Vascular Endothelial Growth Factor Induction of the Angiogenic Phenotype Requires Ras Activation*

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We investigated the role of Ras in vascular endothelial growth factor (VEGF)-mediated signal transduction and the promotion of angiogenic changes primary endothelial cells. We find that VEGF potently induces Ras activation and that this step is essential for the stimulation by VEGF of several cellular changes associated with angiogenesis, including proliferation, migration, and branching morphogenesis in three-dimensional culture. Inhibition of Ras signaling induced subtle changes in the actin architecture but had no effect on the phosphatidylinositol 3-kinase (PI3K) or p38 signaling pathways. In contrast, activation of ERK was largely dependent on Ras. Although inhibiting ERK activity completely suppressed cell proliferation and partially blocked in vitro differentiation, neither ERK nor PI3K activity was required for VEGF-induced migration. These data provide the first direct demonstration that inhibition of Ras signal transduction is anti-angiogenic. Interestingly, VEGF signal transduction bifurcates both upstream and downstream of Ras, with different Ras-dependent signals controlling endothelial cell proliferation and migration, essential components of the angiogenic response.

Angiogenesis, the sprouting of new capillaries from existing endothelial cells, is essential for vascular development, wound healing, and normal reproduction. In addition, angiogenesis is a critical component of several human diseases, including cancer, diabetic microvascular disease, rheumatoid arthritis, and psoriasis (1, 2). Although a number of soluble factors have been described as angiogenic, e.g. platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor, most of these factors and their cognate receptors have a wide tissue distribution and modulate many cell types (3). In contrast, the receptors for VEGF (VEGFR-1, VEGFR-2) are largely restricted to endothelial cells (4, 5). This has given rise to the idea that VEGF may be a critical component of the angiogenic response (1, 2). This notion is supported by several lines of investigation: 1) VEGF is essential for vascular development as evidenced by mice deficient in VEGF, which die at 8.5–9 days gestation with abnormalities in both vasculosgenisis and angiogenesis (6, 7); 2) mice lacking either VEGFR-1 (8) or VEGFR-2 (9) also demonstrate abnormal vascular development and early gestational lethality (days 8.5–9); 3) striking co-localization of VEGF and VEGFRs can be demonstrated by in situ hybridization, at areas of vascular development in the developing embryo (4, 5) and regions of neovascularization in the adult (10); and 4) VEGF expression is also markedly induced by decreases in oxygen tension (11), thereby providing a tissue with the means to compensate for hypoxic stress by stimulating new vessel growth. Furthermore, inhibitors of VEGF’s action markedly blunt angiogenesis (12, 13). Despite the biological importance of VEGF, the molecular signaling mechanisms responsible for its effects remain largely unclarified.

There are two high affinity VEGF receptors, VEGFR-1 (Flt-1) (14) and VEGFR-2 (Flk-1/KDR) (15). These receptors are 45% identical to each other and most homologous to the PDGF-receptor family. The VEGF receptors have intrinsic tyrosine kinase activity, and ligand binding to these receptors induces dimerization and phosphorylation of the receptors (16), ultimately resulting in the recruitment of Src homology (SH)-2 domain containing proteins. Several SH2-domain proteins are reported to be tyrosine-phosphorylated in response to VEGF. These include p120GAP (GTPase Activating Protein) (17), phosphatidylinositol 3’-kinase (PI3K), phospholipase C, and the SH-2/SH-3 adapter protein Nck (18). In addition, activation of mitogen-activated protein kinase (MAPK) or extracellular regulated kinase (ERK) has also been described (19–21). The activation of ERK and PI3K, reorganization of actin, and the tyrosine phosphorylation of p120GAP are cellular changes often associated with the activation of Ras signal transduction. These changes occur in response to a number of mitogenic growth factors (22). Although the suggestion has recently been made that inhibition of Ras might be an effective anti-angiogenic therapy (23, 24), a direct role for Ras in VEGF-mediated signal transduction remains ambiguous (20, 21).

