Anti-Flt1 Peptide, a Vascular Endothelial Growth Factor Receptor 1–Specific Hexapeptide, Inhibits Tumor Growth and Metastasis

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Abstract

Purpose: The purpose of this study was to develop antagonists specific for the vascular endothelial growth factor receptor 1 (VEGFR1) and to investigate the effects of the antagonists on the VEGF-induced endothelial cell functions and tumor progression.

Experimental Design: Hexapeptides that inhibit binding of VEGFR1 and VEGF were identified through screening of synthetic peptide library. A selected peptide, anti-Flt1, was investigated for binding specificity with various receptors and ligand peptides. Effects of the peptide on proliferation, cell migration, and fibrin gel–based angiogenesis of endothelial cells were also investigated. The activity of anti-Flt1, *in vivo*, was evaluated for inhibition of tumor growth and metastasis in VEGF-secreting cancer cell–implanted mice by s.c. injections of the peptide.

Results: Here, we report on a short peptide that binds to VEGFR1 and prevents binding of VEGF. A hexapeptide, anti-Flt1 (Gly-Asn-Gln-Trp-Phe-Ile or GNQWFI), was identified from peptide libraries. The anti-Flt1 peptide shows specificity toward binding to VEGFR1 and it inhibits binding of VEGF, placental growth factor (PlGF), and VEGF/PlGF heterodimer to VEGFR1. This peptide does not inhibit the proliferation of endothelial cells induced by VEGF and VEGF/PlGF heterodimer but it effectively blocks VEGF-induced migration of endothelial cells and their capacity to form capillary-like structures on fibrin gel–based *in vitro* angiogenesis system. Furthermore, growth and metastasis of VEGF-secreting tumor cells were also significantly inhibited by s.c. injections of anti-Flt1 peptide in nude mice. Accordingly, VEGF-induced migration and capillary formation are mediated through VEGFR1, and these processes may play an important role in the growth and metastasis of VEGF-secreting tumors.

Conclusions: We show that a peptide (anti-Flt1) specific for VEGFR1 inhibits growth and metastasis of tumor that secretes VEGF. The effects on endothelial cell functions, *in vitro*, indicate that the anticancer activity of anti-Flt1 peptide with reduced blood vessel density could also be due to the blocking of VEGFR1-mediated endothelial cell migration and tube formation. Although the effects of anti-Flt1 peptide still remain to be further characterized, the receptor 1–specific peptide antagonist, anti-Flt1, has potential as a therapeutic agent for various angiogenesis-related diseases, especially cancer.

Vascular endothelial growth factor (VEGF) is an evolutionarily conserved homodimeric glycoprotein and the most potent endothelial cell–specific mitogen that plays a critical role in angiogenesis and vasculogenesis. VEGF induces various intracellular signaling and physiologic responses that are essential for angiogenesis, such as intracellular Ca^{2+} influx, chemotaxis (migration), expressions of plasminogen activators, urokinase receptor, collagenases, and vascular permeability (1). Its biological effects are elicited through two high-affinity receptor tyrosine kinases [i.e., VEGF receptors 1 (VEGFR1) and 2 (VEGFR2)], which are mainly expressed in endothelial cells (2, 3). The significance of VEGF in vasculogenesis and angiogenesis has been shown by gene deletion studies of VEGF and its receptors (4–6). Whereas endothelial cells overgrow and blood vessels are disorganized in VEGFR1 null mice (5), the development of endothelial and hematopoietic cells is impaired in VEGFR2 null mice (6). These facts indicate that these receptors have different functions during development and the various activities of VEGF are segregated between the two receptors. However, genetic approaches, such as the knockout of *VEGF* and *VEGFR* genes, have been restricted to their roles only during development because of their embryonic lethality.

Several biologically relevant agents and conditions have been shown to induce VEGF expression in various cell types, such as interleukin-1 and interleukin-6, keratinocyte growth factor, transforming growth factor-β, platelet-derived growth factor, and tissue hypoxia (7–13). In addition, the inactivation of p53 or von Hippel-Lindau tumor suppressor genes (14, 15), as well as the activation of oncogenes, such as *ras, raf,* or *src*, enhance

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VEGF expression (16–18). The studies on the roles of individual VEGFRs in VEGF-induced endothelial functions are complicated, in part, by the observation that most endothelial cells simultaneously express both VEGFR1 and VEGFR2 (19). It is widely accepted that VEGFR2 is the receptor that mediates the proliferation of endothelial cells. Mutant forms of VEGF that lack a binding ability to VEGFR1 are able to stimulate growth, suggesting that there is no significant biological role for VEGFR1 (20). However, in spite of the essential roles in embryogenesis, the function of VEGFR1 is poorly understood in the adult stage (21). During embryonic development, VEGFR1 has a negative regulatory function in physiologic angiogenesis, possibly with its stronger VEGF-trapping activity (5, 22). Although individual VEGFRs are ectopically expressed and phosphorylated in heterologous cells, it is unclear whether these signaling events also occur in primary endothelial cells because the activities of VEGFRs are different according to the cellular environment in which they are expressed (23, 24).

MVEGFR1 also functions as a positive regulator using its weak tyrosine kinase. It was shown that the activation of VEGFR1 correlates with endothelial cell migration and growth *in vitro* and angiogenesis *in vivo* (25–27). Recent studies also indicate that VEGFR1-mediated signaling may have a significant role in VEGF-related pathologic angiogenesis, such as the ability of VEGFR1-specific VEGF-B to regulate plasminogen activator (28), up-regulation of VEGFR1 in endothelial cells under hypoxic conditions that stimulate angiogenesis (29), suppression of angiogenesis and tumor growth by genetic truncation of the VEGFR1 tyrosine kinase domains (30), or antisense-mediated down-regulation of VEGFR1 (31). Furthermore, an anti-VEGFR1 monoclonal antibody, which blocks binding of VEGF and placental growth factor (PlGF) to VEGFR1, suppresses tumor angiogenesis, arthritis, and atherosclerosis (32). These findings suggest that alternate pathways for disease-related angiogenesis may exist and VEGFR1 may have a restricted angiogenic activity in pathologic conditions. Therefore, we investigated the therapeutic potential of VEGFR1 antagonist for the inhibition of tumor growth and metastasis, which are angiogenesis dependent.

In this report, a VEGFR1-selective hexapeptide, Gly-Asn-Gln-Trp-Phe-Ile (GNQWFI, named as anti-Flt1), has been identified from peptide libraries [positional scanning-synthetic peptide combinatorial library (PS-SPCL)] using a screening system involving purified recombinant VEGFs and chimeric receptors. The VEGFR1-specific anti-Flt1 peptide inhibits binding of various VEGFR1 ligands. This peptide does not inhibit the VEGF-induced proliferation of endothelial cells; rather, it inhibits VEGF-induced endothelial cell migration and morphogenesis. Furthermore, this peptide inhibits growth and metastasis of tumor cells in mice. Although the exact mechanism of anti-Flt1 peptide on VEGF signaling in endothelial cells still remains to be further investigated, anti-Flt1 will serve as a useful tool in further elucidating the roles of VEGFR1. Anti-Flt1 is also a potential candidate for a therapeutic agent in various angiogenic disorders, such as cancer, rheumatoid arthritis, retinal ischemia, and atherosclerosis.

Materials and Methods

Cell culture. Adhesion- and suspension-cultured *Sf9* insect cells were maintained in TC100 medium supplemented with 10% fetal bovine serum, antibiotics, and 0.1% pluronic acid at 27°C. Human umbilical vein endothelial cells (HUVE cells, Bio Whittaker Molecular Applications, Walkersville, Maine) were cultured as previously described (33) and used between passages 3 and 6. SW480 and HM7 human colon cancer cell lines were maintained with DMEM containing 10% fetal bovine serum and antibiotics.

