Vascular Endothelial Growth Factor (VEGF)-D and VEGF-A Differentially Regulate KDR-mediated Signaling and Biological Function in Vascular Endothelial Cells*

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Vascular endothelial growth factor (VEGF)-D binds to VEGF receptors (VEGFR) VEGFR2/KDR and VEGFR3/Flt4, but the signaling mechanisms mediating its biological activities in endothelial cells are poorly understood. Here we investigated the mechanism of action of VEGF-D, and we compared the signaling pathways and biological responses induced by VEGF-D and VEGF-A in endothelial cells. VEGF-D induced KDR and phospholipase C-γ tyrosine phosphorylation more slowly and less effectively than VEGF-A at early times but had a more sustained effect and was as effective as VEGF-A after 60 min. VEGF-D activated extracellular signal-regulated protein kinases 1 and 2 with similar efficacy but slower kinetics compared with VEGF-A, and this effect was blocked by inhibitors of protein kinase C and mitogen-activated protein kinase kinase. In contrast to VEGF-A, VEGF-D weakly stimulated prostacyclin production and gene expression, had little effect on cell proliferation, and stimulated a smaller and more transient increase in intracellular [Ca²⁺]. VEGF-D induced strong but more transient phosphatidylinositol 3-kinase (PI3K)-mediated Akt activation and increased PI3K-dependent endothelial nitric-oxide synthase phosphorylation and cell survival more weakly. VEGF-D stimulated chemotaxis via a PI3K/Akt- and endothelial nitric-oxide synthase-dependent pathway, enhanced protein kinase C-α and PI3K-dependent endothelial tubulogenesis, and stimulated angiogenesis in a mouse sponge implant model less effectively than VEGF-A. VEGF-D-induced signaling and biological effects were blocked by the KDR inhibitor SU5614. The finding that differential KDR activation by VEGF-A and VEGF-D has distinct consequences for endothelial signaling and function has important implications for understanding how multiple ligands for the same VEGF receptors can generate ligand-specific biological responses.

The vascular endothelial growth factor (VEGF) family comprises VEGF-A, placental growth factor, VEGF-B, VEGF-C, and VEGF-D, and VEGF-E. VEGF-A is a prime regulator of vasculogenesis and angiogenesis in development and disease, and the other members of this family have also been implicated in the regulation of angiogenesis (1, 2). VEGF-A binds and activates two related receptor tyrosine kinases, VEGFRI/Flt1 and VEGFR2/KDR (1, 3, 4). The precise function of Flt1 in mediating VEGF-A biological responses is still not clear, although current evidence indicates that a key role of Flt1 in development is the regulation of KDR activity partly by acting as a decoy (1) and partly via direct regulatory effects on KDR (5). KDR is activated through ligand-stimulated receptor dimerization and trans(auto)phosphorylation of at least six tyrosine residues as follows: Tyr-951 and Tyr-996 in the kinase insert; Tyr-1054 and Tyr-1059 in the kinase domain; and Tyr-1175 and Tyr-1214 in the C-terminal tail (3, 4). KDR activation causes stimulation of an array of signal transduction pathways and subsequent biological responses including mitogenesis, migration, and survival of endothelial cells (1, 3, 4). Activation of phospholipase Cγ (PLCγ) results in mobilization of intracellular Ca²⁺, protein kinase C (PKC) activation, and PKC-mediated activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) cascade that in turn mediates VEGF-A-induced endothelial cell proliferation (6, 7) and prostacyclin (PGI₂) production (8). The PI3K pathway has an important function in regulating cell survival via activation of the anti-apoptotic kinase Akt (9) and also mediates phosphorylation and activation of constitutive endothelial nitric-oxide synthase (eNOS) (10, 11). PI3K/Akt-dependent eNOS phosphorylation and focal adhesion kinase phosphorylation mediated via Src have also been implicated in the stimulation of cell migration and the reorganization of the actin cytoskeleton by VEGF-A (12–14).

VEGF-D (15), also known as c-Fos-induced growth factor (16), is structurally and functionally related to VEGF-C (17). Like VEGF-C, VEGF-D is a ligand for VEGFR2 and VEGFR3/Flt4 and is initially synthesized as a disulfide-linked prepropeptide containing N- and C-terminal extensions not found in other VEGF polypeptides, flanking a central receptor-binding VEGF homology domain (18). The unprocessed full-length form of VEGF-D preferentially binds Flt4 and has low affinity

AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraakis(acetoxymethyl ester); EBM, endothelial cell basal medium; eNOS, endothelial nitric-oxide synthase; PKC, protein kinase C; HUVECs, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PG, prostaglandin; RT, reverse transcriptase; PLCγ, phospholipase Cγ; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PI3K, phosphatidylinositol 3-kinase; PBS, fetal bovine serum; VEGFR, VEGF receptor.

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for KDR (19). The N- and C-terminal domains are proteolytically cleaved, releasing the VEGF homology domain during or after secretion to generate a fully processed mature form, which forms noncovalent homodimers of ~21 kDa and binds KDR with greatly increased affinity (19). VEGF-D has been shown to be mitogenic for endothelial cells and promotes angiogenesis in vitro and in rabbit cornea, rat cremaster muscle, skin, and rabbit hindlimb skeletal muscle models of angiogenesis in vivo (20–22). VEGF-D also stimulates lymphangiogenesis in mice when overexpressed in skin keratinocytes and tumors (23, 24) and induces Akt activation, cell survival, and migration in lymphatic endothelial cells (25). However, in comparison to VEGF-A, little is known about the biological activities and signal transduction pathways initiated by VEGF-D in vascular endothelial cells, and the mechanisms underlying the biological effects of VEGF-D are poorly understood.

