A Vascular Endothelial Growth Factor High Affinity Receptor 1-specific Peptide with Antiangiogenic Activity Identified Using a Phage Display Peptide Library*

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Vascular endothelial growth factor (VEGF) is known to play a predominant role in tumor angiogenesis and metastasis formation that is mediated by its interactions with two tyrosine kinase receptors, VEGFRI (Flt-1) and VEGFRII (KDR). Inhibition of VEGF-dependent events in tumor tissues is known to enhance apoptosis and to suppress tumor growth. A novel peptide, SP5.2, which selectively binds Flt-1 and inhibits a broad range of VEGF-mediated events, was identified using a phage-display library screening. The fluorescein-labeled SP5.2 specifically bound to VEGF-stimulated primary human cerebral endothelial cells (HCECs), whereas non-stimulated HCECs, as well as human neuroblastoma cells (ShyY) did not show any interaction with the peptide. SP5.2 prevented proliferation of cultured primary human umbilical vein endothelial cells induced by recombinant human VEGF$_{165}$ with an IC$_{50}$ of 5 μM. SP5.2 was also shown to antagonize VEGF- and PLGF-induced, but not basic fibroblast growth factor-induced proliferation of HCECs. In contrast to "scrambled" peptide, SP5.2 was also found to selectively inhibit VEGF-stimulated migration of HCECs. The *in vitro* analysis of antiangiogenic activity of SP5.2 using a capillary-like tube formation assay showed that VEGF-induced angiogenesis of HCECs grown on Matrigel™ was completely inhibited in the presence of 10 μM SP5.2. Further studies demonstrated that SP5.2 prevented VEGF-induced permeability increase in HCECs monolayers. To explore whether SP5.2 can be used as a targeting agent, chemical and recombinant conjugates of SP5.2 with reporter proteins (peroxidase and β-galactosidase) were produced. The resulting products showed significant increases (200-fold for SP5.2-β-gal and 400-fold for SP5.2-peroxidase) in binding affinity to recombinant Flt-1 compared with the original synthetic SP5.2, suggesting that conjugate with therapeutic activity in nanomolar range could potentially be developed based on SP5.2 structure.

Vascular endothelial growth factor (VEGF)[1] and its receptors are the focus of intense interest because of their role in blood vessel formation (angiogenesis and vasculogenesis) in a variety of physiological and pathophysiological processes, including embryogenesis, development of the fetal cardiovascular system, wound healing, tumor growth, proliferative retinopathies, and chronic inflammatory diseases such as rheumatoid arthritis (1–3). VEGF is unique among the growth factors in being an endothelial cell-specific mitogen that promotes the proliferation and migration of endothelial cells, remodeling of the extracellular matrix, formation of capillary tubules and vascular leakage (4).

VEGF activities are mediated through binding to two high affinity receptors, human kinase domain receptor (KDR) and Fms-like tyrosine kinase receptor (Flt-1) (5), both of which are selectively expressed on endothelial cells during embryogenesis and VEGF-related pathologies (6). Both of these receptors are class III tyrosine kinases (7, 8) that undergo ligand-induced dimerization that triggers signal transduction. Studies in mice have shown that the expression of KDR reaches the highest levels during embryonic vasculogenesis and angiogenesis (6). In contrast, low Flt-1 mRNA levels were found during fetal growth, moderate during organogenesis, and high in newborn mice (9). The third VEGF receptor from the tyrosine kinase family, Flt-4, is largely confined to the lymphatic vasculature and has a role in lymph angiogenesis (10). Flt-4 binds VEGF-C and -D, but not VEGF (11). Neuropilin-1 (NRP-1) was recently identified as a new receptor for VEGF (12). It is expressed in the enocardium, coronary vessels, myocardial capillaries, and epicardial blood vessels of human fetal heart (12). Experiments with knockout mice deficient in Flt-1 or KDR receptor revealed that KDR is essential for the development of endothelial cells, whereas Flt-1 is necessary for the organization of embryonic vasculature (13, 14). The VEGF-Flt-1 receptor system also plays an important role in the simulation of tumor angiogenesis, which makes Flt-1 an interesting target for antiangiogenic drugs (15).

Human Flt-1 receptor is composed of seven extracellular Ig-like domains containing the ligand binding region, a single short membrane-spanning sequence, and an intracellular region containing the tyrosine kinase domain. The amino acid sequences of Flt-1 and KDR show ~45% identity; however, Flt-1 has a higher affinity for VEGF (K_D = 10–20 pM) compared

[1] The abbreviations used are: VEGF, vascular endothelial growth factor; Flt-1, tyrosine kinase receptor VEGFRI; KDR, tyrosine kinase receptor VEGFRII; NRP-1, neuropilin-1; HRP, horseradish peroxidase; HCEC, human cerebral endothelial cell; HUVEC, human umbilical vein endothelial cell; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Flt-MP, Flt-1-coated magnetic particle; ELISA, enzyme-linked immunosorbent assay; cfu, colony-forming unit(s); bFGF, basic fibroblast growth factor; CMV, cytomegalovirus; SPDP, *N*-succinimidy 1,3-(2-pyridylthio)propionate; EC, endothelial cell; AB, antibody; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); PLGF, placenta growth factor.

with KDR (K_D = 75–125 pM). The activation of Flt-1 receptor by VEGF regulates interactions of endothelial cells with each other or the basement membrane on which they reside (16, 17). Alternative splicing of the Flt-1 receptor results in two forms, the full-length membrane-spanning receptor and a soluble form, denoted sFlt-1. Isolated sFlt-1 retains specific high affinity binding for VEGF and fully inhibits VEGF-stimulated endothelial cell mitogenesis by dominant negative mechanism (18). Furthermore, it was suggested that sFlt-1 might form heterodimeric complexes with KDR with potentially negative effect on KDR signal transduction (19).

Because VEGF receptors are implicated in several pathologies, pharmacological interference with the VEGF/VEGF receptor system antagonists is clinically attractive. Humanized neutralizing antibodies that interact with VEGF near the KDR and Flt-1 binding sites (20–22) and systemic evolution of ligands by exponential enrichment (SELEX)-derived RNA molecules (23) that selectively bind to Flt-1 have been shown to block tumor growth dependent on vascularization of adjacent normal tissue (24). Similarly, anti-KDR monoclonal antibodies inhibited VEGF-induced signaling and demonstrated a high antitumor activity (25). Soluble Flt receptor (26), fragments of VEGF, as well as small molecule inhibitors of the VEGF receptors tyrosine kinase activity, such as PTK787/ZK222584 (27) and ZD4190 (28), have been shown to inhibit angiogenesis *in vivo*. Anti-VEGF antisense oligonucleotide has been shown to inhibit VEGF expression, VEGF-induced neovascularization, and tumor implantation and growth (29–31). However, no truly antagonistic compounds that would selectively discriminate between Flt-1 and KDR receptors are available yet.

In the present study, a new peptide motif that inhibits VEGF binding to Flt-1 has been identified using a phage-displayed peptide library. A random 16-mer peptide library displayed on the surface of the filamentous phage M13 was screened against the extracellular domain of Flt-1. This screening resulted in a peptide (SP5.2) that competed with VEGF for the Flt-1 binding and inhibited a broad range of VEGF-induced events in cultured endothelial cells. Potential use of SP5.2 as a targeting agent was evaluated using both synthetic and genetically constructed conjugates of the peptide and reporter proteins.

EXPERIMENTAL PROCEDURES

Materials—A recombinant Flt-1 receptor chimera consisting of the six N-terminal extracellular domains of human Flt receptor and Fc fragment of human IgG was obtained from R&D Systems (Minneapolis, MN). The goat polyclonal anti-hFlt-1 antibody, rhVEGF$_{165}$, hKDR/Fc chimera, mNRP-1/Fc chimera, hsICAM-1, mFlt-1/Fc chimera, and mFlk-1/Fc chimera were obtained from R&D Systems. Biotin-labeled anti-human IgG (Fc-specific) rabbit polyclonal antibody was purchased from ICN (Costa Mesa, CA). The mouse monoclonal anti-β-galactosidase clone Gal-13 and anti-mouse IgG peroxidase conjugate developed in goat and absorbed with rat serum proteins were obtained from Sigma (Oakville, Ontario, Canada). The anti-histidine HRP conjugate antibody was purchased from Invitrogen (Burlington, Ontario, Canada). Scrambled peptide was synthesized at Supratek (Laval, Quebec, Canada).

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were purchased from BioWhittaker Inc. (Walkersville, MD). The cells were cultured in the endothelial growth medium, Clonetics media EGM & EGM BulletKit™ (BioWhittaker Inc.). The cells were used for the experiments at their 3rd or 4th growth passage.

