0023-6837/03/8312-1873$03.00/0
LABORATORY INVESTIGATION
Vol. 83, No. 12, p. 1873, 2003
Printed in U.S.A.

$VEGF_{121}$ and $VEGF_{165}$ Regulate Blood Vessel Diameter Through Vascular Endothelial Growth Factor Receptor 2 in an In Vitro Angiogenesis Model

Martin N. Nakatsu, Richard C. A. Sainson, Sofía Pérez-del-Pulgar, Jason N. Aoto, Mark Aitkenhead, Kevin L. Taylor, Philip M. Carpenter, and Christopher C. W. Hughes

Department of Molecular Biology and Biochemistry (MNN, RCAS, SPdP, JNA, MA, KLT, CCWH), University of California Irvine, Irvine, and Department of Pathology (PMC), University of California, Irvine Medical Center, Orange, California

SUMMARY: Vascular endothelial growth factor (VEGF) is essential for the induction of angiogenesis and drives both endothelial cell (EC) proliferation and migration. It has been suggested that VEGF also regulates vessel diameter, although this has not been tested explicitly. The two most abundant isoforms, $VEGF_{121}$ and $VEGF_{165}$, both signal through VEGF receptor 2 (VEGFR-2). We recently optimized a three-dimensional in vitro angiogenesis assay using HUVECs growing on Cytodex beads and embedded in fibrin gels. Fibroblasts provide critical factors that promote sprouting, lumen formation, and vessel stability. Using this assay, we have examined the role of VEGF in setting vessel diameter. Low concentrations of both $VEGF_{121}$ and $VEGF_{165}$ promote growth of long, thin vessels, whereas higher concentrations of VEGF remarkably enhance vessel diameter. Placental growth factor, which binds to VEGFR-1 but not VEGFR-2, does not promote capillary sprouting. Moreover, specific inhibition of VEGFR-2 signaling results in a dramatic reduction of EC sprouting in response to VEGF, indicating the critical importance of this receptor. The increase in vessel diameter is the result of cell proliferation and migration, rather than cellular hypertrophy, and likely depends on MEK1-ERK1/2 signaling. Both phosphatidylinositol 3-kinase and p38 activity are required for cell survival. We conclude that the diameter of new capillary sprouts can be determined by the local concentration of VEGF and that the action of VEGF on angiogenic EC in this assay is critically dependent on signaling through VEGFR-2. (*Lab Invest 2003, 83:1873–1885*).

Angiogenesis is a multistep process whereby new blood vessels are formed from existing vessels in response to angiogenic stimuli (Carmeliet, 2000; Folkman, 1975, 1985; Risau, 1997; Yancopoulos et al, 2000). It involves endothelial cell (EC) degradation of the adjacent extracellular matrix, migration (sprouting) into the surrounding tissue, proliferation, alignment, tube formation, anastomosis, recruitment of parenchymal cells, and a return to quiescence (Conway et al, 2001; Folkman, 1975, 1985; Yancopoulos et al, 2000). The importance of angiogenesis in solid tumor growth and survival is well established, and solid tumors larger than a few millimeters in size uniformly induce a vascular supply (Folkman, 1975). In many respects, angiogenesis in the adult is a replay of morphogenetic events that occur during development. In the developing embryo, in response to poorly understood cues, a primitive vascular plexus formed by the process of vasculogenesis is then remodeled by processes similar to those seen in adult angiogenesis (Conway et al, 2001). Pruning, sprouting, and growth give rise to the familiar network of larger vessels ramifying into smaller and smaller vessels down to the level of capillaries. How the phenotypes of different vessels are determined is not known; neither is it known how the diameter of vessels is determined. Angiogenic growth factors that induce proliferation and migration of ECs, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), have been identified (Friesel and Maciag, 1995; Gale and Yancopoulos, 1999; Yancopoulos et al, 2000), as have factors such as the angiopoietins, which regulate maturation of developing vessels (Maisonpierre et al, 1997; Suri et al, 1996). Recently, the Ephrin/Eph and the Notch/Delta/Jagged systems have also been implicated in regulating vascular specification and network organization during development (Adams et al, 1999; Cheng et al, 2002; Gale et al, 2001; Gridley, 1997; Taylor et al, 2002).

VEGF is a crucial angiogenic factor, involved in all stages of blood vessel formation, from lineage commitment, through EC migration and proliferation, to

DOI: 10.1097/01.LAB.0000107160.81875.33

Received October 23, 2003.

This work was supported by a grant from the National Institutes of Health (RO1 HL-60067). Current addresses: Dr. Sofia Pérez-del-Pulgar, Hepatic Hemodynamic Laboratory, Liver Unit, Hospital Clínic, Barcelona, Spain; Dr. Mark Aitkenhead, Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, CA 90822; Dr. Kevin Taylor, Taussig Cancer Center, Cleveland Clinic, Cleveland, OH 44195

Address reprint requests to: Dr. Christopher C. W. Hughes, Department of Molecular Biology and Biochemistry, 3219 McGaugh Hall, University of California Irvine, Irvine, CA 92697. E-mail: cchughes@uci.edu

vessel assembly (Dvorak et al, 1995). In many systems, it is able to induce angiogenic sprouting and the formation of immature vessels in the absence of other exogenously added factors. There is also considerable evidence for the importance of VEGF in angiogenesis later in development, and its importance in inducing new vessel growth into tumors has led to a growing interest in VEGF and its receptors as therapeutic targets (Dvorak et al, 1991). Transgenic mice that express VEGF under the control of a keratin promoter show hypervascularity of the skin (Detmar et al, 1998; Larcher et al, 1998), whereas exogenous VEGF has been shown to induce malformed, fused vessels, with enlarged lumens (Drake and Little, 1999). The VEGF family consists of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (P/GF). VEGF-A is the predominant mitogen, and embryonic lethality in embryos that lack even a single allele attests to its critical importance and also indicates that fine regulation of VEGF expression levels is essential for normal vascular development (Carmeliet et al, 1996).

Five isoforms of VEGF-A are known, and these differ in their interactions with heparan sulfate proteoglycans and with the VEGF receptors (Shima et al, 1996; Tischer et al, 1991). Mice that express only the $VEGF_{164}$ isoform are normal and healthy (Stalmans et al, 2002), whereas mice that express exclusively $VEGF_{120}$ exhibit severe defects in vascular growth and patterning and die from cardiac failure as a result of impaired myocardial angiogenesis (Carmeliet et al, 1999). Mice that express only $VEGF_{188}$ show defects in arterial but not venular development (Stalmans et al, 2002). It has been suggested that $VEGF_{188/189}$ decreases vessel diameter whereas $VEGF_{164/165}$ and $VEGF_{120/121}$ increase vessel diameter (Carmeliet, 2000; Conway et al, 2001), although this has not been tested explicitly. In quail embryos, $VEGF_{165}$ has been shown to induce vessels with large lumens by stimulating vessel fusion (Drake and Little, 1999).

Many models for angiogenesis, both in vivo and in vitro, have been published, and all have strengths and weaknesses. In vivo models have clear relevance; however, it is hard to manipulate genetically the EC, and direct versus indirect effects on the EC are often hard to separate. Various in vitro assays of angiogenesis have been developed that allow easier manipulation of the ECs but often fail to mimic all—or even most—of the discrete steps. Because of these disadvantages, we have recently developed a new three-dimensional (3-D) in vitro angiogenesis assay that is a modification of a previously described fibrin gel assay (Nehls and Drenckhahn, 1995a, 1995b). The original protocol does not promote good growth of HUVECs, a widely used model for vascular ECs, and the induced vessels tend to be thin and immature, often with narrow, incomplete lumens (Nehls and Drenckhahn, 1995a, 1995b). We have modified the assay so that we can now reproducibly obtain growth of HUVECs into mature, long-lived, capillary-like vessels with well-developed lumens (Nakatsu et al, 2003). This system models sprouting, elongation, lumen formation, branching, and anastomosis into complex networks.

We have used this system to investigate the role of VEGF concentration in determining vessel diameter and to identify important downstream signaling components. Our results show a critical role for VEGF concentration in vascular growth consistent with in vivo data demonstrating haploinsufficiency for VEGF (Carmeliet et al, 1996; Ferrara et al, 1996).

