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Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor

(endothelial cells/mitogenic inhibitor/alternative transcription/angiogenesis)

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ABSTRACT **Vascular endothelial cell growth factor, a mitogen selective for vascular endothelial cells *in vitro* that promotes angiogenesis *in vivo*, functions through distinct membrane-spanning tyrosine kinase receptors. The cDNA encoding a soluble truncated form of one such receptor, fms-like tyrosine kinase receptor, has been cloned from a human vascular endothelial cell library. The mRNA coding region distinctive to this cDNA has been confirmed to be present in vascular endothelial cells. Soluble fms-like tyrosine kinase receptor mRNA, generated by alternative splicing of the same pre-mRNA used to produce the full-length membrane-spanning receptor, encodes the six N-terminal immunoglobulin-like extracellular ligand-binding domains but does not encode the last such domain, transmembrane-spanning region, and intracellular tyrosine kinase domains. The recombinant soluble human receptor binds vascular endothelial cell growth factor with high affinity and inhibits its mitogenic activity for vascular endothelial cells; thus this soluble receptor could act as an efficient specific antagonist of vascular endothelial cell growth factor *in vivo*.**

Angiogenesis, the formation of new blood vessels, is critical for embryonic development, subsequent growth, and tissue repair. Neoangiogenesis is associated with multiple diseases such as rheumatoid arthritis, psoriasis, diabetic retinopathy, and cancer (1). Vascular endothelial cell growth factor (VEGF)* is a particularly selective mitogen for vascular endothelial cells, promotes angiogenesis (2–5) and, under some circumstances, induces vascular permeability (6). This growth factor has been isolated from several tumor cell lines and implicated as a tumor angiogenesis factor in some human gliomas (7). VEGF expression has also been found to be induced by hypoxia (8), so it could be elevated in a wide variety of hypoxic solid tumors and function as a general tumor angiogenesis factor. Inhibition of VEGF function by anti-VEGF monoclonal antibodies recently has been shown to inhibit tumor growth in immune-deficient animals (9).

VEGF is a homodimeric glycoprotein of two 23-kDa subunits exhibiting sequence homology with platelet-derived growth factor A and B chains (5, 6, 10, 11) and placenta growth factor (12). The homologous tyrosine kinase receptors fms-like tyrosine kinase receptor (FLT) and kinase insert domain-containing receptor (KDR) function as high-affinity VEGF receptors (13, 14). KDR (15) and, presumably, FLT are selectively expressed by vascular endothelial cells. Both FLT and KDR are membrane-spanning receptors that each contain seven immunoglobulin-like domains in the extracellular ligand-binding region and cytoplasmic tyrosine kinase domains. We report the identification of a vascular endothelial cell-derived cDNA sequence encoding a secreted soluble form of the VEGF FLT receptor. Recombinant human soluble FLT (sFLT), purified on the basis of its avid binding to immobilized heparin, specifically binds VEGF with high affinity and inhibits its mitogenic activity for vascular endothelial cells in culture. Therefore, this endogenously encoded soluble receptor functions as a specific high-affinity antagonist of VEGF function.

EXPERIMENTAL PROCEDURES

sFLT Cloning. A 587-bp DNA probe for *flt* (16) was obtained by the PCR amplification of a human umbilical vein endothelial cell (HUVEC) λgt10 cDNA phage library (Clontech) using the primers flt-1 (nt 1808–1827; 5′-GCACCTTGGTTGTGGCTGAC-3′) and flt-2 (nt 2377–2394; 5′-TGGAATTCGTGCTGCTTCCTGGTCC-3′). The resulting DNA fragment was cloned into pGEM3Z as a *Xba* I–*Eco*RI fragment. The probe was prepared by the Megaprime system (Amersham) at a specific activity of 1×10^7 cpm/ng. The HUVEC cDNA library was plated, and 1×10^6 plaques were screened by hybridization as described (17). Inserts were cloned into pGEM3Z (Promega) for DNA sequence analysis. The 5′ end of the *flt* coding sequence was also cloned from this cDNA library by PCR using the 5′ primer flt-6 (nt 240–258; 5′-GGAATTCCGCGCTCACCATGGTCAGC-3′) and the 3′ primer flt-7 (nt 711–728; 5′-TTTGAATTCACCCGGCAGGGAATGACG-3′). The PCR fragment produced with this set of primers was cloned into the unique *Sac* I site of the sFLT λ cDNA clone as an *Eco*RI–*Sac* I fragment to generate the full-length coding sequence of sFLT. Presence of the entire contiguous 2.3-kb coding sequence of sFLT mRNA in HUVEC was confirmed by using reverse transcriptase (RT)-PCR. Poly(A)$^+$-selected RNA was isolated from confluent HUVEC using the PolyATtract system 1000 kit (Promega), and 100 ng was used to generate single-strand cDNA with the SuperScript preamplification system (GIBCO/BRL). RT-PCR was done as follows: 50 ng of the primers flt-6 and sflt-3 (nt 2537–2554; 5′-CAACAAACACAGAGAAGG-3′) was added; the mixture was incubated first at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 4 min.

