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Vascular Endothelial Growth Factor Receptor-1 Modulates Vascular Endothelial Growth Factor-Mediated Angiogenesis via Nitric Oxide

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The known responses of vascular endothelial growth factor (VEGF) are mediated through VEGF receptor-2 (VEGFR-2/KDR) in endothelial cells. However, it is unknown whether VEGFR-1 (Flt-1) is an inert decoy or a signaling receptor for VEGF during physiological or pathological angiogenesis. Here we report that VEGF-stimulated nitric oxide (NO) release is inhibited by blockade of VEGFR-1 and that VEGFR-1 via NO negatively regulates of VEGFR-2-mediated proliferation and promotes formation of capillary networks in human umbilical vein endothelial cells (HUVECs). Inhibition of VEGFR-1 in a murine Matrigel angiogenesis assay induced large aneurysm-like structures. VEGF-induced capillary growth over 14 days was inhibited by anti-VEGFR-2-blocking antibody as determined by reduced tube length between capillary connections ($P < 0.0001$) in an *in vitro* angiogenesis assay. In contrast, loss of VEGFR-1 activity with a neutralizing anti-VEGFR-1 antibody resulted in an increase in the accumulation of endothelial cells ($P < 0.0001$) and a dramatic decrease in the number of capillary connections that were restored by the addition of NO donor. Porcine aortic endothelial (PAE) cells expressing human VEGFR-1 but not VEGFR-2 plated on growth factor-reduced Matrigel rearranged into tube-like structures that were prevented by anti-VEGFR-1 antibody or a cGMP inhibitor. VEGF stimulated NO release from VEGFR-1- but not VEGFR-2-transfected endothelial cells and placenta growth factor-1 stimulated NO release in HUVECs. Blockade of VEGFR-1 increased VEGF-mediated HUVEC proliferation that was inhibited by NO donors, and potentiated by NO synthase inhibitors. These data indicate that VEGFR-1 is a signaling receptor that promotes endothelial cell differentiation into vascular tubes, in part by limiting VEGFR-2-mediated endothelial cell proliferation via NO, which seems to be a molecular switch for endothelial cell differentiation. *(Am J Pathol 2001, 159:993–1008)*

In the adult male life angiogenesis seldom occurs and the turnover of endothelial cells is very low. The process occurs normally as part of the body's repair processes, as in wound healing and bone fracture, and in the female reproductive system angiogenesis occurs in monthly cycles. Unrestrained angiogenesis promotes pathological conditions such as atherosclerosis, diabetic retinopathy, rheumatoid arthritis, and solid tumor growth. Vascular endothelial growth factor (VEGF) is a potent soluble growth factor that is a major positive regulator of both physiological and pathological angiogenesis.[1] However, our knowledge of the molecular mechanisms of VEGF and its receptor interaction in postnatal blood vessel formation are poorly understood. Moreover, very little is known about the spatial cues guiding endothelial cells to assemble into three-dimensional networks. Effective therapeutic angiogenesis requires a better understanding of VEGF receptor function in normally differentiated endothelium.

The known biological responses of VEGF in endothelial cells are reported to be mediated by the activation of VEGF tyrosine kinase receptor-2 (VEGFR-2).[1,2] Transfection of human VEGFR-1 and VEGFR-2 into porcine aortic endothelial (PAE) cells showed that human recombinant VEGF was able to stimulate chemotaxis and proliferation in VEGFR-2-transfected and not in VEGFR-1-transfected cells.[3] Only a few functions of VEGF have been attributed to VEGFR-1, including stimulation of peripheral blood monocyte migration and tissue factor expression,[4] nitric oxide (NO) release in trophoblasts,[5] and up-regulation of matrix metalloproteinases in vascular smooth muscle cells.[6] Placenta growth factor (PlGF) that binds to VEGFR-1 and not VEGFR-2 also stimulates monocyte migration.[4]

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Knockout studies demonstrate that both VEGFR-1 and VEGFR-2 are essential for normal development of the embryonic vasculature.[7,8] Mice lacking VEGFR-2 fail to develop a vasculature and have very few mature endothelial cells,[7] whereas mice engineered to lack VEGFR-1 seem to have excess formation of endothelial cells that abnormally coalesce into disorganized tubules.[8] More recently, Fong and colleagues[9] showed that increased mesenchymal-hemangioblast transition is the primary defect in VEGFR-1 knock-out mice, whereas the formation of disorganized vascular channels is a secondary phenotype because of the overcrowding of the endothelial population. However, it is unclear how VEGFR-1 prevents overcrowding. As truncation of VEGFR-1 at the tyrosine kinase domain does not impair embryonic angiogenesis, this led to the suggestion that VEGFR-1 acts as an inert decoy by binding VEGF and thereby regulating the availability of VEGF for activation of VEGFR-2.[10] However, this does not negate the involvement of VEGFR-1 signaling in adult endothelia. Indeed, there is now a considerable body of evidence that on the contrary supports this notion[5,11,12] and the role of this receptor has been implicated in both physiological[13] and pathological angiogenesis.[10,14]

Angiogenesis is initiated by vasodilatation, a NO-mediated process. Originally identified as endothelium-derived relaxing factor, NO has profound vasomotor regulatory effects on the vasculature.[15] In addition to its potent vasodilatory function, NO inhibits platelet aggregation, leukocyte adherence, and smooth muscle proliferation and migration, supporting its role in the maintenance of vascular integrity. Synthesis of NO is under tight regulation of a family of NO synthase (NOS) isoenzymes that convert L-arginine to L-citrulline in the presence of molecular oxygen yielding free NO.[16] Ziche and co-workers[17] established the first line of evidence that NO can induce angiogenesis *in vivo*. We and others have shown that VEGF stimulates the release of NO from human umbilical vein endothelial cells (HUVECs)[5] and from intact rabbit arterial strips.[18] Further studies have shown that NO generated in response to VEGF induces angiogenesis both *in vitro*[19] and *in vivo*.[20] The most compelling evidence that NOS is a downstream effector of VEGF-mediated angiogenesis comes from studies in eNOS knockout mice in which VEGF fails to induce angiogenesis.[21] However, it is not clear at what stage in the process of blood vessel formation that NO exerts its influence. Accordingly, we tested the hypothesis that VEGFR-1 negatively regulates VEGFR-2 action via NO that functions to inhibit endothelial cell growth and to promote vascular tube formation.

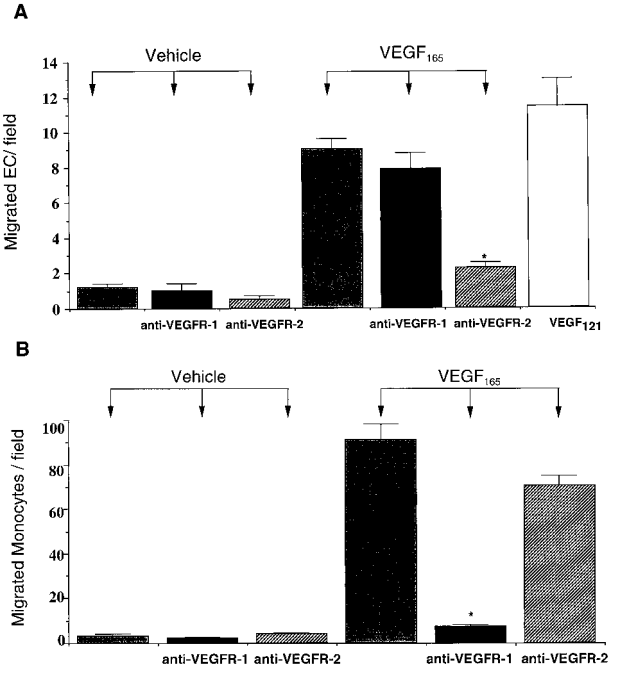


Figure 1. Effect of anti-VEGFR antibodies on VEGF-induced migration of HUVECs and monocytes. Migration of HUVECs (**A**) was performed using the modified Boyden's chamber and migration of monocytes (**B**) using the 48-well microchemotaxis chamber, as described in Materials and Methods. Cells (2×10^5) were preincubated for 30 minutes with the vehicle alone (**gray bars**) or with the anti-VEGFR-1 antibody (100 ng/ml) (**black bars**) or with the anti-VEGFR-2 antibody (100 ng/ml) (**hatched bars**) and then seeded in the upper compartment. Cells that migrated across the polycarbonate filters in response to the vehicle alone or to 20 ng/ml $VEGF_{165}$ were counted after a 4-hour incubation. Chemotaxis in response to 20 ng/ml $VEGF_{121}$ (**white bar**) was used as a control for VEGFR-2-dependent migration of HUVECs. Results are expressed as the mean (±SEM) HUVECs counted per 10 fields (original magnification, ×200) or monocytes counted in four fields (original magnification, ×200) in representative experiments performed in duplicate and quadruplicate, respectively. Five similar experiments were performed on different cell cultures with similar results. *, $P < 0.05$ *versus* VEGF.

Materials and Methods

Reagents

Recombinant $VEGF_{165}$, $VEGF_{121}$, and VEGF:PlGF heterodimer were purchased from Strathmann Biotech GmbH (Hanover, Germany). The soluble VEGFR-1 ectodomain (sVEGFR-1), a VEGF antagonist, was kindly provided by Dr. H. Weich (GBF, Braunschweig, Germany). The neutralizing monoclonal anti-VEGF antibody was a gift from Dr. N. Ferrara (Genetech, San Francisco, CA). The neutralizing polyclonal anti-VEGFR-1 and anti-VEGFR-2 antibodies were raised as previously described[5] and validated as shown in Figure 1. Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). Polycarbonate filters (8-μm pore size, polyvinylpyrrolidone-free) were obtained from Nucleopore Corporation (Pleasanton, CA). Sodium nitroprusside, *N*-(b-D-glucopyranosyl)-*N*2-acetyl-*S*-nitroso-D,L-penicillaminamide (Glyco-SNAP-1), N^G monomethyl L-arginine (L-NNA), 8-bromo-cGMP, LY 83583, and tyrosine kinase inhibitors were from Calbiochem Novabiochem Corporation (La Jolla, CA). All cell culture reagents were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK).

Cell Culture

HUVECs were isolated, characterized, and cultured as previously described.[5] Experiments were performed on second or third passage HUVECs. PAE cells that were

transfected with either human VEGFR-1 ($PAE_{VEGFR-1}$) or VEGFR-2 ($PAE_{VEGFR-2}$) were obtained from Dr. Johannes Waltenberger (Ulm, Germany). Scatchard analysis of receptor binding of VEGF to $PAE_{VEGFR-1}$ and $PAE_{VEGFR-2}$ showed that the binding and expression of these receptors are comparable to HUVEC.[3] Peripheral blood monocytes were isolated from buffy coats using gradient centrifugation over Ficoll (histopaque 1077) and subsequent on plastic dishes.

