VEGF$_{145}^*$, a Secreted Vascular Endothelial Growth Factor Isoform That Binds to Extracellular Matrix*

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A vascular endothelial growth factor (VEGF) mRNA species containing exons 1–6 and 8 of the VEGF gene was found to be expressed as a major VEGF mRNA form in several cell lines derived from carcinomas of the female reproductive system. This mRNA is predicted to encode a VEGF form of 145 amino acids (VEGF$_{145}^*$). Recombinant VEGF$_{145}^*$ induced the proliferation of vascular endothelial cells and promoted angiogenesis in vivo. VEGF$_{145}^*$ was compared with previously characterized VEGF species with respect to interaction with heparin-like molecules, cellular distribution, VEGF receptor recognition, and extracellular matrix (ECM) binding ability. VEGF$_{145}^*$ shares with VEGF$_{165}^*$ the ability to bind to the KDR/flk-1 receptor of endothelial cells. It also binds to heparin with an affinity similar to that of VEGF$_{165}^*$. However, VEGF$_{145}^*$ does not bind to two additional endothelial cell surface receptors that are recognized by VEGF$_{165}^*$ but not by VEGF$_{121}^*$. VEGF$_{145}^*$ is secreted from producing cells as are VEGF$_{121}^*$ and VEGF$_{165}^*$. However, VEGF$_{121}^*$ and VEGF$_{165}^*$ do not bind to the ECM produced by corneal endothelial cells, whereas VEGF$_{145}^*$ binds efficiently to this ECM. Basic fibroblast growth factor (bFGF)-depleted ECM containing bound VEGF$_{145}^*$ induces proliferation of endothelial cells, indicating that the bound VEGF$_{145}^*$ is active. The mechanism by which VEGF$_{145}^*$ binds to the ECM differs from that of bFGF. Digestion of the ECM by heparinase inhibited the binding of bFGF to the ECM and released prebound bFGF, whereas the binding of VEGF$_{145}^*$ was not affected by heparinase digestion. It therefore seems that VEGF$_{145}^*$ possesses a unique combination of biological properties distinct from those of previously characterized VEGF species.

The vascular endothelial growth factor (VEGF) isoforms display a limited structural similarity to platelet-derived growth factor and are important regulators of angiogenesis and blood vessel permeability (1–3). The human VEGF isoforms are generated by alternative splicing from a single gene (4–6). The domain encoded by exons 1–5 contains information required for the recognition of the known VEGF receptors KDR/flk-1 and flt-1 (7) and is present in all VEGF isoforms. The amino acids encoded by exon 8 are also present in all the VEGF splice variants. The VEGF isoforms are distinguished by the presence or the absence of the peptides encoded by exons 6 and 7 of the VEGF gene. VEGF$_{121}^*$ is 121 amino acids long and lacks both exons. VEGF$_{165}^*$ contains the exon 7-encoded peptide, whereas VEGF$_{189}^*$ contains both exon 6- and exon 7-encoded peptides (6, 8). VEGF$_{121}^*$ and VEGF$_{165}^*$ promote angiogenesis, cause permeabilization of blood vessels, and induce proliferation of vascular endothelial cells (10–14). VEGF$_{189}^*$ has not yet been purified, but studies with cells expressing VEGF$_{189}^*$ indicate that it may induce endothelial cell proliferation (8, 9). Low levels of a mRNA corresponding in size to a mRNA encoding a putative VEGF variant of 145 amino acids (VEGF$_{145}^*$) containing exon 6 but lacking exon 7 were detected previously in reverse PCR experiments, but the protein encoded by this mRNA has not yet been characterized (15, 16).

The different VEGF isoforms differ in their heparin binding ability. VEGF$_{121}^*$ does not bind to heparin, whereas VEGF$_{165}^*$ and VEGF$_{189}^*$ do (8, 17–19). The heparin binding affinity of VEGF$_{189}^*$ was reported to be higher than that of VEGF$_{165}^*$, suggesting that exon 6 contributes to the heparin binding ability of VEGF$_{189}^*$. VEGF$_{165}^*$ and VEGF$_{121}^*$ are secreted efficiently from producing cells and do not bind efficiently to the ECM produced by CEN4 cells (8, 9). In contrast, VEGF$_{189}^*$ is retained on the cell surface and in the ECM, from which it can be released by prolonged incubation with heparin. The peptide encoded by exon 7 also seems to affect the receptor recognition patterns of VEGF isoforms. VEGF$_{121}^*$ recognizes a single VEGF receptor in endothelial cells that was identified as the KDR/flk-1 VEGF receptor (20, 21). VEGF$_{165}^*$ also binds to this receptor but recognizes two additional VEGF receptors of unknown structure that are found in endothelial cells and in several transformed cell types (21, 22).