In the present study, we compared the biological functions and signaling pathways stimulated by VEGF-A and the fully processed form of VEGF-D in human umbilical vein endothelial cells (HUVECs), and we assessed the role of VEGFR2/KDR in endothelial functions of VEGF-D. The results show that although VEGF-D and VEGF-A exhibit some overlap in their endothelial biological effects, they also have marked differential effects on KDR phosphorylation, KDR-mediated signaling, and a range of biological responses including PGI2 production, eNOS phosphorylation, gene expression, proliferation, survival, migration, tubulogenesis, and angiogenesis in vitro. These results indicate that KDR is differentially regulated by VEGF-A and VEGF-D in vascular endothelial cells and have implications for understanding the basis for the generation of distinct ligand-specific cellular responses through activation of KDR.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human VEGF-A165 and fully processed VEGF-D were obtained from R & D Systems. SU5614, LY294002, wortmannin, PKC inhibitors, and 1,2-bis-(oligo(dT)12–18) primer using the SuperScript first-strand synthesis system (Invitrogen) and diluted for real time PCR. Primers for real time PCR (Sigma Genosys) were designed by Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi) by using the 3′- untranslated region of the gene sequence to ensure the specificity of the fragment and ensuring that amplified fragments were 200–250 bp (Table 1). Amplification of predicted fragments was verified by conventional RT-PCR. Real time PCR was performed using the LightCycler- FastStart DNA Master SYBR Green I kit and Lightcycler System (Roche Diagnostics). For each primer pair, a melting curve was used to identify a temperature where only amplicon, and not primer dimers, with the indicated concentration of 125I-VEGF-A165 (1200–1800 Ci/mmol, Amersham Biosciences). After 2 h of incubation at 4°C, the medium was aspirated and washed four times with cold PBS. The cells were lysed with 0.25 M NaOH, 0.5% SDS solution, and the bound radioactivity of the lysates was measured. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled VEGF-A.

Measurement of [Ca2+]i—Confluent HUVECs plated on gelatin-coated glass coverslips were incubated in EBDM containing 10% fetal bovine serum and Fluo-4 AM (4 μM; Molecular Probes) for 20 min; the cells were then washed to remove extracellular Fluo-4 AM and incubated in PBS. In some experiments, cells were incubated in Cd2+-free PBS containing 10 μM EDTA. Cells were mounted on the stage of a Zeiss 510CLSM microscope, and images of fluorescence were obtained before and after application of VEGF or VEGF-D (λexcitation 488 nm, λemission 505–550 nm).

PGL3 Assay—Following treatments, the PGL3 content of the cell supernatants was measured by an enzyme immunoassay of its stable metabolite, 6-keto-prostaglandin F1α, using a kit (Amersham Biosciences) according to the manufacturer’s instructions. Cell Proliferation—HUVECs were seeded at a density of 105 cells per well in 24-well plates. 24 h after plating, the medium was replaced with fresh EBDM containing 0.5% FBS and 25 ng/ml VEGF-A165 or 1 μg/ml VEGF-D. After 3 days, the cell numbers were determined by using a Sysmex CDA-500 cell counter.

Cell Migration—Cell migration was measured in a modified Boyden chemotaxis chamber (NeuroProbe Inc., Cabin John, MD) as described (12). Growth factors in M199, 1% BSA were placed in the bottom wells of the chamber; polycarbonate filters with 8-μm pores (Osmonten Poretics) were placed between the bottom and top chambers. Cells were trypsinized, washed, and resuspended in M199, 1% BSA to give a final cell concentration of 3 × 105/ml. 15,000 cells or without inhibitors as indicated were placed into each well of the top chamber, and the chemotaxis chambers were incubated at 37°C for 4 h. After the incubation, unemigrated cells were removed from the top side of the filters, and migrated cells were stained with Diff-Quik. The stained cells from each well were counted at ×100 magnification using an eyepiece indexed graticule.

Apoptosis—Subconfluent HUVECs in 6-well plates were washed twice with serum-free M199 medium and incubated with the indicated additions for 24 h. The cells were then trypsinized, collected by centrifugation, and stained with fluorescein-conjugated annexin V and propidium iodide (Roche Diagnostics). After staining, the cells were analyzed by flow cytometry using a FACScan (BD Biosciences). Annexin V-positive staining cells were counted as apoptotic cells.

RNA Preparation and Real Time RT-PCR—Total RNA was extracted using a RNeasy kit (Qiagen). After DNase I treatment, single-stranded cDNA was synthesized from 2 μg of total RNA with oligo(dT)12–18 primer using the SuperScript first-strand synthesis system (Invitrogen) and diluted for real time PCR. Primers for real time PCR (Sigma Genosys) were designed by Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi) by using the 3′ untranslated region of the gene sequence to ensure the specificity of the fragment and ensuring that amplified fragments were 200–250 bp (Table 1). Amplification of predicted fragments was verified by conventional RT-PCR. Real time PCR was performed using the LightCycler-FastStart DNA Master SYBR Green I kit and Lightcycler System (Roche Diagnostics). For each primer pair, a melting curve was used to identify a temperature where only amplicon, and not primer dimers, were accounted for SYBR green-bound fluorescence. Real time RT-PCR was conducted in duplicate for each sample using RNA preparations from at

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**Table 1**

| Gene   | Forward 5′ to 3′ | Reverse 5′ to 3′ |
|--------|------------------|------------------|
| GAPDH  | GTCACTTGTTGGACCTGCACCT | CCGTTGCTCGTGAAGCAAAAT |
| NR4A1  | GCTGCGAGATTGACTCCACCG | AGCAGCACTGGGGCTTA |
| NR4A2  | TGCAACGCCTTGGACCTG  | GCCCTGACTGTCAGCTATC |
| NR4A3  | GGCTTACAGCTTGACGCTATC | AGGAAAACCTATGGGGAATG |
| EGR3   | GTCACTTGTTGGACCTGCACCT | CCGTTGCTCGTGAAGCAAAAT |

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least two independent experiments. Data were normalized to the reference gene GAPDH and presented as the mean fold change (±S.D.) compared with control.