Splice-Site Analysis. The site of sequence divergence between membrane-spanning FLT and sFLT was analyzed by RT-PCR of HUVEC mRNA, as described above, except that the 72°C extension time was shortened to 2 min. The primers flt-1, flt-2, sflt-3, sflt-4 (nt 2223–2240; 5′-GCACTGCAACAAAAAGGC-3′), and flt-5 (nt 2162–2180; 5′-CCAGGAATGTATACACAGG-3′), targeted toward FLT and sFLT se-

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Abbreviations: VEGF, vascular endothelial cell growth factor; FLT, fms-like tyrosine kinase receptor; sFLT, soluble FLT; KDR, kinase insert domain-containing receptor; HUVEC, human umbilical vein endothelial cell(s); Sf9, *Spodoptera frugiperda* clone 9; FGF, fibroblast growth factor; IL, interleukin; RT, reverse transcriptase.

*The C-terminally truncated sequence of human soluble fms-like tyrosine kinase receptor reported in this paper has been deposited in the GenBank data base (accession no. U01134).

quences on either side of the splice site, were used. The corresponding region of human genomic DNA (300 ng) was also analyzed with this PCR amplification protocol and some of these same primers. The identity of all PCR products spanning the FLT/sFLT splice site was confirmed by either restriction-site analysis or direct DNA sequencing.

Expression. The full-length sequence encoding sFLT was cloned as an *Eco*RI–*Bam*HI fragment into pGEM3Z. The *Eco*RI site was then modified to a *Bam*HI site and cloned into pBlueBac III (Invitrogen) downstream from the polyhedrin promoter. This plasmid was transfected into *Spodoptera frugiperda* clone 9 (Sf9) insect cells using a liposome transfection system (Invitrogen). Virus was plaque-purified in soft agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside at 150 μg per ml, and recombinant plaques were identified by blue color. High-titer viral stocks were prepared as instructed in the Invitrogen baculovirus manual. Protein expression was accomplished by infecting Sf9 cells at a density of 2×10^6 cells per ml with the high-titer viral stock at a multiplicity of infection of 5–10. Cells were changed to serum-free SF900II medium (GIBCO/BRL) 24 hr after infection, incubated for an additional 48 hr, and removed by centrifugation; the conditioned medium was then collected.

Protein Purification. The sFLT-containing Sf9-conditioned culture medium (50 ml) was loaded onto a 0.7 × 5 cm heparin-Sepharose CL-6B column (Pharmacia) equilibrated in 10 mM sodium phosphate buffer, pH 6.2/0.15 M NaCl. The column was washed with the same buffer containing 0.6 M NaCl, and sFLT was eluted as a single peak with 1.0 M NaCl in phosphate buffer. sFLT protein was analyzed by SDS/12.5% PAGE and visualized by staining with Coomassie blue R250.

Iodination of VEGF. Purified recombinant human VEGF was radiolabeled with $Na^{125}I$ (Amersham) by the chloramine-T method (18) to a specific activity of $0.5–1 \times 10^6$ cpm/ng. Labeled VEGF was separated from free ^{125}I by gel filtration on a 0.7 × 15 cm Sephadex G-25 column equilibrated in phosphate-buffered saline containing gelatin at 1 mg/ml.

Covalent Crosslinking of VEGF-sFLT Complexes. Human ^{125}I-labeled VEGF (0.2 ng) was incubated for 3 hr at 23°C in binding buffer [Dulbecco's modified Eagle's medium (DMEM)/25 mM Hepes, pH 7.5/0.3% gelatin] with 10 ng of purified sFLT. Complexes were covalently crosslinked with 1 mM bis(sulfosuccinimidyl)suberate (Pierce) for 15 min at 23°C, the reaction was terminated with 3 μl of 0.1 M Tris·HCl, pH 7.4, the products were separated by SDS/7.5% PAGE, and labeled bands were visualized with a phosphoImager (Molecular Dynamics).

