Selective Binding of VEGF<sub>121</sub> to One of the Three Vascular Endothelial Growth Factor Receptors of Vascular Endothelial Cells*

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VEGF<sub>121</sub> and VEGF<sub>165</sub> are vascular endothelial growth factor splice variants that promote the proliferation of endothelial cells and angiogenesis. VEGF<sub>165</sub> contains the 44 additional amino acids encoded by exon 7 of the VEGF gene. These amino acids confer upon VEGF<sub>165</sub> a heparin binding capability which VEGF<sub>121</sub> lacks. 125I-VEGF<sub>165</sub> bound to three vascular endothelial growth factor (VEGF) receptors on endothelial cells, while 125I-VEGF<sub>121</sub> bound selectively only to the flk-1 VEGF receptor which corresponds to the larger of the three VEGF receptors. The binding of 125I-VEGF<sub>121</sub> to flk-1 was not affected by the removal of cell surface heparan sulfates or by heparin. Both VEGF<sub>165</sub> and VEGF<sub>121</sub> inhibited the binding of 125I-VEGF<sub>121</sub> to a soluble extracellular domain of the flk-1 VEGF receptor in the absence of heparin. However, heparin potentiated the inhibitory effect of VEGF<sub>121</sub> by 2-3-fold. These results contrast with previous observations which have indicated that the binding of 125I-VEGF<sub>165</sub> to the flk-1 receptor is strongly dependent on heparin-like molecules. Further experiments showed that the receptor binding ability of VEGF<sub>165</sub> is susceptible to oxidative damage caused by oxidants such as H<sub>2</sub>O<sub>2</sub> or chloramine-T. VEGF<sub>121</sub> was also damaged by oxidants but to a lesser extent. Heparin or cell surface heparan sulfates restored the flk-1 binding ability of damaged VEGF<sub>165</sub> but not the receptor binding ability of damaged VEGF<sub>121</sub>. These observations suggest that alternative splicing can generate a diversity in growth factor signaling by determining receptor recognition patterns. They also indicate that the heparin binding ability of VEGF<sub>121</sub> may enable the restoration of damaged VEGF<sub>165</sub> function in processes such as inflammation or wound healing.

Four vascular endothelial growth factor (VEGF)<sup>1</sup> forms are produced by alternative splicing from the VEGF gene (1–3). The 121-amino acid form (VEGF<sub>121</sub>) lacks a heparin binding ability, while VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub> bind efficiently to heparin. All forms are mitogenic to vascular endothelial cells and induce permeabilization of blood vessels (4, 5). VEGF<sub>165</sub> induces angiogenesis in vivo (6) and plays a central role in the process of tumor angiogenesis (7–9). Binding and cross-linking experiments have shown that VEGF<sub>165</sub> binds to three VEGF receptors on the cell surface of vascular endothelial cells (10–12). Two VEGF receptors have recently been identified and cloned (13–16). These include the mouse flk-1 receptor and its human homologue KDR, and the human fit-1 receptor. The flk-1 receptor was shown to transduce a VEGF<sub>165</sub> mitogenic signal (9, 17), while activation of the fit-1 receptor does not seem to result in a similar mitogenic response (16, 18).

The binding of 125I-VEGF<sub>165</sub> to various VEGF receptors and the effects of heparin on the binding have been characterized extensively (10–12, 19), while the interaction of VEGF<sub>121</sub> with the VEGF receptors of vascular endothelial cells has not yet been studied. We show that VEGF<sub>121</sub> binds selectively to the larger of the three VEGF receptors of human umbilical vein-derived endothelial cells (HUE) and that this receptor probably corresponds to the human homologue of the flk-1 VEGF receptor. We also show that both VEGF<sub>165</sub> and VEGF<sub>121</sub> are susceptible to oxidative damage and that heparin restores the receptor binding ability of damaged VEGF<sub>165</sub>, but not the receptor binding ability of damaged VEGF<sub>121</sub>.

EXPERIMENTAL PROCEDURES

Materials—VEGF<sub>165</sub> and VEGF<sub>121</sub> were produced using the baculovirus system as described for VEGF<sub>165</sub> (20). VEGF<sub>189</sub> and VEGF<sub>206</sub> were purified as described previously (19, 21). The flk-1/SEAP soluble receptor was produced as described (19). Anti-flk-1 and anti-flt-1 antibodies directed against peptides derived from the intracellular domains of the respective receptors were purchased from Santa Cruz Biotechnology Inc. Anti-alkaline phosphatase antibodies were purchased from Dako. Tissue culture media and reagents were from Biological Industries (Beth-Haemek Biological Industries, Kibbutz Beth Haemek, Israel) and tissue culture plasticware from Nunc. Disuccinimidyl suberate was from Pierce Chemical Co., 125I-sodium from DuPont NEN, and heparin-Sepharose from Pharmacia. Recombinant bacterial heparinase-1 was kindly provided by Dr. Zimmermann (Ibex Technologies, Montreal, Canada). High molecular mass protein markers were obtained from Bio-Rad. Porcine mucosa-derived heparin (H-7005) was purchased from Sigma as were all of the other chemicals.

