Vascular Endothelial Cell Growth Factor–Driven Endothelial Tube Formation Is Mediated by Vascular Endothelial Cell Growth Factor Receptor-2, a Kinase Insert Domain–Containing Receptor

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Abstract—Vascular endothelial cell growth factor (VEGF) binds to 2 related receptor tyrosine kinases, known as kinase insert domain–containing receptor (KDR) and fms-like tyrosine kinase (Flt-1). The KDR has been shown to mediate VEGF-stimulated endothelial cell mitogenesis, migration, and permeability. The Flt-1 receptor has been suggested to mediate VEGF-stimulated endothelial branching morphogenesis, a process whereby endothelial cells, in the presence of a 3D milieu composed of extracellular matrix components and a mixture of growth factors, undergo a morphological transition into a tubular network with many lumina. In the present study, we have used 2 independent endothelial cell tube formation models and highly selective VEGF mutants for the KDR and Flt-1 receptors. We demonstrate that KDR, not Flt-1, stimulation is responsible for the induction of endothelial tubulogenesis. In addition, we demonstrate a modulatory role for Flt-1 in VEGF-mediated tube formation. We also report that VEGF-driven endothelial tube formation is inhibited by selective inhibitors of mitogen-activated protein kinase activation and p38 protein kinase. (Arterioscler Thromb Vasc Biol. 2001;21:1934-1940.)

Key Words: angiogenesis ■ lumen ■ kinase insert domain–containing receptor ■ vascular endothelial cell growth factor ■ endothelium

Vascular endothelial cell growth factor (VEGF), a potent regulator of angiogenesis and vasculogenesis, has been shown to play an essential role in physiological and pathological angiogenesis.1,2 A unique feature of VEGF, compared with other growth factors that are involved in the regulation of new vessel formation, is the high degree of specificity for endothelial cells. VEGF treatment of cultured endothelial cells has been shown to promote cell survival, proliferation, migration, calcium influx, and branching morphogenesis.3 In vivo, VEGF treatment also elicits new vessel growth, endothelial proliferation, increased vascular permeability, and vasodilation.3 The biological effects of VEGF are mediated by the activation of 2 receptor tyrosine kinases: (1) fms-like tyrosine kinase (Flt-1), also known as VEGF receptor (VEGFR)-1, and (2) kinase insert domain–containing receptor (KDR), also known as VEGFR-2. Gene knockout experiments have confirmed a critical role in angiogenesis for Flt-1 and KDR.4,5 A null mutation of the VEGFR-2 gene causes failure of vasculogenesis, the process of de novo formation of blood vessels from undifferentiated mesenchyme.5 The VEGF-2 knockout embryos are unable to form blood islands and to generate hematopoietic precursors. In contrast, targeted mutation of the VEGFR-1 (Flt-1) gene does not affect the differentiation of endothelial cells but causes a disorganized assembly of the developing vasculature. The vascular abnormalities caused by deletion of either VEGFR-1 and VEGFR-2 result in embryonic lethality between days 8.5 and 9.5.

Several groups have reported that KDR is the receptor that mediates endothelial cell proliferation, NO synthase expression, calcium flux, and cell survival,6,7 but the role of the Flt-1 receptor has remained elusive. Flt-1 differs from KDR in that it displays a higher affinity for VEGF but lower kinase activity, suggesting the importance of its extracellular domain. Mice with an Flt-1 mutation that results in the deletion of the kinase domain without affecting the ligand-binding region developed normal vessels and survived (study of Hiratsuka et al8). On the basis of that study, some investigators view Flt-1 as a negative regulator of VEGF activity, acting as a “decoy” but having little or no transducing activity.8,9 However, other studies suggest there may be Flt-1–specific biological activities in endothelial cells. For

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example, Maru et al.\textsuperscript{10} created a constitutively active form of Flt-1, called BCR-FL-Tm. Sinusoidal endothelial cells transfected with BCR-FL-Tm formed tubes with fenestrated structures in vitro, leading these authors to speculate that the morphogenic responses of endothelial cells to VEGF could be mediated by the activation of Flt-1.\textsuperscript{10} An Flt-1–specific ribozyme has antiangiogenic activity in vivo,\textsuperscript{11} and mice deficient in placental growth factor (PLGF, a naturally occurring Flt-1 ligand) were born with retinal angiogenesis after ischemia and in tumors.\textsuperscript{12} Kanno et al.\textsuperscript{13} using receptor-selective antibodies, concluded that Flt-1 mediated VEGF-induced endothelial migration but not proliferation.

