Identification of a 190-kDa Vascular Endothelial Growth Factor 165 Cell Surface Binding Protein on a Human Glioma Cell Line

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Vascular endothelial growth factor (VEGF) is an angiogenesis factor for which two signaling protein tyrosine kinase receptors, Flt1 and KDR, have been identified. We describe here a 190-kDa component present on a human glioma cell line that binds VEGF165 with high affinity. In contrast, VEGF121 is bound only with low affinity, suggesting that the C-terminal part of VEGF165 is important for interaction with the 190-kDa component. No internalization or stimulation of tyrosine phosphorylation was recorded after ligand binding to the 190-kDa component, suggesting that it may not be directly involved in signaling; its function may be to present ligand or stabilize ligand binding to signaling receptors.

Vascular endothelial growth factor (VEGF) is a dimeric molecule related to platelet-derived growth factor and occurs as four different isoforms with 121, 165, 189, and 206 amino acid residues, as a result of different splicing of the gene (2–5). Whereas VEGF121 does not bind heparin, VEGF165 binds heparin with medium affinity, and VEGF189 and VEGF206 bind heparin with high affinity (6–8).

VEGF binds to two structurally similar protein tyrosine kinase receptors, designated Flt1 and KDR (9–12). Activation of the KDR receptor has been shown to lead to mitogenicity, chemotaxis, and morphological changes (13, 14). The role of Flt1 in signal transduction is less well characterized, but Flt1 has been shown to mediate e.g. tissue factor induction in endothelial cells (15). VEGF was originally identified as a growth factor specific for endothelial cells; however, recent observations have suggested that the VEGF receptors KDR and Flt1 are not exclusively expressed on endothelial cells (16). Recently, cell surface proteins of 120–130 kDa were identified on a breast cancer cell line and on endothelial cells that were shown to bind the exon 7-encoded sequence of VEGF165; VEGF121, which lacks this sequence, did not bind the 120–130-kDa components (17, 18). In this report, we describe a 190-kDa component present on a human glioma cell line, which binds VEGF165 but not VEGF121 with high affinity.

EXPERIMENTAL PROCEDURES

Materials—Human VEGF165, and a rabbit antisera against human VEGF were purchased from Pepro Tech Inc. Human VEGF121 was a kind gift from Dr. Gera Neufeld (16, 19). A rabbit antiserum raised against the intracellular domain part of KDR has been described previously (13). An Flt1 antiserum was purchased from Santa Cruz Bio-technology Inc., and an anti-phosphotyrosine monoclonal antibody (PY20) was purchased from Transduction Laboratories. The anti-phosphotyrosine polyclonal antiserum (PY6) has been described previously (20).

Endoglycosidase F/peptide N-glycosidase F was purchased from Boehringer Mannheim. Induction of human VEGF165 and VEGF121 were performed using the Bolton and Hunter method (22); 125I-labeled VEGF was separated from free 125I using Sephadex G-25 and kept in phosphate-buffered saline with 5 mg/ml bovine serum albumin at 4 °C. The specific activities of the labeled VEGF165, and VEGF121 were about 6 × 106 cpm/ng and 3 × 107 cpm/ng, respectively.

Cell Culture—The porcine aortic endothelial (PAE) cell line and PAE cell lines transfected with cDNA for KDR (PAE/KDR) and Flt1 (PAE/Flt1) were cultured in Ham’s F-12 medium (15). U-178MG human glioma cells (gift of B. Westerman, Department of Pathology, Uppsala, Sweden) (21) and MDA MB231 cells (purchased from American Type Culture Collection) were cultured in Eagle’s minimum essential medium and Dulbecco’s modified essential medium, respectively. All cell lines were cultured in 10% fetal calf serum, 100 units/ml penicillin, 50 mg/ml streptomycin, and 4 mM l-glutamine.

