Kinase Insert Domain Receptor (KDR) Extracellular Immunoglobulin-like Domains 4–7 Contain Structural Features That Block Receptor Dimerization and Vascular Endothelial Growth Factor-induced Signaling*

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The vascular endothelial growth factor (VEGF) receptor tyrosine kinase subtype kinase insert domain receptor (KDR) contains seven extracellular Ig-like domains, of which the three most amino-terminal contain the necessary structural features required for VEGF binding. To clarify the functional role of KDR Ig-like domains 4–7, we compared VEGF-induced signaling in human embryonic kidney and porcine aortic endothelial cells expressing native versus mutant receptor proteins in which Ig-like domains 4–7, 4–6, or 7 had been deleted. Western blotting using an anti-receptor antibody indicated equivalent expression levels for each of the recombinant proteins. As expected, VEGF treatment robustly augmented native receptor autophosphorylation. In contrast, receptor autophosphorylation, as well as downstream signaling events, were VEGF-independent for cells expressing mutant receptors. 125I-VEGF₁₆₅ bound with equal or better affinity to mutant versus native receptor, although the number of radioligand binding sites was significantly reduced because a significant percentage of mutant, but not native, receptors were localized to the cell interior. As was the case for native KDR, 125I-VEGF₁₆₅ binding to the mutant receptors was dependent upon cell surface heparan sulfate proteoglycans, and 125I-VEGF₁₆₅ bound with an affinity equal to that of 125I-VEGF₁₆₅ to the native and mutant receptors. It is concluded that KDR Ig-like domains 4–7 contain structural features that inhibit receptor signaling by a mechanism that is independent of neuropilin-1 and heparan sulfate proteoglycans. We speculate that this provides a cellular mechanism for blocking unwanted signaling events in the absence of VEGF.

Vascular endothelial growth factor (VEGF)¹ has for a number of reasons received attention as a key angiogenesis activator (for review, see Ref. 1). Its expression correlates both temporally and spatially with the onset of angiogenesis in several normal and pathophysiological situations (2–5), VEGF elicits a strong angiogenic response in a variety of in vivo experimental models (6, 7), and its actions are largely restricted to vascular endothelial cells (8, 9). An essential role for VEGF in tumor angiogenesis and ischemia-related retinal disorders has been demonstrated by the findings that neutralizing VEGF antibodies or dominant-negative VEGF receptors inhibit both angiogenesis and the progression of these diseases (10–12).

VEGF exhibits high affinity binding to two distinct endothelial cell receptor tyrosine kinases, the fms-like tyrosine kinase Flt1 (13, 14) and the kinase insert domain containing receptor KDR (15, 16). Both receptors possess insert sequences within their catalytic domains and seven immunoglobulin-like domains in the extracellular regions, and they are related to the PDGF family of receptor tyrosine kinases. Although expression of both VEGF receptor types occurs in adult endothelial cells including human umbilical vein endothelial cells, recent findings suggest that KDR and not Flt-1 is able to mediate the mitogenic and chemotactic effects of VEGF (17, 18). VEGF binding to KDR stimulates other cellular responses including enhancement of expression of matrix-degrading enzymes (19), inhibition of apoptosis (20), and regulation of nitric-oxide synthase expression (21). A number of cell signaling proteins that participate in the diverse biological functions of VEGF have been identified, including NCK, PLCγ, mitogen-activated protein kinase, phosphoinositide 3-kinase, focal adhesion kinase, and paxillin (22, 23).

The very earliest steps in KDR-mediated signal transduction, and the KDR structural features that allow those steps, are just starting to be clarified. Based upon the wealth of experimental results obtained using other receptor tyrosine kinases, it is generally accepted that the molecular mechanism by which VEGF initiates signaling is through receptor dimerization followed by receptor autophosphorylation (for review, see Ref. 24). VEGF is a covalent dimer, and part of the mechanism by which it causes KDR dimerization is by binding two receptor monomers simultaneously (25–27). Kit and PDGF receptors are growth factor receptors structurally similar to KDR, and in addition to the interactions between ligand and receptor, receptor-receptor interactions are necessary for receptor dimerization (28, 29). The fourth Ig-like domains of these receptors mediate the receptor-receptor interactions. Whether receptor-receptor interactions are necessary for KDR dimerization is not known.

