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Inhibition of Vascular Endothelial Growth Factor (VEGF)-induced Endothelial Cell Proliferation by a Peptide Corresponding to the Exon 7-Encoded Domain of $VEGF_{165}$*

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Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells (EC) *in vitro* and a major regulator of angiogenesis *in vivo*. $VEGF_{121}$ and $VEGF_{165}$ are the most abundant of the five known VEGF isoforms. The structural difference between these two is the presence in $VEGF_{165}$ of 44 amino acids encoded by exon 7 lacking in $VEGF_{121}$. It was previously shown that $VEGF_{165}$ and $VEGF_{121}$ both bind to KDR/Flk-1 and Flt-1 but that $VEGF_{165}$ binds in addition to a novel receptor (Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996) *J. Biol. Chem.* 271, 5761–5767). The binding of $VEGF_{165}$ to this $VEGF_{165}$-specific receptor ($VEGF_{165}R$) is mediated by the exon 7-encoded domain. To investigate the biological role of this domain further, a glutathione *S*-transferase fusion protein corresponding to the $VEGF_{165}$ exon 7-encoded domain was prepared. The fusion protein inhibited binding of ^{125}I-$VEGF_{165}$ to $VEGF_{165}R$ on human umbilical vein-derived EC (HUVEC) and MDA-MB-231 tumor cells. The fusion protein also inhibited significantly ^{125}I-$VEGF_{165}$ binding to KDR/Flk-1 on HUVEC but not on porcine EC which express KDR/Flk-1 alone. $VEGF_{165}$ had a 2-fold higher mitogenic activity for HUVEC than did $VEGF_{121}$. The exon 7 fusion protein inhibited $VEGF_{165}$-induced HUVEC proliferation by 60% to about the level stimulated by $VEGF_{121}$. Unexpectedly, the fusion protein also inhibited HUVEC proliferation in response to $VEGF_{121}$. Deletion analysis revealed that a core inhibitory domain exists within the C-terminal 23-amino acid portion of the exon 7-encoded domain and that a cysteine residue at position 22 in exon 7 is critical for inhibition. It was concluded that the exon 7-encoded domain of $VEGF_{165}$ enhances its mitogenic activity for HUVEC by interacting with $VEGF_{165}R$ and modulating KDR/Flk-1-mediated mitogenicity indirectly and that exon 7-derived peptides may be useful VEGF antagonists in angiogenesis-associated diseases.

Angiogenesis, the process in which new blood vessels sprout from pre-existing vessels, normally occurs during reproduction, embryonic development, and wound repair. On the other hand, pathological processes such as tumor progression may lead to aberrant angiogenesis (reviewed in Refs. 1–4). The discovery that tumor growth is angiogenesis-dependent has led to the identification of a number of angiogenesis-promoting factors such as basic (bFGF)[1] and acidic fibroblast growth factor, vascular endothelial growth factor (VEGF), tumor necrosis factor-α, transforming growth factor-β, platelet-derived endothelial cell growth factor, and interleukin-8 (reviewed in Refs. 2, 4, and 5). Concomitant with the discovery of positive regulators of angiogenesis, inhibitors of angiogenesis have been identified including thrombospondin-1, interferon-γ, thalidomide, AGM-1470, the 16-kDa fragment of prolactin, cartilage-derived inhibitor, angiostatin, and endostatin (reviewed in Refs. 2, 4, and 5).

There is mounting evidence that VEGF may be a major regulator of angiogenesis (reviewed in Refs. 6–8). VEGF was initially purified from the conditioned media of folliculostellate cells (9) and from a variety of tumor cell lines (10, 11). VEGF was found to be identical to vascular permeability factor, a regulator of blood vessel permeability that was purified from the conditioned medium of U937 cells at the same time (12). VEGF is a specific mitogen for endothelial cells (EC) *in vitro* and a potent angiogenic factor *in vivo*. The expression of VEGF is up-regulated in tissues undergoing vascularization during embryogenesis and the female reproductive cycle (13, 14). High levels of VEGF are expressed in various types of tumors, but not in normal tissue, in response to tumor-induced hypoxia (15–18). Treatment of tumors with monoclonal antibodies directed against VEGF resulted in a dramatic reduction in tumor mass due to the suppression of tumor angiogenesis (19).

VEGF exists in five different isoforms that are produced by alternative splicing from a single gene containing eight exons (6, 20–22). Human VEGF isoforms consist of monomers of 121, 145, 165, 189, and 206 amino acids, each capable of making an active homodimer (22, 23). The $VEGF_{121}$ and $VEGF_{165}$ isoforms are the most abundant. $VEGF_{121}$ is the only VEGF isoform that does not bind to heparin and is totally secreted into the culture medium. $VEGF_{165}$ is functionally different than $VEGF_{121}$ in that it binds to heparin and cell surface heparan sulfate proteoglycans (HSPGs) and is only partially released into the culture medium (24, 25). The remaining isoforms are entirely associated with cell surface and extracellular matrix HSPGs

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[1] The abbreviations used are: bFGF, basic fibroblast growth factor; 231 cells, MDA-MB-231 cells; EC, endothelial cells; GST, glutathione *S*-transferase; HSPG, heparan sulfate proteoglycan; HUVEC, human umbilical vein-derived endothelial cells; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor; $VEGF_{165}R$, $VEGF_{165}$ receptor; FCS, fetal calf serum; PAE, porcine endothelial cells.

(24, 25).