Results

Skin Fibroblasts Provide Factors that Stabilize Vessels and Promote Maturation

When HUVECs were placed into a fibrin gel assay and cultured with bFGF and VEGF, sprout formation was limited, in contrast to the robust growth seen with microvascular ECs (Montesano et al, 1993; Nehls and Drenckhahn, 1995a, 1995b; Villaschi and Nicosia, 1994). HUVEC cultures yielded a mixture of detached but migrating ECs, some sprouting, but with vessels that seemed immature (Fig. 1A). In many cases, short and distinct sprouts formed early, but later in the assay, cells began to separate from the leading edge of the vessels and migrate away. Vessels often formed, only to regress after 3 to 4 days. In the limited number of vessels that did persist, lumens were difficult to distinguish, often seeming to be no more than discontinuous slits, and anastomosis between vessels was minimal. These observations suggested that critical factors required for mature, stable vessels to form were absent from the cultures.

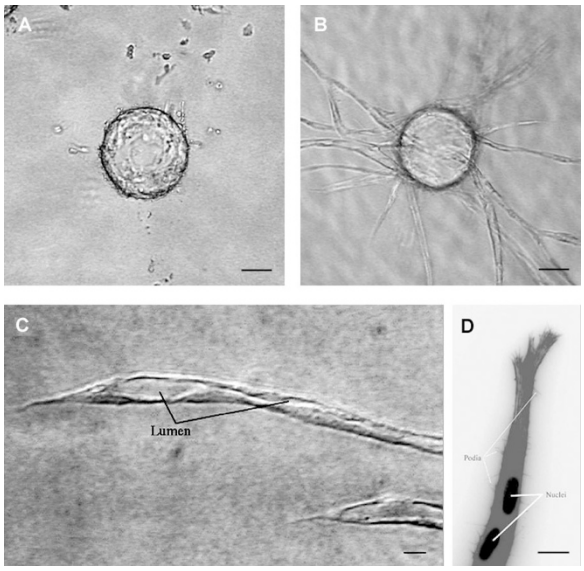

Figure 1.
Skin fibroblasts (SFs) stabilize vessel formation. HUVEC-coated bead in the absence of SFs (A) and in the presence of SFs (B). Cultures were established as described in "Materials and Methods" and photographed at Day 7. Note the enhanced organization of cells and lumen formation in the presence of SFs. Scale bars for A and B represent 50 μm. C, Phase contrast image of two vessels showing tip cells. D, Vessels were fixed and stained for F-actin with TRITC-phalloidin and for DNA with 4', 6-diamidino-2-phenylindole. Actin-rich filopodia were observed at the tips of the vessels. No vessel lumens were visualized within the tip cells. Scale bars for C and D represent 20 μm.

Earlier studies suggested that stromal/peri-ECs, such as pericytes, smooth muscle cells, and fibroblasts, may provide factors that stabilize vessels and induce maturation (Greenwood, 1991; Hurwitz et al, 1993; Montesano et al, 1993; Nehls et al, 1994; Villaschi and Nicosia, 1994). We therefore tested the ability of skin fibroblasts (SFs) to sustain vessel development and maturation. When SFs were plated on top of the fibrin gels, ECs sprouting from the beads were apparent within 2 to 3 days. By 5 days, numerous long and morphologically complex vessels had extended from the beads (Fig. 1B). Free migrating ECs (not part of a growing vessel) were absent, and lumens were easily distinguishable. Lumens were clearly not slit-like or composed of coalescing intracellular vacuoles but rather were surrounded by, presumably, polarized cells with luminal and abluminal faces. As has been reported previously, lumen formation occurred behind the leading tip of the vessel (Folkman, 1985), which was composed of one or two cells with numerous filopodia extending forward into the matrix (Fig. 1, C and D). These tip cells were strongly reminiscent of neural growth cones in their morphology. Figure 2 follows a single bead and shows the progression of sprouts at a concentration of 2.5 ng/ml of $VEGF_{165}$ in the presence of fibroblasts, over the course of 7 days. We observed that at Day 1 (Fig. 2A), small buds emerged from the beads and most developed into recognizable vessels with lumens at approximately Day 3 (Fig. 2B). A small number of buds regressed and eventually disappeared, but the majority continued to lengthen over the course of the assay (Fig. 2C). By Day 7, most vessels had clearly defined lumens (Fig. 2D). By approximately Days 13 to 14, lengthening had usually stopped and vessels from adjacent beads showed clear anastomoses. Networks were stable with no signs of EC apoptosis for >2 weeks (Nakatsu et al, 2003, and data not shown).

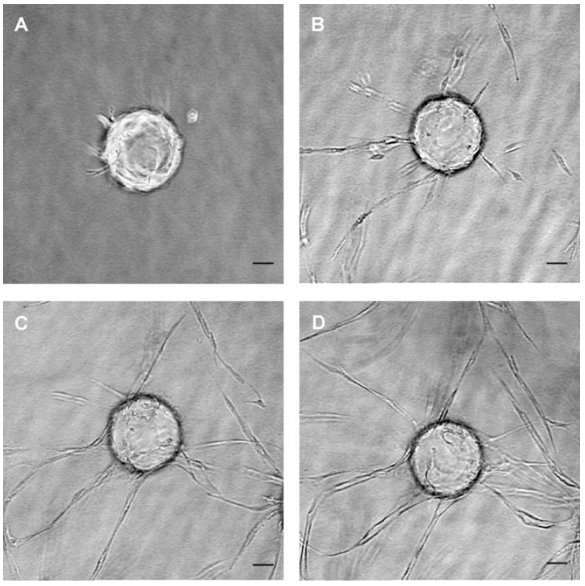

Figure 2.

Time course of sprouting. A fibrin bead assay was established in the presence of 2.5 ng/ml of $VEGF_{165}$ and SFs. A single bead is shown at multiple time points. A, Day 1. B, Day 3. C, Day 5. D, Day 7. Lumen formation begins at approximately Days 3 to 4, and by Day 7, the majority of vessels have lumens. Scale bars represent 50 μm.

Although SFs began to migrate into the gels at later times, contact between SFs and ECs was not required for induction of vessel maturation. SFs separated from the ECs by a Transwell were still effective, although the time course was somewhat delayed (data not shown). The SF cells express angiopoietin-1; however, angiopoietin-1 alone is not sufficient to support vessel maturation (Nakatsu et al, 2003; and data not shown). Fibroblasts also express VEGF, especially under hypoxic conditions, and we have confirmed this in our SF cells (data not shown). Smooth muscle cells, in contrast to SFs, did not provide support, although they did form cords similar to those made by ECs (Nehls and Drenckhahn, 1995a, 1995b; Nehls et al, 1994). These experiments demonstrate that fibroblasts are capable of providing signals that promote maturation of vessels in vitro as evidenced by tight control of EC migration; formation of multicellular structures; development of large, contiguous, and patent lumens; and stabilization of networks over several weeks.

$VEGF_{165}$ Concentration Determines Vessel Morphology

Previous reports have suggested that $VEGF_{165}$ not only drives angiogenic sprouting but may also play a role in determining lumen diameter, as well as vessel length (Carmeliet, 2000; Conway et al, 2001). Therefore, to test this idea, we added increasing concentrations of $VEGF_{165}$ into fibrin gel assays and quantified the number of sprouts, sprout length, vessel diameter, and shape index. SFs under these conditions do not produce significant quantities of VEGF. A dose-response titration was performed in which 0 ng/ml to 35 ng/ml of $VEGF_{165}$ was added to each well, in the presence of a fixed amount of bFGF (5 ng/ml). Cultures were monitored over the course of 7 days, and images were captured every other day. At 7 days, there was a sharp peak of five sprouts per bead at 2.5 ng/ml $VEGF_{165}$, whereas at concentrations <1 or >10 ng/ml, there was an average of only one sprout per bead (Fig. 3A). The average length per sprout was also highest at 2.5 ng/ml $VEGF_{165}$ (Fig. 3B), although this peak was considerably less dramatic and higher concentrations made little difference to sprout length. It is interesting that the progression of sprouts at 25 ng/ml was slowed compared with 2.5 ng/ml of $VEGF_{165}$ as sprouts did not emerge until Day 3, compared with Day 1 for the lower concentration. Remarkably, despite the dramatic decrease in number of sprouts at higher $VEGF_{165}$ concentrations, we observed a robust increase in the average shape index of sprouts per bead at these concentrations (Fig. 3C), with vessels having significantly larger diameters than those growing in lower concentrations of growth factor (Fig. 3, D and E). Overall, at low $VEGF_{165}$ concentrations, sprouts appeared earlier and tended to be slightly longer, although lumens were still easily distinguishable. In contrast, sprouts that grew at higher concen-