Synthesis of peptide library (positional scanning-synthetic peptide combinatorial library) and peptides. The peptide library (PS-SPCL) was synthesized at the Peptide Library Support Facility of Pohang University of Science and Technology (Postech) in Korea, according to the method suggested by Pinilla et al. (34). All of the peptides described in this study were synthesized by Peptron, Inc. (Daejon, Korea).

Purification of dimeric recombinant proteins $VEGF_{165}H_6$, $VEGF_{121}H_6$, $VEGFR1\text{-}FcH_6$, $VEGFR2\text{-}FcH_6$, and FcH_6. $VEGF_{165}$ and $VEGF_{121}$ cDNAs without their signal sequence and stop codon were amplified from human liver cDNA. The entire ectodomains of human VEGFR2 or soluble VEGFR1 (soluble Flt1) without their signal sequences were amplified by reverse transcription-PCR from RNA extracted from HUVE cells and fused with the Fc fragment of human IgG1. The resulting DNA fragments were cloned into a baculoviral expression vector containing the mellitin signal sequence at NH_2 terminal and hexa-histidine tag at COOH terminal. Each of the five expression vectors and linearized baculoviral DNA (BD PharMingen, San Diego, CA) were cotransfected into *Sf9* insect cells with LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. The transfected cells were incubated at 27°C for 3 days (for protein expression) or 7 days (for virus amplification). High-titered recombinant viruses (1×10^8-5×10^8 plaque-forming units/mL) were used for infection of the adhesion culture of *Sf9* insect cells (1-5 multiplicities of infection) in serum-free medium (HyQ SFX-Insect, Hyclone, Inc., Logan, UT) for the production of recombinant proteins. Concentrated supernatant (molecular weight cutoff, M_r 5,000, Millipore Corp., Billerica, MA) was applied onto a metal affinity column (HiTrap Chelating HP, Amersham Biosciences, Piscataway, NJ) and the bound proteins were eluted by 0.1 to 0.3 mol/L imidazole. The desired protein fractions were dialyzed against distilled water (for VEGFs) or 40 mmol/L NaH_2PO_4/Na_2HPO_4 (pH 8.0)/500 mmol/L NaCl (for VEGFRs). The purity and dimeric conformation of proteins were assessed by reduced and nonreduced SDS-PAGE. The molecular identity was verified by immunobloting with each protein-specific antibody or anti-human Fc. The purified proteins were quantitated with a protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA) and stored at −70°C until use.

Binding of $VEGFR1\text{-}FcH_6$ and $VEGFR2\text{-}FcH_6$ to VEGF, PlGF, or VEGF/PlGF heterodimer. The ligand receptor binding activity of recombinant proteins and the effects of various peptides on the interaction of VEGFR1 with various ligands [VEGFs, PlGF, or VEGF/PlGF heterodimer (R&D Systems, Minneapolis, MN)] were examined. Ninety-six-well plates were coated with ligands [recombinant $VEGF_{121}$ (40 ng), $VEGF_{165}$ (25 ng), PlGF (25 ng), or VEGF/PlGF heterodimer (25 ng)] in 50 μL PBS at 4°C overnight, blocked with 100 μL of blocking buffer [PBS/3% bovine serum albumin (BSA)] at room temperature for 2 hours, and then 1 to 10 pmol/L of receptor (10-100 ng $VEGFR1\text{-}FcH_6$, $VEGFR2\text{-}FcH_6$, or FcH_6) in blocking buffer was incubated with the coated ligands at room temperature for 2 hours. This was followed by detection with each protein-specific antibody or a mouse antihexahistidine antibody (R&D Systems). The plates were washed with 150 μL PBS containing 0.2% Tween 20 thrice and reacted with 3,3′,5,5′-tetramethyl benzidine liquid substrate system for ELISA (Sigma-Aldrich, Inc., St. Louis, MO). The color intensity was read at 405 nm. Binding of the proteins of interest to blank wells was determined and the value was considered as background.

Screening of peptide libraries. For the identification of the VEGFR1-specific antagonist from the screening of peptide libraries, 50 ng receptor was preincubated with various concentrations of peptide library pools or peptides at room temperature for 1 hour and then these mixtures were allowed to interact with coated VEGFs at room

temperature for 2 hours. The amount of bound VEGF receptors was determined with anti-human Fc-horseradish peroxidase conjugate.

Binding of the purified recombinant proteins to immobilized peptide. The anti-Flt1 peptide (100 ng) was coated at 4°C overnight in an ELISA plate. The wells were blocked with 100 μL blocking buffer at room temperature for 2 hours. To each well, various recombinant proteins (100 ng in 50 μL PBS/0.1% BSA) were added at room temperature for 2 hours. After incubation, each well was washed thrice with PBS containing 0.2% Tween 20 at room temperature. The amount of bound recombinant proteins was detected with mouse antihexahistidine antibody.

Effects of anti-Flt1 on the interaction of [^{125}I]VEGF$_{165}$ with its receptors receptors on human umbilical vein endothelial cells. Seeded HUVE cells (5×10^4 cells/well) were incubated at 37°C overnight and washed with a warm binding buffer [25 mmol/L HEPES (pH 7.4)/0.1% BSA in serum-free Medium 199] at 37°C for 2 hours. The cultures were then preincubated with various concentrations of antagonistic peptides in 200 μL binding buffer at 37°C for 1 hour and then transferred onto an oscillating platform at 4°C and set at 1 cycle/s. [^{125}I]VEGF (1,903 Ci/mmol, 20 nCi/well, Amersham Pharmacia Biotech) with or without antagonists were added and the plate was incubated at 4°C for 3 hours. The nonspecific binding of [^{125}I]VEGF to the cells was determined in the presence of a 100-fold excess of nonlabeled VEGF. After washing twice with a cold binding buffer followed by washing in cold PBS/0.1% BSA, the cells were solubilized by the addition of 0.25 mL of 20 mmol/L Tris-HCI (pH 7.4) containing 1% Triton X-100 at room temperature for 20 minutes on an oscillating platform set at 2 cycles/s. The receptor-bound radioactivity was determined in a gamma counter.

Endothelial cell proliferation assay. HUVE cells (5×10^3 cells/well) were seeded on a gelatin-coated 96-well culture plate in Medium 199/10% fetal bovine serum/10 mmol/L HEPES (pH 7.4). After preincubation of the cells with various concentrations of peptides or VEGF antagonists at 37°C for 1 hour, VEGF (5 ng/mL) or VEGF/PlGF heterodimer (100 ng/mL) was added. After 2 days, the cells were further incubated with methyl-[^{3}H]thymidine (0.5 μCi/well, 76 Ci/mmol, Amersham Pharmacia Biotech) for 1 day. Cells were washed thrice with PBS containing 0.1% BSA, solubilized with 100 μL of 0.4 N NaOH at room temperature for 20 minutes, and then neutralized with 20 μL of 2.0 N HCl. Radioactivity was determined in a liquid scintillation counter. All experiments were carried out in triplicate.

Cell migration assay. Culture inserts (8.0 μm) were used. HUVE cells (10^5 cells/well) in 5% fetal bovine serum/Medium 199 were plated in the upper chamber. VEGF (50 ng/mL) was placed in the lower chamber with or without various concentrations of peptide or a VEGF neutralizing antibody. Cells were allowed to migrate at 37°C overnight. Unmigrated cells were removed from the upper side of the membrane by scraping with a cotton swab. The migrated cells on the bottom side of the membrane were determined according to the manufacturer's instructions (Chemicon International, Inc., Temecula, CA).

In vitro angiogenesis assay. A fibrin gel–based *in vitro* angiogenesis system (Chemicon International) was used. HUVE cells (5×10^4 cells/well) in 5% BSA/Medium 199 were plated on the surface of preformed fibrin gel in 24-well culture plates. VEGF (50 ng/mL) with or without antagonistic peptides or an anti-VEGF neutralizing antibody were used to induce angiogenesis of HUVE cells at 37°C for 8 hours. The results were recorded as two photographs per each well and evaluated by two independent persons (double-blind manner, 100× magnification). Each experiment was repeated at least thrice and consistent results were obtained.

