KDR activation is crucial for VEGF\textsubscript{165}-mediated Ca\textsuperscript{2+} mobilization in human umbilical vein endothelial cells

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Cunningham, Sonia A., Tuan M. Tran, M. Pia Arrate, Robert Bjercke, and Tommy A. Brock. KDR activation is crucial for VEGF\textsubscript{165}-mediated Ca\textsuperscript{2+} mobilization in human umbilical vein endothelial cells. Am. J. Physiol. 276 (Cell Physiol. 45): C176–C181, 1999.—We have prepared a polyclonal mouse antibody directed against the first three immunoglobulin-like domains of the kinase insert domain-containing receptor (KDR) tyrosine kinase. It possesses the ability to inhibit binding of the 165-amino acid splice variant of vascular endothelial cell growth factor (VEGF\textsubscript{165}) to recombinant KDR in vitro as well as to reduce VEGF\textsubscript{165} binding to human umbilical vein endothelial cells (HUVEC). These results confirm that the first three immunoglobulin-like domains of KDR are involved in VEGF\textsubscript{165} interactions. The anti-KDR antibody is able to completely block VEGF\textsubscript{165}-mediated intracellular Ca\textsuperscript{2+} mobilization in HUVEC. Therefore, it appears that binding of VEGF\textsubscript{165} to the fms-like tyrosine kinase (Flt-1) in these cells does not translate into a Ca\textsuperscript{2+} response. This is further exemplified by the lack of response to placental growth factor (PIGF), an Flt-1-specific ligand. Additionally, PIGF is unable to potentiate the effects of submaximal concentrations of VEGF\textsubscript{165}. Surprisingly, the VEGF-PIGF heterodimer was also very inefficient at eliciting a Ca\textsuperscript{2+} signaling event in HUVEC. We conclude that KDR activation is crucial for mobilization of intracellular Ca\textsuperscript{2+} in HUVEC in response to VEGF\textsubscript{165}.

Vascular endothelial cell growth factor (VEGF), a mitogen that promotes angiogenesis, vasculo genesis, and endothelial differentiation, is crucial for embryonic development. This is exemplified by VEGF-deficient embryos that do not survive beyond midgestation even in the heterozygous state (6, 13). VEGF binds to and activates the kinase insert domain-containing receptor/fetal liver kinase (KDR/Flk-1) and fms-like tyrosine kinase (Flt-1) (30). In endothelial cells, where both receptors are expressed simultaneously, it is not clear whether VEGF stimulation results in redundant signaling. However, unique signaling pathways and/or expression patterns are apparent, since knockout mice reveal differing lethal phenotypes for each receptor. Whereas Flk-1 is essential for embryonic endothelial cell differentiation and vasculogenesis (25), Flt-1 plays a crucial role in the organization of the developing vasculature (14).

Several studies concerning VEGF signaling have been performed in endothelial cells expressing endogenous receptors. Thus VEGF promotes phosphorylation of phosphatidylinositol 3-kinase, rasGAP, and Nck in bovine aortic endothelial cells (17). Most recently, VEGF has been shown to stimulate tyrosine phosphorylation of p125 focal adhesion kinase and paxillin in human umbilical vein endothelial cells (HUVEC; Ref. 1). However, it is not possible to delineate the individual pathways activated by each receptor in these studies.

Our understanding of the separate signaling pathways utilized by KDR/Flt-1 has mainly come from recombinant expression of these receptors in heterologous cells. Expression in porcine aortic endothelial cells (PAEC) defines a role for KDR but not Flt-1 in VEGF-induced cell proliferation (31). This is further supported by the lack of proliferation obtained by expression and activation of Flt-1 in NIH/3T3 cells (24). VEGF also appears to be a mitogen for pancreatic ductal epithelium that expresses the KDR but not the Flt-1 receptor (21). Other functions attributed to KDR in PAEC are changes in cell morphology, actin reorganization, membrane ruffling, and chemotaxis (31). In addition to endothelial cells, recent reports have described Flt-1 expression in pericytes (18, 28) and monocytes (3, 8). However, it is not clear whether the signaling mechanisms utilized by Flt-1 in this background are modified by the expression of specific intracellular signaling proteins.