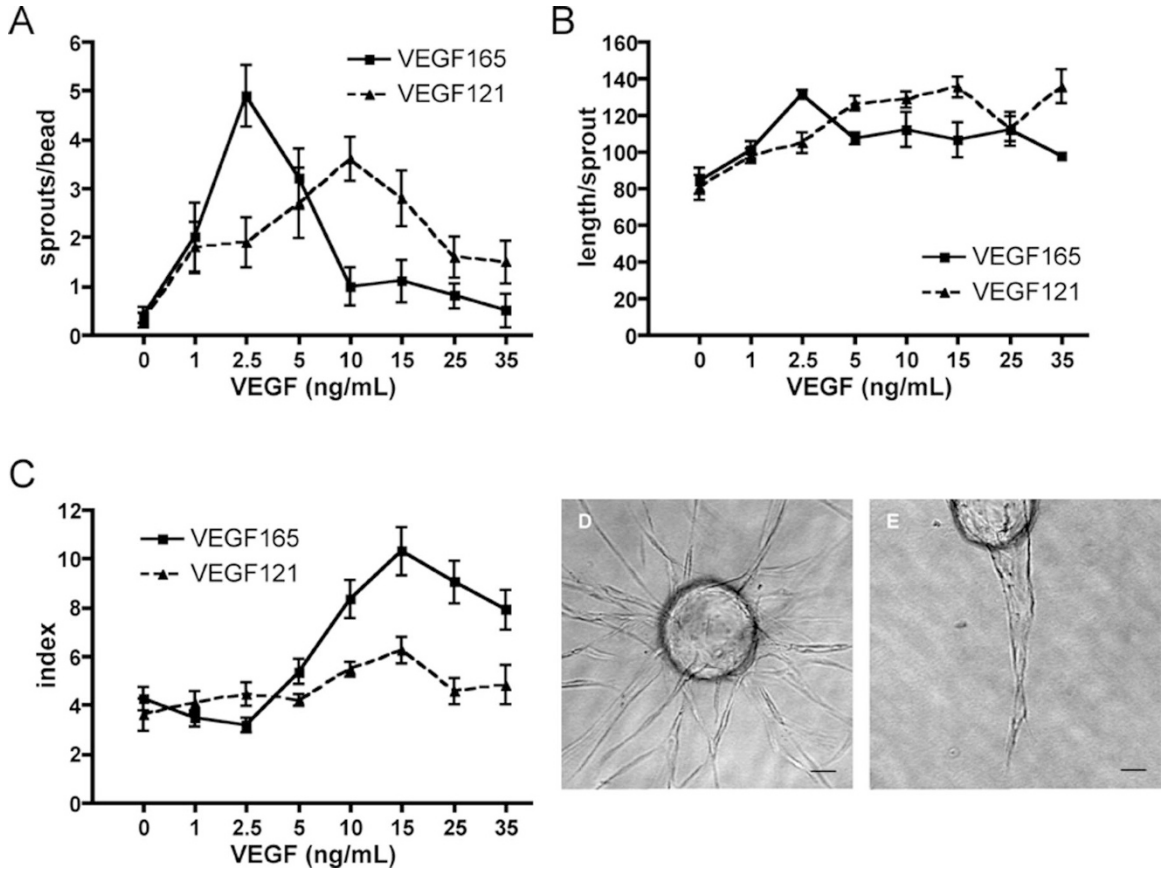

Figure 3.

Concentration of $VEGF_{165}$ and $VEGF_{121}$ regulates vessel morphology in vitro. Fibrin bead assays were established at various $VEGF_{165}$ and $VEGF_{121}$ concentrations between 0 ng/ml and 35 ng/ml in the presence of SFs. Sprouts were quantified at Day 7. Data represent one experiment. Similar results were obtained in three similar experiments. Values represent mean ± SEM. A, Sprouts per bead. B, Average sprout length. C, Vessel shape index. D, Vessel morphology at 2.5 ng/ml of $VEGF_{165}$. E, Vessel morphology at 15 ng/ml of $VEGF_{165}$. Scale bars represent 50 μm.

trations of $VEGF_{165}$ were slower to appear and were wide and slightly shorter. Similar experiments using different concentrations of bFGF did not yield a relationship between growth factor concentration and vessel diameter (data not shown). These data indicate that $VEGF_{165}$ concentration can directly affect vessel morphology, in particular, vessel diameter.

$VEGF_{121}$ Also Increases Vessel Diameter

It has been suggested that the different isoforms of VEGF-A have different effects on developing vessels and that $VEGF_{121}$ and $VEGF_{165}$ increase, whereas $VEGF_{189}$ decreases vessel diameter (Carmeliet, 2000; Conway et al, 2001). We therefore tested in our in vitro system whether $VEGF_{121}$ was also capable of increasing vessel diameter. As predicted, varying the concentration of $VEGF_{121}$ had similar effects on vessel diameter, as $VEGF_{165}$, increasing vessel diameter in a dose-dependent manner, although less dramatically than $VEGF_{165}$ (Fig. 3C). The effects of $VEGF_{121}$ on inducing sprout formation and inducing vessel lengthening were similar to those of $VEGF_{165}$, although the number of sprouts per bead and maximum length both peaked somewhat higher (10 to 15 ng/ml) with $VEGF_{121}$ (Fig. 3, A and B). Thus, $VEGF_{121}$ acts similarly to $VEGF_{165}$ in this assay. Recombinant $VEGF_{189}$ is not readily available.

Increased Vessel Diameter Is Due to Increased Cell Number, Rather Than to Cell Hypertrophy

The increase in vessel diameter that we observed in response to increasing concentrations of VEGF may be explained by increasing cell number or by cellular hyperplasia. To resolve these two possibilities, we calculated mean internuclear distance, as a surrogate for cell size, for vessels growing in low (2.5 ng/ml) or high (15 ng/ml) concentrations of VEGF. We found no difference in this parameter between the two groups, demonstrating that cells under each condition are the same size (data not shown). Thus, increased vessel diameter is a result of increased recruitment of ECs into the developing vessel (hyperplasia), rather than an increase in cell size (hypertrophy).

Cell Proliferation Can Account for Increased Vessel Diameter

To confirm that EC proliferation is, indeed, occurring in these cultures, we stained paraffin sections that contained vessels with mAb Ki-67, which recognizes a

nuclear antigen in proliferating cells, and then counterstained with hematoxylin. Positive staining for Ki-67 is observed within the vessel (Fig. 4, *thick black arrow*), usually in cells close to the bead (*black arrowheads*). Nonproliferating cells were also observed within the vessel (*white arrows*). When we harvested the vessels from cultures grown for 7 days in either 2.5 ng/ml or 15 ng/ml of VEGF, we found a 75% increase in the number of cells from cultures grown at the higher VEGF concentration (450,000 versus 790,000). These data suggest, therefore, that the increase in vessel size can be accounted for by cellular proliferation.

Sprouting and Growth of Vessels In Vitro Is Dependent on VEGFR-2 Signaling

The major VEGF receptor for vascular EC during angiogenesis is thought to be VEGFR-2, although VEGFR-1 is also expressed. To determine whether VEGFR-2 is necessary for sprouting and vessel development in this system, we used a well-characterized inhibitor that competes with ATP in the active site of VEGFR-2 (Sun et al, 2000). We performed a dose-response experiment using a range of concentrations from 0.001 μM to 1 μM and quantified sprouting and vessel growth. We first observed inhibition of sprouting at 0.01 μM of drug, with inhibition increasing to complete abolition of sprouting at 1 μM (Fig. 5A). Sprouting was normal in control wells treated with carrier. VEGF also binds to VEGFR-1, and we confirmed expression of this receptor in our cultured cells by RT-PCR (data not shown). Although the exact function of VEGFR-1 is still unclear, it has been shown that VEGFR-1 knockout mice die in utero, as a result of increased angioblast proliferation and the eventual disorganization of the developing vasculature (Fong et al, 1995, 1999). To address whether signaling occurred through VEGFR-1 in the fibrin gel bead assay, we treated cultures with 35 ng/ml of P/GF in the presence or absence of 1 μM of VEGFR-2 inhibitor and in the presence or absence of 2 ng/ml of VEGF. P/GF binds to VEGFR-1 but not to VEGFR-2 and should, therefore, in the presence of the VEGFR-2 inhibitor reveal a possible role for VEGFR-1 signaling in this system. Our results revealed, however, that P/GF had little to no effect on sprout formation in the presence or absence of VEGFR-2 inhibitor (Fig. 5B). In the presence of inhibitor, only a few, very rudimentary, sprouts formed in response to P/GF, indicating that P/GF (and thus VEGFR-1) alone cannot support vessel formation. When used in conjunction with VEGF, in the absence of inhibitor, we did not see an increase in either length or shape index of the vessels, suggesting that VEGFR-1 does not contribute to vessel formation in this system. These findings confirm, therefore, the importance of VEGFR-2 in mediating proangiogenic signaling by VEGF in this system and suggest that signaling through VEGFR-1, if it occurs, is not sufficient.