We have observed that VEGF$_{145}^*$ is one of the main VEGF isoforms expressed by several cell lines derived from carcinomas of the female reproductive system. We have characterized VEGF$_{145}^*$ and have compared its biological properties with those of other VEGF forms. Recombinant VEGF$_{145}^*$ expressing the exon 6-derived peptide binds to heparin but behaves like VEGF$_{121}^*$ with regard to its receptor recognition ability. In contrast to VEGF$_{121}^*$ and VEGF$_{165}^*$, it binds to a basement membrane like ECM produced by corneal endothelial cells in a biologically active form. VEGF$_{145}^*$ therefore represents a VEGF form possessing distinct biological characteristics.

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EXPERIMENTAL PROTOCOLS

Materials—Human recombinant VEGF165 and VEGF121 were purified from SF-9 insect cells, whereas recombinant human bFGF was produced in bacteria as described previously (19, 23, 24). A rabbit polyclonal antibody directed against VEGF165 (23) and a mouse monoclonal IgM antibody (M-35) directed against full-length VEGF165 were produced in our laboratory using standard techniques. Heparninas type 13 and II-A were kindly donated by Dr. J. Zamenhof (SomaChem Technologies, Montreal, Canada). The biceratonic mammalian expression vector MIRB was kindly provided to us by Dr. Craig MacArthur (Washington University, St. Louis, MO) (25). Anti-VEGF monoclonal antibody clone 26503.11 and peroxidase- and alkaline phosphatase-conjugated anti-rabbit IgG antibodies were from Sigma. Disuccinimidyl suberate was from Pierce. Sodium alginate was from Fluka. Heparin-Sepharose was purchased from Pharmacia Biotech Inc. (23). Sambroodin was obtained from New England Nuclear. Anti-mouse IgM antibodies conjugated to alkaline phosphatase were from Southern Biotechnology Associates Inc. (Birmingham, AL). Tissue culture plasticware was from Nunc, and 96-well dishes for enzyme-linked immunosorbent assays were bought from Corning. Grace’s medium was obtained from Life Technologies, Inc. All other tissue culture reagents were from Biological Industries Inc. (Kibbutz Beth Haemek, Israel).

Identification of VEGF145 mRNA in Cancer Cells—Total mRNA was prepared from OC-238 human epithelial ovarian carcinoma cells (26). Complementary DNA was synthesized from 300 ng of total RNA using oligo(dT) as a primer and avian myeloblastosis virus reverse transcriptase. PCR amplification was carried out in the presence of a [32P]dCTP tracer (2 µCi in a 100-µl reaction volume), 1 mM of each dNTP, 200 µM of MgCl2, and 2.5 units of polymerase. 25 amplification cycles were used, each consisting of a 1-min incubation at 94 °C, a 2-min incubation at 65 °C, and a 3-min incubation at 72 °C. The VEGF-specific oligonucleotides used were GGAGGATGAGCTCTTACAG and TCACCGCTTTGCTTGTCACA, corresponding to amino acids 92–98 and to the six carboxyl-terminal amino acids of VEGF, respectively. A pair of primers from the L19 ribosomal protein were included in some reactions as an internal control. Amplified fragments were resolved in a 6% nondenaturing polyacrylamide gel and were visualized by autoradiography. The band corresponding to the VEGF145 mRNA (see Fig. 1B) was excised, reamplified, and sequenced.

A similar procedure using different primers was used to detect VEGF145 mRNA in A431 and HeLa cells. The VEGF-specific primers used here were derived from the 5’ of the coding region (amino acids 13–20) and from the 3’ region encompassed by the last 6 amino acids of VEGF including the translation stop codon of the VEGF sequence.

Construction of VEGF145 Encoding Expression Vectors—In order to produce recombinant VEGF145, we prepared a VEGF145 cDNA construct by deleting the oligonucleotides encoded by exon 7 out of the VEGF165 cDNA. Primers used to amplify exons 1–6 of the VEGF cDNA were the external primer GCTTCCGCTATGTATGTTGCGG, corresponding to nucleotides 2534–2551 of the VEGF coding sequence, and the internal primer ACCTGCCAGGATCTTACAG, corresponding to a sequence at the 3’ end of exon 6. Primers used to amplify the 3’ end of the VEGF cDNA were complementary to the puc118 sequence GTTAACGCCAGGTCCCTTCAAGG and to the 3’ end of the exon 6 sequence (underlined) and to the start of exon 8 (GCCGTTATTCCTCTGAGCTGTATGCGAACGCGGAGGCGG-TGA). Following amplification, the PCR products were precipitated, and the products were reamplified using only the puc118-derived external primers. The product was gel purified, subcloned into the PCR-II vector, and sequenced using the Sequenase-II kit from U. S. Biochemical Corp. This cDNA was further used for protein expression studies.

Production and Purification of Recombinant VEGF145—The VEGF145 cDNA was subcloned into the BamHI site of the MIRB expression vector (25). Following transfection into BHK-21 cells and selection with 0.6 mg/ml G418, VEGF145-expressing cells were identified using anti-VEGF antibodies. The VEGF145 cDNA was also subcloned into the transfer plasmid pVL-1393 (Invitrogen) downstream from the polyhedrin promoter to yield pVL-1393/145. This plasmid and baculovirus wild-type DNA were co-transfected into SF9 cells using the calcium-phosphate co-precipitation method, and recombinant baculoviruses were isolated as described (23).

