Heparin Modulates the Interaction of VEGF\textsubscript{165} with Soluble and Cell Associated flk-1 Receptors*

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The 165-amino acid form of vascular endothelial growth factor (VEGF\textsubscript{165}) is a mitogen for vascular endothelial cells and a potent angiogenic factor. Expression of a chimeric receptor containing the extracellular domain of the \textit{flk}-1 receptor fused to the transmembrane and intracellular domains of the human \textit{c-fms} receptor in NIH-3T3 cells, resulted in the appearance of high affinity binding sites for 125I-VEGF\textsubscript{165} on transfected cells. The binding of 125I-VEGF\textsubscript{165} to the \textit{flk}-1/SEAP fusion protein specifically inhibited VEGF\textsubscript{165}-induced autophosphorylation of the chimeric receptors that were inhibited in the presence of low concentrations of heparin (1–10 \textmu g/ml). In contrast, similar concentrations of heparin potentiated the binding of 125I-VEGF\textsubscript{165} to the endogenous VEGF receptors of the transfected cells, indicating that to some extent, the effect of heparin on 125I-VEGF\textsubscript{165} binding is receptor type-dependent.

A soluble fusion protein containing the extracellular domain of \textit{flk}-1 fused to alkaline phosphatase (\textit{flk}-1/SEAP) was used to study the effects of heparin on the binding of 125I-VEGF\textsubscript{165} to \textit{flk}-1 in a cell-free environment. The fusion protein specifically inhibited VEGF\textsubscript{165}-induced proliferation of vascular endothelial cells, but bound 125I-VEGF\textsubscript{165} inefficiently in the absence of heparin. Addition of low concentrations of heparin or heparan sulfate (0.1–1 \textmu g/ml) resulted in a strong potentiation of 125I-VEGF\textsubscript{165} binding, whereas higher heparin or heparan sulfate concentrations inhibited the binding. The effect of heparin on the binding of 125I-VEGF\textsubscript{165} to \textit{flk}-1/SEAP could not be mimicked by desulfated heparin or by chondroitin sulfate. Both bFGF and aFGF inhibited the binding when low concentrations of heparin were added to the binding reaction. However, higher concentrations of heparin abolished the inhibition, indicating that the inhibition is probably caused by competition for available heparin. Taken as a whole, these results indicate that heparin-like molecules regulate the binding of VEGF\textsubscript{165} to its receptors in complex ways which depend on the heparin binding properties of VEGF\textsubscript{165} on the specific VEGF receptor type involved, and on the amount and composition of heparin-like molecules that are present on the cell surface of VEGF receptor containing cells.

Vascular endothelial growth factor (VEGF)\textsuperscript{1} is a secreted heparin-binding glycoprotein that displays some structural homology with PDGF. Five forms of human VEGF mRNA encoding VEGF proteins of 121, 145, 165, 189, and 206 amino acids are produced from a single gene as a result of alternative splicing (1, 2). The best characterized VEGF species is the 165-amino acid long form (VEGF\textsubscript{165}). The active form of VEGF\textsubscript{165} is a homodimer of 47 kDa (1, 3) that induces angiogenesis and blood vessel permeabilization \textit{in vivo} and displays a mitogenic activity that seems to be restricted to vascular endothelial cells (1, 4–8). Several recent reports indicate that VEGF may play an important role in the process of tumor angiogenesis (9–11). VEGF\textsubscript{165} binds to specific cell surface receptors which are found on vascular endothelial cells, and on several types of non-endothelial cells such as NIH-3T3 cells and melanoma cells which do not seem to respond to VEGF\textsubscript{165} with a mitogenic response (12–16). Cell surface-associated heparin-like molecules are required for the interaction of VEGF\textsubscript{165} with the three VEGF receptor types observed on vascular endothelial cells (15) and with the two receptor types present in WW94 melanoma cells (16).

The protein encoded by the \textit{flt} gene was recently reported to be a VEGF receptor (17, 18), belonging to the PDGF receptor subfamily of the tyrosine kinase receptors (19). The \textit{flt}-1 gene was isolated from an embryonic liver-derived cell population enriched with primitive hematopoietic stem cells (20, 21) and encodes a tyrosine kinase receptor that is homologous to \textit{flt}. The product of the \textit{flk}-1 gene and the product of its human homologue KDR also bind VEGF and undergo autophosphorylation in response to VEGF. However, it is not clear yet whether \textit{flt}-1 or \textit{flk}-1 can transduce a VEGF induced mitogenic signal (22, 23). Both \textit{flt} and \textit{flk}-1 contain seven immunoglobulin-like loops in their extracellular domains, whereas other members of the PDGF receptor family such as \textit{c-kit} or \textit{c-fms} contain only five immunoglobulin-like loops (20).