The role of Ras in regulating the angiogenic phenotype in...
duced by VEGF is unknown. However, several lines of evidence suggest that Ras signal transduction may be critical in regulating endothelial cell function. Notably, the VEGF receptors are of the same family as the PDGF, colony-stimulating factor, and stem cell factor receptors, which all require Ras to transmit mitogenic signals, as do many growth factors (22). Furthermore, addition of an activated Ras allele to an SV-40 large T-antigen immortalized endothelial line resulted in an angiogenic switch and the formation of endothelialiomas (25). In another study, microinjection of an activated Ras protein resulted in altered endothelial cell motility, and microinjection of blocking antibodies to the Ras protein blocked endothelial cell migration in response to bFGF (26). In genetic experiments, mice that are deficient in p120GAP (a protein that negatively regulates Ras signaling) showed abnormal endothelial cell organization and failed to form a vascular network (27). In addition, mice deficient in B-Raf, a downstream kinase normally activated by Ras GTPases, showed marked endothelial cell apoptosis (28). At this point, it is unclear whether any of the effects observed in mice are the direct result of disruption of endothelial pathways, perturbations to the surrounding stroma, or a combination of both. When taken collectively, however, these experiments suggest that Ras has the potential to modulate mitogenesis, endothelial cell motility, cell survival, as well as the organization and differentiation into a vessel. These actions are all critical to angiogenesis, and all are effects that are induced by VEGF. However, to date, a direct evaluation of the role of Ras-mediated signal transduction and its relationship to the control of the angiogenic phenotype by VEGF has not been undertaken. This study investigates the role of Ras and several downstream signal transduction pathways in regulating proliferation, migration, and in vitro differentiation following VEGF stimulation of primary endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) from pooled donors were purchased from VEC Technologies (Troy, NY) and cultured in M199 media supplemented with 20% FBS, 1% penicillin/streptomycin, 10 mM Hepes, and 50 μg/ml heparin, and 50 μM EDTA, 5 mM NaF, and Complete anti-protease mixture (Roche Biochemical, Indianapolis, IN), 600 μl per pair of plates. Lysates were clarified by centrifugation at 14,000 × g for 10 min. The extracts were incubated with 3 μg of GST-RBD (amino acids 53–132 of human Raf-1) (31) for 45 min at 4 °C. The pellet was washed in medium salt buffer, followed by several washes in phosphate-buffered saline. The pellets were resuspended in 1 × SDS-PAGE gel. Samples were transferred to nitrocellulose and Western blotted with anti-pan Ras antibody (Ab-1, Oncogene Science) as described above.

**Generation of Recombinant Adenovirus**—A hemagglutinin (HA) tag was added to the N terminus of RasN17 by PCR with custom primers encoding the amino acids YPYDVPDYA (primer sequences available upon request). The RasN17 cDNA was a generous gift of Larry Feig (Tufts University School of Medicine, Boston MA). The resulting PCR product was cloned into pcDNA3 (Stratagene), and the sequence was confirmed by automated dyeoxy sequencing at the Albany Medical College sequencing facility (Albany, NY), using primers to the T7 and M13 sites in pcDNA3. The AdEasy system was used to generate recombinant adenoviruses for the FasTagging for the HA-tagged negative RasN17, essentially as previously described (32). HA-RasN17 was released from pcDNA3 by digestion with BamHI and NotI and ligated into the pAdTrack-CMV shuttle vector digested with BglII and NotI. To generate Ad.HA-RasN17, the plasmid, HA-RasN17/Adtrack-CMV, was linearized with the restriction endonuclease Pmel. The linear plasmid was cotransformed into Escherichia coli B8139 cells with the pAdEasy-1 plasmid containing an E1- and E3-deleted Ad5 genome. Clones undergoing homologous recombination were selected using resistance to kanamycin and confirmed by restriction endonuclease analysis. Once the presence of the inserted gene was established, the recombinant plasmid was linearized with PvuI and transfected into a viral packaging cell line, HKE-293 (ATCC), using LipofectAMINE Plus (Invitrogen, Rockville, MD). Foci producing recombinant viruses were amplified by serial passage, and purified high titer viruses were generated using cesium chloride density centrifugation. Viral titers were estimated by absorbance at 260 nm using the established value of 1 × 1012 viral particles, equal to an absorbance of 1.0, and the estimate that the ratio of infectious particles/total particles was 20:1. Infection efficiency was monitored under a microscope by visualizing the green fluorescent protein (GFP) marker expressed from the AdEasy recombinants (32). Typical experiments used an estimated multiplicity of infection (~50) after preliminary experiments in both HUVEC and BLMVEC cultures demonstrated that this level of infection with Ad.GFP resulted in visible expression of GFP in nearly 100% of the cells without any overt consequences to cell morphology or survival for up to 2 weeks following infection (not shown).