HCECs were isolated using previously described protocols (32). Purity of HCEC cultures generated by these procedures was routinely assessed by the immunocytochemical staining for Factor VIII-related antigen and the lack of staining for smooth muscle β-actin and was estimated to be >95%. The morphological, phenotypic, biochemical, and functional characteristics of these HCEC cultures have been described in detail previously (32, 33). For endothelial permeability studies, HCECs were used as an *in vitro* blood-brain barrier model described previously (33).

Bacterial Strains and Bacteriophages—K91Kan cells obtained from G. Smith were grown on LB agar supplemented with kanamycin (100 μg/ml). Cells were made competent using the protocol described previously by Smith (34). M15 was purchased from Qiagen (Mississauga, Ontario, Canada) cells made competent following the protocol from Qiagen. pQE16 vector purchased from Qiagen and pCMVb vector from Clontech (Palo Alto, CA).

The phage library containing the 16-amino acid peptide was constructed essentially as previously described, using fUSE5 as the phage vector (35). This linear library consisted of 10^9 independent recombinant phages recovered as tetracycline-resistant colonies. Sequencing of randomly selected clones indicated that the majority of these phages (>90%) contain inserts.

Screening the Phage Display Library with Flt-1-coated Magnetic Particles—The receptor, rhFlt-1 chimera (10 μg in 50 μl of 0.1% BSA/PBS), was immobilized on streptavidin-coated magnetic particles (Roche Applied Science, Laval, Canada) using biotinylated anti-hIgG(Fc) antibody. The Flt-1-coated magnetic particles (Flt-MPs) were blocked with 3% BSA/PBS for 2 h at room temperature. For selection, phage (1×10^{11} cfu) from the linear 16-amino acid random peptide phage display library diluted in 0.1% BSA/PBS were added to Flt-MP and incubated overnight at 4 °C. After extensive washing with 0.1% BSA/PBS, the bound phage were either eluted with a low pH buffer (0.2 M glycine-HCl, pH 2.2) or displaced from Flt-MP with 10 μg/ml rhVEGF$_{165}$ for 1 h at 22 °C. Recovered phages were amplified using competent K91Kan *Escherichia coli* cells and then subjected to four subsequent rounds of selection on Flt-MP. Phage binding was quantified by counting the phage titer in eluted aliquots from Flt-MP as described earlier (34). Phages from selected clones were sequenced at Sheldon Biotechnology center (McGill, Montreal).

ELISA for the Displacement of Phage Binding—The rhFlt-1 receptor chimera were immobilized on Streptavidin-coated microtiter plates (total binding capacity for biotin-labeled AB = 1.5 μg/well, Roche Applied Science). Biotin-labeled anti-human FC antibody (ICN), at a concentration of 10 μg/ml in 0.1% BSA/PBS, were added into each well of the plates. The plates with assay mixtures were incubated at 4 °C for 8 h in a humidified container and then washed four times with 0.1% BSA/PBS. Recombinant human Flt-1/Fc chimera (1 μg/well in 0.1% BSA/PBS, R&D Systems, Minneapolis, MN) were added into each well, and the plates were incubated overnight at 4 °C in a sealed container to allow the receptor to attach to the ligand. Unbound receptor was washed away with 2 ml of 0.1% BSA/PBS. Each well was then filled with 250 μl of blocking buffer (2% nonfat dry milk in PBS) and incubated at room temperature for 2–3 h. As a negative control, three of the wells were blocked with the blocking buffer (2% nonfat milk in PBS). Phage particles (5×10^9 to 1×10^{10} cfu/well) were added to each well and incubated for 2 h at room temperature. In competition experiments, phage suspension was premixed with various concentrations of competing agent (SP5.2 peptide, rhVEGF$_{165}$) and then added to immobilized receptor. Wells were washed with 0.1% BSA/PBS, and the amount of bound phages was detected with peroxidase-conjugated anti-M13 antibody (Amersham Biosciences). After the addition of the substrate, ABTS, and H_2O_2, antibody reaction was analyzed in a microtiter plate reader at 405 nm.

Production of Mutant Variants of SP5.2 Phage—Several mutations in SP5.2 coding oligonucleotide inserts were carried out to identify the amino acids involved in the receptor binding site domain. The single point mutations in V5.2 insert coding sequence were produced as described previously (36). Briefly, a series of SP5.2-coding oligonucleotides, with a particular amino acid coding triplet replaced with GCT (alanine-coding triplet), was synthesized by Invitrogen (Ontario, Canada). The mutant oligonucleotides were cloned into fUSE5 phage vector as described (35). All mutants were purified and verified by DNA sequencing.

Peptide Synthesis—Peptide amides were synthesized manually on solid Rink amide resin (Nova Biochem) using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protocol and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation. The fluorescein residue was introduced into the peptide by standard coupling of N-terminal-deprotected peptide with fluorescein-5-carboxylic acid. Peptide amides were cleaved from the resin with trifluoroacetic acid:water: ethanedithiol:triisopropylsilane (95:5:2.5:2.5, v/v) and were recovered by precipitation with ice-cold diethyl ether. Crude products were purified by high performance liquid chromatography on a Vydac C18 column using a linear gradient of 30% to 70% acetonitrile/water (0.1% trifluoroacetic acid), for 60 min at a 5-ml/minute flow rate. The identity of peptides was verified by electrospray mass spectrometry (PE Sciex API III Biomolecular Mass Analyzer, Applied Biosystems, CA).

Binding Competition for Flt-1 Receptor—Fluorescein-labeled peptide in 0.1% BSA/PBS buffer, pH 8.5, was added at various concentrations (0, 0.1, 1, 10, 50, and 100 μM) into wells of an ELISA plate coated with immobilized Flt-1 receptor. In competition experiments, the various concentrations of phage or VEGF$_{165}$ were mixed and added into wells. After 2-h incubation, microtiter plates were washed 10 times with 0.1% BSA/PBS buffer, pH 8.5, and the bound peptide was measured using a microplate fluorescence reader (FL600, BioTek) (λ_{ex} = 485 nm; λ_{em} = 530 nm).

Flt-1 Receptor Immunochemistry—HCECs were grown on glass coverslips in a 24-well dish for 3 days until sub-confluent. Media was aspirated out; cells were washed two times in PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were subsequently washed three times in PBS, incubated in blocking solution containing 5% goat serum in PBS for 1 h at room temperature, and exposed to the primary antibody (5 μg/ml goat anti-human Flt-1 in blocking solution; R&D Systems Inc) for 1 h at room temperature. After washing three times in blocking solution, cells were exposed to the secondary antibody (1:20 dilution of donkey anti-goat IgG-immunogold in blocking solution; Accurate Chemical and Scientific Corp.) for 30 min at room temperature. Cells were then extensively washed (three times with blocking solution and three times with distilled water) and incubated in silver enhancing solution for 30 min (1:1 dilution of reagents A and B). After washing with distilled water (two times for 5 min), slides were viewed under a microscope.

SP5.2 Binding to Cells—Fluorescein-labeled SP5.2 peptide was added to HCECs cells at different concentrations, (1, 10, and 50 μM) and incubated for 30 min. The neuroblastoma ShyY cell line was used as a negative control. Media were aspirated, and cells were washed in phenol red-free M199 and observed under a fluorescence microscope.

Endothelial Cell Proliferation Assay—HUVECs were placed in 96-well plates (Costar) at 10^4 cells per well in 200 μl of EGM-2 medium (Clonetics) supplemented with 0.5% heat-inactivated fetal bovine serum. Cells were incubated for 24 h at 37 °C in 5% CO$_2$. HCECs were placed in 12-well plates and allowed to grow for 3 days until sub-confluent. Cells were then washed once by PBS and incubated in a serum- and glucose-free DMEM for 24 h to suppress cell growth. Both HCECs and HUVECs were then exposed to various treatments (*i.e.* 10% fetal bovine serum, VEGF$_{165}$ (20 ng/ml), PLGF (100 ng/ml), bFGF (10 ng/ml) in the absence or presence of SP5.2 peptide). Scrambled peptide NGSAIAASSAVTHGMS at the same concentration was used as control. After 48 h of incubation, 1 μCi of [*methyl*-^{3}H]thymidine (20 Ci/mmol; ICN) was added in each well, and plates were incubated for an additional 24 h. The cells were then placed on ice, washed twice with EGM-2 medium containing 10% fetal bovine serum, and fixed for 10 min in ice-cold 10% trichloroacetic acid. After washing with ice-cold water, cells were lysed and DNA was solubilized in 50 μl of 2% SDS. [^{3}H]Thymidine incorporation was determined by scintillation counting.