VEGF145 was produced in SF9 cells as described for VEGF165 (23). The conditioned medium contained approximately 5 mg of VEGF145/ml. The conditioned medium was concentrated by precipitation with 70% ammonium sulfate at 4 °C for 12 h. The precipitate was solubilized in 20 mM Tris, pH 7, and 0.1 M NaCl, dialyzed extensively against this buffer at 4 °C, and applied to a heparin-Sepharose column. The column was washed with the same buffer containing 0.3 M salt, followed by elution with the same buffer containing 0.8 M NaCl. A small residual amount also eluted at 2 M NaCl. The 0.8 M salt eluant was further purified by reverse phase high pressure liquid chromatography on an Applied Biosystems Brownlee C-8 column using a linear gradient of acetonitrile (20–80%) containing 0.1% trifluoroacetic acid. VEGF145 was eluted at 48% acetonitrile. The trifluoroacetic acid in the eluant was neutralized using Tris base, and the acetonitrile was removed using a SpeedVac evaporator at room temperature.

Enzyme-linked Immunosorbent Assays—Increasing concentrations of VEGF in 50 µl of coating buffer (20 mM K2HPO4, 10 mM KH2PO4, 1 mM EDTA, 0.1 M NaCl, pH 7.2) were adsorbed to 96-well dishes for 3 h at 25 °C. Free VEGF was aspirated, and the wells were blocked with coating buffer containing 1% bovine serum albumin for 1 h. The wells were extensively washed with wash buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.1% Tween 20), incubated with the anti-VEGF M-35 monoclonal antibody for 2 h at 25 °C, washed again, and incubated for 1 h with an alkaline phosphatase-conjugated secondary antibody. After final washing, the amount of bound antibody was determined using para-nitrophenylphosphate as substrate.

Cell Culture and Production of ECM-coated Dishes—Human umbilical vein-derivated endothelial cells (HUVECs) were prepared from umbilical veins and cultured as described previously in M199 medium supplemented with 20% fetal calf serum, vitamins, 1 ng/ml bFGF, and antibiotics (21, 27). Proliferation assays were done as described previously (11). Bovine corneal endothelial (BCE) cells were isolated from steer eyes and cultured as described previously (28). ECM-coated dishes were prepared from cells grown in the presence of the absence of 20 mM chlorate as described previously (28, 29).

Binding of VEGFs to HUVECs and to ECM-coated Dishes—The binding and the cross-linking of [125I]-VEGF145 to confluent layers of HUVECs grown in 5-cm dishes in the presence or the absence of various competitors was done essentially as described. VEGF145 was purified from infected SF9 cells and iodinated as described (19, 21, 23, 30).

Binding of VEGFs to ECM-coated 96- or 24-well dishes was performed at room temperature. The ECM-coated wells were washed with rinse buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.1% Tween 20). Non-specific sites were blocked with binding buffer (20 mM K2HPO4, 10 mM KH2PO4, 1 mM EDTA, 0.8% NaCl, 1 mg/ml bovine serum albumin, pH 7.2) for 1 h at room temperature. The binding buffer was aspirated and iodinated, or unlabeled growth factors were incubated with the ECM-coated wells in binding buffer for 2 h at 24 °C. Free growth factors were removed by aspiration, and the ECM was washed twice with rinse buffer. In binding experiments, 0.2 mM NaOH was added to dissociate bound growth factors, and aliquots were counted in a γ counter or neutralized using Tris base and analyzed by SDS-PAGE followed by autoradiography. Alternatively, the wells were further incubated with various anti-VEGF monoclonal antibodies in binding buffer for 2 h and washed. Bound antibody was detected with appropriate secondary antibodies coupled to alkaline-phosphatase, using para-nitrophenylphosphate as substrate. Our M-35 anti-VEGF monoclonal antibody and commercial anti-VEGF monoclonal antibodies produced identical results in these assays.

For biological activity experiments, 15,000 HUVECs were seeded on ECM containing various amounts of adsorbed VEGF in a final volume of 1 ml of growth medium. Cells were trypsinized and counted after 3 days in a coulter counter. All experiments were repeated at least twice with similar results.

