Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) achieves its multiple functions by activating two receptor tyrosine kinases, Flt-1 (VEGF receptor-1) and KDR (VEGF receptor-2), both of which are selectively expressed on primary vascular endothelium. To dissect the respective signaling pathways and biological functions mediated by these receptors in primary endothelial cells with these two receptors intact, we developed a chimeric receptor system in which the N terminus of the epidermal growth factor receptor was fused to the transmembrane domain and intracellular domain of KDR (EGFR) and Flt-1 (EGLT). We observed that KDR, but not Flt-1, was responsible for VPF/VEGF-induced human umbilical vein endothelial cell (HUVEC) proliferation and migration. Moreover, Flt-1 showed an inhibitory effect on KDR-mediated proliferation, but not migration. We also demonstrated that the inhibitory function of Flt-1 was mediated through the phosphatidylinositol 3-kinase (PI-3K)-dependent pathway because inhibitors of PI-3K as well as a dominant negative mutant of p85 (PI-3K subunit) reversed the inhibition, whereas a constitutively activated mutant of p110 introduced the inhibition to HUVEC-EGDR. We also observed that, in VPF/VEGF-stimulated HUVECs, the Flt-1/EGLT-mediated down-modulation of KDR/EGFR signaling was at or before intracellular Ca2+ mobilization, but after KDR/EGFR phosphorylation. By mutational analysis, we further identified that the tyrosine 794 residue of Flt-1 was essential for its antiproliferative effect. Taken together, these studies contribute significantly to our understanding of the signaling pathways and biological functions triggered by KDR and Flt-1 and describe a unique mechanism in which PI-3K acts as a mediator of antiproliferation in primary vascular endothelium.

To grow beyond minimal size, tumors must generate a new vascular supply for purposes of gas exchange, cell nutrition, and waste disposal (1–4). They do so by secreting angiogenic cytokines that induce the formation of new blood vessels (3–6).

Tumor-secreted angiogenic cytokines include fibroblast growth factor, platelet-derived growth factor B, and vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) (6–9). VPF/VEGF is likely the most important of these because it is expressed abundantly by a wide variety of human and animal tumors and because of its potency, selectivity for endothelial cells, and ability to regulate most and perhaps all of the steps in the angiogenic cascade (5, 6, 10–13). Moreover, a number of other angiogenic cytokines act, at least in part, by up-regulating VPF/VEGF expression (5, 14). VPF/VEGF extensively reprograms endothelial cell expression of proteases, integrins, and glucose transporters, stimulates endothelial cell migration and division, protects endothelial cells from apoptosis and senescence, and induces angiogenesis in both in vitro and in vivo models (for review, see Refs. 5, 6, 13, and 15). In addition, VPF/VEGF is the only angiogenic cytokine identified thus far that renders microvessels hyperpermeable to circulating macromolecules, a characteristic feature of angiogenic blood vessels (8, 10–12, 16).

Most of the biological activities of VPF/VEGF are thought to be mediated by its interaction with two high-affinity receptor tyrosine kinases, Flt-1 (VEGF receptor-1) and KDR (VEGF receptor-2; flk-1 in mice) (17–21). A third receptor, neuropilin, has been recognized, but little is known about its capacity to initiate endothelial cell signaling (22, 23). Both Flt-1 and KDR are selectively expressed on vascular endothelium but bind VPF/VEGF with different affinities; thus, Flt-1 binds VPF/VEGF with a \( K_d \) of ~10 pM, whereas the \( K_d \) for KDR binding is 400–900 pM (24, 25). Both receptors possess tyrosine kinase domains, potential ATP-binding sites, and long kinase insert regions that contain phosphorylation sites with binding capacity for different signaling molecules. Flt-1 and KDR also have different ligand specificities. Thus, Flt-1 interacts with VPF/VEGF (also known as VEGF-A) and with two other members of the VPF/VEGF family, PIGF and VEGF-B. KDR, on the other hand, interacts with VEGF-C and VEGF-D, in addition to VPF/VEGF (26). Both Flt-1 and KDR are essential for normal vascular development (17, 20).

At present, the signaling cascades following VPF/VEGF interaction with cultured endothelial cells (ECs) are only par-
tially understood but are known to involve a series of protein phosphorylations, beginning with receptor phosphorylation and subsequently with tyrosine phosphorylation of phospholipase C-γ (PLC-γ) and phosphatidylinositol 3-kinase (PI-3K) (for review, see Refs. 26 and 27). Like other endothelial cell agonists such as thrombin and histamine, VPF/VEGF activates protein kinase C, increases [Ca²⁺], and stimulates inositol-1,4,5-triphosphate accumulation (28).

Because most cultured endothelial cells express both Flt-1 and KDR, it has been difficult to delineate the distinct signaling pathways and biological functions triggered individually by each, and much of our current information comes from studies with cell lines, particularly porcine aortic endothelial (PAE) cells, which do not normally express detectable levels of either KDR or Flt-1 and do not respond to VPF/VEGF. However, when PAE cells were engineered to express KDR, VPF/VEGF induced striking changes in cell morphology and behavior including actin reorganization, membrane ruffling, cell division, and chemotaxis (25). Less is known about the consequences of VPF/VEGF interaction with Flt-1. In PAE cells engineered to express Flt-1 but not KDR, VPF/VEGF stimulated tissue factor expression but not cell migration or proliferation (25).