Saturation Binding. Binding of VEGF to sFLT was done essentially as described by Duan *et al.* (19). Purified sFLT (≈200 ng) was diluted to 10 ml in 25 mM Tris, pH 7.4/100 mM NaCl/20 mM NH_4HCO_3. Aliquots (100 μl) were absorbed to the surface of 96-well plates (Dynatech) for 18 hr at 4°C. The plates were then washed twice with blocking buffer (DMEM/25 mM Hepes, pH 7.5/0.5% gelatin), nonspecific sites were blocked in the same buffer for 2 hr at 23°C, and the plates were washed twice in DMEM/Hepes/gelatin binding buffer used in the crosslinking experiment. Increased amounts of ^{125}I-labeled VEGF were added to each well and incubated for 3 hr at 23°C. The wells were washed three times with 100 μl of binding buffer, the bound protein was solubilized with 100 μl of 1% SDS/0.5% bovine serum albumin per well, and radioactivity was counted in a γ counter.

sFLT Inhibition of VEGF Activity. HUVEC (Clonetics, San Diego) were plated in DMEM/10% fetal calf serum (GIBCO/BRL) on gelatin-coated 96-well plates at a density of 4000 cells per well. After 16 hr the appropriate wells were pretreated for 15 min with various amounts of sFLT (1.4–50 ng/ml) followed by addition of VEGF at 10 ng/ml. After an additional 24 hr [*methyl*-^3H]thymidine at 0.64 μCi per well (20 Ci/mmol, Amersham; 1 Ci = 37 GBq) was added; the cells were incubated for 72 hr at 37°C, washed, and solubilized with 100 μl of lysis buffer (0.2% Na_2CO_3/0.1 M NaOH); and [^3H]DNA was quantified by scintillation counting.

RESULTS

Cloning and Structure. We have isolated and characterized four independent cDNA clones of the FLT VEGF receptor from a human vascular endothelial-cell cDNA library identified by hybridization with a DNA probe derived from the extracellular domains of this receptor (16). On the basis of sequence analysis, two of these clones appear to be derived from the previously identified membrane-spanning receptor, whereas the other two clones encode a C-terminally truncated form of the FLT VEGF receptor. The two truncated FLT clones are also independent of each other because they terminate at different 5′ and 3′ sites. The shorter 1.8-kb clone begins at bp 803, whereas the longer 2.6-kb clone starts at bp 597 of the known *flt* coding sequence. The longer clone spans the coding sequence from the second immunoglobulin-like domain to the 3′ noncoding region. The cDNA coding sequences of both the truncated *flt* clones are identical to full-length *flt* up to bp 2218, after which they encode a distinctive hydrophilic C-terminal 31-aa residue sequence, containing two cysteine residues but not resembling an immunoglobulin-like domain, before the open reading frame is terminated by TAA (Fig. 1*A*).

The presence of HUVEC-derived mRNA containing the entire 2.3-kb coding sequence of sFLT, including the secretory leader sequence and first immunoglobulin-like domain, was established by RT-PCR and confirmed by restriction

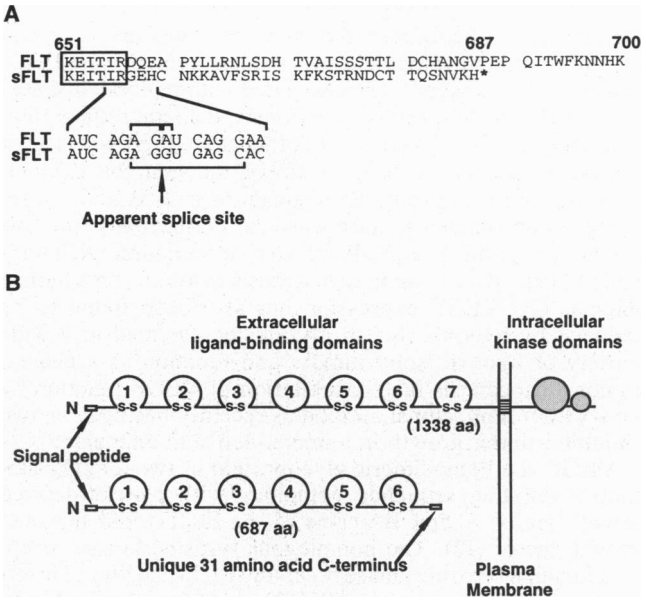

FIG. 1. Deduced protein sequence of human sFLT. (*A*) Alignment of aa 651–700 of membrane-spanning FLT and C-terminal aa 651–687 of sFLT. Sequences N terminal to aa 651 are identical, as are those in box. *, Translational stop site. mRNA sequences spanning the site of divergence are shown below the corresponding protein sequences. The characteristic donor AG and acceptor G/A sequences flanking the apparent splice site of FLT and the full donor splice-site sequence of sFLT are bracketed. (*B*) Domain structures of membrane-spanning FLT and sFLT are schematically illustrated. Immunoglobulin-like domains in the extracellular ligand-binding regions are numbered from the N termini with the characteristic immunoglobulin-fold disulfide-bonded cysteine-residue sulfur atoms (S) marked.

