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 125I-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 125I-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 thrombospordin-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.

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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 VEGF121 and VEGF165 bind to KDR/Flk-1 and Flt-1, only VEGF165 binds to the new receptor. Thus, this is an isoform-specific receptor and has been named as the VEGF165 receptor (VEGF165R).

VEGF165R has a molecular mass of approximately 130 kDa, and it binds VEGF165 with a $K_d$ of about $2 \times 10^{-10}$ M, compared with approximately $5 \times 10^{-12}$ M for KDR/Flk-1. In structure-function analysis, it was shown directly that VEGF165 binds to VEGF165R via its exon 7-encoded domain which is absent in VEGF121 (35).

VEGF165 is a more potent mitogen for EC than is VEGF121 (36). One possible explanation is that the interaction of VEGF165 with VEGF165R enhances KDR/Flk-1-mediated VEGF165 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 VEGF165) was prepared. The GST-exon 7 fusion protein inhibited the binding of 125I-VEGF165 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 VEGF165 mitogenic activity for HUVEC and that exon 7-derived peptides are potential antagonists of VEGF mitogenic activity for EC.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant VEGF165 and VEGF121 were produced in SF21 insect cells infected with recombinant baculovirus encoding human VEGF165 or VEGF121 as described previously (35, 37). VEGF165 was purified from the conditioned medium of infected SF21 cells by heparin affinity chromatography, and VEGF121 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. Porcine intestinal mucosal-derived heparin was purchased from Bio-Rad Laboratories (Richmond, CA). Human recombinant VEGF 165 and VEGF 121 were purchased from R&D Systems (Minneapolis, MN). Molecular weight marker was purchased from Amersham Corp. (Arlington Heights, 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. MDA-MB-231 (231) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and grown in DMEM supplemented with 10% FCS and GPS as described (32). MDA-MB-231 (231) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and grown in DMEM supplemented with 10% FCS and GPS.

**Differential Receptor Binding and Mitogenic Activities of VEGF165 and VEGF121**—Binding and cross-linking experiments using 125I-VEGF165 and 125I-VEGF121, 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.

**RESULTS**

**Differential Receptor Binding and Mitogenic Activities of VEGF165 and VEGF121 for HUVEC**—VEGF165 and VEGF121 differ in their ability to interact with VEGF receptors expressed on HUVEC (35, 39). VEGF121 binds to KDR/Flk-1 to form a 240-kDa labeled complex (Fig. 1, lane 2), whereas VEGF165, 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 VEGF165 receptor (VEGF165R). These differential receptor binding properties suggest that VEGF165 and VEGF121 might also have differential mitogenic activities. Accordingly, the ability of the two VEGF isoforms to stimulate HUVEC proliferation was tested. VEGF165 was a more potent mitogen for HUVEC than was VEGF121 (Fig. 2). VEGF165 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 VEGF121 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 VEGF121 compared with VEGF165 was needed to attain half-maximal stimulation, and VEGF121-induced proliferation was inhibited 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.

**Binding and Cross-linking of 125I-VEGF**—Binding and cross-linking experiments using 125I-VEGF165, and 125I-VEGF121, 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. 125I-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 + (Ex 7–+), CGGATCCCCGGGCTCGTCCCTCTGTTGTACAAGAT and GGAATTCTTAACATCTGCAAGTACGTT; exon 7 with residues 1–10 deleted (Ex 7d-(1–10)), CGGATCCCCGTCCCGTGCTTTCTGTTGTACAAGAT and GGAATTCCTGAATCTCTCTTGTTGTACAAGAT; exon 7 with residues 1–21 deleted (Ex 7d-(1–21)), CGGATCCCTGTGTTGTACAAGAT and GGAATTCCTGTTGTACAAGAT. The amplified products were digested with BamHI and EcoRI restriction enzymes and cloned into the vector pGEX-2TK (Pharmacia Biotech Inc.) encoding GST (38) to yield the plasmid p2TK-exon 7 and its derivatives. Exon 7 was cloned with p2TK-exon 7 and derivatives to produce GST fusion proteins (see Fig. 3B for sequences). The amplified products were digested with BamHI and EcoRI restriction enzymes and cloned into the vector pGEX-2TK (Pharmacia Biotech Inc.) encoding GST (38) to yield the plasmid p2TK-exon 7 and its derivatives. Exon 7 was cloned with p2TK-exon 7 and derivatives to produce GST fusion proteins (see Fig. 3B for sequences). Bacterial lysates were subsequently separated by a glutathione-agarose affinity chromatography (38). Samples eluted from glutathione-agarose affinity chromatography were analyzed by 15% SDS-PAGE and silver staining. GST fusion proteins were further purified on a TSK-heparin column as described previously (35).
Our previous studies indicated that HUVEC and 231 Cells—

