Vascular Endothelial Growth Factor (VEGF) Expression in Human Coronary Atherosclerotic Lesions
Possible Pathophysiological Significance of VEGF in Progression of Atherosclerosis

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Background—Vascular endothelial growth factor (VEGF) is an important angiogenic factor reported to induce migration and proliferation of endothelial cells, enhance vascular permeability, and modulate thrombogenicity. VEGF expression in cultured cells (smooth muscle cells, macrophages, endothelial cells) is controlled by growth factors and cytokines. Hence, the question arises of whether VEGF could play a role in atherogenesis.

Methods and Results—Frozen sections from 38 coronary artery segments were studied. The specimens were characterized as normal with diffuse intimal thickening, early atherosclerosis with hypercellularity, and advanced atherosclerosis (atheromatous plaques, fibrous plaques, and totally occlusive lesions). VEGF expression as well as the expression of 2 VEGF receptors, flt-1 and Flk-1, were studied with immunohistochemical techniques in these samples at the different stages of human coronary atherosclerosis progression. The expression of VEGF mRNA was also studied with reverse transcription–polymerase chain reaction. Normal arterial segments showed no substantial VEGF expression. Hypercellular and atheromatous lesions showed distinct VEGF positivity of activated endothelial cells, macrophages, and partially differentiated smooth muscle cells. VEGF positivity was also detected in endothelial cells of intraplaque microvessels within advanced lesions. In totally occlusive lesions with extensive neovascularization, intense immunostaining for VEGF was observed in accumulated macrophages and endothelial cells of the microvessels. Furthermore, VEGF mRNA expression was detected in atherosclerotic coronary segments but not in normal coronary segments. The immunostainings for flt-1 and Flk-1 were detected in aggregating macrophages in atherosclerotic lesions and also in endothelial cells of the microvessels in totally occlusive lesions.

Conclusions—These results demonstrate distinct expression of VEGF and its receptors (flt-1 and Flk-1) in atherosclerotic lesions in human coronary arteries. Considering the multipotent actions of VEGF documented experimentally in vivo and in vitro, our findings suggest that VEGF may have some role in the progression of human coronary atherosclerosis, as well as in recanalization processes in obstructive coronary diseases. (Circulation. 1998;98:2108-2116.)

Key Words: angiogenesis ■ atherosclerosis ■ coronary disease ■ immunohistochemistry ■ pathology

In 1989, a new factor involved in angiogenesis was isolated, known as vascular endothelial growth factor (VEGF) or vascular permeability factor. It may play a role in tumor growth, wound healing, age-related macular degeneration, rheumatoid arthritis, diabetic retinopathy, and collateral formation in ischemic tissue. VEGF is reported to potently induce migration and proliferation of endothelial cells (ECs), enhance vascular permeability, and modulate thrombogenicity. VEGF was considered unique among angiogenic growth factors, because its 2 high-affinity receptors with tyrosine kinase domains (flt-1 and Flk-1/kinase insert domain receptor) were thought to be confined to ECs; consequently, VEGF could act as an EC-specific mitogen. Studies on knockout mice of VEGF or its receptors have revealed that VEGF plays a critical role in the development and formation of blood vessel networks. However, recent studies have demonstrated that the gene for the VEGF receptor flt-1 is expressed in human monocytes as.

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well, and the VEGF-induced chemotactic response of human monocytes is mediated via this receptor. 7,8 Claus et al9 also documented that VEGF induces monocyte activation and migration. These data strongly suggest that VEGF plays a role in the chemotaxis of monocytes/macrophages, which is crucial in inflammatory reactions and in wound-repair processes. Recently, we demonstrated that VEGF strongly suppresses the secretion of C-type natriuretic peptide, 10 a member of the natriuretic peptide family that acts as a vasoactive peptide secreted by vascular ECs. This, then, raised the possibility that VEGF could indirectly modulate the contraction and growth of smooth muscle cells (SMCs).

