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Endogenous Regulation of Angiogenesis in the Rat Aorta Model

Role of Vascular Endothelial Growth Factor

Roberto F. Nicosia,* Yuzhong J. Lin,† Darcie Hazelton,* and XiaoHua Qian*

From the Department of Pathology and Laboratory Medicine, Allegheny University of the Health Sciences, Philadelphia, Pennsylvania and Cascade Biologics,† Portland, Oregon*

The purpose of this study was to investigate the role of vascular endothelial growth factor (VEGF) in the rat aorta model of angiogenesis. Freshly cut aortic rings generated microvascular outgrowths in serum-free collagen gel culture. Angiogenesis was reduced to 10% when the explants were embedded in collagen 10 to 14 days after excision from the animal. Immunochemical studies of conditioned medium demonstrated secretion of VEGF by the aortic cultures. Levels of VEGF decreased during the second week of culture when the explants became quiescent and microvessels stopped growing. Treatment of quiescent aortic rings with exogenous VEGF stimulated angiogenesis and restored microvascular growth to values observed in cultures of freshly cut explants. Reverse transcriptase polymerase chain reaction of vasoformative collagen gel cultures of rat aorta demonstrated the expression of the alternatively spliced isoforms $VEGF_{165}$, $VEGF_{189}$, and the high affinity VEGF receptor *flk-1*. Reverse transcriptase-polymerase chain reaction of rat aorta-derived cell strains confirmed the presence of $VEGF_{165}$ and $VEGF_{189}$ in endothelial cells, smooth muscle cells, and fibroblasts. The *flk-1* receptor was expressed by endothelial cells but not by fibroblasts or smooth muscle cells, which is consistent with the endothelial target specificity of VEGF. The spontaneous angiogenic response of freshly cut aortic rings was inhibited by 70% with a neutralizing antibody against VEGF, whereas nonimmune IgG had no effect ($P < 0.001$). These findings provide evidence for a VEGF-mediated autocrine/paracrine regulation of angiogenesis in the rat aorta model. ***(Am J Pathol 1997, 151:1379–1386)***

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is a complex multistep process requiring endothelial cell migration, proliferation, proteolytic activity, and morphogenesis. Endothelial cells form new blood vessels in response to angiogenic factors secreted by a variety of normal and transformed cell types.[1] The cells of the vessel wall, ie, the intimal endothelial cells, the medial smooth muscle cells, and the adventitial fibroblasts, are all capable of producing angiogenic factors.[2–4] Thus, blood vessels have the potential to promote their own angiogenic response through an autoregulated process mediated by autocrine/paracrine mechanisms. Autoregulation of angiogenesis is observed when rings of rat aorta are cultured in biomatrix gels.[5,6] In this model, the rat aortic endothelium exposed to a three-dimensional matrix of collagen or fibrin switches to a microvascular phenotype generating branching networks of microvessels.[5,6] Angiogenesis is triggered by the injury of the dissection procedure and does not require stimulation by exogenous growth factors. Therefore, the rat aorta model can be used to investigate the endogenous mechanisms by which blood vessels regulate angiogenesis during wound healing. Because it enables the study of the angiogenic activity of the arterial wall, this model may also contribute to our understanding of artery-related angiogenic events such as the neovascularization of atherosclerotic plaques, the recanalization of thrombi, and the development of collateral circulation.

Recent evidence suggests that rat aortic angiogenesis is promoted by paracrine interactions among endothelial cells, smooth muscle cells, and fibroblasts.[5–8] The molecular mediators regulating this system, however, have not been fully characterized. We recently found that the angiogenic response of the rat aorta is stimulated by vascular endothelial growth factor (VEGF).[9] Studies by others have shown that VEGF is produced by endothelial cells, smooth muscle cells, and fibroblasts.[10–12] On this basis, we hypothesized that VEGF is among the growth factors that regulate angiogenesis in the rat aorta model.

VEGF is a 32 to 45 kd homodimer with endothelial target specificity.[13,14] Genetic ablation of VEGF or its receptor *flk-1* in mice results in the absence of vasculogenesis and the early intrauterine death of the em-

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Address reprint requests to Roberto F. Nicosia, Department of Pathology and Laboratory Medicine, Hahnemann Division, Mail Stop 435, Allegheny University of Health Sciences, Broad and Vine Streets, Philadelphia, PA 19102.

bryo.[15,16] VEGF plays a critical role in tumor angiogenesis because experimental tumors fail to grow when animals are treated with a neutralizing anti-VEGF antibody[17] or with a dominant-negative form of *flk-1*, which makes endothelial cells unresponsive to VEGF.[18] VEGF is also involved in wound healing-associated angiogenesis because its expression is up-regulated after injury.[19] In this study, we present evidence that endogenous VEGF plays an important role in the angiogenic response of rat aortic rings cultured in collagen gels. These findings support the hypothesis that vascular-derived VEGF contributes to the mechanisms that regulate angiogenesis during vascular wound healing.