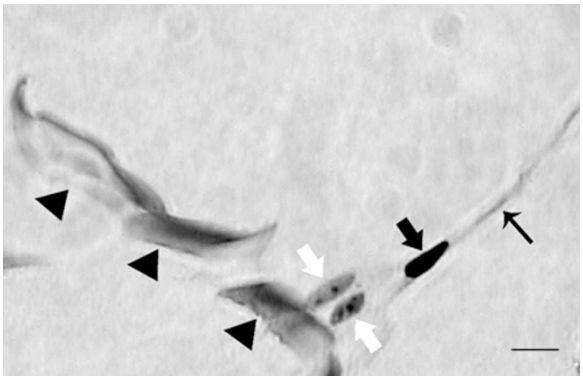

Figure 4.
Proliferation of ECs in sprouts in the presence of SFs. Fibrin bead assays were established in the presence of SFs. Gels were embedded in paraffin and sectioned. Staining with Ki67 antibody, a marker for cells undergoing division, reveals a proliferating cell within a spout (*thick black arrow*). The thin black arrows indicate hematoxylin control staining of the sprout. White arrows indicate nonproliferating cells. The black arrowheads demarcate the surface of the bead. Scale bar represents 20 μm.

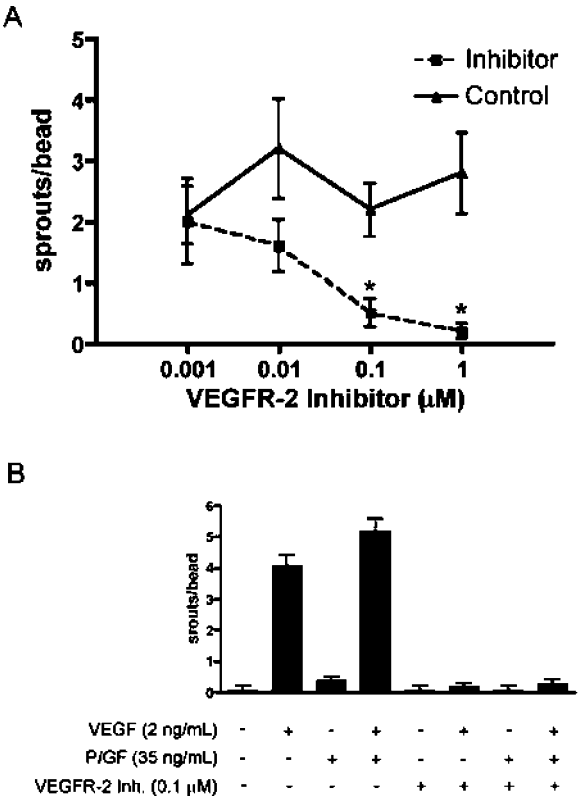

Figure 5.
Sprouting is dependent on VEGFR-2 signaling. A, Fibrin bead assays were established in the presence of SFs and 2.5 ng/ml of VEGF and treated with a VEGFR-2 inhibitor for 7 days at doses ranging from 0.001 μM to 1 μM. Cultures were then photographed, and the number of sprouts per bead was counted for inhibitor versus control. Values represent mean ± SEM. Data represent one experiment. Similar results were obtained in three separate experiments. B, Bead cultures were treated in the presence or absence of 2 ng/ml of VEGF, 35 ng/ml of P/GF, and 0.1 μM of VEGFR-2 inhibitor as indicated. Cultures were photographed after 7 days, and the number of sprouts per bead was counted. Values represent mean ± SEM. Data represent one experiment. Similar results were obtained in two separate experiments.

Inhibitors of MEK1-ERK1/2 Activation Block Increased Vessel Diameter

Signaling through VEGFR-2 promotes both cell survival and cell proliferation (Gerber et al, 1998; Gratton

et al, 2001; Rousseau et al, 2000). Numerous downstream targets of VEGFR-2 are known to be phosphorylated upon activation of the receptor, including phosphatidylinositol 3-kinase (PI3K), MAP and ERK Kinase-1 (MEK-1), p38 mitogen-activated protein kinase (MAPK), phospholipase C-γ (PLC-γ), Ras GTPase-activating protein, and several others (Bernatchez et al, 2001; Gerber et al, 1998; Meadows et al, 2001; Rousseau et al, 2000; Takahashi et al, 1999).

It is possible that the increased diameter of vessels in response to higher concentrations of VEGF represents a graded response of second messenger pathways to increased VEGFR-2 signaling. Alternatively, higher VEGF concentrations may induce qualitative changes in signaling, reflected in the induction of new pathways at higher concentrations. To investigate signaling downstream of VEGFR-2 in sprouting capillaries, we first examined tyrosine phosphorylated protein levels in vessels cultured in low or high concentrations of VEGF. Western blotting revealed eight distinct bands present in both the 2.5 ng/ml and 15 ng/ml lanes, with the majority of bands showing increased intensity at 15 ng/ml compared with 2.5 ng/ml (Fig. 6A, *thick arrows*). We observed only one band, at 120 kD, that decreased in intensity from 2.5 ng/ml to 15 ng/ml (*thin arrow*) and one other band, at 86 kD, that showed no change in phosphorylation (*arrowhead*). More important, there did not seem to be a qualitative difference in the pattern of tyrosine phosphorylated proteins, suggesting that previously activated pathways are increasing their throughput, rather than there being induction of new, previously quiescent signaling modules.

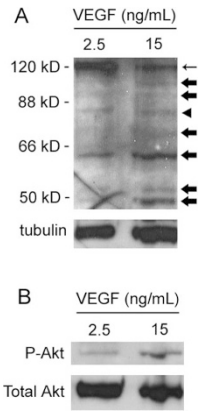

Figure 6.

PI3K/Akt and other tyrosine kinase pathways are activated through VEGFR-2 during vessel growth. Fibrin bead assays were established in the presence of SFs and low (2.5 ng/ml) or high (15 ng/ml) concentrations of VEGF for 7 days. Cell extracts were isolated as described in "Materials and Methods" and immunoblotted with phospho-Akt and phospho-tyrosine antibodies. The membranes were stripped and reprobed with total Akt or α-tubulin antibodies to demonstrate equal loading. A, Phospho-tyrosine blot. Thick black arrows at 105 kD, 95 kD, 73 kD, 62 kD, 51 kD, and 46 kD represent an increase in phosphorylation from 2.5 ng/ml to 15 ng/ml of VEGF. The thin arrow at 120 kD represents a decrease in phosphorylation from 2.5 ng/ml to 15 ng/ml of VEGF. The arrowhead at 85 kD represents no change in phosphorylation from 2.5 ng/ml to 15 ng/ml of VEGF. B, Phospho-Akt blot. One of three similar experiments.

PI3K and its immediate target, Akt, are well-characterized downstream effectors of VEGFR-2 signaling (Gerber et al, 1998; Rousseau et al, 2000) and have been reported to mediate enhanced EC survival, a possible explanation for enhanced EC numbers at higher levels of VEGF, so we looked for Akt activation in narrow tubes—cultured in 2.5 ng/ml of VEGF—and in wider tubes—cultured in 15 ng/ml of VEGF. As seen in Figure 6B, phospho-Akt is detectable at low concentrations of VEGF, and its level is increased at higher concentrations, as expected. Thus, activation of PI3K and phosphorylation of Akt correlate with increased VEGF concentration and increased vessel diameter.

To determine whether the PI3K pathway is necessary for sprouting and control of vessel diameter, we used a specific inhibitor of this enzyme, LY294002. We also tested specific inhibitors of the MEK-1/MAPK kinase (PD98059) and p38 MAPK (PD169316) pathways. Cultures were grown for 7 days, at which time inhibitors were added at the indicated concentrations. Twenty-four hours later, the number of sprouts per bead was counted and the morphologic status of the cells was assessed. Inhibitors of PI3K, MEK-1, and p38 MAPK all significantly reduced the number of sprouts per bead (Fig. 7). The p38 MAPK and PI3K inhibitors had the most dramatic effect, reducing sprout number by $>$60% at 30 μM. Remarkably, however, the effects of the inhibitors on cell and sprout morphology were different. When PI3K was blocked, sprouts disintegrated and ECs showed fragmented nuclei and seemed to be undergoing apoptosis (Fig. 7, B and C), consistent with the reported role of PI3K in cell survival and protection from apoptosis (Gerber et al, 1998; Minshall et al, 1996; Yao and Cooper, 1995). The phenotype of cells that were treated with the p38 inhibitor was essentially similar (Fig. 7, F and G). In sharp contrast, inhibition of the MEK-ERK1/2 pathway induced regression of shorter sprouts but no apparent signs of cell death or nuclear fragmentation/apoptosis (Fig. 7, D and E). To determine whether this pathway affected vessel diameter, we calculated the average shape index as before, for cultures grown in low or high VEGF, in the presence or absence of the MEK-1 inhibitor PD98059. As shown in Figure 8, PD98059 significantly blocked the increased vessel diameter promoted by higher VEGF concentrations—in this experiment by 65%.