Li et al.\textsuperscript{14} developed highly receptor-selective VEGF variants with a minimal number of sequence changes compared with the wild-type VEGF (VEGFwt). One VEGF165 mutant (Flt-sel), with 4 amino acid changes, binds with native affinity to Flt-1 and (≈128-fold weaker) to KDR compared with VEGFwt. A second variant (KDR-sel), with 3 changes from the wild-type protein, has wild-type affinity for KDR but 2000-fold reduced affinity for Flt-1. Gille et al.\textsuperscript{15} used these receptor-selective mutants to demonstrate that KDR mediates VEGF-induced endothelial proliferation and migration in vitro and new blood vessel growth in vivo. In the present study, we have used these 2 mutants, as well as VEGFwt and PLGF, to determine which VEGF receptor mediates endothelial branching morphogenesis and tube formation. By selectively activating Flt-1 or KDR in primary cultures of human macrovascular and bovine microvascular endothelial cells, we have examined the role of each individual receptor in endothelial tube formation. We demonstrate that KDR activation alone is sufficient for endothelial morphogenesis into tubelike structures.

**Methods**

**Endothelial Tube Assays**

Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics and grown in Clonetics EGM medium supplemented with 10% FBS and endothelial cell growth supplements provided by the manufacturer. Cells from passages 4 to 7 were used throughout the study. Three-dimensional collagen gels containing cells were prepared as described previously.\textsuperscript{16} After gelation at 37°C for 30 minutes, the gels were overlaid with 1× basal medium (described in detail by Yang et al.\textsuperscript{16}) supplemented with PBS (control), hepatocyte growth factor (HGF), VEGF, PLGF, VEGF mutant, or the combination of HGF and VEGF, PLGF, or VEGF mutant at the indicated concentrations.

Bovine skeletal muscle endothelial cells (BSMEs) were obtained from Vectech (Renssalaer) and grown in MG132 medium (Sigma Chemical Co) supplemented with 10% FBS and endothelial cell growth supplement (Clonetics). Cells from passages 4 to 7 were used throughout the study. Three-dimensional collagen gels containing BSMEs were prepared as described above for the HUVECs. After gelation at 37°C for 30 minutes, the gels were overlaid with 1× basal medium supplemented with PBS (control), VEGF, PLGF, VEGF mutant, or VEGF mutant at the indicated concentrations.

**Blot Analysis**

Proteins were extracted from KDR-PAE cells lysed with lysis buffer (1% deoxycholate, 1% Triton X-100, and 0.25% SDS), and lysed with proteinase K. The proteins were boiled, separated by SDS-PAGE, and transferred to nitrocellulose. The blots were blocked for nonspecific binding by using 5% nonfat milk in PBS for 1 hour at room temperature, followed by addition of primary antibodies to either KDR (MAKD-5, Genentech) or Flt-1 (MAFL-6, Genentech). The blots were washed with PBS overnight, with shaking at 4°C, blocked for 30 minutes, and the primary antibody was added onto a nitrocellulose membrane and used for analysis. Blots were incubated with anti-mouse IgG (H+L) conjugated to horseradish peroxidase (Amersham) and visualized with ECL solution (Amersham). The blots were photographed using the Openlab software as described above. Two to 3 separate experiments were repeated for each antibody.

**RT-PCR Analysis**

Reverse transcription (RT)–polymerase chain reaction (PCR) was performed on mRNA made from BSMEs and HUVECs according to the manufacturer's protocol (Qiagen). Because of limited sequence information on the bovine genes, primers were designed on the basis of regions of high homology between different species.
primer (5'-AAGAGGGCCCTTTGGGAAAGT-3') and reverse primer (3'-AAACTGTAGAAATCAAGTGTTTTCAT-5') and KDR forward primer (5'-AGACTGGTTCTGGGACAC-3') and reverse primer (3'-GATCACATAGCTGGGAACTA-5'). The cycle was programmed as follows: 50°C for 30 minutes, 95°C for 15 minutes, 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute (25 total cycles for the last 3 steps), and 72°C for 10 minutes. Loading dye was added to 20 μL of product, and samples were run on a 1% agarose gel.