One of the first cell-based signaling assays to be analyzed for VEGF was the mobilization of intracellular Ca\textsuperscript{2+} in endothelial cells (16). Activation of phospholipase C (PLC) results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate into d-myo-inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol. The elevated IP\textsubscript{3} levels cause release of Ca\textsuperscript{2+} from the endoplasmic reticulum. Both autophosphorylated KDR and Flt-1 are capable of binding to the src homology 2 (SH2) domains of PLC-γ (10, 23), and tyrosine phosphorylation of PLC-γ occurs following VEGF stimulation of cells transfected with Flt-1 (23) and KDR (29). This would suggest that the Ca\textsuperscript{2+} mobilization in response to VEGF stimulation of endothelial cells is due to redundant signaling through both the Flt-1 and KDR receptors. To test this, we raised neutralizing mouse polyclonal antiserum against the VEGF binding region of KDR. With this tool, we directly addressed the involvement of endog-
enously expressed KDR with respect to Ca\(^{2+}\) signaling in primary cultures of endothelial cells.

**METHODS**

Preparation of polyclonal mouse serum. Immunoglobulin domains 1–3 encompassing amino acids 1–338 of KDR were fused to the carboxy-terminal end of murine IgG2A as previously described for Flt-1 (amino acids 1–348; Ref. 11). The construct was subcloned into the pBakPak vector (Clontech), and recombinant baculovirus was prepared according to standard procedures. SF21 cells were infected with virus, and the serum-free medium containing the secreted fusion proteins was harvested after 72 h. Recombinant protein was purified over protein A Sepharose columns (KDR) and used for injection into mice. Mice were injected with 50 µg of antigen diluted 1:1 in PBS and complete Freund's adjuvant on day 0. Animals were boosted with 25 µg of antigen diluted 1:1 in PBS and incomplete Freund's adjuvant on days 21 and 45. The serum used for these experiments was collected after 10 days after each boost. Further boosts and bleedings were performed as necessary.

C61 culture. HUVEC (Cascade Biologics) in this study were used between passages 1 and 5. Cells were grown on gelatin-coated plates in medium 199, 2 mM L-glutamine, 0.2 µg/ml heparin (Sigma), and 100 µg/ml endothelial mitogen (Biomedical Technologies).

\(^{125}\)I-labeled 165-amino acid VEGF receptor binding on recombinant protein. Both Flt-1 and KDR-Fc fusions were immobilized on Immulon 4 plates using a goat anti-mouse capture antibody. \(^{125}\)I-labeled 165-amino acid splice variant of VEGF (VEGF\(_{165}\)) at 4 ng/ml was bound to the receptor fusions at room temperature for 90 min in binding buffer consisting of medium 199 (BRL), 1% BSA, and 25 mM HEPES (pH 7.4). Nonspecific counts were estimated using unlabeled VEGF\(_{165}\) at 400 ng/ml to compete. For inhibition, various dilutions of antisera against KDR-Fc were preincubated with receptor for 60 min before radiolabeled VEGF\(_{165}\) addition. Wells were washed three times with cold PBS and directly counted in a gamma counter.

\(^{125}\)I-labeled VEGF\(_{165}\)-receptor binding on endothelial cells. HUVEC were plated on 48-well plates at 10,000 cells/well. Binding was performed essentially as described for the recombinant protein after 4 days of culture. After the PBS wash, cells were detached from the plate with 150 µl of 0.1 M NaOH and counted in a gamma counter.

Endothelial Ca\(^{2+}\) mobilization assay. HUVEC were detached from monolayers and loaded with 2 µM Fura 2-AM for 30 min at 37°C in PBS. Cells were washed and resuspended at a final count of 0.5 × 10\(^6\) cells/ml. For antibody studies, polyclonal serum at dilutions of 1:100 to 1:400 was incubated with cells at 37°C for 30 min before stimulation with VEGF\(_{165}\). Fluorescence was measured at 340 and 380 nm, and intracellular Ca\(^{2+}\) concentration was estimated as described previously (16). For each experiment, a baseline resting fluorescence was measured for 100 s before stimulation with 10 ng/ml VEGF\(_{165}\), 190 ng/ml placental growth factor (PIGF; 149 amino acids; R&D), or 100–500 ng/ml VEGF-PIGF heterodimer.

**RESULTS**

From our previous work and that of others focusing on the Flt-1 receptor, we reasoned that the VEGF binding determinants of KDR would reside in the first three immunoglobulin-like domains (4, 11, 12, 15). Therefore, we injected a recombinant KDR encompassing this region into mice as an immunogen to produce polyclonal antiserum.