These data are consistent with previous reports showing a role for the MEK-ERK1/2 pathway downstream of VEGF in regulating cell proliferation but not necessarily survival (Meadows et al, 2001; Takahashi et al, 1999). Our data suggest, therefore, that whereas cell survival seems to be dependent on PI3K and p38, vessel diameter regulation by VEGF is more directly dependent on cell proliferation, likely through a pathway involving MEK-ERK1/2 activation.

Discussion

The mechanism of vessel diameter determination is not well understood but is likely to involve interactions

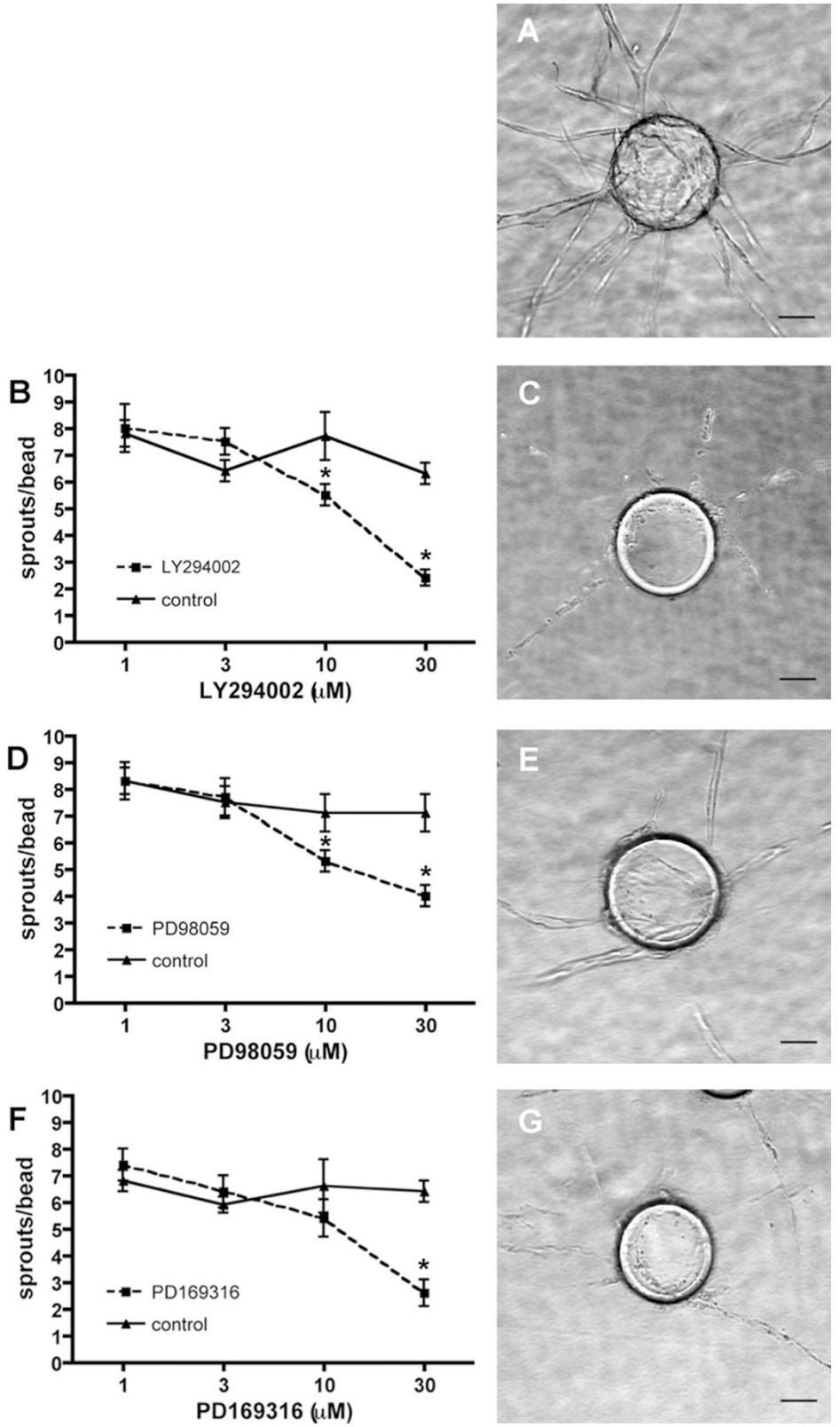

Figure 7.

Sprouting is reduced through inhibition of the PI3K, MEK-ERK1/2, and p38 MAPK pathways. Fibrin bead assays were established in the presence of SFs. Various inhibitors were added to the cultures at Day 6 as indicated. Beads were photographed the following day and quantified as described. A, Control culture. B, Sprouts per bead for cultures treated with the PI3K inhibitor LY294002 versus control. C, HUVEC-coated bead in the presence of SFs and 30 μM of LY294002. D, Sprouts per bead for cultures treated with the MEK-1 inhibitor PD98059 versus control. E, HUVEC-coated bead in the presence of SFs and 30 μM of PD98059. F, Sprouts per bead for cultures treated with the p38 inhibitor PD169316 versus control. G, HUVEC-coated bead in the presence of SFs and 30 μM of PD169316. Data represent one experiment. Similar results were obtained in three separate experiments. Values represent mean $\pm$ SEM, $^*p < 0.05$. Scale bar represents 50 μm.

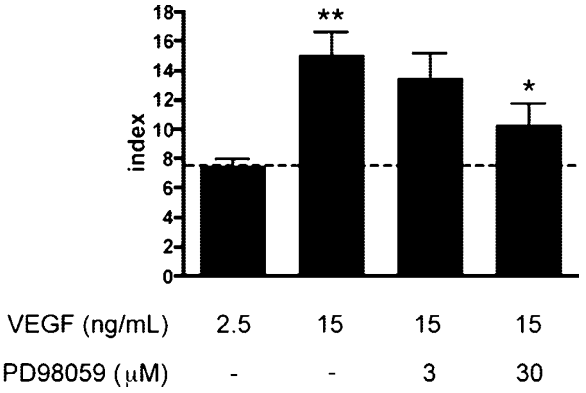

Figure 8.

Inhibition of the MEK-ERK1/2 pathway blocks increased vessel diameter. Fibrin bead assays were established at low (2.5 ng/ml) and high (15 ng/ml) concentrations of $VEGF_{165}$ in the presence of SFs. Various concentrations of the MEK-1 inhibitor PD98059 were added to the cultures at Day 6. Beads were photographed and quantified the following day. Dashed line indicates the baseline index at 2.5 ng/ml of VEGF. Data represent one experiment. Similar results were obtained in two separate experiments. Values represent mean ± SEM; $^{**}p < 0.05$ for 2.5 vs 15 ng/ml of VEGF in the absence of inhibitor; $^{*}p < 0.05$ for 15 ng/ml of VEGF in the presence of 0 μM vs 30 μM of PD98059.

among soluble factors, hemodynamics, and genetic programming. We have shown here that at a basic level, vessel diameter can be controlled simply by varying the concentration of VEGF available to the growing vessel. Previous studies in whole animals have reported that overexpression of VEGF results in vessels with dilated or enlarged lumens (Drake and Little, 1999; Larcher et al, 1998). Drake and Little also reported "hyperfusion," whereby numerous capillaries were seen to fuse into larger vessels. In these studies, it was not possible to determine a precise mechanism for vessel enlargement. In the embryo, possibilities include effects of VEGF on recruitment of angioblasts, proliferation of precommitted ECs, or the induction of morphologic abnormalities such as vascular fusion. Similarly, in the adult, VEGF may dilate vessels as a result of EC thinning and hemodynamic forces, or it may increase EC proliferation. By reducing the process of vessel sprouting to a simplified in vitro model, we have now generated evidence that vessel diameter can be programmed by VEGF acting at the level of EC proliferation.