Binding Experiments—In some experiments 125I-VEGF165 purchased from Amersham Corp. was used. Binding experiments with 1 ng/ml 125I-VEGF104 and 125I-VEGF121 in the absence or the presence of various concentrations of unlabeled ligands were performed as described before, using U-178MG cells, as well as nontransfected and transfected PAE cell lines in 24-well dishes (13). To investigate ligand-dependent internalization of VEGF receptors, cells were preincubated and kept in phosphate-buffered saline with 5 mg/ml bovine serum albumin and incubated for 1 min with binding buffer supplemented with 20 mM acetic acid, pH 3.75, to dissociate cell surface receptor-bound VEGF, and then subjected to 125I-VEGF165 binding assays (13).

Immunoprecipitations of Metabolically Labeled Receptors—Cells were metabolically labeled with [35S]methionine for 3 h. After extraction with lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 1 mM benzamidine, and 5 μg/ml leupeptin) and centrifugation at 10,000 × g for 15 min, the receptors were immunoprecipitated with antiserum against KDR or Flt1. After SDS gel electrophoresis using 7% polyacrylamide gels, the labeled proteins were analyzed using a phosphoimager (FUJIX BAS 2000, Fuji). For immunoprecipitation with anti-VEGF antisera, cells labeled for 3 h with [35S]methionine were incubated with 100 ng/ml VEGF165 for 1 h at 4 °C and then analyzed as described above.

Cross-linking Experiments—U-178MG cells, MDA MB231 cells, as well as nontransfected and transfected PAE cell lines (cultured in 10-cm dishes) were washed twice with phosphate-buffered saline supplemented with 1 mg/ml bovine serum albumin and incubated for 90 min on ice with 10 ng/ml 125I-VEGF165. After three washes with phosphate-buffered saline, ligand-receptor complexes were cross-linked by incubation in 0.1 mM bis(sulfosuccinimidyl) suberate for 30 min at room temperature. After incubation in 70 mM methylammonium chloride for 10 min, cell lysates were prepared as described above and subjected to immunoprecipitation with antisera against KDR, Flt1, or VEGF. Some of these samples were heat-denatured in the presence of 0.2% SDS and 2% 2-mercaptoethanol and deglycosylated with endoglycosidase F/peptide N-glycosidase F (0.2 units) overnight at 37 °C in 0.1 mM potassium phosphate buffer, pH 6.5, containing 10 mM EDTA and 2% n-octyl-β-D-glycoside. Samples were analyzed by SDS gel electrophoresis using 4–12% gradient or 7% homogenous polyacrylamide gels, followed by autoradiography using a phosphoimager.
The amount of cell-associated radioactivity was determined in a g mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 10,000× g for 15 min, and the supernatants were immunoprecipitated with 125I-VEGF165 (10 ng/ml) was bound and cross-linked to confluent cultures of PAE cells (open circles), PAE/KDR cells (closed circles), PAE/Flt1 cells (open squares), and U-178MG cells (closed squares) were incubated with 1 ng/ml 125I-VEGF165 in the absence or the presence of various concentrations of unlabeled VEGF165 for 2 h at 4 °C. After washing three times, the amount of cell-associated radioactivity was determined in a γ-counter. The number of VEGF binding sites on each cell and their Kd were obtained from Scatchard analysis.

Tyrosine Phosphorylation and Immunoblotting—Cells were stimulated with 100 ng/ml VEGF165 for 60 min at 4 °C, washed with Tris-buffered saline containing 0.5 mM Na3VO4, 1 mM dithiothreitol, and solubilized in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM Na3VO4, 1 mM dithiothreitol, 1% Trasylol, and 1 mM phenethylmethylsulfonyl fluoride. The cell lysates were centrifuged at 10,000× g for 15 min, and the supernatants were immunoprecipitated with a pan-phosphotyrosine polyclonal antiserum (PY6). After SDS gel electrophoresis, samples were blotted onto nitrocellulose membranes (Hybond C extra, Amersham Corp.). Membranes were incubated with an anti-phosphotyrosine polyclonal antiserum (PY20, and immune complexes were detected using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (ECL, Amersham Corp.).