It has been shown that VEGF is produced not only by malignant tumor cells of several types but also by cultured SMCs, 11 macrophages, 12 and ECs. 13 The expression of VEGF in these cultured cells appears to be controlled by a variety of growth factors and cytokines. VEGF gene expression is upregulated not only by hypoxia but also by transforming growth factors and cytokines. VEGF gene expression is documented that VEGF induces monocyte activation and migration. These data strongly suggest that VEGF plays a role in inflammatory reactions and in wound-repair processes. Recently, we demonstrated that VEGF strongly suppresses the secretion of C-type natriuretic peptide, 10 a member of the natriuretic peptide family that acts as a vasoactive peptide secreted by vascular ECs. This, then, raised the possibility that VEGF could indirectly modulate the contraction and growth of smooth muscle cells (SMCs).

Thus far, however, we are unaware of any studies that have looked into the potential significance of VEGF in human atherosclerosis. For this reason, we have studied the expression of VEGF as well as its 2 receptors, flt-1 and Flk-1, in human coronary arteries using immunohistochemical analysis and the occurrence of VEGF mRNA using the reverse transcription–polymerase chain reaction (RT-PCR) method.

Methods

Materials

Thirty-eight coronary artery segments were harvested at autopsy within 3 hours after death (15 patients; age range, 21 to 72 years). Twelve segments contained diffuse intimal thickening (DIT) but were otherwise normal. Twenty-six contained atherosclerotic lesions characterized histologically either as early atherosclerotic lesions with hypercellularity (n=7) or as advanced atherosclerotic lesions (n=19). 14–17 The hypercellular atherosclerotic lesion was defined as a cell-rich intimal lesion composed predominantly of SMCs and macrophages but without an appreciable extracellular lipid core. The advanced atherosclerotic lesions were divided into atheromatous (n=8), fibrous (n=7), and totally occluded lesions (n=4).

The coronary arteries were dissected from the epicardial surface, and a 2-mm slice from each segment was snap-frozen and stored at −80°C. The snap-frozen samples were sectioned serially at 6-μm thickness and fixed in acetone. Every first section was stained with hematoxylin and eosin; the other sections were used for immunohistochemical staining.

Immunohistochemical Staining

The source and specificity of all antibodies used in this study are summarized in the Table. For the identification of VEGF, a rabbit polyclonal antibody was used that was directed against the 20 amino-terminal residues of human VEGF (Santa Cruz Biotechnology). The antibody was demonstrated to neutralize VEGF activity and specifically react with native and denatured (reduced) VEGF by Western blot. 18 For the identification of flt-1 and Flk-1, rabbit polyclonal antibodies were used (Santa Cruz Biotechnology). Both antibodies react with flt-1 or Flk-1 of mouse, rat, and human cell origin but do not cross-react with other tyrosine kinase membrane receptors.

HLA-DR antibody was used to examine the activation state of cells. 19 To identify SMCs and to evaluate differences in the state of differentiation, 2 anti-actin markers, 1A4 and CGA7, were used. 20–23

Single Staining

Sections were incubated with the primary antibodies, either overnight at 4°C or for 1 hour at room temperature. The sections were then subjected to a 3-step staining procedure, with the use of streptavidin-biotin complex with horseradish peroxidase for color detection. Tris-buffered saline was used for washing between the subsequent incubation steps. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole, and the sections were faintly counterstained with hematoxylin.

The specificity and results obtained with anti-VEGF, flt-1, and Flk-1 antibodies were checked by omitting the primary antibodies and using nonimmune rabbit serum (Dako) as negative control. Human renal tissues served as a positive control.

Immunodouble Staining

For the simultaneous identification of SMCs and macrophages, sections were double-stained with 1A4 and EBM11, according to procedures previously reported. 24 In this immunodouble staining, we visualized the enzymatic activity of β-galactosidase for 1A4 in turquoise (BioGenex Kit) and the activity of alkaline phosphatase for EBM11 in red (New Fuchsin Kit, Dako).

