic changes in mouse aortas. The results are in line with results from recent clinical

### Statistical Analysis

Calculations of the en face lesion areas, cross-sectional lesion areas, and areas with neovascularization were performed independently 3 times, and average values are reported. Data are expressed as mean±SD and were compared between the groups by ANOVA, followed by modified t test (SPSS 7.5, SPSS Inc). A value of \( P<0.05 \) was considered significant.

## Results

Tail-vein injections of adenoviruses led to transgene expression primarily in the liver.18 Growth factors were secreted into the systemic circulation as detected by ELISA assays. Human VEGF-A, -B, -C, and -D were still detectable in the circulation 4 to 6 weeks after the gene transfer, although the peak expression was achieved 4 days after the gene transfer (Figure 1). Human VEGF-A protein was detectable only a few minutes after the intravenous injection of rhVEGF-A and was already below the detection limit after 15 minutes (data not shown). Serum levels of the endogenous mouse VEGFs in nontransduced control mice or in mice transduced with LacZ adenovirus were not detectable with the human assays. We also analyzed the levels of human VEGF-A 5 days after the

<table>
<thead>
<tr>
<th>Groups</th>
<th>Triglycerides, mmol/L</th>
<th>Total Cholesterol, mmol/L</th>
<th>Total Lesion Area/Total Aorta, %</th>
<th>12 Weeks on Diet</th>
<th>22 Weeks on Diet</th>
<th>Cross-Sectional Lesion, %</th>
<th>Macrophages/Total Lesion Area, %</th>
<th>12 Weeks on Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>2.0±0.9 (11)</td>
<td>35.2±5.9 (11)</td>
<td>15.6±2.0 (12)</td>
<td>31.7±5.6 (7)</td>
<td>53.2±4.1 (7)</td>
<td>56.2±10.4 (7)</td>
<td></td>
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</tr>
<tr>
<td>VEGF-B</td>
<td>2.4±0.8 (11)</td>
<td>37.6±6.4 (12)</td>
<td>14.5±2.2 (12)</td>
<td>33.0±4.7 (6)</td>
<td>48.7±2.2 (6)</td>
<td>54.8±11.1 (6)</td>
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<tr>
<td>VEGF-C</td>
<td>1.5±0.4 (8)</td>
<td>33.1±5.1 (8)</td>
<td>15.1±3.1 (8)</td>
<td>34.4±4.8 (4)</td>
<td>ND</td>
<td>55.0±11.1 (4)</td>
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</tr>
<tr>
<td>VEGF-D</td>
<td>1.6±0.4 (11)</td>
<td>33.2±7.0 (12)</td>
<td>15.7±1.9 (11)</td>
<td>33.7±5.3 (4)</td>
<td>51.5±4.8 (5)</td>
<td>53.9±11.9 (4)</td>
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<tr>
<td>LacZ</td>
<td>1.9±0.8 (12)</td>
<td>33.4±7.9 (9)</td>
<td>14.8±2.5 (7)</td>
<td>32.1±3.6 (8)</td>
<td>54.9±4.0 (6)</td>
<td>57.6±14.7 (8)</td>
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<td></td>
</tr>
<tr>
<td>RhVEGF-A</td>
<td>1.8±0.6 (10)</td>
<td>34.2±4.3 (10)</td>
<td>15.2±2.1 (10)</td>
<td>31.8±3.8 (4)</td>
<td>57.7±6.7 (6)</td>
<td>54.5±10.2 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>2.4±1.1 (12)</td>
<td>38.0±5.8 (9)</td>
<td>14.6±2.9 (9)</td>
<td>34.3±5.6 (6)</td>
<td>58.7±3.4 (6)</td>
<td>54.3±13.7 (6)</td>
<td></td>
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</table>

ND indicates not determined. n of the analyzed samples is shown in parentheses.
Figure 2. Effect of VEGF gene transfers on atherosclerosis. A, Total en face lesion areas of the aorta (%) after 12 weeks on diet. Aortas from the arch to the bifurcation were opened longitudinally, pinned on a black surface, and analyzed for the presence of macroscopic lesions. B and C, Cross-sectional lesion areas (%) after 12 weeks (B) and 22 weeks (C) on diet. Areas were quantified by evaluation of the total lesion areas at the aortic sinus level, which is recognized by 3 valve cusps. The results for individual mice are presented as scatterplots, and the average of each group is indicated as a horizontal bar.