Measurement of DNA Synthesis and Proliferation

Quiescent HUVECs or transfected $PAE_{VEGFR-1}$ and $PAE_{VEGFR-2}$ cells were incubated with test substances and assayed for DNA synthesis by measuring [^{3}H]-thymidine incorporation[5] and cell proliferation by counting the cells in a Coulter Counter (Coulter Electronics Ltd., Hialeah, FL).[22]

Measurement of NO Release

For concentration-dependence and time-course experiments, stimulations were initiated in confluent and quiescent HUVECs and PAE cells in serum-free culture medium. For the inhibition studies, cells were preincubated for 30 minutes with test substances as indicated, and stimulation then initiated with 50 ng/ml VEGF in a final volume of 0.5 ml at 37°C for a further 60 minutes. The reaction was terminated by removal of the supernatant that was centrifuged and immediately stored at −80°C for NO analysis within 1 week. Because increasing intracellular cGMP levels cannot be ascribed solely to NOS activation,[23] we measured NO directly in the gas phase using a Sievers NOA 280 chemiluminescence analyzer (Analytix, Sunderland, UK) as previously described.[5] Results are corrected for background levels of NO present in culture medium alone in all of the experiments excepts in low calcium buffer studies.

In Vitro *Migration Assay*

Chemotaxis of HUVECs and monocytes to VEGF was assessed using a modified Boyden's chamber. Briefly, HUVECs (2×10^5) or monocytes (1.5×10^6/ml) were seeded in the upper chamber and their migration across a polycarbonate filter in response to 20 ng/ml of VEGF was investigated. The upper surface of the filter was scraped and filters were fixed and stained with Diff-Quik (Harleco, Gibbstown, NJ). Ten random fields at ×200 magnification were counted and the results expressed as mean (±SEM) number per field.

Tube Formation

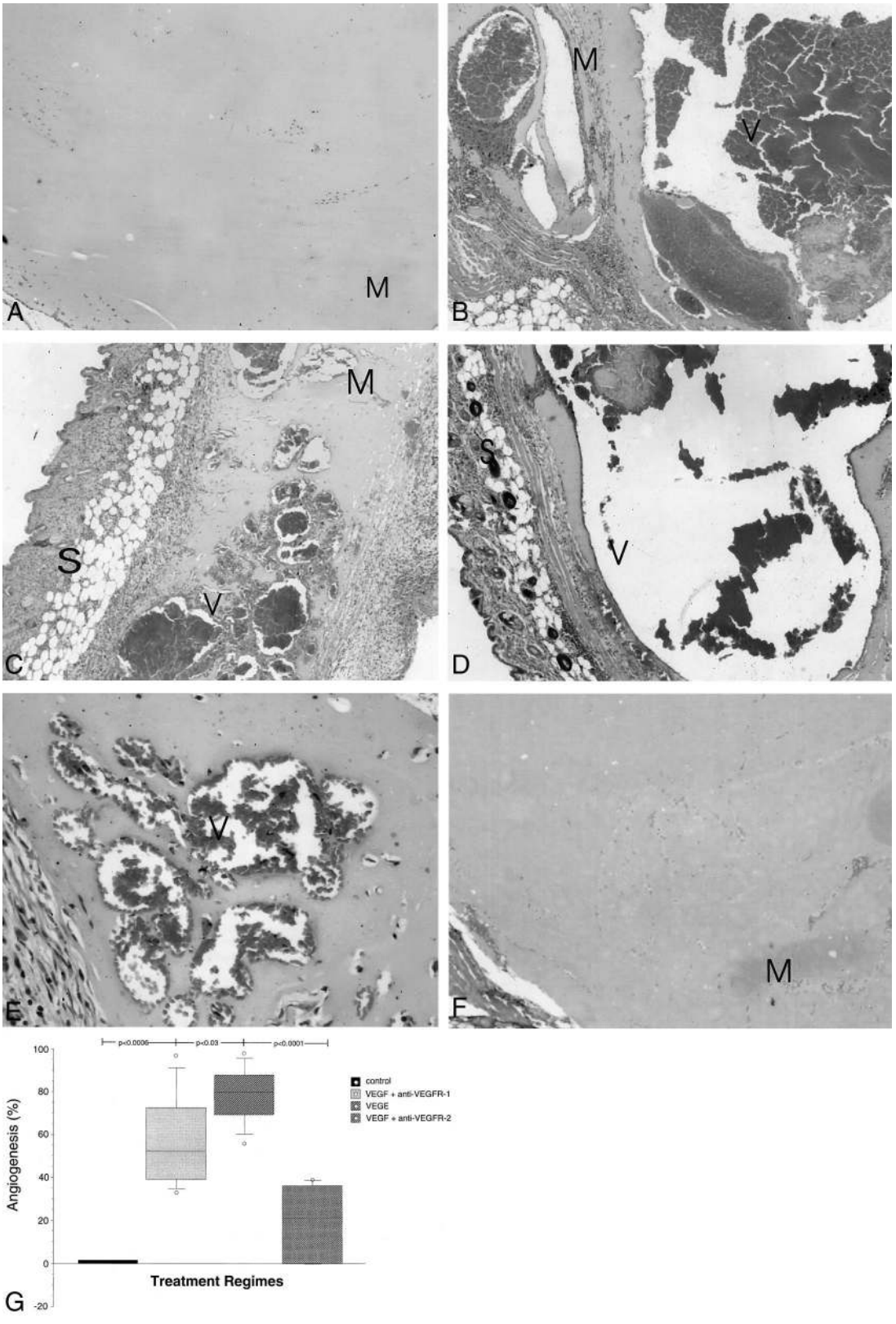
In vitro formation of tubular structures was studied on growth factor-reduced Matrigel diluted 1:1 in ice-cold Dulbecco's modified Eagle's medium, as previously described.[24] PAE-transfected cells (5×10^4 cells/well) were seeded onto Matrigel-coated wells in culture medium containing 10% fetal calf serum (FCS). After cells had attached to the Matrigel (2 hours at 37°C), the FCS-containing media was removed and $VEGF_{165}$ (25 ng/ml) or the vehicle alone in media containing 0.2% bovine serum albumin was added. Cells were pretreated with anti-VEGFR antibodies (200 ng/ml) or 8-bromo-cGMP or LY 83583 at 37°C for 30 minutes prior to stimulation with $VEGF_{165}$. In parallel experiments, Trypan Blue exclusion showed that cell viability was >90%. Cells were observed with a Nikon inverted microscope and experimental results recorded with an Optimas image analysis software (Microscope Service, Surrey, UK).

In Vitro *Co-Culture Angiogenesis Assay*

In vitro angiogenesis was assessed as formation of capillary-like structures of HUVECs co-cultured with matrix-producing cells that had been UV irradiated before plating of primary HUVECs. The experimental procedure followed the manufacturer's protocol provided with the *In Vitro* angiogenesis kit (TCS Biologicals, Buckingham, UK). Briefly, cells were stimulated with the test substances at day 3 and medium was replaced at day 5, 8, and 10. At day 13, the cells were fixed and HUVECs were stained using an anti-CD31 antibody (TCS Biologicals) according to the instructions provided with the kit. To measure the formation of the capillary network, the number of connections between three or more capillary-like structures was counted and expressed as the number of capillary connections per field. Furthermore, the average thickness of the tube or cell overcrowding, the total length of tubes per field, and the average length (distance) of tube between connections were quantified by image analysis at ×4 magnification with a MicroImage analysis system (Cast Imaging srl, Venice, Italy) calibrated with an Olympus micrometer slide. Four (0.5 mm^2) different fields were analyzed per well.

Murine Matrigel Angiogenesis Assay

In vivo angiogenesis was assayed in mice as growth of blood vessels from subcutaneous tissue into a Matrigel plug containing the test sample, according to Passaniti and colleagues.[25] Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. Matrigel (8.13 mg/ml) in liquid form at 4°C was mixed with 64 U/ml heparin and VEGF (40 ng/ml) or vehicle alone (saline containing 0.25% bovine serum albumin). In selected experiments, the neutralizing anti-VEGFR-1 or anti-VEGFR-2 antibodies were included in the Matrigel plug at a final concentration of 200 mg/ml. Matrigel was injected (0.5 ml) into the abdominal subcutaneous tissue of female C57 mice (6 to 8 weeks old) along the peritoneal mid-line. At day 6, mice were sacrificed and plugs were recovered and processed for histology. Typically, the overlying skin was removed, and gels were cut out by retaining the peritoneal lining for support, fixed in 10% buffered formalin, and embedded in paraffin. Sections (3



μm) were cut and stained with hematoxylin and eosin (H&E) and examined under a light microscope system (Nikon UK Limited Instrument Division, Kingston, UK). Vessel area and the total Matrigel area were planimetrically assessed from cross-sections of Matrigel plugs as described by Kibbey and colleagues.[26] The mean area of H&E-stained vessels per field from 10 to 20 fields ($\times$40 magnification) was evaluated using the computing inte-

gral area calculation of Lucia digital system (Nikon UK Limited). Blood vessels defined as those structures possessing a patent lumen and containing red blood cells were identified blind by an independent observer. Positive immunofluorescence staining for vWF, an endothelium-specific antigen, was shown to correlate with the presence of vessels detected by light microscopy.[26] Results were expressed as percentage of the vessel area to the total Matrigel area.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was performed by analysis of variance with Dunnett's or Newmann-Keul's multiple comparison test or by Mann-Whitney test where appropriate.

Results

Functional Specificity of VEGF Receptor Subtype-Selective Antibodies

$VEGF_{165}$ stimulates chemotaxis in transfected $PAE_{VEGFR-2}$ and not $PAE_{VEGFR-1}$ cells demonstrating that VEGFR-2 activation is required for endothelial migration.[3] In contrast, VEGF stimulates monocyte chemotaxis via VEGFR-1.[4] To test the functional specificity of the neutralizing anti-VEGF receptor antibodies, the chemotactic effect of VEGF was evaluated on both HUVECs and monocytes. The anti-VEGFR-2 antibody (100 ng/ml) but not the anti-VEGFR-1 antibody significantly inhibited the effect of $VEGF_{165}$ (20 ng/ml) on migration of HUVECs (Figure 1A; $P < 0.05$). The VEGFR-2 agonist, $VEGF_{121}$,[27] induced a chemotactic response comparable to VEGF. Basic fibroblast growth factor (10 ng/ml)-induced HUVEC migration was unaffected by the anti-VEGFR antibodies (data not shown). In contrast to HUVECs, preincubation of monocytes (that only express VEGFR-1) with anti-VEGFR-1 antibody (50 ng/ml) but not with the anti-VEGFR-2 antibody (50 ng/ml) completely inhibited the VEGF-mediated migration (Figure 1B; $P < 0.05$), thus confirming the specificity of the two antibodies.