mapping of the PCR product. Therefore, the entire 687-aa-truncated receptor is composed of the secretory leader sequence and the N-terminal six extracellular immunoglobulin-like domains present in the previously described membrane-spanning receptor fused to the specific C-terminal polypeptide sequence. The receptor is missing the membrane-proximal seventh immunoglobulin-like domain, the transmembrane-spanning sequence, and the kinase domains (Fig. 1*B*) and, thus, is a soluble form of FLT.

Alternative Splicing. The different 3′ sequences encoding membrane-spanning and sFLT arise by alternative mRNA splicing. The base pairs flanking the apparent splice site (Fig. 1*A*) incorporate the characteristic splice-site donor (AG) and acceptor (G>A) nucleotides (20). PCR analysis confirmed that the soluble receptor is generated by an alternatively spliced message. RT-PCR reactions using HUVEC poly(A)$^+$-selected RNA as a template were done with a primer derived from the extracellular domain of FLT (flt-1) common to both forms of the receptor and primers specific for either the membrane-spanning form (flt-2) or the soluble form (sflt-3) of FLT (Fig. 2*A*). The expected 587-bp band (Fig. 2*B*, lane 1) was produced using the primers for the membrane-spanning form of FLT. The PCR reaction done with primers that flank the putative splice site of the soluble receptor generates the predicted 747-bp fragment (lane 2), and primers specific for the soluble receptor (sflt-3 and sflt-4) produce the expected 332-bp band (lane 3). These PCR products were not detected in control reactions without RT (data not shown), eliminating the possibility of contamination by genomic DNA.

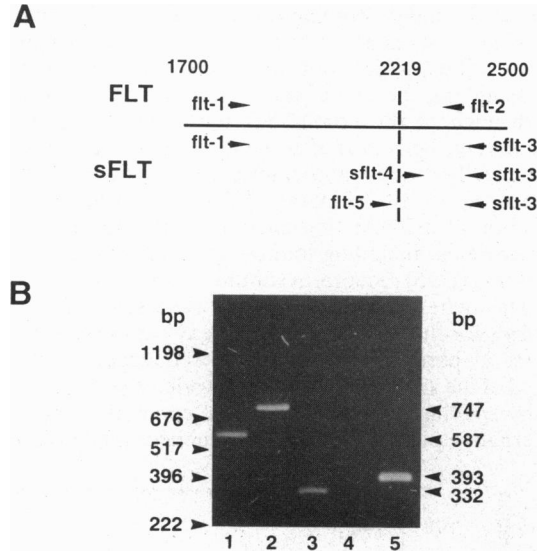

FIG. 2. Alternative splicing of FLT mRNA. (*A*) Coding region from nt 1700–2500 spanning the splice site preceding nt 2219. Relative locations of PCR primers used to amplify sequences encoding membrane-spanning FLT and sFLT are labeled. Nucleotide sequences at left of vertical dashed line encode amino acid sequences common to both membrane-spanning FLT and sFLT, whereas those at right of this dashed line are specific to either one or the other sequence. Primers that hybridize with the coding regions either common to FLT and sFLT or unique to FLT are denoted flt, whereas those that hybridize to nucleotide sequences unique to sFLT are designated sflt. (*B*) With these primers, RT-PCR products of the expected size were generated, spanning the splice site encoding membrane-spanning FLT (flt-1/flt-2, 587 bp, lane 1) and sFLT (flt-1/flt-3, 747 bp, lane 2) along with the sFLT primers both 3′ of the splice site (flt-4/flt-3, 332 bp, lane 3). PCR products of human genomic DNA designed to amplify the sequence around the putative splice site of membrane-spanning FLT (flt-5/flt-2, 233 bp, lane 4) and sFLT (flt-5/sflt-3, 393 bp, lane 5) are shown. Positions of DNA fragment standards and PCR product sizes in bp are labeled at left and right, respectively.

PCR analysis of RNase-treated human genomic DNA using flt primers is also shown in Fig. 2*B* (lanes 4 and 5). The reaction with flt-5, a primer common to both receptor forms located 38 bp 5′ to the putative splice site, and flt-2, a primer specific for the membrane-spanning receptor, does not produce the predicted 233-bp fragment (lane 4), inferring the existence of an intervening intron. In contrast, the reaction with flt-5 and the sFLT-specific primer sflt-3 generates the expected 395-bp fragment (lane 5), suggesting that sFLT mRNA is generated by direct read-through "splice skipping" at this site. Therefore, either membrane-spanning FLT or sFLT mRNAs are generated, depending on whether either intron excision or read-through occurs at this position.