**RESULTS**

**High Affinity Binding of VEGF to U-178MG Cells**—High specific binding of 125I-VEGF165 was found on the human glioma cell line U-178MG. To estimate the binding affinity, the binding of 125I-VEGF165 was determined in the presence of various concentrations of unlabeled VEGF165; half maximal competition of specific binding was observed at approximately 10 ng/ml (Fig. 1). Scatchard analysis revealed that about 84000 VEGF binding sites on U-178MG cells. In parallel, similar binding studies were performed on PAE cells stably transfected with KDR and Flt1 (PAE/KDR and PAE/Flt1, respectively). Untransfected PAE cells did not bind VEGF. The affinity of binding of 125I-VEGF165 to PAE/KDR cells was similar to the binding to U-178 MG cells with half-maximal competition at about 100 ng/ml, whereas half-maximal competition for the binding to Flt1 cells was observed at approximately 15 ng/ml of VEGF165. The higher binding affinity for VEGF165 to PAE/Flt1 cells as compared with PAE/KDR cells is in agreement with previous studies using the same cell lines (13).

**The VEGF Binding Protein on U-178MG Cells Is Not Flt1 or KDR**—To investigate if the binding of VEGF165 was mediated by KDR or Flt1 receptors, immunoprecipitation experiments using KDR and Flt1 antisera were performed (Fig. 2). PAE, PAE/KDR, PAE/Flt1, and U-178MG cells were incubated on ice with 125I-VEGF165, and bound ligand was cross-linked to the receptors by incubation with bis(sulfosuccinimidyl)suberate. After lysis of the cells, immunoprecipitations were performed with KDR antiserum on PAE, PAE/KDR, and U-178MG cells and with Flt1 antibodies on lysates from PAE, PAE/Flt1, and U-178MG cells. Immune complexes were then analyzed by SDS-polyacrylamide gel electrophoresis and visualized by phosphoimager analysis. As shown in Fig. 2, the expected cross-linked monomeric and dimeric KDR and Flt1 receptor forms were recovered from the transfected cells (lanes 2 and 5). A component of about 130 kDa was also seen in the PAE/Flt1 cells (lane 5); this component was not seen in the untransfected PAE cells (lane 4) and may represent a degraded form of Flt1. However, no KDR (lane 3) or Flt1 (lane 6) receptors were precipitated from U-178MG cells using the respective antiserum. A faint component of about 190 kDa was sometimes seen after precipitation with KDR antiserum, but this component was clearly smaller than KDR. The notion that U-178MG cells do not contain any KDR protein was also strengthened by the lack of signal in immunoblot assays performed on anti-KDR immune complexes derived from U-178MG cells (data not shown). Neither was any KDR or Flt1 mRNA detected in Northern blot analysis of U-178MG cells (data not shown). We thus conclude that the high affinity VEGF binding to U-178MG cells is not due to the presence of KDR or Flt1 receptors.

**VEGF Binding to the U-178MG Cells Is Dependent on the VEGF Exon 7-encoded Sequence**—A VEGF165 binding protein, distinct from Flt1 and KDR, of 233 kDa was recently identified on MDA MB231 cells (18). Interestingly, VEGF165 did not bind to this receptor. A component of similar size and with similar binding specificity was also identified on human...
umbilical vein-derived endothelial cells (17). To explore if this pattern of splice form-specific binding was also a property of the VEGF binding protein on U-178MG cells, we performed binding experiments with 125I-labeled forms of VEGF 165 and VEGF121. For comparison the same type of binding experiments were also performed on PAE/KDR and PAE/Flt1 cells.

As shown in Fig. 3A, the binding of 125I-VEGF165 to PAE/KDR and PAE/Flt1 cells was efficiently competed for with high concentrations of both VEGF165 and VEGF121. In contrast, 125I-VEGF165 binding to U-178MG was only inhibited to about 50% of maximum binding by 625 ng/ml by VEGF 121, whereas almost complete competition was obtained by the same concentration of VEGF165. When 125I-VEGF121 was used, binding was readily detected to PAE/KDR and PAE/Flt1 cells (Fig. 3B). In contrast, very low specific binding was observed to U-178MG cells. Together these data demonstrate a clear difference with regard to the dependence on the exon 7-coded sequence for high affinity binding between KDR and Flt1 on one hand and the VEGF binding proteins on U-178MG cells (Fig. 3) and the 120–130-kDa proteins detected on MDA MB231 (18) and umbilical vein-derived endothelial cells (17).