RESULTS

VEGF145 Is Expressed as a Major VEGF Splice Variant in Several Tumorigenic Cell Lines Originating in the Female Reproductive System—Reverse PCR analysis of mRNA from OC-238 human epithelial ovarian carcinoma cells (Fig. 1B), HeLa cells, and A431 cells (Fig. 1C) detected a VEGF mRNA containing a coding region smaller than that of VEGF121 but larger than that of VEGF121. The size of the coding region corresponds to the expected size of a mRNA encoding a VEGF form containing exons 1–6 and 8 and should lead to the production of a VEGF form containing 145 amino acids (VEGF145) (15). In all these cell lines the VEGF145 mRNA seemed to be expressed at levels comparable with those of VEGF165. The VEGF145 mRNA was not detected in several other transformed cell lines including C6 glioma cells and U937 cells. Sequence analysis of the PCR product from the OC-238 cells showed that this mRNA was
VEGF<sub>145</sub>, a Secreted VEGF Isoform That Binds to ECM

**Fig. 1.** A, the structure of the VEGF splice variants. The peptides encoded by the various exons of the human VEGF gene are shown in boxes but are not drawn to scale. The number of amino acids in each of the exon-encoded peptides is shown at the top. The exon structure of VEGF<sub>145</sub> is shaded. B, expression of VEGF<sub>145</sub> mRNA in OC-238 human epithelial ovarian carcinoma cells. Total RNA from OC-238 cells was translated into cDNA and amplified by PCR using radioactively labeled nucleotides. PCR products were separated on a polyacrylamide gel as described under “Experimental Procedures.” Shown is an autoradiogram of the gel. The amplified species of VEGF and L19 cDNA are indicated. C, expression of VEGF<sub>145</sub> and VEGF<sub>165</sub> cDNA in A431 and HeLa cells. Total RNA from HeLa and A431 cells was translated into cDNA and amplified by PCR using radioactively labeled nucleotides. PCR products were separated on a polyacrylamide gel as described under “Experimental Procedures.” Plasmids containing the VEGF<sub>121</sub> cDNA, the VEGF<sub>145</sub> cDNA, and the VEGF<sub>165</sub> recombinant cDNA were included in separate PCR reactions using the primers described under “Experimental Procedures.” Shown is an autoradiogram of the gel.

was indeed generated by alternative splicing and that it contains exons 6 and 8 but not exon 7 of the VEGF gene.

To study the properties of VEGF<sub>145</sub>, we have expressed the VEGF<sub>145</sub> cDNA in Sf9 insect cells using the baculovirus expression system (23). Most of the VEGF<sub>145</sub> produced by the infected Sf9 cells was found in the conditioned medium as a homodimer of ~41 kDa, with small amounts of monomeric VEGF<sub>145</sub> (Fig. 2B). The VEGF<sub>145</sub> dimers dissociated into monomers upon reduction with dithiotreitol (Fig. 2A). VEGF<sub>145</sub> was partially purified by heparin-Sepharose affinity chromatography. VEGF<sub>145</sub> was eluted from heparin-Sepharose columns using a stepwise salt gradient. Most of the VEGF<sub>145</sub> eluted at 0.6–0.7 M NaCl, indicating that the heparin binding affinity of VEGF<sub>145</sub> is similar to that of VEGF<sub>165</sub> (data not shown) (17, 23). The recombinant VEGF<sub>145</sub> was biologically active and induced the proliferation of HUVECs. The ED<sub>50</sub> of VEGF<sub>145</sub> was 30 ng/ml, whereas VEGF<sub>165</sub> was 6-fold more active than VEGF<sub>145</sub> in this assay (Fig. 3).

**VEGF<sub>145</sub> Induces Angiogenesis in Vivo**—To determine whether VEGF<sub>145</sub> can induce angiogenesis in vivo, the VEGF<sub>145</sub> cDNA was subcloned into the BamHI site of the mammalian bicistronic expression vector MIRB (25). The MIRB/VEGF<sub>145</sub> plasmid was transfected into BHK-21 cells (31), and stable cell lines producing VEGF<sub>145</sub> were isolated. The VEGF<sub>145</sub> produced by the mammalian cells was biologically active and was secreted into the growth medium. A stable clone producing 0.1 µg of VEGF<sub>145</sub> per 10<sup>6</sup> cells was isolated. The VEGF<sub>145</sub> expressing cells were embedded in alginate beads, and the beads were implanted under the skin of BALB/c mice (32). The pellets containing the alginate beads were removed after 4 days and photographed. Clusters of alginate beads containing VEGF<sub>145</sub> expressing cells were dark red with blood, whereas beads containing cells transfected with vector alone had a much lower content of blood (Fig. 4). When examined under higher magnification, pellets containing VEGF<sub>145</sub> producing cells appeared much more vascularized than pellets containing control cells.