Expression and Purification. To analyze the ligand-binding and biological properties of this truncated form of the receptor, the sFLT protein was generated by using a baculovirus expression system. Recombinant human sFLT present in conditioned medium of Sf9 insect cells infected with baculovirus encoding sFLT binds to heparin-Sepharose and is step-eluted from 0.6 to 1.0 M NaCl. The eluted protein migrates on SDS/PAGE as a single major band of 85–90 kDa (Fig. 3). The polypeptide mass of mature sFLT, generated by cleavage of the secretory leader sequence, is deduced from the cDNA sequence to be 75 kDa, which is presumably increased by carbohydrate attachment to some or all of the 12 N-linked glycosylation-site consensus sequences. The somewhat broadened band width of this putative glycoprotein could be attributable to the heterogeneity of its constituent oligosaccharides. Amino acid sequence analysis (data not shown) reveals a single N terminus starting at Ser-27 of the full-length translation product consistent with the signal-peptide-cleavage site predicted by the method of von Heijne (21).

VEGF Binding and Inhibition of Mitogenic Activity. Initially, complex formation between purified recombinant human ^{125}I-labeled VEGF and sFLT in unfractionated culture medium from the baculovirus-infected Sf9 insect cells was shown by the appearance of large radiolabeled complexes that eluted at the void volume of a Sephacryl S200 gel filtration column (data not shown). To determine the masses of these complexes, ^{125}I-labeled VEGF and purified sFLT were covalently crosslinked with bis(sulfosuccinimidyl)-suberate and characterized by SDS/PAGE and autoradiography (Fig. 4, lane 1). A broad band ranging from 115 to 145 kDa and a narrower band of 220 kDa are seen. The broad band is presumably composed of complexes containing one molecule of sFLT crosslinked to single 23-kDa VEGF subunits and 46-kDa homodimers. The mass of the 220-kDa band corresponds to a VEGF dimer linked to two soluble receptor molecules. This higher mass complex indicates that each subunit of dimeric VEGF probably can bind one molecule of sFLT, perhaps promoting soluble receptor dimerization. These bands are eliminated by excess unlabeled VEGF (lane

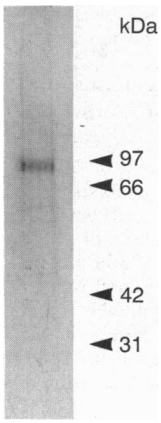

FIG. 3. Purity of recombinant human sFLT. Recombinant human sFLT (0.4 μg), eluted with 1 M NaCl as a single peak from a heparin-Sepharose column, was analyzed by SDS/12.5% PAGE and visualized by staining with Coomassie blue R250. Positions of standards are marked in kDa.

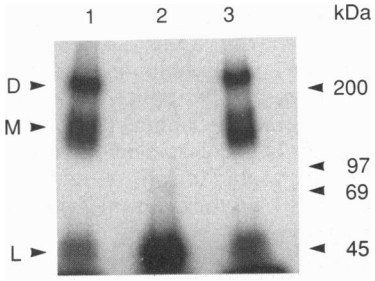

FIG. 4. Covalent crosslinking of VEGF–sFLT complexes. Human ^{125}I-labeled VEGF (0.2 ng) was incubated for 3 hr at 23°C with 10 ng of purified sFLT (lane 1) plus 200 ng of either unlabeled VEGF (lane 2) or unlabeled basic FGF (lane 3). Complexes were covalently crosslinked, and the products were separated by SDS/7.5% PAGE. Arrowheads mark positions of complexes with masses corresponding to VEGF bound to two (dimer, D) and one (monomer, M) molecule of sFLT and free homodimeric ^{125}I-labeled VEGF ligand (L). Positions of standards in kDa are identified.

2) but not by human basic fibroblast growth factor (FGF, lane 3), demonstrating the specificity of complex formation.

