Characterization of Novel Vascular Endothelial Growth Factor (VEGF) Receptors on Tumor Cells That Bind VEGF\textsubscript{165} via Its Exon 7-encoded Domain\textsuperscript{*}

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Vascular endothelial growth factor (VEGF), a potent angiogenic factor, uses two receptor tyrosine kinases, FLK/KDR and FLT, to mediate its activities. We have cross-linked \textsuperscript{125}I-VEGF\textsubscript{165} to the cell surface of various tumor cell lines and of human umbilical vein endothelial cells. High molecular mass (220 and 240 kDa) and/or lower molecular mass (165 and 175 kDa) labeled complexes were detected depending on the cell type. The 220- and 240-kDa labeled complexes were shown to contain FLT and FLK/KDR receptors, respectively. On the other hand, the 165- and 175-kDa complexes did not seem to contain FLK/KDR or FLT but instead appeared to contain novel VEGF receptors with relatively low molecular masses of approximately 120 and 130 kDa. These receptors were further characterized in breast cancer MDA MB 231 cells (231), which did not form the high molecular mass complexes and which did not express detectable amounts of flk/kdr or flt mRNA. The 231 cells displayed one VEGF\textsubscript{165} binding site, with a $K_d$ of $2.8 \times 10^{-10}$ M and 0.95-1.1 \times 10^{10}$ binding sites per cell. By comparison, human umbilical vein endothelial cells had two binding sites, one with a $K_d$ of $7.5 \times 10^{-12}$ M, presumably FLK/KDR, and the other with a $K_d$ of $2 \times 10^{-10}$ M, a value similar to the VEGF\textsubscript{165} binding sites on 231 cells. These lower affinity/molecular mass receptors on 231 cells cross-linked \textsuperscript{125}I-VEGF\textsubscript{165} but not \textsuperscript{125}I-VEGF\textsubscript{121}. Accordingly, exon 7 of VEGF, which encodes the 44 amino acids present in VEGF\textsubscript{165} that are absent in VEGF\textsubscript{121}, was fused to glutathione S-transferase (GST). The GST-VEGF-exon 7 fusion protein bound to heparin-Sepharose with a similar affinity as VEGF\textsubscript{165} and inhibited the binding of \textsuperscript{125}I-VEGF\textsubscript{165} to 231 cells. Cross-linking of \textsuperscript{125}I-GST-VEGF-exon 7 to 231 cells resulted in the formation of 150- and 160-kDa labeled complexes that presumably contained the 120- and 130-kDa lower affinity/molecular mass VEGF\textsubscript{165} receptors. It was concluded that certain tumor-derived cell lines express novel surface-associated receptors that selectively bind VEGF\textsubscript{165} via the exon 7-encoded domain, which is absent in VEGF\textsubscript{121}.

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\textsuperscript{¶}The abbreviations used are: VEGF, vascular endothelial growth factor; EC, endothelial cells; GST, glutathione S-transferase; HUVEC, human umbilical vein-derived endothelial cells; PAGE, polyacrylamide gel electrophoresis.
lines have been shown to synthesize FLT (29). Efficient binding of 125I-VEGF to its receptors on EC requires the presence of heparin-like molecules on the cell surface (20). Heparin has differential effects on the binding of 125I-VEGF to FLK/KDR and FLT. Soluble heparin seems to enhance the binding of 125I-VEGF to EC synthesizing FLK/KDR (20, 30) but has the opposite effect on the binding of 125I-VEGF to melanoma cells synthesizing FLT (28, 29). 125I-VEGF binds to κ2 macroglobulin and as a consequence is not able to bind its receptors on HUVEC. Heparin interferes with this binding and is able to restore the receptor binding capability of 125I-VEGF (30, 31).