Tubule Formation—Human endothelial cell-derived tubule formation was determined in a co-culture assay (TCS CellWorks). Growth factors and inhibitors were added at the start of the experiment and at each medium change at days 4, 7, and 9. At day 11, the cultures were fixed with 70% ethanol for 30 min at room temperature and subsequently immunostained for the endothelial cell adhesion molecule CD31. Fixed cells were incubated with the primary antibody (mouse anti-human CD31) for 1 h at 37 °C, washed three times in blocking buffer (PBS containing 1% BSA), and then incubated with the secondary antibody (goat anti-mouse IgG conjugated to alkaline phosphatase) for 1 h at 37 °C. After three washes in distilled water, the cells were finally incubated with the substrate solution of alkaline phosphatase for 10 min at 37 °C. Images of immunostained tubules were obtained with an Openlab Improvision system using a ×5 objective lens, and endothelial cell-derived tubule formation was quantified by Openlab image analysis software. In each experiment, control wells with VEGF-A or no addition were included.

Murine Sponge Model—Sterile 8-mm polyurethane sponges were inserted under the dorsal skin of anesthetized black C57 female mice on day 0 (5 mice per treatment group). VEGF-D (100 µl of either 0.5, 1, or 2 µg/ml) or VEGF-A (100 µl of 25 ng/ml) recombinant proteins were injected into the sponges on days 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19. Mice were sacrificed on day 21, and sponges with surrounding tissue were excised and fixed in 3.5% paraformaldehyde. Sections (6 µm) of paraffin-embedded sponges were stained with hematoxylin and eosin, and blood vessels, scored as luminal structures containing red blood cells, were counted in a minimum of 5 fields per treatment group.

Data Analysis—Data were analyzed using Prism (version 3.0) statistical packages. Comparisons of two sets of continuous variables were performed using the Student’s *t* test. Differences in qualitative variables (apoptotic frequencies or percentages) between two groups were assessed by the χ² test. Differences between treatment groups in the sponge implant assay were evaluated using a Kruskall-Wallis nonparametric analysis. A value of *p* < 0.05 was taken as statistically significant.

RESULTS

Phosphorylation and Activation of KDR and PLC-γ1 by VEGF-D—The ability of VEGF-D to activate KDR was examined by comparing the effects of VEGF-A and VEGF-D on KDR phosphorylation at Tyr-1054 and Tyr-1059 located within the catalytic domain, two residues required for full KDR activation (26). Immunoblotting of whole-cell lysates with an antibody against KDR phosphorylated at Tyr-1054 and Tyr-1059 showed that VEGF-A induced a striking increase in KDR phosphorylation after 5 min, which thereafter decreased rapidly although remaining above the control level after 60 min; in contrast, VEGF-D had no effect after 5 min, produced a detectable increase after 10 min, and induced a maximum increase in KDR phosphorylation after 30 min that persisted for up to 60 min (Fig. 1A). Treatments with different VEGF-D concentrations indicated that VEGF-D induced KDR phosphorylation at 500 ng/ml and 1 µg/ml after stimulation for either 10, 30, or 60 min, producing a much smaller response compared with VEGF-A after 10 min and similar effects to those of VEGF-A after 30 and 60 min (Fig. 1B). Although Flk-4 was expressed in HUVECs, VEGF-D caused no detectable increase in Flk-4 tyrosine phosphorylation as assessed by anti-phosphotyrosine immunoblot of Flk-4 immunoprecipitates; in parallel cultures, VEGF-A induced a striking increase in KDR tyrosine phosphorylation assessed by anti-phosphotyrosine immunoblot of KDR immunoprecipitates (results not shown).

The weak effect of VEGF-D on KDR tyrosine phosphorylation in HUVECs prompted us to examine how well VEGF-D competed with VEGF-A for binding to high affinity receptor binding sites in these cells. As shown in Fig. 1C, VEGF-D caused only partial inhibition of specific ¹²⁵I-VEGF binding to HUVECs with 20% inhibition of binding at 0.5 and 1 µg/ml. Because HUVECs express KDR, Flt-1, and NP-1 receptors for
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The results are representative of three independent experiments yielding very similar results.

VEGF, the weak inhibition of specific $^{125}$I-VEGF binding to these cells by VEGF-D is at least partly due to the inability of this factor to bind to Flt-1 and NP-1.

Increased tyrosine phosphorylation and activation of PLC-γ leading to PKC activation and mobilization of intracellular Ca$^{2+}$ stores is a key early response directly mediated by activation of KDR (7, 8). VEGF-A induces tyrosine phosphorylation of PLC-γ at tyrosine 783 after 5 and 10 min that declined at later times. In contrast, VEGF-D caused no detectable increase in PLC-γ tyrosine phosphorylation after 5 and 10 min but induced a significant increase after 60 min that was similar to the effect of VEGF-A at the same time of treatment (Fig. 2A). Treatment with different VEGF-D concentrations for 60 min showed that VEGF-D induced PLC-γ tyrosine phosphorylation at 500 ng/ml and 1 μg/ml similar to the concentration dependence observed for VEGF-D-induced KDR phosphorylation (Fig. 2B).