The affinity of VEGF for sFLT was evaluated with an assay in which the purified soluble receptor was adsorbed to the surface of 96-well plates and incubated with increased amounts of ^{125}I-labeled VEGF (Fig. 5*A*). Scatchard analysis (22) of receptor affinity reveals a high-affinity binding site with a K_d of 20 pM (Fig. 5*B*), which is consistent with the high-affinity human VEGF-binding site reported on human vascular endothelial cells (23). Because the soluble form of the receptor is missing the immunoglobulin-like domain adjacent to the transmembrane-spanning region, it is not required for ligand binding. In culture, the soluble receptor competes with HUVEC receptors for binding of ^{125}I-labeled VEGF (data not shown). Furthermore, sFLT eliminates

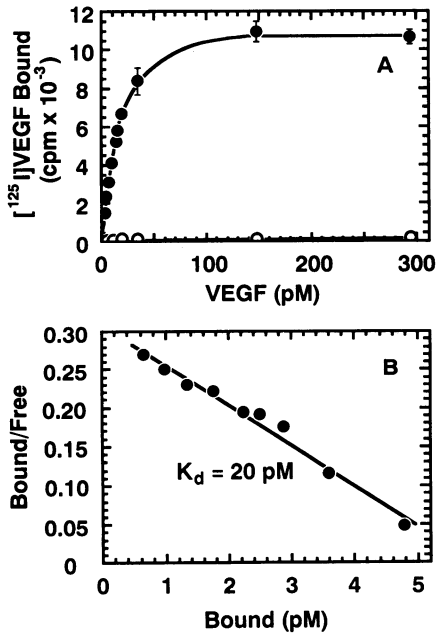

FIG. 5. Saturation binding and Scatchard analysis of VEGF binding to sFLT. (*A*) Purified sFLT (≈2 ng per well) was absorbed to the surface of 96-well plates. Increased amounts of ^{125}I-labeled VEGF were added to each well and incubated for 3 hr at 23°C. The wells were rinsed, bound protein was solubilized, and radioactivity was counted (●). Nonspecific binding was determined by incubation of ^{125}I-labeled VEGF in the presence of 100-fold excess of unlabeled VEGF (○). All data points are the average of quadruplicate determinations ±1 SD. (*B*) Scatchard analysis of these saturation binding data reveals a single high-affinity binding site.

VEGF-induced mitogenesis of HUVEC in a dose–response manner (Fig. 6) but, consistent with the covalent crosslinking results, has no inhibitory effect on basic FGF-stimulated [*methyl*-^{3}H]thymidine incorporation into DNA (data not shown) and, thus, specifically inhibits VEGF function.

DISCUSSION

Alternative splicing of FLT pre-mRNA generates two distinct products encoding the full-length membrane-spanning receptor that mediates VEGF mitogenic activity and a truncated soluble form that inhibits this same activity. The cDNA encoding sFLT was found in two of four independent FLT clones, so the corresponding mRNA is neither an artifact of cloning nor likely to be rare compared with the amount of mRNA encoding the membrane-spanning receptor. By RT-PCR analysis, the entire sFLT coding sequence and the expected sequences characteristic of the membrane-spanning form are both present in cultured confluent human vascular endothelial cells.

The soluble and membrane-spanning FLT mRNAs both contain the consensus AG donor splice-site nucleotides immediately 5′ of the site of sequence divergence. These identical donor splice-site sequences are followed by guanine and adenine, respectively, which are the most and second-most frequent nucleotides, respectively, on the acceptor side of spliced sequences (20). The sequence 3′ of the sFLT mRNA splice site (GUGAGC), however, resembles an unprocessed donor site (GURAGU). The lack of intron excision at the site between the region common to FLT and sFLT encoding the ligand-binding domains and the specific sFLT C-terminal sequence has been confirmed by genomic PCR analysis. Thus, sFLT mRNA arises from the absence of processing this potential splice site, resulting in a direct read-through of the contiguous intron sequence that can, alternatively, be removed to generate mRNA encoding the mitogenically functional membrane-spanning FLT receptor.

Similar splice-site skipping of 3′ introns accounts for the generation of mRNAs that encode soluble forms of several other proteins, including immunoglobulin heavy chains, interleukin-5 (IL-5) receptor α subunits (24) and FGF receptors (25). The switch between the membrane-spanning and soluble forms for the immunoglobulin heavy chains seems to be, at least in part, regulated by the sequence, position and context of the secretion-specific polyadenylylation site rather than by splicing efficiency (26). The competitive processing of alternative polyadenylylation sequences might represent a