The binding of VEGF to VEGF receptors on non-EC does not seem to induce cell proliferation. Rather, VEGF induces motility of monocytes (32), differentiation of osteoblasts (33), production of insulin by beta cells (34), and disorganization of actin stress fibers in Balb/c 3T3 cells (23). Recent studies with cells expressing endogenous or transfected flk and kdr have suggested that VEGF activities, e.g. mitogenicity, chemotaxis, and morphological changes are mediated by FLK/KDR but not through FLT, even though both receptors undergo phosphorylation upon binding of VEGF (35–38). Placenta growth factor, a recently purified VEGF homologue (39) that binds to FLT, has no effect on the proliferation rate of EC (36, 40). These results suggest that although VEGF can bind to multiple receptors on the cell surface, the subsequent VEGF-mediated responses may be different. Cross-linking of 125I-VEGF to EC and non-EC results in the formation of multiple 125I-VEGF-VEGF receptor complexes, with higher molecular masses of about 220–240 kDa and lower molecular masses of about 165–175 kDa (19, 20, 22). However, to date it has not been clear which of the known VEGF receptors are contained in the various labeled complexes. In this report, we demonstrate (i) that the high molecular mass complexes found in EC and melanoma cells contain FLK or FLT; (ii) that the lower molecular mass complexes do not appear to contain FLK or FLT but rather VEGF receptors of relatively lower affinity; (iii) that the lower affinity/molecular mass receptors found on tumor cells are isoform-specific in that they bind 125I-VEGF but not 125I-VEGF, in agreement with previous results obtained for HUVEC (41); and (iv) that the binding of 125I-VEGF to these receptors is mediated by the VEGF exon 7-encoded domain, which is present in VEGF but not VEGF.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant VEGF and VEGF were produced by SF-9 insect cells infected with a baculovirus-based vector expressing VEGF and VEGF cDNAs, as described previously (29, 42). VEGF was purified from the conditioned medium of the infected SF-9 cells by heparin affinity chromatography followed by anion exchange chromatography as described (29, 42). Placenta growth factor was kindly provided by Dr. Y. Cao (Children's Hospital, Boston, MA). Basic fibroblast growth factor was kindly provided by Dr. L. Willson (University of Cambridge, Cambridge, MA). Heparin-Sepharose, heparitinase-agarose, Sephadex CL-4B, Protein G-coupled Sephadex CL-4B, Protein G-Sepharose, Sigma, were purchased from Sigma Chemical Co. Heparin-Sepharose was kindly provided by Dr. S. Kerr (University of Cambridge, Cambridge, MA). Heparin-Sepharose was kindly provided by Dr. S. Kerr (University of Cambridge, Cambridge, MA). Heparin-Sepharose was kindly provided by Dr. S. Kerr (University of Cambridge, Cambridge, MA). Heparin-Sepharose was kindly provided by Dr. S. Kerr (University of Cambridge, Cambridge, MA).

RESULTS

Cross-linking of 125I-VEGF to the Surface of HUVEC and Tumor-Derived Cell Lines—125I-VEGF was cross-linked to cells in the presence of the absence of 1 μg/ml heparin (Fig. 1). As depicted in Fig. 1, lane 1, cross-linking of 125I-VEGF to HUVEC resulted in the formation of a 240-kDa labeled complex (Fig. 1, lane 1). In the presence of heparin, two additional labeled complexes of approximately 165 and 175 kDa were detected (Fig. 1, lane 2). Cross-linking of 125I-VEGF to the breast cancer cell line MDA-MB-231 (231 cells) (Fig. 1, lanes 3 and 4) and to the prostate tumor cell line LNCaP (Fig. 1, lanes 5 and 6) produced similar complexes of approximately 165–175 kDa but not the 240-kDa labeled complex. In contrast, the breast cancer cell line MDA-MB-453 (Fig. 1, lanes 7 and 8)
Receptors for VEGF<sub>165</sub>

FIG. 1. Cross-linking of 125<sup>I</sup>-VEGF<sub>165</sub> to the surface of HUVEC and tumor-derived cell lines. 125<sup>I</sup>-VEGF<sub>165</sub> (5 ng/ml) was bound and cross-linked to subconfluent cultures of HUVEC (lanes 1 and 2), 231 cells (lanes 3 and 4), LNCaP cells (lanes 5 and 6), and MDA-MB-453 cells (lanes 7 and 8) in 6-cm dishes. The binding was carried out in the presence (lanes 2, 4, 6, and 8) or the absence (lanes 1, 3, 5, and 7) of 1 μg/ml heparin. The cells were lysed, and proteins were resolved by 6% SDS-PAGE as described under "Experimental Procedures."