Endothelial Cell Migration—Migration of calcein-AM labeled (Molecular Probes, Eugene, OR, 10 μM for 20 min at 37 °C) HCECs stimulated by VEGF$_{165}$ were tested in the absence or presence of SP5.2 peptide or scrambled peptide using a ChemoTx #101-5 assembly (Neuro Probe, Inc., Gaithersburg, MD) consisting of a 96-well plate and a polycarbonate filter membrane, as described in a previous study (37). Briefly, the wells of the plates were loaded with buffer containing various chemoattractants or phenol red- and serum-free media conditioned by cancer cells. The framed filter membrane was positioned on top, and 50,000 calcein-AM-labeled HCECs were suspended in 20 μl of matching buffer, or media was applied on the top of each membrane/well. The assembly was incubated for 120 min at 37 °C, and the number of endothelial cells transmigrated into the wells was quantified by measuring the intensity of fluorescence (excitation/emission: 485/530 nm) in a CytoFluor 2350 reader (Millipore, Bedford, MA).

Capillary Tube Formation in Matrigel^{TM}—HCECs were seeded (8 × 10^4 cells) on a semi-solid basement membrane matrix, Matrigel^{TM} (Collaborative Biomedical Products, Bedford, MA)-coated 24-well plates and exposed to serum-free media containing various treatments. Cells were treated with 10 nM VEGF$_{165}$ or glioblastoma cell (U-87MG, ATCC, Rockville, MD)-conditioned media (serum-free Dulbecco's modified Eagle's medium conditioned for 48–72 h) in the absence or presence of SP5.2 or control peptide for 24 h. HCECs were then labeled with a vital dye, calcein-AM (2 μM) for 30 min. Cells were then washed in PBS and capillary-like tube formation, a measure of *in vitro* angiogenesis, was observed using a Zeiss LSM 410 inverted laser scanning microscope (Thornwood, NY) with an argon/krypton ion laser and a Zeiss LD achroplan 5×, 0.6-numerical aperture objective. Confocal apertures for each recorded wavelength were adjusted to a full-width half maximum of 20 μm. The emitted light was collected through a 515- to 540-nm

band-pass filter using an excitation of 488 nm. 8–32 individual optical sections were collected, and the average was calculated. Standard image processing was performed to enhance brightness and contrast using ImageSpace software (Molecular Dynamics Inc., Sunnyvale, CA).

SP5.2-β-gal Fusion Construction—The prokaryotic expression vector, pQE-16, purchased from Qiagen, was used to construct the fusion protein SP5.2-β-gal, containing SP5.2 peptide on the N terminus and His$_6$ on the C terminus of β-galactosidase. For this the vector pCMV-β from Clontech (Palo Alto, CA) was digested with BamH1 (New England Biolabs), the resulting β-galactosidase gene was purified from agarose gel and modified by PCR as so to introduce BglII at the 3′-end for cloning into pQE16 instead of dihydrofolate reductase, which has been removed by digestion of plasmid with BamHI and BglII. The SP5.2 insert, containing the EcoRI site at the 5′-end and the BamHI site at the 3′-end, was prepared by annealing the two single-stranded oligonucleotides synthesized by INRS-IAF (Laval, Quebec, Canada) and inserting them into the pQE16-β-gal vector. pQE16-SP5.2-β-gal was used to transform competent *E. coli* M15 cells. The resulting clones were sequenced to verify the fusion gene.

Fusion Protein Expression and Purification—An overnight culture of a single colony was grown in 400 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin to an optical density of ∼0.6 at 600 nm. The culture was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37 °C, centrifuged, and the resulting pellet was stored at −80 °C. The expression of β-galactosidase and the presence of His tag were verified by Western blot using anti-β-galactosidase (Sigma, Ontario, Canada) and anti-His-HRP antibody (Invitrogen, Ontario, Canada). The pellet was thawed and resuspended in lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0), and lysozyme was added to a concentration of 1 mg/ml. After incubation for 30 min at 4 °C, 400 μl of 50% nickel-nitrilotriacetic acid resin (Qiagen, Ontario, Canada) was added to the supernatant and incubated 1 h shaking at 4 °C. The lysate/nickel-nitrilotriacetic acid mixture was loaded into a column (Qiagen), washed with washing buffer (50 mM NaH$_2$PO$_4$, 10 mM NaCl, 20 mM imidazole, pH 8.0), and the protein SP5.2-β-gal was eluted with the elution buffer (50 mM NaH$_2$PO$_4$, 10 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was washed by PBS to decrease imidazole concentration and concentrated by Centricons (Fisher) to get a final concentration of ∼1 μg/ml.

SP5.2-β-gal Fusion Protein Binding Assay—Streptavidin-coated microtiter plates (total binding capacity for biotin-labeled AB = 1.5 μg/well, Roche Applied Science) were used to immobilize the rhFlt-1 receptor chimera. Biotin-labeled anti-human Fc antibody (ICN) 10 μg/ml in 0.1% BSA/PBS were added into each well of streptavidin-coated plate. The microtiter plates were incubated at 4 °C for 16 h in a humidified container and then washed for four times with 0.1% BSA/PBS. Recombinant human Flt-1/Fc chimera (1 μg/well in 0.1% BSA/PBS, R&D Systems) was added into each well. Plates were incubated at 4 °C 8 h in sealed container to let receptor to immobilize. Unbound receptor was washed away with 2 ml of 0.1% BSA/PBS. Then each well was filled with 350 μl of blocking buffer (2% nonfat dry milk in PBS) and blocked for 16 h at 4 °C. As a negative control, three wells with immobilized anti-Fc antibody were blocked with 2% nonfat milk in PBS. SP5.2-β-gal fusion protein and recombinant β-galactosidase from Sigma (Oakville, Ontario, Canada), used as control, were added to each well and incubated for 2 h at room temperature. Wells were washed with 0.1% BSA/PBS, and the amount of bound protein expressing SP5.2-β-gal was detected with anti-β-gal (Sigma) and IgG anti-mouse HRP conjugate (Sigma). After substrate addition (ABTS containing H$_2$O$_2$), wells were analyzed in microtiter plate reader at 405 nm.

Synthesis of SP5.2-Peroxidase-conjugated Peptide—3 mg of peroxidase (ICN) was dissolved in 1 ml of phosphate buffer (0.1 M Na$_2$HPO$_4$, 0.1 M NaCl, 1 mM EDTA, pH 8.5). *N*-Succinimidy 1,3-(2-pyridylthio)propionate (SPDP; Sigma) was dissolved in dimethylformamide in a proportion of 0.234 mg of SPDP/39 μl of dimethylformamide. The solution of SPDP was added to the solution of peroxidase and stirred 30 min at room temperature. The solution of activated peroxidase was applied to Sephadex G-25 column (Fisher) and eluted with 50 ml of phosphate buffer, fractions of 1 ml were collected and detected at 280 nm at a sensitivity level of 50, lamp intensity 0.005, and recorder prints at 5 mm/min. The fractions containing modified peroxidase were selected and combined. 1 mg of SP5.2 peptide synthesized at Supratek was dissolved in phosphate buffer, added to active peroxidase, and incubated stirring for 24 h at room temperature. The reaction was controlled by UV detection at 343 nm. The conjugate was applied on G-25 Sephadex column for purification, and the conjugate fractions were collected and combined. Activity of conjugate was determined by checking peroxidase activity itself using ABTS substrate containing H$_2$O$_2$. Cysteine

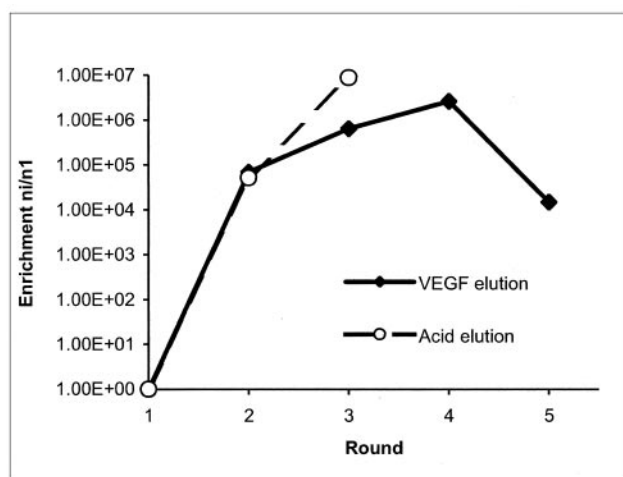*An Antiangiogenic Ligand Against Flt-1 Receptor*

FIG. 1. **Phage library enrichment during selection.** A random 16-mer peptide phage display library was screened for binding to immobilized Flt-1 receptor. Phages were recovered either by acid elution (0.2 M glycine-HCl, pH 2.2) or by 10 μg/ml rhVEGF₁₆₅ displacement. A pronounced enrichment in the phage binding was marked with each panning reaching the maximum after the fifth round of the panning.