VEGF receptor tyrosine kinases, KDR/Flk-1 and/or Flt-1, are expressed by EC and by several types of non-EC such as NIH 3T3, Balb/c 3T3, human melanoma, and HeLa cells (26–30). It appears that VEGF activities such as mitogenicity, chemotaxis, and induction of morphological changes are mediated by KDR/Flk-1 but not Flt-1, even though both receptors undergo phosphorylation upon binding of VEGF (31–34). Recently, we have characterized a new VEGF receptor which is expressed on EC and various tumor-derived cell lines such as breast cancer-derived MDA-MB-231 (231) cells (35). Although both $VEGF_{121}$ and $VEGF_{165}$ bind to KDR/Flk-1 and Flt-1, only $VEGF_{165}$ binds to the new receptor. Thus, this is an isoform-specific receptor and has been named as the $VEGF_{165}$ receptor ($VEGF_{165}R$). $VEGF_{165}R$ has a molecular mass of approximately 130 kDa, and it binds $VEGF_{165}$ with a K_d of about 2×10^{-10} M, compared with approximately 5×10^{-12} M for KDR/Flk-1. In structure-function analysis, it was shown directly that $VEGF_{165}$ binds to $VEGF_{165}R$ via its exon 7-encoded domain which is absent in $VEGF_{121}$ (35).

$VEGF_{165}$ is a more potent mitogen for EC than is $VEGF_{121}$ (36). One possible explanation is that the interaction of $VEGF_{165}$ with $VEGF_{165}R$ enhances KDR/Flk-1-mediated $VEGF_{165}$ bioactivity. To address this hypothesis, a glutathione *S*-transferase (GST) fusion protein containing a peptide corresponding to the 44 amino acids encoded by exon 7 (amino acids 116–159 of $VEGF_{165}$) was prepared. The GST-exon 7 fusion protein inhibited the binding of ^{125}I-$VEGF_{165}$ to receptors on human umbilical cord vein-derived EC (HUVEC) and on 231 cells. The inhibitory activity was localized to the C-terminal portion of the exon 7-encoded domain. Furthermore, the fusion protein inhibited VEGF-induced proliferation of HUVEC. These results suggest that the exon 7-encoded domain contributes to the enhanced $VEGF_{165}$ mitogenic activity for HUVEC and that exon 7-derived peptides are potential antagonists of VEGF mitogenic activity for EC.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant $VEGF_{165}$ and $VEGF_{121}$ were produced in Sf-21 insect cells infected with recombinant baculovirus encoding human $VEGF_{165}$ or $VEGF_{121}$ as described previously (35, 37). $VEGF_{165}$ was purified from the conditioned medium of infected Sf-21 cells by heparin affinity chromatography, and $VEGF_{121}$ was purified by anion exchange chromatography. Basic FGF was kindly provided by Dr. Judith Abraham (Scios, Sunnyvale, CA). Cell culture media were purchased from Life Technologies, Inc. ^{125}I-Sodium was purchased from NEN Life Science Products. Disuccinimidyl suberate and IODO-BEADS were purchased from Pierce. Glutathione-agarose, NAP-5 columns, and pGEX-2TK plasmid were purchased from Pharmacia Biotech Inc. TSK-heparin columns were purchased from TosoHaas (Tokyo, Japan). Molecular weight marker was purchased from Amersham Corp. IL). Porcine intestinal mucosal-derived heparin was purchased from Sigma.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and grown on gelatin-coated dishes in M-199 medium containing 20% fetal calf serum (FCS) and a mixture of glutamine, penicillin, and streptomycin (GPS). Basic FGF (1 ng/ml) was added to the culture medium every other day. Porcine endothelial cells (PAE), parental and transfected to express KDR/Flk-1 (PAE-KDR), were kindly provided by Dr. Lena Claesson-Welsh and grown in F12 medium containing 10% FCS and GPS as described (32). MDA-MB-231 (231) cells were obtained from ATCC and grown in Dulbecco's modified Eagle's medium containing 10% FCS and GPS.

Endothelial Cell Proliferation Assay—HUVEC were seeded in gelatin-coated 96-well dishes at 4,000 cells/200 μl/well in M-199 containing 5% FCS and GPS. After 24 h, VEGF isoforms and VEGF exon 7-GST fusion proteins were added to the wells at the same time. The cells were incubated for 72 h, and [^{3}H]thymidine (1 μCi/ml) was added for 10–12 h. The medium was aspirated, and the cells were trypsinized and harvested by an automatic cell harvester (TOMTEC) and loaded onto Filtermats (Wallac). The Filtermats were scanned and cpm were determined by a MicroBeta counter (Wallac). The results represent the average of samples assayed in triplicate, and the standard deviations were determined. All experiments were repeated at least three times and similar results were obtained.

Radioiodination of VEGF—The radioiodination of $VEGF_{165}$ and $VEGF_{121}$ was carried out using IODO-BEADS according to the manufacturer's instructions. Briefly, one IODO-BEAD was rinsed with 100 μl of 0.1 M sodium phosphate, pH 7.2, dried, and incubated with ^{125}I-sodium (0.2 mCi/μg protein) in 100 μl of 0.1 M sodium phosphate, pH 7.2, for 5 min at room temperature. VEGF (1–3 μg) was added to the reaction mixture, and after 5 min the reaction was stopped by removing the bead. The solution containing ^{125}I-VEGF was adjusted to 2 mg/ml gelatin and purified by size exclusion chromatography using a NAP-5 column that was pre-equilibrated with PBS containing 2 mg/ml gelatin. Aliquots of the iodinated proteins were frozen on dry ice and stored at −80 °C. The specific activity ranged from 40,000 to 100,000 cpm/ng protein.

Binding and Cross-linking of ^{125}I-VEGF—Binding and cross-linking experiments using ^{125}I-$VEGF_{165}$ and ^{125}I-$VEGF_{121}$ were performed as described previously (29, 35). VEGF binding was quantified by measuring the cell-associated radioactivity in a γ-counter (Beckman, Gamma 5500). The counts represent the average of three wells. All experiments were repeated at least three times, and similar results were obtained. ^{125}I-VEGF cross-linked complexes were resolved by 6% SDS-PAGE, and the gels were exposed to a phosphor screen and scanned after 24 h by a PhosphorImager (Molecular Dynamics). Subsequently, the gels were exposed to x-ray film.