Taken together, these results suggest that there might be a correlation between the enhanced mitogenic activity of VEGF165 for EC compared with VEGF121 and the ability of VEGF165 to compete for 125I-VEGF165 binding to VEGF165R. However, the fusion protein did not inhibit the binding of 125I-VEGF165 to PAE-KDR cells which do not express VEGF165R (Fig. 3C). GST protein alone even at concentrations of 20 μg/ml had no significant effect on the binding of 125I-VEGF165 to any of the cell types. Taken together, these binding studies suggested that GST-Ex 7+8 competes for 125I-VEGF165 binding by interacting directly with VEGF165R but not with KDR.

These binding experiments were extended to analyze the effects of GST-Ex 7+8 on 125I-VEGF165 binding to the individual VEGF receptor species by cross-linking (Fig. 4). Cross-linking of 125I-VEGF165 to 231 cells resulted in the formation of labeled complexes with VEGF165R (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). 125I-VEGF165 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 VEGF165R (35) (Fig. 4, lane 1). GST-Ex 7+8 markedly inhibited the formation of the 165–175-kDa labeled complexes containing VEGF165R (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 125I-VEGF165 to KDR/Flk-1 on the PAE/KDR cells (not shown). Taken together, since (i) VEGF165 binds to KDR/Flk-1 via the amino acids encoded by exon 7 (VEGF amino acids 116–159) which is present in VEGF165 but absent in VEGF121 (35). This finding suggested that an excess of exon 7-encoded peptide might inhibit VEGF165 binding to VEGF165R. 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 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 VEGF165R on 231 cells (35). It also binds directly to VEGF165R on HUVEC but not to KDR/Flk-1 on HUVEC (Fig. 1, lane 3). The ability of the GST-VEGF165 exons 7- and 8-encoded peptide (GST-Ex 7+8) to compete with 125I-VEGF165 binding to HUVEC, which express both KDR/Flk-1 and VEGF165R, to PAE-KDR cells which express only KDR/Flk-1 (32), and to 231 cells which express only VEGF165R (35) was tested (Fig. 3). Increasing concentrations of GST-Ex 7+8 markedly inhibited the binding of 125I-VEGF165 to HUVEC by about 85–95% (Fig. 3A) and to 231 cells by 97–98% (Fig. 3B). However, the fusion protein did not inhibit the binding of 125I-VEGF165 to PAE-KDR cells which do not express any VEGF165R (Fig. 3C). GST protein alone even at concentrations of 20 μg/ml had no significant effect on the binding of 125I-VEGF165 to any of the cell types. Taken together, these binding studies suggested that GST-Ex 7+8 competes for 125I-VEGF165 binding by interacting directly with VEGF165R but not with KDR.