Effect of Blockade of VEGF Receptors on in Vivo *Angiogenesis*

The relative role of VEGF receptors in the process of angiogenesis was investigated *in vivo* to determine whether the subtype-selective blocking antibodies could replicate some of the murine knockout phenotypes. The histological and morphometric analyses of Matrigel plugs containing VEGF (40 ng/ml) revealed a diffuse angiogenic process, characterized by canalized vessels and microaneurysm-like structures (Figure 2, B and G; $P < 0.0006$ *versus* control). The blockade of VEGFR-2 reduced VEGF-induced angiogenic process as there were small branched vessels (Figure 2; C, E, and G; $P < 0.003$ *versus* VEGF) suggesting impairment in endothelial cell invasion of the Matrigel. In contrast, the blockade of VEGFR-1 by anti-VEGFR-1 antibody caused the formation of large aneurysm-like structures (Figure 2, D and G; $P < 0.03$ *versus* VEGF). This angiogenic process covered >80% of the Matrigel in the majority of mice treated with VEGF in the presence of anti-VEGFR-1 antibody (Figure 2G). No angiogenesis was present in control plugs containing the vehicle alone, heparin (64 U/ml) plus vehicle (Figure 2A), or the antibodies alone (Figure 2F).

Effect of Blockade of VEGF Receptors on in Vitro *Angiogenesis*

The relative role of VEGF receptors in the process of angiogenesis was investigated *in vitro* using the angiogenesis kit assay to determine the underlying mechanism. Quantitative analysis is shown for the average thickness of the tube or cell overcrowding (Figure 3A), the total length of tubes per field (Figure 3B), the number of capillary connections per field (Figure 3C), and the average length (distance) of tube between connections (Figure 3D). The basal formation of capillary-like structures was increased after stimulation with $VEGF_{165}$ (20 ng/ml). There was a significant increase in tube thickness (Figure 3A; $P < 0.05$, $n = 4$), the total length of tubes per field (Figure 3B; $P < 0.04$, $n = 4$), number of capillary connections (Figure 3C; $P < 0.05$, $n = 4$), and the average length of tube between connections (Figure 3D; $P < 0.003$, $n = 4$). Anti-VEGFR-1 antibody inhibited VEGF-induced total tube length by threefold (Figure 3B; $P < 0.0001$, $n = 4$), capillary connections by a dramatic sevenfold (Figure 3C; $P < 0.0001$, $n = 4$), and the average length of tube between connections were also reduced (Figure 3D; $P < 0.0001$, $n = 4$), whereas tube thickness was increased by almost fourfold (Figure 3, A and G; $P < 0.04$, $n = 4$) compared with VEGF alone. HUVECs remained in isolated islands, proliferated, and accumulated in large aggregates in the presence of anti-VEGFR-1 (Figure 3G). In contrast, blockade of VEGFR-2 by anti-

Figure 2. Effect of selective inhibition of VEGF receptor subtypes on VEGF-induced angiogenesis in murine Matrigel model. **A:** No angiogenesis was present in Matrigel containing vehicle alone. **B:** Canalized vessels containing blood erythrocytes were present in Matrigel with 40 ng/ml of VEGF. **D:** Massive angiogenesis with aneurysm-like structures involving the total Matrigel area in the presence of 40 ng/ml VEGF plus 200 ng/ml of anti-VEGFR-1 antibody. **C** and **E:** Small canalized vessels and microaneurysm-like structures in the presence of 40 ng/ml of VEGF plus anti-VEGFR-2 antibody (200 ng/ml). **F:** Control with the anti-VEGFR-1 alone (100 ng/ml). Original magnifications: ×40 (**A–D** and **F**), ×250 (**E**). M, Matrigel; V, vessels; and S, mouse skin. **G:** Morphometric analysis of the angiogenesis within Matrigel. Matrigel plugs containing VEGF (40 ng/ml, $n = 8$), or VEGF (V) and anti-VEGFR-2 antibody (200 ng/ml, $n = 8$), or VEGF plus anti-VEGFR-1 antibody (200 ng/ml, $n = 10$), or the anti-VEGFR-1 antibody alone (control, $n = 4$) were explanted 6 days after beginning the experiments. Quantitation of neovascularization was performed on H&E-stained histological sections as described in Materials and Methods. The results are expressed as percentage of the vessel area to the total Matrigel area. The data are represented as box plot, where the **bar** indicates the mean, the **box** the intercortile range, and the **whisker** the spread of the data. Statistical significance was determined using the unpaired Student's *t*-test.

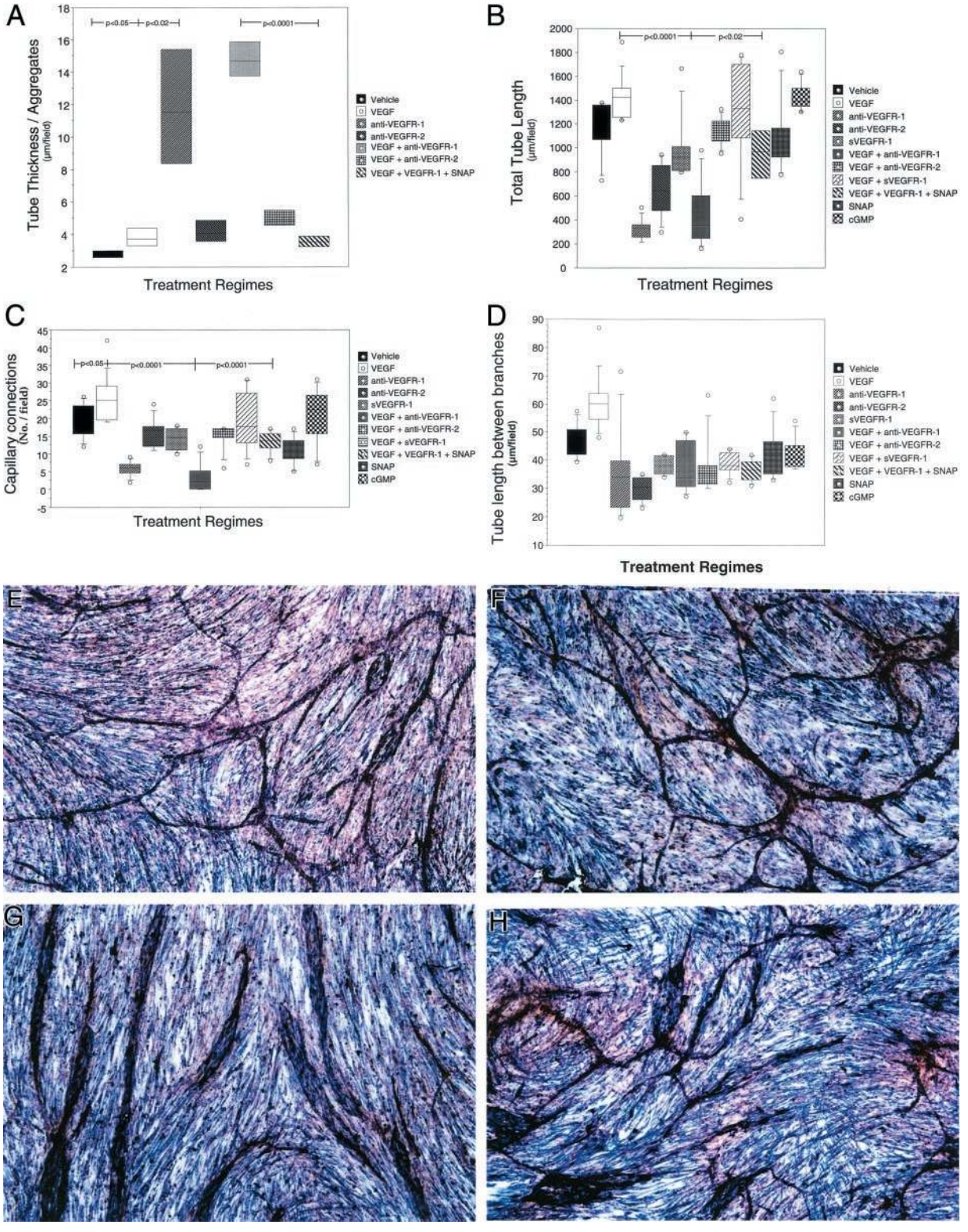


Figure 3. Effect of inhibition of VEGF receptors on the capillary-like network in a co-culture angiogenesis kit. Formation of capillary-like structures of HUVECs cultured with matrix-producing cell types was observed after 11 days. **A–D:** The quantitative analysis for the average thickness of the tube or cell overcrowding (**A**), the total length of tubes per field (**B**), the number of capillary connections per field (**C**), and the average length (distance) of tube between connections (**D**) in the *in vitro* angiogenesis assay. **E–H:** Cells were stimulated with 20 ng/ml VEGF in the absence or presence of the anti-VEGFR-1 antibody (100 ng/ml) or the anti-VEGFR-2 antibody (100 ng/ml) or sVEGFR-1 (50 ng/ml). **E–H** is a representative example of the morphology of CD31-positive endothelial structures detected in the *in vitro* angiogenesis assay. **E:** VEGF (20 ng/ml) induced a diffuse network of capillary structures in the *in vitro* angiogenesis assay. **F:** 8-Bromo-cGMP induced a network of capillaries similar to VEGF. **G:** In the presence of VEGF plus the anti-VEGFR-1 antibody (100 ng/ml) HUVECs remained in isolated islands and accumulated in large aggregates (**H**). In the presence of VEGF plus the anti-VEGFR-2 antibody (100 ng/ml) only short capillaries were detectable. All are at an original magnification of ×10. Endothelial structures were stained using an anti-CD31 antibody. The data are represented as box plot, where the **bar** indicates the mean, the **box** the intercortile range, and the **whisker** the spread of the data. Statistical significance was determined using the unpaired Student's *t*-test.

VEGFR-2 antibody (100 ng/ml) did not cause a significant increase in VEGF-induced tube thickness (Figure 3A) but did inhibit both total tube length (Figure 3B; $P < 0.002$, $n = 4$) and tube length between connections (Figure 3D; $P < 0.0001$, $n = 4$). Capillary connections per field were also inhibited by the anti-VEGFR-2 antibody (100 ng/ml) when compared with 20 ng/ml $VEGF_{165}$ (Figure 3C; $P < 0.0004$, $n = 4$) but under basal conditions, anti-VEGFR-2 antibody alone did not inhibit capillary connections (Figure 3C). Interestingly, the anti-VEGFR-1 antibody did not significantly inhibit tube length between connections under basal conditions whereas anti-VEGFR-2 caused a marked reduction in tube length between connections (Figure 3D; $P < 0.0001$, $n = 4$). In the presence of $VEGF_{165}$ and anti-VEGFR-2 antibody, short tubes were detectable that failed to form capillary connections (Figure 3H). When the NO donor glyco-SNAP (10^{-5} mol/L) was added to cells stimulated with $VEGF_{165}$ in the presence of anti-VEGFR-1 antibody, the ability of HUVECs to form capillary-like structures was partially restored as determined by the increase in total tube length (Figure 3B; $P < 0.02$, $n = 4$) and capillary connections (Figure 3C; $P < 0.0001$, $n = 4$) and decrease in tube thickness (Figure 3A; $P < 0.0001$, $n = 4$). Basal capillary network was inhibited by soluble VEGFR-1 that blocks VEGF activity indicating that VEGF is involved in the process under basal conditions. Addition of 8-bromo-cGMP alone induced a diffuse capillary network (Figure 3, B and F) comparable to VEGF (Figure 3E).