Cell Culture—HUE cells were grown in gelatin-coated dishes in M-199 medium supplemented with 20% fetal calf serum, 4 mM glutamine, antibiotics, and 1 ng/ml bFGF which was added to the cells every other day.

KOR; HUE, human umbilical vein-derived endothelial cells; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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†The abbreviations used are: VEGF, vascular endothelial growth factor; VEGF<sub>165</sub>, 165-amino acid form of vascular endothelial growth factor; VEGF<sub>121</sub>, 121-amino acid form of vascular endothelial growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; flk-1, mouse homologue of the human VEGF receptor.
VEGF121 and VEGF165 were produced and purified from the conditioned medium of recombinant baculovirus-infected Sf9 cells and iodinated as described (19, 21). The purified labeled VEGF forms (Fig. 1) were used further in binding and cross-linking experiments. When a saturating concentration of 125I-VEGF121 (20 ng/ml) was bound and cross-linked to HUE cells, only one 125I-VEGF121-receptor complex of about 225 kDa could be detected. The formation of the 225-kDa 125I-VEGF121-receptor complex was completely inhibited when the binding of 125I-VEGF121 was performed in the presence of either 2 μg/ml unlabeled VEGF121 or 2 μg/ml unlabeled VEGF165 (Fig. 2, lanes 2 and 3), indicating that this receptor binds both VEGF121 and VEGF165.

Addition of exogenous heparin (1 μg/ml) to the binding reaction, or removal of cell surface-associated heparan sulfates using heparinase-1, did not affect significantly the formation of the 125I-VEGF121-receptor complex (Fig. 2, lanes 4 and 5, respectively). Additional 125I-labeled VEGF121-receptor complexes were not detected when heparin was added to the binding reaction. In contrast, when 5 ng/ml 125I-VEGF165 were bound to the cells in the presence of 1 μg/ml heparin, two smaller 125I-VEGF165-receptor complexes were observed (Fig. 2, compare lane 7 to lane 8). 125I-VEGF165 also bound to the two smaller VEGF receptors in the absence of added exogenous heparin provided that higher 125I-VEGF165 Concentrations were used (11). In addition, larger 125I-VEGF165 Containing cross-linked complexes of about ~400 kDa were observed (Fig. 2, lane 8). Such high molecular weight complexes were observed by us in the past (23), and they may represent dimerized receptors. Competition with 2 μg/ml VEGF121 inhibited the formation of the 225-kDa 125I-VEGF165-receptor complex but did not affect the formation of the two smaller 125I-VEGF165-receptor complexes (Fig. 2, lane 10, and Fig. 3). The formation of the two smaller 125I-VEGF165-receptor complexes could not be inhibited significantly even when VEGF121 concentrations as high as 20 μg/ml were added to a binding reaction in which 125I-VEGF165 was bound to the cells in the presence of heparin (not shown). It therefore seems that VEGF121 is not able to bind to
the two smaller VEGF receptors, or alternatively, that the affinity of VEGF121 to these receptors is much lower than that of VEGF165. The experiments therefore suggest that VEGF121 and VEGF165 differ not only with regard to their heparin binding ability, but also in their ability to recognize various species of VEGF receptors.

The binding of 125I-VEGF121 to the larger VEGF receptor of the endothelial cells was not affected by the removal of cell surface heparin-like molecules (Fig. 2, lane 5). This was perhaps to be expected as 125I-VEGF121 does not bind to heparin. This observation also indicates that heparin-like molecules do not affect the VEGF121 binding ability of the larger VEGF receptor of the endothelial cells. However, not all of the VEGF receptors that are capable of 125I-VEGF121 binding behave similarly. The 125I-VEGF121 binding ability of the VEGF receptors of YU-ZAZ6 melanoma cells is inhibited upon the removal of cell surface heparin-like molecules by heparinase digestion or by the addition of exogenous heparin, suggesting that heparin-like molecules can modulate the VEGF121 binding ability of the YU-ZAZ6 VEGF receptors (21).