Figure 3. Representative examples of aortic lesion histology in transduced mice. All genes were transduced after 6 weeks on diet, and mice were euthanized 6 weeks after the gene transfers. Lesion composition in Ad-VEGF-A (A and G), Ad-VEGF-B (B and H), Ad-VEGF-C (C and I), Ad-VEGF-D (D and J), AdLacZ (E and K), and saline control mice (F and L) was similar. Serial sections from aortic arch: Movat pentachrome (A–F); immunostainings for macrophages (G–L). Bar=100 μm.
The results are in line with findings from recent phase II/III clinical trials. The results are also compatible with the vasculoprotective properties of the low levels of VEGF that have been demonstrated in animal studies. It is obvious that angiogenesis and atherogenesis share some common denominators, it is important to recognize that angiogenesis is a different process from atherogenesis.22,23

Previous studies were performed predominantly in apoE-knockout mice9,10 by use of systemic delivery of either rhVEGF-A165 protein or Ad-VEGF-A165 or VEGF-A165 plasmid. Even though angiogenesis and atherogenesis share some common denominators, it is important to recognize that atherogenesis is a different process from angiogenesis and that lipids, smooth muscle cell proliferation, and matrix accumulation still play major roles in the development of atherosclerotic lesions.22,23

Most of the cholesterol is carried in chylomicron and VLDL particles,24 and the lack of the ability of macrophages to produce apoE protein has a significant impact on atherogenesis.25 It is generally accepted that atherogenesis in apoE-knockout mice is heavily dependent on monocyte-macrophages and hence very susceptible to potential effects of the growth factors on bone marrow. Also, Celletti et al used only thoracic aorta for the evaluation of atheroembolic lesions. According to our experience, lesions in the thoracic aorta are usually more random and less predictable than elsewhere in the aortic tree. Also, increased levels of VEGF-A in plasma are very difficult to achieve after intraperitoneal or intramuscular injections with rhVEGF proteins. In our study, after the tail-vein injection of the recombinant protein, rhVEGF-A was detectable in the circulation for only a few minutes. Thus, it is likely that at least some of the results published earlier9,10 may be a result of the special properties of the apoE-knockout mouse as a model for atherogenesis.

We used LDLR/apoB48-deficient mice in which the majority of cholesterol is transported in apoB100-containing lipoproteins (ie, LDL fraction). Also, in these mice, apoE production in macrophages is normal, and the lesion development is not as greatly affected by the inability of macrophages to secrete apoE. Thus, we believe that LDLR/apoB48-knockout mice will represent human atherosclerosis at least equally as well as apoE-knockout mice. According to the results, no major changes in atherogenesis were found in these mice after transient gene transfer of any of the members of the VEGF gene family. Also, no changes were found in angiogenesis in atherosclerotic lesions or thoracic adventitia. Our results show that in this mouse model, transient expression of VEGF growth factors, as verified by increased levels of the transduced proteins in peripheral blood and aortas, does not enhance atherogenesis in large arteries.

In conclusion, no evidence of increased atherogenesis was found in LDLR/apoB48-deficient mice after adenovirus-mediated systemic gene transfers of VEGF-A, -B, -C, and -D. The results are in line with findings from recent phase II/III clinical trials. The results are also compatible with the vasculoprotective properties of the low levels of VEGF that have been demonstrated in animal studies. It is obvious that the possibility of enhanced atherogenesis should be carefully monitored in forthcoming clinical trials involving VEGF recombinant proteins or genes. However, the present results do not suggest any increased proatherogenic effects of the members of the VEGF gene family when expressed transiently after systemic adenoviral gene transfer.

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References
CLINICAL PERSPECTIVE

VEGF has been used in phase II/III clinical trials as recombinant protein (VIVA Trial) or gene therapy (KAT Trial, Euroinject One Trial). Because VEGFs induce angiogenesis, they could accelerate atherosclerosis, as has been shown recently in apoE-knockout mice. Even though no increased atherosclerosis or restenosis has been observed in VEGF clinical trials, this safety concern is of significant importance, because several members of the VEGF family are currently in clinical testing. We conducted a large safety study to evaluate possible proatherogenic effects of various VEGFs. We used LDLR/B48 double-knockout mice, because their cholesterol is carried primarily in LDL, whereas previously conducted studies were performed in apoE-knockout mice, in which the majority of cholesterol is carried in larger lipoprotein particles. Also, in these mice, macrophages cannot properly protect arterial wall, because they are deficient in apoE.

We found no evidence of increased atherosclerosis after systemic adenovirus-mediated gene transfer of VEGFs in either early atherosclerotic lesions or more advanced lesions. Interestingly, recent results from anticancer treatments with anti-VEGF antibody (Avastin) have shown an increased incidence of thrombotic and cardiovascular complications. Thus, it seems that VEGF is needed for normal function of large arteries in adults. Although mice may not fully represent human pathophysiology, our results do not suggest any significant proatherogenic effects of VEGFs after a transient 2- to 3-week treatment period. Testing of VEGFs for their potential therapeutic effects in cardiovascular diseases should proceed without exaggerated concerns of accelerated atherosclerosis as a side effect of VEGF therapy.

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