To determine the neutralizing capacity of the polyclonal antibody, we performed an in vitro \(^{125}\)I-VEGF\(_{165}\) binding assay against the recombinant receptor. Figure 1 shows that, at a 1:100 dilution and a receptor coating of 50 ng/well, the anti-KDR serum was able to inhibit VEGF\(_{165}\) binding to KDR by ~70%. A full inhibition was not achieved, and this was probably due to the high levels of recombinant receptor used in this in vitro assay. The extent of inhibition was reduced with progressive dilutions. Thus the polyclonal serum possesses a proportion of antibodies against regions in the extracellular domain that are important for ligand binding. Flt-1 and KDR display 33% identity in the first three immunoglobulin domains as defined by our construct. Because both receptors bind VEGF\(_{165}\) with high affinity, we tested for the ability of the anti-KDR serum to cross-neutralize VEGF\(_{165}\) binding to Flt-1. In experiments performed in parallel, Flt-1 bound 8,261 ± 1,435 counts/min (cpm) per well, whereas KDR bound 3,135 ± 470 cpm/well (n = 4). Figure 1 shows that the KDR antibody at dilutions of 1:100 and greater has no effect on VEGF\(_{165}\) binding to Flt-1 and thus defines its specificity for KDR.

Having demonstrated the neutralizing capability and specificity of the polyclonal serum on recombinant protein, we performed similar binding assays on the surface of HUVEC. All experiments were performed on cells of low passage number and similar confluencies. Figure 2 depicts the average results from four experiments; it is clear that the anti-KDR serum inhibits a proportion of the \(^{125}\)I-VEGF\(_{165}\)-binding sites available on the cell surface. Inhibition starts to plateau at serum dilutions of 1:3.

Fig. 1. Specificity of anti-kinase insert domain-containing receptor (anti-KDR) polyclonal serum: inhibition of \(^{125}\)I-labeled 165-amino acid splice variant of vascular endothelial cell growth factor (VEGF\(_{165}\)) binding to KDR but not to fms-like tyrosine kinase (Flt-1) in vitro. Immobilized recombinant receptors were preincubated with dilutions of anti-KDR or nonimmune serum at 1:100, 1:300, and 1:900 for 60 min before addition of 4 ng/ml \(^{125}\)I-VEGF\(_{165}\). Results are expressed as %inhibition of VEGF\(_{165}\) binding. KDR 1–3 and Flt 1–3, first 3 immunoglobulin-like domains of KDR and Flt-1, respectively.
dilutions of ~1:100. A maximum inhibition of ~60% was recorded at 1:50 dilution. Limited serum supply did not allow the use of lower dilutions. Nonimmune serum had negligible effects. The serum is useful for detection of recombinant KDR by Western blot analysis in addition to immunocytochemistry. However, it is unable to detect the low levels of expression of KDR in total cell lysates of HUVEC (data not shown).

We and others have shown that PLC-γ can bind to autophosphorylated KDR and Flt-1 receptors (10, 23, 29). Furthermore, we have identified the phosphotyrosine binding sites on each receptor that are important for this interaction. A consequence of PLC-γ activation is the mobilization of intracellular Ca^{2+}. We assessed the ability of our neutralizing KDR antibody to inhibit VEGF_{165}-induced Ca^{2+} mobilization in HUVEC. A representative experiment is shown in Fig. 3 (n = 3). The first trace depicts the response to 10 ng/ml VEGF_{165}. After a short delay, a rapid transient rise of intracellular Ca^{2+} concentration, peaking at ~100 nM within 60 s, is observed following VEGF_{165} addition. Preincubation of cells with the anti-KDR serum attenuated this rapid Ca^{2+} rise at dilutions of 1:100. At 1:50 dilution of antibody, no further effect was recorded (data not shown). With further dilutions of the antibody, the inhibition was gradually lost. Nonimmune serum at similar dilutions was without effect.

Figure 4 shows that acute addition of a 1:100 dilution of the anti-KDR polyclonal serum itself does not activate the KDR receptor and result in Ca^{2+} mobilization. Both normal mouse serum (Fig. 4A) and anti-KDR serum (Fig. 4B) do, however, elicit an equivalent but minimal Ca^{2+} response that occurs immediately after serum addition. This could be due to a variety of agonists found in serum. Nevertheless, this stimulation does not inhibit further stimulation by VEGF_{165} after either acute exposure (Fig. 4, A and B) or preincubation unless specific antibodies against KDR are present (Fig. 3). Figure 4 also demonstrates that a 30-min preincubation with the anti-KDR serum is required to specifically attenuate the VEGF_{165}-mediated Ca^{2+} mobilization.

