Analysis of Biological Effects and Signaling Properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2)

A REASSESSMENT USING NOVEL RECEPTOR-SPECIFIC VASCULAR ENDOTHELIAL GROWTH FACTOR MUTANTS*

Endothelial cells express two related vascular endothelial growth factor (VEGF) receptor tyrosine kinases, KDR (kinase-insert domain containing receptor, or VEGFR-2) and Flt-1 (fms-like tyrosine kinase, or VEGFR-1). Although considerable experimental evidence links KDR activation to endothelial cell mitogenesis, there is still significant uncertainty concerning the role of individual VEGF receptors for other biological effects such as vascular permeability. VEGF mutants that bind to either KDR or Flt-1 with high selectivity were used to determine which of the two receptors serves to mediate different VEGF functions. In addition to mediating mitogenic signaling, selective KDR activation was sufficient for the activation of intracellular signaling pathways implicated in cell migration. KDR stimulation caused tyrosine phosphorylation of both phosphatidylinositol 3-kinase and phospholipase Cγ in primary endothelial cells and stimulated cell migration. KDR-selective VEGF was also able to induce angiogenesis in the rat cornea to an extent indistinguishable from wild type VEGF. We also demonstrate that KDR, but not Flt-1, stimulation is responsible for the induction of vascular permeability by VEGF.

Angiogenesis is an important developmental process and is also critically involved in several pathological conditions such as rheumatoid arthritis, diabetic retinopathy, and growth of solid tumors (1, 2). VEGF1 is a major mediator of normal and abnormal angiogenesis (1). VEGF is secreted by tumor cells and their surrounding stroma and is causally involved in the progression of the majority of solid tumors (1). Inhibition of VEGF activity using various inhibitors results in suppression of tumor growth in vivo (3–6). VEGF is also up-regulated in ischemic tissues and has been implicated as a potential therapy for peripheral and myocardial ischemia (7).

VEGF is expressed as at least four different splice isoforms (1). The most abundantly expressed variant is VEGF165, which contains a heparin-binding domain. Two VEGF receptor tyrosine kinases, Flt-1 (VEGFR-1) and KDR (VEGFR-2), are expressed on proliferating and quiescent endothelial cells (8). Although Flt-1 exhibits higher affinity for VEGF165, its function in the adult vasculature is still poorly understood. Mice lacking the complete Flt-1 gene display an increased number of endothelial progenitors and vascular disorganization and die in utero at embryonic day 9 (9, 10). However, animals homozygous for a deletion of the cytoplasmic domain are fertile and do not display any obvious defects (11). Mice in which the flk-1/KDR gene has been inactivated also die at embryonic day 9. They are deficient in vasculogenesis and also lack blood island formation (12).

Placenta growth factor (PIGF) shares 53% identity with the platelet-derived growth factor-like domain of VEGF (13). It binds Flt-1 with high affinity but is unable to interact with KDR (14). PIGF exerts minimal effects on cell growth and migration, suggesting that binding to Flt-1 alone is not sufficient to mediate these activities. In some instances, however, effects of PIGF on mitogenicity and MAP kinase activation have been reported (15). In recent studies, the effects of PIGF on endothelial cells have been compared with those of another VEGF-like molecule, VEGF_{ort}. Members of the VEGF_{ort} gene family have been isolated from the Orf parapoxvirus, and the encoded proteins bind to KDR but not Flt-1 (16, 17). VEGF_{ort} mediates migration of KDR-expressing PAE cells and corneal angiogenesis to an extent that is comparable with VEGF (17, 18). However, the residues mediating VEGF_{ort} binding to KDR are known because the three basic amino acids comprising the major determinants of VEGF binding to the KDR receptor are absent in VEGF_{ort}. In addition, VEGF_{ort} does not possess the heparin-binding domain found on VEGF. These differences may complicate conclusions about VEGF function, drawn from studies using VEGF_{ort} proteins. VEGF binding to KDR leads to receptor phosphorylation and activation of MAP kinases as well as tyrosine phosphorylation of PI3K and PLCγ. Although Flt-1 becomes phosphorylated and can activate several signaling molecules when expressed in heterologous cells (19), it is unclear whether these signaling events also occur in endothelial cells in response to Flt-1 engagement.