Preparation of GST-VEGF Exon 7 and 8 Fusion Proteins—Different segments of exons 7 and 8 of VEGF were amplified by the polymerase chain reaction from human VEGF cDNA using the following primers: exon 7 + 8 (Ex 7+8), CGGGATCCCCCTGTGGGCCTTGCTC and GGAATTCTTACCGCCTCGGCTTGTC; exon 7 (Ex 7), CGGGATCCCCCTGTGGGCCTTGCTC and GGAATTCTTAACATCTGCAAGTACGTT; exon 7 with residues 1–10 deleted (Ex 7d-(1–10)), CGGGATCCCATTTGTTTGTACAAGAT and GGAATTCTTAACATCTGCAAGTACGTT; exon 7 with residues 1–21 deleted (Ex 7d-(1–21)), CGGGATCCTGTTCCTGCAAAAACACAG and GGAATTCTTAACATCTGCAAGTACGTT; exon 7 with residues 1–22 deleted (Ex 7d-(1–22)), CGGGATCCTGCAAAAACACAG and GGAATTCTTAACATCTGCAAGTACGTT, and exon 7 with residues 30–44 deleted (Ex 7d-(30–44)), CGGGATCCCCCTGTGGGCCTTGCTC and GGAATTCTAGTCTGTGTTTTTGCA. The amplified products were digested with *Bam*HI and *Eco*RI restriction enzymes and cloned into the vector pGEX-2TK (Pharmacia Biotech Inc.) encoding GST (38) to yield the plasmid p2TK-exon 7+8 and its derivatives. *Escherichia coli* (DH5α) were transformed with p2TK-exon 7+8 and derivatives to produce GST fusion proteins (see Fig. 5*B* for sequences). Bacterial lysates were subsequently separated by a glutathione-agarose affinity chromatography (38). Samples eluted from glutathione-agarose were analyzed by 15% SDS-PAGE and silver staining. GST fusion proteins were further purified on a TSK-heparin column as described previously (35).

RESULTS

Differential Receptor Binding and Mitogenic Activities of $VEGF_{165}$ and $VEGF_{121}$ for HUVEC—$VEGF_{165}$ and $VEGF_{121}$ differ in their ability to interact with VEGF receptors expressed on HUVEC (35, 39). $VEGF_{121}$ binds to KDR/Flk-1 to form a 240-kDa labeled complex (Fig. 1, *lane 2*), whereas $VEGF_{165}$, in addition to forming this size complex, also forms a lower molecular mass complex of 165–175 kDa (Fig. 1, *lane 1*). This isoform-specific receptor has been named the $VEGF_{165}$ receptor ($VEGF_{165}R$). These differential receptor binding properties suggest that $VEGF_{165}$ and $VEGF_{121}$ might also have differential mitogenic activities. Accordingly, the ability of the two VEGF isoforms to stimulate HUVEC proliferation was tested. $VEGF_{165}$ was a more potent mitogen for HUVEC than was $VEGF_{121}$ (Fig. 2). $VEGF_{165}$ stimulated half-maximal DNA synthesis at 1 ng/ml and maximal stimulation at 4 ng/ml resulting in an 8-fold increase over control. On the other hand, 2 ng/ml $VEGF_{121}$ were required for half-maximal stimulation and 20 ng/ml for maximal stimulation resulting in a 4-fold increase in HUVEC proliferation over control. Thus, twice as much $VEGF_{121}$ compared with $VEGF_{165}$ was needed to attain half-maximal stimulation, and $VEGF_{121}$-induced proliferation

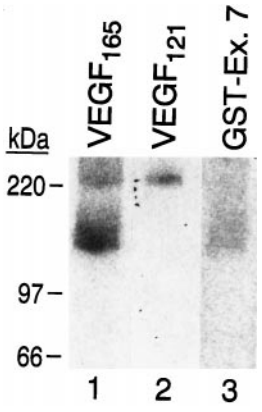

FIG. 1. **Cross-linking of ^{125}I-VEGF$_{165}$, ^{125}I-VEGF$_{121}$, and ^{125}I-GST-EX 7 to HUVEC.** ^{125}I-VEGF$_{165}$ (5 ng/ml) (*lane 1*) or ^{125}I-VEGF$_{121}$ (10 ng/ml) (*lane 2*) or ^{125}I-GST-EX 7 (50 ng/ml) (*lane 3*) were bound to subconfluent cultures of HUVEC in 6-cm dishes. The binding was carried out in the presence of 1 μg/ml heparin. At the end of a 2-h incubation, each ^{125}I-VEGF isoform was chemically cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE. The polyacrylamide gel was dried and exposed to x-ray film.

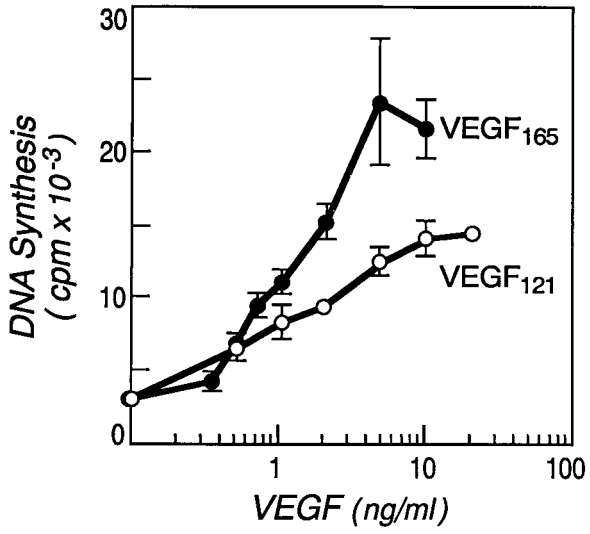

FIG. 2. **HUVEC proliferation in response to VEGF$_{165}$ and VEGF$_{121}$.** HUVEC were cultured in 96-well dishes (5,000 cell/well) for 24 h. Increasing amounts of VEGF$_{165}$ (*closed circles*) or VEGF$_{121}$ (*open circles*) were added to the medium, and the cells were incubated for 3 more days. DNA synthesis based on the incorporation of [^{3}H]thymidine into HUVEC DNA was measured as described under "Experimental Procedures." The results represent the average counts in three wells, and the standard deviations were determined.

was saturated at about one-half the level induced by VEGF$_{165}$. Taken together, these results suggest that there might be a correlation between the enhanced mitogenic activity of VEGF$_{165}$ for EC compared with VEGF$_{121}$ and the ability of VEGF$_{165}$ to bind to an additional receptor (VEGF$_{165}$R) on HUVEC.