Materials and Methods

Collagen Gel Cultures of Freshly Cut Aortic Rings

Thoracic aortas were excised from 2- to 3-month-old Fischer 344 male rats, rinsed in serum-free MCDB 131 growth medium (Clonetics, San Diego, CA) containing 50 μg/ml gentamicin, cleaned of periadventitial fibroadipose tissue, and cross-sectioned at ~1-mm intervals. Freshly cut aortic rings were rinsed in serum-free MCDB 131 medium and each ring was embedded in a collagen gel as described.[5] Collagen gel cultures were transferred to the 18-mm wells of four-well dishes (Nunc Inc., Napezville, IL) and grown at 35.5°C in 0.5 ml of serum-free MCDB 131 that was changed three times a week. For antibody blocking experiments, cultures were treated with 40 μg/ml neutralizing polyclonal goat anti-VEGF antibody (R & D Systems, Minneapolis, MN). Negative controls included untreated cultures and cultures treated with nonimmune IgG.

Collagen Gel Cultures of Quiescent Aortic Rings

In a separate set of experiments, rat aortic rings were cultured in suspension for 10 to 14 days at 35.5°C before being embedded in collagen gels. Each aortic ring was housed in an 18-mm agarose-coated well (Nunc) containing 0.5 ml of serum-free MCDB 131. The purpose of this preincubation step was to obtain quiescent explants. The wells were coated with agarose to avoid attachment of the rings and growth of cells that might have shed from the explants over time. During this preincubation period the medium was changed four times. After the preincubation period, the aortic rings were rinsed four times with serum-free medium, embedded in collagen gels, and incubated in serum-free medium at 35.5°C as described above for the freshly cut explants. Cultures of quiescent aortic rings were treated with increasing concentrations of recombinant VEGF (R & D Systems) or left untreated.

Quantitation of Angiogenesis

Angiogenesis was quantitated by counting the number of neovessels according to published criteria.[5] Each experimental condition included three to four cultures. Experiments were repeated two to three times to confirm results.

Immunoblot and Enzyme-Linked Immunosorbent Assay Studies

VEGF secretion in the aortic cultures was evaluated by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Two types of cultures were studied, suspension cultures and collagen gel cultures. Both types of cultures used freshly cut aortic rings. Suspension cultures were carried out in 18-mm agarose-coated wells (Nunc). Each well housed seven rings suspended in 0.5 ml of serum-free medium. Collagen gel cultures contained four rings per gel. Both suspension and collagen gel cultures were prepared with several explants to increase the yield of VEGF secreted in the medium. Conditioned medium was collected every 2 to 3 days and stored at −70°C.

For immunoblot studies, conditioned medium was blotted onto nitrocellulose paper as described.[20] The paper was then air dried, rehydrated in 20 mmol/L Tris, 500 mmol/L NaCl buffer, pH 7.8 (TBS), blocked for 2 hours with 3% dry milk (Carnation) in TBS, and incubated for 2 hours with affinity-purified goat anti-VEGF antibody (R & D Systems) diluted in blocking buffer (TTBS: 1% milk and 0.05% Tween 20 in TBS). It was then washed with TTBS, reacted with a biotinylated rabbit anti-goat IgG diluted 1:1000 in TTBS, incubated for 1 hour in alkaline phosphatase-conjugated avidin diluted 1:1000 in blocking buffer, washed in TTBS, and developed with the bromochloroindolylphosphate-nitro blue tetrazolium substrate.

ELISA for VEGF was performed according to the manufacturer's recommendations (Cytokit Red VEGF ELISA, CytImmune Sciences, College Park, MD). In this assay, VEGF of the sample competes with biotinylated VEGF, which results in an inverse relationship between the amount of VEGF and the optical density at 490 nm. Previous studies have demonstrated cross-reactivity with rat VEGF.[21] The standard curve was prepared with recombinant VEGF (CytImmune Sciences).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on collagen gel cultures of rat aorta and on isolated cell strains. The endothelial cells, smooth muscle cells, and fibroblasts used for RT-PCR were isolated and characterized in previously published studies.[7,22,23]

For RT-PCR, two collagen gel cultures, each containing four aortic ring outgrowths to maximize RNA yield, were transferred to an Eppendorf tube and centrifuged for 2 minutes at 8000 rpm. The fluid extracted from the gels after centrifugation was discarded. The gel pellets were then lysed by vortexing in 350 μl of lysis buffer RLT (Qiagen, Chatsworth, CA). The lysate was pipetted directly into the QIAshredder column, and the RNA was