**Determination of the M<sub>r</sub> of the VEGF Binding Protein on U-178MG Cells**—To determine the M<sub>r</sub> of the VEGF receptor on U-178MG cells conventional affinity labeling techniques were tried but were found to give too high a background. Two different alternative strategies, both involving VEGF antibody-mediated precipitation of the complex between ligand and binding protein, were therefore employed (Fig. 4).

In the first approach, PAE, PAE/KDR, and U-178MG cells were metabolically labeled with [35S]methionine and subsequently incubated on ice in the absence or the presence of unlabeled VEGF165 (Fig. 4A). After lysis of cells, anti-VEGF immunoprecipitations were performed on lysates, and immune complexes were subjected to SDS-polyacrylamide gel electrophoresis. Recovered components were then visualized by phosphomager analysis. The feasibility of this approach was demonstrated by the VEGF-dependent recovery of the 200-kDa KDR protein from PAE/KDR cells (Fig. 4A, lane 4). Inspection of the lanes corresponding to U-178MG lysates revealed one single VEGF-binding protein on U-178MG cells (Fig. 4A, lane 6). Both A and B samples were analyzed by SDS gel electrophoresis; labeled proteins were detected with a phosphomager. KDR and the VEGF binding proteins on U-178MG cells are indicated by filled and open arrows, respectively.

**The VEGF binding U-178MG component has a size of 190 kDa**. A, PAE cells (lanes 1 and 2), PAE/KDR cells (lanes 3 and 4), and U-178MG cells (lanes 5 and 6) were labeled metabolically with [35S]methionine and then incubated with VEGF165. The cells were then lysed, and lysates were precipitated with VEGF antiserum. B, PAE cells (lanes 1 and 4), PAE/KDR cells (lanes 2 and 5), and U-178 MG cells (lanes 3 and 6) were incubated with 125I-VEGF165 and cross-linked by bis(sulfosuccinimidyl)suberate. The cells were then lysed and precipitated with control serum (lanes 1–3) or VEGF antiserum (lanes 4–6). In both A and B samples were analyzed by SDS gel electrophoresis; labeled proteins were detected with a phosphomager. KDR and the VEGF binding proteins on U-178MG cells are indicated by filled and open arrows, respectively.
FIG. 5. The 190-kDa VEGF binding protein on U-178MG cells is not sensitive to treatment with N-glycosidases. Confluent cultures of U-178MG cells (lanes 1–4) and MDA MB231 cells (lanes 5–8) were incubated with $^{125}$I-VEGF<sub>165</sub> and bound VEGF was cross-linked by bis(sulfosuccinimidyl)suberate. The cells were then lysed and precipitated with control serum or VEGF antiserum as indicated. Half of the immunoprecipitated material were then subjected to treatment with the N-glycosidases endoglycosidase-F (Endo-F) and peptide N-glycosidase F (PNGaseF). Samples were analyzed by SDS gel electrophoresis, and radioactivity was detected by a phosphorimager.

220-kDa complex from U-178MG cells, and in addition, a component of 140 kDa (Fig. 4B, lane 6). No component corresponding to the lower molecular mass component was observed in the experiment using metabolically labeled cells (Fig. 4A). It may therefore correspond to a component interacting with VEGF<sub>165</sub> with lower affinity that only can be seen after covalent cross-linking. A complex of about 220 kDa was recovered from PAE/KDR cells (Fig. 4B, lane 5). Because these complexes were not recovered as anti-VEGF precipitates in untransfected PAE cells (lane 4) nor when preimmune sera were used on the lysates (lanes 1–3), we conclude that they represent $^{125}$I-VEGF<sub>165</sub> cross-linked to the KDR receptor on PAE/KDR cells and to the novel binding protein on U-178MG cells. Because the contribution of cross-linked VEGF<sub>165</sub> to the mass of the complexes should be 23 or 45 kDa, depending on if one or two subunits of the VEGF dimer was present in the cross-linked complex, these experiments suggest a mass of 180–200 kDa for the U-178MG VEGF binding protein, which is in good agreement with the size detected from the immunoprecipitation of metabolically labeled cells (Fig. 4A). The estimated size of KDR from the cross-linking experiment is consistent with previous results (23). Thus, we conclude that the major VEGF binding protein on U-178MG cells has a size of about 190 kDa.