Recent studies have shown that KDR signaling results in endothelial nitric-oxide synthase up-regulation and activation (20). Nitric oxide plays a critical role in the VEGF-induced endothelial cell proliferation, migration, and tube formation, as well as increased vascular permeability, hypotension, and angiogenesis in vivo (21–24). VEGF stimulates the formation of vasodilator prostaglandins, which have been implicated as me-
Diators of VEGF-induced vascular permeability but not new blood vessel formation (21, 25). Recently, it was shown that a VEGF mutant chimeric protein that is unable to trigger KDR activation can cause vascular permeability to an extent that is similar to that of wild type VEGF (26). This led the authors to conclude that either Flt-1 or another as yet unknown receptor is responsible for the increase in vascular permeability in response to VEGF administration.

**Fig. 1. Specificity and activity of KDR- and Flt-selective VEGF mutants.** Competition of VEGF wild type and mutant protein binding with $^{125}$I-VEGF to KDR. Binding of $^{125}$I-labeled VEGF was performed in the presence of the indicated concentrations of cold ligand. A, KDR binding activities. B, Flt-1 binding activities. C, KDR phosphorylation. The ability of mutant and wild type VEGF proteins to induce KDR phosphorylation was analyzed over the indicated concentration rage. D, Flt-sel VEGF is biologically active. This mutant protein displays the same potency as VEGF, in a competitive displacement of $^{125}$I-VEGF from transfected PAE cells expressing Flt(ANGG) (top panel). Flt-sel VEGF also promotes chemotaxis of PAE-Flt(ANGG) cells as efficiently as wild type VEGF, while KDR-sel VEGF is ineffective (bottom panel). Growth factors were added at the indicated concentrations as described under “Experimental Procedures.” Cells were allowed to migrate for 14–16 h. Flt(ANGG) is a mutant Flt-1 receptor that, unlike wild type Flt-1, is able to mediate a motility response to VEGF (30). Data are expressed as stimulation index of quadruplicate wells relative to basal, factor-independent migration. Bars represent the means ± S.E.
We sought to address these issues using novel, highly selective, VEGF mutants generated by phage-display technology (27). Structure-function studies have demonstrated that VEGF interacts differently with its cognate receptors KDR and Flt-1. Hence, it has been possible to generate VEGF mutants that show a strong preference for binding either one or the other receptor. Compared with an earlier generation of receptor specific mutants (28), the novel mutants display a substantially increased selectivity and thus are more likely to yield relevant results pertaining to the function of each individual VEGF receptor. By selectively activating Flt-1 or KDR in primary endothelial cells, we have studied the involvement of each individual receptor in mediating VEGF signaling and cell migration. KDR activation alone is sufficient for the activation of signal transducers involved in mitogenesis and cell migration. Selective KDR engagement also induces in vivo angiogenesis and vascular permeability.

EXPERIMENTAL PROCEDURES

Reagents—HUVEC were purchased from Cell Systems (Kirkland, WA). LY 294002 was purchased from Biomol. The construction and characterization of Flt-1- and KDR-selective VEGF are described by Li et al. (27). The mutations are as follows: KDR-selective VEGF D63S/G65M/L66R and Flt-selective VEGF I43A/I46A/Q79A/I83A. The mutations were introduced into VEGF165 and wild type VEGF, and the receptor-selective mutants were expressed in Escherichia coli and purified as described (29). The endotoxin content of the purified material did not exceed 0.2 Eu/mg.