There are other reasons for suspecting that the extracellular domain of KDR contains structural features that participate in receptor activities that are independent of ligand binding. VEGF is expressed as five alternatively spliced isoforms (30,
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In this study we investigated the consequence of deleting amino acid sequences contained within KDR Ig-like domains 4–7 on receptor function. The experimental results led to the conclusion that this region contains structural features that inhibit receptor dimerization and signaling, and the binding of VEGF to KDR relieves that inhibition, allowing for receptor activation. We propose that this mechanism acts to prevent unwanted receptor dimerization in the absence of growth factor.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Transfected porcine aortic endothelial (PAE) and human embryonic kidney epithelial (HEK293) cells were cultured in DMEM containing 10% newborn calf serum, 0.4 μg/ml puromycin, 10 units/ml penicillin, and 10 μg/ml streptomycin.

**Antibodies**—Rabbit anti-KDR antibody was isolated in our laboratory and targets a polypeptide sequence within the KDR cytosolic domain and targets a polypeptide sequence within the KDR cytosolic domain and targets a polypeptide sequence within the KDR cytosolic domain (35). Anti-NCK, anti-PLCγ, and anti-phosphotyrosine (PY20) monoclonal antibodies were from Transduction Laboratories. Peroxidase-conjugated donkey anti-rabbit and sheep antmouth immunoglobulins were from Amersham Pharmacia Biotech.

**Site-directed Mutagenesis of the KDR cDNA**—The experimental strategy for constructing mutant KDR cDNAs involved the introduction of NofI restriction sites in both the region of KDR between Ig-like domain 7 and the membrane-spanning domain (nucleotides 2195–2202) and the regions between either Ig-like domain 3 and 4 (nucleotides 968–975) or Ig-like domains 6 and 7 (nucleotides 1961–1969). Mutagenesis was done using the native KDR cDNA cloned into an expression vector (36) and a U.S.E. mutagenesis kit (Amersham Pharmacia Biotech.). The resulting plasmids were digested with NofI and religated.

**Results**

**Iodination of VEGF121**—

**Anti-KDR antibody** was isolated in our laboratory and targets a polypeptide sequence within the KDR cytosolic domain (35). Anti-NCK, anti-PLCγ, and anti-phosphotyrosine (PY20) monoclonal antibodies were from Transduction Laboratories. Peroxidase-conjugated donkey anti-rabbit and sheep antmouth immunoglobulins were from Amersham Pharmacia Biotech.

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tion in cells expressing native and mutant receptors was then examined (Fig. 3). The protocol for this experiment involved treating cells with or without 50 ng/ml VEGF, immunoprecipitation with anti-Tyr(P) antibodies, and immunoblotting with the anti-KDR antibody. For HEK293 and PAE cells expressing native KDR, there is no tyrosine phosphorylation of receptor in the absence of VEGF, and growth factor treatment induces a robust response (Fig. 3, A and B). Unexpectedly, KDR(Ig1–3), KDR(Ig1–6), and KDR(Ig1–3, 7) are heavily phosphorylated in control cells, and VEGF does not enhance that phosphorylation (Fig. 3, A, C, and D). For KDR(Ig1–3)-expressing cells, VEGF treatment results in some experiments in a decrease in the level of receptor phosphorylation (Fig. 3 A); the reason for this is not known. For the experiment shown in Fig. 3C, two molecular weight proteins are visualized in samples prepared from KDR(Ig1–3)-expressing PAE cells; this was not observed for all experiments. The structural differences between these proteins are not known, although they could represent receptor degradation or receptor proteins containing different degrees of tyrosine phosphorylation.