How might VEGF regulate vessel diameter in vivo? It is intriguing that mice show haploinsufficiency for VEGF (Carmeliet et al, 1996; Ferrara et al, 1996), which suggests that the local concentration of this factor needs to be finely tuned for vascular development to occur normally. Our findings that small changes in VEGF concentration have a dramatic effect on vessel morphology and diameter in vitro suggest one potential explanation for haploinsufficiency. Moreover, our data suggest that vessel diameter in vivo may be regulated, as it is in vitro, at least in part by the local concentration of VEGF. The level of VEGF is set, in turn, by local hypoxia, and we have modeled this in our system. The SFs when exposed to hypoxic conditions express VEGF (data not shown), likely by mobilizing HIF-1α, which binds to the hypoxia response element in the VEGF promoter (Pugh and Ratcliffe, 2003). Thus, chronic local hypoxia will induce VEGF expression in tissues, leading to local EC proliferation, an increase in vessel diameter, and a subsequent increase in blood flow and O_2 delivery.

In addition to the mechanism described above, there may well be other, overlapping mechanisms, depending on location and time of development. Formation of the great vessels at the midline of developing embryos seems to involve recruitment of newly formed ECs from the surrounding tissue in response to VEGF (Cleaver and Krieg, 1998). A focusing of ECs at the midline along with enhanced EC proliferation in response to local VEGF secretion might be sufficient to stimulate assembly of a large-diameter vessel. During vascular remodeling in the embryo and in the adult, it is likely that in addition to local secretion of VEGF, local blood flow plays a major role in programming vessel diameter, as well as triggering recruitment of smooth muscle cells.

The simple model that we have described for setting of vessel diameter raises another question: why in this in vitro system does an increase in EC number result in increased diameter, rather than an increase in the length of the vessel? We suspect that it is the result of local mechanical forces. After cell division, the ECs remain closely apposed and exert force on adjacent cells as they reestablish strong contacts with the underlying matrix and begin to flatten. Simply put, there are fewer cells to move out of the way in a circumferential direction than there are in a longitudinal direction, and thus the vessel expands in diameter.

Recent studies suggest that ECs respond to proangiogenic signals in subtly different ways. For example, different integrins protect ECs from proapoptotic signals, generated either through intrinsic or extrinsic pathways or through induction of different classes of apoptosis inhibitors (Stupack and Cheresh, 2002). Similar differences are likely to be seen with different growth factors. Our results and those of others suggest that although monolayer cultures of ECs respond to both bFGF and VEGF by proliferating, VEGF provides additional information, as varying the concentration of bFGF had no effect on vessel diameter in our system.

In this context, it is interesting to consider the roles of the different VEGF isoforms. The higher molecular weight forms ($VEGF_{165}$ and $VEGF_{189}$), which have heparin-binding domains, are less freely diffusible than $VEGF_{121}$, which lacks these domains. As such, they are likely to have more localized effects and may mediate localized enlargement of vessels. In our assay, we did not see a difference in the effects of $VEGF_{121}$ and $VEGF_{165}$, indicating that both molecules carry all of the necessary information for programming vessel diameter. Our results also suggest that neuropilin (NP) is not essential for determination of vessel diameter, as $VEGF_{121}$ does not bind this co-receptor. Although NP does not signal directly, as it lacks a tyrosine kinase domain, the NP-1 and NP-2 knockout mice indicate their importance in regulating vascular development (Kawasaki et al, 1999; Takashima et al,

2002). It is likely, therefore, that the NPs alter the dose-response curve to VEGF in vivo, without qualitatively affecting the nature of the VEGF signal (Takashima et al, 2002).

VEGFR-2 is a critical VEGF receptor, both during development and during adult angiogenesis. It also proved to be critical in our in vitro assays. Addition of a VEGFR-2 inhibitor to the fibrin bead assay completely abrogated sprouting and vessel formation. Treatment of cultures with P*l*GF, a VEGF family member that binds to VEGFR-1 but not to VEGFR-2, in combination with the VEGFR-2 inhibitor, induced few if any sprouts, confirming that although VEGFR-1 can mediate cell migration in some assays, it is not sufficient to support even rudimentary sprouting of ECs in fibrin gels. A similar study of EC sprouting in fibrin matrices confirmed our findings: specific blocking antibodies to VEGFR-2, in the presence of $VEGF_{165}$/TNF-α, inhibited vessel formation, whereas blocking antibodies to VEGFR-1, in the presence of $VEGF_{165}$/TNF-α, had no effect (Koolwijk et al, 2001). Previous reports have suggested that VEGFR-1–mediated signaling is relatively weak and that VEGFR-1 may act as a "sink" for VEGF, thereby trapping available VEGF and limiting the interaction between VEGF and VEGFR-2 (Ferrara et al, 2003; Park et al, 1994). Our results are consistent with this interpretation.

Although several signaling pathways downstream of VEGFR-2 have been identified, there is still debate over which are important for each of the distinct processes modulated by VEGF, such as proliferation, migration, and induction of gene expression. Although part of the confusion may lie in the use of ECs from different species and different vascular beds, a consensus is emerging that the MEK-ERK1/2 pathway is involved in proliferation but less so in migration, whereas the PI3K–Akt and p38 pathways are more important for cell migration and survival.

Several reports have demonstrated a critical role for ERK downstream of MEK and VEGFR-2 in mediating DNA synthesis and cell proliferation (Kanno et al, 2000; Meadows et al, 2001; Takahashi et al, 1999); however, PD98059, a specific inhibitor of MEK-1, has consistently failed to block EC migration in response to VEGF. In our system, PD98059 treatment led to a decrease in the shape index of the capillary sprouts (Fig. 8), indicating that the sprouts were thinner than in controls, longer, or both. Further analysis of the data (not shown) indicates that once drug treatment begins, treated vessels continue to extend (migrate) but do not increase in diameter (and may even become slightly thinner). Conversely, control vessels extend to a similar length but also become wider. In aggregate, our data suggest that this is due to cell proliferation.

PI3K has been implicated in several studies as being critical for EC survival; thus, it was not surprising that the PI3K inhibitor LY294002 led to regression of sprouts with widespread cell death—likely by apoptosis. It has been suggested that PI3K exerts its protective effects in HUVECs downstream of VEGF by Akt-mediated inactivation of p38 (Gratton et al, 2001). Furthermore, a recent report suggested that inhibiting p38 through the use of a different inhibitor (SB203580) actually enhances tube formation and promotes cell proliferation in an in vitro angiogenesis assay (Matsumoto et al, 2002). It was surprising, therefore, that the p38 inhibitor PD169316 led to cell death, rather than protection from apoptosis. We currently have no explanation for this finding; however, we do note that most previous studies on p38 signaling in ECs have been performed in monolayer cultures, whereas ECs in this study are embedded in 3-D fibrin gels, which may result in previously unidentified cross-talk between pathways that could confound more simple interpretations. Supporting this hypothesis is a report that VEGFR-2 interacts with the integrin $\alpha_{v\beta3}$ to transduce signals to FAK and p38 that regulate cell movement (Masson-Gadais et al, 2003) and a second report suggesting that $\alpha2\beta1$ integrins may signal through p38 to modulate focal adhesions (Sweeney et al, 2003). ECs in 3-D gels will likely be triggering many integrins through matrix interactions, and loss of integrin-mediated focal adhesions in the absence of active p38 may trigger cell apoptosis (anoikis). In addition to its role in regulating EC survival, p38 MAPK can function in mural cell recruitment. Inhibition of p38 activity with SB203580 blocked mural cell recruitment and its subsequent interaction with ECs, although tube formation was not affected (Zhu et al, 2003). However, we have shown that the mural cells in our fibrin gel system are not recruited to the developing vessels; neither do they even make visible contacts with the ECs. Cross-talk between ECs and SFs thus is through secreted, diffusible factors. The differences between our data and those of others are likely due to differences in the type of gels used (fibrin versus collagen), the geometry of the cultures (2-D versus 3-D), and the presence or absence of closely apposed mural cells.

The maturation of blood vessels correlates with the down-regulation of VEGFR-2 expression on ECs in vivo (Kremer et al, 1997), and we have recently shown that Notch signaling through the basic helix-loop-helix transcription factor HESR-1 inhibits activity of the VEGFR-2 promoter and reduces responsiveness of ECs to VEGF (Henderson et al, 2001; Taylor et al, 2002). Notch activity correlates with a mature, quiescent vascular phenotype, and constitutive activation of the pathway in ECs prevents migration and proliferation of cells (Taylor et al, 2002). These data suggest that the onset of Notch signaling in maturing vessels may down-regulate VEGFR-2 and therefore prevent further EC proliferation and increases in vessel diameter.