Activation of ERK1/2 and Intracellular Ca$^{2+}$ Signaling—VEGF-A induces ERK via a PKC-dependent pathway, and this pathway is essential for VEGF-A stimulation of endothelial cell proliferation (7) and mediates activation of cytosolic phospholipase A$_2$ leading to increased PGL$_2$ production (8, 27). VEGF-D induced a strong concentration-dependent activation of ERK1/2 comparable with the effect of 25 ng/ml VEGF-A that was maximal at 500 ng/ml and 1 μg/ml (Fig. 3A). However, VEGF-D activated ERK1/2 more slowly than VEGF-A. VEGF-A induced a 14-fold increase in ERK1/2 activation after 2.5 min, whereas ERK activation stimulated by $1 \mu g/ml$ VEGF-D was 4-fold after 2.5 min, increasing to 12-fold after 5 min, and a maximum of 17-fold similar to the maximum effect of VEGF-A after 10 min (Fig. 3A). Pretreatment of HUVECs with either the KDR inhibitor SU5614, two structurally unrelated PKC inhibitors, GF109203X and calphostin C, or the MEK inhibitor, U0126, blocked VEGF-D-induced ERK1/2 activation, similar to their effects on VEGF-A-induced ERK activation (Fig. 3B).

In contrast to VEGF-A, VEGF-D induced a much weaker, slower, and more transient increase in endothelial PGI$_2$ production and had little detectable effect on proliferation (results not shown). The finding that VEGF-D induced strong, albeit slower, ERK activation but had little effect on two biological responses known to be mediated, at least in part, by ERK, could be due to an inability of VEGF-D to activate other intracellular signals required for proliferation and endothelial PGI$_2$ production. Because mobilization of intracellular Ca$^{2+}$ is required for VEGF-induced PGL$_2$ production (8) and is also an essential early event in the stimulation of mitogenesis (28), we compared the effects of VEGF-A and VEGF-D on Ca$^{2+}$ signaling. Treatment with 1 μg/ml VEGF-D induced a delayed increase in intracellular Ca$^{2+}$ as determined by measuring fluorescence of the intracellular Ca$^{2+}$ indicator Fluo-4 AM. An increase in [Ca$^{2+}$]$_i$ occurred with a latency that varied between 50 and 180 s after treatment with VEGF-D and characteristically comprised repeated transient increases in [Ca$^{2+}$]$_i$ (Fig. 4, A and C). The mean peak response of cells responding to VEGF-D was 4.4-fold above the control fluorescence signal (S.E. 0.35, n = 61 cells from three preparations). In contrast, treatment of the same cultures with 25 ng/ml VEGF-A caused a brisk, almost synchronous, large peak response within 10–20 s of VEGF-A addition, followed by a sustained plateau (Fig. 4, B and C). The peak response to VEGF-A was 7.6-fold above the basal level (S.E. 0.43, n = 59 cells from three preparations), markedly higher than that induced by VEGF-D. The phase of sustained intracellular Ca$^{2+}$ elevation induced by VEGF-A was abolished by removal of Ca$^{2+}$ from the extracellular medium, whereas the VEGF-D-induced Ca$^{2+}$ transients were independent of extracellular Ca$^{2+}$ (results not shown). These findings indicate that VEGF-A and VEGF-D have strikingly different effects on the mobilization of intracellular Ca$^{2+}$ in endothelial cells, and stimulate Ca$^{2+}$ fluxes via distinct mechanisms.

The role of intracellular Ca$^{2+}$ signaling in the mitogenic response to VEGF-A was investigated by examining the effects of the cell-permeant Ca$^{2+}$-chelating agent BAPTA/AM on proliferation of HUVECs. Treatment of cells with BAPTA/AM at 5 μg/ml, a concentration that inhibits VEGF-induced mobilization of intracellular Ca$^{2+}$ and PGL$_2$ production without lowering the basal [Ca$^{2+}$]$_i$ (8), completely blocked the stimulation of proliferation by VEGF-A (Fig. 4D). In parallel cultures, VEGF-D caused no increase in proliferation compared with control, unstimulated cells.

Activation of Endothelial PI3K/Akt Signaling—Treatment with VEGF-D for 10 min induced a concentration-dependent Akt activation that was detectable at 500 ng/ml, reached a maximum at 1 μg/ml, and was similar to activation by 25 ng/ml VEGF-A (Fig. 5A). The effect of VEGF-D was also time-dependent but slower and more transient than that of VEGF-A with detectable activation at 10 min that persisted for up to 30 min (Fig. 5A). Similar to VEGF-A, VEGF-D-induced Akt activation was blocked by SU5614, wortmannin, and LY294002 (Fig. 5B). Akt activation mediates two major endothelial responses to VEGF-A as follows: inhibition of apoptosis and activation of eNOS via Akt-dependent phosphorylation at Ser-1177 (9, 10). As shown in Fig. 5C, 1 μg/ml VEGF-D decreased the frequency of apoptotic cells (30–40%) compared with the effects of serum deprivation of HUVECs (50–60%), but the anti-apoptotic effect of VEGF-D was consistently weaker than that of VEGF-A (20–28% apoptotic cells). The anti-apoptotic effects of VEGF-D and VEGF-A were similarly prevented by pretreatment with SU5614, LY294002, and wortmannin (Fig. 5C). VEGF-D in-

