Modulation of Endothelial Cell Growth Arrest and Apoptosis by Vascular Endothelial Growth Inhibitor

Jingyi Yu, Song Tian, Linda Metheny-Barlow, Li-Jin Chew, Andrew J. Hayes, Hongguang Pan, Guo-Liang Yu, Lu-Yuan Li

Abstract—Vascular endothelial growth inhibitor (VEGI), a new member of the tumor necrosis factor family, is an endothelial cell–specific gene and a potent inhibitor of endothelial cell proliferation, angiogenesis, and tumor growth. We report here that VEGI mediates the following two activities in endothelial cells: early G₁ arrest in G₀/G₁ cells responding to growth stimuli, and programmed death in proliferating cells. G₀/G₁-synchronized bovine aortic endothelial cells were treated with VEGI before and after the onset of the growth cycle. When the cells were stimulated with growth conditions but treated simultaneously with VEGI, a reversible, early-G₁ growth arrest occurred, evidenced by the lack of late G₁ markers such as hyperphosphorylation of the retinoblastoma gene product and upregulation of the c-myc gene. Additionally, VEGI treatment led to inhibition of the activities of cyclin-dependent kinases CDK2, CDK4, and CDK6. In contrast, VEGI treatment of cells that had entered the growth cycle resulted in apoptotic cell death, as evidenced by terminal deoxytransferase labeling of fragmented DNA, caspase 3 activation, and annexin V staining, all of which were lacking in nonproliferating cells treated with VEGI. Additionally, stress-signaling proteins p38 and JNK were not as fully activated by VEGI in quiescent as compared with proliferating populations. These findings suggest a dual role for VEGI, the maintenance of growth arrest and induction of apoptosis, in the modulation of the endothelial cell cycle. (Circ Res. 2001;89:1161-1167.)

Key Words: angiogenesis ■ apoptosis ■ cell cycle ■ cytokine ■ endothelial

The control of endothelial cell growth cycle is critical to the function of the cardiovascular system. The endothelium performs vital secretory, synthetic, metabolic, and immunologic functions, and is involved in tissue homeostasis, fibrinolysis and coagulation, blood-tissue exchange, and neovascularization.1,2 These roles require that the endothelium be stable but at the same time be able to undergo remodeling as needed. The endothelium in a mature vasculature under physiological conditions is a highly quiescent tissue.3 The proliferation of endothelial cells in physiological angiogenesis as seen in utero, in wound healing, or in the female reproductive system is apparently well controlled by a balance between positive and negative regulators of angiogenesis.4,5 Although endothelial cell proliferation is important for angiogenesis, induction of apoptosis, which severely destabilizes an existing vasculature, is also necessary for vascular remodeling.6–8 and has been shown to precede tumor neovascularization.9 Aberrant apoptosis of the endothelium has been shown to influence atherosclerotic plaque stability,10 congestive heart failure,11 coronary diseases,12 and ischemic neuronal loss.13

We recently reported the discovery of an endothelial cell–specific gene, vascular endothelial growth inhibitor (VEGI).14,15 The protein consists of 174 amino acids and exhibits a 20% to 30% sequence homology to members of the tumor necrosis factor (TNF) superfamily. The VEGI gene is expressed predominantly in endothelial cells, and the VEGI mRNA is detectable in many adult human organs, suggesting a physiological role of the gene in a normal vasculature. Recombinant VEGI inhibited endothelial cell proliferation with remarkable potency, but had no effect on the growth of any other types of cells examined under similar experimental conditions. The protein also inhibited the formation of capillary-like structures by endothelial cells in collagen gels, and the growth of capillaries induced by either basic fibroblast growth factor or vascular endothelial cell growth factor into collagen gels placed on the chick chorioallantoic membrane. Overexpression of a secreted form of VEGI in murine colon cancer cells (MC-38) strongly inhibited the ability of these cells to form tumors in syngenic C57/BL mice. Moreover, coinoculation of either human breast cancer or prostate cancer cells with Chinese hamster ovary cells overexpressing the secreted form of VEGI led to marked inhibition of the growth of the xenograft tumors in athymic nude mice. These findings suggest that VEGI is a negative regulator of angiogenesis.
We report here that the effect of VEGI activity on endothelial cells is cell cycle–dependent. Treatment of G₀/G₁-synchronized adult bovine aortic endothelial (ABAE) cells with VEGI gave rise to a reversible growth arrest in the early G₁ phase of the cell cycle. On the other hand, exposure of proliferating ABAE cells to VEGI resulted in apoptotic cell death. These data suggest that VEGI may play an important role in endothelial cell cycle control.