Tumor growth assay in nude mice. Confluent SW480 and HM7 human colon cancer cell cultures were harvested and resuspended in serum-free DMEM. The presence of single-cell suspensions was confirmed by phase-contrast microscopy, cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used. Viable tumor cells [0.5×10^6 (HM7) or 5×10^6 (SW480) in 100 μL] were injected s.c. over the right scapular region of pathogen-free, 4-week-old male athymic nude mice (BALB/c/*nu*/*nu*, Charles River Laboratories, Yokohama, Japan). When the tumor volume reached ~110 mm^3, mice received s.c. injections of each peptide (100 μg/d) or normal physiologic saline (PBS) for 15 days. Mice were surveyed regularly, and the tumor was measured with a caliper. Tumor volumes were determined using the following formula: volume = 0.5 × (width)^2 × length. Each experimental group consisted of seven (HM7) or eight (SW480) animals.

Immunohistochemical staining for CD31 and microvessel density. After the mice had been sacrificed, tumors were removed and frozen. The cryosections were stained with a monoclonal rat anti-mouse CD31 (platelet/endothelial cell adhesion molecule 1; BD PharMingen) at a dilution of 1:50. Visualization of the antigen-antibody reaction was carried out using an anti-rat immunoglobulin horseradish peroxidase detection kit (BD PharMingen) according to the manufacturer's recommendations. Vessel density was determined by counting the stained vessels at 200×.

Tumor metastasis assay in nude mice. HM7 cells were resuspended in serum-free DMEM (10^7 cells/mL). Four-week-old male athymic nude mice were anesthetized with diethyl ether by inhalation, and the spleen was exteriorized through a flank incision. One million cells were slowly injected into the lower polar side of the splenic pulp through a 27-gauge needle followed by a splenectomy 1 minute later. On the following day, mice received s.c. injections of each peptide (100 μg) or the normal physiologic saline control (PBS) for 2 weeks. Animals were sacrificed 26 days later, the livers were removed and weighed, and then the tumor nodules were counted. All experiments were done with six animals per group.

Cytotoxicity assay. Cells (5×10^3 cells/well) were plated on a 96-well culture plate. After a 24-hour incubation at 37°C, varying concentrations of peptides diluted in 100 μL culture medium were added to each well. Following an incubation for 2.5 days, the number of viable cells was determined using a Cell Titer 96 nonradioactive proliferation assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Each experiment was carried out in triplicate.

Statistical analysis. Values of results are expressed as means and SD or SE, and significance was established by Student's *t* test. In all analyses, the level of statistical significance was more than the 95% confidence level ($P < 0.05$). *, **, or *** means $P < 0.05$, $P < 0.01$, or $P < 0.001$, respectively.

Results

The peptide library used in this study was the PS-SPCL originally developed by Houghten et al. (34). In this library system, each position of a hexapeptide pool is fixed with a known amino acid, and the other five positions are coupled with random mixtures of 20 amino acids. For each position, 20 peptide pools are possible and a total of 120 peptide pools can be made for a hexapeptide library. By analyzing 120 pools, the amino acids important at each position of active peptides can be determined. Based on this information, the reiterative synthesis of peptides can be conducted to identify the sequence of the most active peptides.

Purification of active recombinant vascular endothelial growth factor and vascular endothelial growth factor receptor proteins. Two recombinant VEGFR fusion proteins with hexahistidine at the COOH terminal (fusion between the ectodomain of the receptor and the Fc fragment of human IgG1; VEGFR1-FcH_6 and VEGFR2-FcH_6) and VEGFs with hexahistidine at the COOH terminal (VEGF$_{121}H_6$ and VEGF$_{165}H_6$) have been purified as biologically active dimeric forms. The Fc fragment of the human IgG1 subclass was fused for the artificial dimerization of the ectodomain of the receptor, which mimics the ligand-bound receptor tyrosine kinase on the surface of endothelial cells *in vivo* (35). All of the purified recombinant proteins from

Sf9 insect cells were glycosylated and dimeric proteins, and they showed specific receptor-ligand interactions as expected (data not shown). The interactions between VEGFs and VEGFRs were saturable, concentration-dependent, and abolished by the presence of an excess amount of free VEGF (data not shown). Therefore, all of the used recombinant proteins were biologically active. The binding between $VEGF_{121}$ and VEGFR was used as an assay for the identification of a VEGFR1-specific peptide, and to exclude any possible effects of the heparin-binding domain of other VEGF isoforms, such as $VEGF_{165}$.

Identification of VEGFR1-selective peptide from peptide libraries. Immobilized $VEGF_{121}$ was incubated with a mixture of VEGFR1 and an antagonist, which was prepared by premixing VEGFR1 and various concentrations of peptide library pool or a hexapeptide with a defined sequence. The soluble hexapeptide library PS-SPCL was prepared with 19 amino acids lacking cysteine because the presence of cysteine causes inter-cross-linking and intra-cross-linking of peptides. Thus, the libraries (2.8×10^8 hexapeptides) are composed of 19×6 pools. Each pool contains 2.5×10^6 sequence diversities. The general patterns of inhibition of the binding of VEGF to VEGFR1 by PS-SPCL are shown (Fig. 1), and the amino acids at each position of the hexapeptide library that show a significant contribution to the inhibition are listed (black columns with a single letter code in Fig. 1 and the single letter codes of amino acids in parenthesis in Table 1). Two to four candidate amino acids were selected for each position for subsequent synthesis of secondary library.

As shown in Table 1, 24 subpeptide library pools (T_1-T_{24}) were synthesized by combinations of the candidate amino acids, and each pool was tested for its effect on the binding of $VEGF_{121}$ to its receptor again (data not shown). Based on the