Receptor autophosphorylation allows for the recruitment of cellular signaling proteins to the receptor and subsequent signal transduction. We therefore tested whether cell signaling pathways that are stimulated in endothelial cells by VEGF are activated in the mutant KDR-transfected cells in the absence of growth factor. Transfected HEK293 cells were incubated with and without VEGF, cell extracts were immunoprecipitated with anti-Tyr(P) antibodies, and immunoblotting was done using antibodies to two signaling proteins (NCK and PLCγ) that are activated by growth factor in endothelial cells (22) (Fig. 4). Neither PLCγ (Fig. 4A) nor NCK (Fig. 4B) is tyrosine phosphorylated in KDR-expressing cells, and VEGF stimulates a robust response. For KDR(Ig1–3)-expressing cells, both of the signaling proteins are phosphorylated in the absence of growth factor. As is found for KDR(Ig1–3) autophosphorylation (Fig. 3A), VEGF treatment decreases the level of tyrosine phosphorylation of both NCK and PLCγ; the mechanisms that account for this are not known.

As shown in Fig. 5, the activation of more downstream signaling events is also independent of VEGF in cells transfected with mutant receptors. VEGF-induced assembly of focal adhesions plays a necessary role in the signaling pathway leading to endothelial cell migration (38). Fig. 5, A and B, demonstrates that this effect is observed in KDR-transfected PAE cells because VEGF stimulates the number and increases the size of focal adhesions as visualized by immunofluorescent staining using the anti-Tyr(P) antibody. In the absence of VEGF treatment, both the number and size of focal adhesion complexes in...
The affinity of binding to transfected HEK293 cells was 85 pM for KDR(Ig1–3)-expressing PAE cells. The results shown are representative of at least 10 different fields observed in each experiment and of three similar independent experiments.

KDR(Ig1–3)-expressing cells are identical to that seen for VEGF-treated KDR-expressing cells (Fig. 5C). VEGF had no effect on focal adhesion complex assembly in cells expressing KDR(Ig1–3) (data not shown).

$^{125}$I-VEGF$_{165}$ Binding to Native and Mutant Receptors—The region of KDR which is involved in direct VEGF binding has been mapped to Ig-like domains 1–3 (39). To determine whether amino acid sequences in Ig-like domains 4–7 can regulate growth factor binding, we performed $^{125}$I-VEGF$_{165}$ radioligand binding assays to cells expressing native and mutant receptors. KDR-expressing HEK293 and PAE cells exhibit a high number of specific $^{125}$I-VEGF$_{165}$ binding sites (Fig. 6). The affinity of binding to transfected HEK293 cells was 85 pm (Fig. 6A), which is similar to that which has been reported for endothelial cells expressing endogenous KDR (40, 41). The affinity of binding to transfected PAE cells is significantly weaker (1,200 pm) (Fig. 6B). This result was observed for several individual expanded cell lines derived from two separate transfection protocols (data not shown). The molecular reasons why KDR expressed in these cells exhibits this weak affinity is not clear, although other investigators have made similar observations (42).

$^{125}$I-VEGF$_{165}$ binds to KDR(Ig1–3)-expressing HEK293 cells with an affinity similar to that observed for native receptor (Fig. 6A). In contrast, $^{125}$I-VEGF$_{165}$ binds to KDR(Ig1–3) and KDR(Ig1–6)-expressing PAE cells with affinities that are significantly greater than that observed for native receptor (Fig. 6B). Radioligand bound to KDR(Ig1–3) with a greater affinity than to KDR(Ig1–6), and at an affinity that is similar to that observed for the KDR(Ig1–3) and KDR-transfected HEK293 cells. In addition, the number of $^{125}$I-VEGF$_{165}$ binding sites is reduced significantly in PAE cells expressing mutant, versus native, receptor, a result that was found when analyzing several separate stably transfected cell lines.