To identify cell types that express VEGF, we also performed immunodouble staining for 1A4/VEGF, EBM11/VEGF, and EN-4/VEGF in some sections. In this staining, alkaline phosphatase was visualized with fast blue BB (blue, 1A4, EBM11, and EN-4) and peroxidase with 3-aminophenol–ethanethiol (red, VEGF).

Area Quantification of VEGF-Positive Cells and Statistical Analysis

The total surface area containing VEGF-positive cells was quantified by use of computer-aided planimetry and expressed as a percentage of the total surface area of the intima, the so-called VEGF area. In addition, the surface area occupied by VEGF-positive ECs—the so-called VEGF EC area—was quantified similarly and expressed as a percentage of the total surface area occupied by ECs as shown by von Willebrand factor (vWF) staining. The luminal surface area occupied by vWF-positive ECs was also estimated as a percentage of the whole luminal surface area. Results are expressed as mean±SD. Statistical comparisons between groups were performed by the Kruskal-Wallis test followed by multiple comparison with Scheffe’s test. Values of P<0.05 were considered significant.
RT-PCR Southern Blot Analysis

Normal coronary artery segments with DIT (n=3) and segments with atherosclerosis containing atheromatous plaques (n=3) were used. The segments were selected to be directly adjacent to the ones used for immunohistochemical investigations. RNAs were isolated from these coronary segments by the guanidine thiocyanate/CsCl method. cDNA was synthesized from 5 μg of total RNA primed with oligo-dT by use of reverse transcriptase (Super Script II) at 42°C for 1 hour. cDNA (0.5 μg) was subjected to VEGF PCR with 50 fmol of primers. Primer sequences were 5'-GGACATCTTCCAGGAGTA-3' (human VEGF165 cDNA nucleotides 232 to 250) and 5'-TGCAACGCGAGTCTGTGT-3' (nucleotides 555 to 573) for detecting human VEGF mRNA. Amplification was carried out for 40 cycles with 30 seconds of incubation at 94°C, 30 seconds at 55°C, and 1 minute at 72°C. A 10-μL aliquot of each RT-PCR reaction mixture was electrophoresed on a 1.5% agarose gel (Figure 1). VEGF staining was found occasionally in macrophages and occasionally also in some HLA-DR-positive ECs. The VEGF\(^+\) cell area was 0.8±1.1%; the VEGF\(^-\) EC area was 0.4±1.1%. In normal coronary arteries with DIT, flt-1 or Flk-1 staining was not detected.

Atherosclerotic Plaques (n=26; 10 Patients)

**Hypercellular Atherosclerotic Lesions (n=7; 4 Patients)**

Three of the 7 lesions were composed almost solely of SMCs, with only occasional macrophages scattered throughout the intima. The vast majority of the SMCs stained positive with both 1A4 and CGA7. HLA-DR was expressed by macrophages, but only few SMCs and ECs showed HLA-DR positivity. VEGF-positive staining was seen in some of the ECs and in some macrophages, particularly those in the superficial layers of the intima. SMCs showed no VEGF positivity (data not shown).

The 4 other hypercellular lesions were characterized by numerous foci of clustered macrophages (Figure 2). Of the SMC markers, only 1A4 was positive; CGA7 was negative. HLA-DR positivity was found in macrophages and SMCs associated with the inflammatory cells. VEGF was distinctly expressed at sites that contained clustered macrophages (Figure 2). The immunodouble staining for macrophages (or SMCs) and VEGF-positive cells showed that VEGF positivity was detected in macrophages and some SMCs as well as ECs. Vasa vasorum were not observed in the media and atherosclerotic intima of either one of these hypercellular lesions.

The VEGF\(^+\) cell area was 29.4±18.3%; the VEGF\(^-\) EC area was 26.4±26.6%. Occasional aggregating macrophages showed positivity for flt-1 and Flk-1 (Figure 2).