**VEGF<sub>145</sub> binds to the KDR/flk-1 receptor but not to the two smaller VEGF receptors of HUVECs**—<sup>125</sup>I-VEGF<sub>165</sub> forms high molecular weight complexes with three types of VEGF receptors following cross-linking to HUVECs (Fig. 5, lane 1), whereas <sup>125</sup>I-VEGF<sub>121</sub> only binds to the larger of these receptors. The common receptor to which both VEGF<sub>121</sub> and VEGF<sub>165</sub> bind is the KDR/flk-1 VEGF receptor (Fig. 5, open arrow) (21). In order to compare the receptor recognition pattern of VEGF<sub>145</sub> with those of VEGF<sub>165</sub>, <sup>125</sup>I-VEGF<sub>165</sub> was bound to HUVECs in the presence of 1 µg/ml of heparin and increasing concentrations of VEGF<sub>145</sub>. Bound <sup>125</sup>I-VEGF<sub>165</sub> was subsequently covalently cross-linked to the VEGF receptors. VEGF<sub>145</sub> inhibited the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the KDR/flk-1 receptor of the HUVECs (Fig. 5). This result was verified in a cell-free binding experiment in which VEGF<sub>145</sub> competed with <sup>125</sup>I-VEGF<sub>165</sub> for binding to a soluble fusion protein containing the extracellular domain of the flk-1 receptor (data not shown) (33). In contrast, VEGF<sub>145</sub> did not effectively inhibit the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the two smaller VEGF receptors of the HUVECs (Fig. 5, filled arrow), indicating that the affinity of VEGF<sub>145</sub> toward these two receptors is substantially lower than that of VEGF<sub>165</sub>. This behavior resembles the behavior of VEGF<sub>121</sub> (21) and indicates that the presence of exon 6 is not sufficient to enable efficient binding of VEGF<sub>145</sub> to these two receptors, despite the heparin binding properties that exon 6 confers on VEGF<sub>145</sub>.

**VEGF<sub>145</sub> binds to the ECM produced by corneal endothelial**
fact that VEGF189 binds heparin with high affinity led to the
CEN4 cells, whereas VEGF165 binds to it very weakly (9). The
binding of VEGF165 was marginal (Fig. 6
VEGF189 binds efficiently to the ECM produced by
Cells—VEGF189 binds efficiently to the ECM produced by
CEN4 cells, whereas VEGF165 binds to it very weakly (9). The
VEGF145 binds to an ECM produced by bovine corneal endothelial cells
dependent on ECM-associated heparan sulfates (8, 9). The heparin
binding affinities of VEGF145 and VEGF165 are similar and
substantially lower than the heparin binding affinity of
VEGF189 (8). We therefore expected VEGF145 to bind poorly to
ECM. Unexpectedly, experiments in which VEGF 145 was
bound to an ECM produced by bovine corneal endothelial cells
showed that VEGF145 bound efficiently, whereas the
binding of VEGF145 was marginal (Fig. 6A). In these experiments the binding was monitored with anti-VEGF antibodies,
but similar results were obtained when binding to the ECM
was assayed directly using 125I-VEGF145 (30 ng/ml) or 125I-
VEGF165 (50 ng/ml) (Fig. 6B, first and third lanes). The binding
of 125I-VEGF145 to the ECM was substantially but not
completely inhibited by 10 µg/ml heparin (Fig. 6B, second lane). The
125I-VEGF145 used in these experiments contained some
impurities (Fig. 6C), but the major iodinated protein that was
recovered from the ECM had a mass corresponding to that of
125I-VEGF145 (Fig. 6B, first lane). To make sure that 125I-
VEGF145 binds to the ECM and not to exposed plastic surfaces,
the ECM was scraped off and washed by centrifugation, and
the amount of adsorbed 125I-VEGF145 in the pellet was
determined. The ECM contained ~70% of the adsorbed 125I-
VEGF145. It therefore appears that the presence of the exon
6-derived peptide in VEGF145 enables efficient binding to the
ECM, whereas the exon 7 derived peptide of VEGF165 does not
suffice to confer this ability on VEGF165.

VEGF145 Binds to the ECM Using a Mechanism That Is Not
Dependent on ECM-associated Heparan Sulfates—The interaction
of bFGF with the ECM is mediated by the heparan sulfate
moieties of ECM associated proteoglycans (35). It was of interest
to determine if VEGF145 uses a similar mechanism. When
125I-VEGF145 was bound to ECM-coated dishes in the presence of
10 µg/ml heparin, the binding was inhibited by ~60% (Fig.
7A). Under the same conditions the binding of 125I-bFGF to the
FIG. 2. Production of VEGF145 in Sf9 cells. VEGF145, and VEGF165
were produced in Sf9 insect cells as described under "Experimental
Procedures." Conditioned medium containing recombinant VEGF was
collected, and 10-µl aliquots were either reduced using 0.1 M dithiothre-
tol (A) or not reduced (B). Proteins were separated by SDS-PAGE (12% gel)
and transferred by electroblotting to nitrocellulose. Filters were
washed three times with TBST, and incubated with anti-rabbit IgG
peroxidase-conjugated antibodies for 1 h at room temperature. Bound
antibody was visualized using the ECL detection system.

FIG. 3. VEGF145 stimulates the proliferation of endothelial
cells. HUVECs were seeded in 24-well dishes (20,000 cells/well), and
increasing concentrations of VEGF145 ( ), VEGF165 (■), and VEGF189 (□) were added every other day as described under "Experimental
Procedures." Cells were counted in a Coulter counter after 4 days.