Recently, in PAE cells overexpressing KDR and Flt-1, it was reported that Flt-1 repressed KDR-mediated proliferation (29). Most press Flt-1 but not KDR, VPF/VEGF stimulated tissue factor expression but not cell migration or proliferation (25). Most is known about the consequences of VPF/VEGF on KDR or Flt-1 and do not respond to VPF/VEGF. However, when PAE cells were engineered to express KDR, VPF/VEGF induced striking changes in cell morphology and behavior including actin reorganization, membrane ruffling, cell division, and chemotaxis (25). Less is known about the consequences of VPF/VEGF interaction with Flt-1. In PAE cells engineered to express Flt-1 but not KDR, VPF/VEGF stimulated tissue factor expression but not cell migration or proliferation (25). As such, in most cultured endothelial cells expressing Flt-1, we engineered chimeric constructs of both receptors, replacing the extracellular domain of each with the extracellular domain of EGFR, creating actin reorganization, membrane ruffling, cell division, and chemotaxis (25). Less is known about the consequences of VPF/VEGF interaction with Flt-1. In PAE cells engineered to express Flt-1 but not KDR, VPF/VEGF stimulated tissue factor expression but not cell migration or proliferation (25). As such, in most cultured endothelial cells expressing Flt-1, we engineered chimeric constructs of both receptors, replacing the extracellular domain of each with the extracellular domain of EGFR, creating actin reorganization, membrane ruffling, cell division, and chemotaxis (25). Less is known about the consequences of VPF/VEGF interaction with Flt-1. In PAE cells engineered to express Flt-1 but not KDR, VPF/VEGF stimulated tissue factor expression but not cell migration or proliferation (25).

Available data suggest that KDR and Flt-1 have different expression but not cell migration or proliferation (25). Most press Flt-1 but not KDR, VPF/VEGF stimulated tissue factor expression but not cell migration or proliferation (25). As such, in most cultured endothelial cells expressing Flt-1, we engineered chimeric constructs of both receptors, replacing the extracellular domain of each with the extracellular domain of EGFR, creating actin reorganization, membrane ruffling, cell division, and chemotaxis (25). Less is known about the consequences of VPF/VEGF interaction with Flt-1. In PAE cells engineered to express Flt-1 but not KDR, VPF/VEGF stimulated tissue factor expression but not cell migration or proliferation (25). As such, in most cultured endothelial cells expressing Flt-1, we engineered chimeric constructs of both receptors, replacing the extracellular domain of each with the extracellular domain of EGFR, creating actin reorganization, membrane ruffling, cell division, and chemotaxis (25).
for 3 min. Cells were resuspended in 10 µl of the same buffer containing 1 µg of mouse anti-EGFR-N antibody or mouse IgG and incubated at 4 °C for 1 h. Cells were centrifuged at 1300 rpm for 3 min. Cell pellets were washed twice with the same buffer, resuspended in 10 µl of the same buffer containing 2.5 µg/ml FITC-conjugated anti-mouse IgG antibody, and centrifuged at 4 °C for 4 h. Cells pellets were then washed twice and resuspended in 400 µl of the same buffer. FACS analysis was carried out using a FACS Calibur instrument (Becton Dickinson) with CellQuest software.

**Proliferation Assays**—2 × 10⁶ HUVECs/well (with or without retrovirus infection) were seeded in 24-well plates. After 2 days, cells were serum-starved (0.1% serum) for 24 h and then stimulated with 10 ng/ml VEGF or EGF for 20 h. 1 µCi/ml [³H]thymidine was added to each well, and 4 h later, cells were washed three times with cold PBS, fixed with 100% cold methanol for 15 min at 4 °C, precipitated with 10% cold trichloroacetic acid for 15 min at 4 °C, washed with water three times, and lysed with 200 µl of 0.1 N NaOH for 30 min at room temperature. [³H]Thymidine incorporation was measured in scintillation solution. For growth inhibition experiments, various inhibitors (always in quadruplicate) were added in final concentrations from 0.01× 10⁻⁷ to 10⁻⁴ M. Values were normalized to control without inhibitors. Data are expressed as the mean ± SD of quadruplicate values.

**Migration Assays**—Serum-starved HUVECs (with or without retrovirus infection) were detached from tissue culture plates as described in FACScan analysis, washed twice with endothelial cell basic medium containing 0.1% fetal bovine serum, and seeded (1 × 10⁵ cells/well) into the transwells coated with vitronectin (30 µg/ml) and the transwells were inserted in a 24-well plate containing 1 ml of the same medium. Cells were counted on a range of 3 × 10⁵ to 1 × 10⁶ cells/well. Cells were seeded in a 96-well plate for standard curve. Cells were incubated at 37 °C for 1 h to allow the cells to attach, and then VPF/VEGF or EGF was added at a final concentration of 10 ng/ml. After incubation for an additional 2 h, cells remaining on the upper surface of the transwell filter membrane were wiped off with a cotton tip. The total transwell membrane was cut out and placed in an individual well of the 96-well plate that contained the cells for standard counts. 200 µl of Cyquant DNA stain was added to each well, and the plate was kept at 4 °C overnight to reach room temperature, stained cells were counted in a spectrophotometer (SpectraFluor, TECAN) with Delta Soft 3 software. Data are expressed as the mean ± SD of triplicate values.

**Intracellular Ca²⁺ Release**—Serum-starved HUVECs (with or without retrovirus infection) were detached from plates as described in FACScan analysis. Cell pellets were resuspended in 2 ml of Ca²⁺ buffer (5 mM NaCl, 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin, 0.25 mM saponin, and 10 mM HEPES, pH 7.5) and centrifuged at 1100 rpm for 3 min and resuspended in 2 ml of Ca²⁺ buffer for stimulation with VPF/VEGF or EGF. In some experiments, cells were preincubated with inhibitors at 30 °C before VPF/VEGF stimulation. Intracellular Ca²⁺ concentrations were measured with the DeltaScan Illumination System (Photon Technology International) using Flexi software.

**Immunoprecipitation and Western Blotting**—Serum-starved cells were stimulated with 10 ng/ml VPF/VEGF or EGF for different lengths of time, as indicated. Stimulation was halted by the addition of ice-cold PBS, and the protein pellets were washed three times with cold PBS and lysed with cold radioimmunoprecipitation buffer (20 mM Tris-Cl, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₃, 1 mM EGTA, 1 µg/ml leupeptin, 0.5% aprotinin, and 2 µg/ml pepstatin A). Cells lysates were collected after centrifugation for 15 min at 4 °C. 500 µg of lysate protein was incubated with 1 µg of different antibodies (as indicated) for 1 h, and protein A-conjugated agarose beads at 4 °C for another hour. Beads were washed three times with radioimmunoprecipitation buffer, and immunoprecipitates were resuspended in 2× SDS sample buffer for Western blot analysis.