**Western Blotting**—HUVECs were grown to confluence in 35-mm dishes or 12-well plates. They were serum-deprived and stimulated with vehicle or VEGF (R&D, Minneapolis, MN) as indicated. Whole cell lysates were collected using Laemml sample buffer (30), followed by boiling the samples for 5 min. Samples were loaded onto an SDS-PAGE gel and run at 150 V for ~1 h. The proteins were then transferred onto nitrocellulose paper at 300 mA for 1.5 h, followed by Western blot analysis. Blots were blocked with either 5% dry milk, 0.1% Tween 20 in PBS, or 2% bovine serum albumin, 0.1% Tween 20 in PBS for 1 h at room temperature. The primary antibodies, anti-phosphotyrosine (RC20:HRPO, Transduction Laboratories, Lexington, KY), anti-phospho-ERK, anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HA (BAbCO, Richmond, CA), anti-phospho-p38, anti-phospho-Akt-B, O. anti-ph-r38, and anti-Akt (New England BioLabs, Beverly, MA) were used at a dilution of 1:1000; anti-pan Ras antibody (Ab-1, Oncogene Science), anti-phospho-Raf, 1:200; HA antibody was added for 1.5 h at room temperature or overnight at 4 °C. After washing, the appropriate secondary antibodies (Pierce, Rockford, IL) were added at a dilution of 1:5000 for 1 h at room temperature. After extensive washing, blots were developed with Super Signal enhanced chemiluminescence kit (Pierce) and visualized on Kodak-AR film, following automated development.

**Measurement of Ras Activation**—HUVECs were grown to confluence in 100-mm dishes, and two plates per assay point. They were serum-deprived overnight and stimulated with VEGF (50 ng/ml) for 5 min where indicated. Cells were lysed in medium salt buffer (1% Triton X-100, 100 mM KCl, 20 mM Tris, pH 7.5, 5 mM MgCl2, 10% glycerol, 1 mM EDTA, 5 mM NaF, and Complete anti-protease mixture). An equal amount of 1012 viral particles, equal to an absorbance of 1.0, and the estimate that the ratio of infectious particles/total particles was 20:1. Infection efficiency was monitored under a microscope by visualizing the green fluorescent protein (GFP) marker expressed from the AdEasy recombinants (32). Typical experiments used an estimated multiplicity of infection (~50) after preliminary experiments in both HUVEC and BLMVEC cultures demonstrated that this level of infection with Ad.GFP resulted in visible expression of GFP in nearly 100% of the cells without any overt consequences to cell morphology or survival for up to 2 weeks following infection (not shown).

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**Measurement of DNA Synthesis**—HUVECs were seeded at 2.5 × 10^4 cells per 24 wells and allowed to adhere overnight. Cells were infected with Ad.GFP or Ad.HA-RasN17 for 16 h in reduced serum MCD-131 and stimulated with or without 50 ng/ml VEGF for an additional 16 h. HUVECs were serum-starved overnight and pretreated with or without 10 μM PD98059 or 1 μM U0126 for 15 min prior to VEGF stimulation. Cells were pulsed with 10 μM 5′-bromo-2-deoxyuridine (BrdUrd) for 3 h, and fixed with 3.7% formaldehyde. Cells were stained with an anti-BrdUrd antibody followed by a Texas Red anti-mouse secondary antibody to visualize the BrdUrd incorporation. Cells were counterstained with 1 μg/ml Hoechst dye to label the
cell nuclei. A fluorescence microscope equipped with a Spot-2 digital camera (Diagnostic Instruments Inc., Sterling heights, MI) was used to collect three random 10× fields, and the percentage of positive cells per 10× field was calculated using ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Results were normalized to control and are reported as the average fold increase in BrdUrd-labeled cells.

**Cellular Migration**—Transwell tissue culture inserts (Costar, Corning, NY) containing 8-µm pores were coated with 10 µg/ml collagen for 2 h at 37 °C. HUVECs infected with adenovirus or uninfected cells were serum-starved for 4 h, trypsinized, and seeded in serum-free M199 media at 1 × 10⁶ cells per insert onto the top surface in serum-free media. The lower chamber contained VEGF at a concentration of 50 ng/ml. Cells were fixed in 3.7% formaldehyde/PBS for 15 min, and stained with 0.5% crystal violet. Non-migratory cells adhered to the top surface were removed with cotton swabs. Cells adhered on the bottom surface were permeabilized using 0.1% Triton X-100 in PBS for 15 min and stained with Hoechst dye at a concentration of 1 µg/ml for 20 min. Cells were visualized with a fluorescence microscope, and three random 10× fields were collected using a digital camera. The average cell number per field was computed following automated counting of the digitized images in ImagePro Plus.