FIG. 4. VEGF145 stimulates angiogenesis in vitro. The angiogenic
activity of VEGF145 was determined using the alginate assay (32).
Stable clones of BHK-21 cells transfected with the MIRB expression
vector (MIRB) or with the VEGF145 expression vector MIRB/VEGF145
were trypsinized and suspended in Dulbecco’s modified Eagle’s medium
to a concentration of 2.7 × 10⁶ cells/ml. Sodium alginate (1.2%, 0.66 ml)
was mixed with 1.33 ml of cell suspension. Beads of 1-µl diameter were
formed by contact with a solution of 80 mM CaCl₂. The beads were
washed three times with saline. Each BALB/c mouse out of a group of
four was injected subcutaneously with 400 µl of packed beads containing
a given cell type. Clusters of beads were excised after 4 days and
photographed. Blood-rich areas appear as dark areas in the
photograph.

FIG. 5. Effect of VEGF145 on 125I-VEGF145 binding to endothe-
lial cells. 125I-VEGF165 (10 ng/ml) was bound to confluent HUVECs
grown in 5-cm dishes for 2 h at 4 °C in the presence of 1 µg/ml heparin
and the following concentrations of VEGF145 (µg/ml): lane 1, 0; lane 2, 0.05, lane 3, 0.1; lane 4, 0.25; lane 5, 0.5; lane 6, 1; lane 7, 2; lane 8, 3, Lane 9 received 2 µg/ml of VEGF145. Bound 125I-VEGF165 was
subsequently cross-linked to the cells using disuccinimidyl suberate, and
cross-linked complexes were visualized by autoradiography.
ECM was inhibited by 80% (Fig. 7A). The binding of 125I-bFGF to the ECM was also inhibited by 80% in the presence of 0.8 M salt, indicating that the interaction is probably not hydrophobic (data not shown). These results are compatible with the expected behavior of proteins that bind to the ECM via heparin-like molecules. However, 125I-VEGF145 also bound efficiently to an ECM that was digested with heparinase-II (36).

In contrast, there was almost no binding of 125I-bFGF to heparinase-II-digested ECM (Fig. 7A) (37, 38).

In order to further investigate the mode of interaction of VEGF145 with the ECM, we measured the heparin- or heparinase-II-induced release of prebound VEGF from the ECM. When the ECM-coated wells were incubated for 2 h at 37 °C with buffer, only 20% of the bound 125I-bFGF and 13%
VEGF145 may be more restricted compared with other VEGF isoforms that do not express this mRNA, indicating that the expression of VEGF145 is restricted to the female reproductive system. It therefore seems that the simultaneous presence of both exon 6 and exon 7 is required for efficient binding of VEGF to cell surfaces, whereas the presence of each of these exons on its own does not confer this property on VEGF.

Unlike VEGF121, VEGF145 was able to bind to heparin-Sepharose columns, indicating that the exon 6 encoded peptide acts as an independent heparin binding domain. The affinity of VEGF145 for heparin was similar to that of VEGF165, even though the structures of the heparin binding domains of the two isoforms differ. All the VEGF isoforms tested to date bind to the KDR/flk-1 receptor of endothelial cells (21), and VEGF145 was no exception. However, unlike VEGF165, VEGF121, and VEGF145, do not bind efficiently to two additional VEGF receptors on HUVECs (21). The fact that VEGF121 and VEGF145 are both mitogenic and angiogenic indicates that these two receptors do not play a central role in the angiogenic and mitogenic response of endothelial cells. The biological function and the molecular structure of these two additional receptors is unknown. They appear to be novel VEGF receptors because they are not recognized by VEGF121 receptors, whereas both KDR/flk-1 and flk-1 are recognized by VEGF121. In addition these two receptors are not immunoprecipitated by antibodies directed against the intracellular domains of the known VEGF receptors (21), and they can be detected in tumor cells that do not express detectable levels of KDR/flk-1 or flk-1 mRNA (22). Our results suggests that specific exon 7 sequences that are not present in the exon 6-derived peptide are required for the interaction of VEGF165 with these two receptors. The heparin binding ability conferred on VEGF165 by the exon 7 peptide may not play a central role in the recognition of these receptors by VEGF165 because VEGF145 and VEGF165 bind with similar affinities to heparin yet differ in their ability to recognize these two receptors.

VEGF189 is not found in the conditioned medium of produc-

**Fig. 8.** VEGF<sub>145</sub> bound to the ECM produced by BCE cells promotes proliferation of endothelial cells. Wells of 24-well dishes were coated with an ECM produced by BCE cells cultured in the presence of 30 mM chlorate as described (29). The ECM-coated wells were incubated with increasing concentrations of VEGF<sub>145</sub> (■) or VEGF<sub>165</sub> (□) as indicated and washed extensively as described. HUVECs (15,000 cells/well) were seeded in the ECM-coated wells in growth medium lacking growth factors. Cells were trypsinized and counted after 3 days. The numbers represent the average number of cells in duplicate wells. The experiment was repeated twice with similar results. Variation within duplicates did not exceed 10%.