Real-Time PCR Analysis (Taqman)

Real-time PCR was performed as described previously by use of the Taqman model 7700 Sequence Detector (ABI, Perkin-Elmer). Expression levels for bovine HGF gene were normalized to bovine GAPDH, which was unaffected in the different treatment groups. Bovine liver mRNA was used to construct the standard curve for HGF.

Antisera

Blocking antisera to Flt-1 (MAFL-1 and MAFL-6) and KDR (MAKD-1 and MAKD-5) were from Genentech and have been described previously. Polyclonal antisera to KDR (AF 357) and Flt-1 (SC316) were from R&D Systems and Santa Cruz Biotechnology, respectively. The phosphotyrosine antibody, PY20, was from BD Transduction Laboratories.

Results

KDR-sel but Not Flt-sel VEGF Mutants Promote Endothelial Tube Formation in 3D Collagen Gels

We and others have reported that HUVECs, when suspended in 3D type 1 collagen gels, undergo rapid apoptotic death unless the culture medium is supplemented with phorbol 12-myristate 13-acetate. The growth factors VEGF and basic fibroblast growth factor, alone or in combination, are not sufficient to support endothelial morphogenesis in this 3D model of angiogenesis. However, we have recently reported that HGF, in combination with VEGF, could stimulate endothelial tube formation 3D collagen gels. The morphology and time course of tube formation in this model have been described recently in more detail. The ability of VEGF, in combination with HGF, to support endothelial tube formation in 3D collagen gels presented the opportunity to determine which VEGF receptor mediated endothelial tube formation. To evaluate the activities of VEGFwt, PLGF, and VEGF mutants on HUVECs, we tested the proteins in combination with 2.5 nmol/L HGF. As shown in Figure 1 and Figure 2A, VEGFwt and KDR-sel promoted endothelial tube formation. In contrast, neither Flt-sel nor PLGF exhibited any tube-promoting activity at 10 nmol/L (Figures 1 and 2A). Higher concentrations (200 nmol/L) of Flt-sel and PLGF were also tested and were ineffective (not shown).

In contrast to HUVECs, the BSMEs will form branching networks when stimulated with VEGFwt (Figure 1). These tubelike structures contain lumenlike structures, apparently formed by vacuole coalescence, similar to those previously described by others using HUVECs in collagen gels. Therefore, we evaluated VEGF, PLGF, KDR-sel, and Flt-sel in this second in vitro model system. As shown in Figures 1 and 2B, VEGFwt and KDR-sel but not Flt-sel or PLGF (all tested at 10 nmol/L) stimulated endothelial network formation.

BSMEs and HUVECs Express mRNA and Protein for KDR and Flt-1

A possible explanation for the lack of response to the Flt-sel was the absence of Flt-1 expression in the endothelial cells.
used in these studies. mRNA was prepared from the BSMEs and HUVECs, and the expression of KDR and Flt-1 mRNA was assessed by RT-PCR. Both endothelial cell types expressed both VEGF receptor mRNAs, in agreement with previous publications22,23 (not shown). Surface expression of KDR and Flt-1 in HUVECs was assessed by FACS analysis (Figure 4A), and protein expression of the 2 VEGF receptors in the BSMEs and HUVECs was confirmed by Western blotting (Figure 4B and 4C). Lysates prepared from stably transfected PAE cell lines expressing either human KDR or Flt-1 were used as positive controls. Tyrosine phosphorylation of Flt-1 and KDR receptors was observed in the BSMEs, HUVECs, and PAE cells incubated with 10 nmol/L VEGFwt for 10 minutes (Figure 4B and 4C, lower panels).

VEGFwt-Driven Endothelial Tubulogenesis IsBlocked by Anti-KDR but Not Anti-Flt Antibodies

Additional data to support a role of KDR in the mediation of endothelial tubulogenesis was obtained from receptor-blocking studies. As shown in Figure 5, blocking antibodies to KDR,18 but not Flt-134 (10 μg/mL), when coincubated with VEGFwt and HGF in the HUVEC 3D collagen gel model, blocked endothelial tube formation.