A Fusion Protein Containing the Exons 7- and 8-encoded Domains Inhibits the Binding of ^{125}I-VEGF$_{165}$ to Receptors on HUVEC and 231 Cells—Our previous studies indicated that the binding of VEGF$_{165}$ to VEGF$_{165}$R is mediated by the 44 amino acids encoded by exon 7 (VEGF amino acids 116–159) which is present in VEGF$_{165}$ but absent in VEGF$_{121}$ (35). This finding suggested that an excess of exon 7-encoded peptide might inhibit VEGF$_{165}$ binding to VEGF$_{165}$R. GST fusion proteins containing a peptide encoded by VEGF exon 7 or by VEGF exons 7 and 8 were prepared. The 6 amino acids encoded by exon 8 which is C-terminal to exon 7 were included to facilitate

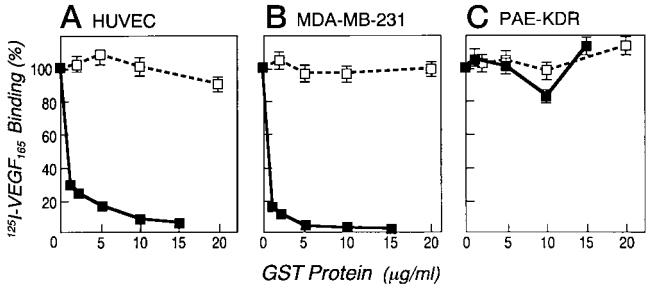

FIG. 3. **Inhibition of ^{125}I-VEGF$_{165}$ binding to HUVEC, MDA MB 231 cells, and PAE-KDR cells by GST-Ex 7+8.** ^{125}I-VEGF$_{165}$ (5 ng/ml) was bound to subconfluent cultures of HUVEC (*A*), MDA MB 231 cells (*B*), and PAE-KDR cells (*C*) in 48-well dishes in the presence of increasing amounts of GST-Ex 7+8 (*closed squares*) or control GST protein (*open squares*). At the end of a 2-h incubation, the cells were washed and lysed, and the cell-associated radioactivity was determined with a γ counter. The counts obtained are expressed as the percentage of the counts obtained in the presence of PBS without addition of GST or fusion protein.

the preparation of the fusion protein but did not affect the results in any way (data not shown). The exon 7 fusion protein binds directly to VEGF$_{165}$R on 231 cells (35). It also binds directly to VEGF$_{165}$R on HUVEC but not to KDR/FLK-1 on HUVEC (Fig. 1, *lane 3*). The ability of the GST-VEGF$_{165}$ exons 7- and 8-encoded peptide (GST-Ex 7+8) to compete with ^{125}I-VEGF$_{165}$ binding to HUVEC, which express both KDR/Flk-1 and VEGF$_{165}$R, to PAE-KDR cells which express only KDR/Flk-1 (32), and to 231 cells which express only VEGF$_{165}$R (35) was tested (Fig. 3). Increasing concentrations of GST-Ex 7+8 markedly inhibited the binding of ^{125}I-VEGF$_{165}$ to HUVEC by about 85–95% (Fig. 3*A*) and to 231 cells by 97–98% (Fig. 3*B*). However, the fusion protein did not inhibit the binding of ^{125}I-VEGF$_{165}$ to PAE-KDR cells which do not express any VEGF$_{165}$R (Fig. 3*C*). GST protein alone even at concentrations of 20 μg/ml had no significant effect on the binding of ^{125}I-VEGF$_{165}$ to any of the cell types. Taken together, these binding studies suggested that GST-Ex 7+8 competes for ^{125}I-VEGF$_{165}$ binding by interacting directly with VEGF$_{165}$R but not with KDR.

These binding experiments were extended to analyze the effects of GST-Ex 7+8 on ^{125}I-VEGF$_{165}$ binding to the individual VEGF receptor species by cross-linking (Fig. 4). Cross-linking of ^{125}I-VEGF$_{165}$ to 231 cells resulted in the formation of labeled complexes with VEGF$_{165}$R (Fig. 4, *lane 3*). The formation of these complexes was markedly inhibited in the presence of 15 μg/ml GST-Ex 7+8 (Fig. 4, *lane 4*). ^{125}I-VEGF$_{165}$ cross-linking to HUVEC resulted in the formation of labeled complexes of higher molecular mass with KDR/Flk-1 and lower molecular mass complexes with VEGF$_{165}$R (35) (Fig. 4, *lane 1*). GST-Ex 7+8 markedly inhibited the formation of the 165–175-kDa labeled complexes containing VEGF$_{165}$R (Fig. 4, *lane 2*). Unexpectedly, GST-Ex 7+8 also inhibited markedly the formation of the 240-kDa labeled complex in HUVEC containing KDR/Flk-1 (Fig. 4, *lane 2*). On the other hand, the fusion protein did not inhibit cross-linking of ^{125}I-VEGF$_{165}$ to KDR/Flk-1 on the PAE/KDR cells (not shown). Taken together, since (i) VEGF$_{165}$ binds to KDR/Flk-1 via the amino acids encoded by exon 4 (40), (ii)) VEGF$_{165}$ binds to VEGF$_{165}$R via the amino acids encoded by exon 7, and (iii) GST-Ex 7+8 binds to VEGF$_{165}$R but not to KDR (Fig. 1 and Fig. 3), these results suggested that by interfering directly with the binding of ^{125}I-VEGF$_{165}$ to VEGF$_{165}$R, GST-Ex 7+8 also inhibits indirectly the binding of ^{125}I-VEGF$_{165}$ to KDR/Flk-1.