**DISCUSSION**

Alternative splicing represents an important mechanism for the generation of diversity in growth factors and in their receptors. The alternative splice forms generated from the VEGF gene share angiogenic properties and are active as mitogens for endothelial cells. The VEGF<sub>145</sub> mRNA has been previously detected as a rare VEGF mRNA species in placenta (15, 16). We have found expression of the VEGF<sub>145</sub> mRNA in several tumorigenic cell types originating from the female reproductive system at levels comparable with the expression levels of VEGF<sub>165</sub>. Several transformed cell lines from other sources did not express this mRNA, indicating that the expression of VEGF<sub>145</sub> may be more restricted compared with other VEGF forms (6). However, it remains to be seen whether production of VEGF<sub>145</sub> is restricted to the female reproductive system.

To study the properties of VEGF<sub>145</sub>, we have produced recombinant VEGF<sub>145</sub> in mammalian and in insect cells. VEGF<sub>145</sub> was found to induce endothelial cell proliferation and in vitro angiogenesis, in agreement with previous studies that have indicated that these functions are not dependent on the presence of either exon 6 or exon 7 (10) and seem to be associated with the ability to bind to the KDR/flk-1 VEGF receptor (41, 42). However, VEGF<sub>145</sub> seemed to be somewhat less active than VEGF<sub>165</sub>, and it is possible therefore that the presence of exon 6 in VEGF<sub>145</sub> can subtly alter the conformations of the protein at the KDR/flk-1 binding site (7). The VEGF<sub>145</sub> protein was secreted into the growth medium of producing cells. VEGF<sub>145</sub>, VEGF<sub>140</sub>, and VEGF<sub>165</sub> are secreted into the medium by producing cells, whereas VEGF<sub>189</sub> and VEGF<sub>206</sub> are sequestered by cell surface heparan sulfates (8). It therefore

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2 G. Neufeld, unpublished results.
ing cells and is sequestered on cell surface heparan sulfates and in the ECM. In view of the strong interaction of VEGF145 with heparin, it was suggested that VEGF145 binds tightly to cell surface and ECM localized heparan sulfates, allowing the sequestration of VEGF145 in the ECM and on cell surfaces. This was supported by experiments that showed that heparin can dissociate bound VEGF145 from the ECM, although long incubation times and high heparin concentrations were required (9). In contrast, VEGF165 interacts weakly at best with cell surface heparan sulfates and with the ECM and is released into the medium of producing cells (8, 9). We expected VEGF145 to bind weakly to cell surfaces and to the ECM because VEGF165 and VEGF145 appear to bind to heparin with similar affinities. Indeed, we have found that VEGF145 is secreted as expected into the medium of VEGF145 producing cells. However, VEGF165 bound to the ECM produced by cornelia endothelial cells much better than either VEGF145 or VEGF121. The lowest VEGF145 concentration at which binding to the ECM was observed was about an order of magnitude lower than the concentration at which binding of VEGF165 to the ECM was detected. In addition we have observed that VEGF145 that is bound to ECM is able to promote proliferation of endothelial cells. These observations prompted us to compare the ECM binding behavior of VEGF145, with that of bFGF, a growth factor that binds specifically to ECM-associated heparan sulfate moieties (38). Unexpectedly, our observations suggested that VEGF145 and bFGF do not bind to common binding sites on the BCE cell-derived ECM. Digestion of the ECM with heparinases releases bound bFGF but does not release bound VEGF145. It is possible that VEGF145 binds to a heparan sulfate subpopulation that is not recognized by bFGF or by the heparinases used in the present study. However, VEGF145 was also able to bind efficiently to an ECM produced in the presence of chlorate, an inhibitor of glycosaminoglycan sulfation (43) and to ECM digested with a mixture of three different heparinases resulting in a greater than 95% depletion of ECM-associated sulfated groups. These experiments therefore indicate that VEGF145 can bind to ECM components distinct from the heparan sulfate side chains of proteoglycans. A similar observation was reported for transforming growth factor-β1, a heparin binding protein (44) that binds to the core protein of the ECM-associated chondroitin sulfate/dermatan sulfate proteoglycan decorin (45) rather than to ECM-associated heparan sulfate moieties.

All the splice variants induce angiogenesis in vivo, so why are five VEGF variants produced? Angiogenesis is often initiated under adverse conditions, such as the conditions encountered during wound healing. Many cell types produce several VEGF forms simultaneously (6, 46), and it is possible that each form offers advantages in different situations. The simultaneous production of several different VEGF forms may therefore ensure a balanced angiogenic response under diverse circumstances. When the properties of the VEGF variants are examined, it is apparent that the differences in their heparin binding abilities may affect their diffusion from a VEGF producing source to target blood vessels. VEGF145 does not bind to either heparan sulfates or to the ECM and should therefore diffuse more readily than the heparin and ECM binding VEGF forms. The ECM may serve as a storage depot for the VEGF forms that bind efficiently to the ECM, and these forms may dissociate slowly from the ECM providing prolonged angiogenic stimulation or be released from the ECM as a result of the activity of proteases (8). The balance may tip toward the production of preferred VEGF isoforms under certain conditions (46). Production of VEGF145 may be such a case because the variety of cell types that produce VEGF145 appears to be limited com-

pared with the range of cell types producing VEGF121 or VEGF165. However, the mechanism that determines what VEGF forms should be produced by a given cell type remains to be elucidated.