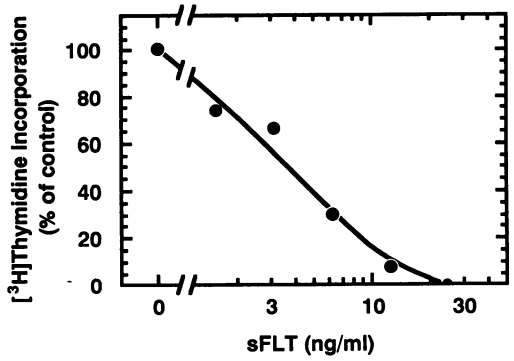

FIG. 6. sFLT inhibition of VEGF-induced mitogenesis of vascular endothelial cells. HUVEC plated in DMEM/10% fetal calf serum were pretreated with increased amounts of sFLT followed by addition of VEGF at 10 ng/ml. After 24 hr [*methyl*-^{3}H]thymidine was added, the cells were incubated for an additional 72 hr, and [^{3}H]DNA was quantified by scintillation counting. Control wells devoid of sFLT were treated with VEGF at 10 ng/ml. Results are the average of triplicate determinations at each sFLT concentration.

general mechanism for generating soluble forms of membrane-bound receptors, regulating their expression and consequently modulating the biological responses mediated by these genes. If so, then an AATAAA polyadenylylation consensus signal sequence identified in the 3′ noncoding region near the end of the 2.6-kb sFLT cDNA clone could, in part, regulate the FLT/sFLT switch.

Production of alternatively transcribed soluble receptors appears to represent a biological mechanism for selectively and efficiently inhibiting cellular responsiveness to specific mitogenic and differentiation factors. Analogous native soluble forms of membrane-bound receptors have been described not only for IL-5 and FGF but also for IL-4 (27), IL-7 (28), granulocyte/macrophage colony-stimulating factor (29), and epidermal growth factor (30). These soluble receptors bind their cognate ligands and thus could function as specific high-affinity antagonists *in vitro*. Furthermore, soluble homologues of several receptors, such as those for IL-1 (31), interferon γ (32), and the tumor necrosis factor (33), are expressed by viruses and increase virulence apparently by depressing the host cytokine response to the invading pathogens (31, 33), showing the function of such antagonists *in vivo*.

Purified recombinant sFLT not only binds to VEGF with high affinity comparable to the membrane-spanning receptor but also efficiently inhibits VEGF-induced mitogenesis of vascular endothelial cells in culture. Although we have not yet rigorously identified the endogenous sFLT protein, a naturally occurring soluble VEGF-binding protein of the expected sFLT mass has been found in conditioned medium of these same human vascular endothelial cells by VEGF affinity labeling (unpublished results). Like recombinant sFLT, the endogenous VEGF-binding protein adheres tightly to immobilized heparin, thus perhaps facilitating its partitioning onto cell surface and basement-membrane heparan proteoglycans. This protein could be either endogenous sFLT or, perhaps, a functionally similar inhibitory ligand-binding fragment generated by proteolysis of membrane-spanning VEGF receptors.

The inhibitory activity of sFLT could reflect either simply the sequestration of VEGF or the formation of heterodimers of membrane-spanning and soluble FLT. If, as is proposed for a variety of tyrosine kinase receptors including those of the homologous platelet-derived growth factor (34), ligand-mediated receptor dimerization is required to assemble functional intracellular receptor tyrosine kinase dimers, then FLT/sFLT heterodimers would be nonfunctional. In this case, sFLT would not simply compete with membrane-spanning FLT but actually have a dominate negative function. Also in analogy with the platelet-derived growth factor α- and β-subunit receptor systems (35), different VEGF receptors, such as FLT and KDR, might form functional heterodimers. If so, then sFLT could also inhibit the KDR-mediated activity of VEGF. Inhibition of VEGF function *in vivo* by sFLT could (*i*) promote quiescence in confluent endothelial-cell monolayers, (*ii*) act as a feedback mechanism to terminate angiogenesis and vascular permeability, and (*iii*) prevent blood vessel growth into normally avascular tissues, such as cornea and hyaline cartilage. To the extent that disease-related neovascularization, such as tumor angiogenesis, is mediated by VEGF, exogenous sFLT could be a therapeutically useful agent for specifically and efficiently inhibiting pathological blood vessel growth and, perhaps, blood-borne tumor metastasis (9, 36–38).