A Fusion Protein Containing the Exons 7- and 8-encoded Domains Inhibits the Binding of 125I-VEGF165 to Receptors on HUVEC and 231 Cells—Our previous studies indicated that the binding of VEGF165 to VEGF165R is mediated by the 44 amino acids encoded by exon 7 (VEGF amino acids 116–159) which is present in VEGF165 but absent in VEGF121 (35). This finding suggested that an excess of exon 7-encoded peptide might inhibit VEGF165 binding to VEGF165R. 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 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 VEGF165R on 231 cells (35). It also binds directly to VEGF165R on HUVEC but not to KDR/Flk-1 on HUVEC (Fig. 1, lane 3). The ability of the GST-VEGF165 exons 7- and 8-encoded peptide (GST-Ex 7+8) to compete with 125I-VEGF165 binding to HUVEC, which express both KDR/Flk-1 and VEGF165R, to PAE-KDR cells which express only KDR/Flk-1 (32), and to 231 cells which express only VEGF165R (35) was tested (Fig. 3). Increasing concentrations of GST-Ex 7+8 markedly inhibited the binding of 125I-VEGF165 to HUVEC by about 85–95% (Fig. 3A) and to 231 cells by 97–98% (Fig. 3B). However, the fusion protein did not inhibit the binding of 125I-VEGF165 to PAE-KDR cells which do not express any VEGF165R (Fig. 3C). GST protein alone even at concentrations of 20 μg/ml had no significant effect on the binding of 125I-VEGF165 to any of the cell types. Taken together, these binding studies suggested that GST-Ex 7+8 competes for 125I-VEGF165 binding by interacting directly with VEGF165R but not with KDR.

These binding experiments were extended to analyze the effects of GST-Ex 7+8 on 125I-VEGF165 binding to the individual VEGF receptor species by cross-linking (Fig. 4). Cross-linking of 125I-VEGF165 to 231 cells resulted in the formation of labeled complexes with VEGF165R (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). 125I-VEGF165 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 VEGF165R (35) (Fig. 4, lane 1). GST-Ex 7+8 markedly inhibited the formation of the 165–175-kDa labeled complexes containing VEGF165R (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 125I-VEGF165 to KDR/Flk-1 on the PAE/KDR cells (not shown). Taken together, since (i) VEGF165 binds to KDR/Flk-1 via the amino acids encoded by exon 7 (40), (ii) VEGF165 binds to VEGF165R via the amino acids encoded by exon 7, and (iii) GST-Ex 7+8 binds to VEGF165R but not to KDR (Fig. 1 and Fig. 3), these results suggested that by interfering directly with the binding of 125I-VEGF165 to VEGF165R, GST-Ex 7+8 also inhibits indirectly the binding of 125I-VEGF165 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...
whether a core inhibitory region exists, deletions were made at the N and C termini of exon 7, and the effects on \( ^{125} \text{I}-\text{VEGF}_{165} \) 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 \( ^{125} \text{I}-\text{VEGF}_{165} \) binding to HUVEC by 80% at 2 \( \mu \text{g/ml} \) fusion protein (Fig. 5). Inhibition of \( ^{125} \text{I}-\text{VEGF}_{165} \) 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 \( \mu \text{g/ml} \) 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 7d-(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\textsuperscript{137} in VEGF, is crucial for maintaining a specific structure required for the inhibition. GST-Ex 7+8 Inhibits VEGF\textsubscript{165}-induced Proliferation of HUVEC—The inhibition of VEGF\textsubscript{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\textsubscript{165} mitogenicity since KDR/Flk-1 mediates VEGF mitogenic activity (32). Addition of 1–5 \( \mu \text{g/ml} \) VEGF\textsubscript{165} to HUVEC resulted in a 5.5-fold increase in the proliferation rate, peaking at 2.5 \( \mu \text{g/ml} \) (Fig. 6). When 15 \( \mu \text{g/ml} \) GST-Ex 7+8 was added in addition to VEGF\textsubscript{165}, HUVEC proliferation was reduced by about 60%. GST protein prepared in a similar way did not inhibit HUVEC proliferation even at 25 \( \mu \text{g/ml} \). GST protein prepared in a similar way did not inhibit HUVEC proliferation even at 25 \( \mu \text{g/ml} \).