extracted according to the Qiagen's RNeasy protocol. For the reverse transcription, first strand cDNA was synthesized from total RNA using oligo-dT primers. The following components were added to a nuclease-free microcentrifuge tube: 1 μl of Oligo (dT) (500 μg/ml) (Promega, Madison, WI), one-fourth of the RNA extracted from the aortic cultures (~ 1 μg), and sterile distilled water to 12 μl. The mixture was heated to 70°C for 10 minutes, quickly chilled on ice, and supplemented with 4 μl of 5× first strand buffer (Life Technologies, Inc., Grand Island, NY), 2 μl of 0.1 mol/L dithiothreitol, and 1 μl of a dNTP mix (10 mmol/L dATP, dGTP, dCTP, and dTTP, Promega). The contents of the tubes were mixed and incubated at 42°C for 2 minutes. After adding 1 μl (200 units) of Superscript II (Life Technologies, Inc), the reaction mixture was incubated at 42°C for 50 minutes. The reaction was inactivated by heating at 70°C for 15 minutes. For the PCR, the following reagents were added to a PCR tube to a final volume of 25 μl: polymerase chain reaction buffer (Fisher, Pittsburgh, PA), 2.5 mmol/L $MgCl_2$, 1.25 mmol/L dNTP mix, 0.2 μmol/L primers for VEGF or *flk-1*, 2 μl of cDNA template, and sterile distilled water. The reaction mixture was heated to 95°C for 5 minutes before 0.25 μl of *Taq* polymerase was added. Thirty-five cycles were performed at the following conditions: denaturation, 94°C for 45 seconds; primer annealing, 55°C for 1 minute; and primer extension, 72°C for 1 minute. VEGF primers were designed from the rat VEGF cDNA sequence[24] to amplify a 308-bp region between nucleotides 151–458 of exons 3 and 7, respectively.[25] The VEGF primers are 5′ CTA TTG CCG TCC GAT TGA GA 3′ and 5′ AAT GCT TTC TCC GCT CTG A 3′. Because the sequence of the rat *flk-1* was not known, the primers were designed from consensus sequences of the mouse *flk-1*[26] and the homologous human *KDR*[27] to amplify a 399-bp region of the intracellular domain of the receptor. The *flk-1* primers are 5′ ATG GAA GAG GAT TCT GGA 3′ and 5′ CAC GGT GGT GTC TGT GTC 3′. The specificity of the amplified products was confirmed by restriction enzyme digestion and sequence analysis. PCR products were cloned in a TA vector (Invitrogen, San Diego, CA) and sequenced at the Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA.

Statistical Analysis

Data obtained from aortic cultures and ELISA assay were analyzed with SPSS/PC+ statistical software. The Student's *t*-test or analysis of variance were used to evaluate the significance of differences among experimental groups. Probability values < 0.05 were required for statistical significance.

Results

Angiogenic Response of Freshly Cut Aortic Rings

Collagen gel cultures of freshly cut aortic rings generated branching microvascular networks, as reported.[5] The angiogenic response of the explants was self-limited as microvessels stopped growing after 8 to 9 days of culture (Figure 1).

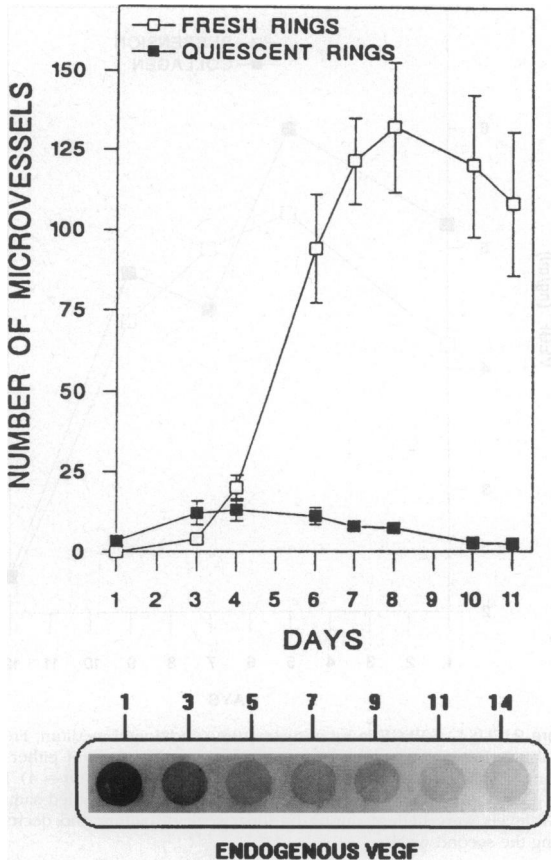

Figure 1. Angiogenesis in collagen gel culture of rat aorta. Rings of rat aorta were embedded in collagen gels immediately after excision from the animal (fresh rings) or 11 days after excision (quiescent rings). Freshly cut aortic rings generated a prominent angiogenic response. Conversely, quiescent rings had a markedly reduced capacity to generate microvessels ($P < 0.001$). $N = 4$. Data are expressed as means ± SEM. Immunoblot staining of rat aorta-conditioned medium with an anti-VEGF antibody demonstrated secretion of VEGF by freshly cut aortic rings (endogenous VEGF). VEGF secretion decreased over time becoming low during the second week of culture when microvessels stopped growing.