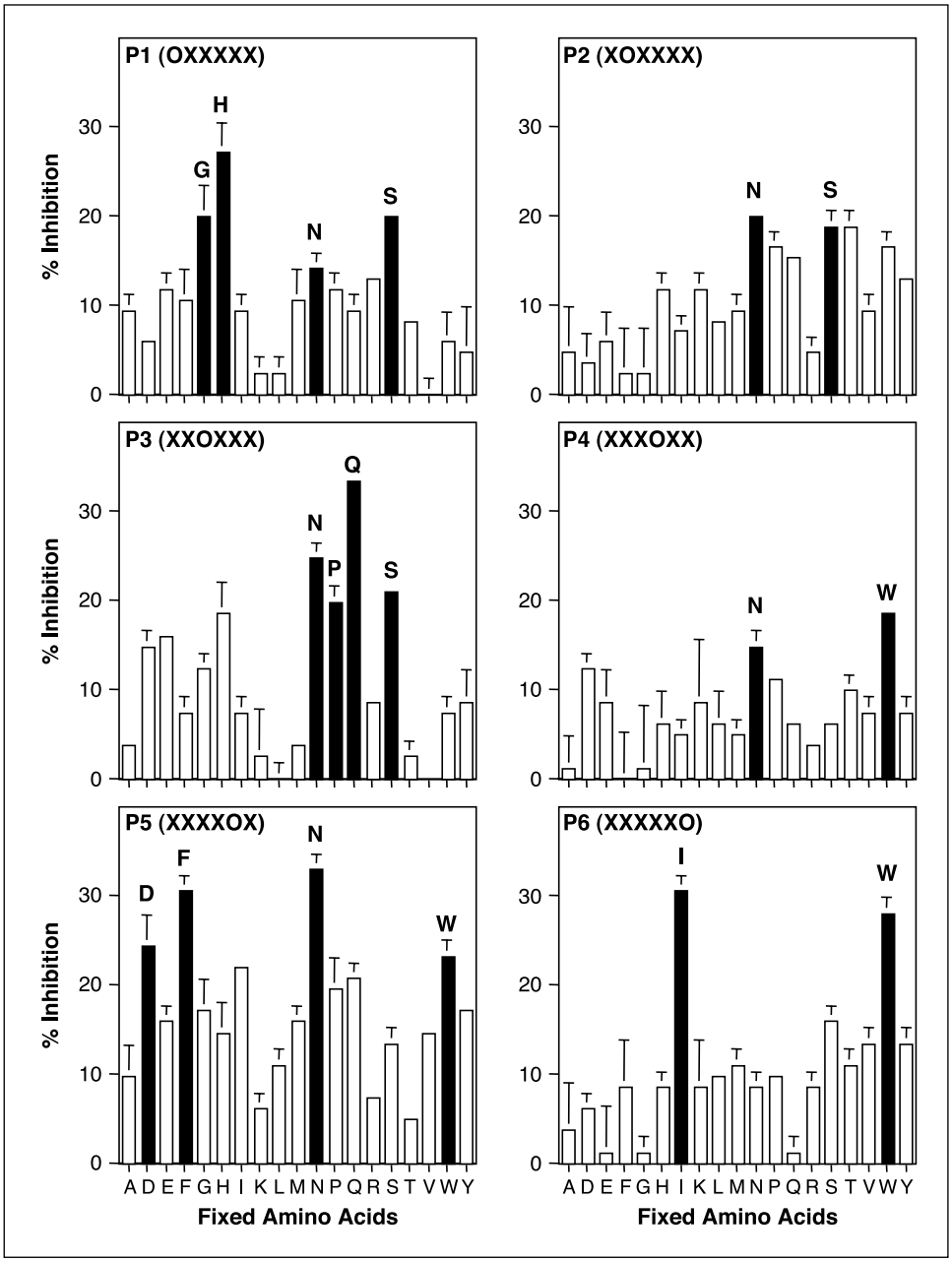


Fig. 1. Initial screening of PS-SPCL for its ability to inhibit the binding of VEGFR1 to coated VEGF. A soluble peptide library, PS-SPCL, was used as the source of an antagonist. X, a mixture of 19 amino acids except cysteine; O, a known amino acid. By assaying 114 pools, the amino acids that are important at each position of active peptides can be determined. Mixtures of VEGFR1 and each library pool (3 nmol/L/sequence) were added to plate wells coated with $VEGF_{121}$, and the binding of VEGFR1 to VEGF was determined. The inhibitory activity of peptide pools (19 for each position) on the VEGF/VEGFR1 interaction was compared for each position. Single-letter codes of amino acids on the *X*-axis indicate the amino acids fixed at each position. *Y*-axis, percent of inhibition of VEGF/VEGFR1 binding. Black columns, amino acids selected for the synthesis of sublibraries.

Table 1. Results of initial library screening and synthesis of 24 sublibrary pools with two defined sequences

Pool name	P1-N (H, G, S, N)*	P2 (N, S)	P3 (Q, N, P, S)	P4 (W, N)	P5 (N, F, D, W)	P6-C (I, W)	Sequence (N → C)
T1	H	N	Mix† of Q, N, P, S	Mix of W, N	Mix of N, F, D, W	Mix of I, W	H N X‡ XXX
T2	G	N					G N XXXX
T3	S	N					S N XXXX
T4	N	N					N N XXXX
T5	H	S					H S XXXX
T6	G	S					G S XXXX
T7	S	S					S S XXXX
T8	N	S					N S XXXX
...	...	...	...	...	...	...	...
T17	Mix of H, G, S, N	Mix of N, S	Mix of Q, N, P, S	Mix of W, N	N	I	XXXX N I
T18					F	I	XXXX F I
T19					D	I	XXXX D I
T20					W	I	XXXX W I
T21					N	W	XXXX N W
T22					F	W	XXXX F W
T23					D	W	XXXX D W
T24					W	W	XXXX W W

NOTE: The most effective candidate amino acids at different positions are shown. H, G, S, and N were selected for NH_2-terminal position 1 (P1-N); N and S for position 2 (P2); Q, N, P, and S for position 3 (P3); W and N for position 4 (P4); N, F, D, and W for position 5 (P5); and I and W for COOH-terminal position 6 (P6-C). Twenty-four sublibrary pools were synthesized by combinations of the selected candidate amino acids (named as T1 to T24). For example, positions 1 and 2 were fixed with one of the candidate amino acids and the rest of the four positions were synthesized with a mixture of candidate amino acids for the T1 sublibrary pool.
*Candidate amino acids for each position from PS-SPCL library screening.
†Positions in which a mixture of candidate amino acids was used for the synthesis.
‡X, positions synthesized with mixtures of amino acids mentioned in the above footnote.

results obtained from the reiterative screening of the original library and subpeptide library pools, the most important amino acid at the second, fourth, and sixth position was determined to be asparagine, tryptophan, and isoleucine, respectively (Table 2). The important amino acids at the first position were those amino acids with small side chains (glycine and serine). The important amino acids for the third position were amide derivatives of acidic amino acids (glutamine and asparagine), and, finally, for the fifth position, aromatic amino acids (phenylalanine and tryptophan) were the most important. These three positions still remain to be further characterized. Therefore, eight defined sequences of hexapeptides were synthesized (anti-Flt1-8; Table 2) and their inhibitory effects were tested at various concentrations (Fig. 2). For all of the eight hexapeptides, their inhibitory effects on the binding of VEGFR1 to $VEGF_{121}$ were saturated in a concentration-dependent manner. The three peptides, anti-Flt1 (GNQWFI), anti-Flt4 (SNNWFI), and anti-Flt5 (GNQWWI), had the successful inhibitory effect among them. The IC_{50} values were within micromolar range. The nonspecific binding of VEGFR1 was determined in the absence of coated VEGF. There was no sequence homology between the amino acid sequences of the selected peptides and those of VEGF and VEGF receptor family members. The most effective anti-Flt1 peptide (GNQWFI, Gly-Asn-Gln-Trp-Phe-Ile) was selected for further characterization.

Table 2. Synthesis of eight hexapeptides with defined sequences

Peptide name	P1-N (G, S)*	P2 (N)	P3 (Q, N)	P4 (W)	P5 (F, W)	P6-C (I)	Sequence (N → C)
Anti-Flt1	G	N	Q	W	F	I	GNQWFI
Anti-Flt2	S	N	Q	W	F	I	SNQWFI
Anti-Flt3	G	N	N	W	F	I	GNNWFI
Anti-Flt4	S	N	N	W	F	I	SNNWFI
Anti-Flt5	G	N	Q	W	W	I	GNQWWI
Anti-Flt6	S	N	Q	W	W	I	SNQWWI
Anti-Flt7	G	N	N	W	W	I	GNNWWI
Anti-Flt8	S	N	N	W	W	I	SNNWWI

NOTE: Based on the results obtained from the reiterative screening of the original library and subpeptide library pools (data not shown), the most important amino acids at the second, fourth, and sixth positions were found to be N, W, and I, respectively, and the sequence of amino acids at the first (G and S), third (Q and N), and fifth (F and W) positions still remain to be further determined. By the combination of the candidate amino acids, eight hexapeptides with defined sequences were synthesized and named as anti-Flt1 to anti-Flt8. The most effective hexapeptide for the inhibition of binding of coated VEGF was anti-Flt1 peptide (GNQWFI, Fig. 2).
*Selected amino acid(s) for each position from the screening of the 24 subpeptide library pools.