**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. \( ^{125} \text{I}-\text{VEGF}_{165} \) (5 \( \mu \text{g/ml} \)) 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 \( \gamma \) 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\textsubscript{165}-stimulated HUVEC proliferation. HUVEC were cultured in 96-well dishes (5,000 cell/well) as in Fig. 2. Increasing concentrations of VEGF\textsubscript{165} (open circles), together with 15 \( \mu \text{g/ml} \) GST-Ex 7+8 (closed circles) or 25 \( \mu \text{g/ml} \) 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\textsubscript{121}-induced Proliferation of HUVEC—GST-Ex 7+8 inhibits the level of VEGF\textsubscript{165}-induced mitogenicity, about 2-fold, to about the level of VEGF\textsubscript{121}-in-
Inhibition by VEGF Exon 7

The exon which is present in VEGF 165 but not VEGF 121. In VEGF165 but not VEGF121 (35). VEGF165 binds to this receptor, HUVEC and tumor cells that is specific in that it binds characterized a novel 130-kDa VEGF receptor found on the surface of HSPG (23–25) and that VEGF165 is a more potent EC mitogen than VEGF121 (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 VEGF165, but not VEGF121 (35). VEGF165 binds to this receptor, termed VEGF165R, via the 44 amino acids encoded by exon 7, the exon which is present in VEGF165 but not VEGF121. In contrast KDR/Flk-1 and Flt-1 bind both VEGF165 and VEGF121 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 VEGF165 activity, in particular mitogenicity for HUVEC, and by what mechanism. To do so, we developed a strategy of inhibiting the binding of VEGF165 to VEGF165R 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 VEGF165R, but not to KDR/Flk-1. The exon 7 fusion protein was found to be a potent inhibitor of 125I-VEGF165 binding to 231 cells which express VEGF165R alone, by 98%, and to HUVEC which express both KDR/Flk-1 and VEGF165R, by 85–95%. It did not, however, inhibit at all the binding of 125I-VEGF165 to HUVEC resulted in the formation of 240-kDa labeled complexes (Fig. 8, lane 2). It was concluded that GST-Ex 7+8 inhibits VEGF121-induced mitogenicity possibly by inhibiting its binding to KDR/Flk-1. The GST-Ex 7+8 fusion protein inhibited VEGF165-induced proliferation of HUVEC by about 60%, to a level equivalent to that induced by VEGF121 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 125I-VEGF165 to VEGF165R 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 VEGF165 interacts with KDR/Flk-1 via its exon 4-encoded domain (40). Thus it appears that the binding of 125I-VEGF165 to VEGF165R 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 VEGF165R are co-localized in close proximity on the cell surface. In this model, a VEGF165 dimer interacts simultaneously with KDR/Flk-1 via the exon 4 domain and with VEGF165R via the exon 7 domain, generating a three-component complex. The GST-Ex 7+8 fusion protein by competing directly with the binding of VEGF165 to VEGF165R impairs indirectly the ability of VEGF165 to bind to the signaling receptor, KDR/Flk-1. Thus, an efficient binding of VEGF165 to KDR/Flk-1 might be dependent in part on successful interaction with VEGF165R. 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 VEGF165 from binding to cell-surface HSPGs that are required for efficient binding of VEGF165 to its receptors (29).

Surprisingly, GST-Ex 7+8 also inhibited the mitogenic activity of VEGF121 for HUVEC, by about 50%, even though VEGF121 does not bind to VEGF165R (35). A possible explanation is that VEGF165R and KDR/Flk-1 are in proximity on the cell surface and that excess GST-Ex 7+8 bound to VEGF165R

**FIG. 7.** GST-Ex 7+8 fusion protein inhibits VEGF165 and VEGF121-stimulated HUVEC proliferation. Increasing concentrations of VEGF165 (circles) or VEGF121 (squares) with 15 μg/ml GST-Ex 7+8 (closed symbols) or without GST-Ex 7+8 (open symbols) were added to HUVEC, and [3H]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.