These results indicate that HUVEC and EP-mel cells synthesize predominantly FLK/KDR, whereas RU-mel cells synthesize FLT. These anti-VEGF receptor antibodies failed to immunoprecipitate the 165- and 175-kDa complexes from any of the cell lines (Fig. 2), suggesting that these complexes contain VEGF-receptors that are different than FLK/KDR or FLT. For further analysis of the 165- and 175-kDa complexes, we chose 231 cells because they did not produce the higher molecular mass complexes containing FLK/KDR or FLT, thus facilitating analysis of the lower molecular mass complexes. In addition, the expression of flk/kdr or flt mRNA could not be detected by a Northern blot analysis of 231 cell-derived RNA (not shown).

Based on the molecular weight of a VEGF<sub>165</sub> dimer (45 kDa), the receptors forming the 165- and 175-kDa labeled complexes were estimated to have molecular masses of approximately 120 and 130 kDa, respectively.

Analysis of VEGF<sub>165</sub> Binding Sites on 231 Cells—To determine the affinity of VEGF<sub>165</sub> for the 120- and 130-kDa receptors, 231 cells and HUVEC were incubated with increasing concentrations of 125<sup>I</sup>-VEGF<sub>165</sub> (Fig. 3A). Binding of 125<sup>I</sup>-VEGF<sub>165</sub> to 231 cells was carried out in the presence or the absence of heparin. The specific binding of 125<sup>I</sup>-VEGF<sub>165</sub> to 231 cells increased in a dose-dependent manner and reached a plateau at approximately 1.9 × 10<sup>-10</sup> M (8.5 ng/ml). Heparin (1 μg/ml) induced an 80% increase in the binding of 125<sup>I</sup>-VEGF<sub>165</sub> to 231 cells, in agreement with the heparin-induced augmentation of binding shown in Fig. 1 (lanes 3 and 4). The binding results were used to generate Scatchard plots (Fig. 3, B–D), which were further analyzed by the LIGAND program (45).

The program predicted the presence of a single class of binding sites on 231 cells with a K<sub>d</sub> of 2.8 × 10<sup>-12</sup> M and 0.95–1.1 × 10<sup>5</sup> binding sites per cell (Fig. 3C). Heparin had no significant effect on the affinity of VEGF<sub>165</sub> for its binding sites on 231...
and 175-kDa complexes on HUVEC and 231 cells as shown in the lower molecular mass VEGF receptors that form the 165-affinity sites on HUVECs suggest that these sites may represent a factor enhances the binding of 125I-VEGF165 by increasing the number of available binding sites on 231 cells rather than by changing the affinity of VEGF165 for its binding sites. In comparison, HUVEC displayed two classes of binding sites for VEGF165, the higher affinity binding sites had a \( K_d \) of 7.5 \( \times \) 10^{-12} and approximately 2 \( \times \) 10^{-12} binding sites/cell, and the lower affinity binding sites had a \( K_d \) of 2.0 \( \times \) 10^{-10} M and 2.5 \( \times \) 10^{-10} binding sites/cell (Fig. 3B). The similar \( K_d \) values of VEGF165 for its binding sites on 231 cells and for the lower affinity sites on HUVEC suggest that these sites may represent the lower molecular mass VEGF receptors that form the 165- and 175-kDa complexes on HUVEC and 231 cells as shown in Fig. 1.

VEGF165 but Not VEGF121 Binds to Receptors on 231 Cells—To test the specificity of the interaction between VEGF165 and the lower affinity/molecular mass receptors, 125I-VEGF165 was bound to 231 cells and HUVEC in the presence of 400 ng/ml of unlabeled VEGF165, VEGF121, PIGF, or platelet-derived growth factor. After binding, cells were washed and lysed, and the cell-associated radioactivity was determined using a \( y \)-counter. The counts obtained are expressed as the percentage of the counts obtained compared with a phosphate-buffered saline (PBS) control.

cells \( (K_p = 2.7 \times 10^{-10}) \) but induced a 2-fold increase in their number \( (1.9-2.0 \times 10^{3} \) binding sites/cell) (Fig. 3D). Thus, heparin enhances the binding of 125I-VEGF165 by increasing the number of available binding sites on 231 cells rather than by changing the affinity of VEGF165 for its binding sites. In comparison, HUVEC displayed two classes of binding sites for VEGF165, the higher affinity binding sites had a \( K_d \) of 7.5 \( \times \) 10^{-12} and approximately 2 \( \times \) 10^{2} binding sites/cell, and the lower affinity binding sites had a \( K_d \) of 2.0 \( \times \) 10^{-10} M and 2.5 \( \times \) 10^{2} binding sites/cell (Fig. 3B). The similar \( K_d \) values of VEGF165 for its binding sites on 231 cells and for the lower affinity sites on HUVEC suggest that these sites may represent the lower molecular mass VEGF receptors that form the 165- and 175-kDa complexes on HUVEC and 231 cells as shown in Fig. 1.