Antibodies Directed against the flk-1 VEGF Receptor Immunoprecipitate 125I-VEGF121-Receptor Complexes—In order to identify the 125I-VEGF121-receptor complexes seen in the crosslinking experiments, immunoprecipitation experiments were performed. The larger 225-kDa 125I-VEGF165- or 125I-VEGF121-labeled receptor was specifically immunoprecipitated by antibodies that recognize the intracellular domain of the flt-1 receptor (Fig. 4A, lane 4, and Fig. 4B, lane 2, respectively). In contrast, none of the labeled complexes could be precipitated by antibodies that recognize the intracellular domain of the flk-1 receptor or by an irrelevant antibody (Fig. 4). The inability to immunoprecipitate 125I-VEGF-receptor complexes with anti-flt-1 antibodies was expected since the mRNA encoding the flt-1 VEGF receptor is not very abundant in HUE cells (21). The two smaller 125I-VEGF165-receptor complexes were not precipitated by any of these antibodies and may represent either novel VEGF receptors or truncated versions of known VEGF receptors which are not recognized by the antibodies used.

125I-VEGF121 Binds to a Soluble Fusion Protein Containing the Extracellular Domain of flk-1, and the Binding Is Inhibited by VEGF121 and VEGF165 Even in the Absence of Heparin—The 225-kDa 125I-VEGF121 receptor complex of HUE cells appears to contain the KDR/flk-1 receptor. To verify that 125I-VEGF121 can indeed bind to the flk-1 VEGF receptor, the interaction of VEGF121 and VEGF165 with a soluble fusion protein containing the entire extracellular domain of flk-1 fused to soluble alkaline phosphatase (flk-1/SEAP) was examined (Fig. 5, A–C) (19). This soluble fusion protein was adsorbed to ELISA dishes coated with an antibody to alkaline phosphatase and used in quantitative binding assays. As expected, 125I-VEGF121 bound to flk-1/SEAP, and the binding was inhibited by unlabeled VEGF121 (Fig. 5B). The binding was effective even in the absence of exogenous heparin, and the addition of increasing concentrations of heparin did not affect the binding (not shown). However, to our surprise, we have found that unlabeled VEGF165, was able to compete with 125I-VEGF121 for binding to the flk-1/SEAP fusion protein in the absence of exogenous heparin (Fig. 5A). Addition of heparin modulated the concentration at which a half-maximal displacement of bound 125I-VEGF121 was observed, shifting it to VEGF165 concentrations 2–3-fold lower than those seen in the absence of heparin (not shown). Similar results were obtained when similar binding experiments were performed using heparinase-digested HUE cells (Fig. 5D). These observations are not in agreement with experiments which have indicated that unless
VEGF<sub>121</sub> Binding to Endothelial Cells

**Fig. 5.** Heparin restores the receptor binding ability of oxidized VEGF<sub>165</sub>, but not the receptor binding ability of oxidized VEGF<sub>121</sub>. A, 125I-VEGF<sub>121</sub> (25 ng/ml) was bound to ELISA dishes coated with flk-1/SEAP fusion protein in a final volume of 50 μl as described under “Experimental Procedures.” The binding was performed in the presence (▲) or absence (□) of heparin (1 μg/ml) and in the presence of increasing concentrations of either untreated VEGF<sub>165</sub> (▲) or VEGF<sub>165</sub> which was treated with chloramine-T as described under “Experimental Procedures” (▲, ▲). The binding was performed for 2 h at room temperature, after which the dishes were washed 3 times with buffer containing 0.1% Tween 20 as described. Bound 125I-VEGF<sub>121</sub> was solubilized using 0.5 n NaOH, and aliquots were counted in a γ-counter. 100% of 125I-VEGF<sub>121</sub> binding corresponds to 10,000 cpm. B, 125I-VEGF<sub>121</sub> (25 ng/ml) was bound to ELISA dishes coated with flk-1/SEAP fusion protein in a final volume of 50 μl as described. The binding was performed in the presence (▲) or absence (□) of heparin (1 μg/ml) and in the presence of increasing concentrations of either untreated VEGF<sub>121</sub> (▲) or VEGF<sub>121</sub> which was treated with chloramine-T as described (▲, ▲). The binding was performed as described under A. 100% of 125I-VEGF<sub>121</sub> binding corresponds to 5500 cpm. C, 125I-VEGF<sub>121</sub> (25 ng/ml) was bound to ELISA dishes coated with flk-1/SEAP fusion protein as described. The binding was performed in the presence (hatched columns) or in the absence (empty columns) of 1 μg/ml heparin. The VEGF<sub>121</sub> and VEGF<sub>165</sub> concentration used for competition was 1 μg/ml. VEGF<sub>165</sub> and VEGF<sub>121</sub> were treated or not with H<sub>2</sub>O<sub>2</sub> (1%) as described under “Experimental Procedures.” D, 125I-VEGF<sub>165</sub> (20 ng/ml) was bound and cross-linked to confluent heparinase 1-digested HUE cells grown in 10-cm dishes in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of 1 μg/ml heparin. Unlabeled VEGF<sub>165</sub> was added to a final concentration of 2 μg/ml to some of the binding reactions (lanes 3 and 4). The heparinase digestion and the visualization of cross-linked complexes were done as described in Fig. 2.