Effect of VEGF on Tube Formation in VEGFR-1- or VEGFR-2-Transfected Cells

$PAE_{VEGFR\text{-}1}$ cells, plated on a Matrigel substratum, elongated and connected in basal conditions (Figure 4A). When stimulated with $VEGF_{165}$ (25 ng/ml), cells organized into complete tubular structures (Figure 4B). The blockade of VEGF with the neutralizing anti-VEGF antibody (50 ng/ml) inhibited all these morphological rearrangements (data not shown) suggesting a specific response. Preincubation with anti-VEGFR-2 antibody (200 ng/ml) had no effect on tube formation (Figure 4C), whereas anti-VEGFR-1 antibody (200 ng/ml) inhibited both the basal and the VEGF-mediated tubular network (Figure 4D). Similar results were obtained with HUVECs (data not shown). cGMP inhibitor LY83583 (2.5 μmol/L) completely inhibited the $PAE_{VEGFR\text{-}1}$ cell organization (Figure 4, E and F) supporting the role of NO released via VEGFR-1 in endothelial cell differentiation. In contrast to $PAE_{VEGFR\text{-}1}$ cells, the $PAE_{VEGFR\text{-}2}$ cells were unable to establish a network of tubular-like structures on Matrigel under basal conditions or when stimulated with $VEGF_{165}$ (25 ng/ml) (Figure 5, A and B). No significant change from basal or VEGF was observed after the addition of anti-VEGFR-2 (Figure 5C) or anti-VEGFR-1 (Figure 5D) antibodies. Interestingly the addition of 8-bromo-cGMP stimulated the formation of a network in $PAE_{VEGFR\text{-}2}$ cells in the absence (Figure 5E) or in the presence of 25 ng/ml $VEGF_{165}$ (Figure 5F). Wild-type PAE_{WT} seeded on Matrigel did not display the ability to form tubular structures both under basal conditions and when stimulated with $VEGF_{165}$ (data not shown). Cell viability after stimulation with cGMP or LY 83583 was >90% as assessed by Trypan Blue exclusion.

VEGF Stimulates eNOS Activity via VEGFR-1

To investigate the mechanism, by which VEGFR-1 promotes capillary morphogenesis, we examined the effect of VEGF on NO production in HUVECs. Addition of $VEGF_{165}$ to quiescent HUVECs resulted in a dose- and time-dependent release of NO (Figure 6A). At the highest concentration tested (50 ng/ml), $VEGF_{165}$ caused a 258.66 ± 14.75% increase in NO release above control (Figure 6A; $P < 0.001$, $n = 3$). Interestingly, VEGF had no significant effect on NO release within the concentration range of 0.5 to 5 ng/ml that is known to promote maximal endothelial cell proliferation.[1] This response was specific to VEGF, because the neutralizing monoclonal anti-VEGF antibody[28] inhibited $VEGF_{165}$-stimulated NO release by 89.7 ± 1.6% (Figure 6B; $P < 0.01$, $n = 3$). Several experimental approaches were undertaken to identify the receptor subtype responsible for VEGF-mediated NO release. To investigate the role of VEGFR-2 in VEGF-mediated NO release, cells were stimulated with increasing concentrations of $VEGF_{121}$ and VEGF:PlGF heterodimer that bind with high affinity to VEGFR-2.[27] Neither $VEGF_{121}$ or VEGF:PlGF stimulated a significant amount of NO release as compared with $VEGF_{165}$ (Figure 6A). To further investigate the role of the VEGF receptors, quiescent HUVECs were exposed to neutralizing anti-VEGFR-1 or anti-VEGFR-2 antibodies (250 ng/ml) for 30 minutes before stimulation with 50 ng/ml $VEGF_{165}$ for 60 minutes (Figure 6C). $VEGF_{165}$-stimulated NO release was completely inhibited by anti-VEGFR-1 antibody ($P < 0.001$, $n = 3$; Figure 6C) whereas the anti-VEGFR-2 antibody had no effect (Figure 6C). Specific activation of eNOS was confirmed by the attenuation of $VEGF_{165}$-stimulated NO release by L-NNA (Figure 6D) as well as by the complete inhibition obtained by the removal of extracellular calcium ($P < 0.001$, $n = 3$; Figure 6E). Both genistin (30 μmol/L) and lavendustin A (25 μmol/L) inhibited 50 ng/ml VEGF-induced NO release ($P < 0.05$; data not shown) and PlGF-1 but not PlGF-2 stimulated NO release ($P < 0.01$, $n = 3$; Figure 6F).

Effect of VEGF on NO Release in VEGFR-1- or VEGFR-2-Transfected Cells

To conclusively demonstrate that VEGF effect on NO release was mediated via VEGFR-1, we used PAE cells transfected with human VEGFR-1 or VEGFR-2. $VEGF_{165}$ stimulated NO release in a concentration-dependent manner from $PAE_{VEGFR\text{-}1}$ cells (Figure 7A). The effect of VEGF on NO release was more potent and efficacious in the $PAE_{VEGFR\text{-}1}$ cells compared with HUVECs. $PAE_{VEGFR\text{-}1}$ cells displayed an increased sensitivity to $VEGF_{165}$ in mediating NO release compared with HUVECs as VEGF induced a twofold increase in NO production at low concentrations

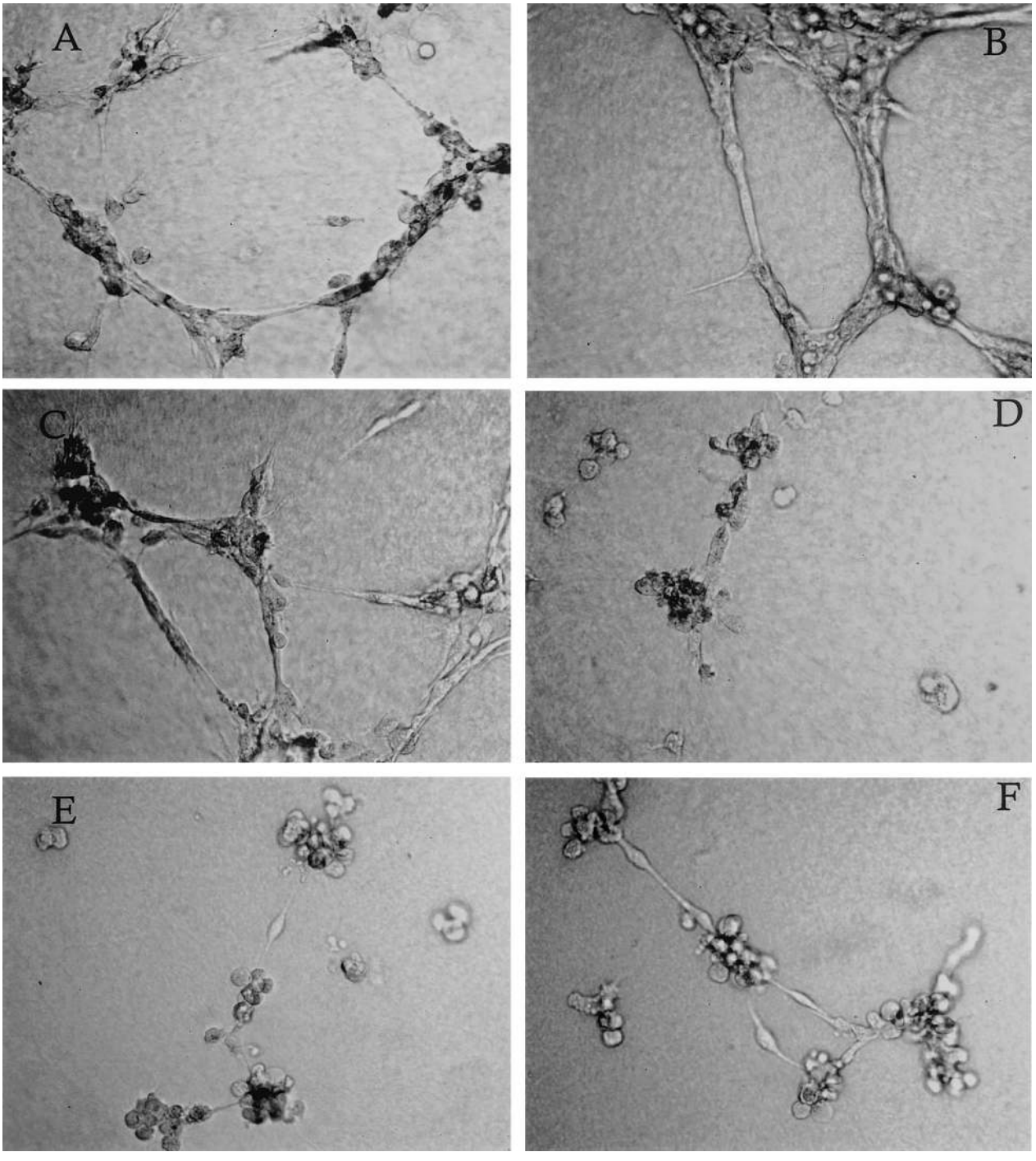


Figure 4. Effect of VEGF on *in vitro* tube formation in VEGFR-1-transfected cells. $PAE_{VEGFR-1}$ cells (5.0×10^4 cells/well) on growth factor-reduced Matrigel were stimulated with VEGF (25 ng/ml) in M199 containing 0.2% bovine serum albumin in the absence or presence of anti-VEGF receptor subtype antibodies. Cells were observed at 3 hours after stimulation and results recorded with a digital system. Under basal conditions $PAE_{VEGFR-1}$ cells formed a network of tubes (**A**) that was enhanced when stimulated with VEGF (25 ng/ml) (**B**). **C:** Tube formation persisted in the presence of anti-VEGFR-2 antibody (200 ng/ml). **D:** Tube formation was completely inhibited with VEGFR-1 antibody (200 ng/ml). Inhibition of both the spontaneous (**E**) -and VEGF (**F**)-induced tube formation in $PAE_{VEGFR-1}$ cells was noted in the presence of the cGMP inhibitor LY 83583 (2.5 μmol/L).