Comparison of VEGF Binding Proteins on U-178MG Cells and MDA MB231 Cells—VEGF binding proteins of 120–130 kDa have been described on the breast cancer cell line MDA MB231 and on endothelial cells (17, 18). To explore the relatedness of these components with the VEGF binding proteins on U-178MG cells, anti-VEGF immunoprecipitations of $^{125}$I-VEGF<sub>165</sub> cross-linked to MDA MB231 and U-178MG cells were compared. To investigate the glycoprotein nature of the components, the effect of treatment of the immunoprecipitates with N-glycosidases was also investigated. As shown in Fig. 5, the 190-kDa component of U-178MG cells did not shift in size after glycosidase-treatment, suggesting that this component contains no or low amounts of N-linked carbohydrate. A less abundant component of similar size, which does not either appear to be a glycoprotein, was also seen in MDA MB231 cells. It is unlikely that the 190-kDa VEGF binding protein on MDA MB231 cells corresponds to the tyrosine kinase receptors KDR or Flt1, because these are known to be glycoproteins. In contrast, the 120–130-kDa VEGF binding component of MDA MB231 decreased in size about 10 kDa after glycosidase treatment; the similarly sized component of U-178MG cells also showed a similar shift in size. These observations suggest that the 120–130-kDa components of MDA MB231 cells and U-178MG cells may be related and distinct from the 190-kDa component of U-178MG cells, which may be related to a component of similar size present on MDA MB231 cells.

Characterization of the VEGF Binding Protein on U-178MG Cells—Exposure of high concentration of ligand to cells carrying signaling growth factors receptors is in most cases followed by loss of binding sites as a consequence of ligand-induced receptor internalization and down-regulation (24–26). We therefore investigated if the U-178MG VEGF binding protein was down-regulated after exposure to high concentrations of VEGF<sub>165</sub>. Identical experiments were performed in parallel on PAE/KDR cells. Cells were first incubated for 1 h at 37 °C in the absence or the presence of 100 ng/ml VEGF<sub>165</sub> for 60 min at 37 °C in culture medium. After washing with binding buffer supplemented with 20 mM acetic acid, pH 3.75, to dissociate cell surface receptor-bound VEGF, cells were subjected to $^{125}$I-VEGF<sub>165</sub> binding assays in the absence or the presence of 100 ng/ml unlabeled VEGF<sub>165</sub>. The amount of cell-associated radioactivity was determined in a γ-counter.

FIG. 6. The VEGF binding component on U-178MG cells is not down-regulated after incubation with ligand at 37 °C. PAE/KDR cells and U-178 MG cells were preincubated in the absence or the presence of 100 ng/ml VEGF<sub>165</sub> for 60 min at 37 °C in culture medium. After washing with binding buffer supplemented with 20 mM acetic acid, pH 3.75, to dissociate cell surface receptor-bound VEGF, cells were subjected to $^{125}$I-VEGF<sub>165</sub> binding assays in the absence or the presence of 100 ng/ml unlabeled VEGF<sub>165</sub>. The amount of cell-associated radioactivity was determined in a γ-counter.