Localization of the Core Inhibitory Region within the Exon 7-encoded Domain—The GST-Ex 7 fusion protein encompasses the entire 44 amino acid exon 7-encoded domain. To determine

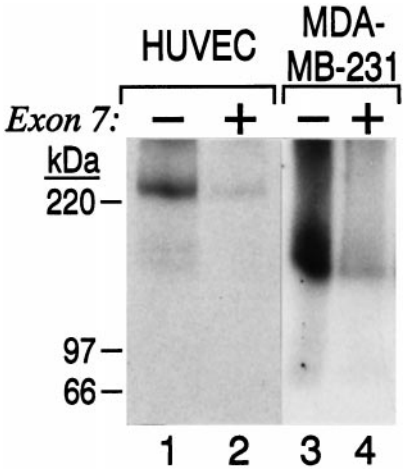

FIG. 4. **GST-Ex 7+8 fusion protein inhibits cross-linking of ^{125}I-VEGF$_{165}$ to VEGF$_{165}$R and to KDR/Flk-1.** ^{125}I-VEGF$_{165}$ (5 ng/ml) was bound to subconfluent cultures of HUVEC (*lanes 1* and *2*) and MDA-MB-231 cells (*lanes 3* and *4*) in 6-cm dishes. The binding was carried out in the presence (*lanes 2* and *4*) or the absence (*lanes 1* and *3*) of 15 μg/ml GST-Ex 7+8. Heparin (1 μg/ml) was added to each dish. At the end of a 2-h incubation, ^{125}I-VEGF$_{165}$ was chemically cross-linked to the cell surface. The cells were lysed, and proteins were resolved by 6% SDS-PAGE. The gel was dried and exposed to x-ray film.

whether a core inhibitory region exists, deletions were made at the N and C termini of exon 7, and the effects on ^{125}I-VEGF$_{165}$ binding to HUVEC were measured (Fig. 5). In these experiments a fusion protein containing the exon 7-encoded domain plus the cysteine residue at position 1 of exon 8 was used as the parental construct. The cysteine residue of exon 8 was included to keep the number of cysteine residues in the VEGF portion of the fusion protein even. The GST-Ex 7 fusion protein inhibited ^{125}I-VEGF$_{165}$ binding to HUVEC by 80% at 2 μg/ml fusion protein (Fig. 5). Inhibition of ^{125}I-VEGF$_{165}$ binding to HUVEC and 231 cells was comparable to that of GST-Ex 7+8 (data not shown). Deletion of the first 10 (GST-Ex 7d-(1–10)) or 21 (GST-Ex 7d-(1–21)) N-terminal amino acids did not reduce the inhibitory activity of the fusion proteins. Actually, 1 μg/ml of GST-Ex 7d-(1–21) had a greater inhibition activity than the same concentration of GST-Ex 7 suggesting that there may be a region within exon 7 amino acids 1–21 that interferes with the inhibitory activity. On the other hand, deletion of the cysteine residue at position 22 in exon 7 (GST-Ex 7 d-(1–22)) resulted in a complete loss of inhibitory activity. Deletion of the 15 C-terminal amino acids (GST-Ex 7 d-(30–44)) also resulted in a complete loss of inhibitory activity (Fig. 5). These results indicated that the inhibitory core is found within amino acids 22–44 of exon 7. Moreover, it seems that the cysteine residue at position 22 in exon 7, which is Cys^{137} in VEGF, is crucial for maintaining a specific structure required for the inhibition.

GST-Ex 7+8 Inhibits VEGF$_{165}$-induced Proliferation of HUVEC—The inhibition of VEGF$_{165}$ binding to KDR/Flk-1 by the GST-Ex 7+8 fusion protein as shown in Fig. 4 suggested that it might also be an inhibitor of VEGF$_{165}$ mitogenicity since KDR/Flk-1 mediates VEGF mitogenic activity (32). Addition of 1–5 ng/ml VEGF$_{165}$ to HUVEC resulted in a 5.5-fold increase in the proliferation rate, peaking at 2.5 ng/ml (Fig. 6). When 15 μg/ml GST-Ex 7+8 was added in addition to VEGF$_{165}$, HUVEC proliferation was reduced by about 60%. GST protein prepared in a similar way did not inhibit HUVEC proliferation even at 25 μg/ml indicating that the inhibitory effect was due solely to the presence of the exon 7+8-encoded domain within the fusion protein. It was concluded that exon 7+8 peptide-mediated inhibition of VEGF$_{165}$ binding to VEGF receptors on HUVEC correlates with the inhibition of HUVEC proliferation.