(1 and 5 ng/ml), ineffective in HUVECs (Figure 7A). In contrast, $VEGF_{165}$ failed to stimulate NO release from $PAE_{VEGFR-2}$ cells (Figure 7A). Basal NO levels in $PAE_{VEGFR-2}$ cells, however, were elevated compared with $PAE_{VEGFR-1}$. The time period during which $VEGF_{165}$ caused changes in eNOS activity in $PAE_{VEGFR-1}$ cells is shown in Figure 7B. VEGF (10 ng/ml) stimulated an increase in eNOS activity that was significant at 20 minutes ($P < 0.016$, $n = 3$), and peaked at 60 minutes to reach a maximum of 190 ± 3.8% more than basal levels ($P < 0.001$, $n = 3$; Figure 7B). The addition of 50 ng/ml of sVEGFR-1 (VEGF antagonist) after 35 minutes of stimulation with $VEGF_{165}$ (10 ng/ml) completely inhibited VEGF-evoked NO release within 35 minutes ($P < 0.001$,

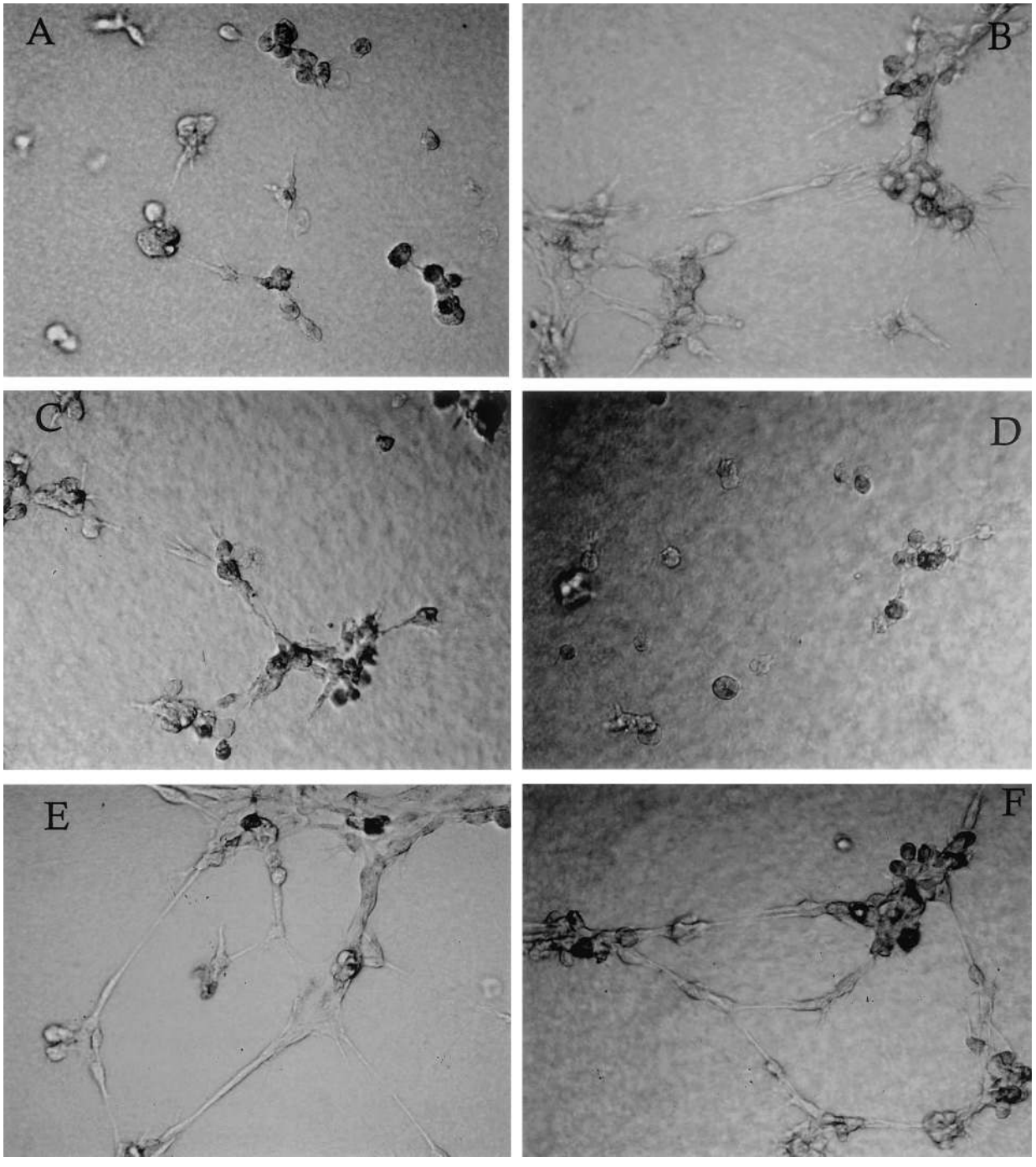


Figure 5. Effect of VEGF on *in vitro* tube formation in VEGFR-2-transfected cells. $PAE_{VEGFR-2}$ cells stimulated with the vehicle alone (**A**) or with 25 ng/ml of VEGF (**B**). VEGF only induced cell connections but not a complete network of tubes. **C:** In the presence of anti-VEGFR-2 antibody (200 ng/ml) plus VEGF (25 ng/ml), $PAE_{VEGFR-2}$ cells were elongated and connected. **D:** Anti-VEGFR-1 antibody (200 ng/ml) completely inhibited VEGF effect on $PAE_{VEGFR-2}$ cells. A complete network of tubes was observed when $PAE_{VEGFR-2}$ cells were stimulated with 100 μmol/L of 8-bromo-cGMP alone (**E**) or with cGMP with 25 ng/ml of VEGF (**F**). At least four experiments were performed with similar results.

$n = 3$; Figure 7B) indicating that VEGF-mediated NO release is sustained during this period. Likewise, the addition of 50 ng/ml of monoclonal anti-VEGF antibody completely inhibited $VEGF_{165}$-evoked NO release (data not shown). Activation of eNOS by VEGF was confirmed by the complete attenuation of $VEGF_{165}$-mediated NO release by the removal of extracellular calcium ($P < 0.001$, $n = 3$; Figure 7C). Premixing $VEGF_{165}$ with excess (1 μg/ml) heparin sulfate proteoglycan that saturates the heparin-binding sites, significantly reduced the VEGF-dependent release of NO (78 $\pm$ 11% reduction, $P < 0.001$, $n = 4$). Heparin sulfate proteoglycan alone had no effect. The results in HUVECs and transfected PAE cells conclusively demonstrate that activation of VEGFR-1 mediates $VEGF_{165}$-stimulated NO release in endothelial cells.

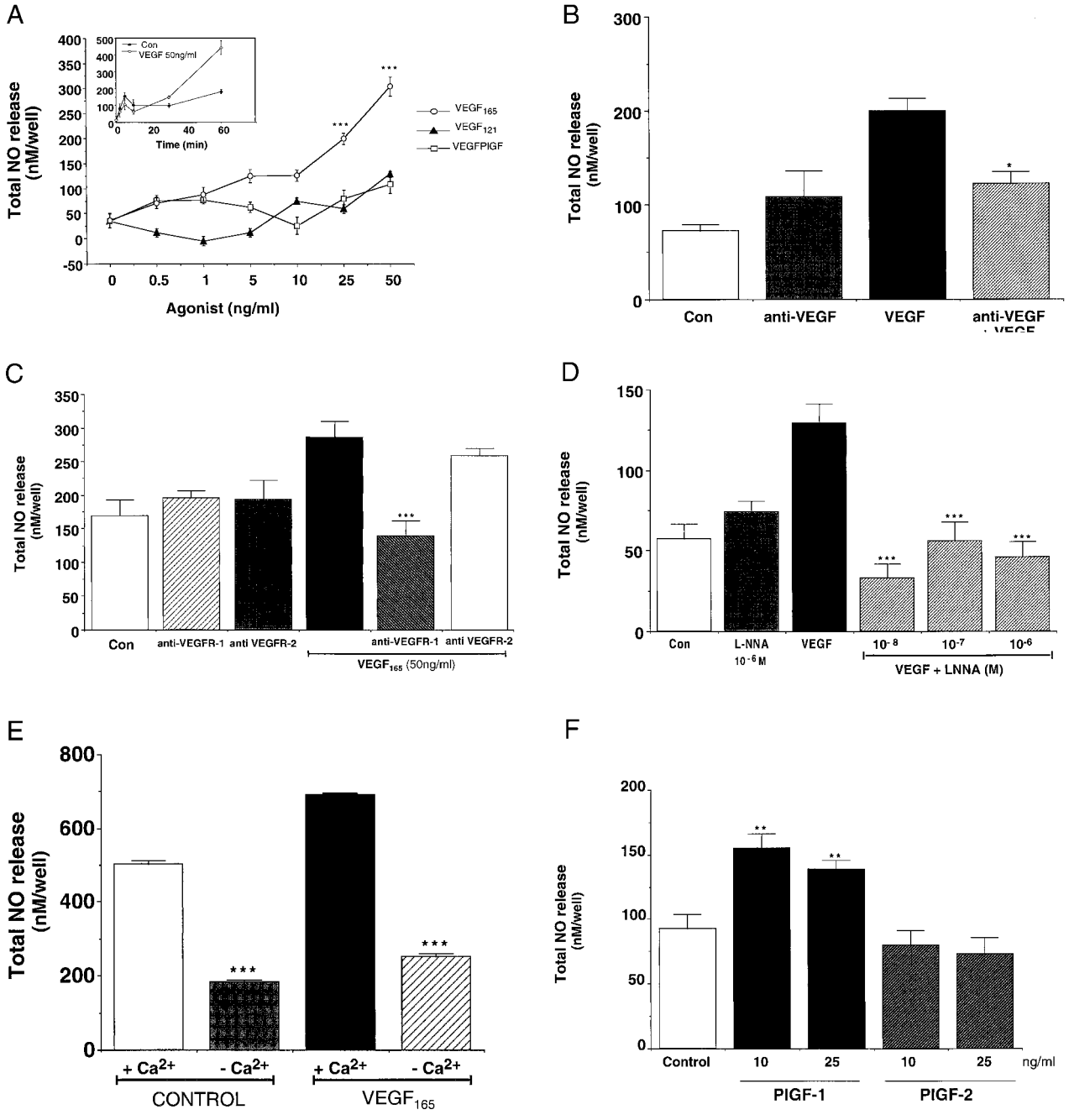


Figure 6. VEGF stimulates NO release through VEGFR-1 in HUVECs. Confluent quiescent HUVECs were stimulated with VEGF (1 to 50 ng/ml) for 1 hour. **A:** Dose-dependant release of total NO in response to $VEGF_{165}$, $VEGF_{121}$, or VEGF: PlGF heterodimer (1 to 50 ng/ml). $VEGF_{165}$ but not $VEGF_{121}$ or the VEGF: PlGF heterodimer stimulated a significant NO release after a 60-minute incubation as compared to the basal levels. **Inset** in **A** is the time-dependent effect of VEGF on NO release. **B:** Cells were stimulated with 50 ng/ml of $VEGF_{165}$ alone or in the presence of 50 ng/ml of anti-VEGF monoclonal antibody preincubated for 30 minutes. The anti-VEGF monoclonal antibody significantly inhibited VEGF-stimulated NO release. **C:** Effect of preincubation of the cells with 250 ng/ml of the anti-VEGFR-1 antibody or the anti-VEGFR-2 antibody on NO levels in the presence or absence of VEGF (50 ng/ml). Neutralization of the VEGFR-1 significantly inhibited VEGF-stimulated NO release whereas anti-VEGFR-2 antibody had no effect. **D:** Inhibition of VEGF-stimulated NO release when cells were preincubated with L-NNA. **E:** $VEGF_{165}$ mediated NO release in normal extracellular calcium buffer (Ca^{2+}, **black bar**) and low calcium buffer (150 nmol/L, Ca^{2+}; **hatched bar**). Removal of extracellular calcium significantly reduced VEGF-stimulated NO release. **F:** Effect of PlGF-1 and PlGF-2 on NO release. The results are expressed as a mean (±SEM) of three independent experiments performed ($n = 9$) and the data expressed as nmol/L per well NO and were corrected for background levels of endogenous NO present in cell culture medium alone, except in **E**. Analysis of variance with Dunnett's multiple comparison test was performed: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *versus* control (**A**) or *versus* VEGF in all other panels.