Materials and Methods

Cell Culture

Human umbilical vein (HUVE), human aortic (HAE), and fetal bovine heart (FBHE) endothelial cells (Clonetics) were grown in endothelial growth medium (EGM-2, Clonetics). Human dermal microvascular (HMVE), human coronary artery (HCAE), and bovine capillary endothelial (EJG; American Type Culture Collection) cells were grown in EGM2-MV (Clonetics). ABAE, mouse brain endothelial (bEND.3), and mouse heart endothelial (HSMV) cells were gifts from Dr Peter Böhlen (ImClone Inc, New York, NY). Human vascular coronary smooth muscle cells (HVSM cells; Clonetics) and ABAE cells were cultured in IMEM (Biofluids Biosource International), 10% FBS, and 1 ng/mL basic fibroblast growth factor (Promega). EAHy926, a human endothelial cell–derived hybridoma, was a gift from Dr Cora-Jean Edgell (University of North Carolina, Chapel Hill, NC). These cells, as well as bEND.3 and HSMV, were maintained in IMEM with 10% FBS.

Ribonuclease Protection Assays (RPAs)

A VEGI (GenBank accession No. AF039390) fragment (bases 1128 to 1360) was cloned into pcDNA3 between the EcoRI and NotI sites (Invitrogen). A mouse β-actin fragment (bases 859 to 939, accession No. X03672) was cloned into pSP72 between the HindIII and BamHI sites (Promega). A 220-bp human 36B4 gene fragment was cloned into pGEM4Z at the PstI site. The antisense probes were 32P-labeled and prepared by using the MAXIscript kit (Ambion). Total RNAs (15 to 20 μg) were hybridized with 2 to 4 × 105 cpm of each probe using the RPA III kit (Ambion). The mixtures were digested and subjected to PAGE and autoradiography. The intensities of the mRNA bands, relative to 36B4, were determined using a PDI densitometer (PDI Inc) and fitted to the following simple exponential equation:

\[ y = y_0 + a \times \exp(b \times x) \]

Where \( R = 0.999 \). The cell number data were fitted to the following sigmoidal regression equation:

\[ y = y_0 + a \times \frac{1}{1 + \exp \left(-\left(x-x_0\right)/b\right)} \]

Protein Expression and Purification

The VEGI protein was purified by affinity chromatography, as described. The activity of VEGI was determined by measuring the inhibition of ABAE cell proliferation. The purified protein either was subjected to immunoprecipitation, by using a monoclonal antibody (5-10B) to VEGI, or was subjected to boiling and centrifugation; in either case, removal of the VEGI protein resulted in inactivation of the preparation, as determined by cell proliferation or by 3H incorporation (data not shown).

Cell Synchronization and Proliferation

Early passages (passages 4 to 9) of primary cell cultures of ABAE cells were allowed to become confluent and remain so for >3 days. Cells were considered synchronized in G₀/G₁ phase if no more than 5% of the cells were incorporating 3H thymidine. The cells were also subjected to propidium iodine (PI) staining and fluorescence-activated cell sorting (FACS) analysis, as described, to confirm that ~90% of the cells were in G₀/G₁. The rate of cell proliferation was determined by counting the number of cells using a Coulter counter, as described.

Cyclin-Dependent Kinase (CDK) Activities

G₀/G₁-synchronized ABAE cells were cultured in the presence or absence of VEGI, washed, incubated in lysis buffer for 15 minutes on ice, and then centrifuged. Protein concentrations were determined by using the BCA assay (Bio-Rad Laboratories). Cell lysates were analyzed for CDK activities, as described, using antibodies against CDK2, CDK4, or CDK6 (Santa Cruz Biotechnology). Recombinant human retinoblastoma protein (pRB) C-terminus fusion protein (RB-C; New England Biolabs) was the substrate for CDK4 and CDK6, and histone H1 (Calbiochem Corp) for CDK2.

Antibodies for Western Blotting

Antibodies to pRB, c-Myc, and β-actin were from Zymed. Caspase 3 antibody was from R&D Systems. Anti-α/β integrin antibodies were from Promega. Antibodies that detect total p38 and JNK were from Cell Signaling Technology.