FIG. 2. **Comparison of phage clones binding to Flt-1 receptor by ELISA.** The binding affinity of each selected clone at a concentration of 1×10^9 cfu/ml was tested by ELISA. The V5.2 phage clone demonstrated the strongest binding with the Flt-1 receptor.

TABLE I

Peptide sequences bound to Flt-1

Twenty clones were isolated and analyzed by DNA sequencing after five rounds of panning. A1 and A2 were selected with acidic elution; V5.2, V5.10, V40, and V5.8 were isolated with VEGF displacement. A2 and V5.2 sequences were identical.

Clone	Insert peptide sequence	Frequency
Elution with acid pH		
A1	WFLLTM	8
A2	NGYEIEWYSWVTHGMY	3
Elution with 100 nM rhVEGF₁₆₅		
V5.8	RIKYHVGNFMYFLAKL	3
V5.2	NGYEIEWYSWVTHGMY	3
V5.10	FVGGWLVPEDERLYPE	2
V40	PEPEVRLSPPGHIQSL	1

conjugated to peroxidase was synthesized at Supratek to be used as a negative control.

SP5.2-Peroxidase Conjugate Binding Assay—Streptavidin-coated microtiter plates (total binding capacity for biotin-labeled AB = 1.5 μg/well, Roche Applied Science) were used to immobilize the rhFlt-1 receptor chimera. Biotin-labeled anti-human FC antibody (ICN, 10 μg/ml in 0.1% BSA/PBS) was added into each well of streptavidin-coated plate. The microtiter plates were incubated at 4 °C for 16 h in a humidified container and then washed for four times with 0.1% BSA/PBS. Recombinant human Flt-1/Fc chimera (1 μg/well in 0.1% BSA/PBS, R&D Systems) was added into each well. Plates were incubated at 4 °C 8 h in a sealed container to let receptor to immobilize. Unbound receptor was washed away with 2 ml of 0.1% BSA/PBS. Then each well was filled with 350 μl of blocking buffer (2% nonfat dry milk in PBS) and blocked for 16 h at 4 °C. As a negative control, three wells with immobilized anti-Fc antibody were blocked with 2% nonfat milk in PBS. SP5.2-peroxidase conjugate and Cys-peroxidase, used as control, were added to each well and incubated for 2 h at room temperature. Wells were washed with 0.1% BSA/PBS, and the amount of bound conjugate was detected with peroxidase activity. After substrate addition (ABTS containing H_2O_2), wells were analyzed in a microtiter plate reader at 405 nm.

RESULTS

Identification of Peptide Sequences That Bind to Human Flt-1 Receptor—In this study, the phage display technique was used to identify peptide sequences that bind to Flt-1 and inhibit VEGF binding to the receptor. A random 16-mer peptide phage display library composed of $\sim 1 \times 10^9$ independent peptide
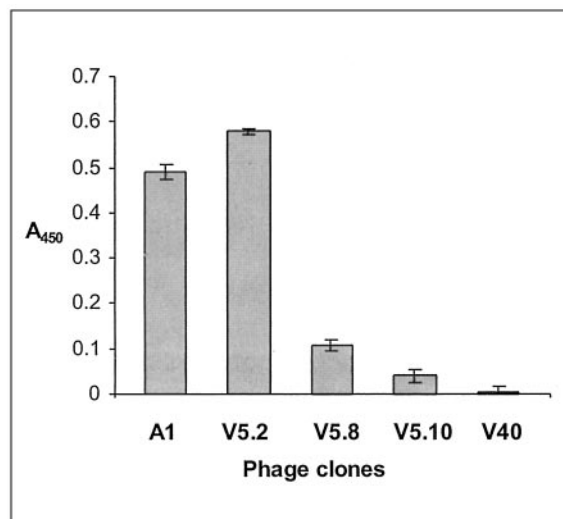
sequences was screened for binding to immobilized human Flt-1 receptor fused with Fc fragment of human IgG antibody (Flt-1/Fc chimera). In one setting, the Flt-1-bound phages were recovered by elution with acidic buffer (0.2 M glycine-HCl, pH 2.2). In another protocol, used to identify phages that interact with the VEGF binding site of Flt-1, 10 ng/ml rhVEGF₁₆₅ was used to displace the bound phages. A marked enrichment in the Flt-1-binding phage relative to bovine serum albumin (BSA), used as a control target protein, was detected with each consecutive panning round, reaching the maximum (2500-fold in the case of acidic elution and 400-fold in the case of elution with VEGF) (Fig. 1) after the fourth panning round. At the end of the selection protocols, a total of 20 clones were isolated and analyzed. DNA sequencing of these clones showed that two independent peptide sequences (A1 and A2) were selected with acidic elution, and four homologous peptide sequences (V5.2, V5.10, V40, and V5.8) were selected with VEGF displacement. A2 and V5.2 sequences, isolated in both protocols, were identical (Table I).

The binding of each of the selected phage clones to Flt-1 receptor was tested using enzyme-linked immunosorbent assay (ELISA) on immobilized Flt-1/Fc chimera (Fig. 2). Among clones tested, the V5.2 phage clone demonstrated highest affinity to Flt-1 receptor at the concentration of 1×10^9 cfu/ml and was chosen for the further studies (Fig. 2).

To analyze the selectivity of V5.2 phage clone for Flt-1 receptor, the binding of V5.2 phage, at the concentration of 1×10^9 cfu/ml was tested by ELISA to two other human endothelial receptors, human low affinity VEGF receptor 1 (KDR) and ICAM-1, both fused with human IgG Fc fragments, as well as with the irrelevant protein, human IgG (Fig. 3). No significant interaction of the V5.2 phage with either KDR or other control proteins was observed (Fig. 3A). In subsequent experiments, V5.2 phage was also found to bind murine analog of Flt-1 (Fig. 3A), to mNRP-1 (Fig. 3B) and does not interact with Flk-1 receptor (murine analog of KDR) (Fig. 3A).

Evaluation of the Synthetic SP5.2 Peptide Binding to Flt-1 Receptor—A linear peptide (SP5.2), with the sequence corresponding to that of V5.2 phage clone peptide insert, was synthesized. The SP5.2 peptide was labeled with fluorescein at the N terminus. The SP5.2 peptide binding to immobilized Flt-/Fc chimera was analyzed using assay similar to that used for the phage binding, except that the read-out was SP5.2-bound flu-

(A)

(B)
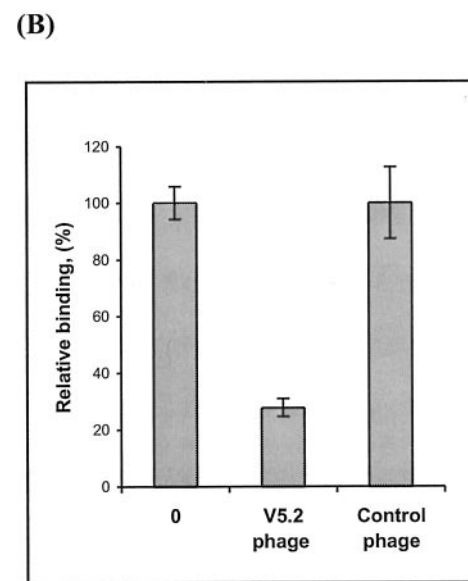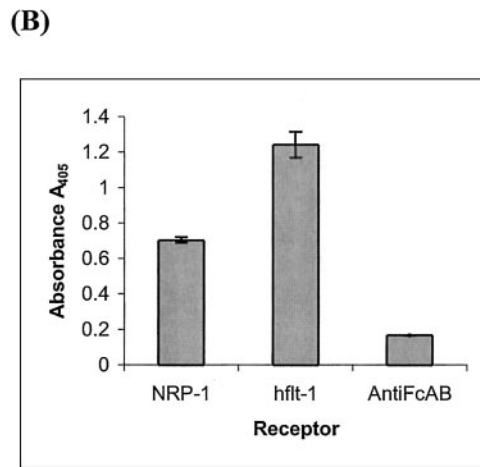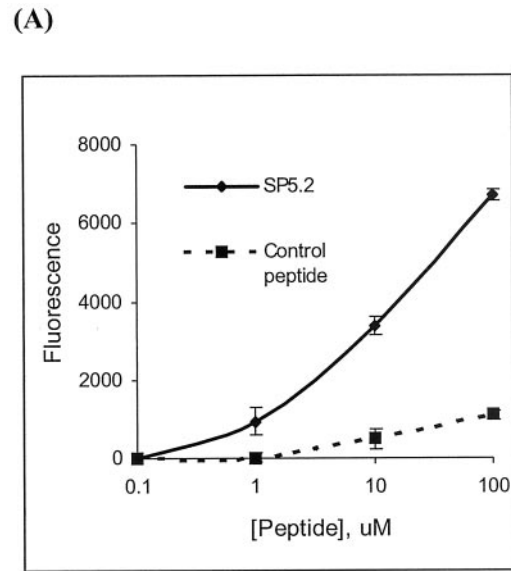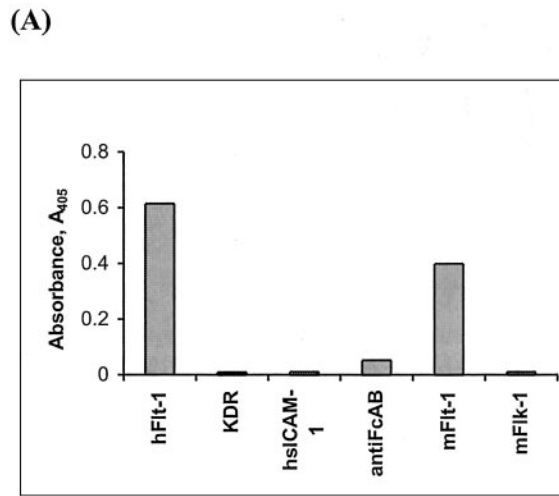
FIG. 3. **Specificity of V5.2 phage to human and murine Flt-1 receptor and to mNRP-1 receptor.** Selectivity of V5.2 phage to Flt-1 receptor was analyzed by ELISA. *A*, two other human endothelial receptors, KDR and ICAM-1, were used, as well as another irrelevant protein, human IgG. V5.2 at a concentration of 1×10^9 cfu/ml showed significant interaction to human Flt-1 and its murine analog, but no significant interaction with KDR and other control protein was observed. *B*, also, V5.2 showed significant interaction to mNRP-1 at a concentration of 1×10^{10} cfu/ml, which is ~50% of the V5.2-hFlt-1 interaction.