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REFERENCES

1. Tischer, E., Gospodarowicz, D., Mitchell, R., Silva, M., Schilling, J., Lai, K., Gitay-Goren, H., Leff, J. A., and Abraham, J. A. (1989) Biochem. Biophys. Res. Commun. 165, 1198–1206
2. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1339–1344
3. Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connelly, D. T. (1989) Science 246, 1330–1312
4. Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. A., and Abraham, J. A. (1989) J. Biol. Chem. 264, 11583–11584
5. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991) Mol. Endocrinol. 5, 1806–1814
6. Neufeld, G., Cohen, T., Gitay-Goren, H., Poltorak, T., Tessler, S., Gengrinovitch, S., and Levi, B. (1996) Cancer Metastasis Rev. 15, 153–158
7. Keyt, B. A., Nguyen, H. V., Berleau, L. T., Duarte, C. M., Park, J., Chen, H., and Ferrara, N. (1996) J. Biol. Chem. 271, 5638–5646
8. Houck, K. A., Leung, D. W., Rowland, A. M., Winer, J., and Ferrara, N. (1992) J. Biol. Chem. 267, 26031–26037
9. Park, J. E., Keller, G. A., and Ferrara, N. (1993) Mol. Biol. Cell 4, 1317–1326
10. Nisato, S., Matsumoto, T., Yoneyama, Y., Ohmori, I., and Suzuki, H. (1995) Biochem. Biophys. Acta 1243, 195–202
11. Gengrinovitch, S., Greenberg, S. M., Cohen, T., Gitay-Goren, H., Rockefeller, P., Maine, T. E., Levi, B.-Z., and Neufeld, G. (1995) J. Biol. Chem. 270, 15059–15065
12. Ferrara, N., Winer, J., Burton, T., Rowland, A., Siegel, M., Phillips, H. S., Terrell, T., Keller, G. A., and Levinson, A. D. (1993) J. Clin. Invest. 91, 160–170
13. Kim, K. J., Li, B., Winer, J., Armanini, M., Gitay-Goren, H., and Ferrara, N. (1993) Nature 362, 841–844
14. Millauer, B., Sasse, U. R., Plate, K. H., Risau, W., and Ullrich, A. (1994) Nature 367, 576–579
15. Charnock-Jones, S. D., Sharkey, A. M., Rajput-Williams, J., Burch, D., Schofield, J. P., Fournier, S. A., Boocock, C., and Smith, S. K. (1993) Biol. Reprod. 48, 1120–1127
16. Cheung, C. Y., Singh, M., Elbahn, M. J., and Blake, R. A. (1995)Am. J. Obstet. Gynecol. 173, 753–759
17. Gospodarowicz, D., Abraham, J. A., and Schilling, J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7311–7315
18. Ferrara, N., and Hengst, W. J. (1989) Biochem. Biophys. Res. Commun. 161, 451–458
19. Cohen, T., Gitay-Goren, H., Sharan, R., Shibusawa, M., Halaban, R., and Neufeld, G. (1995) J. Biol. Chem. 270, 11322–11326
20. Terman, B. I., Dougher-Vermazen, M., Carrion, E. M., Dimitrov, D., Armeanco, D. C., Gospodarowicz, D. H., and Boltien, P. (1992) Biochem. Biophys. Res. Commun. 187, 1579–1586
21. Gitay-Goren, H., Cohen, T., Tessler, S., Sofer, S., Gengrinovitch, S., Rockefeller, P., Klagsbrun, M., Levi, B.-Z., and Neufeld, G. (1996) J. Biol. Chem. 271, 5519–5523
22. Sofer, S., Tessler, H., Neufeld, G., and Klagsbrun, M. (1996) J. Biol. Chem. 271, 5761–5767
23. Cohen, T., Gitay-Goren, H., Neufeld, G., and Levi, B. (1992) Growth Factors 7, 130–138
24. Tessler, S., and Neufeld, G. (1998) J. Cell. Physiol. 175, 310–317
25. MacArthur, C. A., Lawam, A., and Abraham, J. A. (1992) J. Cell Physiol. 150, 255–257
26. Desai, U. R., Wang, H. M., and Linhardt, R. J. (1993) Arch. Biochem. Biophys. 306, 461–468
27. Bashkin, P., Neufeld, G., Gitay-Goren, H., and Vlodavsky, I. (1992) J. Cell. Physiol. 151, 126–137
28. Bashkin, P., Doctrow, S., Klagsbrun, M., Swahn, C. M., Folkman, J., and Vlodavsky, I. (1989) Biochemistry 28, 1737–1743
29. Kerner, G., Bjorsson, T. D., and Vlodavsky, I. (1993) J. Cell. Physiol. 154,
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46. Bacic, M., Edwards, N. A., and Merrill, M. J. (1995) Growth Factors 12, 11–15