**Statistics**—Cell proliferation and migration data were subjected to analysis of variance and to post hoc testing with the Tukey-Kramer multiple comparison test.

**RESULTS**

**Construction, Transduction, and Expression of Chimeric Receptor in HUVECs**—To elucidate the specific signaling events and biological activities mediated by KDR and Flt-1 in early-passage HUVECs, we prepared two chimeric receptors, EGDR and EGLT, by fusing the EGFR extracellular domain to the transmembrane and cytoplasmic domains of either KDR or Flt-1 (Fig. 1a). Each receptor (or both receptors in some experiments) was then transduced into HUVECs with a retroviral expression vector (pMMP). Cells transduced with chimeric receptors or with LacZ as a control were designated EGDR/HUVEC, EGLT/HUVEC, LacZ/HUVEC, and EGRD-EGLT/HUVEC, respectively. Each cell type was then stimulated with EGF or VPF/VEGF, allowing us to study separately the signaling pathways triggered by each chimeric receptor and to compare the results obtained with those that followed stimulation of the wild-type receptors. This approach was possible because HUVECs did not express detectable endogenous EGFR by Western blotting or FACS analysis and did not respond to EGF under the conditions of our experiments (see below).

Immunoblots performed directly on EGDR/HUVEC or EGLT/HUVEC lysates demonstrated clear specific bands with antibodies to the C terminus of KDR (mouse monoclonal antibody) or Flt-1 (rabbit polyclonal antibody), respectively (Fig. 1b, A). The blotting antibodies specifically recognized EGDR and EGLT without detectable cross-reactivity. Neither EDGR nor EGLT was recognized in LacZ/HUVEC. To test whether these proteins contained the EGFR N terminus, lysates of transduced HUVECs were immunoprecipitated with antibodies to the EGFR N terminus, lysates of transduced HUVECs were immunoprecipitated with antibodies to the EGFR N terminus before immunoblotting with antibodies against the C terminus of KDR or Flt-1, respectively (Fig. 1b, B). The chimeric proteins expressed in HUVECs reacted specifically with antibodies to KDR and Flt-1, and both of them contained the N terminus of EGFR.

To determine the fraction of transduced cells that expressed these proteins on their surface, we performed FACS analysis with a mouse monoclonal antibody specific for the N terminus of EGFR. Fig. 1c shows that more than 80% of EGDR/HUVEC and EGLT/HUVEC expressed the expected receptor on their surface. EGFR expression was undetectable in parental HUVECs and in LacZ/HUVEC.

**Function of Transduced Chimeric Receptors**—Stimulation of HUVECs with VPF/VEGF led to time-dependent phosphorylation of both KDR and Flt-1, but with different kinetics (Fig. 1d, A). Phosphorylation of KDR was maximal and equivalent at 5 and 10 min; however, phosphorylation of Flt-1 was maximal and equivalent at 1 and 5 min and had markedly decreased by 10 min. The decline in Flt-1 phosphorylation was not attributable to unequal protein loading because Flt-1 levels were found to be equal when blots were stripped and reprobed with antibody against Flt-1. The differences in phosphorylation therefore likely reflect differences in the kinetics of receptor dephosphorylation.

When transduced HUVECs were stimulated with 10 ng/ml EGF, both EGDR and EGLT underwent phosphorylation and did so with kinetics that mimicked those of KDR and Flt-1 after stimulation with VPF/VEGF (Fig. 1d, B). As expected, LacZ/HUVEC did not express EGFR (Fig. 1d, B). Taken together, these data established the specificity and functionality of our chimeric system, permitting us to use it to investigate the signaling pathways induced by KDR and Flt-1 in early-passage EC cultures.

**EGDR, not EGLT, Mediates VPF/VEGF-induced HUVEC Proliferation and Migration**—As shown in Fig. 2a, VPF/VEGF stimulated an ∼2× increase in incorporation of trichloroacetic acid-precipitable [³H]thymidine in serum-starved HUVECs, regardless of whether or not these were transduced by either one or both of the chimeric receptors or by LacZ. EGDR/HUVEC and EGDR-EGLT/HUVEC were also stimulated to incorporate increased amounts of [³H]thymidine by EGF, whereas native parental HUVECs or LacZ/HUVEC or EGLT/HUVEC was not.
so stimulated by EGF. Of particular interest, EGDR/HUVEC cells responded to EGF with significantly greater (\( >50\%\); \( p < 0.001 \)) thymidine incorporation than they did in response to VPF/VEGF; however, EGDR-EGLT/HUVEC cells gave an equivalent (\( \approx 2\)-fold) response to both VPF/VEGF and EGF. These data provide evidence that KDR/EGDR was solely responsible for ligand-induced HUVEC proliferation and that proliferation was significantly inhibited when Flt-1 or EGLT was present and co-activated.

Similar experiments were performed to determine which receptor(s) was responsible for mediating VPF/VEGF-induced HUVEC migration. As shown in Fig. 2b, VPF/VEGF stimulated migration to an equivalent extent in parental HUVECs and in HUVECs transduced with LacZ, EGDR, or EGLT. As was the case with thymidine incorporation, EGF stimulated the migration of HUVECs that had been transduced with EGDR or with EGDR and EGLT but not that of native HUVECs or HUVECs transduced with LacZ or EGLT. However, in contrast to our experiments measuring HUVEC proliferation (Fig. 2a), the migration response of HUVECs co-transduced with EGDR and EGLT was not reduced compared with cells transduced with EGDR alone. Taken together, these data indicate that, as with proliferation, HUVEC migration was mediated through KDR (or EGDR) and not through Flt-1 (or EGLT). However, in contrast to the proliferation experiments, the presence of Flt-1 (or EGLT) did not inhibit VPF/VEGF (or EGF)-stimulated HUVEC migration.