Apoptosis

Cells were stained with an APO-BRDU kit (Phoenix Flow Systems) to measure the incorporation of bromodeoxyuridine (BrDU) into 3'-hydroxyl of fragmented DNA, as described. PI was used to stain total DNA, as described. The cells were analyzed by using a FACStar-Plus flow cytometer (Becton-Dickinson). With TACS apoptosis detection kit, annexin V-FITC binding and PI staining were carried out according to manufacturer protocol (Trevigen). To exclude necrotic cells, only cells that were annexin V positive and PI negative were counted for early stages of apoptosis.

Results

Detection of VEGI Expression

We previously reported that VEGI is expressed primarily by endothelial cells among >20 different types of human primary cells and cancer cell lines. We report here that VEGI was found in a variety of endothelial cells of human, bovine, and murine origin. These include human umbilical cord vein (HUVE), aorta (HAE), dermal microvessel (HMVE), and coronary artery (HCAE) (Figure 1A). VEGI was also found in an immortalized human endothelial hybridoma, EAHy926. Whereas VEGI mRNA was not detectable in ABAE cells by Northern blotting, it was detected by the more sensitive RPA. In addition, VEGI expression was detected in other bovine endothelial cells, FBHE and EJG, and in the murine endothelioma line bEND.3, but not in cultured human smooth muscle (HVSM) cells or murine endothelioma H5V cells.

VEGI has been shown to inhibit HUVE and ABAE cell growth. To examine regulation of VEGI mRNA under growth-inhibitory conditions, we treated a panel of endothelial cells with recombinant TNF-α at levels that cause inhibition of endothelial growth. TNF-α is a well-characterized inflammatory cytokine. It has angiogenic properties in vivo, but generally inhibits cultured endothelial cell proliferation. All of the cultured human primary endothelial cells responded to TNF-α with an upregulation of VEGI expression. The upregulation was not apparent in bovine cells or in EAHy926 cells (Figure 1A). We further examined the relationship between VEGI expression and the growth status of HUVE cells. HUVE cells synchronized in G₀/G₁ were reseeded under normal growth conditions and were then harvested daily until the cells were again confluent, and RNA levels were analyzed (Figure 1B). VEGI expression was found to be relatively low in proliferating HUVE cells, as seen in the first 4 days after seeding. When the cultures approached confluence, however, the VEGI mRNA levels
increased markedly, reaching a level that is several times higher than that seen with growing cells. This is consistent with the inhibitory role of VEGI on these cells.

**Endothelial Cell Response to VEGI Is Cell Cycle–Dependent**

To investigate the mechanism of action of VEGI, we chose to use ABAE cells, which express low levels of VEGI but are highly responsive to this cytokine.15 Interestingly, we found that growth stage of cells had a significant impact on the response to VEGI. Density-arrested ABAE cells were reseeded under growth conditions. The cells entered the cell cycle and began to divide within ~20 hours. The cell number doubled approximately every 20 hours until the cells were confluent once again in 6 to 8 days, at which time most of the cells again became contact-inhibited (Figure 2A). VEGI was added to the cell cultures at various intervals during this period of time, namely, at the beginning when the G0/G1 cells were being reseeded, in the middle of the growth phase of the cells in culture, and at the end of growth period when the cells were nearly confluent. The cell numbers were determined in
24-hour intervals after the addition of VEGI. The cell numbers remained largely unchanged when the G0/G1-synchronized ABAE cells were reseeded in the presence of VEGI at indicated concentrations. [3H]Thymidine was added to media in 16 hours. Amount of incorporated [3H]thymidine (solid bars) and number of cells (open bars) were then determined in 6 hours. Data are pooled from two independent experiments and are mean±SD (n=6). *P<0.01 and **P<10^-4 (ANOVA). B, Growth inhibition is reversible. G0/G1-synchronized ABAE cells were reseeded in growth media in the absence (●) or presence (○ and ⌂) of VEGI (30 ng/mL). Media were replaced with fresh media on day 3. VEGI was removed from media of one of the experimental groups (○), whereas the media of the other experimental group continued to contain VEGI (●). Number of cells per well was counted at indicated time points. Values are mean±SD.