Binding Assays—The determination of binding affinities of the receptor-selective VEGF mutants was carried out in solution as described (29) utilizing soluble IgG-Fc fusions of KDR and Flt-1 (14). To further characterize the activities of the Flt-sel mutant, 125I-VEGF binding assays were performed in PAE cells transfected with the mutant Flt-1 receptor using an image analysis software (NIH Image®). The PAE cell line lacks endogenous VEGF receptors (31). Kinase Receptor Activation Assay—KDR phosphorylation was analyzed in CHO cells stably expressing KDR with a C-terminal epitope tag. Kinase receptor activation assay assays were performed as described recently (32).

Cell Culture—Passage 4–7 HUVEC were maintained in CS-C medium (Cell Systems, Kirkland, WA) containing 10% fetal bovine serum and growth factors on gelatin-coated dishes and made quiescent by 14 h of starvation in 0.2% fetal bovine serum. Cells were treated as indicated and washed once in ice-cold phosphate-buffered saline containing 0.1 mM sodium orthovanadate. Cells were lysed in 0.5–1 ml of RIPA buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mg/ml ovalbumin for 30 min (1 mg/ml) and used at doses of 0, 0.3, 1, 3, 10, 30, and 100 ng/site. The dose of all other proteins was adjusted to inject equimolar amounts of protein. Sixty minutes after injections were made, animals were euthanized with pentobarbital (160 mg/kg IV), and their skin was removed, cleaned from connective tissues, and photographed. Quantification of the dye extravasation area was carried out on the pictures using an image analysis software (NIH Image®). For each dose, measurements were made in triplicate and averaged to minimize measurement errors.

RESULTS

Characterization of Receptor-selective Mutants—We set out to determine the different contributions of KDR and Flt-1 to VEGF signaling in primary endothelial cells. Because endothelial cells express both VEGF receptors, we made use of two novel VEGF mutants engineered by the use of a phage display selection process to bind selectively to either KDR or Flt-1 (27). These mutations were introduced into the VEGF165 cDNA and recombinant proteins were produced in E. coli. The full-length VEGF mutant proteins were analyzed for their ability to bind to either Flt-1 or KDR in solution. Figure 1A demonstrates the relative inability of the Flt-1-selective VEGF mutant (Flt-sel) to compete efficiently with wild type 125I-VEGF for binding to KDR. The reduction of binding of the KDR-selective mutant (KDR-sel2) to Flt-1 is shown in Fig. 1B. Table I summarizes the Kd values and relative affinities of the individual mutants to Flt-1 and KDR. Binding of the KDR-selective variant to Flt-1 is reduced by a factor of 2000-fold, whereas Flt-selective VEGF binds KDR 128-fold less well. To further characterize the specificity of the mutants, we tested them with respect to their ability to induce KDR phosphorylation in intact cells (Fig. 1C). KDR-selective VEGF was essentially as potent and effective as wild type VEGF. Surprisingly, the ability of Flt-sel VEGF to...
induce KDR phosphorylation was reduced by at least 4 orders of magnitude. However, as illustrated in Fig. 1D, Flt-sel VEGF was fully capable of competing for $^{125}$I-VEGF binding to the Flt-1 receptor even in the context of intact cells and was as potent and effective as VEGF$_{165}$ in promoting motility of PAE cells expressing Flt(ANGG). Unlike wild type Flt-1, this receptor mutant is able to mediate a KDR-like motility response to VEGF because of the removal of a repressor motif in the juxtamembrane region (30). These findings argue against the possibility that the inability of Flt-sel VEGF to induce KDR phosphorylation may be due to inherent instability and/or lack of activity of the mutant protein.

**Activation of MAP Kinases**—Earlier work by this and other laboratories has generally shown that PlGF binding to Flt-1 is not able to cause marked mitogenesis in endothelial cells (14, 19), although evidence to the contrary has also been reported in transfected cell lines (15). We therefore tested whether KDR-selective VEGF was capable of mediating mitogenic signaling. As expected, activation by KDR-selective VEGF triggered phosphorylation of ERK1 and ERK2 in HUVEC (Fig. 2A). The extent of phosphorylation was indistinguishable from that obtained using wild type VEGF. Flt-1-selective VEGF at the highest concentration used resulted in minimal phosphorylation of ERK2. The homodimeric VEGF mutants utilized in this study are not expected to promote receptor heterodimer formation. Hence Flt-1 does not contribute to MAP kinase activation.