**Advanced Atherosclerotic Plaques**

**Atheromatous (n=8; 5 Patients)**

Advanced atheromatous plaques, characterized by a distinct lipid core and fibrous cap, contained abundant macrophages and SMCs (Figure 3). The vast majority of these cells expressed HLA-DR. The SMCs within the fibrous cap stained positive with 1A4 but did not stain with CGA7. The immunodouble staining revealed that in the fibrous cap, VEGF positivity was seen in macrophages and SMCs adjacent to inflammatory cells as well as a good number of ECs (Figure 3), most of which appeared to colocalize with HLA-DR positivity. In the plaque-free wall of eccentric atheromatous lesions, ECs were frequently positive for VEGF, whereas the underlying SMCs stained negative for VEGF (Figure 3). Although there were some EC-denuded areas, the immunodouble staining for ECs and VEGF-positive cells revealed that almost all ECs were uniformly positive for VEGF, and there were no VEGF-positive SMCs or macrophages underlying these EC-denuded areas (Figure 4). Five of the 8 atheromatous lesions contained microvessels within the plaque, 4 of which showed VEGF positivity of the ECs of the vessels (Figure 5).

The VEGF\(^+\) cell area was 24.4±8.0%; the VEGF\(^-\) EC area was 18.8±21.3%. In these plaques, clustered macrophages were positive for flt-1 and Flk-1 (Figure 3).

**Fibrous (n=7; 6 Patients)**

In fibrous plaques, characterized by dense collagen without an appreciable lipid core and with only sparse and scattered
inflammatory infiltrates, only a few cells were VEGF-positive (data not shown). Microvessels were found in 6 of the 7 fibrous plaques; only 2 plaques showed VEGF staining in ECs of the microvessels.

The VEGF$^+$ cell area was 2.6±5.2%; the VEGF$^+$ EC area was 0.8±1.8%.

**Total Occlusion (n=4; 2 Patients)**

Under this subheading, we have also included cases with subtotal occlusion (luminal stenosis of >90%).

Two sites from 1 patient both showed an advanced fibrous plaque with extensive proliferation of microvessels extending from the adventitia through the media and the
plaque into the occluded luminal area. The central preexistent luminal remnant was lined by macrophages (Figure 6). Areas within the plaque that contained conspicuous microvessels also showed accumulation of macrophages (Figure 6). There were only a few 1A4-positive SMCs within the plaque. VEGF staining revealed strong positivity in macrophages and in the ECs of the microvessels (Figure 6); these positive sites appeared to colocalize with those of HLA-DR–positive ECs and macrophages. The immunodouble staining for ECs and VEGF-positive cells demonstrated that most of the ECs of the microvessels within the plaque showed VEGF positivity (Figure 6). The HLA-DR–positive ECs and macrophages were also positive for both flt-1 and Flk-1 immunostainings (Figure 6).
The 2 remaining occluded lesions (from another patient) were also basically fibrous in nature, although with fewer macrophages and with fewer and smaller microvessels with narrowed lumen. In these instances, staining for VEGF provided only few positive macrophages and only occasionally some positivity of ECs of the microvessels.

**Area Quantification and Statistical Analysis**

The VEGF$^+$ cell area was significantly higher ($P<0.05$) in hypercellular atherosclerotic lesions and in advanced atheromatous plaques than in DIT (Figure 7A). The VEGF$^+$ EC area in hypercellular atherosclerotic lesions was significantly higher than in DIT ($P<0.05$) (Figure 7B).

The luminal surface area occupied by vWF-positive ECs did not differ among 4 groups with $92.5\pm4.7\%$ ($93.9\pm4.3\%$ in DIT, $93.1\pm5.8\%$ in hypercellular atherosclerotic lesions, $90.6\pm4.0\%$ in advanced atheromatous plaques, $91.8\pm4.9\%$ in advanced fibrous plaques).

