Journal of the American College of Cardiology
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Published by Elsevier Science Inc.

Vol. 37, No. 8, 2001
ISSN 0735-1097/01/$20.00
PII S0735-1097(01)01301-8

EXPERIMENTAL STUDIES

Effect of Human Recombinant Vascular Endothelial Growth Factor$_{165}$ on Progression of Atherosclerotic Plaque

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OBJECTIVES This study was designed to evaluate the impact of recombinant human vascular endothelial growth factor$_{165}$ (rhVEGF) on atherosclerotic plaque progression.

BACKGROUND Therapeutic angiogenesis represents a promising treatment for ischemic diseases. However, angiogenesis may impact atherosclerosis.

METHODS Albumin or rhVEGF was administered by a single intramuscular injection (2 μg/kg body weight) to New Zealand White rabbits fed with a 0.25% cholesterol diet beginning three weeks before therapy. Subsets of rabbits from each group underwent perfusion-fixation and harvesting of the thoracic aorta for morphometric and immunohistochemical analyses at 7 or 21 days.

RESULTS The mean plaque area was 15.75 ± 2.28% and 22.00 ± 3.24% with VEGF and 0.67 ± 0.22% and 1.17 ± 0.34% with albumin at 7 and 21 days, respectively. The plaque circumference was 13.00 ± 2.58% and 23.75 ± 2.86% with VEGF and 2.50 ± 0.65% and 6.25 ± 1.88% with albumin at 7 and 21 days, respectively. The maximal plaque thickness was 0.11 ± 0.002 and 0.15 ± 0.007 mm with VEGF and 0.04 ± 0.009 and 0.07 ± 0.003 mm with albumin at 7 and 21 days, respectively. The endothelial density (reported as percent total plaque area) was 31.75 ± 4.42% and 63.00 ± 8.45% with VEGF and 7.75 ± 1.65% and 12.75 ± 1.93% with albumin at 7 and 21 days, respectively. The macrophage density was 4.5 ± 0.86 and 19.25 ± 1.54 with VEGF and 4.26 ± 0.75 and 6.00 ± 1.08 with albumin at 7 and 21 days, respectively.

CONCLUSIONS Recombinant human VEGF increases the rate and degree of atherosclerotic plaque formation in the thoracic aorta in a cholesterol-fed rabbit model. (J Am Coll Cardiol 2001;37:2126–30)

Vascular endothelial growth factor (VEGF), a multifunctional cytokine for endothelial cells expressing VEGF receptor-1 (*flt-1*) and 2 (*flk-1*/KDR) (1–3), can increase the development of collateral arteries. Thus, it has become an exciting new therapeutic agent for vascular diseases (4).

Although it is relatively endothelial cell-specific, VEGF also affects monocyte activation and migration through a primary effect on receptor *flt-1* in bone marrow and peripheral blood (5–8). Macrophages, in turn, contribute to angiogenesis by production of factors such as VEGF, tumor necrosis factor-alpha and thymidine phosphorylases (9). In addition, macrophages, the most numerous inflammatory cells present in the atherosclerotic lesion, also elaborate growth factors and cytokines, which play a central role in atherogenesis (9–12).

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Preclinical and clinical studies indicate that angiogenic growth factors can stimulate the development of collateral vessels (4). However, recent studies have suggested that angiogenesis may also be necessary for plaque progression (13).

Based on these studies and on the described effects of VEGF on endothelial cells and macrophages, we evaluated the impact of remote administration of rhVEGF on progression of atherosclerotic plaque in the thoracic aorta of cholesterol-fed rabbits.

METHODS

Animals and experimental design. Albumin or rhVEGF was administered by a single intramuscular injection (2 μg/kg body weight) to 16 New Zealand White male rabbits (3.00 to 3.2 kg) fed with a 0.25% cholesterol diet beginning 21 days before treatment. This animal model was selected because a large body of previous work demonstrated consistent formation of arterial plaques, with a composition and morphology resembling human lesions (14,15). The dose of rhVEGF was selected because it is one of the lowest dose regimens previously shown to be therapeutic in any animal model (16).

At 7 and 21 days after treatment, four rabbits each underwent total body perfusion fixation. Then, the thoracic aorta, from the aortic root to the diaphragm, was harvested

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Manuscript received August 11, 2000; revised manuscript received February 9, 2001, accepted March 14, 2001.

Abbreviations and Acronyms

IgG = immunoglobulin G
rhVEGF = recombinant human vascular endothelial growth factor$_{165}$

and post-fixed in 10% neutral buffered formalin for 12 h before undergoing morphometric analysis, as described subsequently.