Our results from Fig. 3 suggested that Flt-1 was unable to mediate Ca^{2+} mobilization in HUVEC. To confirm this, we stimulated the endothelial cells with PIGF, a specific ligand for Flt-1. Figure 5 represents one of three experiments and shows that addition of up to 190 ng/ml PIGF was without effect. Additionally, PCR analysis confirmed the coexpression of both receptors in our cell cultures (data not shown). Thus it appears that binding of PIGF to Flt-1 is not sufficient to trigger an effective response. It has previously been reported that PIGF can potentiate mitogenesis and permeability changes elicited by submaximal VEGF_{165} stimulation (19). In this assay, we determined that 0.4 ng/ml VEGF_{165} was sufficient to elicit a perceptible Ca^{2+} mobilization. However, PIGF was unable to potentiate the response to this suboptimal VEGF_{165} Concentration.

VEGF can dimerize with itself to form homodimers or with PIGF to form heterodimers. Although PIGF homodimers are specific for Flt-1, the heterodimer is also capable of interacting with KDR. To our knowledge, the effect of VEGF-PIGF heterodimer on Ca^{2+} signaling has not been previously documented. We find that the

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**Fig. 2.** Inhibition of ^{125}I-VEGF_{165} binding to primary cultures of endothelial cells. Human umbilical vein endothelial cells (HUVEC) were preincubated with dilutions of anti-KDR or nonimmune serum at 1:50, 1:100, 1:300, and 1:900 for 60 min in medium 199. ^{125}I-VEGF_{165} at 4 ng/ml was added. Nonspecific counts were determined with 100-fold excess of unlabeled VEGF_{165}.

**Fig. 3.** Anti-KDR serum inhibits VEGF-induced intracellular Ca^{2+} signaling. HUVEC (~1 × 10^6) were loaded with fura 2-AM, and intracellular Ca^{2+} concentration ([Ca^{2+}]_i) was monitored. VEGF_{165} (10 ng/ml) was added at arrow to cells preincubated with or without anti-KDR serum (KDR ab) at 1:100 and 1:400 dilutions (dil). NMS, nonimmune serum at 1:100. Approximate [Ca^{2+}]_i was calculated according to Ref. 16.
heterodimer is unable to elicit a Ca\(^{2+}\) response at 10 ng/ml, a dose that is maximal for the VEGF\(_{165}\) homodimer. Because the heterodimer binds to KDR with a lower affinity than Flt-1, we raised the concentration to 10- and 50-fold. Figure 6 shows that 100 ng/ml was ineffective and that only a barely perceptible response was achieved at 500 ng/ml.

DISCUSSION

In this study, we show that specific antiserum raised against the first three immunoglobulin-like domains of KDR can inhibit VEGF\(_{165}\) binding to this receptor and consequent signaling though Ca\(^{2+}\) in primary cultures of endothelial cells. Our data confirm the location of the VEGF\(_{165}\) binding determinants on KDR that was recently reported by Kaplan et al. (15). In addition, they are also consistent with the results of Davis-Smyth et al. (12), who demonstrated that ligand binding activity was maintained on Flt-1 by replacement of the second immunoglobulin-like domain with that of KDR. Although both receptors interact with VEGF at high affinity, of the 21 amino acids on Flt-1 that make contact with VEGF in the crystal structure, only 1 is identical in KDR (32). It could be predicted that a neutralizing antiserum raised against KDR would not inhibit VEGF binding to Flt-1. Our data support this prediction.

With reports of additional uncharacterized VEGF binding proteins on endothelial cells, monocytes, and pericytes, it is difficult to assign specific signaling pathways to each receptor (27, 28). To address this, several laboratories have constructed stable cell lines expressing either Flt-1 alone or KDR alone. Although this approach has proven very informative, it has drawbacks. One of the prerequisites for the choice of cell line is an absence of basal expression of these receptors. This also means that they may be missing other key endothelial cell-specific signaling factors that engage with Flt-1/KDR upon receptor activation. Indeed, this is the conclusion of Takahashi and Shibuya (29), who compared transduction of signals from KDR to mitogen-activated protein kinase in endothelial cells vs. NIH/3T3 fibroblast stable cell lines. We chose to address this concern by preparing specific neutralizing antiserum against KDR and asking what effects the specific blockade of this receptor has on VEGF\(_{165}\) signaling in primary cultures of endothelial cells. In this setting, our data show that the binding of VEGF\(_{165}\) to
Flt-1 should remain unperturbed. Furthermore, it is unlikely that this antibody would inhibit the recently described interactions of VEGF with the unrelated neuropilin-1 receptor (27).