**Flt-1 Mediates a Signaling Pathway That Inhibits VPF/VEGF-induced, KDR-mediated HUVEC Proliferation, but Not Migration**—To better characterize the inhibitory role of Flt-1/ EGLT on KDR/EGDR-mediated proliferation, we co-infected HUVECs with a constant number of retrovirus particles ex-
was significantly muted when EGLT was co-expressed with EGDR. Significantly greater than that induced by VPF/VEGF in EGDR/HUVEC cells. Moreover, stimulation was significantly increased proliferation only in HUVECs transduced with EGDR. However, induced significantly increased proliferation only in HUVECs transduced with EGDR. Moreover, stimulation was significantly greater than that induced by VPF/VEGF in EGDR/HUVEC cells but was significantly muted when EGLT was co-expressed with EGDR. Results were similar to those shown in a for proliferation, except that EGF stimulation of migration was not greater than that induced by VPF/VEGF in EGDR/HUVEC cells, nor was migration diminished by co-expression of EGLT. Prosing EGDR while varying the number of retrovirus particles containing EGLT. Retrovirus expressing LacZ was used as a supplement so that in each condition, cells were transduced with the same total number of viral particles. Fig. 3a demonstrates that, as expected, the transduced cells expressed constant levels of EGDR but varying levels of EGLT. These co-transduced HUVECs were stimulated with EGF, and proliferation was inhibited progressively as the relative levels of EGLT increased, so that at a 1.0 EGLT:EGDR ratio, thymidine incorporation was inhibited by 50% (Fig. 3b). Even low levels of EGLT transduction (EGLT:EGDR = 0.1) significantly inhibited cell proliferation (p < 0.001), and further inhibition occurred with higher ratios (p < 0.001 for 0.1 versus 1.0; p < 0.5 for 0.1 versus 0.2). These results suggested that activation of Flt-1 down-regulated the proliferative response that was induced by VPF/VEGF through KDR. However, when HUVECs infected with the same ratios of EGLT:EGDR were stimulated with EGF, EGLT did not exert a detectable inhibitory effect on migration (Fig. 3c). Therefore, the inhibitory action of Flt-1 on KDR affected cell proliferation but not migration.

One explanation for these findings is that the Flt-1 effect results from ligand trapping, i.e. that Flt-1 or EGLT binds VPF/VEGF or EGF, reducing the amount of ligand available for interaction with KDR or EGFR. Contrary to this interpretation, however, increasing expression of EGLT affected only EGF-mediated EGDR/HUVEC proliferation and had no effect on EGDR/HUVEC migration. Therefore, we considered other possibilities. One was that KDR and Flt-1 formed heterodimers that in some way modulated signaling. To test this possibility, cell extracts from HUVECs that had been stimulated with VPF/VEGF or HUVECs co-transduced with EGDR and EGLT and stimulated with EGF were immunoprecipitated with antibodies against KDR-C or Flt-1-C and immunoblotted with antibodies against Flt-1-C or KDR-C, respectively. We did not detect KDR-Flt-1 or EGDR-EGLT complexes in either situation (data not shown).

We considered a third possibility, that the signal transduction pathway stimulated through Flt-1/EGLT negatively regulated that stimulated through KDR/EGDR. As shown in Fig. 3d, proliferation of HUVECs was progressively highly significantly inhibited by up to 50% when EGF was added in increasing amounts to EGLT/HUVEC cells 2 min before the addition of VPF/VEGF. In control LacZ/HUVEC cells, EGF did not affect VPF/VEGF-stimulated proliferation (Fig. 3d). On the other hand, EGF did not affect migration stimulated by VPF/VEGF in EGLT/HUVEC or LacZ/HUVEC cells (Fig. 3e). These data strongly suggested that Flt-1/EGLT mediates an inhibitory pathway that affects HUVEC proliferation, but not HUVEC migration, and that this inhibitory effect is not attributable to Flt-1 or EGLT trapping ligand or to the formation of KDR-Flt-1 or EGDR-EGLT heterodimers.

**The Role of PI-3K in the Flt-1-mediated Inhibitory Effect on VPF/VEGF-induced HUVEC Proliferation and Migration**—To investigate the signaling pathways involved in VPF/VEGF-induced HUVEC proliferation and migration, we made use of a number of different signaling inhibitors. Treatment with U73122, an inhibitor of PLC, inhibited both proliferation and migration induced by VPF/VEGF in parental HUVECs (Fig. 4a and c); similar dose-dependent inhibition of proliferation and migration was observed in EGDR/HUVEC cells stimulated with EGF (Fig. 4b and d). On the other hand, U73343, an inactive analog of U73122, did not inhibit proliferation or migration even at a concentration of 10 μM (Fig. 4c). Mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059 inhibited proliferation (Fig. 4a and b) but had no effect on cell migration (Fig. 4c and d) in both VPF/VEGF-stimulated parental HUVECs and in EGDR/HUVEC cells stimulated with EGF. These experiments indicate for the first time that phosphorylation of MAPK is not required for VPF/VEGF-mediated HUVEC cell migration.

Interestingly, wortmannin, a PI-3K inhibitor, significantly (p < 0.001) increased HUVEC proliferation in response to VPF/VEGF, whereas in EGDR/HUVEC, wortmannin did not affect EGF-stimulated cell proliferation (Fig. 4a and b). Our data suggest that wortmannin removes an inhibitory effect in VPF/VEGF-stimulated HUVECs, an inhibitory effect that apparently does not exist in EGDR-mediated HUVEC cells. Taken together, these results suggest that the inhibitory pathway mediated by Flt-1 is regulated by PI-3K. Consistent with this hypothesis, wortmannin had no effect on migration of VPF/VEGF-treated HUVECs or of EGF-treated EGDR/ HUVEC cells (Fig. 4c and d).