VEGF has been reported to stimulate the stress-activated p38 MAP kinase (37, 38). To determine which VEGF receptor is involved, the phosphorylation status of p38 was analyzed after stimulation with wild type Flt- and KDR-selective VEGF. Fig. 2B demonstrates that only KDR-selective VEGF was able to stimulate p38 phosphorylation. However, as above indicated, Flt-selective VEGF was as effective as wild type VEGF$_{165}$ in inducing migration of PAE cells expressing Flt(ANGG), demonstrating that this mutant is biologically active. Furthermore, in agreement with a previous report that implicates Flt-1 activation in metalloproteinase release (39), Flt-selective VEGF stimulated the release of increased amounts of matrix metalloproteinase-9 proteolytic activity from human vascular smooth muscle cells (27).

**PI3K and PLCγ Phosphorylation**—PLCγ phosphorylation and activation has been implicated in VEGF signaling. PLCγ binding to both KDR (40, 41) and Flt-1 (19, 42, 43) has been reported. To determine which VEGF receptor(s) are involved in PLCγ activation in primary endothelial cells, HUVEC were treated with VEGF or VEGF receptor-selective mutants, and PLCγ phosphorylation was assessed after immunoprecipitation (Fig. 3A). Both wild type and KDR-selective VEGF were able to stimulate PLCγ phosphorylation to a similar extent. Flt-selective VEGF did not increase PLCγ phosphorylation over background levels, arguing against a role for Flt-1 in PLCγ activation in HUVEC.

PI3K has been demonstrated to transmit survival signals through the activation of Akt in several cell types (44). VEGF also acts as a survival factor for endothelial cells, and this signal requires PI3K and Akt kinase activity (45). In a variety of cell types, PI3K activity has been demonstrated to be involved in cytoskeletal changes following growth factor stimu-

| Mutants | KDR binding | FLT-1 binding |
|---------|-------------|---------------|
|         | $K_d$ | Relative to VEGF$_{165}$ | $K_d$ | Relative to VEGF$_{165}$ |
| V$_{sel}$ | $97$ | 1 | $37$ | 1 |
| KDR-sel2 | $100$ | 1 | $87,000$ | 2,000 |
| Flt-sel | $12,416$ | 128 | $98$ | 2.6 |

**Fig. 2. Activation of MAP kinases.** Quiescent HUVEC were either left untreated or stimulated with VEGF or VEGF mutant proteins for 5 min. A, Western blots were probed for phosphorylated ERK1 and ERK2. B, the phosphorylation state of p38 stress-activated MAP kinase was assessed with a phospho-specific antiserum. *wt*, wild type.
lation as well as cell migration (46, 47). Therefore, the ability of the VEGF proteins to cause phosphorylation of the p85 regulatory subunit of PI3K was assessed after immunoprecipitation. Only wild type and KDR-selective VEGF were capable of causing phosphorylation the PI3K regulatory subunit (Fig. 3B).

Effect of Receptor-selective VEGF Mutants on Endothelial Cell Migration—One of the central aspects of VEGF action on endothelial cells is its ability to act as a chemoattractant and stimulate the migration of endothelial cells. Fig. 4A shows the effect of receptor-selective VEGF on HUVEC in a modified Boyden chamber assay. In several independent experiments, VEGF caused a 4–5-fold increase in HUVEC migration. KDR-selective VEGF is as effective as wild type VEGF in the promotion of HUVEC migration. Flt-selective VEGF is unable to increase cell migration over background levels.