Angiogenic Response of Quiescent Aortic Rings

The self-limited nature of angiogenesis in cultures of freshly cut explants suggested that the vasoformative capacity of the rat aorta was restricted to the first days after the dissection procedure. To evaluate this possibility, we embedded aortic rings in collagen gels 10 to 14 days after excision of the aorta from the animal. Cell viability during this period was preserved by keeping the aortic rings suspended in repeated changes of serum-free medium. Ten- to 14-day-old aortic rings became quiescent and had a markedly reduced angiogenic response. Quiescent explants generated 10% of the neovessels produced by freshly cut explants (Figure 1). The few neovessels formed in these cultures were also much shorter than those produced in cultures of freshly cut explants. These findings suggested that the endog-

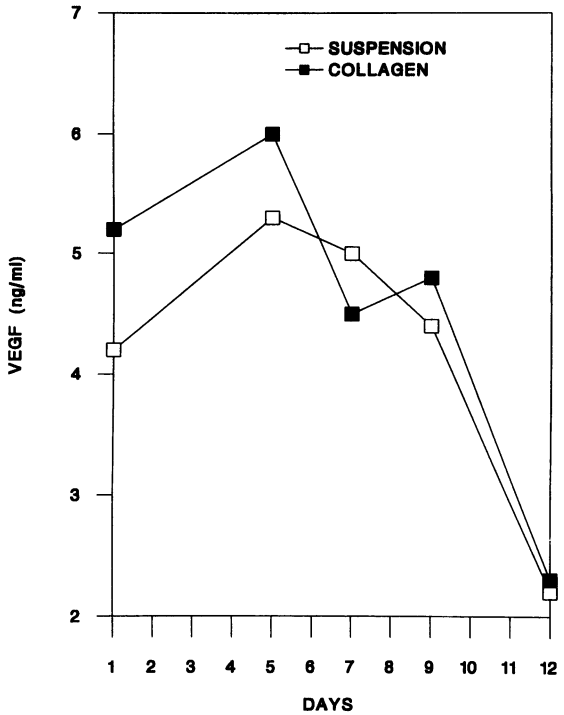

Figure 2. ELISA of VEGF in rat aortic culture-conditioned medium. Freshly cut aortic rings were cultured in serum-free growth medium either free floating in suspension ($n = 6$) or embedded in collagen gels ($n = 4$). Each data point represents the average VEGF concentration in pooled samples. VEGF levels were highest during the first week of culture and decreased during the second week.

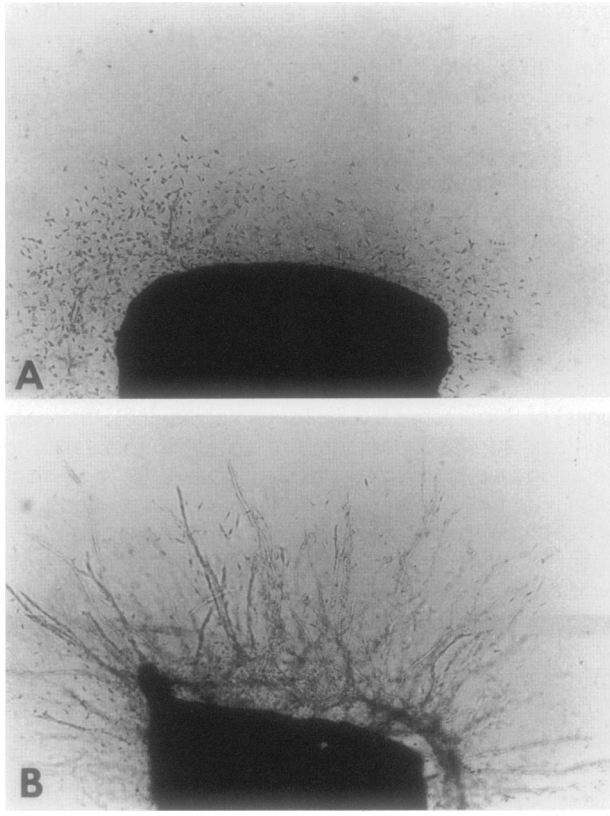

Figure 3. Effect of exogenous VEGF in collagen gel cultures of quiescent rat aortic rings. Treatment with 10 ng/ml VEGF for 7 days (**B**) induced a marked angiogenic response from an aortic ring embedded in collagen 11 days after excision from the animal. There was no significant angiogenesis in an untreated control culture (**A**). Magnification, ×30.

enous stimuli responsible for the angiogenic response of the rat aorta were significantly reduced over time.