**Role of PI-3K in the Flt-1-mediated Inhibitory Effect on VPF/VEGF-induced HUVEC Proliferation**—Because wortmannin, a PI-3K inhibitor, removed the inhibitory effect of the Flt-1/EGLT pathway on KDR/EGDR-mediated HUVEC proliferation (Fig. 4), we performed additional experiments to elucidate the mechanisms by which PI-3K exerted its antiproliferative effect. PI-3K contains a kinase subunit (p110) and an inhibitory subunit (p85) and functions in tyrosine kinase receptor signaling pathways (33). Ligand-activated receptors interact with the
p85 subunit, releasing the p110 subunit in an active form. Also, experiments with the yeast two-hybrid system had suggested that Flt-1 associates with the p85 subunit of PI-3K (34).

To test whether PI-3K is activated by the KDR and/or Flt-1 pathways in HUVECs, we stimulated serum-starved HUVECs with VPF/VEGF and demonstrated that Flt-1, but not KDR, formed an immunocomplex with p85 \( (\text{Fig. 5a}) \). We next used a dominant negative mutant of the p85 subunit of PI-3K (p85(DN)) to determine whether inhibition of p85 blocked the antiproliferative activity of Flt-1. Fig. 5b shows that p85(DN)-transduced HUVECs proliferated to a significantly greater extent than did Lac Z-transduced or p110CAAX (the activated form of PI-3K)-transduced HUVECs in response to VPF/VEGF in HUVECs transduced with EGT or LacZ and stimulated with varying amounts of EGF (5, 10, 50, or 100 ng/ml) 2 min before the addition of VPF/VEGF (10 ng/ml). EGF pretreatment at even the lowest concentration (5 ng/ml) significantly \( (p < 0.001) \) diminished proliferation in EGT/HUVEC cells but had no effect on LacZ/HUVEC cells. In contrast, EGF pretreatment did not affect VPF/VEGF-induced migration in either EGT/HUVEC or in LacZ/HUVEC cells.

Flt-1/EGLT Inhibits Intracellular \( \text{Ca}^{2+} \) Mobilization and Thereby Inhibits KDR/EGDR-mediated HUVEC Proliferation—It has long been known that VPF/VEGF stimulates intracellular \( \text{Ca}^{2+} \) mobilization (28), and we have now shown that this mobilization is activated through KDR or EGDR in response to VPF/VEGF and EGDR, d, and e, proliferation (d) and migration (e) response to VPF/VEGF in HUVECs transduced with Flt or LacZ and stimulated with varying amounts of EGF (5, 10, 50, or 100 ng/ml) 2 min before the addition of VPF/VEGF (10 ng/ml). EGF pretreatment at even the lowest concentration (5 ng/ml) significantly \( (p < 0.001) \) diminished proliferation in EGT/HUVEC cells but had no effect on LacZ/HUVEC cells. In contrast, EGF pretreatment did not affect VPF/VEGF-induced migration in either EGT/HUVEC or in LacZ/HUVEC cells.
To test this hypothesis, we measured \([Ca^{2+}]_i\) after EGF stimulation of HUVECs that expressed constant levels of EGFR but different levels of EGLT. Fig. 6b shows that EGLT expression inhibits intracellular Ca\(^{2+}\) mobilization and does so in a dose-dependent manner.

Additional experiments indicated that the PLC inhibitor U73122 completely inhibited intracellular Ca\(^{2+}\) mobilization induced by VPF/VEGF in HUVECs, whereas the PI-3K inhibitor wortmannin increased both the initial slope and overall magnitude of intracellular Ca\(^{2+}\) mobilization (Fig. 6c). Moreover, p110-CAAX, a constitutively activated mutant form of PI-3K, down-regulated KDR/EGDR-mediated intracellular Ca\(^{2+}\) mobilization in EGF-stimulated EGFR/HUVEC cells (Fig. 6e), much as did Flt-1 activation. Taken together, Flt-1/EGLT inhibited cell proliferation mediated by KDR at least in part by activating PI-3K, which inhibited KDR-mediated intracellular Ca\(^{2+}\) mobilization. It follows that the Flt-1/EGLT signaling pathway intersects that mediated by KDR/EGFR at or up-stream of intracellular Ca\(^{2+}\) mobilization.

**DISCUSSION**

VPF/VEGF is an important, multifunctional angiogenic cytokine that exerts a variety of biological activities on vascular endothelium. These include induction of microvascular hyper-

![Fig. 4. Effects of various inhibitors on VPF/VEGF- or EGF-stimulated proliferation and migration.](http://www.jbc.org/)

**Mutational Analysis of Flt-1 to Determine the Domain(s) That Inhibits KDR-induced HUVEC Proliferation**—We generated three different EGLT mutants to more precisely define the region of Flt-1 that inhibited KDR-mediated HUVEC proliferation. EGLT(824stop), a truncation mutant immediately before the Flt-1 kinase domain, was engineered by changing the Glu\(^{825}\) codon to a stop codon (GAG to TAG). Two other mutants were EGLT(793stop), in which a stop codon was introduced at amino acid 794 (TAC to TAA), and EGLT(Y794F), a point mutant in which a tyrosine residue was changed to phenylalanine (TAC to TTC). These three mutants were transduced into HUVECs with our retroviral system and shown expressed on the cell surface by FACS analysis (Fig. 7A).