Inhibition of VEGFR-1 Promotes HUVEC Proliferation

Addition of $VEGF_{165}$ (10 ng/ml) to quiescent HUVECs for 48 hours caused an increase in cell proliferation by 58.44 ± 11.91% compared with basal (1% FCS) cell growth (Figure 8A). The response was specific to VEGF as $VEGF_{165}$-mediated endothelial cell proliferation was completely inhibited in HUVECs preincubated with 50 ng/ml of neutralizing anti-VEGF monoclonal antibody

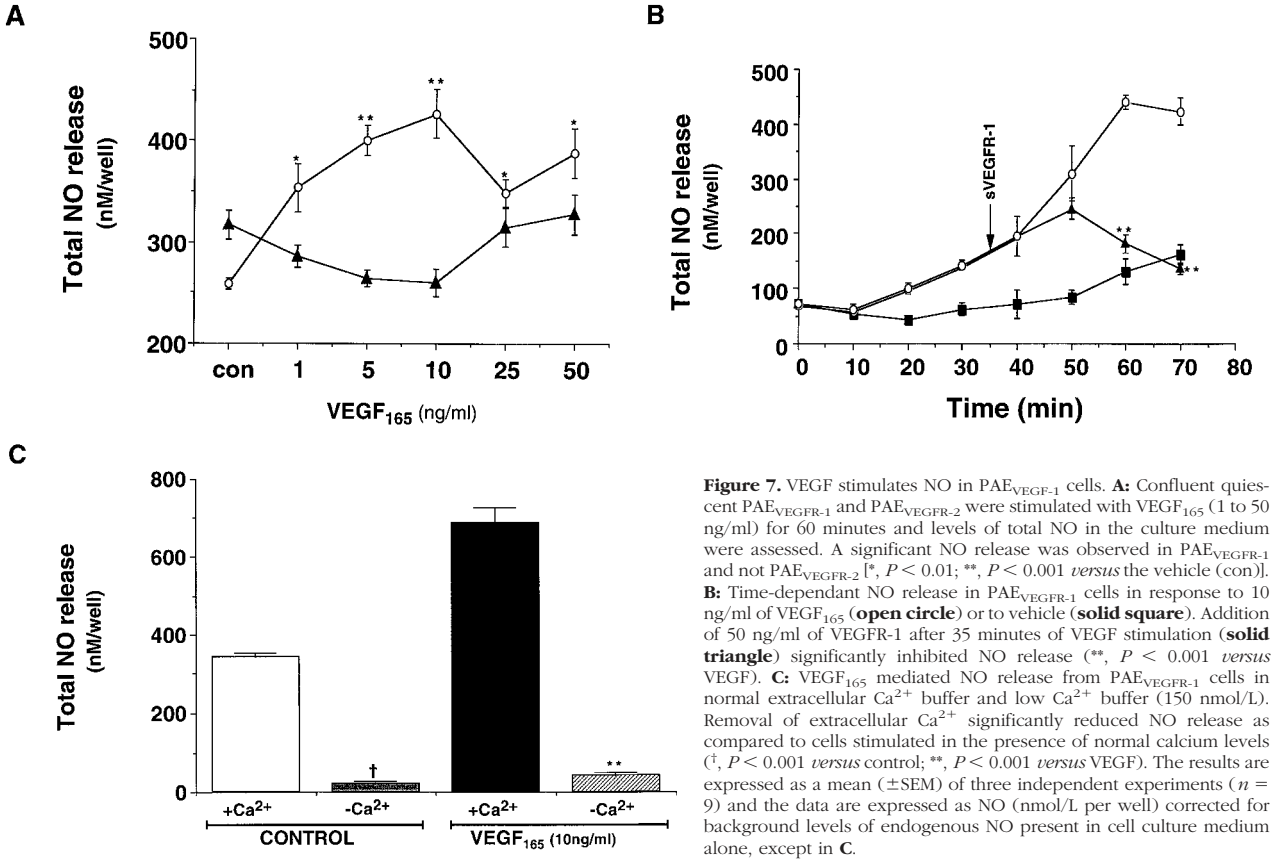


Figure 7. VEGF stimulates NO in $PAE_{VEGFR-1}$ cells. **A:** Confluent quiescent $PAE_{VEGFR-1}$ and $PAE_{VEGFR-2}$ were stimulated with $VEGF_{165}$ (1 to 50 ng/ml) for 60 minutes and levels of total NO in the culture medium were assessed. A significant NO release was observed in $PAE_{VEGFR-1}$ and not $PAE_{VEGFR-2}$ [*, $P < 0.01$; **, $P < 0.001$ *versus* the vehicle (con)]. **B:** Time-dependant NO release in $PAE_{VEGFR-1}$ cells in response to 10 ng/ml of $VEGF_{165}$ (**open circle**) or to vehicle (**solid square**). Addition of 50 ng/ml of VEGFR-1 after 35 minutes of VEGF stimulation (**solid triangle**) significantly inhibited NO release (**, $P < 0.001$ *versus* VEGF). **C:** $VEGF_{165}$ mediated NO release from $PAE_{VEGFR-1}$ cells in normal extracellular Ca^{2+} buffer and low Ca^{2+} buffer (150 nmol/L). Removal of extracellular Ca^{2+} significantly reduced NO release as compared to cells stimulated in the presence of normal calcium levels (†, $P < 0.001$ *versus* control; **, $P < 0.001$ *versus* VEGF). The results are expressed as a mean (±SEM) of three independent experiments ($n = 9$) and the data are expressed as NO (nmol/L per well) corrected for background levels of endogenous NO present in cell culture medium alone, except in **C**.

($P < 0.001$, $n = 3$) or 50 ng/ml of sVEGFR-1 ($P < 0.001$, $n = 3$; Figure 8A). To investigate the relative roles of two high-affinity VEGF receptor subtypes on endothelial cell growth, VEGF-induced proliferation was studied in the absence or presence of anti-VEGFR1 or anti-VEGFR-2 antibodies in HUVECs. In the presence of the neutralizing anti-VEGFR-1 antibody (30 ng/ml) alone, HUVEC proliferation increased by 51.36 ± 2.09% more than basal levels ($P < 0.01$, $n = 3$; Figure 8B) and was comparable with that achieved with 1 ng/ml $VEGF_{165}$. This increase in cell proliferation was inhibited in the presence of anti-VEGF antibody ($P < 0.01$, $n = 3$; Figure 8B) indicating endogenous VEGF action. Addition of 1 ng/ml $VEGF_{165}$ to HUVECs incubated with the anti-VEGFR-1 antibody lead to a weak increase in cell proliferation ($P < 0.05$, $n = 3$; Figure 8B). In contrast, the proliferative response of endothelial cells to the higher concentrations of VEGF (10 to 50 ng/ml) was significantly enhanced by the addition of the anti-VEGFR-1 antibody (Figure 8C). The anti-VEGFR-2 antibody completely inhibited $VEGF_{165}$-mediated endothelial cell proliferation ($P < 0.001$, $n = 3$; Figure 8B). Cell viability, assessed by Trypan Blue exclusion was >95% in all experiments.

Effect of NO on VEGF-Stimulated HUVEC Proliferation

To investigate the role of VEGFR-1-mediated NO production on HUVEC proliferation, quiescent subconfluent HUVECs were incubated with the anti-VEGFR-1 antibody (30 ng/ml) in the presence of NO donors. The increase in DNA synthesis observed in the presence of anti-VEGFR-1 antibody was significantly attenuated by the addition of glyco-SNAP-1 (10^{-4} to 10^{-6} mol/L) (Figure 9A). However, under basal conditions, glyco-SNAP at 10^{-6} to 10^{-8} mol/L significantly increased endothelial cell proliferation as assessed by cell number compared with control, whereas at 10^{-4} mol/L it had no effect (Figure 9B). In contrast, $VEGF_{165}$ (10 ng/ml)-induced proliferation was significantly attenuated by glyco-SNAP-1 at the doses of 10^{-4} to 10^{-6} mol/L (Figure 9B). Significant levels of NO, comparable with that obtained with 50 ng/ml VEGF, were produced when glyco-SNAP-1 was added to HUVECs at concentrations more than 10^{-5} mol/L (data not shown). This result is consistent with the concentration dependency of glyco-SNAP inhibition of endothelial cell proliferation. As the addition of exogenous NO suppressed $VEGF_{165}$-mediated endothelial cell proliferation, it is proposed that inhibition of endogenous NO would promote cell growth. Indeed, incubation of HUVECs with increasing concentrations of L-NNA potentiated $VEGF_{165}$-stimulated, at the dose of 10^{-4} mol/L L-NNA, endothelial cell proliferation by 49 ± 14% ($P < 0.05$, $n = 3$) as compared to stimulation with $VEGF_{165}$ alone (Figure 9C). In addition, $VEGF_{165}$-mediated DNA synthesis was completely blocked by the addition of 10^{-4} mol/L 8-bromo-cGMP ($P < 0.02$, $n = 3$; Figure 9D).