**RT-PCR Southern Blot Analysis of VEGF mRNA Expression**

As shown in Figure 8, human renal tissue, used as positive control, gave 2 distinct signals with 428 and 356 bp, which corresponded to mRNA for VEGF$^{189}$ and VEGF$^{165}$, respectively. Advanced atheromatous plaques gave 1 distinct band with 356 bp. In contrast, normal coronary arteries gave substantially no signals in RT-PCR Southern blot analysis.

**Discussion**

In humans, in vivo studies have demonstrated the expression of VEGF mRNA or VEGF protein in the normal kidney, in a variety of tumors, in heart transplants, and in rheumatoid synovial tissue. However, to the best of our knowledge, this is the first study to demonstrate the expression of VEGF protein and VEGF mRNA in the progression of human coronary atherosclerosis.

The present study clearly showed that positive immunostaining for VEGF in human coronary atherosclerotic plaques was always related to the occurrence of abundant HLA-DR$^+$ positive cells. Our quantitative analysis unequivocally demonstrated a significantly higher VEGF$^+$ cell area in hypercellular atherosclerotic lesions and in atheromatous plaques than in normal coronary arteries with DIT. Moreover, the present study also showed distinct expression of VEGF mRNA in human coronary atherosclerotic plaques but not in normal coronary arteries. Previous studies have revealed that a marked increase in the number of HLA-DR$^+$ positive cells in human atherosclerotic lesions is linked closely to inflammatory processes within the atherosclerotic plaques and clinically to progressive disease. Hence, our findings strongly suggest that VEGF is closely related to the progression of coronary atherosclerosis in humans.

Ross et al$^{14}$ promoted the concept that dysfunction of ECs, with subsequent infiltration of circulating monocytes/lymphocytes, is the primary event for atherogenesis. It is of interest, therefore, that ECs of normal coronary arteries, albeit with DIT, in our study did not show immunostaining for VEGF. Conversely, once activated (HLA-DR$^+$) ECs were identified in atherosclerotic lesions, positive immunostaining of ECs was also found. Indeed, the VEGF$^+$ EC area in atherosclerotic lesions was significantly higher than that of normal coronary arteries. It is tempting to consider these
differences as an integral part of the change in EC function in relation to atherogenesis. This is more likely because VEGF has been recognized not only to stimulate EC proliferation, increase vascular permeability, and alter thrombogenicity but also to induce migration of human mononuclear phagocytes/monocytes and stimulate their expression of tissue factor.

In our samples, there were some EC-denuded areas. The luminal surface area occupied by ECs, however, did not differ among 4 groups in different stages of atherosclerosis (91% to 94%). Because we did not detect any thrombus formation in these denuded areas, we consider that the observed endothelial denudation possibly occurred during our sampling, sectioning, and immunohistochemical staining procedure. Although the extent of endothelial denudation was almost the same in each stage of atherosclerosis, endothelial expression of VEGF differed significantly, as shown in Figure 7. In addition, the immunodouble staining for ECs and VEGF-positive cells re-

Figure 6. Sections of advanced atherosclerotic plaque with almost total occlusion of lumen. A, Immunodouble staining with 1A4 (turquoise) and EBM11 (red) shows accumulation of macrophages lining vestige of lumen (L). Macrophage infiltration is also found diffusely within plaque and is accompanied by extensive proliferation of intraplaque microvessels. M indicates media. B, Staining for ECs. C, Staining for VEGF reveals distinct positivity of macrophages and ECs of intraplaque microvessels. D, Immunodouble staining with EN-4 (blue) and anti-VEGF antibody (red) shows that ECs of microvessels are positive for VEGF. E, Staining for flt-1. F, Staining for Flk-1. Macrophages and ECs of microvessels are positive for Flk-1 and flt-1. Magnification: A, ×52; B and C, ×129; D, ×645; E and F, ×129.
revealed that there seemed to be no apparent effect of endothelial denudation on VEGF positivity for the bordering ECs and the underlying SMCs or macrophages (Figures 3B and 4).