A comparison of the results shown in Figs. 2 and 6 indicates that although the number of mutant KDR proteins expressed in PAE cells is similar to that for native KDR proteins, the number of $^{125}$I-VEGF$_{165}$ binding sites is significantly less for cells expressing mutant receptor. It is known that VEGF$_{165}$ stimulates the internalization of KDR in endothelial cells by a mechanism that requires receptor autophosphorylation (35). In view of the finding that KDR(Ig1–3) autophosphorylation and signaling are VEGF-independent (Figs. 3, 4, and 5), we reasoned that KDR(Ig1–3) internalization may also be VEGF-independent. This would account for the decreased number of $^{125}$I-VEGF$_{165}$ binding sites in cells expressing mutant receptor because the radioligand does not penetrate the cell membrane and only binds to surface receptors.

This hypothesis was tested using immunofluorescence protocols to examine receptor distribution in KDR- and KDR(Ig1–3)-expressing PAE cells. In cells expressing native receptor, there is weak KDR staining in the cell cytoplasm, and the cell periphery is clearly outlined, suggesting cell surface receptor (Fig. 7A). In contrast, staining of KDR(Ig1–3)-expressing cells indicates an intense spotty receptor distribution within the cytoplasm, and the line of fluorescence marking the cell periphery is not visible (Fig. 7B). These results are consistent with the hypothesis that the low number of $^{125}$I-VEGF$_{165}$ binding sites in cells expressing mutant receptor is because these receptors are contained in the distinct domains within the cell interior and a relatively less number at the cell surface.

KDR(Ig1–3) Dimerization Is VEGF-independent—Receptor tyrosine kinase dimerization is the earliest step in the signaling pathway by which growth factors activate cells. In view of the finding that KDR(Ig1–3) autophosphorylation and signaling are observed in the absence of VEGF treatment, we asked whether KDR(Ig1–3) might exist constitutively in the dimeric form. VEGF-treated and control KDR- and KDR(Ig1–3)-expressing HEK293 cells were solubilized and then subjected to cross-linking analysis using disuccinimidyl suberate. The solubilization step was included because of the results indicating that the majority of KDR(Ig1–3) is internal.

The results from affinity cross-linking KDR-expressing cells did not reveal the presence of covalent receptor dimers, even after VEGF treatment (Fig. 8, lanes 1–4). There may be two reasons for receptor dimers not being observed in the presence of growth factor. First, the SDS-polyacrylamide gels were run in the presence of 2-mercaptoethanol, and so noncovalent receptor dimers will have dissociated during sample preparation. Second, the inefficiency of the cross-linking step may prevent detection of covalently dimerized receptors.

The results were very different for KDR(Ig1–3)-expressing cells, where it was found that greater than 50% of receptors were cross-linked, even in the absence of growth factor (Fig. 8,
cells were scraped from the wells, pelleted, and suspended in 200 μl of PBS containing 0.5% Triton X-100. 15 min later, 1 mM disuccinimidyl suberate (DSS) was added to some samples (lanes 3, 4, 7, and 8), and all samples were incubated at room temperature for 20 min. Gel sample buffer was added, and the samples were boiled for 5 min. Immunoblotting was done using anti-KDR antibody.

lanes 7 and 8). These results suggest that deletion of Ig-like domains 4–7 induces activation of receptor tyrosine kinase activity by allowing receptor dimerization in the absence of VEGF binding.

Requirement of Cell Surface HSPGs for KDR Dimerization—It is concluded from the data described above that Ig-like domains 4–7 contain structural features that inhibit KDR autophosphorylation and signaling, and deletion of this domain relieves that inhibition. We hypothesize further that the molecular mechanism by which VEGF activates KDR is by relieving the inhibitory action of Ig domains 4–7.