We have expressed the \textit{flk}-1 cDNA and a chimeric gene containing the extracellular domain of \textit{flk}-1 and the tyrosine kinase domain encoded by the \textit{c-fms} gene in NIH-3T3 cells, and we report that both the chimera and the native receptor encoded by the \textit{flk}-1 gene bind VEGF\textsubscript{165} with high affinity. We show that heparin concentrations that inhibit the binding of 125I-VEGF\textsubscript{165} to the chimeric receptor, and the VEGF\textsubscript{165}-induced autophosphorylation of the chimeric receptor, potentiate the binding of VEGF\textsubscript{165} to endogenous receptors of NIH-3T3 cells. We also present evidence indicating that the binding of

\textsuperscript{1}The abbreviations used are: VEGF, vascular endothelial growth factor; VEGF\textsubscript{165}, 165-amino acid form of vascular endothelial growth factor; \textit{flt}, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CSF-1, colony stimulating factor 1; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; SEAP, secreted alkaline-phosphatase.

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VEGF<sub>165</sub> to a soluble fusion protein containing the entire extracellular domain of the flk-1 receptor is strongly enhanced by heparin, but not by chondroitin sulfate or desulfated heparin.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant VEGF<sub>165</sub> was purified from the conditioned medium of the c-fms-expressing cells infected with a baculovirus-based expression vector for VEGF<sub>165</sub> as described (24). The factor was highly purified as determined by SDS-PAGE chromatography followed by silver staining, using three purification steps, including hydrophobic chromatography, cation-exchange chromatography, and heparin-Sepharose affinity chromatography. Recombinant human bFGF and aFGF were produced by bacterial expression and were purchased from Amersham. Recombinant PDGF-BB was kindly given by Dr. C. Goeddel (Genentech, San Francisco, CA).

RESULTS

The Receptor Encoded by the flk-1 Gene Binds VEGF<sub>165</sub> with High Affinity—Full-length flk-1 cDNA was subcloned into the moloney murine leukemia virus long terminal repeat expression vector pMFG. This expression vector was stably transfected into NIH-3T3 cells and baby hamster kidney-derived fibroblast (BHK-21 cells) (28). The presence of VEGF binding sites was examined in geneticin-resistant clones of cells using <sup>125</sup>I-VEGF<sub>165</sub> binding (12). The transfection resulted in the appearance of clones which expressed low densities (no more than 600 receptors/cell) of high affinity <sup>125</sup>I-VEGF<sub>165</sub> binding sites (not shown). We could not detect a mitogenic response to VEGF<sub>165</sub> in these cells nor could we detect VEGF<sub>165</sub>-induced autophosphorylation.

To try to overcome these problems we have expressed in NIH-3T3 cells a chimeric cDNA containing the extracellular domain of flk-1 fused to the transmembrane and intracellular domains of the CSR-1 receptor (c-fms). Saturating binding experiments with <sup>125</sup>I-VEGF<sub>165</sub> were bound to NIH-3T3 clone C4 cells expressing the chimeric flk-1/c-fms receptors were analyzed by the method of Scatchard using the ligand program (29). These experiments revealed one class of high affinity binding sites for <sup>125</sup>I-VEGF<sub>165</sub> with a dissociation constant of 1.9 × 10<sup>-11</sup> M. The density of these flk-1/c-fms receptors was 2800 receptors/cell (Fig. 1D).