**Cellular Adhesion**—HUVECs infected with adenovirus or uninfected cells were serum-starved for 4 h and trypsinized, and cells were seeded at 5 × 10⁴ per well of 12-well dishes precoated with 10 µg/ml collagen. Cells were stimulated with or without 50 ng/ml VEGF for 15 min at 37 °C, 5% CO₂. Non-adhered cells were detached by rocking the 12-well plates on a rotator for 60 s. Cells that remained adhered were washed three times with PBS, fixed with 3.7% formaldehyde, and stained with 1 µg/ml Hoechst dye followed by analysis using fluorescence microscopy, as described above.

**Labeling of Actin Cytoskeleton**—HUVECs infected with Ad.GFP and Ad.HA-RasN17 were trypsinized, and 5.6 × 10⁵ cells were seeded onto 12-mm circle glass coverslips that were precoated with 10 µg/ml collagen. Cells were maintained in serum-free media overnight. Fresh media was administered to the cells 2 h prior to stimulation with 50 ng/ml VEGF for 15 min. The cells were washed with cold PBS and fixed in cold 3.7% formaldehyde for 15 min. Cell membranes were permeabilized with 0.1% saponin/PBS for 30 min, incubated with 1% bovine serum albumin for 30 min to reduce nonspecific background staining. Labeling of filamentous actin was attained by staining with 0.5 units/ml of Texas Red-X Phalloidin (Molecular Probes) for 30 min. For visualization, the coverslips containing cells were mounted onto glass slides in Vectashield mounting media containing 4',6-diamidino-2-phenyl-indole (Vector Laboratories). Images were captured using a digital fluorescence microscope.

**Branching Morphogenesis Assay**—Collagen gels were prepared using a neutral solution of 1 mg/ml collagen in 1× Dulbecco's modified Eagle's medium with or without 50 ng/ml VEGF supplemented. The gels were allowed to polymerize in a 24-well plate for 1 h at 37 °C. Uninfected or infected (Ad.GFP or Ad.HA-RasN17) BLMVECs were seeded at 1.5 × 10⁴ per well and were grown in MCDB-131 supplemented with 2.5% FBS and 1% penicillin/streptomycin for 48 h in a humidified 37 °C, 5% CO₂ incubator. In some experiments, cells were cultured in the presence of 10 µM PD98059 or 1 µM U0126. Cells were fixed with 3.7% formaldehyde. After washing, cells were photographed with a Spot-2 digital camera, and the cumulative tube length was measured using ImagePro Plus, essentially as previously described (34).

**RESULTS**

The stimulation of Ras and subsequent activation of its downstream effector molecules is critical in the biological response of many receptor tyrosine kinases. Compelling circumstantial evidence also suggests a potential role for Ras in regulating angiogenesis. Therefore, our initial experiments were directed at determining if Ras becomes activated following stimulation with VEGF in primary human endothelial cells. Two strategies were employed to oppose conclusions regarding the stimulation of Ras by VEGF (20, 21). To maximize sensitivity in our experiments we chose to use the Ras binding domain pull-down method (35, 36) rather than the less sensitive immunoprecipitation method employed in the earlier studies (20, 21). As a positive control for assay specificity, we transfected 293 cells with expression constructs coding for either a constitutively activated Ras (RasV12) or one incapable of binding GTP (RasN17). The clarified lysates were tested for Ras binding to GST-RBD. As shown in Fig. 1A, the binding showed excellent selectivity between the GTP- and GDP-bound forms of Ras, even when highly overexpressed. We next sought to determine if Ras activation was detectable following stimulation with VEGF. Acute stimulation of serum-deprived HUVECs with VEGF resulted in a robust activation of Ras after 5 min of stimulation, as evidenced by binding to GST-RBD. A similar degree of stimulation was found with bFGF (Fig. 1B).

To study the role of Ras in VEGF signal transduction and the VEGF-induced angiogenic phenotype, we wished to genetically manipulate Ras activation. To do this we chose to make a recombinant adenovirus coding for the dominant negative Ras mutant, RasN17. An epitope-tagged HA-RasN17 was cloned into the AdEasy shuttle vector that contains two CMV promoters, one driving expression of HA-RasN17, and the second driving GFP expression. This shuttle vector was used to generate a recombinant adenovirus using the AdEasy system originally described by He et al. (32). Using this recombinant virus, we observed high efficiency infection (essentially 100%) of HUVECs and BLMVECs as assessed by comparing GFP-expressing cells to the total number of cells, visualized by staining with Hoechst dye to label the nuclei (data not shown).