orescence. The SP5.2 peptide binding to Flt-1 was dose-dependent, with a half-maximum value observed at 40 μM (Fig. 4*A*). The control (scrambled) peptide (sequence NGSAIAASSAVTH-GMS) did not show significant binding (Fig. 4*A*). The binding specificity of the SP5.2 peptide to Flt-1 was further confirmed by the competition analysis of the bound SP5.2 with the V5.2 phage (1×10^{10} cfu/ml) (Fig. 4*B*). A non-selected phage library used at the same concentration as V5.2 phage control did not affect SP5.2 peptide binding (Fig. 4*B*). The results showed that the V5.2 phage inhibited SP5.2 peptide binding to Flt-1 by 75% at the conditions used in this experiment (Fig. 4*B*).

Mutation Analysis of SP5.2 Sequence—The single point mutations in the V5.2 phage insert-coding sequence were produced as described under "Experimental Procedures." Briefly, a series of SP5.2 coding oligonucleotides with a particular amino acid coding triplet replaced to GCT (alanine coding triplet) were synthesized. These mutated oligonucleotides were cloned one by one into M13 phage RF (replicative form) DNA vector.

(A)

(B)

FIG. 4. **Binding of fluorescent SP5.2 peptide to immobilized Flt-1 receptor and competition with V5.2 phage.** *A*, a fluorescein-labeled linear peptide was synthesized with the sequence corresponding to that of the V5.2 phage clone peptide insert. The fluorescence was assayed to evaluate the binding activity. The Flt-1-specific binding was dose-dependent and required 40 μM to achieve half-maximum value. *B*, the binding specificity of SP5.2 peptide to Flt-1 was further confirmed in displacement assay using V5.2 phage as a competitor to SP5.2. A non-selected phage library was used as a control. V5.2 phage inhibited the binding of SP5.2 peptide to 25%; however, the control did not affect the binding.
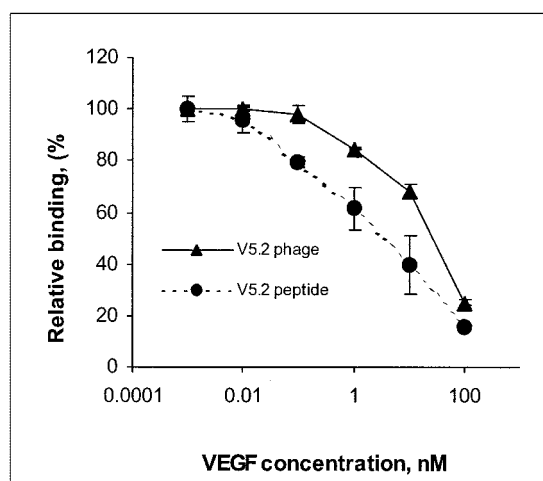
The binding of these mutated clones to Flt-1 receptor was measured by ELISA assay as described above. This assay demonstrated that the substitution of Asn-1, Glu-4, Glu-6, Ile-5, Tyr-8, Trp-10, or Tyr-16 with alanine significantly decreased binding of the SP5.2 peptide to Flt-1 to 60–80%. Other mutations (Trp-7, Ser-9, Thr-12, His-13, and Met-15) had little or no effect on the phage-receptor binding (Fig. 5). Based on these findings, the SP5.2 peptide motif N*XX*EIE*XYX*W*XXXXXX*Y, where *X* represents amino acid residues not involved in the Flt-1 binding, has been deduced.

Competition between SP5.2 and VEGF for Flt-1 Binding—The ability of SP5.2 peptide and V5.2 phage to inhibit VEGF binding to Flt-1 was analyzed using a competitive receptor-
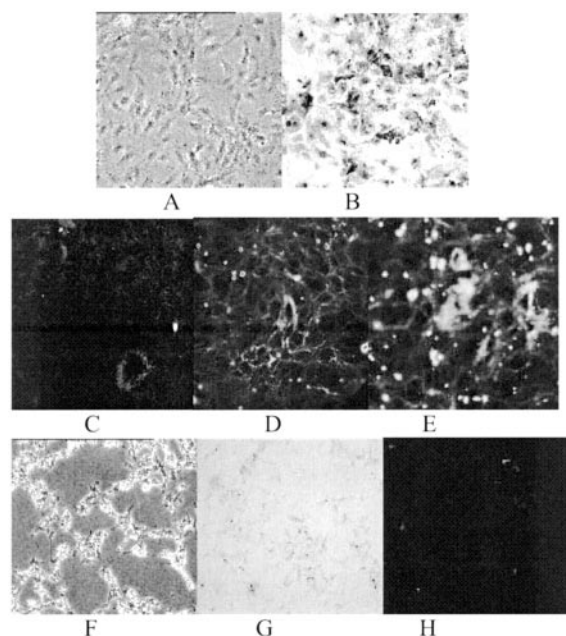
An Antiangiogenic Ligand Against Flt-1 Receptor
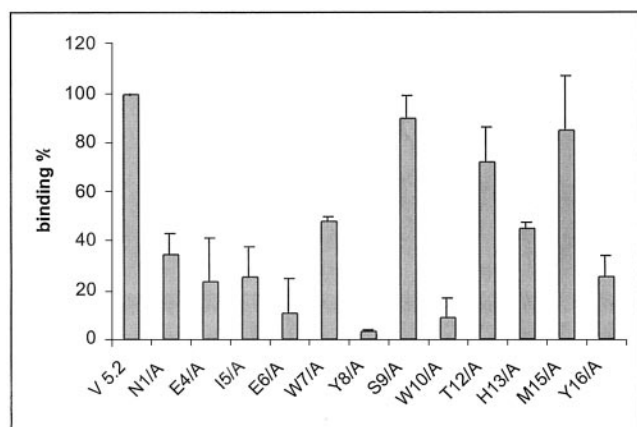
FIG. 5. **Analysis of SP5.2 mutagenesis and their binding affinity to immobilized Flt-1 receptor.** Single point mutations in the V5.2 phage insert were produced. An ELISA assay was used to determine their binding affinity. This assay showed that the exchange of Asn-1, Glu-4, Glu-6, Ile-5, Tyr-8, Trp-10, and Tyr-16 to alanine significantly decreases the binding to Flt-1 to 80%. However, other mutations, Trp-7, Ser-9, Thr-12, His-13, and Met-15 had little or no effect.

FIG. 7. **Binding of fluorescent SP5.2 peptide to Flt-1 expressing cells.** *A*, phase contrast image of HCECs in culture. *B*, immunocytochemical staining for flt-1 receptor in HCECs. *C–E*, binding of fluorescently labeled SP5.2 to HCECs. SP5.2 was added to HCECs at 1 μM (*C*), 10 μM (*D*), and 50 μM (*E*) for 30 min, media were aspirated and replaced with clear M199, and cells were observed under a fluorescence microscope. *F* and *G*, control experiments using the neuroblastoma ShyY cell line. *F*, phase contrast micrograph of ShyY cells; *G*, immunocytochemistry for flt-1 receptor in ShyY cells; *H*, binding of fluorescently labeled SP5.2 (50 μM; 30 min) to ShyY cells. Immunocytochemistry procedures are as described under "Experimental Procedures."