The inhibitory effect of anti-Flt1 peptide is sequence specific and anti-Flt1 peptide binds to VEGFR1. Whereas the inhibitory effect of the anti-Flt1 peptide was concentration-dependent, the reverse-ordered peptide (IFWQNG) did not show any inhibitory effect on the VEGF/VEGFR1 interaction (Fig. 3A). Furthermore, 100 μmol/L of the anti-Flt1 peptide showed a nearly complete inhibition of the VEGF/VEGFR1 interaction, but did not show any effect on the VEGF/VEGFR2 interaction (Fig. 3B). Therefore, the inhibitory effect of the

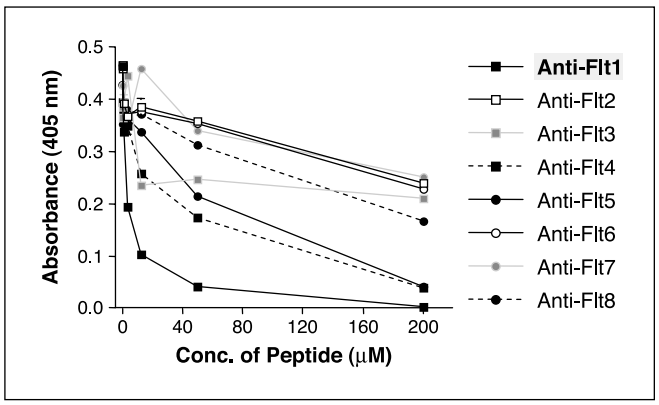


Fig. 2. Effects of eight kinds of anti-VEGFR1 peptides. Coated VEGF was allowed to interact with VEGFR1 in the presence or absence of various concentrations of the respective peptide. Based on the results from the initial and the subsequent screening of the subpeptide libraries (Table 1), eight hexapeptides (anti-Flt1 through anti-Flt8) with defined sequences were synthesized as shown in Table 2. All of the peptides showed concentration-dependent inhibition. Anti-Flt1 was the most effective; the IC_{50} value was 10 μmol/L.

anti-Flt1 peptide seems to be sequence specific and VEGFR1 specific. To investigate if the inhibitory effect of anti-Flt1 is due to a specific binding to VEGFR1, binding of the various recombinant proteins to the anti-Flt1 peptide was determined. Only VEGFR1 bound to the coated anti-Flt1 peptide; other proteins, such as VEGFR2, the Fc fragment (fusion partner), $VEGF_{121}$, and $VEGF_{165}$, did not (Fig. 3C). Anti-Flt1 inhibited the binding of VEGFR1 to both $VEGF_{121}$ and $VEGF_{165}$ (Fig. 3D).

Anti-Flt1 peptide also inhibits binding of other vascular endothelial growth factor family to VEGFR1. To date, several VEGF family members are known to bind to VEGFR1: PlGF, VEGF/PlGF heterodimer, VEGF-B, and VEGF (25–27, 35–37). Therefore, the effects of the anti-Flt1 peptide on the interaction of VEGFR1 with the other VEGFR1-binding VEGF family members were investigated. The anti-Flt1 peptide also showed sequence-specific and concentration-dependent inhibitory activity for binding of VEGFR1 to PlGF (Fig. 4A) and the VEGF/PlGF heterodimer (Fig. 4B). Therefore, the results suggest that VEGF, PlGF, and PlGF/VEGF heterodimer may have a common

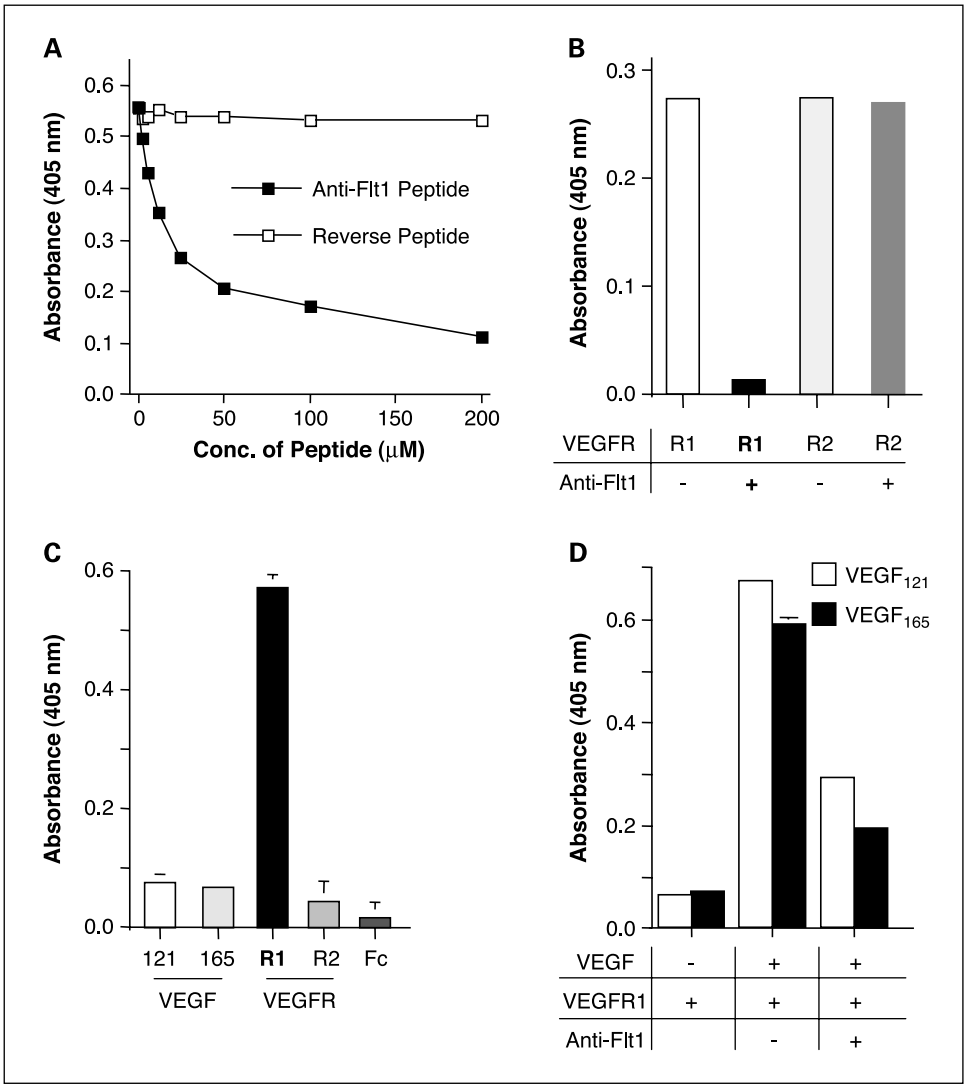


Fig. 3. Anti-Flt1 binds VEGFR1 specifically and inhibits binding of VEGFR1 with various VEGF isoforms. *A*, sequence specificity of the anti-Flt1 peptide. Effect of the anti-Flt1 peptide (GNQWFI, ■) or its reverse-ordered peptide (IFWQNG, □) on the binding of VEGFR1 to immobilized VEGF121 was compared under the same condition described in Fig. 1. *B*, receptor specificity of the anti-Flt1 peptide was examined by comparing the effects of anti-Flt1 (100 μmol/L) on the binding of VEGFR1 and VEGFR2 to $VEGF_{121}$. *C*, binding partner of the anti-Flt1 peptide. Various proteins used in this study were allowed to interact with the immobilized anti-Flt1 peptide, and the bound proteins were detected by an antihistidine antibody. *D*, anti-Flt1 (20 μmol/L) inhibits the binding of VEGFR1 to both the immobilized $VEGF_{121}$ (*white columns*) and $VEGF_{165}$ (*black columns*).