The expression of the dominant negative allele of Ras was confirmed routinely by Western blot analysis using an antibody to the HA epitope (Fig. 2A, see also Fig. 7A). In addition, using the same technique, we demonstrated that expression of Ad.RasN17 in primary endothelial cells did not interfere with VEGF receptor phosphorylation. Cells expressing RasN17 demonstrated a similar elevation in phosphotyrosine levels when stimulated with VEGF when compared to cells expressing GFP alone (Fig. 2B). Thus, the observed changes in responses following Ad.RasN17 infection were not due to a diminished responsiveness of the VEGF receptors to their ligand.

One of the best-characterized angiogenic responses to VEGF is the stimulation of DNA synthesis. Mitogenesis has been strongly associated with Ras activation in other systems, thus we sought to determine if expression of HA-RasN17 would interfere with VEGF-mediated stimulation of DNA synthesis. We decided to use a BrdUrd incorporation assay to measure DNA synthesis, because this gives a result normalized to cell number and, thus, is a true reflection of DNA synthesis rather than cell number. In addition, this methodology ensured we were only assessing effects in infected cells. In cells infected with GFP alone, BrdUrd incorporation was substantially increased in the number of BrdUrd-positive cells. In contrast, infection of cells with Ad.HA-RasN17 completely inhibited this response (Fig. 2B). Infection with Ad.HA-RasN17 in the absence of VEGF also reduced the unstimulated level of BrdUrd incorporation when compared with GFP alone, indicating a role for Ras in the basal level of DNA synthesis in these cells. In addition, it should be noted that there were about 50% of the total number of cells in the Ad.HA-RasN17 infected, unstimulated populations, when

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**FIG. 1. VEGF activates Ras in HUVECs.** A. 293 cells were transfected with plasmids encoding HA-RasN17 or HA-RasV12. Clarified cell lysates were incubated with GST-RBD immobilized on glutathione agarose. After washing, bound proteins were analyzed by SDS-PAGE and Western blot analysis using monoclonal anti-Ras antibody, Y13259. B. serum-deprived HUVECs were stimulated with VEGF or FGF prior to lysis. Control cells were unstimulated. Clarified cell lysates were incubated with GST-RBD as described above. Similar results were obtained in two additional experiments.
RasN17 under serum-free conditions followed by stimulation with VEGF. HUVECs were infected with adenoviruses coding for Ad.GFP or HA-, independent experiments. Determined as fold increase compared to control. Data was collected as the number of positive cells/total cell count. BrdUrd-positive cells were quantified by fluorescence digital microscope. BrdUrd incorporation was visualized with an anti-BrdUrd antibody and recorded with a digital fluorescent microscope, and counted. The results are from a single experiment with values representing the mean ± S.E. Similar results were obtained in three additional experiments (A) or one additional experiment (B).

Another important angiogenic phenotype induced by VEGF is the induction of the angiogenic program in endothelial cells that stimulates proliferation and migration; however, another important aspect of neovascularization is the capacity of endothelial cells to differentiate into vascular structures. Although this process cannot be completely recapitulated in vitro, endothelial cells can be coaxed to undergo a branching morphogenic program, resulting in the formation of tubular structures that are often connected end to end, resembling a vessel (37, 38). To assess the importance of Ras activation in the VEGF stimulation of this response, we measured the branching morphogenic response of BLMVECs plated on a three-dimensional collagen gel. We decided to use BLMVECs for these experiments because the branching morphogenic response of BLiVECs were done with antibodies to HA (A) and phosphotyrosine, RC20:HRPO (B). Cells were seeded into 24 wells were infected with Ad.GFP and Ad.RasN17 in serum-free media for 16 h. Cells were stimulated with VEGF for an additional 16 h; control cells were unstimulated. Cells were pulsed with BrdUrd for 3 h. Following fixation, BrdUrd incorporation was visualized with an anti-BrdUrd antibody and recorded with a fluorescence digital microscope. BrdUrd-positive cells were quantified, and data was collected as the number of positive cells/total cell number. The data were normalized to the control value and are reported as fold increase ± S.E. Similar results were obtained in three independent experiments.

The inhibition of VEGF-stimulated migration and the inability of the Ras-infected cells to branch and survive on the three-dimensional collagen matrix (Fig. 3B). Furthermore, there were no changes in the ability of Ad.HA-RasN17-infected cells to spread on collagen (see for example Fig. 5).