Secretion of VEGF by Rat Aortic Cultures

Because VEGF has been implicated as a major regulator of angiogenesis,[13] we hypothesized that this growth factor was implicated in the angiogenic response of the rat aorta. Immunoblot studies demonstrated VEGF in medium conditioned by aortic rings cultured in suspension. VEGF secretion was highest in the days after the excision of the aorta from the animal and decreased over time (Figure 1). ELISA confirmed these results showing maximal secretion of VEGF during the first week of culture and a reduction during the second week (Figure 2). VEGF levels in suspension cultures decreased from 0.7 ng/ aortic ring at days 5 to 7 to 0.3 ng/aortic ring at day 12. VEGF levels in collagen gel cultures decreased from 1.4 ng/aortic culture at day 5 to 0.6 ng/aortic culture at day 12. The higher values in the collagen gel cultures are attributable to the outgrowths that in these cultures probably contributed to the production of VEGF.

Effect of Exogenous VEGF on the Angiogenic Response of Quiescent Aortic Rings

The pattern of VEGF secretion correlated with the angiogenic response of the aortic cultures. Decreased VEGF secretion in the second week of culture was associated with a markedly reduced angiogenic activity of the aortic rings. To investigate whether a correction of the VEGF deficiency could reverse this phenomenon, 11-day-old aortic rings were embedded in collagen gels and treated with exogenous VEGF. VEGF caused a dose-dependent and saturable stimulation of angiogenesis. Maximal effects were obtained with 10 ng/ml VEGF (Figures 3 and 4). Thus, correction of endogenous VEGF deficiency with exogenous VEGF restored the angiogenic response of quiescent aortic rings to values obtained with freshly cut rings.

Expression of VEGF and flk-1 mRNA in Rat Aortic Cultures

RT-PCR of collagen gel cultures of freshly cut rat aortic rings demonstrated expression of VEGF and its high affinity receptor *flk-1* (Figure 5). The specificity of the reaction products was confirmed by restriction and sequence analysis. RT-PCR of VEGF consistently demonstrated two bands. Based on sequence analysis, the two bands represented the alternatively spliced isoforms $VEGF_{165}$ and $VEGF_{189}$. The higher molecular weight band contained 72 additional basepairs, which corresponded to the exon 6 of $VEGF_{189}$. The lower molecular

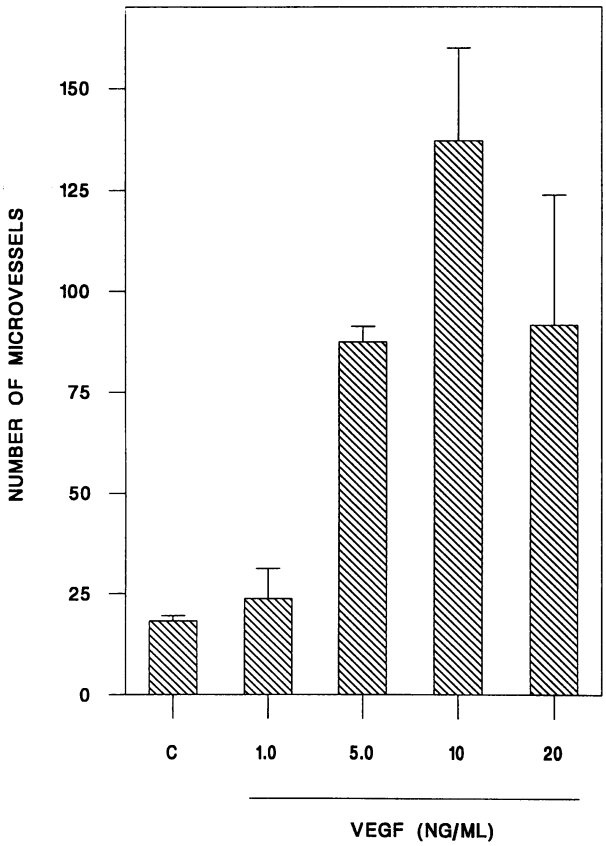

Figure 4. Effect of exogenous VEGF in collagen gel cultures of quiescent rat aortic rings. VEGF stimulated in a dose-dependent manner the angiogenic response of quiescent aortic rings embedded in collagen 11 days after excision from the animal. Maximal effect was obtained with 10 ng/ml VEGF that restored the angiogenic response to values observed in cultures of freshly excised explants ($P < 0.01$). $N = 4$. Data are expressed as means ± SEM.

weight band represented $VEGF_{165}$, which lacks exon 6 (Figure 5).