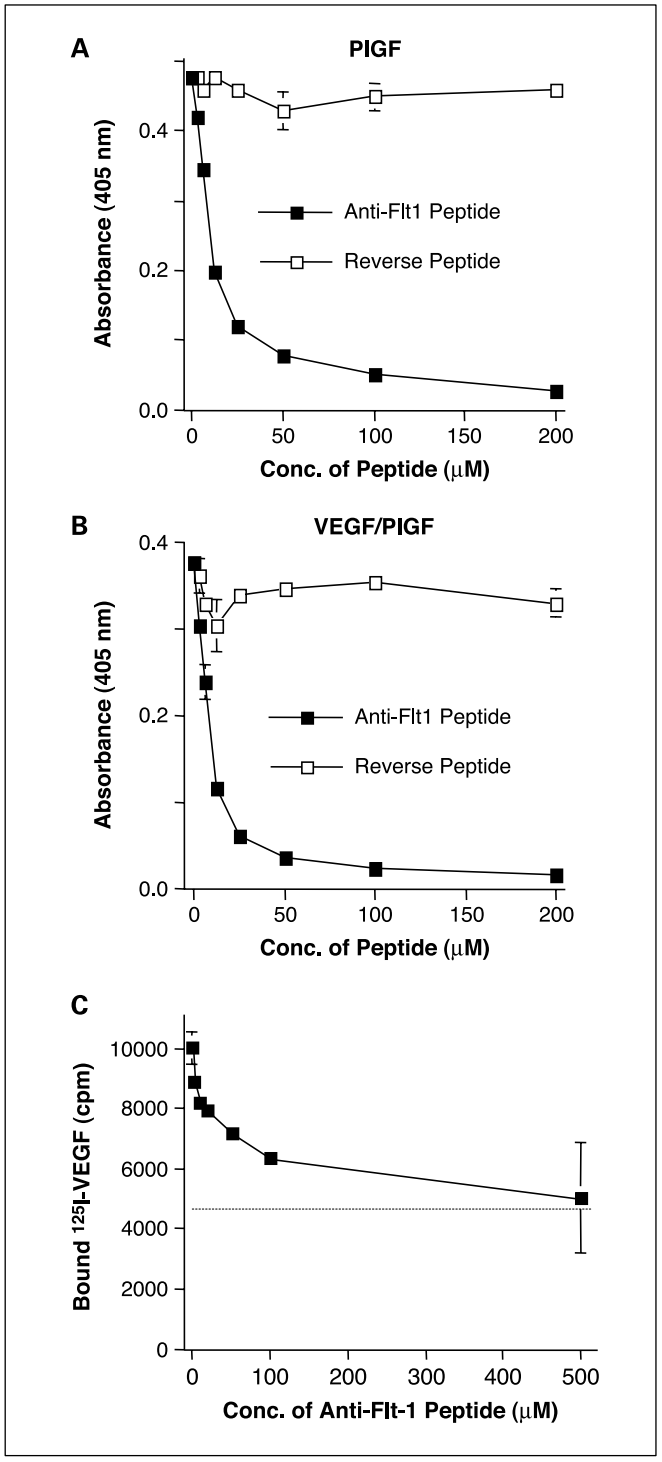


Fig. 4. Anti-Flt1 inhibits the binding of VEGFR1 with PIGF and VEGF/PIGF heterodimer and the binding of [^{125}I]VEGF to HUVE cells. Coated PIGF (*A*) and VEGF/PIGF heterodimer (*B*) were allowed to interact with VEGFR1 in the presence or absence of various concentrations of anti-Flt1 (■) or its reverse peptide (□). *C*, various concentrations of the anti-Flt1 peptide were preincubated with HUVE cells and then [^{125}I]$VEGF_{165}$ was allowed to bind its receptors in the presence of the peptide. The radioactivity of bound [^{125}I]VEGF was determined in a gamma counter.

or at least overlapping binding site on the extracellular domain of VEGFR1. Furthermore, the multiple inhibitory effects of the anti-Flt1 peptide is elicited by direct binding to their common ligand-binding region on the VEGFR1 ectodomain.

Anti-Flt1 peptide inhibits binding of vascular endothelial growth factor to human umbilical vein endothelial cells. We investigated whether the anti-Flt1 peptide also inhibits the binding of VEGF to VEGFR on HUVE cells. There are several VEGF binding sites on endothelial cells, such as VEGFR1,

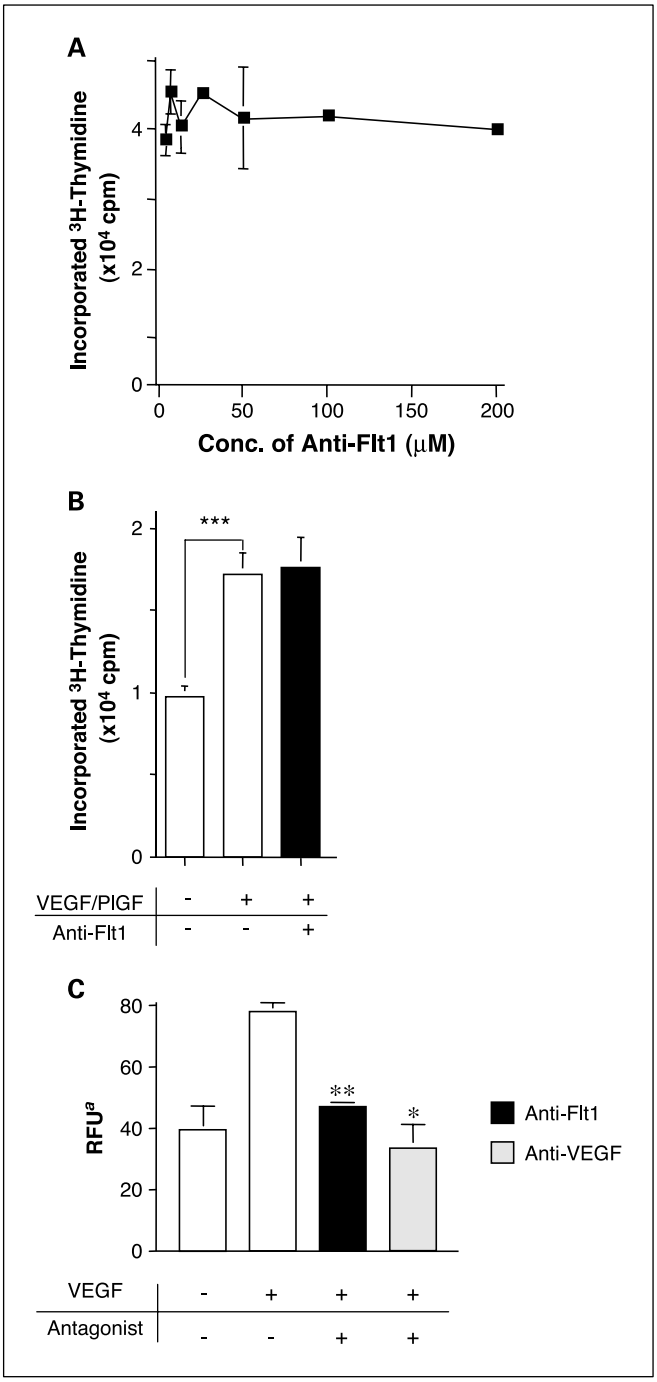
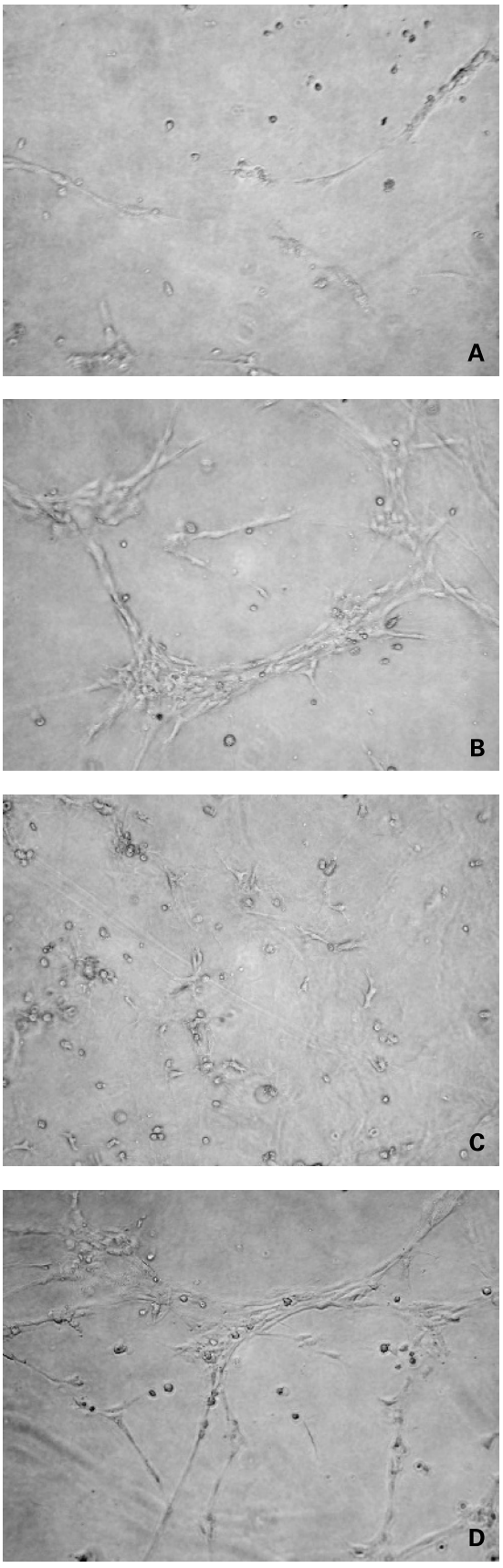