Cross-linking experiments revealed a <sup>125</sup>I-VEGF<sub>165</sub>-contain-
The binding of '25I-VEGF₁₆₅ to flk-1/c-fms-producing cells. A, expression of the flk-1/c-fms chimeric receptor in NIH-3T3 cells. Flk-1/c-fms-producing (lanes 1 and 2) and nonproducing (lane 3) NIH-3T3 cells were grown to subconfluence in 6-cm dishes. The binding of '25I-VEGF₁₆₅ (5 ng/ml) to the cells in the presence (lane 2) or absence (lanes 1 and 3) of 0.5 µg/ml of unlabeled VEGF₁₆₅ and the subsequent cross-linking of bound '25I-VEGF₁₆₅ to the receptors were conducted as described under "Materials and Methods." B, representative saturation binding experiment with flk-1/c-fms expressing NIH-3T3 cells. Cells were grown to subconfluence in 24-multiwell dishes (160,000 cells/well). Increasing concentrations of '25I-VEGF₁₆₅, ranging from 10 pglml to 10 ng/ml, were bound to the cells for 2 h at 4 °C. Nonspecific binding was measured in the presence of 0.5 µg/ml unlabeled VEGF₁₆₅, and the specific binding was calculated by the subtraction of the nonspecific binding from the total binding. At the end of the binding reaction, the cells were washed three times with 1 ml of ice-cold Dulbecco's phosphate-buffered saline containing 1 mg/ml BSA. The cells were then solubilized with 0.5 ml of 0.2 n NaOH. Aliquots were counted in a γ-counter. Shown is a saturation curve in which the amount of specifically bound '25I-VEGF₁₆₅ is plotted as a function of added '25I-VEGF₁₆₅ concentrations and a Scatchard plot derived from the saturation curve (inset).
Methods." The cells were stimulated with VEGF,65 at 5 or 10 ng/ml for 8 min in the absence or presence of the indicated concentrations of heparin. Following stimulation, cells were lysed and the receptors immunoprecipitated with anti-c-fms antibodies as described under "Materials and Methods." Immunoprecipitated material was chromatographed on a SDS-PAGE gel, transferred to nitrocellulose, and tyrosine-phosphorylated proteins were visualized as described under "Materials and Methods.

To study the interaction between VEGF,65 and the flk-1 receptor in a controlled environment in which the composition of glycosaminoglycans can be controlled precisely, we produced a soluble receptor. '125I-VEGF,65 was incubated with the fusion protein and the visualization of cross-linked complexes were done as described under "Materials and Methods." The labeling density of the 125I-VEGF,65/flk-1/SEAP complexes that were formed (see B) was determined using a cliniscan-2 densitometer, and plotted as a function of heparin concentration. B, the effect of native and modified heparins and of various glycosaminoglycans on the binding of 125I-VEGF,65 to flk-1/SEAP. 125I-VEGF,65 (10 ng/ml) was bound to soluble flk-1/SEAP (100 ng/ml) in the presence of the following additions: lanes 1 and 3, no additions; lanes 2 and 4, 0.1 µg/ml heparin; lane 5, 0.1 µg/ml chondroitin sulfate A, lane 6, 0.1 µg/ml chondroitin sulfate C; lane 7, 0.1 µg/ml N/O-desulfated heparin; lane 8, 0.005 µg/ml O-oversulfated heparin. Binding was performed for 1 h at room temperature. The subsequent cross-linking of bound 125I-VEGF,65 to the flk-1/SEAP fusion protein and the visualization of cross-linked complexes were done as described under "Materials and Methods."
The binding of 125I-VEGF<sub>165</sub> to the endogenous VEGF receptors of vascular endothelial cells and to the small number of endogenous VEGF receptors found in NIH-3T3 cells is potentiated by the addition of 1 pg/ml heparin (15). Unexpectedly, both the VEGF<sub>165</sub>-induced autophosphorylation of the chimeric flk-1/c-fms receptors expressed in the NIH-3T3 clone C4 cells and the binding of 125I-VEGF<sub>165</sub> to these receptors were inhibited by heparin concentrations equal or larger than 1 pg/ml, whereas lower heparin concentrations had no effect. It follows that heparin can potentiate the binding of 125I-VEGF<sub>165</sub> to one class of VEGF receptors and inhibit the binding of 125I-VEGF<sub>165</sub> to another class of VEGF receptors simultaneously. These experiments indicate therefore that the effect that heparin will have on the binding of 125I-VEGF<sub>165</sub> to specific VEGF receptors depends not only on the heparin binding ability of the growth factor, but to some extent also on specific characteristics associated with specific VEGF receptor types.