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1. Folkman, J. & Klagsbrun, M. (1987) *Science* **235**, 442–447.
2. Ferrara, N., Houck, K., Jakeman, L. & Leung, D. W. (1992) *Endocr. Rev.* **13**, 18–32.
3. Connolly, D. T. (1991) *J. Cell. Biochem.* **47**, 219–223.
4. Rosenthal, R. A., Megyesi, J. F., Henzel, W. J., Ferrara, N. & Folkman, J. (1990) *Growth Factors* **4**, 53–59.
5. Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V. & Ferrara, N. (1989) *Science* **246**, 1306–1309.
6. Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J. & Connolly, D. T. (1989) *Science* **246**, 1309–1312.
7. Plate, K. H., Breier, G., Weich, H. A. & Risau, W. (1992) *Nature (London)* **359**, 845–848.
8. Shweiki, D., Itin, A., Soffer, D. & Keshet, E. (1992) *Nature (London)* **359**, 843–845.
9. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. & Ferrara, N. (1993) *Nature (London)* **362**, 841–844.
10. Conn, G., Bayne, M. L., Soderman, D. D., Kwok, P. W., Sullivan, K. A., Palisi, T. M., Hope, D. A. & Thomas, K. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2628–2632.
11. Tischer, E., Gospodarowicz, D., Mitchell, R., Silva, M., Schilling, J., Lau, K., Crisp, T., Fiddes, J. C. & Abraham, J. A. (1989) *Biochem. Biophys. Res. Commun.* **165**, 1198–1206.
12. Maglione, D., Guerriero, V., Viglietto, G., Delli Bovi, P. & Persico, M. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9267–9271.
13. de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N. & Williams, L. T. (1992) *Science* **255**, 989–991.
14. Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D. & Bohlen, P. (1992) *Biochem. Biophys. Res. Commun.* **187**, 1579–1586.
15. Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L. & Shows, T. B. (1991) *Oncogene* **6**, 1677–1683.
16. Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H. & Sato, M. (1990) *Oncogene* **5**, 519–524.
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
18. Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496.
19. Duan, D.-S. R., Pazin, M. J., Fretto, L. J. & Williams, L. T. (1991) *J. Biol. Chem.* **266**, 413–418.
20. Mount, S. M. (1982) *Nucleic Acids Res.* **10**, 459–472.
21. von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683–4690.
22. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672.
23. Myoken, Y., Kayada, Y., Okamoto, T., Kan, M., Sato, G. H. & Sata, J. D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5819–5823.
24. Tavernier, J., Tuypens, T., Plaetinck, G., Verhee, A., Fiers, W. & Devos, R. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7041–7045.
25. Johnson, D. E., Lu, J., Chen, H., Werner, S. & Williams, L. T. (1991) *Mol. Cell. Biol.* **11**, 4627–4634.
26. Lassman, C. R. & Milcarek, C. (1992) *J. Immunol.* **148**, 2578–2585.
27. Mosley, B., Beckmann, M. P., March, C. J., Idzerda, R. L., Gimpel, S. D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J. M., Smith, C., Gallis, B., Sims, J. E., Urdal, D., Widmer, M. B., Cosman, D. & Park, L. S. (1989) *Cell* **59**, 335–348.
28. Goodwin, R. G., Friend, D., Ziegler, S. F., Jerzy, R., Falk, B. A., Gimpel, S., Cosman, D., Dower, S. K., March, C. J., Namen, A. E. & Park, L. S. (1990) *Cell* **60**, 941–951.
29. Raines, M. A., Liu, L., Quan, S. G., Joe, V., DiPersio, J. F. & Golde, D. W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8203–8207.
30. Petch, L. A., Harris, J., Raymond, V. W., Blasband, A., Lee, D. C. & Earp, H. S. (1990) *Mol. Cell. Biol.* **10**, 2973–2982.
31. Spriggs, M. K., Hruby, D. E., Maliszewski, C. R., Pickup, D. J., Sims, J. E., Buller, R. M. L. & VanSlyke, J. (1992) *Cell* **71**, 145–152.
32. Upton, C., Mossman, K. & McFadden, G. (1992) *Science* **258**, 1369–1372.
33. Smith, C. A., Davis, T., Wignall, J. M., Din, W. S., Farrah, T., Upton, C., McFadden, G. & Goodwin, R. G. (1991) *Biochem. Biophys. Res. Commun.* **176**, 335–342.
34. Kelley, J. D., Haldeman, B. A., Grant, F. J., Murray, M. J., Seifert, R. A., Bowen-Pope, D. F., Cooper, J. A. & Kazlauskas, A. (1991) *J. Biol. Chem.* **266**, 8987–8992.
35. Seifert, R. A., Hart, C. E., Phillips, P. E., Forstrom, J. W., Ross, R., Murray, M. & Bowen-Pope, D. F. (1989) *J. Biol. Chem.* **264**, 8771–8778.
36. Horak, E. R., Leek, R., Klenk, N., LeJeune, S., Smith, K., Stuart, N., Greenall, M., Stepniewska, K. & Harris, A. L. (1992) *Lancet* **340**, 1120–1124.
37. Teicher, B. A., Sotomayor, E. A. & Huang, Z. D. (1992) *Cancer Res.* **52**, 6702–6704.
38. Weidner, N., Semple, J. P., Welch, W. R. & Folkman, J. (1991) *N. Engl. J. Med.* **324**, 1–8.