**DISCUSSION**

The most abundant of the VEGF isoforms are VEGF165 and VEGF121. 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 VEGF165, but not VEGF121, binds to cell-surface HSPG (23–25) and that VEGF165 is a more potent EC mitogen than is VEGF121 (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 VEGF165, but not VEGF121 (35). VEGF165 binds to this receptor, termed VEGF165R, via the 44 amino acids encoded by exon 7, the exon which is present in VEGF165 but not VEGF121. In contrast KDR/Flk-1 and Flt-1 bind both VEGF165 and VEGF121 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 VEGF165 activity, in particular mitogenicity for HUVEC, and by what mechanism. To do so, we developed a strategy of inhibiting the binding of VEGF165 to VEGF165R 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 VEGF165R, but not to KDR/Flk-1. The exon 7 fusion protein was found to be a potent inhibitor of 125I-VEGF165 binding to 231 cells which express VEGF165R alone, by 98%, and to HUVEC which express both KDR/Flk-1 and VEGF165R, by 85–95%. It did not, however, inhibit all the binding of 125I-VEGF165 to HUVEC.

**FIG. 8.** GST-Ex 7+8 fusion protein inhibits cross-linking of 125I-VEGF165 to KDR/Flk-1 of HUVEC. 125I-VEGF165 (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, 125I-VEGF165 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.

PAE-KDR cells which express KDR/Flk-1 but not VEGF165R. 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 125I-VEGF165-receptor complexes, confirmed that GST-Ex 7+8 markedly inhibited the binding of 125I-VEGF165 to VEGF165R on HUVEC and 231 cells. Taken together, these results indicate that the exon 7 fusion protein interacts directly with VEGF165R and can act as a competitive inhibitor of binding of 125I-VEGF165 to this receptor.

The GST-Ex 7+8 fusion protein inhibited VEGF165-induced proliferation of HUVEC by about 60%, to a level equivalent to that induced by VEGF121 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 125I-VEGF165 to VEGF165R 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 VEGF165 interacts with KDR/Flk-1 via its exon 4-encoded domain (40). Thus it appears that the binding of 125I-VEGF165 to VEGF165R 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 VEGF165R are co-localized in close proximity on the cell surface. In this model, a VEGF165 dimer interacts simultaneously with KDR/Flk-1 via the exon 4 domain and with VEGF165R via the exon 7 domain, generating a three-component complex. The GST-Ex 7+8 fusion protein by competing directly with the binding of VEGF165 to VEGF165R impairs indirectly the ability of VEGF165 to bind to the signaling receptor, KDR/Flk-1. Thus, an efficient binding of VEGF165 to KDR/Flk-1 might be dependent in part on successful interaction with VEGF165R. 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 VEGF165 from binding to cell-surface HSPGs that are required for efficient binding of VEGF165 to its receptors (29).
Inhibition by VEGF Exon 7

sterically inhibits access of VEGF to KDR/Flk-1. Cross-linking analysis did indeed show diminished binding of 121–VEGF 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 to KDR/Flk-1.

GST-Ex 7 + 8 also inhibits VEGF binding to 231 breast cancer cells, which express VEGF-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 binding to these tumor cells.

The coordinate binding of VEGF to a higher and to a lower affinity receptor (KDR/Flk-1 and VEGF-R, respectively) on HUVEC (35) and the inhibitory effects of GST-Ex 7 + 8 fusion protein on the binding of VEGF to these two receptors suggest that there is a dual receptor system at work in mediating VEGF 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 HS PGs and to its signaling receptors (43, 44). It has been suggested that the binding of bFGF to its low affinity receptors (HS PGs) may induce conformational changes in bFGF so that the HS PG-bound bFGF could be efficiently presented to its high affinity, signaling receptors (43, 44). Thus, the binding of VEGF to both VEGF-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. 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 showed that a substitution of Cys to Trp, 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 permeability activity and a total loss of EC mitogenicity (45). The Cys 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 biological activity, such as interaction with VEGF-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-R and therefore a deletion of the N-terminal portion results in enhanced binding to VEGF-R and yields a better competitor of VEGF.