The fibrin gel model that we have recently developed (Nakatsu et al, 2003) and that we use here expands and improves on a number of previously published techniques. In our updated method, the unique combination of ECs growing on beads; a fibrin, rather than collagen gel; and paracrine factors provided by SFs yields a reproducible assay that recapitulates many of the early stages of angiogenesis, including sprouting, elongation, proliferation, lumen formation, branching, and anastomosis. We have used this improved in vitro angiogenesis assay to

show that VEGF, likely by driving EC proliferation through a MEK-ERK1/2–dependent pathway, can contribute to setting the diameter of newly forming vessels.

Materials and Methods

Cell Culture

HUVECs were isolated from umbilical cords. Cords were obtained through local hospitals under Institutional Review Board approval. HUVECs were routinely grown in M199 (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; GIBCO, Grand Island, New York) and endothelial cell growth supplement (BD Biosciences, Bedford, Massachusetts) at 37° C and 5% CO_2. HUVECs between P3 and P4 were used for all experiments. SFs (Detroit 551) were purchased from the American Type Culture Collection (Rockville, Maryland). SFs were routinely grown in M199 supplemented with 10% FBS at 37° C and 5% CO_2. SFs between P15 and P25 were used for all experiments.

Fibrin Bead Assay

HUVECs were mixed with Cytodex 3 microcarriers (Amersham Pharmacia Biotech, Piscataway, New Jersey) at a concentration of 400 HUVECs per bead in 1 ml of EGM-2 (Clonetics, Walkersville, Maryland). Beads with cells were shaken gently every 20 minutes for 4 hours at 37° C and 5% CO_2. After incubating, beads with cells were transferred to a 25-cm^2 tissue culture flask (Falcon, Bedford, Massachusetts) and left for 12 to 16 hours in 5 ml of EGM-2 at 37° C and 5% CO_2. The following day, beads with cells were washed three times with 1 ml of EGM-2 and resuspended at a concentration of 200 cell-coated beads/ml in 2.5 mg/ml of fibrinogen (Sigma, St. Louis, Missouri) with 0.15 units/ml of aprotinin (Sigma). A total of 500 μl of fibrinogen/bead solution was added to 0.625 units of thrombin (Sigma) in one well of a 24-well tissue culture plate. Fibrinogen/bead solution was allowed to clot for 5 minutes at room temperature and then at 37° C and 5% CO_2 for 20 minutes. One milliliter of EGM-2 with 0.15 units/ml of aprotinin was added to one well and equilibrated with the fibrin clot for 30 minutes at 37° C and 5% CO_2. EGM-2 was removed from the well and replaced with fresh 1 ml of EGM-2 with 0.15 units/ml of aprotinin and different treatments. SFs were layered on top of the clot at a concentration of 20,000 cells/well. Medium was changed every other day. $VEGF_{165}$, $VEGF_{121}$, and P*l*GF (R&D Systems, Minneapolis, Minnesota) were used at the indicated concentrations. Inhibitors were as follows: the VEGFR-2 inhibitor (Calbiochem, San Diego, California) was (*Z*)-5-Bromo-3-(4,5,6,7-tetrahydro-1*H*-indol-2-ylmethylene)-1,3-dihydroindol-2-one. It is a membrane permeant indolin-2-1 class of receptor tyrosine kinase inhibitor [IC_{50} = 70 nM for VEGFR-2, 920 nM for PDGFR b, 4.92 mM for p60 c-src, and 13.3 mM for FGFR-1. It does not inhibit EGF-R kinase activity (IC_{50} > 100 mM)]. MEK-1 inhibitor (PD98059), PI3K inhibitor (LY294002), and p38 inhibitor (PD169316) all were from Calbiochem and were used at the indicated concentrations. All were made up in DMSO, which was used as the vehicle control in all experiments.

Quantification of Vessels In Vitro

High-resolution images of beads were captured on an IX70 Olympus microscope with a 4× objective. The advantage of taking images at a lower magnification is the depth of field that enables us to include all sprouts in focus. Images are then magnified in Adobe Photoshop (Adobe Systems, San Jose, California) and analyzed in NIH ImageJ. The number of sprouts per bead was counted. Only sprouts with a length of one bead diameter were included. Sprout length and shape index (area/length) were also measured in arbitrary units. All experiments were replicated at least three times with similar results.

Histology and Immunostaining

For EC nuclei staining, fibrin gel clots were washed twice with 1× PBS and then fixed overnight in 2% paraformaldehyde. Fibrin gel clots were washed twice again with 1× PBS and then stained with 4', 6-diamidino-2-phenylindole (Sigma) or TRITC-phalloidin (a gift from Dr. R. Warrior, UCI). Fluorescence was monitored using an IX70 Olympus microscope with an IX-FLA fluorescence attachment. To calculate the internuclear distance, we determined the ratio of nuclei to area of the sprout and then calculated the square root of the ratio, yielding the internuclear distance. This calculation corrects for the viewing of a 3-D object in two dimensions. For immunoperoxidase methods, a fibrin bead assay was performed as above with the exception that fibrin clots were placed in transwells with fibroblasts seeded into the bottom well. Membranes and clots were removed, fixed in 10% neutral-buffered formalin, and paraffin embedded. Six-micrometer-thick sections were cut and then stained with Ki67 mAb (DAKO, Carpinteria, California) and counterstained with hematoxylin (DAKO).

Western Blot Analysis

Fibrin gels cultured in low or high concentrations (2.5 ng/ml and 15 ng/ml) of VEGF were treated with 10× Trypsin-EDTA (GIBCO) for 2 to 3 minutes to remove the top layer of gel and the SFs. An additional 1 ml of 10×Trypsin-EDTA was added to the fibrin gel to release the HUVEC-coated beads. HUVEC-coated beads were then washed with M199 supplemented with 40% FBS and centrifuged to disrupt the beads and release the HUVECs. Cells were washed twice with 1× PBS and then lysed in 100 μL of Triton X-100 lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) w/phosphatase and protease inhibitor cocktails (1:100; Sigma) for 15 minutes. Lysed cells were centrifuged for 15 minutes at 4° C, and total cell lysates were resuspended in sample buffer. Samples were boiled for 5 minutes, and 10 μg of protein per lane was loaded onto a 10% polyacryl-

amide gel. Proteins were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, Bedford, Massachusetts). Membranes were blocked with 5% milk/TBST for 1 hour and incubated overnight at 4° C with Phospho-Akt (Thr308) rabbit polyclonal (Cell Signaling, Beverly, Massachusetts), phosphotyrosine mouse monoclonal (Cell Signaling), total Akt rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, California), or α-tubulin mouse monoclonal (Sigma) antibodies in 5% BSA/TBST. Membranes were washed in TBST and then incubated with goat anti-rabbit or anti-mouse IgG-HRP secondary antibody (Bio-Rad, Hercules, California) for 1 hour at room temperature. Bands were visualized with an ECL Western Detection Kit (Amersham Biosciences, Piscataway, New Jersey).

Acknowledgements

We thank Dr. David Fruman for providing the phospho-Akt and total Akt antibodies and Ms. Erin Conn for expert technical assistance.

References

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, and Klein R (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: Demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev 13:295–306.

Bernatchez PN, Allen BG, Gelinas DS, Guillemette G, and Sirois MG (2001). Regulation of VEGF-induced endothelial cell PAF synthesis: Role of p42/44 MAPK, p38 MAPK and PI3K pathways. Br J Pharmacol 134:1253–1262.

Carmeliet P (2000). Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389–395.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, and Nagy A (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439.

Carmeliet P, Ng YS, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, Ehler E, Kakkar VV, Stalmans I, Mattot V, Perriard JC, Dewerchin M, Flameng W, Nagy A, Lupu F, Moons L, Collen D, D'Amore PA, and Shima DT (1999). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med 5:495–502.

Cheng N, Brantley DM, and Chen J (2002). The ephrins and Eph receptors in angiogenesis. Cytokine Growth Factor Rev 13:75–85.

Cleaver O and Krieg PA (1998). VEGF mediates angioblast migration during development of the dorsal aorta in Xenopus. Development 125:3905–3914.

Conway EM, Collen D, and Carmeliet P (2001). Molecular mechanisms of blood vessel growth. Cardiovasc Res 49: 507–521.

Detmar M, Brown LF, Schon MP, Elicker BM, Velasco P, Richard L, Fukumura D, Monsky W, Claffey KP, and Jain RK (1998). Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. J Invest Dermatol 111:1–6.