The binding of VEGF165 to KDR is dependent upon HSPGs (32, 33). Although it is generally thought that this effect is mediated by an interaction of HSPG with growth factor, a necessary interaction of HSPG with KDR for receptor dimerization is possible. A heparin binding domain has been identified between KDR Ig domains 6 and 7, and it was suggested that interactions of HSPG with this domain might facilitate VEGF binding and receptor dimerization (33). This hypothesis is made more reasonable by reports demonstrating that HSPG interactions with both bFGF and its receptor participate in growth factor binding (43, 44). It was proposed that the functional role for HSPG binding domains on the bFGF receptor is to prevent receptor dimerization in the absence of growth factor and that HSPG binding to these domains facilitates bFGF-induced signaling (44).

Experiments were designed to test the hypothesis that HSPG interactions with KDR participate in relieving the inhibition of signaling caused by Ig-like domains 4–7, thereby facilitating the ability of VEGF to activate KDR autophosphorylation and cell signaling. Two strategies were taken. First, we tested whether heparin is required for 125I-VEGF121 binding to KDR-expressing cells. The rationale for this was that if HSPG functions through an interaction with KDR, then HSPGs would be required for both VEGF121 and VEGF165 binding to receptor. Second, we asked whether HSPGs are required for 125I-VEGF165 binding to KDR(Ig1–3)- and KDR(Ig1–6)-expressing cells. The rationale for this was that if HSPG binding to Ig-domains 4–7 participates in relieving an inhibitory effect, then deletion of these domains would remove the need for HSPG in 125I-VEGF165 binding.

A preliminary experiment was done to compare the interaction of VEGF121 versus VEGF165 with KDR and mutant KDR-expressing cells. VEGF121 treatment of KDR-expressing HEK293 cells results in receptor autophosphorylation at a degree equivalent to that observed using VEGF165. VEGF121 bound to KDR- and KDR(Ig1–3)-expressing HEK293 cells (data not shown) and PAE cells (Fig. 9B) with an affinity similar to that seen for 125I-VEGF165 (compare Fig. 9B with Fig. 6B).

Commercial heparin can substitute for cellular HSPGs in allowing for 125I-VEGF165 binding to KDR, although an effect of heparin on 125I-VEGF165 binding is maximal only when HSPGs are depleted (32). We took the strategy of blocking HSPG biosynthesis by treating cells with chlorate, an inhibitor of ATP sulfurylase and hence of the production of phosphoadenosine phosphosulfate, the active sulfate donor for sulfotransferases (45). Chlorate abolishes sulfation on proteins and carbohydrate residues in cells without inhibiting protein synthesis or cell growth (46).

KDR-expressing HEK293 cells were cultured to 40% confluence on 10-cm dishes, and 70 mM chlorate was added for 24 h. The cells were then plated on 24-well dishes and cultured for 24 h in medium containing 70 mM chlorate. 125I-VEGF165 radioligand binding indicated that the cells lose 75% of cell surface binding sites compared with control cells not incubated with inhibitor (Fig. 10A). The addition of heparin restored radioligand binding to the chlorate-treated cells. In contrast, chlorate treatment had no effect on 125I-VEGF121 binding to these cells (Fig. 10B), indicating that HSPGs are not required for binding of this isoform.

These results argue against the hypothesis that HSPGs function to interact with sites within Ig-like domains 4–7 and argue that HSPGs do not participate in relieving the inhibitory actions of these domains. This conclusion is supported by the
results of testing for an HSPG requirement in $^{125}\text{I}}$-VEGF$_{165}$ binding to cells expressing KDR(Ig1–3) (Fig. 10C). As observed for cells expressing native receptor, chlorate treatment inhibited radioligand binding to mutant receptor, and heparin restored the binding. Heparin had no effect on $^{125}\text{I}}$-VEGF$_{121}$ binding to KDR(Ig1–3) (Fig. 10D).

DISCUSSION

The accepted model for activation of receptor tyrosine kinases is that ligand-induced receptor dimerization juxtaposes the cytoplasmic tyrosine kinase domains, resulting in trans-phosphorylation of target amino acids and initiating signaling cascades. Our results indicate that there are further molecular interactions that regulate growth factor-induced receptor activation. More specifically, for KDR there are structural features in the extracellular domain which prevent unwanted receptor activation in the absence of VEGF binding.