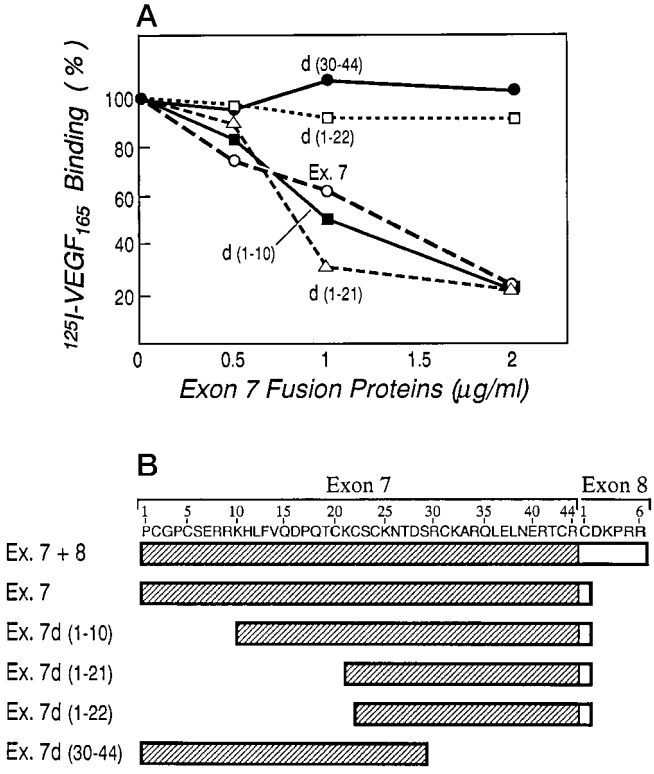

FIG. 5. **Localization of a core inhibitory region within exon 7.** GST-Ex 7 fusion proteins containing full-length exon 7-encoded domain or truncations at the N-terminal and C-terminal ends were prepared as described under "Experimental Procedures." *A*, ^{125}I-VEGF$_{165}$ (5 ng/ml) was bound to subconfluent HUVEC cultures, as described in Fig. 3, in the presence of increasing concentrations of the GST fusion proteins. At the end of a 2-h incubation, the cells were washed and lysed, and the cell-associated radioactivity was determined with a γ counter. The counts obtained are expressed as percentage of the counts obtained in the presence of PBS without fusion protein. *B*, the amino acid sequences of VEGF exon 7 derivatives. These derivatives were prepared to contain the first cysteine residue of exon 8 at their C termini to keep an even number of cysteine residues.

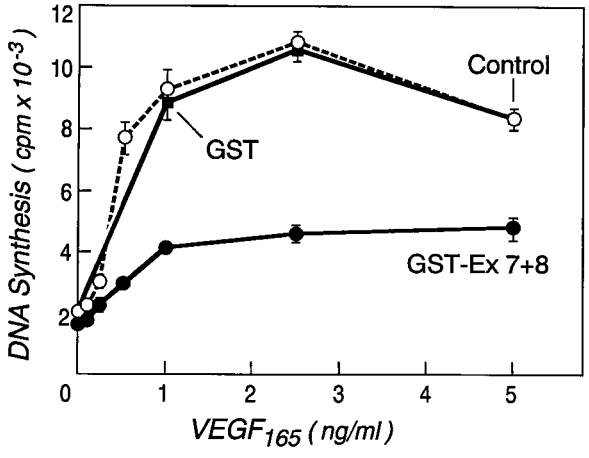

FIG. 6. **GST-Ex 7+8 fusion protein inhibits VEGF$_{165}$-stimulated HUVEC proliferation.** HUVEC were cultured in 96-well dishes (5,000 cell/well) as in Fig. 2. Increasing concentrations of VEGF$_{165}$ (*open circles*), together with 15 μg/ml GST-Ex 7+8 (*closed circles*) or 25 μg/ml GST (*squares*), were added to the medium, and the cells were incubated for 4 more days. DNA synthesis was measured in HUVEC as described in Fig. 2. The results represent the average counts of three wells, and the standard deviations were determined.

GST-Ex 7+8 Inhibits VEGF$_{121}$-induced Proliferation of HUVEC—GST-Ex 7+8 inhibits the level of VEGF$_{165}$-induced mitogenicity, about 2-fold, to about the level of VEGF$_{121}$-in-

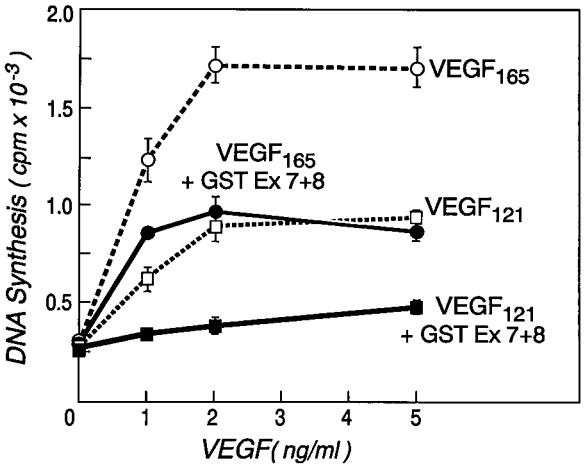

FIG. 7. **GST-Ex 7+8 fusion protein inhibits VEGF$_{165}$ and VEGF$_{121}$-stimulated HUVEC proliferation.** Increasing concentrations of VEGF$_{165}$ (*circles*) or VEGF$_{121}$ (*squares*) with 15 μg/ml GST-Ex 7+8 (*closed symbols*) or without GST-Ex 7+8 (*open symbols*) were added to HUVEC, and [^{3}H]thymidine incorporation into the DNA was measured as in Fig. 2. The results represent the average counts of three wells, and the standard deviations were determined.