**Fig. 2.** Induction of PLC-γ tyrosine phosphorylation by VEGF-D and VEGF-A. A, confluent and quiescent cultures of HUVECs were incubated for the indicated times with medium (control, C), 25 ng/ml VEGF-A (A), or 1 μg/ml VEGF-D (D). Whole-cell lysates were then immunoblotted with antibodies to PLC-γ phosphorylated at Tyr-783 (upper) or total PLC-γ (lower). B, HUVECs were incubated for 60 min with medium (control, C), 25 ng/ml VEGF-A (A), or the indicated concentrations of VEGF-D and were immunoblotted as indicated in A. The results are representative of three independent experiments yielding very similar results.
duced eNOS phosphorylation at Ser-1177 but was noticeably less effective than VEGF-A. Stimulation of eNOS phosphorylation was detectable only at 1 μg/ml VEGF-D, or were incubated for 10 min with medium (C), 25 ng/ml VEGF-A (A), or the indicated concentrations of VEGF-D. Whole-cell lysates were prepared and immunoblotted with antibody to ERKs1/2 phosphorylated at Thr-202/Tyr-204 (upper) or total ERKs1/2 (lower). The results are representative of three independent experiments yielding very similar results. Fold increases in ERK phosphorylation relative to the control level after different times of VEGF-A and -D treatment were quantified by scanning densitometry and are indicated below the respective lanes. B, cells were pretreated for 15 min with medium (C), or 1 μg/ml VEGF-A (A), or 1 μg/ml VEGF-D (D). Lysates were then immunoblotted with antibodies to either ERKs1/2 phosphorylated at Thr-202/Tyr-204 or total ERKs1/2. The results are representative of three independent experiments yielding very similar results.

**Fig. 4. Differential effects of VEGF-D and VEGF-A on intracellular Ca**2+.

A–C, confluent cultures of HUVECs grown on gelatin-coated glass coverslips were incubated for 20 min with the intracellular Ca**2+ indicator Fluo-4 AM and then treated with either 1 μg/ml VEGF-D alone (A), 25 ng/ml VEGF-A alone (B), or 1 μg/ml VEGF-D followed by 25 ng/ml VEGF-A (C). The open and black bars indicate the times of treatment with VEGF-D and VEGF-A respectively. Before and after addition of growth factors, fluorescence was measured continuously by using a Zeiss 510CLSM microscope and software and is represented as the relative Fluo-4 AM fluorescence expressed in arbitrary units. Two typical results obtained from single cells are shown and are representative of results obtained from treatment of three independent cultures. D, HUVECs were plated at 10^4 cells per well and, 24 h after plating, were incubated with fresh EBM containing 0.5% FBS with either no addition (C, control) or supplemented with 25 ng/ml VEGF-A (A), 5 μg/ml BAPTA/AM (BA), 25 ng/ml VEGF plus 5 μg/ml BAPTA/AM (A+BA), or 1 μg/ml VEGF-D (D). Cell numbers were determined 3 days later. BAPTA/AM blocks the stimulation of cell proliferation by VEGF-A; VEGF-D does not increase cell number. The results are representative of four independent experiments. *, p < 0.027 for VEGF-A versus control; †, p < 0.034 for VEGF-A versus VEGF-A+BAPTA/AM.

Regulation of Gene Expression—Endothelial genes regulated by VEGF-A have been identified recently (29) by oligonucleotide array analysis. To compare the abilities of VEGF-A and VEGF-D to induce gene expression in HUVECs, we examined the effects of VEGF-D on mRNA expression of four genes that were strongly up-regulated by VEGF-A, the zinc finger transcription factor EGR3, and three related members of the N4A family of nuclear receptors, NUR77, NURR1, and NOR1 (29). In parallel cultures of HUVECs, treatment with 25 ng/ml
FIG. 6. Effects of VEGF-A and VEGF-D on eNOS phosphorylation. A, confluent and quiescent cultures of HUVECs were incubated for 10 min with medium (control, C), 25 ng/ml VEGF-A (A), or the indicated concentrations of VEGF-D, or were incubated for the indicated times with 25 ng/ml VEGF-A (A) or 1 μg/ml VEGF-D (D). Whole-cell lysates were prepared and immunoblotted with antibody to eNOS phosphorylated at Ser-1177 (upper) or total eNOS (lower). The results are representative of three independent experiments yielding very similar results. B, cells were pretreated for 15 min with either an equivalent volume of Me2SO (control, C), 5 μM SU5614 (SU), 10 μM LY294002 (LY), or 100 nM wortmannin (Wo) and then received no further addition or were treated for a further 10 min with 25 ng/ml VEGF-A (A) or 1 μg/ml VEGF-D (D). Lysates were then immunoblotted with antibody to Akt phosphorylated at Ser-473 (upper) or total Akt (lower). The results are representative of three independent experiments yielding very similar results. C, subconfluent HUVECs were incubated for 24 h with 10% serum (FBS) or were pretreated for 30 min in serum-free medium containing either an equivalent volume of Me2SO (control, C), 5 μM SU5614 (SU), 10 μM LY294002 (LY), or 100 nM wortmannin (Wo) and incubated for a further 24 h with either no addition, with 25 ng/ml VEGF-A (A), or 1 μg/ml VEGF-D (D). The frequency of apoptotic cells was then determined by flow cytometric analysis of annexin V+/PI+ cells and is expressed as the mean percentages ± S.D. The values shown are representative of three independent experiments yielding very similar results. *, p < 0.05 versus control; **, p < 0.01 versus control; ††, p < 0.001 versus control; †††, p < 0.001 versus VEGF-A; ††††, p < 0.001 versus VEGF-A or VEGF-D.