The amplified *flk-1* product was digested with *NaeI* endonuclease, which generated two fragments of 139 and 260 bp, respectively, as expected from published mouse and human sequences. The predicted amino acid sequence from this region was identical to that of the mouse *flk-1* receptor and 98% homologous to the same

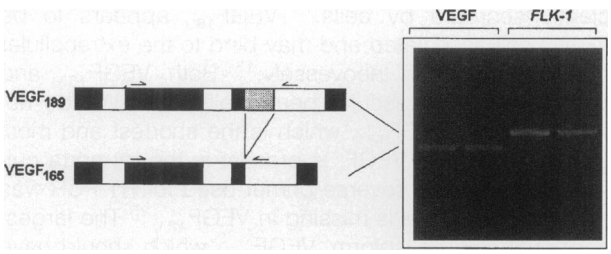

Figure 5. RT-PCR of duplicate sets of collagen gel cultures of rat aorta demonstrated expression of VEGF and its receptor *flk-1*. The specificity of the reaction products was confirmed by sequence analysis. The cultures expressed two alternatively spliced isoforms of VEGF, $VEGF_{165}$ and $VEGF_{189}$. The schematic drawing illustrates the exon composition of VEGF. $VEGF_{189}$ contains eight exons including exon 6 (**shaded box**, 72 bp), which is missing in $VEGF_{165}$. The **small arrows** indicate the location of the forward and reverse primers in exons 3 and 7, respectively.

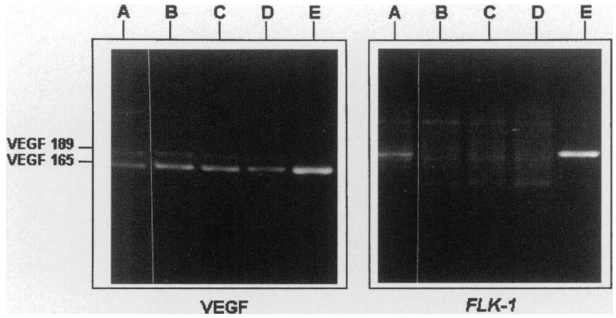

Figure 6. RT-PCR performed on isolated cell strains demonstrated expression of $VEGF_{165}$ and $VEGF_{189}$ by rat aortic endothelial cells (**A**), fibroblasts (**B**), and smooth muscle cells (**C** and **D**). Conversely, *flk-1* was expressed only by endothelial cells. Lanes labeled **E** indicate positive cDNA controls for rat $VEGF_{165}$ and *flk-1*.

region of the human *KDR* receptor. The nucleotide sequence was 98% homologous to that of the mouse receptor and 91% homologous to that of the human receptor.

RT-PCR of isolated cell strains demonstrated expression of $VEGF_{165}$ and $VEGF_{189}$ by rat aortic endothelial cells, fibroblasts, and smooth muscle cells. Conversely, *flk-1* was expressed only by endothelial cells (Figure 6).

Effect of Neutralizing Anti-VEGF Antibody on the Angiogenic Response of Freshly Cut Aortic Rings

To evaluate the importance of endogenous VEGF in the angiogenic response of the rat aorta, collagen gel cultures of freshly cut aortic rings were treated with a neutralizing anti-VEGF antibody. The anti-VEGF antibody caused a 70% inhibition of angiogenesis. Conversely, a nonimmune IgG had no effect (Figures 7 and 8). The effect of the antibody was specific for angiogenesis because the aorta-derived fibroblastic outgrowths showed no evidence of inhibition.

Discussion

Formation of new microvessels in the rat aorta model of angiogenesis is a self-limited process mediated by autocrine/paracrine mechanisms triggered by the injury of the dissection procedure. The present study indicates that VEGF is an important regulator of angiogenesis in this system. This conclusion is based on the following observations: rat aortic cultures express VEGF and its high affinity tyrosine kinase receptor *flk-1*; the arrest of the angiogenic response coincides with a reduced secretion of VEGF by the aortic cultures; quiescent aortic rings that are unable to produce a significant angiogenic response secrete lower amounts of VEGF than freshly cut rings; exogenous VEGF restores the angiogenic response of quiescent aortic rings to values observed in cultures of freshly cut rings; and the angiogenic response of freshly cut aortic rings is significantly inhibited by a neutralizing anti-VEGF antibody.