24-hour intervals after the addition of VEGI. The cell numbers remained largely unchanged when the G0/G1-synchronized ABAE cells were reseeded in the presence of VEGI (day 0; Figure 2A). The cell numbers also remained unchanged when confluent cultures were treated with VEGI (day 6; Figure 2A). However, a substantial number of cells died in cultures that were treated with VEGI on days 2 and 4 after seeding (Figure 2A). Confluent ABAE cells were synchronized with >90% of cells in G1 phase, whereas day 2 cells were growing rapidly and had 20% to 40% of cells in S phase (Figure 2B). These results indicate that VEGI had the following two different effects: growth arrest of G0/G1 cells that were responding to growth stimuli and induction of cell death in cells that had already entered the cell growth cycle. It is important to note that there was no more cell death when ~40% of the cells died, nor was there any significant cell growth thereafter, in the presence of VEGI; this suggests that the remaining cell population had undergone growth arrest.

Growth Arrest Induced by VEGI Is Reversible
We determined the ability of ABAE cells to synthesize DNA in the presence of VEGI while responding to growth stimuli.

Figure 3.

Figure 4.

Figure 5.

Figure 4. VEGI inhibits pRB hyperphosphorylation and c-myc upregulation. G0/G1-synchronized ABAE cells were reseeded in growth media in the absence (-) or presence (+) of VEGI (60 ng/mL). Cells were harvested at indicated time intervals. Total cell lysates were subjected to Western blotting analysis, using a monoclonal antibody to pRB, or to the Myc protein. The same membrane was analyzed for β-actin as a protein loading control. The experiment was repeated three times.

We found that cells treated with VEGI were unable to incorporate [3H]thymidine (Figure 3A). The inhibition was dose-dependent, with a half-maximal inhibition concentration (IC50) of ~30 ng/mL (1.5 nmol/L). VEGI thus prevented the G0/G1-synchronized cells from progressing into the S phase. Furthermore, the inhibition by VEGI was reversible, as the cells resumed growth at a rate comparable with that of the untreated cells once VEGI was removed from the culture media (Figure 3B); this suggests that the arrested cells were unable to proceed to late G1, which is an irreversible event.

VEGI-Treated Cells Lack Markers of Late G1 Phase
We determined the impact of VEGI treatment on typical markers of the G1 phase of the cell cycle to find out whether VEGI treatment causes early G1 arrest. A well-established marker for the late hours of the G1 phase is the hyperphosphorylation of the retinoblastoma gene product, pRB, which is underphosphorylated in G0 or early-G1 cells. To determine the phosphorylation status of the pRB protein, density-arrested ABAE cells were reseeded in the absence or presence of VEGI (60 ng/mL). The cells were harvested at various time points. Values are mean±SD.

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Figure 5.

VEGI inhibits CDK2, CDK4, and CDK6 activation. G0/G1-synchronized ABAE cells in confluent cultures (conf) were reseeded in growth media in the absence (-) or presence (+) of VEGI (60 ng/mL). Cells were harvested after 4 and 24 hours. Protein levels of CDK2, CDK4, and CDK6, detected by Western blotting analysis of the lysates, are shown. Also shown are the extent of phosphorylation of the substrates (histone H1 for CDK2, or RB-C for CDK4 and CDK6) as a measure of the activity of these kinases. Equal loading was controlled by β-actin. The experiment was repeated two times.
intervals and subjected to Western blotting analysis for pRB. The occurrence of a predominant, higher molecular weight species of pRB indicated that the protein was mostly hyperphosphorylated within 24 hours after seeding in the absence of VEGI, whereas in VEGI-treated cells the pRB protein remained largely underphosphorylated, as represented by the lower molecular weight species (Figure 4). Thus, the growth arrest caused by VEGI treatment precedes pRB phosphorylation. To confirm this finding, we determined the expression of the c-myc gene, another well-studied late G1 marker. Western blotting analysis of the Myc protein demonstrated that c-myc gene expression was suppressed by VEGI treatment, whereas the Myc protein level was elevated in the proliferating cells as expected. These data demonstrate that VEGI treatment prevented endothelial cells from proceeding to late G1 phase.

VEGI Inhibits the Activities of CDKs

Because pRB is the substrate of a number of CDKs such as CDK4, CDK6, and CDK2, we investigated the effect of VEGI treatment on the activities of these enzymes. G0/G1-synchronized ABAE cells were reseeded in growth media in the absence or presence of VEGI (60 ng/mL) and kinase activity and protein levels determined at 4 and 24 hours after seeding (Figure 5). CDK2 phosphorylation of histone substrate increased markedly in 4 hours as the control G0/G1 cells began to respond to growth stimuli, which was not seen in VEGI-treated cultures. The CDK2 protein level decreased in VEGI-treated cells in 4 hours and remained low at 24 hours. CDK4 and CDK6 activities in cells entering the growth cycle were similar to that in confluent cells, and increased in 24 hours after reseeding. In the presence of VEGI, however, CDK6 activities were markedly inhibited, as is evident from lowered levels of the phosphorylation of the substrate RB C-terminal fragment. Unlike the CDK2 protein, the CDK4 and CDK6 protein levels increased moderately within 24 hours in the absence of VEGI but remained relatively unchanged in the same period of time in VEGI-treated cells. These observations suggest that VEGI regulates the cell cycle by modulating the protein levels as well as the activities of the CDKs.