Plaque morphometric evaluation. All observers were blinded to the treatment groups and time points for analyses. The arteries were divided into four equal segments, with all sections obtained proximally. Eighty sections (5 to 8 μm in thickness, 20 each for 4 segments), from the aortic root to the mid-portion of the descending thoracic aorta, were prepared. The slides were stained with Verhoeff reagent and hematoxylin. The plaque images were analyzed with the Image Pro plus system (Media Cybernetics, Silver Spring, Maryland). Plaque area, circumference and thickness were evaluated. Plaque area was calculated as the difference between the luminal area and the area delimited by the internal elastic lamina. The results were normalized to total vessel cross-sectional area for each arterial section to eliminate variations due solely to vessel size.

Plaque circumference was evaluated by the ratio of the extension of the cross-sectional circumference of each plaque traced manually to the total luminal circumference. Finally, maximal thickness of the plaque for each axial section was measured.

Immunohistochemistry. ENDOTHELIAL AREA. Six cross-sections (5 μm in thickness) from each animal, two each from the first three segments, were incubated overnight with a primary monoclonal CD31 antibody (20 μg/ml; Pharmingen, San Diego, California). After washing, the slides were incubated with biotinylated anti-mouse immunoglobulin G (IgG) (1:250 dilution; Calbiochem-Novabiochem, La Jolla, California) and then with an avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, California). For all immunohistochemistry studies, the sections incubated with diluent and isotype-matched antibody served as separate negative control samples. The images were analyzed in a blinded manner to determine the total number of positive pixels, with the results normalized to total plaque area to eliminate error due simply to differences in plaque size. This method was selected in lieu of the more traditional lumen-count capillary density to evaluate forming vessels quantitatively, without errors caused by variable microvessel compression and fixation or observer bias.

MACROPHAGES. Six cross sections (5 μm in thickness), as described earlier, were incubated overnight with primary monoclonal antibody to rabbit macrophages (clone RAM-11, 1:800 dilution, Dako, Carpinteria, California). After washing, the slides were incubated with biotinylated anti-mouse IgG (Calbiochem, San Diego, California) and then with an avidin-biotin peroxidase complex (Pierce, Rockford, Illinois). The total number of positive cells was manually counted for each cross section by an observer in a blinded manner.

Table 1. Percentage of Total Cross Section Represented by Plaque Area (Difference Between Lumen Area and Area Delimited by Internal Elastic Lamina) at Either 7 or 21 Days After Treatment with rhVEGF (2 μg/kg by a Single Intramuscular Injection) or Albumin

	7 Days	21 Days
rhVEGF	15.75 ± 2.28 (*p = 0.007)	22.00 ± 3.24 (*p = 0.009)
Albumin	0.67 ± 0.22	1.17 ± 0.34

*Indicates statistical significance. Data are expressed as the mean value ± SE. rhVEGF = recombinant human vascular endothelial growth factor.

Statistical analysis. Data are expressed as the mean value ± SE. Statistical significance for these analyses was determined by two-way analysis of variance (ANOVA; time vs. treatment group) and by one-way repeated measures ANOVA (for each group at each time point), with significance determined at 95% (Fisher, Bonferroni, Tukey-A, Student-Newman-Keuls post hoc testing performed where applicable using Statview or SPSS version 6.1).

RESULTS

Plaque morphometry. PLAQUE AREA. As detailed in Table 1, the mean plaque area is significantly increased compared with that of control samples at both 7 and 21 days after VEGF administration

PLAQUE CIRCUMFERENCE. As detailed in Table 2, circumferential plaque extension is significantly greater compared with that of control samples both at 7 and 21 days after VEGF administration

PLAQUE THICKNESS. Maximal plaque thickness (Table 3) is significantly increased compared with that of control samples at both 7 and 21 days after VEGF administration.

Figures 1 and 2 are representative photomicrographs of Verhoeff-van Gieson/Masson sections depicting an increased degree of atherosclerosis in the VEGF-treated groups.

Immunohistochemistry. ENDOTHELIAL CELLS. As detailed in Table 4, the total CD31 positive area (percent total plaque area) increased significantly compared with that of

Table 2. Percentage of Total Lumen Circumference Represented by Plaque at Either 7 or 21 Days After Treatment With rhVEGF (2 μg/kg by a Single Intramuscular Injection) or Albumin

	7 Days	21 Days
rhVEGF	13.00 ± 2.58 (*p = 0.03)	23.75 ± 2.86 (*p = 0.003)
Albumin	2.50 ± 0.65	6.25 ± 1.88

*Indicates statistical significance. Data are expressed as the mean value ± SE. rhVEGF = recombinant human vascular endothelial growth factor.