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basal and stimulated actin organization, a process believed to be critical to VEGF-stimulated motility (17, 39). The GTP loading of Ras results in the stimulation of several downstream effector pathways that have been implicated in actin reorganization. Notable among these is the activation of PI3K. The PI3K pathway has been linked in various reports to a range of activities in VEGF-stimulated endothelial cells, including cellular migration, proliferation, and cell survival phenotypes (40–42). Therefore, we sought to investigate whether disruption of this pathway contributes to the mechanistic basis for the alterations in the actin cytoskeleton and the inhibition of proliferation, migration, and branching morphogenesis observed following treatment with dominant negative Ras. We first investigated whether PI3K was activated using phospho-Akt as an indicator, because it is well established that activation of Akt in PI3K-dependent in endothelial cells (40–42). We found that Akt was activated in our system following VEGF, as previously reported (40–42), and this was sensitive to low doses of the PI3K inhibitors LY294002 and wortmannin (10 μM and 100 nM, respectively). Interestingly, infection with Ad.RasN17 did not significantly inhibit either the basal or the VEGF-mediated activation of Akt (Fig. 6A), suggesting that PI3K activation is not dependent upon Ras and that disruption of PI3K activity is not responsible for the observed inhibition of the angiogenic phenotypes in response to expression of dominant negative Ras. Because several reports have implicated the PI3K pathway as being important for VEGF-stimulated migration (41, 43), we also investigated the effects of two chemically distinct inhibitors of PI3K, wortmannin, and LY294002. Using doses of PI3K inhibitors sufficient for complete abrogation of Akt activation in response to VEGF (Fig. 6A), we found no inhibition of the VEGF-stimulated migration by either agent (Fig. 6B). Similar negative results were seen in branching morphogenesis assays and cell proliferation assays (data not shown). Thus, from these results we can conclude that: 1) stimulation of PI3K activity is not dependent upon Ras activation; 2) that the inhibition of the angiogenic phenotypes seen following expression of dominant negative Ras is not a result of disruption of the PI3K pathway; and 3) PI3K is not required for the VEGF-mediated changes in the angiogenic phenotypes we have investigated in primary cultures of HUVECs.

Experiments by Rousseau et al. (39, 44) demonstrated that inhibition with the p38 inhibitor, SB203580, inhibits VEGF-induced migration and actin reorganization. These data directly implicate p38 as a critical modulator of migration. In other experiments investigating the PDGF receptor, a VEGF-related receptor tyrosine kinase, Ras, has been reported to be required for the stimulation of p38 MAPK (45). Therefore, we sought to determine whether expression of dominant negative Ras effected the activation state of p38. As shown in Fig. 6C, VEGF-induced p38 activation, as detected with phosphorylation state-specific antibodies. This activation, however, was not affected by infection with Ad.RasN17. These data rule out the possibility that the inhibition of the angiogenic phenotypes that accompanied expression of the dominant negative Ras was the result of diminished p38 activation. In addition, this result, as well as the results investigating phosho-Akt activation are noteworthy in that they demonstrate that disruption of the Ras pathway does not globally inhibit all VEGF-mediated signal transduction and suggests that there are divergent signal transduction pathways activated by VEGF, several of which are upstream of Ras, but that in the absence of Ras signaling these other signals are insufficient to drive the angiogenic phenotype.

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We obtained similar results with both of these alternate methods (data not shown).

These data suggest that both ERK-dependent and ERK-independent aspects of the VEGF-stimulated angiogenic phenotype exist and that, although inhibition of ERK activation by expression of RasN17 accounts for the observed effects on cellular proliferation, it is not sufficient to explain the inhibition of the VEGF-induced migratory response under these conditions.

We next sought to examine the role of the ERK pathway in regulating the branching morphogenesis seen on three-dimensional cultures of collagen gels. This phenotype likely repre-
sents a culmination of several discreet phenotypic changes in the endothelial cell, including survival, migration, alterations in peri-cellular proteolysis, as well as changes in the pattern of gene expression. As shown in Fig. 9A, VEGF treatment converts relatively planar, grouped islands of cells into elongated, branching, scattered arrays of cells that have increased depth of field. This phenotype is not fundamentally altered, in either the control or VEGF-stimulated condition, following inhibition of the ERK pathway. However, visually it appeared as if the phenotype was somewhat blunted, with less cells in the branching tube phenotype than in untreated controls (Fig. 9A). This was revealed to be the case following quantification by digital morphometry (Fig. 9B), when we utilized cumulative tube length as a measure. However, quantitative analysis of the average tube length (Fig. 9C) shows that the ability of the cells to differentiate into tubular structures remains largely intact following ERK inhibition, as assessed by average tube length. Thus, although this model of in vitro angiogenesis was completely dependent upon Ras activation, the relative qualitative aspects of the phenotype are not fundamentally changed following ERK inhibition. Rather, ERK may contribute to a survival or proliferative component of this phenotype, resulting in a quantitatively diminished cumulative response.