Finally, we co-transduced EGFR/HUVEC cells with each of these EGLT mutants and evaluated them for cell proliferation in response to EGF and VPF/VEGF. None of the three mutants affected VPF/VEGF-induced cell proliferation. However, as shown in Fig. 7B, EGF stimulated \(^{[3]H}\)thymidine incorporation into DNA of EGFR/LacZ cells significantly (1.5-fold) more than did VPF/VEGF. On the other hand, EGF stimulated EGFR-EGLT/HUVEC cell proliferation to the same extent as that induced by VPF/VEGF in parental HUVECs. Neither the EGLT(793stop) nor the EGLT(Y794F) mutant inhibited EGF-stimulated proliferation mediated through EGFR; however, EGLT(824stop) had an inhibitory effect similar to that of wild-type EGLT. These results indicate that tyrosine residue 794 of Flt-1 is essential for inhibiting KDR-mediated proliferation.
permeability, stimulation of proliferation and migration, significant reprogramming of gene expression, endothelial cell survival, and prevention of senescence (for review, see Refs. 5, 6, 13, and 26). All of these functions are thought to be mediated by two receptor tyrosine kinases, KDR and Flt-1, that are selectively expressed on vascular endothelium and up-regulated at sites of VPF/VEGF overexpression as in tumors, healing wounds, chronic inflammation, etc. (for review, see Ref. 6). Because both receptors are expressed on vascular endothelium, it has been difficult to define the respective role of each in mediating the various signaling events and biological activities induced in endothelium by VPF/VEGF. Therefore, current information has been gleaned largely from studies with a cell line, PAE, that does not express either receptor unless engineered to do so (24, 25); from studies with PI GF, a ligand that binds Flt-1 but not KDR (6); from the use of a Flt-1-specific antibody (31); from studies with antisense oligonucleotides that block Flt-1 expression (30); and from VPF/VEGF mutants that specifically bind to Flt-1 (32).

We sought to delineate the respective roles of KDR and Flt-1 in early-passage endothelial cells in which both receptors remained intact and functional. To that end, we engineered chimeric receptors, fusing the extracellular domain of the EGF receptor with the transmembrane and intracellular domains of either KDR or Flt-1. We transduced these constructs (or, as a control, LacZ) into HUVECs with a retroviral vector. This approach was feasible because, under the conditions of our experiments (>80% confluence), HUVECs did not express the EGFR and did not respond to EGF. This strategy was also attractive because endogenous KDR and Flt-1 persisted in HUVECs transduced with either one or both of the chimeric receptors; therefore, the signaling and biological responses induced by VPF/VEGF or EGF could be compared in the same cells.

Transducing our chimeric receptor into HUVECs, we found that KDR, but not Flt-1, mediated VPF/VEGF-induced EC proliferation and migration, extending earlier work that had reached the same conclusion using different approaches. Waltenberger et al. (25) stably transfected KDR and Flt-1 into PAE, an endothelial cell line that uniquely fails to express either KDR or Flt-1. KDR-transfected PAE responded to VPF/VEGF stimulation with strong mitogenic and chemotactic responses, in contrast to Flt-1-transfected PAE, which failed to proliferate or migrate in response to VPF/VEGF. Bernardetz et al. (30) made use of antisense oligonucleotides to demonstrate that repression of Flk-1/KDR, but not of Flt-1 expression, inhibited VPF/VEGF-mediated proliferation and migration in bovine aortic ECs. More recently, Gille et al. (32) used a VPF/VEGF mutant that reacted selectively with KDR to determine that activated KDR can stimulate HUVEC proliferation and migration. Therefore, taken together, similar results have been obtained using different experimental approaches and three different types of endothelium, firmly establishing and generalizing the finding that KDR, but not Flt-1, mediates VPF/VEGF-induced EC proliferation and migration.

The function of Flt-1 has been much less clear. One proposal has been that Flt-1 is a decoy receptor rather than a signal transducer because Flt-1 kinase domain null mice develop normally (35), in contrast to Flt-1 knockout mice, which are embryonic lethal (26). It was also reported that VPF/VEGF failed to stimulate Flt-1 tyrosine phosphorylation in PAE cells engineered to overexpress Flt-1 and did so poorly in HUVECs (25, 36). However, the expression level of Flt-1 in HUVECs is only about one-tenth that of KDR (24, 25), and therefore measurement of Flt-1 phosphorylation is difficult and may have been underestimated. To take account of the large differences in receptor expression, we used three times as many HUVECs for measurement of phosphorylation of Flt-1 as for KDR and had
no difficulty in demonstrating phosphorylation of both receptors (Fig. 1d, A).

We then used our chimeric receptors to elucidate the distinct signaling pathways mediated by KDR and Flt-1. After transduction into HUVECs, both EGDR and EGLT were expressed at equivalent levels, and both underwent equivalent phosphorylation in response to EGF. This result stands in contrast to that reported by the study of Rahimi et al. (29), in which a chimeric receptor generated by fusing the N-terminal domain of colony-stimulating factor-1 receptor to Flt-1 showed no significant tyrosine phosphorylation when it was overexpressed in PAE cells and stimulated with colony-stimulating factor-1. It must be remembered, however, that PAE is a cell line with atypical properties (e.g., it doesn’t normally express KDR or Flt-1) and therefore may be less representative of vascular endothelium than early-passage HUVECs.

Of particular interest was the finding that EGDR/HUVEC cells responded to EGF with a proliferative response that was \( \frac{50}{\text{greater}} \) than that induced by VPF/VEGF. Moreover, when HUVECs were co-transduced with EGLT and EGDR, the proliferation response to EGF was reduced to levels induced by VPF/VEGF in parental HUVECs. EGLT dose-response experiments further supported the inhibitory effect of Flt-1/EGLT on KDR/EGDR-mediated HUVEC proliferation. They also correlated well with the situation in parental HUVECs, where the ratio of KDR:Flt-1 expression is \( \frac{10}{1} \), but the binding affinity of VPF/VEGF to Flt-1 is 10 times higher than that to KDR. In our dose-response experiment, equivalent amounts of EGLT and EGDR were required to achieve the same inhibitory effect seen in parental HUVECs because the binding affinity of EGF to both EGDR and EGLT is similar.

Several possible explanations have been proposed for the inhibitory effect of Flt-1 on KDR signaling. The possibility that Flt-1 sequestered VPF/VEGF, making it unavailable to KDR,
as suggested by Hiratsuka et al. (35) with Flt-1 kinase domain null mice and by Rahimi et al. after activation of the colony-stimulating factor-1/Flt-1 chimera in PAE (29), was excluded by the finding that Flt-1/EGLT inhibited EC proliferation but had no inhibitory effect on migration. Moreover, pretreatment of EGLT/parental HUVECs with EGFR before the addition of VPF/VEGF led to the same inhibitory effect on proliferation observed in parental HUVECs treated with VPF/VEGF alone. Also, co-transduction with EGLT, even in amounts equivalent to those of EGFR that greatly reduced proliferation, had no inhibitory effect on migration. Taken together, these results indicated that Flt-1/EGLT most likely acted by activating an inhibitory signaling pathway that down-modulated KDR/EGLT-mediated HUVEC proliferation.