Fig. 5. Anti-Flt1 inhibits VEGF-induced cell migration, but not proliferation. The effects of anti-Flt1 on the VEGF-dependent (*A*) or VEGF/PIGF heterodimer–dependent (*B*) induction of endothelial cell growth (DNA synthesis) and VEGF-induced endothelial cell migration (*C*) were determined as described in Materials and Methods. *A*, data are corrected for the radioactivity incorporated into DNA in the absence of VEGF (24,518 ± 948 cpm). *B*, 200 μmol/L anti-Flt1 was used. *C*, 50 ng/mL VEGF, 100 μmol/L anti-Flt1 or reverse peptide, and 5 μg/mL anti-VEGF neutralizing antibody were used. [a] Relative fluorescence unit (*RFU*).



VEGFR2, neuropilin 1, or more (19, 35, 36), and it is very difficult to monitor the interaction of a ligand with VEGFR1 alone in this complex system. Various concentrations of the anti-Flt1 peptide were preincubated with HUVE cells; then, [^{125}I]VEGF$_{165}$ was allowed to bind to its receptors on the surface of HUVE cells in the presence of the various concentrations of the anti-Flt1 peptide. The inhibitory activity of the anti-Flt1 peptide was concentration dependent, and only 60% of total VEGF binding was observed even at the concentration of 500 μmol/L anti-Flt1 (Fig. 4C). The binding of [^{125}I]VEGF to HUVE cells was further inhibited by the presence of a VEGFR2-specific neutralizing antibody in a concentration-dependent manner (data not shown). These results suggest that the VEGFR1-specific character of anti-Flt1 peptide verified with purified recombinant proteins (Fig. 3B and C) may still be true in an endothelial cellular context.

Anti-Flt1 peptide does not inhibit VEGF– and VEGF/PlGF–induced endothelial cell proliferation, but effectively inhibits VEGF-induced endothelial cell migration. VEGF induces pro liferation, migration (chemotaxis), and angiogenesis *in vitro* and *in vivo* (1, 35, 36). It was investigated whether the anti-Flt1 peptide inhibits the VEGF-induced DNA synthesis in HUVE cells. As shown in Fig. 5A, the anti-Flt1 peptide (up to 200 μmol/L) did not inhibit the proliferation of HUVE cells induced by VEGF. The same result was observed in the case of VEGF/PlGF heterodimer–induced HUVE cell proliferation (200 μmol/L anti-Flt1; Fig. 5B). However, the anti-Flt1 peptide effectively blocked VEGF-induced HUVE cell migration through a membrane with 8 μm pore size (Fig. 5C). Therefore, the anti-Flt1 peptide does not inhibit the mitogenic effect of VEGF, which is the result of VEGFR2 signaling (20, 21); rather, it inhibits VEGF-induced endothelial cell migration.

Anti-Flt1 peptide inhibits vascular endothelial growth factor–induced in vitro angiogenesis. The central aspects of VEGF action on endothelial cells are its ability to act as a chemoattractant and to stimulate the angiogenic morphogenesis of endothelial cells (*in vitro* angiogenesis; refs. 35, 36). VEGF (50 ng/mL) effectively induced *in vitro* angiogenesis of endothelial cells on fibrin gel (Fig. 6B). The anti-Flt1 peptide completely inhibited VEGF-induced angiogenesis, but the reverse sequence did not (Fig. 6C and D). It was also reported that a repressor sequence in the juxtamembrane of VEGFR1 is related to endothelial cell migration. Thus, it is possible that VEGFR1 signaling is essential or, at least, required for VEGF-induced endothelial cell migration and *in vitro* angiogenesis among various VEGF activities. It is also possible that VEGFR1 signaling is not essential for endothelial cell proliferation, as also reported by others, such as receptor-selective VEGF variants, anti-VEGF or anti-VEGFR1 neutralizing antibodies, and the juxtamembrane domain of VEGFR1 (20, 21, 32, 38, 39).

Anti-Flt1 peptide inhibits **tumor growth and metastasis in nude mice.** Endothelial cell migration and tube formation are required for the completion of the angiogenic process, and the acquisition of an angiogenic phenotype is considered decisive

Fig. 6. Anti-Flt1 inhibits VEGF-induced *in vitro* angiogenesis of endothelial cell. *In vitro* angiogenesis of the endothelial cells grown on the fibrin gel was induced by VEGF in the presence or absence of anti-Flt1, as described in Materials and Methods. Four independent experiments were carried out and the results were similar: no VEGF (*A*), recombinant human VEGF (50 ng/mL; *B*), and recombinant human VEGF (50 ng/mL) plus 100 μmol/L anti-Flt1 peptide (*C*) or its reverse peptide (*D*).