FIG. 6. **Inhibition of V5.2 phage and SP5.2 peptide binding to Flt-1 receptor by VEGF.** 5×10^9 cfu/ml of V5.2 phage or 30 μM fluorescein-labeled SP5.2 peptide and various concentrations of human recombinant VEGF (1 pM to 100 nM) were added to wells with immobilized Flt-1 receptor. Both peptide SP5.2 and phage V5.2 competed with VEGF for receptor binding. The half-maximum inhibition was detected at the presence of 5 nM VEGF for SP5.2 peptide and 50 nM for V5.2 phage.

binding assay. The competition for binding to Flt-1/Fc chimera was assayed using 5×10^9 cfu/ml V5.2 phage or 30 μM fluorescein-labeled SP5.2 peptide and various concentrations (1 pM to 100 nM) of human recombinant VEGF. As shown in Fig. 6, both SP5.2 peptide and V5.2 phage competed with VEGF for the receptor binding. The half-maximum inhibition of SP5.2 peptide binding was achieved with 5 nM VEGF, whereas ten times higher concentration of VEGF was required to inhibit the V5.2 phage interaction with Flt-1 receptor by 50%. Considering the multivalency of V5.2 phage (three to five copies of the P3 protein are displayed on M13 phage filaments), the results suggest that the synthetic SP5.2 peptide has a comparable binding affinity to that of the phage-displayed peptide.

Binding of SP5.2 Peptide to Flt-1-expressing Cells—The binding of the fluorescent SP5.2 peptide to Flt-1-expressing and non-expressing cells was analyzed. The Flt-1 expression in the primary HCECs grown in the presence of 10 ng/ml rhVEGF was confirmed by immunocytochemistry using anti-human Flt-1 antibody (Fig. 7, *A* and *B*). The neuroblastoma ShyY cell

line, in which flt-1 was not detected by immunocytochemistry (Fig. 7*G*), was used as a negative control (Fig. 7, *F* and *G*). Fluorescently tagged SP5.2 bound to VEGF-stimulated HCECs in a concentration-dependent manner (Fig. 7, *C–E*), as determined by fluorescence microscopy assay. Internalization of the receptor-ligand complex was also seen in the receptor-expressing cells. No bound fluorescence was detected in Flt-1-negative ShyY cells at SP5.2 peptide concentration of 50 μM (Fig. 7*H*).

Effects of SP5.2 Peptide on VEGF-mediated Cellular Events—The ability of Sp5.2 to inhibit functional responses induced in endothelial cells by VEGF was tested using various *in vitro* models. "Angiogenic" responses *in vitro* were assessed in three complementary assays: EC proliferation, EC migration, and formation of capillary-like tubes in the basement membrane matrix.

Inhibition of VEGF-mediated Proliferation of Endothelial Cells—The effects of SP5.2 peptide on endothelial proliferation were assessed in HUVECs and HCECs cells stimulated with VEGF using the DNA synthesis rate as a measure of cell proliferation.

HUVECs were stimulated with 10 ng/ml rhVEGF$_{165}$ and then treated with various concentrations of SP5.2 peptide for 48 h. The peptide inhibited the proliferation of HUVECs in a dose-dependent manner with an IC$_{50}$ of 5 μM (Fig. 8*A*).

In HCECs the ability of SP5.2 peptide and control peptide NGSAIAASSAVTHGMS to affect the DNA synthesis, induced by different growth factors, rhVEGF$_{165}$ (20 ng/ml), PLGF (100 ng/ml), and bFGF (10 ng/ml), was analyzed (Fig. 8*B*). At the concentration of 10 μM, SP5.2 peptide inhibited VEGF- and PLGF-induced HCECs proliferation by 70 and 90%, respectively (Fig. 8*B*), and demonstrated no effect on bFGF-induced HCEC proliferation (Fig. 8*B*), suggesting that the SP5.2 peptide effect is the result of its interaction with Flt-1 receptor. At

(A)

(B)
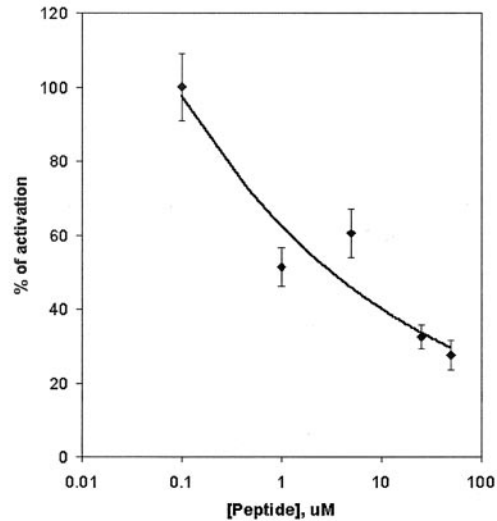
FIG. 8. **Inhibition of HUVEC and HCEC proliferation by SP5.2 peptide.** *A*, HUVECs were stimulated with 10 ng/ml rhVEGF$_{165}$ and then treated with various concentrations of SP5.2 peptide for 48 h. It was demonstrated that the peptide inhibits endothelial cell proliferation in a dose-dependant manner with an IC$_{50}$ of 5 μM. *B*, HCECs were induced by different growth factors, rhVEGF$_{165}$ (20 ng/ml), PLGF (100 ng/ml), and bFGF (10 ng/ml). At the concentration of 10 μM the SP5.2 peptide inhibited VEGF- and PLGF-induced proliferation by 70 and 90%, respectively, however, there was no effect on bFGF-induced proliferation.
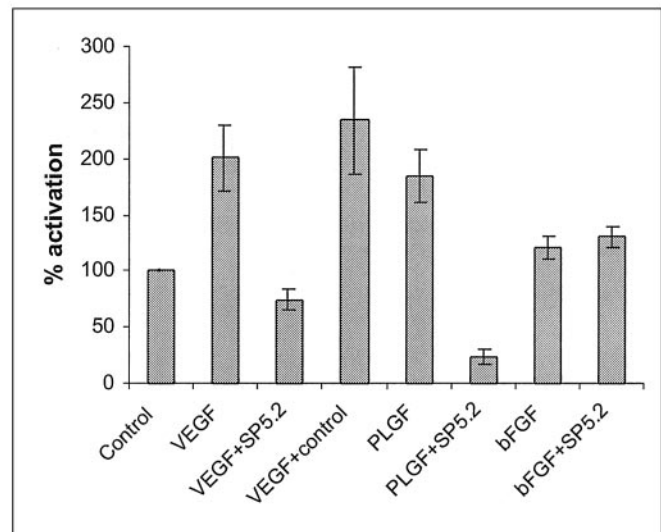
the same concentration, the control peptide did not affect HCEC proliferation (Fig. 8*B*).

Inhibition of VEGF-mediated Migration of HCECs—Migration (chemotaxis) of calcein-AM-labeled HCECs induced by several stimuli, including VEGF$_{165}$ and glioblastoma-conditioned media, was tested in the absence or presence of SP5.2 peptide or scrambled peptide as described previously (37). VEGF stimulated migration of HCECs across the filter by 25% (Fig. 9), and this effect was counteracted by SP5.2 but not by the control peptide (Fig. 9). SP5.2 also inhibited migration of HCECs induced by glioblastoma cell-conditioned media, known to contain high amount of secreted angiogenic factors.
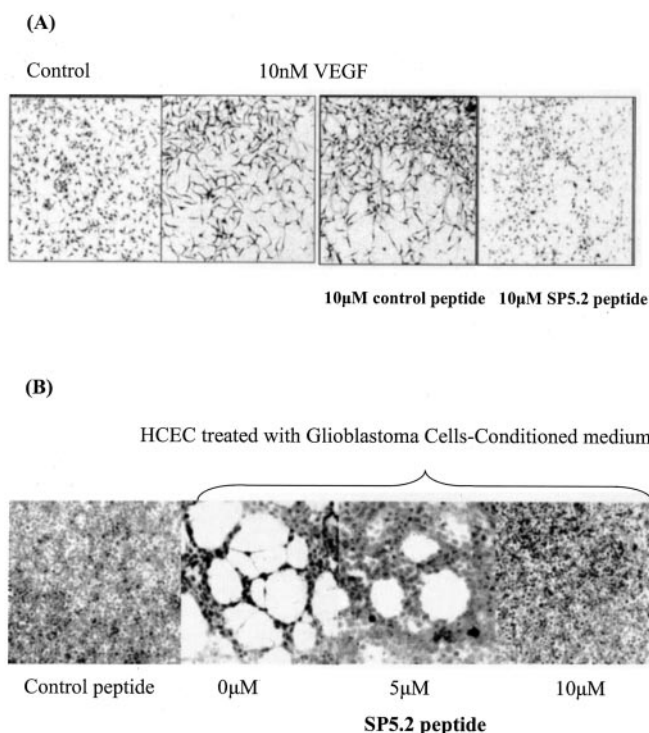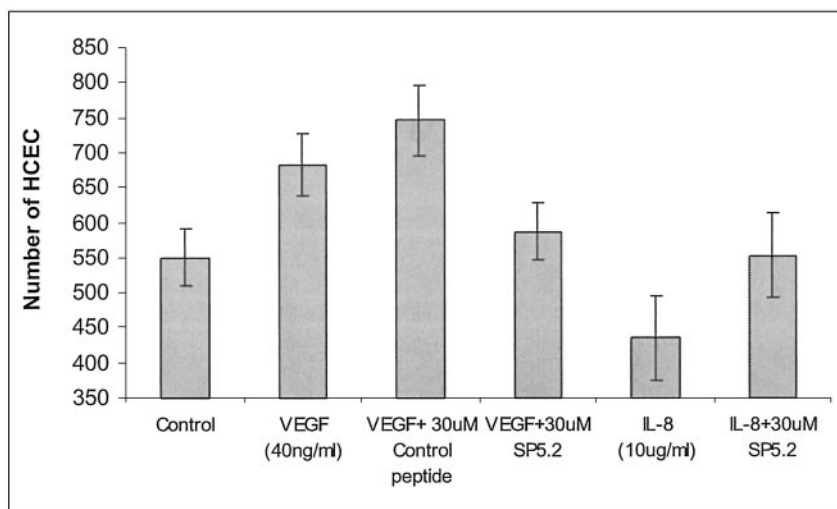
Inhibition of VEGF-induced Capillary Tube Formation—Both VEGF (10 nM) and glioblastoma cell-conditioned media induced capillary tube formation by HCECs grown in Matrigel™ (Fig. 10, *A* and *B*). SP5.2 peptide (10 μM) was found to