FIG. 5. Effects of VEGF-A and VEGF-D on Akt activation and cell survival. A, confluent and quiescent cultures of HUVECs were incubated for 10 min with medium (control, C), 25 ng/ml VEGF-A (A), or the indicated concentrations of VEGF-D, or were incubated for the indicated times with 25 ng/ml VEGF-A (A) or 1 μg/ml VEGF-D (D). Whole-cell lysates were prepared and immunoblotted with antibody to Akt phosphorylated at Ser-473 (upper) or total Akt (lower). The results are representative of three independent experiments yielding very similar results. B, cells were pretreated for 15 min with either an equivalent volume of Me2SO (control, C), 5 μM SU5614 (SU), 10 μM LY294002 (LY), or 100 nM wortmannin (Wo) and then received no further addition or were treated for a further 10 min with 25 ng/ml VEGF-A (A) or 1 μg/ml VEGF-D (D). Lysates were then immunoblotted with antibody to Akt phosphorylated at Ser-473 (upper) or total Akt (lower). The results are representative of three independent experiments yielding very similar results. C, subconfluent HUVECs were incubated for 24 h with 10% serum (FBS) or were pretreated for 30 min in serum-free medium containing either an equivalent volume of Me2SO (control, C), 5 μM SU5614 (SU), 10 μM LY294002 (LY), or 100 nM wortmannin (Wo) and incubated for a further 24 h with either no addition, with 25 ng/ml VEGF-A (A), or 1 μg/ml VEGF-D (D). The frequency of apoptotic cells was then determined by flow cytometric analysis of annexin V+/PI+ cells and is expressed as the mean percentages ± S.D. The values shown are representative of three independent experiments yielding very similar results. *, p < 0.05 versus control; **, p < 0.01 versus control; ††, p < 0.001 versus control; †††, p < 0.001 versus VEGF-A; ††††, p < 0.001 versus VEGF-A or VEGF-D.
VEGF-A caused a rapid and striking increase in expression of EGR3 and NR4A genes, whereas 1 μg/ml VEGF-D induced a much weaker response, equivalent to ~10% or less of the response to VEGF-A in each case (Fig. 7).

Chemotactic Responses to VEGF-D—VEGF-D induced a strong chemotactic response in HUVECs with a detectable effect at 500 ng/ml and a maximum response at 1 μg/ml that ranged between 30 and 50% (n = 4) of the maximum VEGF-A-induced migratory response measured in parallel cell cultures (Fig. 8A), and was blocked by SU5614 (Fig. 8B). Akt-mediated eNOS phosphorylation is strongly implicated in mediating the migratory response to VEGF-A (14). To investigate the role of this pathway in VEGF-D-induced chemotaxis, HUVECs were pretreated with LY294002, wortmannin, and two inhibitors of cNOS, L-NMMA and L-NAME. The results showed that the chemotactic responses to VEGF-D and VEGF-A were both strongly decreased by inhibition of PI3K and eNOS (Fig. 8, C and D).

In Vitro and in Vivo Angiogenesis—The ability of VEGF-D to promote endothelial tubulogenesis was evaluated in cocultures of human vascular endothelial cells and fibroblasts, which develop endothelially derived tubules in response to stimulation with angiogenic growth factors (30). As visualized by immunostaining for CD31, 500 ng/ml and a maximum response at 1 μg/ml that ranged between 30 and 50% (n = 4) of the maximum VEGF-A-induced migratory response measured in parallel cell cultures (Fig. 8A), and was blocked by SU5614 (Fig. 8B). Akt-mediated eNOS phosphorylation is strongly implicated in mediating the migratory response to VEGF-A (14). To investigate the role of this pathway in VEGF-D-induced chemotaxis, HUVECs were pretreated with LY294002, wortmannin, and two inhibitors of cNOS, L-NMMA and L-NAME. The results showed that the chemotactic responses to VEGF-D and VEGF-A were both strongly decreased by inhibition of PI3K and eNOS (Fig. 8, C and D).

To investigate further the relative angiogenic responses of the two growth factors, we also compared the effects of VEGF-D and VEGF-A on neovascularization in vivo by using a mouse sponge implant model. VEGF-D at 1 μg/ml significantly increased the number of vessels in subcutaneously implanted sponges above the saline control group (Fig. 10). A concentration of 2 μg/ml induced a similar increase in angiogenesis, whereas 500 ng/ml VEGF-D had no significant effect. Consistent with the results obtained in the in vitro assay of tubulogenesis, VEGF-A at 25 ng/ml induced a significantly stronger angiogenic response in the sponge implants than VEGF-D at 1 or 2 μg/ml.

**DISCUSSION**

The fully processed form of VEGF-D binds with high affinity to VEGFR2 and VEGFR3, but neither the relative importance of these receptors for mediating biological responses to VEGF-D in endothelial cells nor the signaling pathways involved have been fully elucidated. VEGFR3 is thought to be primarily responsible for mediating effects of VEGF-D in lymphatic endothelial cells, but the role of VEGFR2 in mediating effects of this factor in vascular endothelial cells is less well understood. Because VEGFR2 is the major receptor responsible for mediating endothelial signaling and biological responsiveness to VEGF-A, other VEGFR2 ligands such as VEGF-D might be inferred to have similar consequences for VEGFR2 activity and trigger a spectrum of signaling events and biological functions similar to that induced by VEGF-A. The goals of the present study were therefore to identify the endothelial signaling pathways and downstream biological responses activated by processed VEGF-D, and to investigate any potential differences in signal transduction and biological effects induced by two VEGFR2 ligands by systematically comparing VEGF-D with VEGF-A.