Fig. 11 shows two molecular mechanisms that may mediate this effect. Model A predicts that in the absence of growth factor, there are structural features within the KDR extracellular domain which repel receptor-receptor interactions, thus preventing receptor dimerization. The binding of VEGF to KDR neutralizes these structural features, either by causing a conformational change in the receptor or by VEGF-KDR interactions, thus allowing for receptor dimerization. Model B predicts that in the absence of VEGF, the three-dimensional structure of the KDR monomer is such that interactions between monomers will not allow the catalytic domains to come close enough together to allow for trans-phosphorylation. VEGF causes a conformational change in the receptor monomers so that nec-
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essay structural features within the catalytic domain become sufficiently juxtaposed to allow trans-phosphorylation. Both of these models would be consistent with our results showing that deletion of KDR Ig-like domains 4–7 leads to VEGF-independent receptor activation. For model A, the mutant receptors no longer contain the structural features that repel receptor-receptor interactions, thus allowing for receptor signaling. For model B, the mutant receptors no longer contain the conformational constraints that prevent the catalytic domains to juxtapose, and so the receptor would be active.

Model A predicts that there are specific peptide sequences within the KDR extracellular domain which prevent VEGF-independent signaling. Model B, on the other hand, predicts that the three-dimensional structure of Ig-like domains 4–7 is important, and so mutations throughout this region would most likely disrupt the inhibitory effect. Therefore, our findings are more consistent with model B because all three mutant receptors showed VEGF-independent receptor activation.

The proposed mechanism by which VEGF stimulates KDR is similar to how certain nonreceptor kinases (e.g., protein kinase A and protein kinase C) are activated by their substrates. Both of these kinases contain regulatory and catalytic domains, and binding of either cAMP or diacylglycerol to the regulatory domains causes a conformational change in the protein allowing for catalytic activity (47, 48). Our experimental results indicate that VEGF activates KDR in an analogous manner. This conclusion is strengthened by the fact that separation of the regulatory domain from the catalytic domain of protein kinases A and C, either by expression of recombinant catalytic domains (49, 50), or in the case of protein kinase C, proteolytic digestion (51), leads to substrate-independent kinase activity. In an analogous manner, a recombinant KDR cysteolic domain is catalytically active (52). There are most likely differences in the molecular interactions by which KDR versus the other protein kinases are activated by substrate, as the conformational changes in the regulatory domains caused by cAMP and diacylglycerol allow for the exposure of ATP-binding peptides within the catalytic domains (53, 54). It is more difficult to envision a similar mechanism for VEGF activation of KDR because VEGF and ATP binding to KDR occur on opposite sides of the cell membrane.

Two other receptor tyrosine kinases also contain structural features within their extracellular domains which function to block ligand-independent receptor dimerization. A mutant PDGFA receptor lacking Ig-like domain 3 results in growth factor-independent receptor dimerization and activation (55), and an Ig-like domain contained within the extracellular domain of the TrkA receptor serves a similar function (56). Interestingly, the structural features of the PDGFA receptor A and TrkA receptors that participate in these effects are contained within the receptor’s ligand binding domains. This is different from that seen for KDR because the constitutively active receptor mutants maintain 125I-VEGF binding activity.

PDGFR receptor and cKit are receptor-like tyrosine kinases that contain five extracellular Ig-like domains. Each of these receptors contains structural features within their fourth Ig-like domain which participate in receptor-receptor interactions and are required for receptor dimerization. The deletion of these domains blocks both high affinity growth factor binding as well as receptor signaling. The results shown in Figs. 4, 5, and 6 demonstrating that deletions of KDR Ig-like domains 4–7 have no effect on 125I-VEGF binding and allow for KDR activation, would indicate that this structural feature is not contained within the KDR Ig-like domains 4–7. We cannot rule out that KDR Ig-like domains 1–3 contain structural features that facilitate receptor-receptor interactions, and the results of Fig. 8 demonstrating VEGF-independent dimerization of KDR(Ig1–3) are consistent with this.