To extend these findings, we made use of inhibitors of specific metabolic pathways. The PLC inhibitor U73122 completely inhibited VPF/VEGF-induced HUVEC proliferation and migration, whereas the PI-3K inhibitor wortmannin inhibited VPF/VEGF-induced stimulation in all mutants, whereas co-transduction with EGLT and the mutant EGLT(824stop) significantly inhibited VPF/VEGF-induced stimulation; i.e. the inhibitory activity of EGLT was lost in the EGLT(793stop) and EGLT(Y794F) mutants.

Because Flt-1/EGLT mediated an inhibitory effect on KDR/EGDR-mediated HUVEC proliferation (Figs. 2a and 3), and wortmannin removed an inhibitory effect in VPF/VEGF-stimulated HUVECs that did not exist in EGF-stimulated EGDR/ HUVEC proliferation (Fig. 4, a and b), we considered the possibility that PI-3K was involved in the Flt-1/EGLT-mediated inhibitory pathway. Our results with p85(DN) and the constitutively active PI-3K, p110CAAX, confirmed this hypothesis (Fig. 5, b and c). Treatment with VPF/VEGF induced more extensive proliferation (~3-fold) in p85(DN)-overexpressing HUVECs than in LacZ-transduced HUVECs (~2-fold). On the other hand, overexpression of p110CAAX did not inhibit proliferation further. Whereas p85(DN) had no effect on EGFR-induced proliferation of EGDR/HUVEC, p110CAAX inhibited proliferation in these cells by 50% (Fig. 5c), a finding consistent with the inhibitory effect of Flt-1/EGLT (Fig. 3). p85(DN) also removed the inhibitory effect of EGLT on EGDR-mediated proliferation with EGF (Fig. 5c). However, neither p85(DN) nor p110CAAX had any effect on HUVEC migration induced by VPF/VEGF. Interestingly, Flt-1 was found to interact with the p85 subunit of PI-3K, whereas no association was observed between KDR and p85 (Fig. 5a). These results corroborated the findings of Cunningham et al. (34), which suggested an association between Flt-1 and p85 using the yeast two-hybrid system. Furthermore, PI-3K (p110) knockout mice (Pik3ca<sup>±/±</sup>) demonstrated extravasation blood, suggestive of defective angiogenesis. These embryos are developmentally retarded and die before embryonic day 9.5 and embryonic day 10.5 (38). The similarities of the Pik3ca<sup>±/±</sup> phenotype to that of Flt-1 knockout mice are consistent with our results that PI-3K is involved in Flt-1 signaling pathway.

Intracellular Ca<sup>2+</sup> mobilization is an important consequence of HUVEC stimulation by VPF/VEGF. We have now demonstrated that the Ca<sup>2+</sup> response is mediated by KDR and not by Flt-1 signaling, a finding consistent with that obtained using an antisense approach (30). However, our results also indicated that cells transfected with EGLT or p110CAAX exerted an inhibitory effect on KDR/EGLT-mediated intracellular Ca<sup>2+</sup> mobilization (Fig. 6, b and e), an effect also observed in parental HUVECs. However, inhibition could be relieved by the PI-3K inhibitors wortmannin and LY294002, both of which increased the slope of Ca<sup>2+</sup> mobilization and the overall magnitude (Fig. 6, c and d). We did not detect any difference in EGDR phosphorylation in cells that were or were not transfected with EGLT, and we did not detect any difference in KDR phosphorylation in parental HUVECs that were or were not pretreated with wortmannin. Therefore, we conclude that the Flt-1/EGLT pathway intersects KDR signaling and inhibits KDR-mediated HUVEC proliferation at a step after receptor phosphorylation and at or before the step at which KDR mediates intracellular Ca<sup>2+</sup> mobilization.

Finally, to define the domain(s) of Flt-1 responsible for its antiproliferative activity, we engineered several EGLT mutants, EGLT(Y794F), EGLT(824stop), and EGLT(793stop). We did not detect any difference in Ca<sup>2+</sup> mobilization in cells that were or were not transfected with EGLT, and we did not detect any difference in KDR phosphorylation in parental HUVECs that were or were not pretreated with wortmannin. Therefore, we conclude that the Flt-1/EGLT pathway intersects KDR signaling and inhibits KDR-mediated HUVEC proliferation at a step after receptor phosphorylation and at or before the step at which KDR mediates intracellular Ca<sup>2+</sup> mobilization.

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785 but also introduced a six-amino acid sequence from the following intron. In this six-amino acid sequence, there is a Gln residue, which, like tyrosine, often has an important role in protein function. On the other hand, tyrosine 794 is also six amino acids away from the three serine “repressor” residues that have been described by others (39). One explanation of the difference between our results and those with fltkinase−/− mice is that this 6-amino acid sequence may introduce an unexpected function. Because Flt-1 does not inhibit KDR-mediated migratory signaling, our data fail to support the idea that Flt-1 behaves as a decoy receptor but do support the hypothesis that its inhibitory function reflects a negative signaling event.

In summary, we have engineered chimeric receptors to distinguish the signaling events triggered by KDR and Flt-1 in early-passage HUVECs. These tools have allowed us to identify a number of downstream signaling pathways that are stimulated by activation of KDR and Flt-1. KDR-mediated proliferation and migration involve activation of PLC, whereas Flt-1 was found to exert an inhibitory effect on HUVEC proliferation, but not on migration, through the PI-3K pathway. The Flt-1-mediated antiproliferative pathway acts after KDR phosphorylation but at or before KDR-mediated intracellular Ca2+ mobilization. This study thus represents the first direct analysis of Flt-1 and KDR function in early-passage ECs and has demonstrated cross-talk between the pathways mediated by these two receptors. However, neither the KDR nor Flt-1 pathways are fully defined, and further work is needed to demonstrate the complete complement of signaling steps and pathway regulators that govern such other VPF/VEGF-mediated effects on ECs as increased microvascular permeability, gene expression reprogramming, survival, and prevention of senescence.