To determine the contribution of PI3K to endothelial cell migration, different concentrations of the inhibitor LY 294002 were added to the assay after the cells had been allowed to attach to the membrane. Because of the deleterious effects of PI3K inhibition on endothelial cell survival a short-term assay was performed (see “Experimental Procedures”). Fig. 4B shows that at its highest concentration, and LY 294002 caused a 56% inhibition of HUVEC migration. Thus, PI3K activity contributes significantly to endothelial cell migration.

KDR, but Not Flt-1, Signaling Causes in Vivo Angiogenesis—Because endothelial cells in adult organisms express both KDR and Flt-1 and previous approaches that interfere with in vivo angiogenesis have been targeted at interfering with VEGF binding to both receptors, we sought to identify the receptor(s) responsible for in vivo angiogenesis. Hydron pellets containing 200 ng of growth factors were implanted into rat corneas, and the angiogenic areas were evaluated after 1 week (Fig. 5A). KDR-selective VEGF was as efficient as wild type VEGF in inducing corneal angiogenesis. Although Flt-selective VEGF occasionally induced marginal angiogenesis (Fig. 5A), analysis of the angiogenic surface areas in several animals showed that Flt-selective VEGF was unable to stimulate angiogenesis over control levels. PlGF gave only a marginal response (Fig. 5B). Therefore, only KDR and not Flt-1 is capable of promoting angiogenesis in vivo.
Increased Vascular Permeability Is Mediated through KDR but Not Flt-1 Activation—Similarly, we sought to determine the relative importance of KDR and Flt-1 receptors for the VEGF-induced vascular permeability. Vascular permeability was assessed by the Miles assay, and responses were evaluated 60 min following the intradermal administration of VEGF and VEGF receptor-selective mutants. KDR-selective VEGF induced vascular permeability to a comparable extent as wild type VEGF, whereas Flt-1 receptor-selective VEGF caused essentially no leakage (Fig. 6A). The results obtained in four animals are summarized in Fig. 6B. In additional experiments, we tested the Flt-1-selective mutant at much higher concentrations (up to 30 μg/site), and still no extravasation was observed (data not shown). Therefore, VEGF-induced vascular permeability is mediated by KDR receptor binding. To further confirm these results, we compared the vascular permeability of VEGF165 and the Flt-1-selective ligand PIGF. To rule out the possibility that differences in Flt-1 receptor binding affinity between VEGF and PIGF could explain the lack of vascular permeability induced by PIGF, we used PIGF at equimolar doses and at doses 20-fold higher relative to those of VEGF consistent with previous reports (Refs. 14 and 48 and Fig. 6C). These results demonstrate the critical role of KDR receptor activation in the VEGF-induced vascular permeability response.

**DISCUSSION**

The expression of at least two VEGF receptor tyrosine kinases on endothelial cells has made it difficult to elucidate the individual contributions of each receptor to VEGF signaling. Although PIGF binds preferentially to Flt-1, its use has sometimes led to conflicting results especially when heterologous cell types such as NIH3T3 fibroblasts were used (15, 19). This study explores whether the activities of both Flt-1 and KDR are required to mediate VEGF functions in primary endothelial cells and *in vivo*. To this end, we employed homodimers of receptor-selective VEGF mutants. Flt-selective VEGF was unable to generate a mitogenic signal, as evidenced also by its inability to stimulate ERK activity. This result is in agreement with previous work that examined the abilities of earlier receptor-selective mutants to promote endothelial cell proliferation (28). Interestingly, although the novel Flt-1-selective mutant demonstrated a 128-fold selectivity using soluble IgG chimeric receptors, in a kinase receptor activation assay.
format it was virtually devoid of the ability to induce KDR phosphorylation, even at concentrations exceeding 10,000 ng/ml. Therefore, the receptor binding assays largely underestimated the extent of specificity of this mutant that may be obtained in a biological context. There are various examples of lack of good correlation between binding affinity and biological activity of mutant proteins (49). In the case of our mutant, one of the possible explanations for the discrepancy is that the reduced affinity leads to only a transient existence of ligand-receptor complexes, below a critical threshold for effective receptor phosphorylation and dimerization. Interestingly, a "minimum residency time threshold" for effective phosphorylation has been recently described for the prolactin receptor (50). However, it was important to verify that Flt-sel VEGF is capable of exerting the appropriate biological activity. This mutant is able to compete for $^{125}$I-VEGF binding in PAE cells expressing Flt(ANGG) with a dose response nearly identical to that of VEGF$_{165}$, demonstrating its ability to bind Flt-1 not only in soluble receptor assays but also in the context of intact cells. Furthermore, this binding results in stable receptor ac-