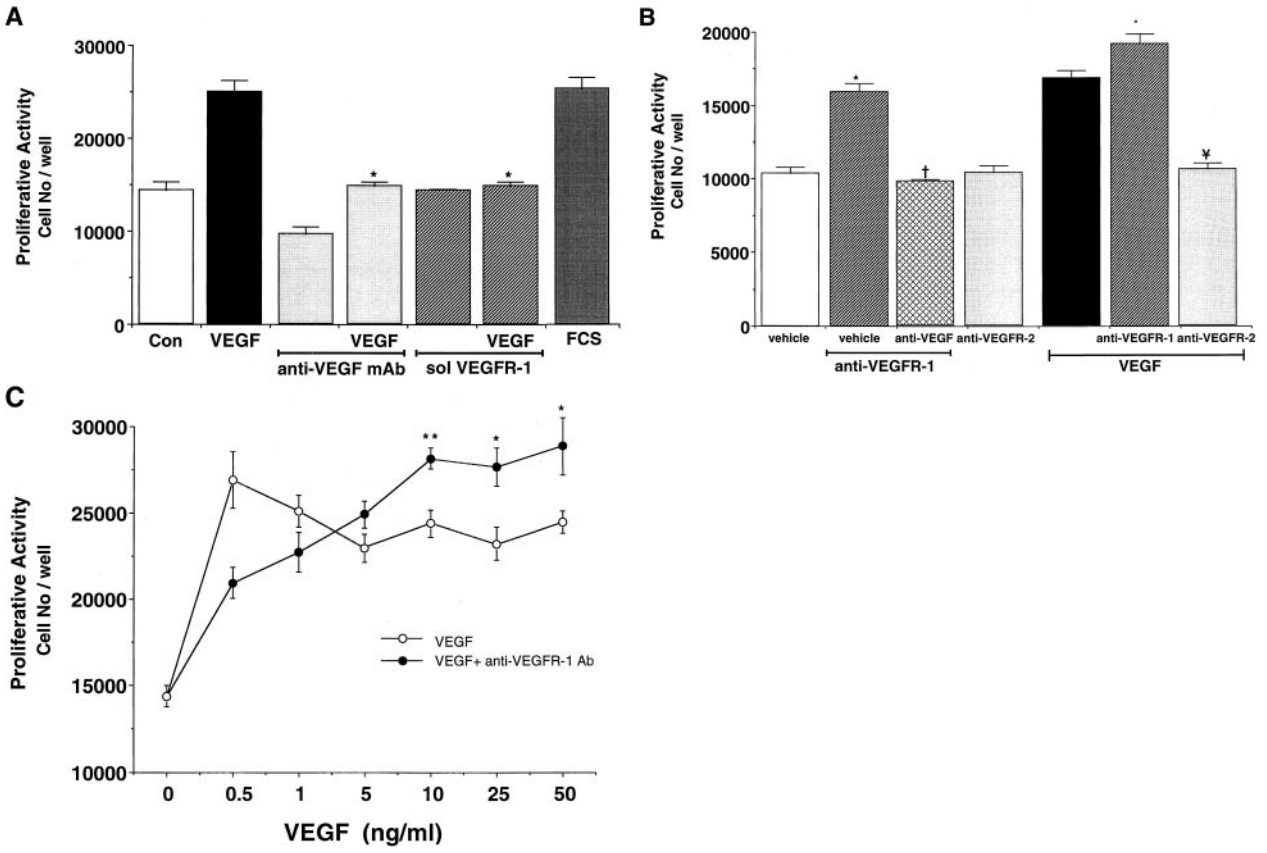


Figure 8. Blockade of VEGFR-1 potentiates VEGF-stimulated HUVEC proliferation. Before stimulation with VEGF, HUVECs were preincubated with vehicle or with the neutralizing antibodies, or sVEGFR-1 for 30 minutes at 37°C in medium containing 1% FCS. **A:** The neutralizing anti-VEGF antibody (50 ng/ml) or the sVEGFR-1 (50 ng/ml) completely inhibited VEGF (10 ng/ml)-stimulated proliferation. FCS (20%) was used as positive control (*, $P < 0.01$ *versus* VEGF). **B:** Incubation of the endothelial cells with 30 ng/ml of anti-VEGFR-1 antibody or anti-VEGFR-2 in the absence or presence of 1 ng/ml of VEGF. The anti-VEGFR-1 stimulated a significant increase in endothelial cell proliferation in the absence of exogenous VEGF as compared to the control ($P < 0.01$, $n = 3$) but the increase in proliferation was only partially increased in the presence of exogenous VEGF as compared with VEGF alone (°, $P < 0.05$; $n = 3$). Addition of the neutralizing anti-VEGF monoclonal antibody to the anti-VEGFR-1-treated cells significantly inhibited the proliferative activity. The anti-VEGFR-2 antibody significantly inhibited VEGF-stimulated HUVEC proliferation. Analysis of variance with Neumann-Keul's multiple comparison test was performed among treatment (vehicle) *versus* treatment plus anti-VEGFR antibodies (*, $P < 0.01$) or among VEGF and VEGF plus anti-VEGFR-1 (°, $P < 0.01$) and anti-VEGFR-2 (¥, $P < 0.01$) or among anti-VEGFR-1 antibody *versus* anti-VEGFR-1 plus anti-VEGF antibody (†, $P < 0.01$). **C:** Incubation of the HUVECs with increasing concentrations (1 to 50 ng/ml) of VEGF alone (**open circle**) and in the presence of a fourfold excess of anti-VEGFR-1 antibody (**solid circle**). The VEGFR-1 antibody significantly potentiated VEGF-induced proliferation at concentrations of 10, 25, and 50 ng/ml of VEGF (*, $P < 0.05$; **, $P < 0.01$ *versus* VEGF). Cell numbers were assessed after a 48-hour incubation by Coulter counter and results are expressed as a mean (±SEM) of three independent experiments ($n = 9$).

Discussion

In this study we demonstrate that activation of VEGFR-1 results in the generation of NO in endothelial cells and identified NO as a cue for negative modulation of VEGFR-2-dependent endothelial cell proliferation and for positive regulation of endothelial cell redifferentiation into capillary-like structures. The selective blockade of VEGFR-1 potentiated HUVEC growth and sustained the maximal proliferative effect of VEGF indicating that VEGFR-1 modulates VEGFR-2-mediated endothelial cell growth. It is clear that VEGFR-1 regulates VEGFR-2 responses via NO as the pharmacological generation of NO inhibited VEGF-mediated HUVEC proliferation. The inhibition of VEGFR-1 with a neutralizing antibody resulted in the failure of endothelial cells to form capillary connections and the overcrowding of these cells into isolated islands and that NO donors prevented this phenotype. This mechanism is confirmed by our observation that the capacity of $PAE_{VEGFR-1}$ to form tubular networks is inhibited by NOS and cGMP inhibitors, whereas the inability of $PAE_{VEGFR-2}$ to form a tubular network can be restored with 8-bromo-cGMP. Thus we provide the first direct evidence that there is cross-talk between the two VEGF receptors that is mediated by NO and that VEGFR-1 activation can be seen as a molecular switch for endothelial cell differentiation.

VEGFR-2 is absolutely critical for the earliest stages of vasculogenesis as blood islands, endothelial cell, and major blood vessels fail to develop in appreciable numbers in embryos lacking VEGFR-2.[7] The reduction of VEGF-induced angiogenesis in the murine Matrigel assay in the presence of the anti-VEGFR-2 antibody confirms that this receptor is essential for angiogenesis. In the VEGFR-1 knockout mouse embryos the vessels do form, but are disorganized.[8] The blockade of the VEGFR-1 with anti-VEGFR-1-neutralizing antibodies prevented vessel stabilization and promoted formation of large aneurysm-like structures in the murine Matrigel angiogenesis assay further supporting a regulatory role for VEGFR-1 in vessel formation.

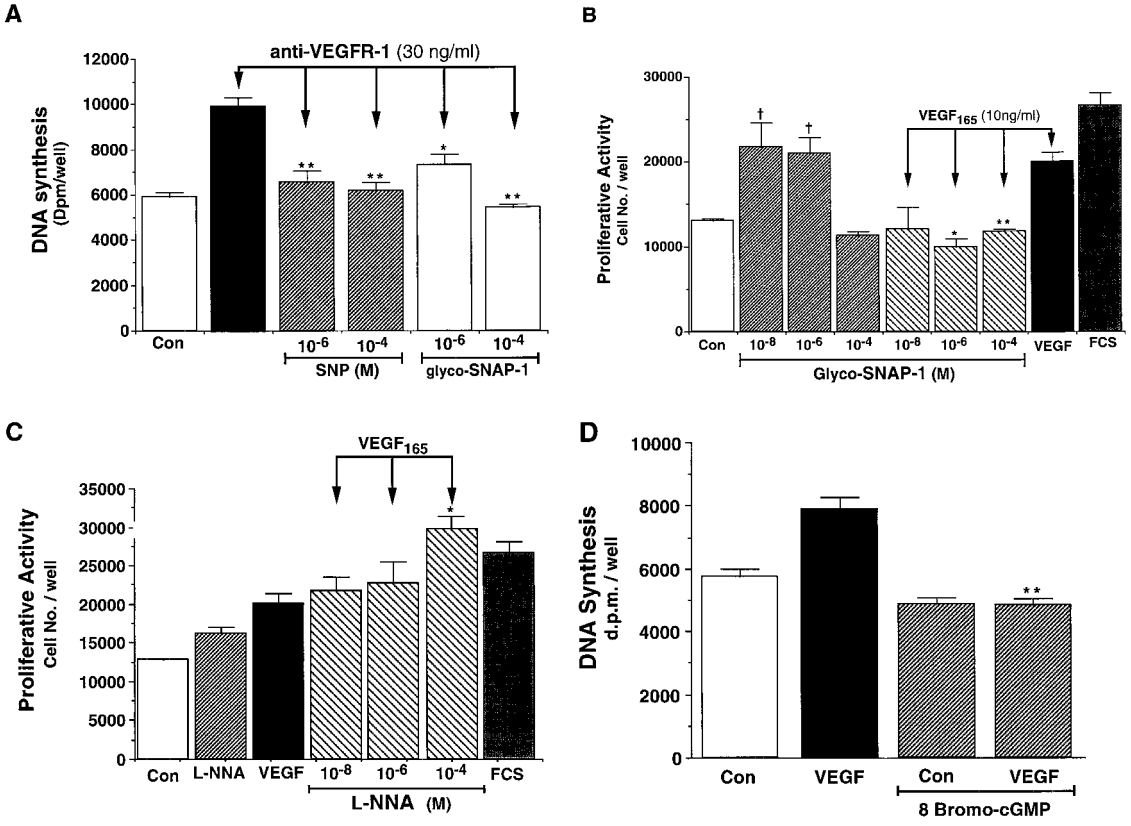


Figure 9. NO suppresses VEGF-stimulated HUVEC proliferation. Subconfluent quiescent HUVECs were incubated with agonists in M199 containing 1% FCS. **A:** Cells were incubated with the 30 ng/ml of anti-VEGFR-1 antibody alone and in the presence of sodium nitroprusside or glyco-SNAP-1. Both NO donors significantly attenuated the increase in DNA synthesis induced by the anti-VEGFR-1 antibody (*, $P < 0.05$; **, $P < 0.01$ *versus* anti-VEGFR-1 alone). **B:** Cell numbers assessed after a 48-hour incubation with glyco-SNAP-1 in the absence (**narrow hatched bars**) or in the presence (**wide hatched bars**) of 10 ng/ml of VEGF. At 10^{-8} and 10^{-6} mol/L, glyco-SNAP-1 alone significantly increased HUVEC proliferation whereas VEGF-mediated cell proliferation in the presence of glyco-SNAP-1 at 10^{-6} and 10^{-4} mol/L was significantly inhibited as compared to VEGF (†, $P < 0.05$ *versus* control; *, $P < 0.01$ *versus* VEGF). **C:** HUVECs were exposed to L-NNA alone (10^{-6} mol/L) or to increasing concentrations of L-NNA in the presence of 10 ng/ml VEGF. VEGF-induced cell proliferation was moderately increased at 10^{-4} mol/L L-NNA (*, $P < 0.05$ *versus* VEGF). **D:** Cells were stimulated with 10 ng/ml of VEGF alone or in the presence of the 8-bromo-cGMP (10^{-4} mol/L). Addition of 8-bromo-cGMP attenuated VEGF-stimulated increase in DNA synthesis (*, $P < 0.02$ *versus* VEGF). Results are expressed as a mean (±SEM) of three independent experiments ($n = 9$).