It is clear that VEGF causes the tyrosine phosphorylation of PLC-γ and mobilizes intracellular Ca²⁺ in HUVEC. Furthermore, VEGF induces membrane translocation of protein kinase C (PKC) isofoms α, βI, and δ in vivo (2). It is not clear whether these signals are propagated simultaneously through Flt-1 and KDR or whether signaling through one receptor predominates in this pathway. In the yeast-two hybrid system, both Flt-1 and KDR autophosphorylate tyrosine residues in the juxta-membrane and carboxy-terminal tail that are capable of binding PLC-γ (16). Furthermore, stable cell lines expressing Flt-1 show that this receptor can indeed utilize PLC-γ as a signaling substrate (23). In contrast, heterologous expression of KDR in PAEC lines does not reveal a role for KDR in Ca²⁺ signaling (31). Nevertheless, KDR is capable of mobilizing Ca²⁺ in Xenopus oocytes (20) and can be co precipitated with PLC-γ when expressed in NIH3T3 cells (29). Most importantly, KDR is capable of associating with PLC-γ following its activation in primary cultures of HUVEC (24).

In this study, we show that the Ca²⁺ response elicited by VEGF165 in primary cultures of endothelial cells derived from the umbilical vein is primarily relayed through the KDR receptor. It has previously been shown that approximately one-half of the VEGF165 binding sites on HUVEC can be competed against with PlGF (19). This suggests that Flt-1 is expressed to levels at least as high as KDR in these cells (19). To our knowledge, the only other study utilizing neutralizing antisera to KDR and primary cultures of endothelial cells focused on tissue factor production (8). In combination with PlGF, these authors concluded that both receptors mediated this physiological response.

It has been reported that the full-length Flt-1 receptor is inefficiently autophosphorylated both when expressed in its native endothelial setting and when recombinantly expressed in other cell lines (19, 24). This is not a property of the intracellular domain per se (10). Therefore, it appears that, despite very high affinity binding to VEGF, Flt-1 is maintained in a repressed state. An inability to efficiently dimerize or inhibition of autophosphorylation by intracellular proteins must account for this phenomenon. An analogy would be inhibition of the catalytic activity of the epidermal growth factor receptor by PKC-mediated serine/threonine phosphorylation (7) or association with caveolin (9). Alternatively, Flt-1 may recruit phosphatases more efficiently. This inherent phenomenon associated with Flt-1 may explain our results. However, because the kinase domain possesses good potential binding sites for PLC-γ, we predict that, under defined conditions, Flt-1 has the capacity to contribute to the Ca²⁺ response. This is apparent in monocytes, in which Flt-1 can phosphorylate PLC-γ and mobilize Ca²⁺ quite efficiently (26). Thus, the background expression of the cell is most important. This is further demonstrated by the ability of PlGF to elicit chemotactic and mitogenic effects to a greater degree in coronary venular endothelial cells than in HUVEC (33).

Most interestingly, it is apparent that Flt-1 signaling is dependent on the ligand with which it is activated. Landgren et al. (17) show that Flt-1-expressing PAEC respond to PlGF with increased DNA synthesis, a response lacking after VEGF stimulation. A weak growth stimulatory effect of PlGF has also been documented in HUVEC (22). Nevertheless, PlGF is unable to mediate a rapid transient intracellular Ca²⁺ mobilization in our studies. Potentiation of VEGF action by PlGF has previously been shown for endothelial cell mitogenesis and permeability (19). This phenomenon does not occur for Ca²⁺ mobilization in HUVEC.

We find furthermore, and to our surprise, that the VEGF-PlGF heterodimer is extremely inefficient in signaling through Ca²⁺ in HUVEC. This ligand can bind both Flt-1 and KDR and induces the autophosphorylation of KDR in HUVEC (5). However, it displays a much lower affinity for KDR, and this translates to a 20- to 50-fold lower potency at inducing HUVEC cell proliferation (5). Nevertheless, even at concentrations of 500 ng/ml, a barely detectable Ca²⁺ response could be measured. Thus, unless the heterodimer locally achieves these high levels in vivo, it is unlikely to be a physiological mediator of Ca²⁺ signaling. This suggests that, like Flt-1, KDR may transmit some signals more efficiently than others according to which ligand it interacts with. Furthermore, because Flt-1 binds with high affinity to the VEGF-PIGF heterodimer, we can conclude that binding does not translate to Flt-1 signaling through Ca²⁺ in HUVEC. Finally, it should be noted that, although we can conclude that KDR autophosphorylation is crucial, we cannot rule out the possibility that the simultaneous activation or heterodimerization of Flt-1 and KDR is required for efficient signaling. To test this theory in the background of primary endothelial cells, either specific neutralizing antisera to Flt-1 or specific activation of KDR alone is required.

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