The results of Fig. 6 indicate that the KDR structural domains that mediate growth factor binding are contained within Ig-like domains 1–3. A similar conclusion was made in certain previous studies measuring 125I-VEGF Ig45 binding to a soluble chimeric protein consisting of various deletion mutants of the KDR extracellular domain fused to the Fc portion of human IgG (39, 40). One previous report (57) used this soluble receptor chimera and concluded that Ig-like domain 4 is also required for VEGF binding. The difference between these results and the others is most likely accounted for by the fact that 22 fewer amino acids were used in defining the Ig-like domain 3-COOH boundary, and so the recombinant KDR Ig-like 1–3 protein used lacked important structural features required for VEGF binding. This hypothesis is supported by the recently reported (58) finding that KDR amino acids 313 and 315, which are contained within the relevant 22-amino acid peptide, interact directly with VEGF and are required for binding.

The experimental results shown in Figs. 6 and 7 indicate that the vast majority of mutant receptors expressed in PAE cells are internal. It was suggested under “Results” that this is because once the receptors autophosphorylate at the cell surface, they internalize. We cannot rule out an alternative conclusion that a majority of newly synthesized mutant receptors never reach the cell surface either because they lack appropriate trafficking domains or because of receptor activation prior to reaching the cell surface. Our results do clearly demonstrate that a certain population of newly synthesized mutant receptors is expressed at the cell surface because we can measure 125I-VEGF binding. Evidence indicating that these cell surface receptors are functional is that focal adhesion complex assembly, which is dependent upon the KDR-mediated recruitment of NCK to the cell surface (59), is VEGF-independent in the KDR(Ig1–3)-expressing PAE cells.

The molecular interactions that account for the lower affinity of 125I-VEGF binding to KDR-transfected PAE versus HEK293 cells is not clear. The fact that high affinity binding is seen for cells expressing mutant receptors suggests that the interactions are mediated, in part, by KDR Ig-like domains 3–7. PAE cells do not express neuropilin-1 (34), but this most likely does not account for the observed effects because neuropilin-1 does not interact with 125I-VEGF (34), which also binds with low affinity to these KDR-expressing PAE cells.

In summary, the results of this study indicate that the role of VEGF in stimulating KDR activation is more complex than simply allowing for receptor monomers to come into close contact with each other. In addition to ligand binding regions within the extracellular domain of KDR, there are also regions that act to prevent VEGF-independent dimerization and thus unwanted signaling events. VEGF functions, in part, to relieve this inhibitory effect. The molecular interactions that allow for VEGF activation of KDR are different from those involved in PDGF and colony-stimulating factor-1 activating their receptors because deletion of KDR Ig-like domain 4 does not prevent high affinity VEGF binding.

REFERENCES

1. Ferrara, N., and Davis-Smyth, T. (1997) Endocr. Rev. 18, 4–25
2. Iruela-Arispe, M. L., and Dvorak, H. F. (1997) Throm. Haemostasis 78, 672–677
3. Carmeliet, P., and Jain, R. K. (2000) Nature 407, 249–257
4. Fava, R. A., Olsen, N. J., Spencer-Green, G., Yeo, T.-K., Yeo, K.-T., Berse, B., Jackman, R. W., Senger, D. R., Dvorak, H. F., and Brown, J. F. (1994) J. Exp. Med. 180, 340–346
5. McLaren, J., Prentice, A., Charmack-Jones, D. S., Millican, S. A., Muller, K. H., Sharkey, A. M., and Smith, S. K. (1996) J. Clin. Invest. 98, 482–489
6. Takeshita, S., Zheng, L., Brogi, E., Kearney, M., Pu, L.-Q., Bunting, S., Ferrara, N., Symes, J. F., and Isner, J. M. (1994) J. Clin. Invest. 93, 662–670
7. Li, J., Brown, L. P., Hibberd, M. G., Grossman, J. D., Morgan, J. P., and...
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