Table 3. Maximal Plaque Thickness (mm) Was Determined for Cross Sections Obtained at Either 7 or 21 Days After Treatment With rhVEGF (2 μg/kg by a Single Intramuscular Injection) or Albumin

	7 Days	21 Days
rhVEGF	0.110 ± 0.002 (*p = 0.001)	0.150 ± 0.007 (*p = 0.003)
Albumin	0.040 ± 0.009	0.070 ± 0.003

*Indicates statistical significance. Data are expressed as the mean value ± SE.
rhVEGF = recombinant human vascular endothelial growth factor.

control samples at both 7 and 21 days after VEGF administration. The total CD31-positive area is presented instead of lumen-count capillary density to evaluate forming vessels quantitatively, without artifactual errors caused by tissue sectioning techniques or observer bias.

MACROPHAGES. As detailed in Table 5, RAM-11–positive cells per cross section are significantly greater compared with those of control samples at 21 days, but not at 7 days after VEGF administration.

DISCUSSION

These results demonstrate that rhVEGF can induce progression of atherosclerotic plaque, although exogenous endothelial cell growth factors are already present in plaque tissue (13). Complementing these findings, a recent study (13) found that inhibition of angiogenesis successfully limited progression of atherosclerosis.

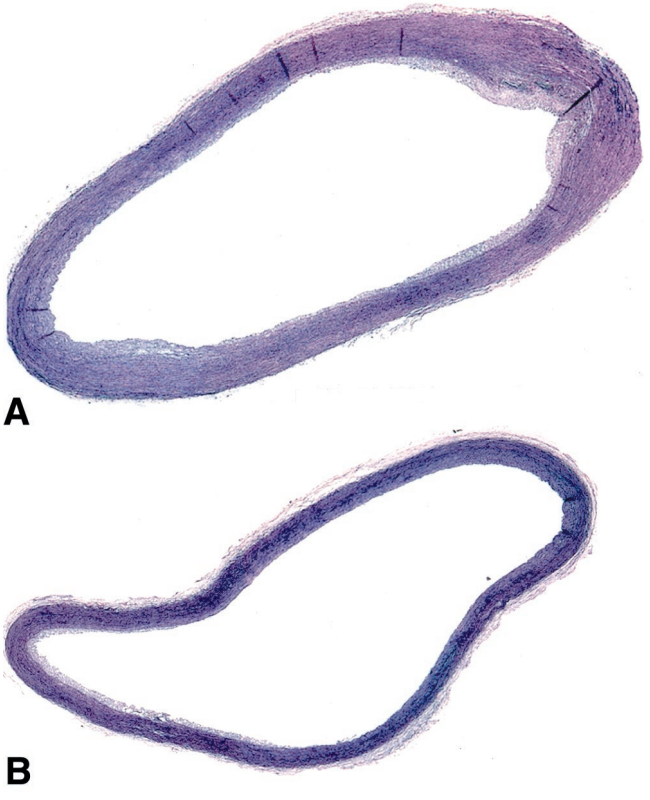

Figure 1. Representative photomicrographs at seven days. Low-magnification (×8, before 50% reduction) photographs of thoracic aortic cross sections stained with Verhoeff and counterstained with hematoxylin from **(A)** a rabbit treated with rhVEGF (2 μg/kg by a single intramuscular injection) and **(B)** an albumin-treated rabbit.

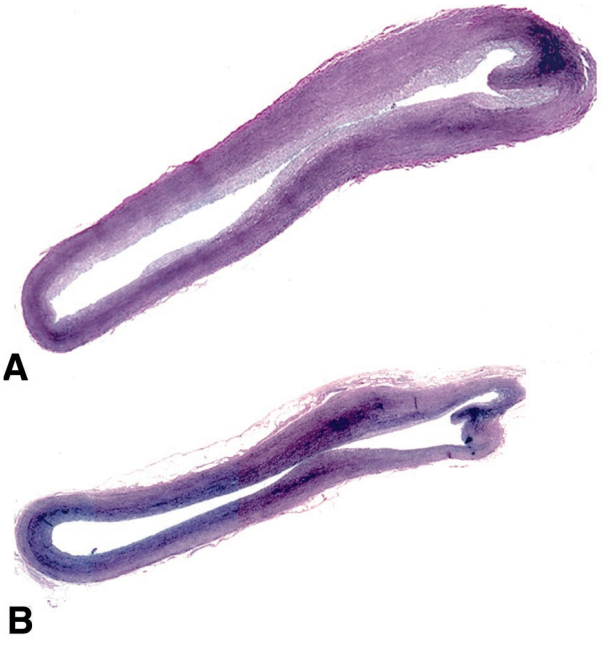

Figure 2. Representative photomicrographs at 21 days. Low-magnification (×8, before 50% reduction) photographs of thoracic aortic cross sections stained with Verhoeff and counterstained with hematoxylin from **(A)** a rabbit treated with rhVEGF (2 μg/kg by a single intramuscular injection) and **(B)** an albumin-treated rabbit.