The results demonstrate that VEGF-D induced KDR tyrosine phosphorylation, tyrosine phosphorylation of PLC-γ, activation of PKC-dependent ERK1/2 and PI3K-dependent Akt pathways, and multiple biological responses including eNOS activation, cell survival, migration, tubulogenesis, and in vivo angiogenesis. The fact that signaling events and biological responses were blocked by the specific KDR inhibitor SU5614, strongly suggests that the biological effects of VEGF-D in HUVECs are mediated largely via VEGFR2. In addition, the concentration range in which VEGF-D activated intracellular signaling events and elicited endothelial cellular functions (0.3–1 μg/ml) showed good agreement with both biologically active VEGF-D concentrations reported in previous studies (23, 25) and affinity constants (4.8–5.6 × 10^-8 M; equivalent to ~1 μg/ml) for binding of the mature VEGF-D form to KDR/VEGFR2 (19, 31). We were unable to detect an effect of VEGF-D on VEGFR3 activity in HUVECs, suggesting that the fully processed form of VEGF-D is also a weak activator of this receptor in endothelial cells. We cannot preclude, however, that VEGF-D does cause some activation of VEGFR3 in HUVECs.
and that this receptor may therefore partly mediate biological effects of VEGF-D.

A major finding of this study is that whereas VEGF-D activates arrays of intracellular signaling cascades and associated biological responses that exhibit similarities to those triggered by VEGF-A, VEGF-D and VEGF-A have strikingly differential effects on KDR activity, signaling, and biological effects in endothelial cells. Thus, direct comparison of the effects of VEGF-D and VEGF-A revealed marked differences in the potency, efficacy, and kinetics displayed by these growth factors in stimulating endothelial signal transduction and biological responses. KDR is activated through ligand-stimulated receptor dimerization and trans/autophosphorylation of tyrosine residues in the cytoplasmic kinase domain. Tyr-1054 and Tyr-1059 in the activation region of the tyrosine kinase domain are essential for maximal activation of the KDR receptor kinase, and mutation of both these residues reduces VEGF-stimulated receptor activation to ~10% of that for the native receptor (26). VEGF-D weakly induced KDR phosphorylation at Tyr-1054 and Tyr-1059 in HUVECs compared with VEGF-A, indicative of a markedly reduced level of KDR activation. Furthermore, compared with VEGF-A, VEGF-D induced KDR phosphorylation with strikingly slower kinetics. Although slower and weaker at early times, the effect of VEGF-D on KDR phosphorylation was more sustained compared with that of VEGF-A, and at later times (60 min), VEGF-D stimulated KDR phosphorylation as effectively as VEGF-A. As phosphorylation at Tyr-1054 and Tyr-1059 is also required for efficient internalization of KDR (26), the more sustained effect of VEGF-D on KDR phosphorylation at Tyr-1054 and Tyr-1059 may be due to slower internal and hence delayed down-regulation of the receptor. It is possible that despite the lower level of KDR activity induced by VEGF-D compared with VEGF-A, the sustained increase in KDR phosphorylation induced by VEGF-D may play an important role in mediating its biological effects in endothelial cells. The reduced efficacy and slower kinetics of VEGF-D-induced KDR tyrosine phosphorylation are most likely to be due to differences in the relative abilities of VEGF-D and VEGF-A to bind to KDR. This possibility is supported by the finding that VEGF-D at concentrations up to 1 μg/ml competed weakly with VEGF-A for binding to high affinity sites in HUVECs. VEGF-D was also unable to compete with VEGF-A binding to NP-1 (results not shown), a co-receptor for VEGF-A that may be required for optimal KDR binding and activation (32).

Despite the relatively weak activation of KDR by VEGF-D, similar to the effects of VEGF-A, this factor induced strong activation of ERK1/2 and Akt, which occurred via PKC- and MEK-mediated and PI3K-dependent mechanisms, respectively. A major difference in the regulation of these pathways by the two factors, however, was the slower kinetics of ERK and Akt activation in response to VEGF-D, a finding that is most likely explained by the slower activation of KDR by VEGF-D. VEGF-D also induced markedly slower PLC-γ and eNOS phosphorylation. The occurrence of low KDR activity and relatively strong downstream signaling displayed by VEGF-D-treated cells nevertheless suggests that a relatively low percentage of receptor occupancy and activation is required for maximum or near-maximum signal generation via the ERK1/2 and Akt pathways.