Flt-sel Enhances VEGFwt-Induced but NotKDR-sel-Induced Tube Formation

To determine a potential modulator role for Flt-1 in VEGF-induced tube formation, we incubated BSMEs with a low concentration of VEGFwt (1 nmol/L) or KDR-sel in the absence and presence of an excess of Flt-sel (80 nmol/L). At these low concentrations of VEGF, tube formation per se does not actually occur, but occasional sprouting cells (defined as those longer than 50 μm) are observed. Therefore, we evaluated the frequency (number of sprouting cells per well) and the average length of the sprouts per well. As shown in Figure 6, compared with VEGFwt alone, the addition of Flt-sel (80-fold higher than VEGFwt) significantly enhanced the number and average length of sprouting cells per well. The most pronounced effect was on the number of sprouting structures. There were very few of these structures in the low-dose VEGFwt or KDR-sel+Flt-sel groups (25 to 30 per well), but there were >200 per well in the VEGFwt+Flt-sel group. Flt-sel had no significant effect on the length or frequency of sprouting structures in response to KDR-sel. Incubation of Flt-sel (80 nmol/L) in the presence of higher concentrations of VEGFwt (10 nmol/L) had no detectable effect on tube formation (not shown).

Effects of Inhibitors of PI-3-Kinase, p38 Kinase, and MAPK Kinase on Endothelial Tubulogenesis

VEGF has been reported to stimulate mitogen-activated protein kinase (MAPK [ERK1/2]) activity,25 and this response has been shown to be mediated by the KDR, not the Flt-1 receptor.15 To determine the roles of these signal transduction pathways in VEGF-mediated tube formation, we evaluated the effects of selective inhibitors by using the simpler model, ie, BSMEs, which only required VEGFwt to induce tube formation. The role of MAPK activation was assessed by using PD98059, a selective inhibitor of the upstream MAPK kinase, which phosphorylates and activates MAPK (ERK1/2).26 Pretreatment of BSME with 30 μmol/L...
PD98059 markedly reduced KDR-sel–induced MAPK activation, as indicated by the reduction in phosphorylated MAPK (ERK1/2) as shown in Figure 4D. The effects of PD98059 on KDR-sel–induced tube formation were significant, with just >50% inhibition at 10 nmol/L and nearly 75% inhibition at 30 nmol/L (Figure 7). The p38 kinase inhibitor, SB203580, has been reported to inhibit VEGF-induced endothelial migration,13 and Gille et al15 demonstrated that VEGF-induced activation of p38 was KDR dependent. Pre-treatment of HUVECs with 30 nmol/L SB203580 inhibited KDR-sel–induced p38 phosphorylation (Figure 4E) and also reduced tube formation elicited by KDR-sel, although the effects of this drug were slightly less potent than those of PD98059 (Figure 7). In addition, the effects of SB203580 and PD98059 appeared to be additive. The combination of 30 nmol/L SB203580 and 30 nmol/L PD98059 nearly completely inhibited tube formation (Figure 7) and was greater than the effect of either drug alone at the same concentration.

Pi-3-kinase has been demonstrated to transmit survival signals through the activation of Akt in several cell types,27 and VEGF survival activity in HUVECs also requires Pi-3-kinase and Akt kinase activity.28 Gille et al15 have recently reported that only VEGFwt and KDR-sel VEGF mutants, and not PLGF or Flt-sel, were capable of causing the phosphorylation of the PI-3-kinase regulatory subunit in HUVECs. To assess the role of PI-3-kinase in endothelial tube formation stimulated by VEGFwt, we tested the ability of the specific PI-3-kinase inhibitor, LY294002,29 to inhibit branching morphogenesis. Although LY294002 dose-dependently inhibited BSME and HUVEC tube formation, with near complete inhibition at 30 nmol/L (not shown), it was difficult to distinguish whether the inhibitory effects of the LY294002 were primary or secondary because of the inhibition of cell survival. Cells treated with LY294002 exhibited fragmented nuclei and other features of cell death (not shown).

**Discussion**

In the present study, we determined whether the activities of Flt-1, KDR, or both are required to mediate VEGF-stimulated endothelial branching morphogenesis in vitro. We used highly selective homodimers of the receptor-selective mutants, KDR-sel and Flt-sel, as well as the naturally occurring Flt-1–specific ligand, PLGF.