**DISCUSSION**

We investigated whether Ras was activated by VEGF and the impact of inhibiting Ras activation on VEGF-mediated signal transduction and induction of the angiogenic phenotype. Our results provide the first comprehensive analysis of the role of Ras and Ras-related signaling pathways in VEGF signal transduction and modulation of the angiogenic phenotype. These data make several important conclusions possible: 1) VEGF is a strong activator of Ras; 2) Ras activation is obligatory for VEGF-induced migration, proliferation, and three-dimensional morphogenesis; 3) VEGF-mediated activation of ERK, but not p38 or Akt, is dependent upon Ras activation; 4) inhibition of Ras activation results in subtle changes in the regulation of the actin cytoskeleton in response to VEGF; 5) activation of ERK is an absolute requirement for VEGF-stimulated proliferation but not migration or branching; 6) PI3K activity is not obligatory for VEGF-stimulated migration; and 7) signal transduction networks bifurcate downstream of Ras to provide unique and independent contributions to the angiogenic phenotype.

While our experiments were in progress, two other reports investigated the role of Ras in the activation of ERK, with differing conclusions. Experiments by Takahashi et al. (20) found no activation of Ras by VEGF using an immunoprecipitation-based assay and no effect of a dominant negative adenovirus (coding for a different mutant) on the activation of ERK. These authors concluded that ERK activation was dependent upon PKC but independent of Ras. In contrast to these results, Doanes et al. (21) demonstrated a VEGF-mediated increase in activated Ras following treatment with VEGF, also...
using an immunoprecipitation-based assay. One possibility for the discrepancy between these previous studies is the low sensitivity and technical difficulty of the immunoprecipitation-based assays of Ras activation. In our studies, we have employed a more sensitive assay for Ras activation based on the high affinity of active Ras for the Ras-binding domain of Raf, a downstream effector of activated Ras (36). Our data indicate a strong activation of Ras by VEGF, in agreement with the findings of Doanes et al. (21). Although the experiments by Doanes et al. (21) found that Ad.RasN17 did not inhibit activation of ERK by VEGF, they did conclude Ras regulated ERK, because the Ras effector antagonist, Ad.Raf 1-149, inhibited the response. The interpretation these authors suggest is that Ras is activated by a protein kinase C (PKC)-dependent, RasN17-insensitive pathway, similar to that used in G1-regulated signaling (35). Taking into account all of the results, the simplest explanation is that VEGF can activate multiple effector pathway leading to Ras activation and subsequent ERK stimulation; some are PKC-dependent and RasN17-insensitive, whereas others are largely sensitive to RasN17. This proposed model is summarized in Fig. 10. Within the context of this model, it is important to note that we never saw complete inhibition of the ERK activation by Ad.RasN17, perhaps because of parallel activation of the proposed PKC-regulated pathway. It may be that in different cell types, under different culture conditions, different regulatory components dominate. Such signal transduction plasticity would impart the angiogenic endothelial cells with considerable advantages in the complex and changing environmental conditions where they naturally function.

Our studies provide the first direct evidence of a critical role for the Ras pathway in regulating VEGF-induced angiogenesis. We investigated proliferation, migration, and three-dimensional morphogenesis, and found all three phenotypes were exquisitely sensitive to inhibition of Ras. Interestingly, in the branching morphogenesis assays on three-dimensional collagen, Ras was absolutely required for the apparent survival of endothelial cells, and this loss of viability could not be rescued by the addition of VEGF. The requirement of Ras activity for survival and maintenance of normal cell shape was quite specific for three-dimensional cultures, because two-dimensional cultures showed normal viability, adhesion, and cell shape up to 72 h following infection with Ad.RasN17. These results are in agreement with the findings of Hegland et al. (50), where expression of Ad.RasN17 had no effect on two-dimensional morphology, but where cells failed to form spontaneous networks on three-dimensional cultures of Matrigel, a laminin-rich tumor-derived extracellular matrix. An important distinction in the two systems is that our differentiation assay is dependent upon VEGF activation where the Matrigel tube formation occurs independent of the addition of any angiogenic factors. Thus, Ras may be a required permissive factor for tubulogenesis, independent of the presence of an angiogenic factor.