Although inducing strong activation of the ERK and Akt pathways, VEGF-D was significantly less effective than VEGF-A in stimulating either PLC-γ tyrosine phosphorylation, mobilization of intracellular Ca²⁺, or downstream biological
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**Fig. 9.** Signaling pathways mediating VEGF-D-induced tubulogenesis. **A,** at the start of the experiment (day 1) and at each medium change (days 4, 7, and 9), the indicated concentrations of VEGF-A or VEGF-D were added to co-cultured human endothelial cells and human fibroblasts. Endothelial tubulogenesis was visualized by immunostaining for CD31 on day 11, and culture wells were photographed at ×50 magnification. **B,** quantification of tubule formation induced by VEGF-A and VEGF-D. Results are presented as mean percentages ± S.E. of control, unstimulated tubule formation. *, p < 0.05; ***, p < 0.001 versus control. **C,** endothelial tubulogenesis was determined in response to 25 ng/ml VEGF-A or 500 ng/ml VEGF-D and in the absence or presence of either an equivalent volume of Me2SO (solvent control), 5 μM SU5614, 10 μM LY294002, or 3 μM GF109203X (GF). **D,** quantification of tubule formation shown in C; **,** p < 0.01; ***, p < 0.001 versus control; ††, p < 0.001 versus control.

responses mediated via ERK and Akt. The differences in Ca2+ signaling were particularly striking. The response of endothelial cells to VEGF-A was rapid, almost synchronous, and comprised a large peak response followed by a sustained plateau. In contrast, VEGF-D induced an increase in intracellular Ca2+ concentration that was typically more delayed in onset, was smaller, transient, and lacked a sustained phase after the initial peak, and displayed considerable variability in latency and amplitude. The finding that the sustained elevation of intracellular Ca2+ induced by VEGF-A was dependent on extracellular Ca2+ demonstrates that these factors regulate Ca2+ fluxes via distinct mechanisms. The sustained increase in intracellular Ca2+ induced by VEGF-A is most likely due to the activation of Ca2+ influx via a pathway that is so far unidentified. VEGF-D also had noticeably weaker effects on eNOS phosphorylation at Ser-1177 and cell survival and was unable to significantly stimulate two responses mediated via the MEK/ERK pathway, endothelial cell proliferation and PGI2 production. A further marked difference between the two factors was the much weaker effect of VEGF-D on expression of EGR3 and NR4A nuclear receptor genes, previously shown to be strongly up-regulated by VEGF-A (29). VEGF-D induced a strong chemotactic response that was blocked by inhibition of either PI3K or eNOS, whereas VEGF-A evoked a stronger migratory response dependent on a similar range of signaling pathways. These findings indicate that VEGF-D is able to induce cell survival and eNOS Ser-1177 phosphorylation via the PI3K/Akt pathway and migration via the PI3K/Akt and eNOS pathways but, in contrast to VEGF-A, is unable to trigger ERK-mediated biological responses such as cell proliferation and PGI2 production (Fig. 11). Because VEGF stimulation of endothelial cell proliferation and PGI2 production are also critically dependent on mobilization of intracellular Ca2+, the inability of VEGF-D to stimulate these biological responses is likely to be due, at least in part, to the smaller and more transient increase in [Ca2+]i, stimulated by VEGF-D. The sustained elevation of intracellular Ca2+ induced by VEGF-A and lacking in the response to VEGF-D is likely to play a key role in mediating longer-term biological functions such as proliferation. The role of ERK1/2 activation in the biological functions of VEGF-D is unclear and warrants further investigation.

The induction of tubulogenesis by both VEGF-D and VEGF-A was dependent on PI3K and PKC, consistent with an essential role of migration, cell survival, and possibly PKC-dependent ERK activation in this process. The weaker angiogenic response stimulated by VEGF-D in vitro and in vivo can be explained as a consequence of its more restricted signaling repertoire and reduced efficacy in generating intracellular signals, and hence its weaker ability to stimulate endothelial cell survival and cell migration, and its inability to promote cell proliferation.

Taken together, these results (summarized in Fig. 11) support the conclusion that whereas VEGF-D activates KDR and stimulates intracellular signaling and cellular functions via KDR, it has distinct consequences for KDR activation and produces a pattern of signaling and biological responsiveness in endothelial cells that differs markedly from that induced by
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VEGF-D signaling is unclear. Tyr-1054 and 1059, increases phosphorylation of PLC-γ by several reports (34) of VEGF-D induces angiogenesis in the porcine heart (33) and elucidating the mechanism of action of VEGF-D is emphasized of this receptor.

VEGF-A in efficacy, kinetics, and range of responses. Identification of these differences will be essential for elucidating the distinct biological activities of VEGF-D and VEGF-A and the underlying mechanisms involved. The practical importance of elucidating the mechanism of action of VEGF-D is emphasized by the finding that adenoviral gene delivery of the mature form of VEGF-D induces angiogenesis in the porcine heart (33) and by several reports (34–36) that VEGF-D is overexpressed in cancers, including colorectal, breast, and ovarian carcinoma. Mechanistically, these results have implications for understanding how multiple ligands for the same VEGF receptors can generate ligand-specific biological responses. The ability of different VEGF family ligands to generate distinct spectra of signaling events through KDR may allow for greater functional versatility and diversity in the biological response to activation of this receptor.

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Fig. 11. VEGF-D signaling pathways in vascular endothelial cells. VEGF-D activates KDR as determined by phosphorylation at Tyr-1054 and 1059, increases phosphorylation of PLC-γ at Tyr-783, and induces mobilization of intracellular Ca2+. PI3K-mediated Akt activation, and PKC/MEK-mediated activation of ERKs1 and -2. The PI3K/Akt pathway also mediates VEGF-D stimulation of eNOS phosphorylation at Ser-1177 and cell survival. VEGF-D stimulation of migration is mediated via the PI3K/Akt pathway and eNOS activation. Signaling pathways mediating effects of VEGF-D on migration and cell survival are essential for the promotion of endothelial tubulogenesis. VEGF-D is a weak inducer of endothelial PGI2 generation and proliferation probably because of the smaller and more transient effect of VEGF-D on intracellular Ca2+ signaling. The biological role of ERK activation in VEGF-D signaling is unclear.