Very high concentrations of 125I-VEGF<sub>165</sub> are used, 125I-VEGF<sub>165</sub> does not bind to flk-1/SEAP in the absence of heparin (19). These experiments also disagree with observations which have shown that the binding of 125I-VEGF<sub>165</sub> to cell surface receptors on vascular endothelial cells requires cell surface heparan sulfates (Fig. 2, lanes 11 and 12) (11).

These experiments imply that the receptor binding ability of VEGF<sub>165</sub> may be impaired during iodination. Subsequent experiments have indicated that the flk-1/SEAP binding ability of VEGF<sub>165</sub> is sensitive to oxidants. VEGF<sub>165</sub>, damaged by oxidizing agents such as chloramine-T or H<sub>2</sub>O<sub>2</sub>, was not able to compete with 125I-VEGF<sub>121</sub> for binding to flk-1/SEAP in the absence of heparin (Fig. 5, A and C). However, the ability to compete with 125I-VEGF<sub>121</sub> for binding to flk-1/SEAP was partially restored by the addition of 1 μg/ml heparin to the binding reaction (Fig. 5, A and C). Similar results were also obtained in analogous experiments performed with heparinase-treated HUE cells (not shown). Oxidized VEGF<sub>121</sub> also lost some of its ability to compete with 125I-VEGF<sub>121</sub> for binding to flk-1/SEAP, although VEGF<sub>121</sub> seemed to be somewhat more resistant than VEGF<sub>165</sub> to oxidation (Fig. 5, B and C). However, in contrast to VEGF<sub>165</sub>, the addition of heparin did not restore the flk-1/SEAP binding ability of damaged VEGF<sub>121</sub> (Fig. 5, B and C).

The relative insensitivity of VEGF<sub>121</sub> to oxidative damage may explain why 125I-VEGF<sub>121</sub>, in contrast to 125I-VEGF<sub>165</sub>, is still able to bind to flk-1/SEAP and to the flk-1 receptor of the vascular endothelial cells in the absence of heparin-like molecules.

The potentiating effect that heparin exerts on the receptor binding ability of untreated VEGF<sub>165</sub> could reflect oxidative damage sustained before or during VEGF<sub>165</sub> purification. Alternatively, it could mean that heparin has a real ability to potentiate the binding of undamaged VEGF<sub>165</sub> to the flk-1 VEGF receptor and perhaps to other types of VEGF receptors as well. Our results imply that the heparin binding ability of VEGF<sub>165</sub> may be required under conditions in which oxidizing agents and free radicals are produced. Such conditions can be encountered in biological processes such as wound healing, hypoxia-induced angiogenesis, or inflammation, processes in which VEGF<sub>165</sub> was shown to play an important role (24–28). Under such conditions, cell surface heparin-like molecules could restore the activity of damaged VEGF<sub>165</sub> molecules. This restorative function of heparin-like molecules could be of critical importance under conditions in which the initial concentration of VEGF<sub>165</sub> is low to begin with. Heparin-like molecules are also able to restore the activity of damaged bFGF and aFGF.
and to protect them from inactivation by heat and oxidation (29, 30). The protective and restorative effects of heparin could perhaps account for some of the opposing conclusions that were obtained in experiments designed to assess the importance of heparin-like molecules in the interaction of bFGF with FGF receptors (31–33). Such a restorative effect would be harder to detect in the case of bFGF since an active bFGF homologue lacking a heparin binding ability (like VEGF$_{121}$) is unavailable.

In conclusion, our experiments indicate that both VEGF$_{121}$ and VEGF$_{165}$ bind to the 180-kDa VEGF receptor of HUE cells forming 220–230-kDa complexes after covalent cross-linking. However, only VEGF$_{165}$ is capable of binding to the two smaller VEGF receptors of the endothelial cells. To the best of our knowledge, this is the first time that splice variants of a growth factor are found to differ in receptor recognition patterns. We have also performed immunoprecipitation experiments which indicate that the 220-kDa $^{125}$I-VEGF-receptor complex contains the KDR/flk-1 VEGF receptor. Competition experiments using a soluble fusion protein containing the extracellular domain of flk-1 have also revealed that heparin is not essential for the binding of VEGF$_{121}$ or VEGF$_{165}$ to flk-1 receptors. These experiments also suggest that the ability to bind heparin-like molecules may help to preserve the biological function of VEGF$_{165}$ under conditions in which oxidants and free radicals are produced.

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