FIG. 6. VEGF-induced vascular permeability is mediated by binding to KDR receptors. A, representative pictures of skin of guinea pigs used in the Miles assay showing the vascular permeability in response to wild type VEGF$_{165}$, and KDR receptor-selective VEGF mutant, and the lack of effect of the Flt receptor-selective VEGF mutant. B, dose-response curves for VEGF and receptor-selective VEGF mutants. Error bars represent S.E. C, comparison of the vascular permeability response obtained with equimolar doses of VEGF and PI GF and a 20-fold higher dose of PI GF over VEGF.

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vation, as assessed by the ability of Flt-1 selective VEGF to effectively promote migration of PAE cells expressing Flt(ANGG) in an overnight assay. Although wild type Flt-1 fails to transmit a motility signal (30, 31), the juxtamembrane mutant Flt(ANGG) is able to mediate VEGF-dependent migration in transfected PAE cells, similar to that mediated by KDR (30). Additionally, as previously mentioned, Flt-selective VEGF has been shown to stimulate the release of matrix metalloprotease-9 proteolytic activity from cultured human vascular smooth muscle cells (27).

Activation of the KDR receptor was also sufficient to cause cell migration in HUVEC. Therefore, we also examined the activation of proteins previously reported to be involved in the migratory response of normal endothelial cells. Both PI3K and PLCγ phosphorylation were stimulated to the same extent by wild type VEGF and KDR-selective VEGF. HUVEC migration was inhibited by treatment with the PI3K inhibitor LY294002, demonstrating a prominent role for PI3K in endothelial cell migration. This is consistent with results obtained for other cell types, such as epithelial cells and fibroblasts, whereas PI3K activity is required for cytoskeletal changes and cell migration (46, 47).

Recently, the properties of novel VEGF family members present in Orf parapoxviruses have been analyzed (VEGForf). VEGForf is a distant relative of VEGF-A. It lacks a heparin-binding domain and binds preferentially to KDR. Additionally, VEGForf features a threonine- and proline-rich C-terminal domain of unknown function that is not present in any other mammalian VEGF. VEGForf mediates endothelial cell mitogenesis and migration of KDR-expressing PAE cells as well as corneal angiogenesis to an extent that is comparable with VEGF(165) (18, 43). Our rationally designed KDR-selective VEGF should be more representative for the functions of mammalian VEGFs, because it is largely unchanged from wild type VEGF, and only its ability to interact with Flt-1 has been removed.

Because our Flt-1-selective VEGF mutant shows substantially reduced binding and little or no ability to activate KDR, we tested whether this highly selective protein could induce dye extravasation. The absence of extravasation following administration of the Flt-1 receptor-selective mutant and the comparable extent of vascular permeability caused by wild type VEGF and the KDR receptor-selective mutant indicates that VEGF-induced vascular permeability is solely mediated through binding of VEGF to the KDR receptor.

However, a chimeric mutant of mouse VEGF, which shows preferential binding to Flt-1, has recently been reported to stimulate vascular permeability, leading the authors (26) to conclude that KDR activation is not required to elicit this biological effect. Our findings ascertain that KDR can be the sole mediator of VEGF-induced migration, angiogenesis and permeability in endothelial cells. Furthermore, these VEGF mutants should provide especially valuable tools to further dissect Flt-1 and KDR function in vivo through the use in transgenic models, where the protein is overexpressed. Their high selectivity over a broad concentration range may be critical to correctly define the VEGF receptor biology in such circumstances.

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