completely inhibit both VEGF- and glioblastoma cell-conditioned media-induced formation of capillary-like tubes, whereas control peptide was ineffective (Fig. 10).

Inhibition of VEGF-induced Permeability—Effect of VEGF on endothelial permeability was assessed in a polarized *in vitro* model of the blood-brain barrier. VEGF has been shown to disrupt tight junctions formed by brain endothelial cells *in vitro* and *in vivo* (38, 39). A 24-h exposure of the HCEC monolayer to VEGF resulted in increased permeability of HCECs for the paracellular diffusion marker, sodium fluorescein. This effect of VEGF was attenuated by SP5.2 peptide added to the cells at the concentration of 5 μM (Fig. 11).
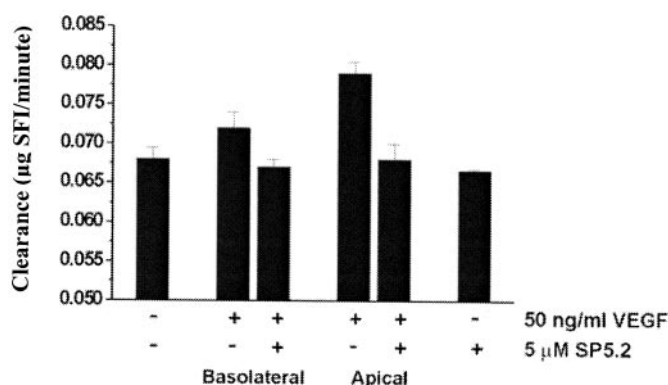
SP5.2-β-gal Fusion Protein—To explore the possibility that the SP5.2 sequence can be used as a targeting moiety for delivery of biologically active agents such as genes, polypeptides and particulate delivery vehicles to Flt-1-expressing cells/

An Antiangiogenic Ligand Against Flt-1 Receptor

FIG. 9. **Inhibition of VEGF-stimulated chemotaxis of HCECs by SP5.2 peptide.** Migration (chemotaxis) of calcein-AM-labeled HCECs induced by several stimuli, including VEGF$_{165}$, and glioblastoma-conditioned media was tested in the absence or presence of SP5.2 peptide or scrambled peptide using a Chemo-Tx#101-5 assembly. VEGF-induced increased migration of HCECs by 25% across the filter that was significantly reduced by SP5.2 and not the control.

(A)

(B)

FIG. 10. **Inhibition of VEGF and tumor-induced angiogenesis by SP5.2 peptide.** HCECs were seeded onto a semi-solid basement membrane matrix, Matrigel™. Cells induced capillary tube formation by either 10 nM VEGF$_{165}$ (*A*) or glioblastoma cell-conditioned media (*B*). Cells were then treated with SP5.2 or scrambled control peptide. SP5.2 was shown to completely inhibit capillary-like tubes formation in a concentration-dependent manner.

FIG. 11. **Inhibition of VEGF-induced permeability of the blood-brain barrier using the HCEC model by SP5.2 peptide.** A 24-h exposure of the HCEC monolayers to VEGF$_{165}$ was found to increase permeability of monolayers, due to a known disrupting effect of VEGF$_{165}$ on the tight junctions formed by HCECs. The addition of 5 μM SP5.2 attenuated this function of VEGF.

tissues, a fusion protein, SP5.2-β-gal, expressing the peptide sequence at the N terminus and the His$_6$ tag at the C terminus of β-galactosidase, was produced and purified by His tag affinity chromatography. The SP5.2-β-gal affinity and selectivity for the Flt-1 receptor was determined by ELISA assay using a recombinant β-galactosidase as control. Fig. 12*A* shows that SP5.2-β-gal construct binds to Flt-1 receptor with an apparent K_D of 200 nM, suggesting that the fusion protein has about 200-fold binding affinity compared with the synthetic SP5.2 peptide. At the same time, SP5.2-β-gal showed no significant interaction to KDR receptor (Fig. 12*B*). This result suggests that SP5.2 can potentially be used as a targeting element for genetically constructed therapeutic proteins to be delivered

into specific Flt-1-rich compartments such as tumor endothelium, inflammation sites, or a damaged blood-brain barrier.

Peroxidase-conjugated Peptide—A possibility that the therapeutic entity can be chemically attached to SP5.2 peptide was also tested. SP5.2 peptide was chemically conjugated with the peroxidase enzyme using SPDP as a heterobifunctional cross-linking agent. Resulting conjugate contained between 3 and 5 molecules of SP5.2 peptide per each peroxidase molecule. The Flt-1 binding of the conjugate was analyzed by measuring the peroxidase activity. Peroxidase conjugated with cysteine was used as a negative control. SP5.2 peroxidase conjugate demonstrated concentration-dependent specific binding to Flt-1, whereas no detectable binding to Flt-1 of the Cys-peroxidase conjugate was observed. The half-maximum binding concentration (100 nM) of the SP5.2 peroxidase conjugate was 400-fold lower than that of the non-conjugated peptide (Fig. 13). The results of this experiment suggest that SP5.2 can potentially be linked chemically to therapeutic entities such as small molecules, proteins, liposomes, micelles, nanoparticles, and other particulate delivery vehicles loaded with a therapeutic agent.

DISCUSSION

Previous studies have shown that antiangiogenic therapy is a promising approach for the treatment of cancer (40–43). Vascular endothelial growth factor (VEGF) and its receptors are the focus of intense interest because of their role in several biological processes that involve angiogenesis. VEGF has also
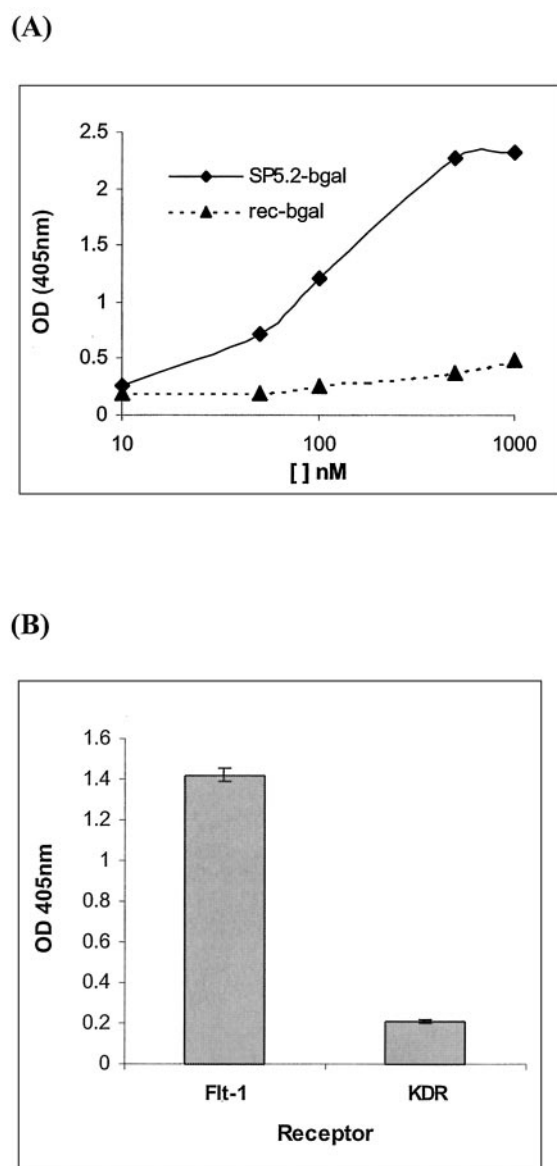
(A)