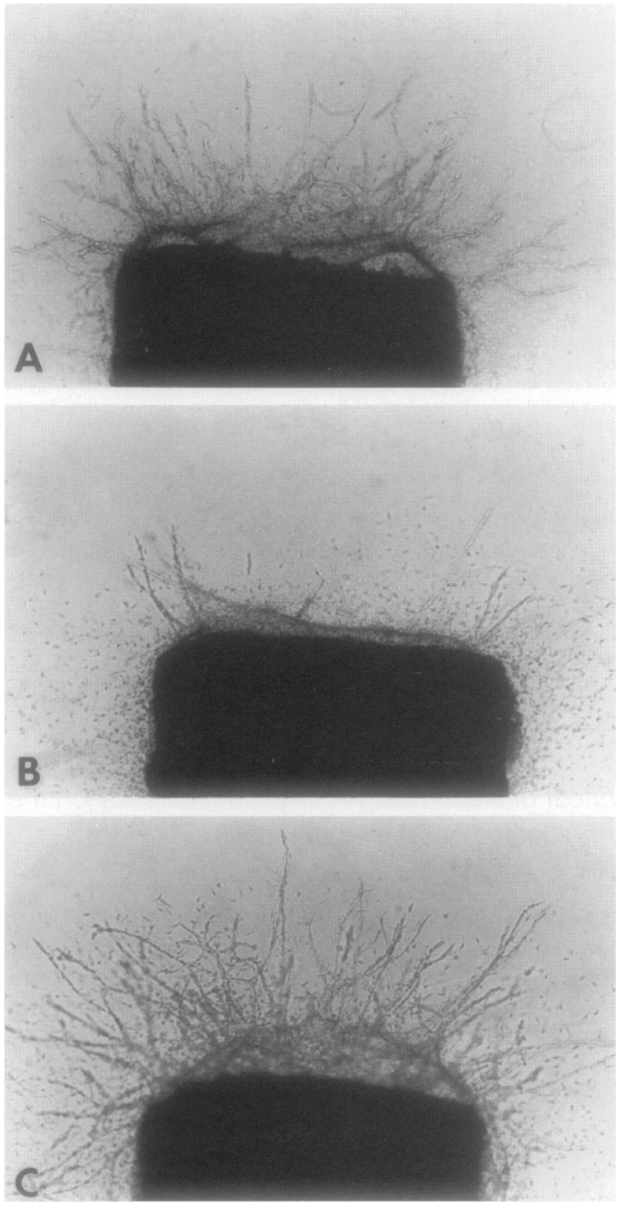

Figure 7. Effect of anti-VEGF neutralizing antibody on angiogenesis in collagen gel cultures of freshly cut aortic rings. Treatment with 40 μg/ml anti-VEGF antibody (**B**) caused a marked reduction in angiogenesis. An untreated culture (**A**) and a culture treated with 40 μg/ml nonimmune IgG (**C**) exhibited the characteristic angiogenic response of freshly cut aortic rings. Magnification, ×30.

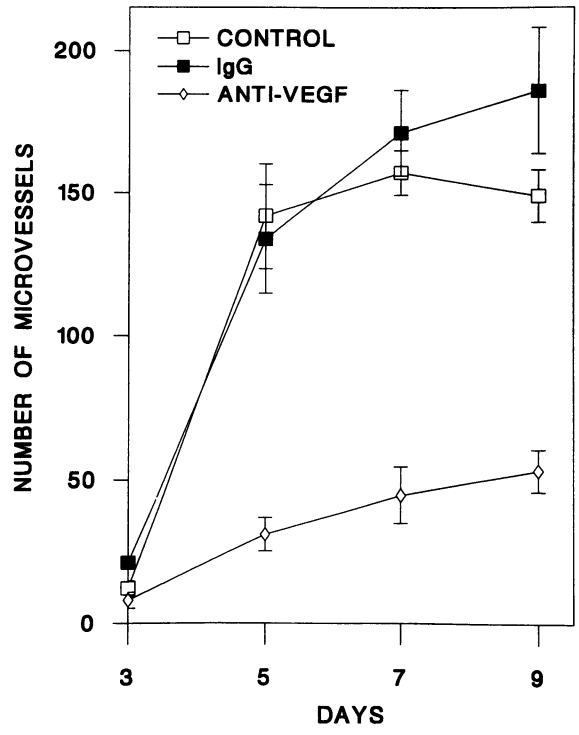

Figure 8. Effect of anti-VEGF neutralizing antibody on angiogenesis in collagen gel cultures of freshly cut aortic rings. Treatment with 40 μg/ml anti-VEGF antibody caused a 70% reduction in the angiogenic response ($P < 0.001$). Cultures treated with 40 μg/ml nonimmune IgG had the same angiogenic response as the untreated controls. $N = 6$. Data are expressed as means ± SEM.

These results indicate that VEGF is among the factors secreted by the vessel wall in response to injury. They also demonstrate that when the vessel wall is embedded in a collagen gel, which promotes endothelial morphogenesis and capillary tube formation, vascular-derived VEGF functions as a major promoter of angiogenesis. The cells of the vessel wall, ie, endothelial cells, smooth muscle cells, and fibroblasts, are all capable of producing VEGF.[10–12] However, the target specificity of VEGF is restricted to the endothelium.[13] RT-PCR studies of rat aorta-derived cell strains are consistent with these observations because they demonstrate that VEGF mRNA is expressed in endothelial cells, smooth muscle cells, and fibroblasts, whereas *flk-1* mRNA is found in endothelial cells but not in smooth muscle cells or fibroblasts. The reduced angiogenic response of the aortic rings over time is apparently not due to a deficiency of VEGF receptors because quiescent rings express the *flk-1* mRNA by RT-PCR (data not shown) and are fully responsive to exogenous VEGF.