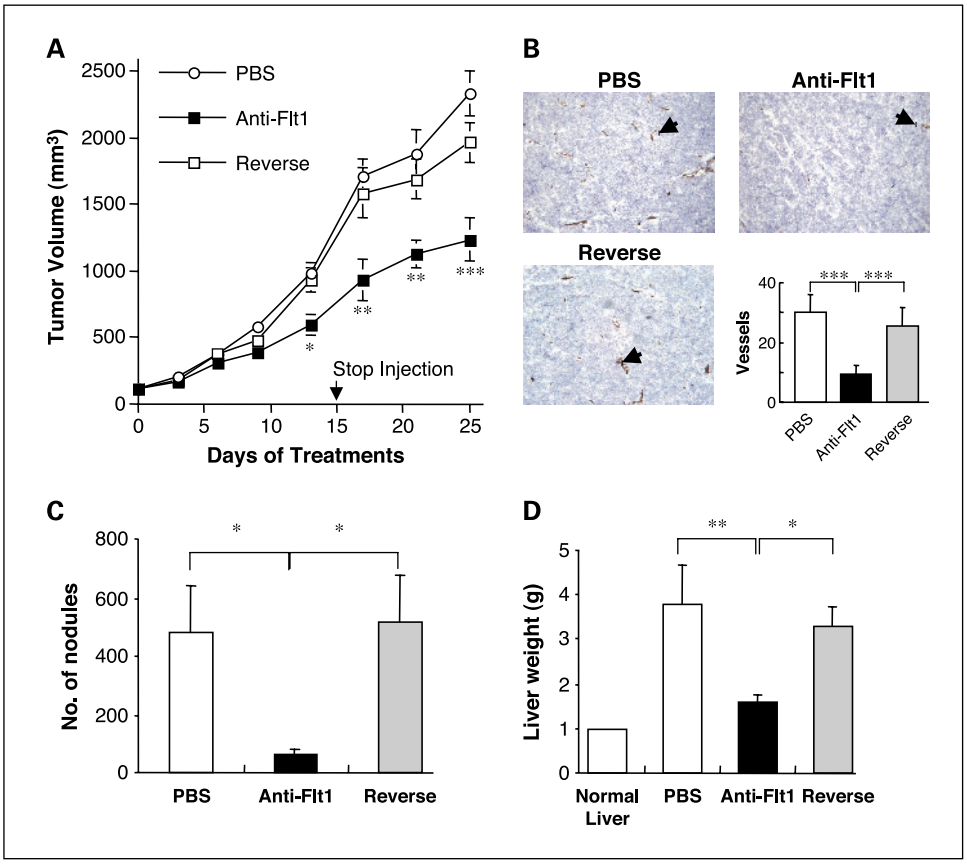


Fig. 7. Anti-Flt1 inhibits the growth and metastasis of VEGF-secreting tumor cells in nude mice. *A*, SW480 human colon cancer cells were s.c. transplanted into nude mice. After the establishment of a solid tumor, mice received a s.c. injection of each treatment [saline solution (PBS; ○), 100 μg reverse peptide (□), or anti-Flt1 peptide (■)] per day for 15 days. Tumor volumes were determined regularly up until the 25th day. *B*, immunohistochemisty of tumor sections for angiogenesis. On 10 days after each treatment from A, histologic sections were prepared as described in Materials and Methods. Tumor vessels were stained with anti-CD31 antibody and vessel density was determined by counting the number of capillary blood vessels per high-power field. *C* and *D*, HM7 cells (10^6 cells) were injected into the spleen of nude mice, and the mice received s.c. injections of each treatment [saline solution (PBS; □), 100 μg reverse peptide (⊠), or anti-Flt1 peptide (■)] per day for 2 weeks. On the 26th day, the livers were removed. *C*, metastatic nodules; *D*, weight of the liver.

for tumor progression (40). Therefore, we have further investigated whether the anti-Flt1 peptide as a VEGFR1-specific antagonist is able to inhibit growth and metastasis of VEGF-secreting tumor cells *in vivo*. VEGF-secreting cell lines, SW480 and HM7 human colon carcinoma cells, were transplanted into nude mice. After the establishment of solid tumors (tumor volume = ~110 mm^3), the anti-Flt1 peptide was s.c. injected at a dose of 100 μg/d for 15 days. The anti-Flt1 treatment significantly inhibited the expansion of SW480 cells (Fig. 7A) in a sequence-specific manner because the reverse sequence showed no effect on the growth of the tumor cells. Similar results were also obtained with HM7 cells (data not shown). With histologic sections of the SW480 tumors grown in nude mice, the effect of anti-Flt1 peptide on tumor angiogenesis was verified by immunohistochemical analysis with an endothelial cell–specific surface marker (CD31). As determined by the number of CD31-stained microvessels (Fig. 7B), tumor angiogenesis was found to be significantly suppressed in the anti-Flt1–treated mice versus control (3.2-fold) mice or mice treated with reverse anti-Flt1 sequence (2.8-fold), respectively.

Activation of angiogenesis is also responsible for the increased tumor cell entry into circulation and metastasis. According to our previous results, halting angiogenesis by the blocking of VEGF signaling suppresses tumor metastasis (40). Here, we investigated whether the anti-Flt1 peptide also blocks metastasis of malignant human colon carcinoma cells, HM7, from the spleen to liver in mice. Anti-Flt1, at 100 μg/d, reduced the number of metastatic nodules (Fig. 7C) and liver weight (Fig. 7D). The reverse peptide did not show inhibitory effects at the same dose. The anti-Flt1 peptide did not show any direct cytotoxic effects on the cancer cells and endothelial cells (data not shown). Therefore, the antimigratory and antiangiogenic activities of this peptide may be responsible for the inhibition of tumor growth and metastasis in animals by suppression of tumor angiogenesis, thus suggesting a crucial role of VEGFR1 signaling in angiogenesis and tumor growth and metastasis *in vivo*.

Discussion

Although the exact role of VEGFR1 still remains to be elucidated, VEGFR1 is involved in various pathologic angiogenesis processes in adults, and a tumor cannot grow and metastasize without suitable angiogenesis (25, 29–32, 40). Here, we investigated the therapeutic potential of a VEGFR1-specific antagonist for cancer treatment. We also tried to define the role(s) of VEGFR1 with the antagonist. The VEGFR1-selective hexapeptide anti-Flt1 (Gly-Asn-Gln-Trp-Phe-Ile, GNQWFI) was identified from the screening of PS-SPCL. It was found that the anti-Flt1 peptide specifically binds to VEGFR1 and inhibits the interaction of VEGF only with VEGFR1 with sequence specificity.

Coordinated movement (cell migration), growth, and differentiation (tube formation) of endothelial cells are essential prerequisites for angiogenesis (36) and angiogenic switch is one of the most important pathologic events during tumorigenesis (40). The anti-Flt1 peptide blocks VEGF-induced endothelial cell migration and *in vitro* angiogenesis (Figs. 5C and 6). Furthermore, together with suppression of microvessel density in tumors, the anti-Flt1 peptide effectively inhibits the growth and metastasis of VEGF-secreting cancer cells *in vivo*

(Fig. 7). Because there was no direct cytotoxicity against the cancer cells and endothelial cells *in vitro* (data not shown), it is possible that the antitumor activity of the anti-Flt1 peptide is caused by reduced angiogenesis because of the blocking of migration and tube formation induced by VEGF.

During development, the primary function of VEGFR1 is a nonsignaling "reservoir" for VEGF (3, 5, 22). However, what is the role of VEGFR1 in adult stage? A recent study showed that the monoclonal VEGFR1 antibody suppress neovascularization in various diseases including tumors and autoimmune arthritis (32). Our results (Figs. 5-7), as reported by others (20, 21, 32, 38, 39), suggest that VEGFR1 signaling is not responsible for VEGF-induced cell proliferation but is essential for other VEGF activities, such as endothelial cell chemotaxis and tube formation. VEGF signaling also plays a positive role in tumor growth and metastasis.

Other VEGFR1-specific VEGF family members, such as PlGF, VEGF/PlGF heterodimer, and VEGF-B are also responsible for various angiogenesis-related events in pathologic conditions (26, 27, 30, 32, 37). The PlGF/VEGFR1 system, while affecting vascular development, is not essential for physiologic angiogenesis during development and reproduction, but it impairs angiogenesis in various pathologic conditions (41, 42). Therefore, it is possible that PlGF and VEGFR1 are, collectively, one of the key regulators of the angiogenic switch. Additionally, the inhibition of VEGFR1 can improve various disease conditions, such as ischemia, inflammation, and cancer. In this study, we found that the anti-Flt1 peptide inhibits the interaction of not only VEGF but also PlGF and VEGF/PlGF heterodimer with VEGFR1 (Figs. 3A and 4A, B). Therefore, it is possible that the anti-Flt1 peptide masks a region of the VEGFR1 ectodomain to which various VEGFR1 ligands interact. The anti-Flt1 peptide can be utilized not only as a lead for the development of an anticancer drug, but also as a useful tool for studies investigating the interactions of VEGFR1 with other VEGFR1 ligands, PlGF, VEGF/PlGF heterodimer, or VEGF-B.

When compared with several known VEGFR1 antagonists for the treatment of angiogenesis-dependent diseases, such as anti-VEGFR1 monoclonal antibodies or RNA antagonists (31, 32, 43), the anti-Flt1 peptide would be advantageous for use from a clinical point of view because it is a short peptide that is easily synthesized by chemical methods and it has low immunogenicity.

In conclusion, the data presented here shows (*a*) the roles of VEGFR1 in endothelial cell migration and *in vitro* angiogenesis as well as positive roles in tumor growth and metastasis and (*b*) an ability of the VEGFR1-specific anti-Flt1 peptide to block tumor growth and metastasis. Moreover, our results give the opportunity to investigate the roles of VEGFR1 in the progress of various angiogenesis-dependent diseases triggered by VEGF and other VEGF family members. Work is in progress to improve the activity of the anti-Flt1 peptide by modifying the structure or length of the peptide.

Acknowledgments

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