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 the peptide encoded by exon 7 of VEGF and possibly a smaller core inhibitory peptide. Since the exon 7-encoded peptide inhibits both VEGF and VEGF-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), angiotatin 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 N-terminal 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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REFERENCES
1. Folkman, J. (1995) Nat. Med. 1, 27–31.
2. Battegay, E. J. (1995) J. Mol. Med. 73, 333–346.
3. Risau, W., and Flamme, I. (1995) Annu. Rev. Cell Dev. Biol. 11, 73–91.
4. Hanahan, D., and Folkman, J. (1996) Cell 86, 353–364.
5. Folkman, J. (1995) N. Engl. J. Med. 333, 1777–1783.
6. Ferrara, N., Houck, K., Jakeman, L., and Leung, D. W. (1992) Endocr. Rev. 13, 18–42.
7. Klagsbrun, M., and Soker, S. (1993) Curr. Biol. 3, 699–702.
8. Klagsbrun, M., and D’Amore, P. A. (1996) Cytokine Growth Factor Rev. 7, 259–270.
9. Ferrara, N., and Henzel, W. J. (1989) Biochem. Biophys. Res. Commun. 161, 851–858.
10. Myoken, Y., Kayada, Y., Okamoto, T., Kan, M., Sato, G. H., and Sato, J. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5819–5823.
11. Plant, J., Schilling, J., and Gospodarowicz, D. (1989) EMBO J. 8, 3801–3806.
12. Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feeder, J., and Cannoy, D. T. (1989) Science 246, 1309–1312.
13. Brier, G., Albert, U., Stürmer, S., and Risau, W. (1992) Development 114, 521–532.
14. Shweiki, D., Itin, A., Neufeld, G., Gitay-Goren, H., and Keshet, E. (1993) J. Clin. Invest. 91, 2235–2243.
15. Foon, E., Chin, S., Soffer, D., and Keshet, E. (1992) Nat Med. 8, 843–845.
16. Dvorak, H. F., Sioussat, T. M., Brown, L. F., Berse, B., Nagy, J. A., Sotrel, A., Manseau, E. J., Van de Water, L., and Senger, D. R. (1991) J. Exp. Med. 174, 1275–1278.
17. Plate, K. H., Breier, G., Millauer, B., Ullrich, A., and Risau, W. (1993) Cancer Res. 53, 5822–5827.
18. Ikeeda, E., Achen, M. G., Breier, G., and Risau, W. (1995) J. Biol. Chem. 270, 17961–17966.
19. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillette, N., Phillips, H. S., and Ferrara, N. (1993) Nature 362, 841–844.
20. Tischer, E., Mitchell, R., Raff, T., Silber, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. (1991) J. Biol. Chem. 266, 11947–11954.
21. Ferrara, N. (1993) Trends Cardio. Med. 3, 244–250.
22. Piltz, R., Cohen, T., Sivan, R., Kamel, S., Spira, G., Vlodavsky, I., Keshet, E., and Neufeld, G. (1997) J. Biol. Chem. 272, 7151–7158.
23. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991) Mol. Endocrinol. 5, 1806–1814.
24. Houck, K. A., Leung, D. W., and Risau, W., and Ferrara, N. (1992) J. Biol. Chem. 267, 20631–20637.
25. Park, J. E., Keller, G. A., and Ferrara, N. (1993) Mol. Cell. Biol. 13, 1317–1326.
26. Tischer, E., Bougher-Vermelho, M. E., Dimitrou, D., Armellino, D. C., Gospodarowicz, D., and Bohlen, P. (1992) Biochem. Biophys. Res. Commun. 187, 1579–1586.
27. Shibuya, M., Yamaguchi, S., Yamane, A., Ikeeda, T., Tojo, A., Matsushima, H., and Sato, M. (1990) Oncogene 5, 519–524.
28. de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992) Science 255, 989–991.
29. Gitay-Goren, H. Soker, S., Vlodavsky, I., and Neufeld, G. (1992) J. Biol. Chem.
Inhibition by VEGF Exon 7