VEGI Induces Apoptosis in Proliferating Cells, but not in Quiescent Cells

To confirm that only proliferating ABAE cells undergo apoptosis in response to VEGI, density-arrested cells were reseeded under growth conditions, and VEGI was added to the media either on day 0 at the time of seeding, on day 2, or on day 6. The cells were harvested 24 hours after the addition of VEGI and the percentage of the cells with extensive nuclear DNA fragmentation, a hallmark of programmed cell death, was determined by terminal deoxynucleotide transferase reactions incorporating BrdU (Figure 6A). It was found that the percentage of apoptotic cells was significantly higher...
cells, but not in confluent cells or cells arrested in early G1.

VEGI-induced programmed death only in proliferating cells. G0/G1-synchronized ABAE cells were reseeded and incubated for 48 hours to allow logarithm growth. These cells and density-arrested confluent cells were then treated with VEGI (200 ng/mL) for times indicated and subsequently submitted to Western analysis for phosphorylated p38 and phosphorylated JNK. The experiment was repeated two times.

in cells treated with VEGI on day 2, when the cells were undergoing proliferation, as compared with that of the cells treated either on day 0, at which time most of the reseeded cells were in G0/G1 phase, or on day 6 when the cell cultures were nearly completely confluent and the cells were resting once again. The percentage of apoptotic cells in the cell cultures treated with VEGI on days 0, 2, and 6 were ~5%, 14%, and 2%, respectively (Figure 6B). These results indicate that VEGI induces programmed death only in proliferating cells, but not in confluent cells or cells arrested in early G1.

Caspase 3 has been implicated in VEGI-mediated apoptosis. To further examine the differential apoptotic response to VEGI in quiescent versus proliferating populations, we analyzed the activation of caspase 3 as represented by the processing of procaspase 3 into the active p17 subunit. No active p17 was seen in confluent cells with or without the addition of VEGI (Figure 6C). By contrast, proliferating cells in response to VEGI showed a marked increase of p17 from a background level. Furthermore, we compared the response of subconfluent cells cultured under normal serum conditions (10%) with those incubated under serum starvation (0.5%) for 48 hours. Interestingly, whereas the background level of apoptosis was high in the serum-starved cultures, VEGI not only did not induce further apoptosis but conferred a protective effect against serum starvation–induced cell death (Figure 6D).

Differential Activation of MAPKs in Proliferating Versus Confluent Cells

Because activation of stress-signaling proteins such as p38 and JNK is required for VEGI-mediated apoptosis of subconfluent BPAE cells, we sought to determine whether the activation of these molecules was different in ABAE in proliferating cells in comparison with confluent cells in response to VEGI. We found that VEGI led to marked and sustained phosphorylation of p38 in proliferating cells, but the effect was only moderate in confluent cells. Phosphorylation of p38 occurred within 10 minutes in proliferating cells, continued to increase through 60 minutes, and remained high at 90 minutes, whereas in confluent cells p38 activation appeared to plateau at 40 minutes (Figure 7). Phosphorylation of JNK, by contrast, was not seen in VEGI-treated confluent cells; however, the p46 JNK became phosphorylated within 10 minutes in proliferating cells and continued to increase through 60 minutes (Figure 7). These results indicate that VEGI was able to activate certain apoptotic pathways in proliferating cells but not in confluent cells.