Plaque progression. In the present study, rhVEGF increases plaque progression at both 7 and 21 days. In the VEGF-treated group, the mean plaque area at 7 and 21 days was 23- and 18-fold greater, respectively, than that of the control samples. Circumferential plaque extension at 7 and 21 days increased 5.2 and 3.8 times, respectively, over that of control samples. Vascular endothelia growth factor$_{165}$ potentially impacts plaque formation through complex stimuli, including endothelialization and macrophage infiltration. In fact, VEGF may increase plaque capillary density and extend the depth of neovascularization to the intima (17–19). The clinical importance of plaque neovascularization is suggested by studies that show a higher capillary density in lesions with mural hemorrhage, plaque rupture or unstable angina (19–21). In addition, factors that stimulate plaque angiogenesis may also precipitate plaque rupture, with subsequent myocardial infarction and ischemic stroke (20).

Table 4. Endothelial-Positive Area: Number of CD31-Positive Cells Normalized to Total Plaque Area (cells/mm^2) at Either 7 or 21 Days After Treatment With rhVEGF (2 μg/kg by a Single Intramuscular Injection) or Albumin

	7 Days	21 Days
rhVEGF	31.75 ± 4.42 (*p = 0.009)	63.00 ± 8.45 (*p = 0.004)
Albumin	7.75 ± 1.65	12.75 ± 1.93

*Indicates statistical significance. Data are expressed as the mean value ± SE.
rhVEGF = recombinant human vascular endothelial growth factor.

Table 5. Macrophage Infiltration: Number of RAM-11–Positive Cells Per Cross Section Obtained at Either 7 or 21 Days After Treatment With rhVEGF (2 μg/kg by a Single Intramuscular Injection) or Albumin

	7 Days	21 Days
rhVEGF	4.50 ± 0.86	19.25 ± 1.54
	(p = 0.60)	(*p = 0.005)
Albumin	4.26 ± 0.75	6.00 ± 1.08

*Indicates statistical significance. Data are expressed as the mean value ± SE.
rhVEGF = recombinant human vascular endothelial growth factor.

Endothelial density. Despite increased plaque area, as described earlier, cross sections of the thoracic aorta from the VEGF-treated group showed a 4- to 4.9-fold increases in the endothelial-positive area after 7 and 21 days, respectively. These results suggest increased vascularity of lesions in the rhVEGF group. Previous work suggests that plaque angiogenesis is frequently associated with increased infiltration of macrophages, T cells and mast cells, and ultimately correlates with the degree of luminal stenosis (8–12).

Macrophage infiltration. Given the potential of VEGF to increase circulating macrophage precursor levels, we quantitatively evaluated local cross-sectional macrophage infiltration. Although all other plaque measurements and cellular variables studied revealed increases at both 7 and 21 days, macrophage infiltration significantly increased in the rhVEGF-treated group only at 21 days (3.2-fold). A number of factors may be involved in this apparent delay in macrophage accumulation relative to all other variables. Less mature macrophages may accumulate earlier and differentiate over time (i.e., not RAM-11–positive initially), or mature macrophages may actually exhibit delayed tissue accumulation. Furthermore, immunohistochemical studies must be undertaken to better assess the relative contribution of each. However, these findings are consistent with previous observations that VEGF affects monocyte activation and migration through a primary effect on receptor *flt-1* in bone marrow and peripheral blood (5–7). In this regard, an enhanced understanding of these observations is crucially important because macrophages elaborate growth factors and cytokines that mediate intimal hyperplasia and may, therefore, mediate atherogenesis (8,9,11,12). Macrophages may also contribute to plaque progression through elaboration of a variety of tissue factors, such as matrix metalloproteinases and other hydrolytic enzymes (22). In addition, monocytes are known to sustain proliferation of monocytes, smooth muscle and endothelial cells (8–11).

Study implications. Recently, it was clinically shown that intra-arterial administration of rhVEGF markedly increases the development of collateral vessels in patients with ischemic syndromes. As a result, VEGF has become a potentially exciting new therapeutic agent for coronary and peripheral occlusive vascular diseases (4). However, it has been reported that in vivo introduction of human $VEGF_{165}$ complementary deoxyribonucleic acid into rabbit carotid arteries by Sendai virus liposomes induces prominent angiomatoid proliferation of enothelial cells and thickening of the intima (23) and that VEGF administration exacerbates neointimal thickening after vascular injury in dogs (24). Consistent with these and other recent studies (25), we have shown that even a modest dose of rhVEGF can enhance progression of atherosclerotic plaques in vivo. Although additional studies are required to better characterize the mechanisms underlying this observation, this study suggests that alternate dosing regimens or delivery strategies must be considered. In addition to evaluating new ways to deliver the drug (i.e., local delivery) to avoid systemic effects, new ways to locally block the impact of VEGF on undesirable pathways may need to be developed.

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