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In recent years, there has been growing evidence that VEGF plays a major role in regulating angiogenesis during normal development and in tumors (6, 11, 13, 38, 46). VEGF binds to specific high affinity receptors, FLK/KDR and FLT, which mediate VEGF responses (26, 27), and which were initially shown to be associated with various types of EC (25, 47, 48). In addition to EC, VEGF also binds to receptors on the surface of cell types such as HeLa, human melanoma, and NIH3T3 (20, 22, 23). Analysis of 125I-VEGF165 VEGF receptor cross-linking patterns have demonstrated that there are multiple VEGF165

**FIG. 4.** The binding of 125I-VEGF165 to 231 cells and HUVEC in the presence of excess growth factors. 125I-VEGF165 (4 ng/ml) was bound to subconfluent cultures of 231 cells (hatched bars) and HUVEC (solid bars) in 48-well dishes in the presence of 400 ng/ml of unlabeled VEGF165, VEGF121, PIGF, or platelet-derived growth factor. After binding, cells were washed and lysed, and the cell-associated radioactivity was determined using a \( y \)-counter. The counts obtained are expressed as the percentage of the counts obtained compared with a phosphate-buffered saline (PBS) control.

**FIG. 5.** Cross-linking of 125I-VEGF165 and 125I-VEGF121 to the surface of 231 cells and HUVEC. 125I-VEGF165 (5 ng/ml) (lanes 1, 2, 7, and 8) or 125I-VEGF121 (10 ng/ml) (lanes 3–6) were bound and cross-linked to subconfluent cultures of 231 cells (lanes 1–4) and HUVEC (lanes 5–8) in 6-cm dishes. The binding was carried out in the presence (lanes 2, 4, 6, and 8) or the absence (lanes 1, 3, 5, and 7) of 1 \( \mu \)g/ml heparin. Cells were lysed, and proteins were resolved by a 6% SDS-PAGE as described in the legend to Fig. 1.

**TABLE**

| VEGF165 | HUVEC | Heparin |
|---------|-------|---------|
| 125I-VEGF165 | - | + |
| 125I-VEGF121 | - | + |

**Discussion**

In recent years, there has been growing evidence that VEGF plays a major role in regulating angiogenesis during normal development and in tumors (6, 11, 13, 38, 46). VEGF binds to specific high affinity receptors, FLK/KDR and FLT, which mediate VEGF responses (26, 27), and which were initially shown to be associated with various types of EC (25, 47, 48). In addition to EC, VEGF also binds to receptors on the surface of cell types such as HeLa, human melanoma, and NIH3T3 (20, 22, 23). Analysis of 125I-VEGF165 VEGF receptor cross-linking patterns have demonstrated that there are multiple VEGF165.
binding sites, for example, on EC and on melanoma cells (19, 20, 22). However, the actual identities of the multiple VEGF receptors in these cross-linked complexes have not been determined, and thus our goal in this study was to characterize the VEGF receptors in these cross-linked complexes. Accordingly, 125I-VEGF165 was cross-linked to the cell surface of several cell types. Analysis of the cross-linking products revealed the presence of several 125I-VEGF165-cross-linked complexes of higher molecular mass. HUVEC formed both the higher and lower molecular mass complexes as shown previously (20, 41), as did several melanoma cell types (22, 29). On the other hand, the breast cancer-derived cell line, MDA MB 231 (231 cells), and the prostate carcinoma-derived cell line, LNCaP, formed only the lower molecular mass complexes. There was no case in which the high but not the low molecular mass complexes were formed.

Immunoprecipitation studies with anti-FLK/KDR and anti-FLT antibodies were used to identify the VEGF165 receptors on the various cell types. The 240-kDa complex formed by 125I-VEGF165 binding to HUVEC was shown to contain FLK/KDR, confirming previous results (41), whereas the 220-kDa complex formed with RU-mel cells was shown to contain FLT. Although EC have been reported to express flt mRNA (47, 48), we have not been able to identify FLT as part of the 125I-VEGF165-cross-linked complexes formed with HUVEC or bovine aortic endothelial cells. On the other hand, immunoprecipitation with anti-FLK/KDR and anti-FLT antibodies showed no cross-reactivity with proteins in the 165- and 175-kDa complexes in 231 cells, consistent with similar results for HUVEC (41), suggesting that these complexes contain VEGF165 receptors that are different than FLK/KDR and FLT. Based on the molecular mass of a VEGF165 dimer, we estimated the size of these lower molecular mass VEGF165 receptors to be 120 and 130 kDa.