RT-PCR of aortic cultures revealed two alternatively spliced isoforms of VEGF, $VEGF_{165}$ and $VEGF_{189}$. The same VEGF isoforms were demonstrated in isolated endothelial cells, smooth muscle cells, and fibroblasts. $VEGF_{165}$, which is the most soluble of the two isoforms, consistently appeared as the most abundant species. $VEGF_{165}$ promotes endothelial cell growth and is efficiently secreted by cells.[13] $VEGF_{189}$ appears to be mostly cell associated and may bind to the extracellular matrix around the neovessels.[13] Both $VEGF_{165}$ and $VEGF_{189}$ induce vascular permeability.[13,14] We do not know whether $VEGF_{121}$, which is the shortest and most soluble isoform of VEGF, is present in the rat aorta cultures because the reverse primer used for RT-PCR was from exon 7, which is missing in $VEGF_{121}$.[25] The largest and least soluble isoform, $VEGF_{206}$, which should have been detected by our primer set, was not observed in either the aortic cultures or the isolated cell strains.

Interestingly, the neutralizing antibody against VEGF inhibited 70% of the angiogenic response but was unable to completely abolish it. It is possible that some VEGF molecules, particularly those bound to the extracellular

matrix, were inaccessible to the antibody. An alternative explanation is that the angiogenic response of the rat aorta is mediated by more than one growth factor. Of particular interest is basic fibroblast growth factor (bFGF) that is released by the rat aorta after injury[20] and has the capacity to stimulate angiogenesis synergistically with VEGF.[28] The previous observation that neutralizing anti-bFGF antibodies reduce rat aortic angiogenesis by 40%[20] indicates that bFGF contributes to the angiogenic response of the aortic rings. However, additional studies are needed to determine whether VEGF and bFGF function independently or share the same angiogenic pathway. An additional mediator may be platelet-derived growth factor because it is produced by the vessel wall in response to injury[29] and stimulates angiogenesis when added as an exogenous component to the aortic cultures.[9] Finally, transforming growth factor-β1, which is among the growth factors up-regulated after injury,[30] may contribute to the angiogenic response by stimulating VEGF production in fibroblasts.[31] However, the role of this growth factor is unclear because exogenous transforming growth factor-β1 inhibits rat aortic angiogenesis.[9] In addition, transforming growth factor-β1 has been shown to down-regulate expression of *flk-1* in endothelial cells.[32]

The mechanisms regulating the production of VEGF by the aortic wall are unknown. Previous studies have demonstrated that VEGF expression is up-regulated by hypoxia.[33] It appears unlikely that hypoxia played a role in the angiogenic response of the rat aorta because the cultures were kept at atmospheric oxygen tension. Rather, our results demonstrate a link among mechanical injury, VEGF secretion, and angiogenesis. This observation is consistent with reports by others that mechanical or immune-mediated injury cause up-regulation of VEGF expression.[19,34] Injury may stimulate VEGF production either directly or indirectly through the action of intermediate factors. For example, platelet-derived growth factor, which is produced by injured endothelial cells,[34] has been shown to stimulate the expression of VEGF in smooth muscle cells.[3]

The finding that VEGF mediates the angiogenic response of aortic endothelial cells to injury is consistent with reports that VEGF promotes the reendothelialization of arteries injured *in vivo* with a balloon catheter.[35] These observations suggest that both impaired angiogenesis and inadequate reendothelialization of denuded arteries may be due to an insufficient local production of VEGF that can be rectified with either exogenous VEGF or VEGF gene therapy.[35,36] An additional implication of these studies is that the type of endothelial response to VEGF depends on the spatial context in which it takes place. Thus, endothelial cells respond to VEGF by forming capillary tubes in the three-dimensional environment of a collagen gel and intimal sheets in the two-dimensional environment of a mechanically denuded artery. The type of endothelial response may be modified by changes in the extracellular matrix environment around the endothelium. In fact, the rat aortic endothelium switches from its native sheet-like configuration to a microvascular phenotype on exposure of its apical surface to a fibrin or collagen gel.[5,6] Conditions similar to the rat aortic cultures may occur *in vivo* as a result of pathological processes. For example, the injured endothelium of a complicated atherosclerotic plaque or of a thrombosed artery may become embedded in collagen or fibrin matrices that promote capillary tube morphogenesis. The VEGF secreted by the injured vessel would in this context stimulate neovascularization by the intimal endothelium, which would reproduce *in vivo* the angiogenic effect that we have observed *in vitro* with the rat aorta model. VEGF may also contribute to the neovascularization of atherosclerotic plaques by promoting neovessel formation from the vasa vasorum of the adventitia.[37] The potential involvement of VEGF in the pathological neovascularization of the vessel wall may have important implications for the fate and stability of atherosclerotic plaques and recanalized thrombi.

In conclusion, our study indicates that VEGF plays a significant role in the autocrine/paracrine mechanisms that regulate the angiogenic response of the rat aorta to injury. This suggests that blood vessel-derived VEGF may represent an important mediator of vascular proliferation in physiological and pathological conditions associated with vascular injury.

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