It was recently reported that the ligand binding ability of fibroblast growth factor receptor-1 is regulated by the direct binding of heparin to the receptor (35). The effect of heparin on 125I-VEGF<sub>165</sub> binding could also be in part the result of a direct interaction between heparin and a subset of VEGF receptors. Alternatively, it is possible that heparin modulates the binding of VEGF<sub>165</sub> to its receptors indirectly through specific cell surface heparin-binding proteins. It was reported that vascular endothelial cells express cell surface heparin receptors (36), and it was shown that cell surface-bound heparin can potentiate the binding of 125I-VEGF<sub>165</sub> to the VEGF receptors of vascular endothelial cells (15). Taken together, the experiments suggest that the effect of heparin on the interaction of VEGF<sub>165</sub> with cell surface VEGF receptors is a complex process that needs to be studied using an experimental setup that will allow precise control of the binding environment.

We have taken a step toward the establishment of such an experimental setup by producing a soluble chimeric VEGF receptor containing the entire extracellular domain of flk-1 fused to soluble alkaline phosphatase (25). This flk-1/SEAP fusion protein turned out to be a specific inhibitor of VEGF<sub>165</sub>-induced cell proliferation. This soluble receptor could perhaps be used in the future as an in vivo VEGF<sub>165</sub> antagonist. The availability of the fusion protein allowed us to conduct binding experiments in a precisely regulated cell free environment. The flk-1/SEAP-soluble receptor did not bind 125I-VEGF<sub>165</sub> efficiently in the absence of heparin, but addition of low heparin concentrations (as low as 5 ng/ml) to the binding reaction strongly potentiated the binding of 125I-VEGF<sub>165</sub> to flk-1/SEAP. The effect of heparin on 125I-VEGF<sub>165</sub> binding could not be mimicked by chondroitin sulfate, but heparan sulfate had a similar effect. The sulfation level of the heparin was important for the potentiating effect, since desulfated heparin had no activity,
whereas oversulfated heparin potentiated the binding of $^{125}$I-VEGF<sub>165</sub> more efficiently than heparin. These experiments, done in a controlled cell free environment, indicate that heparin can directly modulate the binding of $^{125}$I-VEGF<sub>165</sub> to flk-1/SEAP and that the modulation in this case is probably mediated exclusively through the interaction of heparin with $^{125}$I-VEGF<sub>165</sub> since flk-1/SEAP does not appear to bind to heparin-Sepharose.

In the presence of 1 μg/ml heparin, the binding of $^{125}$I-VEGF<sub>165</sub> to the flk-1/c-fms receptors on transfected NIH-3T3 cells is inhibited, whereas the same concentration of heparin potentiates the binding of $^{125}$I-VEGF<sub>165</sub> to the soluble flk-1/SEAP receptor. Since cell surfaces already contain heparin-like molecules, it follows that the effect of exogenous heparin on the binding of $^{125}$I-VEGF<sub>165</sub> to a cell surface located VEGF receptor will be superimposed upon the effect of pre-existing cell surface associated heparin-like molecules. In addition, putative heparin binding cell surface receptors may also modulate the binding, resulting in complex final effects which are avoided by the usage of the soluble flk-1/SEAP receptors.

The binding of $^{125}$I-VEGF<sub>165</sub> to the flk-1/SEAP-soluble receptor could not be inhibited by the growth factors PDGF and EGF (6–8). In contrast, high concentrations of bFGF and aFGF inhibited the binding of $^{125}$I-VEGF<sub>165</sub> to the soluble flk-1/SEAP fusion protein. We found that these growth factors lost their ability to inhibit the binding of $^{125}$I-VEGF<sub>165</sub> to the soluble flk-1/SEAP fusion protein when the binding experiments were conducted in the presence of high concentrations of heparin. It therefore follows that competition for available cell surface heparin-like molecules could perhaps function as an indirect cross-talk mechanism by which a growth factor such as bFGF may modulate the activity of another heparin binding growth factor such as VEGF<sub>165</sub>.

To conclude, our results indicate that heparin affects the interaction of VEGF<sub>165</sub> with various VEGF receptors by more than one mechanism. We have also shown that heparin can modulate the interaction of VEGF<sub>165</sub> with the VEGF binding domain of the flk-1 receptor, even when the binding is done in an environment that contains only flk-1/SEAP receptors, VEGF, and heparin. The results of this study indicate that VEGF may play an important role in the maturation process of hematopoietic cells, since the flk-1 cDNA was originally isolated from a cell population enriched with primitive hematopoietic stem cells (20).

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