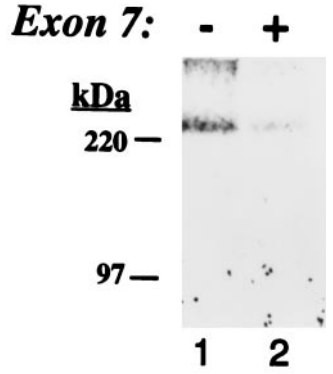

FIG. 8. **GST-Ex 7+8 fusion protein inhibits cross-linking of ^{125}I-VEGF$_{121}$ to KDR/Flk-1 of HUVEC.** ^{125}I-VEGF$_{121}$ (20 ng/ml) was bound to subconfluent cultures of HUVEC in 6-cm dishes. The binding was carried out in the presence (*lane 2*) or the absence (*lane 1*) of 15 μg/ml GST-Ex 7+8. Heparin (1 μg/ml) was added to each dish. At the end of a 2-h incubation, ^{125}I-VEGF$_{121}$ was chemically cross-linked to the cell surface. The cells were lysed, and proteins were resolved by 6% SDS-PAGE. The gel was dried and exposed to x-ray film.

duced mitogenicity (Fig. 7). GST-Ex 7+8, at 15 μg/ml, also inhibited VEGF$_{121}$-mediated HUVEC proliferation, by about 2-fold. This was an unexpected result considering that VEGF$_{121}$ does not contain exon 7. To understand better the nature of the VEGF$_{121}$ inhibition, the effect of GST-Ex 7+8 on the binding of ^{125}I-VEGF$_{121}$ to VEGF receptors was analyzed by cross-linking studies. Cross-linking of ^{125}I-VEGF$_{121}$ to HUVEC resulted in the formation of 240-kDa labeled complexes (Fig. 8, *lane 1*), which have been shown to contain VEGF$_{121}$ and KDR/Flk-1 (35, 39). Formation of these complexes was significantly inhibited by GST-Ex 7+8 at 15 μg/ml (Fig. 8, *lane 2*). It was concluded that GST-Ex 7+8 inhibits VEGF$_{121}$-induced mitogenicity possibly by inhibiting its binding to KDR/Flk-1.

DISCUSSION

The most abundant of the VEGF isoforms are VEGF$_{165}$ and VEGF$_{121}$. An important question in terms of understanding VEGF biology is whether these isoforms differ in their biochemical and biological properties. To date, it has been demonstrated that VEGF$_{165}$, but not VEGF$_{121}$, binds to cell-surface HSPG (23–25) and that VEGF$_{165}$ is a more potent EC mitogen than is VEGF$_{121}$ (36) (Fig. 2). In addition, we recently characterized a novel 130-kDa VEGF receptor found on the surface of HUVEC and tumor cells that is specific in that it binds VEGF$_{165}$ but not VEGF$_{121}$ (35). VEGF$_{165}$ binds to this receptor, termed VEGF$_{165}$R, via the 44 amino acids encoded by exon 7, the exon which is present in VEGF$_{165}$ but not VEGF$_{121}$. In contrast KDR/Flk-1 and Flt-1 bind both VEGF$_{165}$ and VEGF$_{121}$ and do so via the VEGF exons 4 and 3, respectively (40). Our goal in the present study was to determine whether exon 7 modulated VEGF$_{165}$ activity, in particular mitogenicity for HUVEC, and by what mechanism. To do so, we developed a strategy of inhibiting the binding of VEGF$_{165}$ to VEGF$_{165}$R using a GST fusion protein containing the exon 7-encoded domain and examining any subsequent effects on HUVEC proliferation. Cross-linking experiments demonstrated, as expected, that the exon 7 fusion protein could bind to VEGF$_{165}$R but not to KDR/Flk-1. The exon 7 fusion protein was found to be a potent inhibitor of ^{125}I-VEGF$_{165}$ binding to 231 cells which express VEGF$_{165}$R alone, by 98%, and to HUVEC which express both KDR/Flk-1 and VEGF$_{165}$R, by 85–95%. It did not, however, inhibit at all the binding of ^{125}I-VEGF$_{165}$ to PAE-KDR cells which express KDR/Flk-1 but not VEGF$_{165}$R. GST protein alone did not inhibit binding to any of the cell types demonstrating that the inhibition was due solely to the exon 7 portion of the fusion protein. Cross-linking analysis, which demonstrated the formation of specific ^{125}I-VEGF$_{165}$-receptor complexes, confirmed that GST-Ex 7+8 markedly inhibited the binding of ^{125}I-VEGF$_{165}$ to VEGF$_{165}$R on HUVEC and 231 cells. Taken together, these results indicate that the exon 7 fusion protein interacts directly with VEGF$_{165}$R and can act as a competitive inhibitor of binding of ^{125}I-VEGF$_{165}$ to this receptor.

The GST-Ex 7+8 fusion protein inhibited VEGF$_{165}$-induced proliferation of HUVEC by about 60%, to a level equivalent to that induced by VEGF$_{121}$ suggesting that activation of the KDR/Flk-1 tyrosine kinase receptor was somehow being adversely affected. Indeed, cross-linking analysis showed that the fusion protein not only inhibited cross-linking of ^{125}I-VEGF$_{165}$ to VEGF$_{165}$R but to KDR/Flk-1 as well. This result was unexpected since our cross-linking studies show that the exon 7 fusion protein does not bind directly to KDR/Flk-1 consistent with the previous demonstration that VEGF$_{165}$ interacts with KDR/Flk-1 via its exon 4-encoded domain (40). Thus it appears that the binding of ^{125}I-VEGF$_{165}$ to VEGF$_{165}$R via the exon 7-encoded domain modulates indirectly the interaction of the growth factor with KDR/Flk-1. A possible mechanism for this inhibitory effect of GST-Ex 7+8 on HUVEC proliferation is that KDR/Flk-1 and VEGF$_{165}$R are co-localized in close proximity on the cell surface. In this model, a VEGF$_{165}$ dimer interacts simultaneously with KDR/Flk-1 via the exon 4 domain and with VEGF$_{165}$R via the exon 7 domain, generating a three-component complex. The GST-Ex 7+8 fusion protein by competing directly with the binding of VEGF$_{165}$ to VEGF$_{165}$R impairs indirectly the ability of VEGF$_{165}$ to bind to the signaling receptor, KDR/Flk-1. Thus, an efficient binding of VEGF$_{165}$ to KDR/Flk-1 might be dependent in part on successful interaction with VEGF$_{165}$R. An alternative possibility is that the exon 7-encoded domain contains a heparin-binding domain (35) and that an excess of GST-Ex 7+8 prevents VEGF$_{165}$ from binding to cell-surface HSPGs that are required for efficient binding of VEGF$_{165}$ to its receptors (29).