REFERENCES

1. Folkman, J. (1996) Science 275, 150–154
2. Folkman, J. (1971) N. Engl. J. Med. 285, 1182–1186
3. Folkman, J., and Klagsbrun, M. (1987) Science 235, 442–447
4. Folkman, J., Watson, K., Ingber, D., and Hanahan, D. (1989) Nature 339, 58–61
5. Dvorak, H. F., Nagy, J. A., Feng, D., Brown, L. F., and Dvorak, A. M. (1999) Curr. Top. Microbiol. Immunol. 237, 97–132
6. Risau, W. (1997) Nature 386, 671–674
7. Benedetti, M., Vlodavsky, I., Ishai-Michaeli, R., Neufeld, G., and Bar-Shavit, R. (1995) Blood 81, 3524–3531
8. Senzer, D. R., Pervuzzi, C. A., Feder, J., and Dvorak, H. F. (1986) Cancer Res. 46, 5629–5632
9. Vlodavsky, I., Pufe, Z., Ishai-Michaeli, R., Bashkin, P., Levi, E., Korner, G., Bar-Shavit, R., and Klagsbrun, M. (1991) J. Cell. Biochem. 45, 167–176
10. Dvorak, H. F. (1990) Prog. Clin. Biol. Res. 354A, 317–330
11. Dvorak, H. F., Orenstein, N. S., Carvalho, A. C., Churchill, W. H., Dvorak, A. M., Galli, S. J., Feder, J., Bitzer, A. M., Ryppse, J., and Giovinco, P. (1979) J. Immunol. 122, 166–174
12. Dvorak, H. F., Senger, D. R., and Dvorak, A. M. (1984) Dev. Oncol. 22, 96–114
13. Ferrara, N. (1999) Curr. Top. Microbiol. Immunol. 237, 1–30
14. Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rikkin, D. B., and Mignatti, P. (1998) J. Cell Biol. 141, 1659–1673
15. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1306–1309
16. Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983) Science 219, 883–895
17. Feng, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) Nature 376, 66–70
18. Miller, B., Wizigmann-Voss, S., Schnurich, H., Martinez, R., Meller, N. P. H., Risau, W., and Ulrich, A. (1990) Cell 72, 835–846
19. Quinn, T. P., Peters, K. G., De Vries, C., Ferrara, N., and Williams, L. T. (1995) Proc. Natl. Acad. Sci. U. S. A. 70, 7533–7537
20. Shalaby, F., Hsu, J., Stanford, W. L., Fisher, K. D., Schuh, A. C., Schwartz, L., Bernstein, A., and Rossant, J. (1997) Cell 89, 981–990
21. Terman, B., Dougher-Vermazen, M., Carrión, M., Dimitrov, D., Armellino, D., Gospodarowicz, D., and Bohnen, P. (1992) Biochem. Biophys. Res. Commun. 187, 1579–1586
22. Seker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. (1998) Cell 92, 735–745
23. Gagnon, M. L., Bielenberg, D. R., Gechtman, Z., Miao, H. Q., Takashima, S., Soker, S., and Klagsbrun, M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2573–2578
24. Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N., and Alitalo, K. (1997) EMBO J. 16, 3988–3991
25. Waltenberger, J., Claesson-Welsh, L., Sieghahn, A., Shibuya, M., and Heldin, C. H. (1994) J. Biol. Chem. 269, 26988–26995
26. Petrova, T. V., Makinen, T., and Alitalo, K. (1999) Exp. Cell Res. 253, 117–130
27. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) Exp. Cell Res. 253, 255–270
28. Brock, T. A., Dvorak, H. F., and Senger, D. R. (2019) Am. J. Pathol. 138, 213–221
29. Rahimi, N., Dayanir, V., and Lashkari, K. (2000) J. Biol. Chem. 275, 16986–16992
30. Bernatchez, P. N., Seker, S., and Sirois, M. G. (1999) J. Biol. Chem. 274, 31047–31054
31. Kanno, S., Oda, N., Abe, M., Terai, Y., Ito, M., Shitara, K., Tabayashki, K., Shibuya, M., and Sato, Y. (2000) Oncogene 19, 2138–2146
32. Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Ziochbeck, T. F., Pelletier, N., and Ferrara, N. (2001) J. Biol. Chem. 276, 3222–3230
33. Vanhaesebroeck, B., and Waterfield, M. D. (1999) Exp. Cell Res. 253, 239–254
34. Cunningham, S. A., Waxham, M. N., Arrate, P. M., and Brock, T. A. (1995) J. Biol. Chem. 270, 20254–20257
35. Hiratsuka, S., Minowa, O., Kano, J., Noda, N., and Shibuya, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9349–9354
36. de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992) Science 255, 389–391
37. Thakker, G. D., Hajjar, D. P., Muller, W. A., and Rosengart, T. K. (1999) J. Biol. Chem. 274, 10002–10007
38. Bi, L., Okabe, I., Bernard, D. J., Wynshaw-Boris, A., and Nussbaum, R. L. (1999) J. Biol. Chem. 274, 10963–10968
39. Gille, H., Kowalski, J., Yu, L., Chen, H., Pisabarro, M. T., Davis-Smyth, T., and Ferrara, N. (2000) EMBO J. 19, 4064–4073
Vascular Permeability Factor (VPF)/Vascular Endothelial Growth Factor (VEGF) Receptor-1 Down-modulates VPF/VEGF Receptor-2-mediated Endothelial Cell Proliferation, but Not Migration, through Phosphatidylinositol 3-Kinase-dependent Pathways

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