The present study of immunodouble staining demonstrated that macrophages that accumulated in atherosclerotic lesions showed positive immunostaining for VEGF. This finding is in keeping with previous reports, which have shown that activated macrophages in culture12 and macrophages involved in rat and guinea pig wound healing26 produce VEGF. Clauss et al.9 recently demonstrated that VEGF promotes monocyte migration in vitro. Moreover, Shen et al.7 and Barleon et al.8 showed that migration of human monocytes in response to VEGF is mediated via flt-1. We also confirmed that macrophages that accumulated in atherosclerotic lesions expressed flt-1 and Flk-1. The observations in experimental work and our present findings support the hypothesis that VEGF, which originated from activated macrophages, could recruit further macrophages into the lesions through the VEGF receptors.

The present study, using 2 actin markers, IA4 and CGA7, revealed that SMCs in human coronary arteries showed differences in VEGF staining positivity, which related to their phenotypic differentiation as judged by the characters of these 2 different actin markers (see the Table). CGA7-positive (fully differentiated20,21) SMCs in the media did not show VEGF immunoreactivity. Within atherosclerotic lesions, conversely, our immunodouble staining analysis revealed that although CGA7-positive SMCs did not stain for VEGF, less differentiated SMCs (CGA72/IA41)21–23 showed distinct positivity for VEGF. The results indicate that SMCs with a phenotypic shift toward a less differentiated state are capable of expressing VEGF.

The presence of microvessels within human coronary atherosclerotic lesions has been recognized for some time, but their potential role in promoting the progression of the disease has been revealed only recently.15,27 In the present study, we detected intraplaque microvessels in advanced atherosclerotic plaques. We also demonstrated distinct VEGF expression in early atherosclerotic lesions that contained abundant clustered macrophages. This is of considerable interest, because one could argue that VEGF expression within the plaque may be an important phenomenon preceding the formation of the intraplaque microvessels. Recent animal studies have pointed out that VEGF is a key mediator of neovascularization associated with a variety of disorders.28 These experimental data and our present findings suggest that VEGF could be one of the growth factors involved in the development of intraplaque microvessels in human coronary arteries.

In totally occlusive lesions, new vessels are formed as part of the organization and recanalization response. In the present study, 2 different types of the totally occlusive lesions were observed. Obstructive lesions with numerous macrophages and extensive neovascularization throughout the arterial wall, considered to be part of an ongoing process of organization and recanalization, showed intense immunostaining for VEGF, whereas DIT showed no substantial VEGF mRNA expression. Over, Shen et al.7 and Barleon et al.8 showed that migration of human monocytes in response to VEGF is mediated via flt-1. We also confirmed that macrophages that accumulated in atherosclerotic lesions expressed flt-1 and Flk-1. The observations in experimental work and our present findings support the hypothesis that VEGF, which originated from activated macrophages, could recruit further macrophages into the lesions through the VEGF receptors.

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2116 VEGF in Human Coronary Atherosclerosis

Recently, VEGF has been promoted as a potential new therapeutic agent for occlusive vascular diseases. Intra-arterial administration of recombinant human VEGF markedly increased the development of collateral vessels and associated perfusion in a rabbit model with chronic hindlimb ischemia. Gene therapy with VEGF for human vascular diseases has recently started. Our observations of totally occluded coronary arteries may provide further rationale for such clinical trials. At the same time, however, a word of caution should be heard, because our observations also suggest that VEGF itself may promote the process of atherosclerosis. In fact, it has already been reported that the in vivo introduction of human VEGF cDNA into rabbit carotid arteries by the hemagglutinating virus of Japan/liposome method induced prominent angiomatoid proliferation of ECs. Lazarous et al also reported that VEGF administration exacerbated neointimal thickening after vascular injury in dogs. These reports suggest that VEGF is indeed capable of inducing neointimal angiogenesis and intimal hyperplasia.

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