Surprisingly, GST-Ex 7+8 also inhibited the mitogenic activity of VEGF$_{121}$ for HUVEC, by about 50%, even though VEGF$_{121}$ does not bind to VEGF$_{165}$R (35). A possible explanation is that VEGF$_{165}$R and KDR/Flk-1 are in proximity on the cell surface and that excess GST-Ex 7+8 bound to VEGF$_{165}$R

sterically inhibits access of $VEGF_{121}$ to KDR/Flk-1. Cross-linking analysis did indeed show diminished binding of ^{125}I-$VEGF_{121}$ to KDR/Flk-1 in the presence of GST-Ex 7+8 which does not bind directly to KDR/Flk-1, suggesting an indirect effect of the fusion protein on the binding of $VEGF_{121}$ to KDR/Flk-1.

GST-Ex 7+8 also inhibits $VEGF_{165}$ binding to 231 breast cancer cells, which express $VEGF_{165}R$ and not KDR/Flk-1. However, VEGF is not mitogenic for these cells and at present we do not know the consequence of inhibiting $VEGF_{165}$ binding to these tumor cells.

The coordinate binding of $VEGF_{165}$ to a higher and to a lower affinity receptor (KDR/Flk-1 and $VEGF_{165}R$, respectively) on HUVEC (35) and the inhibitory effects of GST-Ex 7+8 fusion protein on the binding of $VEGF_{165}$ to these two receptors suggest that there is a dual receptor system at work in mediating $VEGF_{165}$ activity. Several other growth factors have been shown to bind to high and low affinity receptors. Transforming growth factor-β generates a complex with three receptors; two of them, receptors I and II, are the signaling receptors, whereas transforming growth factor-β receptor III/betaglycan is a lower affinity accessory binding molecule (41). The low affinity receptor for the nerve growth factor family, p75, is part of a complex with the signaling TRK receptors (42). A different type of dual receptor recognition is the binding of bFGF to cell-surface HSPGs and to its signaling receptors (43, 44). It has been suggested that the binding of bFGF to its low affinity receptors (HSPGs) may induce conformational changes in bFGF so that the HSPG-bound bFGF could be efficiently presented to its high affinity, signaling receptors (43, 44). Thus, the binding of $VEGF_{165}$ to both $VEGF_{165}R$ and KDR/Flk-1 appears to be part of a general mechanism wherein two different types of receptors are used to modulate growth factor activity.

Receptor binding studies were used to identify an inhibitory core within the 44 amino acids encoded by exon 7. Deletions were made in both the N-terminal and C-terminal regions of exon 7, and the inhibitory activity was localized to the 23-amino acid C-terminal portion of exon 7 (amino acids 22–44). Of these 23 amino acids, 5 are cysteine residues. The high proportion of cysteine residues suggests that this domain has a defined three-dimensional structure required for efficient binding to $VEGF_{165}R$. The cysteine residue at position 22 of the exon 7 domain is critical for inhibitory activity, probably for maintenance of a necessary three-dimensional structure. A study that examined the role of cysteine residues at different positions in $VEGF_{165}$ showed that a substitution of Cys^{146}, which lies within the core inhibitory domain of exon 7 (at position 31 in exon 7), by a serine residue resulted in a 60% reduction in $VEGF_{165}$ permeability activity and a total loss of EC mitogenicity (45). The Cys^{146} mutation had no effect on the dimerization of VEGF (45). Thus, it appears that this cysteine residue is not involved in the formation of interdisulfide bonds between two VEGF monomers but might rather involve intradisulfide bonding within the monomer. These results support our hypothesis that a three-dimensional structure stabilized by cysteine residues exists in the C-terminal half of exon 7 that contributes to $VEGF_{165}$ biological activity, such as interaction with $VEGF_{165}R$. Interestingly, a fusion protein corresponding to a deletion of the N-terminal 21 amino acid residues encoded by exon 7 was a more potent inhibitor than the intact exon 7-encoded peptide. It may be that the N-terminal portion interferes in part with the interaction of the C-terminal portion with $VEGF_{165}R$ and therefore a deletion of the N-terminal portion results in enhanced binding to $VEGF_{165}R$ and yields a better competitor of $VEGF_{165}$.

Since the identification of VEGF as a major angiogenesis factor and contributor to tumor pathology, numerous attempts had been made to design specific VEGF antagonists. These antagonists include anti-VEGF antibodies (19) and soluble KDR/Flk-1 and Flt-1 ectodomains (46–48). We now add to this group the peptide encoded by exon 7 of VEGF and possibly a smaller core inhibitory peptide. Since the exon 7-encoded peptide inhibits both $VEGF_{165}$- and $VEGF_{121}$-induced mitogenicity for HUVEC, it and its derivatives may be useful as general VEGF inhibitors. The VEGF exon 7-encoded domain is an example of a portion of an EC mitogen being an EC inhibitor. Previously, it has been shown that fragments of SPARC (secreted protein, acidic and rich in cysteine) inhibit EC proliferation while the intact SPARC maintains angiogenic activity (49). Several other EC inhibitors are fragments of larger proteins, which in themselves are devoid of inhibitory activity. These include the 16-kDa fragment of prolactin (50), fragments of laminin (51), plasmin-cleaved fragments of fibronectin (52), angiostatin which is a fragment of plasminogen (53), and endostatin which is a fragment of collagen XVIII (54). Thus, it seems that there are numerous examples of EC inhibitors being generated from larger proteins.

Our identification of the NEGF exon 7-encoded domain as an EC antagonist is based on the analysis of VEGF and VEGF receptor structure-function relationships. In the future, further analysis of the exon 7 domain might be useful for the design of small pharmacological peptides that would serve as VEGF antagonists in angiogenesis-related diseases.

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