In two important studies, Fong and co-workers[8,9] demonstrated that the primary role of VEGFR-1 during vasculogenesis is to limit the differentiation of hemangioblasts into endothelial cells, it was unclear however how this was prevented by VEGFR-1. Increased hemangioblast commitment in the absence of VEGFR-1 resulted in increased numbers of endothelial cells that caused an overcrowded microvascular environment and markedly disorganized vasculature. The absence of VEGFR-1 resulted in mouse embryos and in cystic embryoid bodies derived from embryonic stem cells *in vitro* in large, disorganized vascular channels filled with aggregates of hemangioblasts and endothelial cells. These findings are strikingly similar to those observed in both our *in vitro* and *in vivo* angiogenesis assays. Inhibition of VEGFR-1 *in vivo* resulted in the formation of massive blood vessels lined with aggregates of endothelial cells and unchecked proliferation of cultured endothelial cells led to the collection of large cellular aggregates.

VEGF was reported to stimulate NO release from both cultured HUVECs[5,19] and intact arterial strips.[18] Blockade of VEGFR-2 did not affect VEGF-stimulated NO release in HUVECs. In addition, neither $VEGF_{121}$ nor the VEGF:PlGF heterodimer that specifically binds to VEGFR-2[27,29] were able to induce NO release demonstrating that VEGFR-2 activation is not required for NO release in HUVECs. These results are further supported by the ability of $PAE_{VEGFR-1}$-transfected cells to release NO in response to increasing concentrations of VEGF. Although VEGF up-regulates eNOS expression in $PAE_{VEGFR-2}$,[30] this does not equate to NOS activity. Indeed, it was recently stated that expression of eNOS in bovine endothelial cells does not correlate with activity (Professor Ron R. Magness, University of Wisconsin-Madison, personal communication; and data presented at FASEB Summer Conference, Copper Mountain, CO on June 2000). In our hands, we noted that basal levels of NO were higher in $PAE_{VEGFR-2}$ cells compared with $PAE_{VEGFR-1}$, but when VEGF was added, a significant increase in NO release was only observed in $PAE_{VEGFR-1}$ cells. As sVEGFR-1 inhibited the NO release when added to cells that were previously stimulated for 35 minutes with VEGF, this increase was sustained. These findings however are in contrast to those of Kroll and Waltenberger[31] that reported VEGF caused a transient increase in cGMP levels at 2 minutes in $PAE_{VEGFR-2}$ cells, but at subsequent time points VEGF had no effect. This is surprising as VEGF-

mediated NO release in endothelial cells is sustained.[5,19,20]

Murohara and colleagues[32] reported that VEGF stimulates permeability via NO and prostacyclin through VEGFR-2 based on their observation that PlGF that binds to VEGFR-1 but not VEGFR-2, failed to increase vascular permeability. The negative effect of PlGF often used to conclude a lack of biological response via VEGFR-1 is misleading. Our findings clearly demonstrate that PlGF-1 that lacks a heparin-binding domain is able to stimulate NO release in HUVECs, whereas PlGF-2 has no effect on NO production. The apparent lower potency of PlGF-2 may be explained by the fact that it contains a heparin-binding domain.[33] We suggest that the use of PlGF as a VEGFR-1-selective ligand to assess VEGF receptor function must involve the use of both isoforms. It is also important to remember that agonists that bind to a common receptor do not necessarily transduce the same signals as exemplified by angiopoietin-1 and angiopoietin-2 that bind to Tie-2.[34] Moreover, the fact that PlGF homodimers that bind to VEGFR-1 and PlGF:VEGF heterodimers that bind to VEGFR-2 induced tyrosine phosphorylation of a 38-kd protein in HUVECs whereas VEGF homodimers did not[29] supports the view that PlGF and VEGF can exert different biological actions through the same receptor. Thus an absence of a response to PlGF should not be misconstrued as a lack of VEGFR-1 activity.

The specific blockade of VEGFR-1 with a neutralizing anti-VEGFR-1 antibody potentiating HUVEC proliferation and sustaining the maximal mitogenic effect of VEGF suggests that VEGFR-1 transduces a regulatory signal to control VEGFR-2-dependent endothelial cell proliferation. Similar results were reported in trophoblasts[5] and in PAE cells transfected with VEGF receptor chimeras.[35] However, the underlying mechanism involved in regulating VEGF-mediated endothelial cell proliferation is still unclear, and the role of NO in this process remains controversial. NO may stimulate proliferation of endothelial cells at low concentrations but inhibit their proliferation at higher concentrations by a ribonucleotide-dependent mechanism.[36] In the present study, VEGF-stimulated proliferation was attenuated by glyco-SNAP-1 as well as by 8-bromo-cGMP indicating that activation of cGMP by NO controls VEGF-mediated endothelial cell proliferation. Furthermore, the inhibitory effect of the anti-VEGFR-1 antibody on endothelial cell proliferation was bypassed by treatment of cells with NO donor glyco-SNAP-1. Our data are supported by a recent study showing a role for NO in inhibiting endothelial proliferation and promoting *in vitro* capillary organization induced by basic fibroblast growth factor.[37]

In the studies by Fong and colleagues,[8,9] the primary abnormality in VEGFR-1 null mice was found to be one of differentiation, not increased proliferation of endothelial cell precursors. In contrast, our results clearly indicate a role for VEGFR-1 in the modulation of VEGFR-2-mediated proliferation through the generation of NO. A possible explanation for this difference is that our studies examined the effects of blocking VEGFR-1 during physiological angiogenesis, whereas the observations of Fong and colleagues[8,9] were during vascular development. The similar phenotypes suggest a common role for VEGFR-1 in the modulation of endothelial cell differentiation during both embryonic angiogenesis and adult angiogenesis, one that might involve VEGFR-1-mediated NO production because eNOS is expressed as early as day 4 in the embryo during murine development.[38] Our data demonstrate that VEGFR-1 signal generates NO that counteracts VEGFR-2-mediated endothelial cell proliferation to allow differentiation to occur. Recently, Hiratsuka and co-workers[14] proposed that VEGFR-1 acts as a positive regulator of pathological angiogenesis when levels of PlGF are elevated because murine Lewis lung carcinoma cells overexpressing PlGF-2 grew faster in wild-type mice than in VEGFR-1 tyrosine domain-deficient mice. We have previously shown that addition of PlGF-1 or PlGF-2 to cultured trophoblasts causes tyrosine phosphorylation of VEGFR-2 that is inhibited by anti-VEGF or anti-VEGFR-2 antibodies[22] indicating that VEGFR-1, the signaling receptor for PlGF, can modulate VEGFR-2. If a similar mechanism exists in endothelial cells then the observation that PlGF promotes pathological angiogenesis and tumor growth directly by VEGFR-1[14] could be explained as indirect VEGFR-2 activation. VEGF signaling is very similar in both trophoblast and endothelial cells. We have previously shown that VEGF can induce mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase 1 and 2 (ERK1/2) in trophoblasts[39] that was later confirmed in endothelial cells.[40]

This function of VEGFR-1 may be masked in an *in vivo* environment leading to the suggestion that VEGFR-1 acts as a VEGF-sink.[10] Indeed, Cunningham and colleagues[41] demonstrated an interaction between VEGFR-1 and the p85 subunit of the phosphatidylinositol 3-kinase. Moreover, members of the Src family showed an increased level of phosphorylation after VEGF stimulation in $PAE_{VEGFR-1}$ and not in $PAE_{VEGFR-2}$ cells.[3] Furthermore, VEGFR-1 kinase was shown to display a morphogenic activity as fibroblasts expressing the activated cytoplasmic domain of the VEGFR-1 spontaneously form tubular structures on matrix.[42] Moreover, using the novel *in vitro* co-culture angiogenesis assay, we show that both VEGFR-1 and VEGFR-2 are required for angiogenesis, but it seems that VEGFR-1 may promote vascular connections within the capillary network (branching angiogenesis) whereas VEGFR-2 predominantly promotes tube length between branches. This suggests that endothelial cells stimulated to proliferate and migrate via VEGFR-2 and are unable to organize themselves into vascular structures without VEGFR-1. Indirect evidence that supports this comes from the JunB null mice where the failure to establish proper vascular interactions with the maternal circulation is associated with down-regulation of VEGFR-1. In these mice, fetal blood vessels could only be located in the chorio-allantoic plate and they were not able to penetrate or to branch into the labyrinth trophoblasts.[43]

In summary, our studies show that VEGFR-1 activation releases NO and that NO is the cross-talking second messenger that inhibits VEGFR-2-mediated endothelial cell proliferation and induces these cells to differentiate into capillary-like structures. A model for VEGF action during physiological or pathological angiogenesis

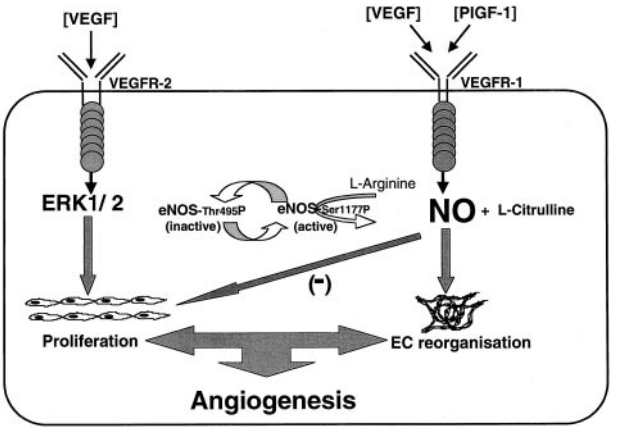


Figure 10. Diagram of the regulatory pathways mediated by VEGFR-1 and VEGFR-2 in endothelial cells during physiological or pathological angiogenesis.

through the regulatory pathways mediated by VEGFR-1 and VEGFR-2 is proposed (Figure 10). Although the activation of VEGFR-2 may be sufficient for embryonic angiogenesis, activation of VEGFR-1-mediated NO release may be required to promote the development of the intricate architecture of the vascular network (branching angiogenesis) during menstruation, luteal development, and wound healing. It is therefore likely that altered expression or activity of VEGFR-1 may cause vascular abnormalities. There is a reciprocal relation between VEGF and NO in the endogenous regulation of endothelial integrity after arterial injury[44] and in eNOS knockout mice VEGF fails to induce angiogenesis.[21] The present study, together with earlier reports on trophoblasts[9] and tumor epithelial cells,[45] provides clear evidence that NO via VEGFR-1 activation leads to vascular morphogenesis by arresting VEGF-induced proliferation and initiating a program of cell differentiation. Thus NO seems to be a molecular switch for endothelial cell differentiation and that induction of eNOS may have therapeutic activity in vascular insufficiencies whereas NOS inhibitors may limit tumor growth.

Acknowledgment

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