Discussion

Our data indicate that VEGI plays a key role in the modulation of endothelial cell proliferation. VEGI-induced growth arrest takes place in the early G1 phase of the cell cycle. This is evident from several lines of observations. Firstly, VEGI-induced growth arrest is reversible. There are two distinguishable periods in the G1 phase, early and late G1. The entering of G0 cells to early G1 is reversible; however, once the cells proceed to late G1 phase, they are obliged to continue into the S phase. Secondly, the pRB protein in VEGI-treated cells did not become hyperphosphorylated. The pRB protein is a key player in the regulation of G1/S transition. Through the early hours of G1, pRB is in an underphosphorylated form. During the last hours of G1, most of the pRB molecules become hyperphosphorylated, giving rise to the inactivation of the growth-inhibitory effect of this protein. The pRB protein apparently was unable to undergo hyperphosphorylation in the presence of VEGI. Thirdly, VEGI prevented the upregulation of the early-response gene c-myc the transcription of which indicates the transition of the late G1 to the S phase. As a positive regulator of G1–specific CDKs, the c-myc gene is activated by mitogenic signals, and is suppressed by growth-inhibitory and differentiation signals. Our data demonstrated that the c-myc gene expression was markedly upregulated in endothelial cells as they underwent the transition from G1 to G2 and subsequently entered the growth cycle. In the presence of VEGI, however, the Myc protein levels gradually diminished.

VEGI inhibition of the activation of CDK2, CDK4, and CDK6 activities is not only consistent with the induction of early G1 growth arrest, but is suggestive of the mechanism of action of this unique cytokine in the control of the cell cycle. Phosphorylation of pRB by cyclin D–, cyclin E–, or cyclin A–dependent kinases has been well-documented. We determined the activities of the cyclin D–dependent kinases CDK4 and CDK6 because the D-type cyclins are induced by growth factor stimulation, whereas in many types of cells cyclin E is found in middle to late G1 phase when pRB has already become extensively phosphorylated, and cyclin A is induced mostly in cells in S phase and diminishes when the cells exit from mitosis. That CDK4 and CDK6 were inactivated in the presence of VEGI indicates a possible involvement of VEGI in the activation of inhibitors of these cyclin D–dependent kinases. In contrast, the inhibition of the activity of the cyclin A– and cyclin E–dependent CDK2 by VEGI treatment appears to have resulted from a decrease of the CDK2 protein. This suggests that the modulation of CDK2 activity by VEGI may take place at the level of gene transcription or protein degradation.

VEGI-induced apoptosis occurred mostly in proliferating cells. At a rate of 14% cell death per 24 hours in the presence of VEGI, a loss of ~36% of the cells would occur in 3 days.
This is consistent with $\sim40\%$ of cell death observed when proliferating cells were treated. The rest of the cells apparently underwent growth arrest, as there was no more cell death or cell growth, presumably because the surviving cells had re-entered the early G1 phase at the time of VEGI treatment. It was reported that treatment of BPAE cells with a similar preparation of VEGI led to apoptosis.25 Cells in subconfluent cultures were used in that study. Because the majority of cells in a subconfluent culture are undergoing proliferation, the results are in agreement with our findings.

VEGI-induced apoptotic pathways appear to be active only when the cells are present in certain cell cycle phases other than early G1. Both p38 and JNK were reported to be activated by VEGI.25 An important finding of that study is that elimination of either p38 or JNK signaling leads to a significant decrease in VEGI-mediated apoptosis, suggesting that activation of both pathways is necessary to signal the significant decrease in VEGI-mediated apoptosis, suggesting an inability of VEGI to induce apoptosis in quiescent endothelial populations.

The upregulation of VEGI expression by TNF-α and the increased VEGI level in resting endothelial cells compared with proliferating cells suggest a possible function of VEGI in modulating vascular stability as well as remodeling. In addition, the initially discovered VEGI was postulated to be a type II membrane protein. In mammalian cells did not result in a soluble protein in the conditioned media and did not have detectable effects on endothelial growth.14,15 A truncated form of VEGI consisting of residues 29 to 174 that apparently possesses antiangiogenic activity has been used in all studies instead. We now have evidence (L.-J. Chew et al., unpublished data, 2001) for the existence of a secreted form of VEGI that can be detected in HUVE cell conditioned media and human sera, and that mimics the angiogenesis and antiangiogenic activities of recombinant, secretable VEGI fusion protein previously described.14 Although these observations strongly support a physiologically significant role for VEGI in vivo, the present study at the cellular level using recombinant VEGI also holds direct relevance to the application of VEGI as an inhibitor of angiogenesis.

Acknowledgments

This work is supported in part by grants from the National Heart, Lung, and Blood Institute (HL06060) and the Department of Defense Breast Cancer Research Program (DAMA17-98-1-8093) (to L.-Y.L.).

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Circ Res. 2001;89:1161-1167; originally published online November 15, 2001;
doi: 10.1161/hh2401.101909

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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