The diminished differentiation response we observed following Ad.RasN17 infection is not the result of inhibiting ERK activation by VEGF. Inhibition of ERK stimulation by VEGF, using either U0126 or PD98059, resulted in cells still undergoing a qualitatively identical differentiation response. Interestingly, when analyzed quantitatively, inhibition of the ERK activation diminished branching when compared with control. This was due to a decrease in the number of cells undergoing this morphogenetic event, rather than the extent of branching tubulogenesis, as revealed by comparative analysis of the individual and cumulative tube length parameter. More detailed investigations will be required to determine if the decrease in the number of differentiating cells is due to inhibition of proliferation, survival, or both; or, alternatively, if it is the result of fewer cells committing to the branching phenotype.

An important outcome of these studies is the realization that discreet phenotypes of the endothelial cell are contributed to by discreet signal transduction pathways. Interestingly, the regulation of these phenotypes bifurcates downstream of Ras activation. The proliferative response to VEGF was completely dependent upon the activation of ERK, similar to earlier data (40, 51). In contrast, the migratory response was completely independent of ERK activation and was not affected by either inhibitor of MEK, under conditions where ERK activation was completely suppressed. The continued elucidation of the contributions of each of the discreet signal transduction responses will ultimately allow more precise regulation of the angiogenic response.

Currently it is unclear what signals are being altered and are responsible for the observed inhibition of the migratory and
differentiation responses following infection with Ad.RasN17. Our data demonstrate that the observed inhibition of migration by Ad.RasN17 is not the result of disruption of ERK, p38, or PI3K signal transduction. Several pieces of data support this conclusion. As mentioned above, inhibiting ERK had no effect on VEGF-mediated migration. Similarly, infection with Ad.RasN17 had no detectable effect on either VEGF-stimulated PI3K/Akt or the p38 pathways. These data suggest that PI3K and p38, along with signals such as phospholipase C and nitric oxide generation, likely originate upstream of Ras and provide parallel signaling inputs to modulate the angiogenic response (see Fig. 10). In further support of this notion, complete inhibition of PI3K with two chemically distinct inhibitors failed to affect VEGF-induced migration, demonstrating that inhibition of this pathway in isolation is not sufficient to inhibit the migration response to VEGF (in contrast to the effects of inhibiting the ERK pathway on cell proliferation). Lastly, although this result does not agree with experiments done in porcine and bovine cells (41, 43), these data are consistent with recent results investigating the contribution of two VEGF receptors (VEGFR-1 and VEGFR-2) in primary HUVECs (52).

Although the exact nature of the signal transduction pathway downstream of Ras that controls migration remains undefined, our data do point to a probable regulatory event, i.e. reorganization of the actin cytoskeleton. We have found subtle changes in the VEGF-stimulated actin reorganization following inhibition of Ras signal transduction. These changes consisted of decreases in the prominence, as well as the organization of the cellular actin fibers, and thus these cells may be compromised in their ability to generate contractile force. The ability to generate cellular tension through actin/myosin interactions is a well recognized component of both cell migration and cell survival phenotypes. The changes we observe in the actin filaments, although not sufficient to disrupt the cell spreading, adhesion, or survival on rigid substrates, may be sufficient to compromise the ability of the cell to generate sufficient tension on the more pliable substrates used in the migration and differentiation assays (filter inserts and collagen gels, respectively). Importantly, these less rigid environments more closely approximate in vivo conditions. If indeed the actin rearrangements are responsible for the observed inhibition of migration and differentiation following expression of dominant...
negative Ras, several excellent candidates exist for Ras-regulated effectors, including the Rho GTPases (53), as well as the Ras binding protein Raf-GDS (54). These possibilities are currently under investigation.

In conclusion, these experiments underscore the importance of the Ras molecule in the regulation of angiogenesis in response to VEGF. Given the previous experiments demonstrating the ability of activated forms of Ras to convert immortalized endothelial cells to an angiogenic phenotype in vitro, it will be interesting to determine if activated Ras is sufficient to induce any of the angiogenic phenotypes in primary endothelial cells. In addition, the critical role of Ras in regulating the angiogenic response revealed in this study suggests that anti-Ras therapeutics currently being developed for inhibiting tumor growth could potentially realize some of their effects by directly inhibiting endothelial cells participating in tumor angiogenesis.

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Fig. 10. A model for VEGF-mediated signal transduction. Signal transduction networks downstream of VEGF and their proposed relationship to the regulation of the angiogenic phenotype are shown in a schematic fashion. Red items indicate molecules inhibiting Ras and MAPK signaling. Molecules in black are components of the Ras-mediated signaling pathway leading to the angiogenic phenotypes obtained in this study (boldface text); signaling molecules in blue text are not likely to be transducing through Ras.