We chose 231 cells to characterize the lower molecular mass VEGF165 receptors because they do not produce FLK/KDR- or FLT-containing complexes and do not express flk/kdr or flt mRNA. Scatchard analysis of the binding of 125I-VEGF165 to 231 cells revealed the presence of one class of binding sites that bind VEGF165 with a Kd of 2.8 × 10^-10 M and 0.95–1.1 × 10^-10 M binding sites/cell. Heparin induced a 2-fold increase in the binding of 125I-VEGF165 to 231 cells by increasing the number of binding sites/cell without significantly changing the affinity for VEGF165. On the other hand, HUVEC displayed two binding sites for VEGF165, with Kd values of 7.5 × 10^-10 M and 2.0 × 10^-10 M, respectively. These results indicate that the VEGF165 binding sites on 231 cells have an affinity for VEGF165 similar to the lower affinity binding sites on HUVEC. Because both cell types produce labeled complexes of 165 and 175 kDa upon cross-linking with 125I-VEGF165, it seems that the lower molecular mass VEGF165 receptors detected on the cell surface of 231 cells may be the same as the lower affinity VEGF165 receptors found on HUVEC. Accordingly, we have designated these binding sites as lower affinity/molecular mass VEGF receptors.

Although excess nonlabeled VEGF165 inhibited 125I-VEGF165 binding to 231 cells, a 100-fold excess of VEGF121 did not. In addition, no radiolabeled complexes, neither of higher nor lower molecular mass, could be detected upon cross-linking of 125I-VEGF121 to 231 cells. As a control, 125I-VEGF121 was shown to be active in that it formed a 240-kDa complex, presumably containing FLK/KDR, upon cross-linking to HUVEC. On the other hand, 125I-VEGF121 did not form 165- and 175-kDa complexes with HUVEC, consistent with the 231 cell results. The ability of 125I-VEGF165 but not 125I-VEGF121 to form 165- and 175-kDa complexes with HUVEC has been re-
ported recently (41). Taken together, these results demonstrate that VEGF165 to 231 cells. To test this hypothesis, a chimeric
protein made of GST and the exon 7-encoded domain of VEGF165 was prepared. This GST-ex7 fusion protein bound to
heparin-Sepharose with a similar affinity as VEGF165 (17), indicating that the heparin-binding domain of VEGF165 is
localized to the exon 7-encoded domain. In addition, GST-ex7 competed with the binding of 125I-VEGF165 to 231 cells and
could bind and be cross-linked directly to the lower affinity/molecular mass receptors. Taken together, these results indi-
cate that the exon 7-encoded domain is responsible for both VEGF165 heparin-binding and the binding to the lower affinity/molecular mass receptors.

An important question to consider is whether the 120- and 130-kDa lower affinity receptors expressed by 231 cells are
novel or whether they may be truncated forms of FLK or FLT lacking about 40–50 kDa in molecular mass. The latter
possibility is unlikely because (i) VEGF121 does not bind to FLT lacking about 40–50 kDa in molecular mass. The latter
receptors mediate other effects of VEGF165 such as migration, protein phosphorylation of 231 cells. It may be that these
receptors via its exon 7-encoded domain suggests that these proteins might contain heparan sulfate because exon 7 con-
tains within it the heparin-binding domain of VEGF165. However, the lower affinity/molecular mass VEGF165 receptors on
231 cells are probably not heparan sulfate-containing proteoglycans because heparin augments rather than inhibits their
125I-VEGF165 binding and the relative sharpness of the 165- and 175-kDa complexes is not characteristic of proteoglycans.

In summary, we have characterized a new class of lower affinity/molecular mass VEGF isoform-specific receptors found
on EC and tumor cell surfaces, that bind VEGF165 but not VEGF121. Their structure and function is, however, unclear at
present. Purification of these receptors is now underway in order to better determine their role in modulating VEGF165 activity.

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