Endothelial cell tube formation in 3D gels occurs as a consequence of a number of necessary biological activities, including cell migration, vacuolization, cell-cell junction formation, and cell elongation. We have recently reported that VEGF, in combination with HGF (neither VEGF nor HGF alone, even at concentrations of 1 μmol/mL, was capable of supporting tube formation), could support HUVEC tube formation,21 and we used this new experimental condition to evaluate the effects of the VEGFR-specific mutants in human endothelial cells. The highly selective KDR-sel VEGF mutant and VEGFwt, but not Flt-sel or PLGF, when combined with HGF, were capable of stimulating endothelial tube formation in the present study. Additionally, the effects of the VEGFwt on tube formation were completely blocked by anti–KDR-specific, not anti–Flt-1-specific, antibodies. We had also noted in preliminary studies that endothelial cells derived from bovine skeletal muscle microvessels (BSMEs) could form branching networks in the 3D collagens in the presence of VEGF. The morphology of the tubelike structures formed by the BSMEs was similar to that observed with HGF and
VEGF, although the length of the tubular structures was longer. Vacuoles and lumenlike structures, similar to those reported previously in other collagen gel models, were also demonstrated. The ability of the BSMEs to form tubes in response to VEGF may be due to the ability of these cells to express low but constitutive levels of HGF. HGF mRNA was detectable in BSMEs and also increased over the 48-hour period of the tube assay (not shown). Similar to our observations with HUVECs, BSMEs formed branching networks when they were stimulated with VEGFwt and KDR-sel but not with PLGF or the Flt-sel VEGF mutant. The presence of KDR and Flt-1 mRNA and protein in the bovine and human endothelial cells was confirmed by PCR, FACS analysis, and Western blotting, clearly demonstrating that the lack of response to Flt-sel or PLGF was not due to the lack of Flt-1 expression on the endothelial cells used in the present study. The biological activity of the Flt-sel mutant has been previously demonstrated in 2 bioassays. In an earlier study, we demonstrated that Flt-sel upregulated matrix metalloproteinase-9 expression in human vascular smooth muscle cells (which express Flt-1 but not KDR). Recently Gille et al. showed that Flt-sel could potently elicit PAE migration by using PAE cells transfected with an Flt-1 mutant receptor in which 3 amino acids of the juxtamembrane region were exchanged with KDR. Finally, the lack of response to PLGF, a naturally occurring VEGF family member that selectively binds Flt-1, further substantiates the differential roles of KDR versus Flt-1 in endothelial tube formation. To evaluate a potential modulatory role of Flt-1 on KDR-mediated endothelial tube formation, we tested the effects of an 80-fold excess of Flt-sel on KDR-sel–mediated and VEGFwt-mediated tube formation in BSMEs. We were unable to detect any significant effect (ie, neither inhibitory nor stimulatory) on KDR-mediated endothelial tube formation. However, high doses of Flt-sel were capable of significantly potentiating the effects of a low concentration of VEGFwt. These data are consistent with the concept that sequestration of VEGF by Flt-1 can occur and may modulate the ability of VEGF to stimulate endothelial cells at lower concentrations. The observation that Flt-sel did not significantly alter the submaximal response to KDR-sel argues against a receptor cross-talk type of modulation. Therefore, we conclude that the binding of VEGF to KDR is necessary and sufficient to induce endothelial differentiation into tube-like structures and that a contribution from Flt-1 is not required, although this receptor may modulate this biological response by acting as a "decoy" target for VEGF. Direct roles mediated by Flt-1 signaling in angiogenesis remain to be convincingly demonstrated. However, the role of Flt-1 expression on monocytes and vascular smooth muscle cells is poorly understood and is a topic worthy of further investigation.

Ilan et al. found that phorbol 12-myristate 13-acetate−induced HUVEC tube formation in 3D collagen gels involved PI-3-kinase and MAPK kinase activation and that inhibitors of these signal transduction pathways were effective inhibitors of endothelial tube formation. VEGF-induced endothelial cell proliferation and migration have also been associated with these 2 signal transduction events, and Gille et al. have recently found that PI-3-kinase activation is selectively induced by the KDR-sel, and not the Flt-sel, VEGF mutant. In the present study, we report that KDR-driven endothelial tubulogenesis also requires MAPK and p38 activation. Although we observed inhibitory effects of the PI-3-kinase inhibitor, the death-inducing effects of this drug precluded conclusions as to the role of this pathway in endothelial tube formation.

In summary, our findings demonstrate that the VEGF receptor, KDR, is the VEGF receptor that mediates VEGF-induced endothelial differentiation into tubelike structures. These findings extend the previous studies of Gille et al. and Li et al. which have demonstrated that KDR mediates VEGF-induced endothelial migration and proliferation in vitro and VEGF-induced permeability and angiogenesis in vivo.

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References
14. Yang et al. KDR-Driven Tube Formation


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