31588

267, 6093–6098

30. Jakeman, L. B., Winer, J., Bennett, G. L., Altar, C. A., and Ferrara, N. (1992) *J. Clin. Invest.* **89**, 244–253.

31. Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., and Ulrich, A. (1993) *Cell* **72**, 835–846.

32. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C.-H. (1994) *J. Biol. Chem.* **269**, 26988–26995.

33. Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S., and Shibuya, M. (1995) *Oncogene* **10**, 135–147.

34. Yoshida, A., Anand-Apte, B., and Zetter, B. R. (1996) *Growth Factors* **13**, 57–64.

35. Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996) *J. Biol. Chem.* **271**, 5761–5767.

36. Keyt, B. A., Berleau, L. T., Nguyen, H. V., Chen, H., Heinsohn, H., Vandlen, R., and Ferrara, N. (1996) *J. Biol. Chem.* **271**, 7788–7795.

37. Cohen, T., Gitay-Goren, H., Neufeld, G., and Levi, B. Z. (1992) *Growth Factors* **7**, 131–138.

38. Smith, D. B., and Johnson, K. S. (1988) *Gene* (Amst.) **67**, 31–40.

39. Gitay-Goren, H., Cohen, T., Tessler, S., Soker, S., Gengrinovitch, S., Rockwell, P., Klagsbrun, M., Levi, B.-Z., and Neufeld, G. (1996) *J. Biol. Chem.* **271**, 5519–5525.

40. 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.

41. Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S., and Massague, J. (1991) *Cell* **67**, 785–795.

42. Barbacid, M. (1995) *Curr. Opin. Cell Biol.* **7**, 148–155.

43. Yayon, A., Klagsbrun, M., Esco, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell* **64**, 841–848.

44. Klagsbrun, M., and Baird, A. (1991) *Cell* **67**, 229–231.

45. Claffey, K. P., Senger, D. R., and Spiegelman, B. M. (1995) *Biochim. Biophys. Acta* **1246**, 1–9.

46. Kendall, R. L., and Thomas, K. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10705–10709.

47. Aiello, L. P., Pierce, E. A., Foley, E. D., Takagi, H., Chen, H., Riddle, L., Ferrara, N., King, G. L., and Smith, L. E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10457–10461.

48. Strawn, L. M., McMahon, G., App, H., Schreck, R., Kuchler, W. R., Longhi, M. P., Hui, T. H., Tang, C., Levitzki, A., Gazit, A., Chen, I., Keri, G., Orfi, L., Risau, W., Flamme, I., Ulrich, A., Hirth, K. P., and Shawver, L. K. (1996) *Cancer Res.* **56**, 3540–3545.

49. Sage, E. H., Bassuk, J. A., Yost, J. C., Folkman, M. J., and Lane, T. F. (1995) *J. Cell. Biochem.* **57**, 127–140.

50. Clapp, C., Martial, J. A., Gezman, R. C., Rentier-Delure, F., and Weiner, R. I. (1993) *Endocrinology* **133**, 1292–1299.

51. Sakamoto, N., Iwahana, M., Tanaka, N. G., and Osada, Y. (1991) *Cancer Res.* **51**, 903–906.

52. Homandberg, G. A., Williams, J. E., Grant, D., Schumacher, B., and Einsenstein, R. (1985) *Am. J. Pathol.* **120**, 327–332.

53. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994) *Cell* **78**, 315–328.

54. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) *Cell* **88**, 277–285.