Drake CJ and Little CD (1999). VEGF and vascular fusion: Implications for normal and pathological vessels. J Histochem Cytochem 47:1351–1355.

Dvorak HF, Brown LF, Detmar M, and Dvorak AM (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 146:1029–1039.

Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Sotrel A, Manseau EJ, Van de Water L, and Senger DR (1991). Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: Concentration in tumor blood vessels. J Exp Med 174:1275–1278.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, and Moore MW (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442.

Ferrara N, Gerber HP, and LeCouter J (2003). The biology of VEGF and its receptors. Nat Med 9:669–676.

Folkman J (1975). Tumor angiogenesis: A possible control point in tumor growth. Ann Intern Med 82:96–100.

Folkman J (1985). Tumor angiogenesis. Adv Cancer Res 43:175–203.

Fong GH, Rossant J, Gertsenstein M, and Breitman ML (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–70.

Fong GH, Zhang L, Bryce DM, and Peng J (1999). Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. Development 126:3015–3025.

Friesel RE and Maciag T (1995). Molecular mechanisms of angiogenesis: Fibroblast growth factor signal transduction. FASEB J 9:919–925.

Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiara TM, McDonald DM, and Yancopoulos GD (2001). Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol 230:151–160.

Gale NW and Yancopoulos GD (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev 13:1055–1066.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, and Ferrara N (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336–30343.

Gratton JP, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K, and Sessa WC (2001). Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. J Biol Chem 276:30359–30365.

Greenwood J (1991). Astrocytes, cerebral endothelium, and cell culture. The pursuit of an in vitro blood-brain barrier. Ann N Y Acad Sci 633:426–431.

Gridley T (1997). Notch signaling in vertebrate development and disease. Mol Cell Neurosci 9:103–108.

Henderson AM, Wang SJ, Taylor AC, Aitkenhead M, and Hughes CC (2001). The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation. J Biol Chem 276:6169–6176.

Hurwitz AA, Berman JW, Rashbaum WK, and Lyman WD (1993). Human fetal astrocytes induce the expression of blood-brain barrier specific proteins by autologous endothelial cells. Brain Res 625:238–243.

Kanno S, Oda N, Abe M, Terai Y, Ito M, Shitara K, Tabayashi K, Shibuya M, and Sato Y (2000). Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. Oncogene 19: 2138–2146.

Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, and Fujisawa H (1999). A requirement for neuropilin-1 in embryonic vessel formation. Development 126:4895–4902.

Koolwijk P, Peters E, van der Vecht B, Hornig C, Weich HA, Alitalo K, Hicklin DJ, Wu Y, Witte L, and van Hinsbergh VW (2001). Involvement of VEGFR-2 (kdr/flk-1) but not VEGFR-1 (flt-1) in VEGF-A and VEGF-C-induced tube formation by human microvascular endothelial cells in fibrin matrices in vitro. Angiogenesis 4:53–60.

Kremer C, Breier G, Risau W, and Plate KH (1997). Up-regulation of flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system. Cancer Res 57:3852–3859.

Larcher F, Murillas R, Bolontrade M, Conti CJ, and Jorcano JL (1998). VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and accelerated tumor development. Oncogene 17:303–311.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, and Yancopoulos GD (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60.

Masson-Gadais B, Houle F, Laferriere J, and Huot J (2003). Integrin $\alpha v\beta 3$, requirement for VEGFR2-mediated activation of SAPK2/p38 and for Hsp90-dependent phosphorylation of focal adhesion kinase in endothelial cells activated by VEGF. Cell Stress Chaperones 8:37–52.

Matsumoto T, Turesson I, Book M, Gerwins P, and Claesson-Welsh L (2002). p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. J Cell Biol 156:149–160.

Meadows KN, Bryant P, and Pumiglia K (2001). Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. J Biol Chem 276:49289–49298.

Minshall C, Arkins S, Freund GG, and Kelley KW (1996). Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. J Immunol 156:939–947.

Montesano R, Pepper MS, and Orci L (1993). Paracrine induction of angiogenesis in vitro by Swiss 3T3 fibroblasts. J Cell Sci 105:1013–1024.

Nakatsu MN, Sainson RCA, Aoto JN, Taylor KL, Aitkenhead M, Pérez-del-Pulgar S, Carpenter PM, and Hughes CCW (2003). Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: The role of fibroblasts and angiopoietin-1. Microvasc Res 66:102–112.

Nehls V and Drenckhahn D (1995a). A microcarrier-based cocultivation system for the investigation of factors and cells involved in angiogenesis in three-dimensional fibrin matrices in vitro. Histochem Cell Biol 104:459–466.

Nehls V and Drenckhahn D (1995b). A novel, microcarrier-based in vitro assay for rapid and reliable quantification of three-dimensional cell migration and angiogenesis. Microvasc Res 50:311–322.

Nehls V, Schuchardt E, and Drenckhahn D (1994). The effect of fibroblasts, vascular smooth muscle cells, and pericytes on sprout formation of endothelial cells in a fibrin gel angiogenesis system. Microvasc Res 48:349–363.

Park JE, Chen HH, Winer J, Houck KA, and Ferrara N (1994). Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 269: 25646–25654.

Pugh CW, and Ratcliffe PJ (2003). Regulation of angiogenesis by hypoxia: Role of the HIF system. Nat Med 9:677–684.

Risau W (1997). Mechanisms of angiogenesis. Nature 386: 671–674.

Rousseau S, Houle F, and Huot J (2000). Integrating the VEGF signals leading to actin-based motility in vascular endothelial cells. Trends Cardiovasc Med 10:321–327.

Shima DT, Kuroki M, Deutsch U, Ng YS, Adamis AP, and D'Amore PA (1996). The mouse gene for vascular endothelial growth factor. Genomic structure, definition of the transcriptional unit, and characterization of transcriptional and post-transcriptional regulatory sequences. J Biol Chem 271:3877–3883.

Stalmans I, Ng YS, Rohan R, Fruttiger M, Bouche A, Yuce A, Fujisawa H, Hermans B, Shani M, Jansen S, Hicklin D, Anderson DJ, Gardiner T, Hammes HP, Moons L, Dewerchin M, Collen D, Carmeliet P, and D'Amore PA (2002). Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest 109:327–336.

Stupack DG and Cheresh DA (2002). Get a ligand, get a life: Integrins, signaling and cell survival. J Cell Sci 115:3729–3738.

Sun L, Tran N, Liang C, Hubbard S, Tang F, Lipson K, Schreck R, Zhou Y, McMahon G, and Tang C (2000). Identification of substituted 3-[(4, 5, 6, 7-tetrahydro-1H-indol-2-yl)methylene]-1, 3-dihydroindol-2-ones as growth factor receptor inhibitors for VEGF-R2 (Flk-1/KDR), FGF-R1, and PDGF-Rβ tyrosine kinases. J Med Chem 43:2655–2663.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, and Yancopoulos GD (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 87:1171–1180.

Sweeney SM, DiLullo G, Slater SJ, Martinez J, Iozzo RV, Lauer-Fields JL, Fields GB, and San Antonio JD (2003). Angiogenesis in collagen I requires $\alpha 2\beta 1$ ligation of a GFP*GER sequence and possibly p38 MAPK activation and focal adhesion disassembly. J Biol Chem 278:30516–30524.

Takahashi T, Ueno H, and Shibuya M (1999). VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 18:2221–2230.

Takashima S, Kitakaze M, Asakura M, Asanuma H, Sanada S, Tashiro F, Niwa H, Miyazaki Ji J, Hirota S, Kitamura Y, Kitsukawa T, Fujisawa H, Klagsbrun M, and Hori M (2002).

Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. Proc Natl Acad Sci U S A 99:3657–3662.

Taylor KL, Henderson AM, and Hughes CC (2002). Notch activation during endothelial cell network formation in vitro targets the basic HLH transcription factor HESR-1 and downregulates VEGFR-2/KDR expression. Microvasc Res 64:372–383.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, and Abraham JA (1991). The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947–11954.

Villaschi S and Nicosia RF (1994). Paracrine interactions between fibroblasts and endothelial cells in a serum-free coculture model. Modulation of angiogenesis and collagen gel contraction. Lab Invest 71:291–299.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, and Holash J (2000). Vascular-specific growth factors and blood vessel formation. Nature 407:242–248.

Yao R and Cooper GM (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003–2006.

Zhu WH, Han J, and Nicosia RF (2003). Requisite role of p38 MAPK in mural cell recruitment during angiogenesis in the rat aorta model. J Vasc Res 40:140–148.