Because all known signaling molecules for VEGF<sub>165</sub> are receptor tyrosine kinases, we also investigated if an alteration in the pattern of tyrosine phosphorylated proteins could be detected in U-178MG cells after stimulation with VEGF<sub>165</sub>. PAE, PAE/KDR, and U-178MG cells were incubated on ice for 1 h in the absence or the presence of 100 ng/ml of VEGF<sub>165</sub>. Cell lysates were immunoprecipitated with phosphotyrosine antibodies; after SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose filters, tyrosine-phosphorylated pro-
with binding buffer containing 0.5 M NaCl. As shown in Fig. 8, binding protein on U-178MG cells, a modified binding experi-
technique was performed after binding of ligand, cells were washed with binding buffer containing 0.5 M NaCl. As shown in Fig. 8, this change in washing conditions did not affect VEGF binding to Flt1 and had only a small effect on VEGF165 binding to KDR. In contrast, almost no binding of VEGF to the binding protein on U-178MG cells was detected after this treatment. To exclude the possibility that the loss of binding to U-178MG cells after the high salt wash reflected dissociation of the binding protein from the cells rather than dissociation of the ligand from the receptor, we also investigated the effect of a high salt wash before the binding experiment. This treatment did not affect the binding of VEGF to the U-178MG cells (Fig. 8). We therefore conclude that the VEGF binding to the 190-kDa binding protein differs from the binding to KDR and Flt1 with regard to sensitivity to increased ionic strength. Furthermore, the fact that the binding capacity of the U-178MG cells was not affected by pretreatment of the cells with 0.5 M NaCl suggests that the binding protein is tightly associated with the cell, possibly as an integral membrane protein rather than as a protein loosely associated with the cell surface.

**DISCUSSION**

We describe in the present communication a 190-kDa VEGF<sub>165</sub> binding protein present on a human glioma cell line. Because the 190-kDa component does not appear to have any intrinsic tyrosine kinase activity or to stimulate tyrosine phosphorylation in the cells, it is possible that it is not directly involved in signaling. This conclusion is supported by the lack of observable effect of VEGF on growth or shape of U-178MG cells, which have the 190-kDa component but lack both KDR and Flt1 receptors (data not shown). It is possible, however, that the 190-kDa protein serves an accessory role in VEGF stimulation of cells with Flt1 or KDR receptors, e.g. by presenting ligand to signaling receptors or forming a complex with signaling receptors with increased ligand binding affinity. Such roles have been suggested, e.g. for betaglycan in transforming growth factor-β signaling (27) and for heparan sulfate proteoglycan in fibroblast growth factor signaling (28).

**FIG. 7.** The VEGF binding protein on U-178MG cells does not mediate increased tyrosine phosphorylation. PAE cells, PAE/KDR cells, and U-178MG cells were stimulated with 100 ng/ml VEGF<sub>165</sub> for 60 min at 4 °C. Cells were then lysed and subjected to immunoprecipitation with affinity purified anti-phosphotyrosine polyclonal antibodies (PY6). After SDS gel electrophoresis, samples were analyzed by immunoblotting using the monoclonal antibodies against phosphotyrosine (PY20); immune complexes were detected using horse-radish peroxidase-linked secondary antibodies and enhanced chemoluminescence. Phosphorylated KDR is indicated by an arrow.

**FIG. 8.** The binding of VEGF165 to U-178MG cells is sensitive to high salt concentrations. PAE/KDR cells, PAE/Flt1 cells, and U-178MG cells were incubated for 2 h at 4 °C with [125I]-VEGF<sub>165</sub> in the absence or presence of various concentrations of unlabeled VEGF<sub>165</sub>. Binding experiments were performed as described above (○), or with inclusion of a high salt wash after the binding experiment (■) or before the binding experiment (□).

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cell line lacking KDR and Flt1 receptors. It should be noted that on cells having KDR or Flt1 receptors the 190-kDa protein may be difficult to discern in conventional cross-linking experiments due to its similarity in size with the tyrosine kinase receptors.

The VEGF binding protein described in the present report was observed on a human glioma cell line. Investigations of the breast carcinoma cell line MDA MB231 (Fig. 5) and three other human glioma cell lines revealed high molecular mass VEGF binding proteins, which may be related to the VEGF binding protein on U-178MG cells. Purification, cDNA cloning, and the establishment of specific antisera will make it possible to further characterize the 190-kDa component and its role in VEGF signaling.

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