(B)

FIG. 12. **Binding affinity and specificity of SP5.2-β-gal to Flt-1 receptor.** *A*, using an ELISA, it was found that SP5.2-β-gal binds to Flt-1 receptor with a K_D of 200 nM, which is 200-fold higher binding affinity compared with fluorescein-labeled SP5.2. *B*, SP5.2-β-gal showed no significant interaction to KDR receptor at a concentration of 50 μM.

been shown to play a role in development of vascular leakiness, blood-brain barrier disruption, brain edema in brain tumors (44) and cerebral ischemia (45). VEGF activities are mediated through its binding to two high affinity receptors Flt-1 and KDR. The VEGF-Flt-1 receptor system plays an important role in the stimulation of tumor angiogenesis, which makes Flt-1 an important target for antiangiogenic drugs. Furthermore, it has been shown that blocking the interaction between VEGF and Flt-1 can result in the regression of murine and human tumors (46, 47). We report here the identification of a new VEGF peptide antagonist isolated by screening of a phage-displayed peptide library against Flt-1.

In this study, five rounds of biopanning of a 16-mer peptide phage display library against Flt-1 immobilized on magnetic particles resulted in identification of five different phage clones capable of binding to immobilized Flt-1. The most efficient of these, a phage V5.2, expressing the SP5.2 peptide (NGYEIEW-

YSWVTHGMY) was chosen for further analysis. Chemically synthesized SP5.2 peptide was tested for its ability to bind other endothelial receptors such as ICAM-1, Flk-1, and KDR, none showed any significant binding compared with Flt-1. However, there was a significant and selective cross interaction with the murine analog of Flt-1, also to murine NRP-1. An alanine scan was used to identify functionally important amino acids in the SP5.2 sequence, and the binding properties of the obtained mutated phages were analyzed by ELISA. Based on these results a critical structural motif of the SP5.2 peptide important for the binding to Flt-1 receptor was identified as: N*XX*EIE*XY X*W*XXXXX*Y, were *X* represents amino acid residues not involved in the Flt-1 binding.

Cell-based experiments have demonstrated that SP5.2 produces inhibition of VEGF-mediated functional events in Flt-1-positive cells such as HUVECs and HCECs. SP5.2 was found to inhibit several components of angiogenic response in these cells induced by VEGF, including proliferation, migration, early angiogenesis, and vascular permeability. All these inhibitory effects were sequence-specific and selective with respect to VEGF compared with other growth factors. SP5.2 was also shown to antagonize some effects of PLGF, known to share more than 50% homology with VEGF and to act through Flt-1 and NRP-1 receptors (48, 49).

Most previous studies, aiming to establish functional roles of Flt-1 and KDR receptors, have produced a general agreement that events mediated through Flt-1 are mainly responsible for early stages of VEGF-induced angiogenesis, such as modulation of cell motility and adhesion of endothelial cells, whereas KDR is more important in regulating endothelial proliferative response (50–52). However, it has recently been demonstrated that Flt-1 and KDR can form heterodimers in which Flt-1 may participate in the regulation of proliferative response to VEGF (43). Also, NRP-1 when coexpressed in cells with KDR may present VEGF$_{165}$ to the KDR receptor in a manner that enhances the effectiveness of KDR-mediated signal transduction (53). SP5.2, although selectively binding to Flt-1 receptor in *in vitro* studies, was capable of inhibiting both Flt-1-mediated cellular events such as capillary-like tube formation, vascular permeability, endothelial migration, and predominantly KDR-mediated endothelial proliferation. This result is in agreement with recent studies suggesting that Flt-1 is involved in the proliferative control of endothelium most likely through its heterodimerization with KDR (43). KDR-mediated signal transduction and its mitogenic activity for endothelial cells can also be inhibited while SP5.2 binding to the NRP-1 receptor occurs. Further studies are required for more detailed mechanistic understanding of the antiproliferative effects of SP5.2.

It is well known that peptides identified during phage display library screening often show much lower potencies when synthesized as individual compounds (54, 55). The two main reasons for this are: (i) small synthetic peptides most often do not have sufficient conformational rigidity that, in the case of phage, is provided by phage filament protein in which the peptide is inserted and (ii) phage particles display multiple copies of inserted peptides (from three to five peptides in the case of P3 protein-based libraries), and this multivalency results in higher avidity compared with the synthetic peptide. The synthetic form of SP5.2 when presented as a single molecule demonstrated a micromolar affinity in a variety of binding assays. A possible strategy to increase binding of the synthetic peptide is to achieve its multivalency by attaching multiple copies to a structurally rigid moiety such as a protein, liposome, polymer, etc. (56–58). To explore a possibility of increasing potency of SP5.2, two derivatives of this molecule were produced. The first was a recombinant fusion of SP5.2 with β-ga-

Fig. 13. **Binding of SP5.2 peroxidase conjugate to Flt-1 receptor.** SP5.2 was chemically conjugated with peroxidase by using SPDP. The Flt-1 binding activity of the conjugate was analyzed by evaluating the peroxidase activity using its substrate ABTS. Peroxidase conjugated to cysteine was used as a negative control. SP5.2 peroxidase conjugate demonstrated concentration-dependent specific binding to Flt-1, whereas no detectable binding was observed in the case of the negative control.
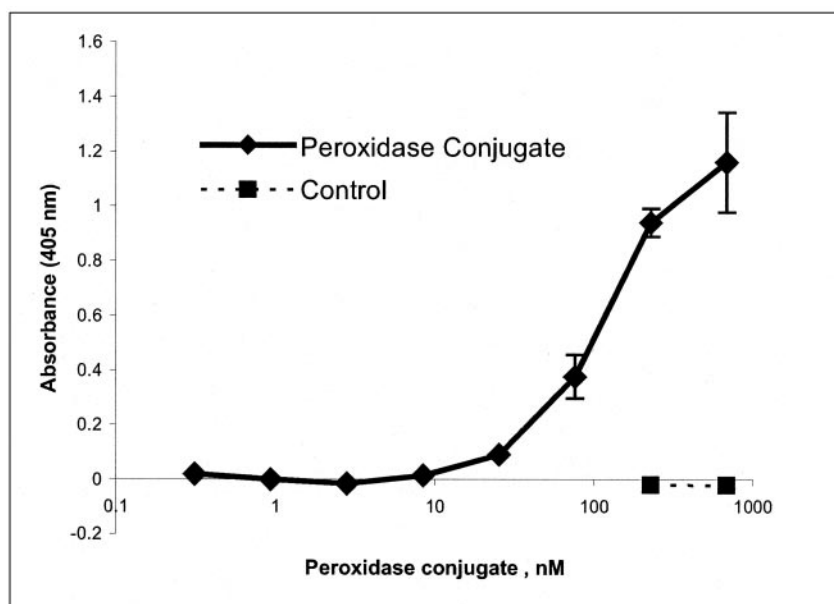
lactosidase in which SP5.2 was present as a single copy at the N terminus of the enzyme that was used as a carrier protein. The second derivative was a chemical conjugate of SP5.2 and peroxidase in which three to five copies of the peptide were attached to the protein. The resulting products showed considerably higher binding potencies to Flt-1 compared with the original synthetic peptide. The apparent dissociation constants established in the Flt-1 binding assays were 200 nM (200-fold lower compared with SP5.2) for the fusion with a single copy of the peptide, and 100 nM (400-fold lower compared with SP5.2) in the case of the chemically linked, polyvalent product. These results suggest that SP5.2 can potentially be used as an efficient targeting component that, as an added benefit, displays inherent antiangiogenic activity. Several possible therapeutic applications of SP5.2 are presently being explored in ongoing studies. These include combining SP5.2 with therapeutic proteins such as cytokines in targeted genetic constructs, as well as combining this peptide with polymeric micelles loaded with cytotoxic drugs.

In summary, this study describes discovery of the new peptide, SP5.2, which is an effective and selective Flt-1 antagonist. This molecule and its derivatives may have future utility in clinical applications for treating cancer, diabetic retinopathy, and other angiogenic or proliferative disorders associated with Flt-1 up-regulation in endothelial cells.

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