Arginine-rich Anti-vascular Endothelial Growth Factor Peptides Inhibit Tumor Growth and Metastasis by Blocking Angiogenesis*

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Tumor angiogenesis is a critical step for the growth and metastasis of solid tumors. Vascular endothelial growth factor (VEGF) is a specific and potent angiogenic factor and contributes to the development of solid tumors by promoting tumor angiogenesis. Therefore, it is a prime therapeutic target for the development of antagonists for treatment of cancer. We identified from peptide libraries arginine-rich hexapeptides that inhibit the interaction of VEGF165 with VEGF receptor (IC50 = 2–4 μM). They have no effect on binding of basic fibroblast growth factor to cellular receptor. The hexapeptides inhibit the proliferation of human umbilical vein endothelial cells induced by VEGF165 without toxicity. The peptides bind to VEGF and inhibit binding of both VEGF165 and VEGF121, suggesting that the peptides interact with the main body of VEGF but not the heparin-binding domain that is absent in VEGF121. The identified peptides block the angiogenesis induced by VEGF165 in vivo in the chick chorioallantoic membrane and the rabbit cornea. Furthermore, one of the hexapeptides, RRRRRR, blocks the growth and metastasis of VEGF-secreting HM7 human colon carcinoma cells in nude mice. Based on our results, the arginine-rich hexapeptides may be effective for the treatment of various human tumors and other angiogenesis-dependent diseases that are related to the action of VEGF and could also serve as leads for development of more effective drugs.

Angiogenesis, the formation of new blood vessels from established vessels, occurs under a variety of normal and pathological conditions. In physiological states such as embryonic development of vascular system, wound healing, and the female reproductive system, neovascularization is turned on for a brief periods and then completely inhibited by a balance of stimulatory and inhibitory factors (1). These finely tuned controls may fail and result in the induction of normal blood vessels to grow at accelerated rate in a harmful way during the development of many diseases including solid tumor growth and metastasis, rheumatoid arthritis, diabetic retinopathy, and various inflammatory disorders (2–4). Angiogenesis is especially essential for the growing tumor because the delivery of blood-borne nutrients to the tumor cells is essential for their survival (5) and for the spread of metastatic tumor cells because the tumor cells come into contact with an increased concentration of blood vessels (6). Induction of angiogenesis precedes the formation of malignant tumors (5). Therefore, angiogenesis could be a rate-determining step not only for tumor expansion but also for the onset of malignancy.

Numerous angiogenic factors have been identified, but many of these factors possess only very little or no direct mitogenicity on vascular endothelial cells (7). In contrast, VEGF is a potent endothelial cell-specific mitogen in vitro (8), enhancing vascular permeability and stimulating angiogenesis in vivo (9, 10) associated with tumor progression. VEGF is a heparin-binding growth factor that has a homodimeric structure and limited sequence homology to the platelet-derived growth factor family (11, 12) and placental growth factor (13). VEGF is produced in four isoforms having 121, 165, 189, and 206 amino acids by alternative splicing (14), and these isoforms form active disulfide-linked homodimers. Among them, only VEGF121 does not have the heparin-binding domain encoded by exon 7 of VEGF gene (11). VEGF exerts its activity through binding to its identified receptors, Flt-1 and KDR/Flk-1, selectively expressed on the surface of endothelial cells (15, 16). Although KDR/Flk-1-deficient mice lack any mature endothelial cells (17), Flt-1 knockout mice are found to contain mature endothelial cells but poorly organized tube formation and no functional blood vessels (18).

The significance of VEGF in vasculogenesis and angiogenesis has been demonstrated by gene deletion studies of VEGF (19, 20) and VEGF-Rs (17, 18), by their enhanced tumorigenic behaviors caused by forced overexpression of VEGF in tumor cells (21), by blockade of tumor growth by neutralizing anti-VEGF monoclonal antibodies (22), and by experiments where tumor growth was blocked by evoking the expression of sFlt (23) and dominant-negative KDR/Flk-1 on cells in the vicinity of growing tumors (24). Thus, the VEGF/VEGF receptor system is an attractive target for inhibition of tumor angiogenesis, tumor growth, and metastasis (25).

The information available to date suggests that direct, structure-based rational design of small molecule inhibitors would be difficult to succeed. However, the development of small molecule drug candidates from combinatorial library of small peptides has proven remarkably successful (26, 27). In this study, we have identified from soluble hexapeptide libraries arginine-rich peptides that can block the interaction between VEGF165 and the VEGF receptors on the surface of endothelial cells. The arginine-rich hexapeptides inhibit VEGF-induced angiogenesis.

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial; CAM, chorioallantoic membrane; hFGF, basic fibroblast growth factor; PS-SPCL, Positional Scanning-Synthetic Peptide Combinatorial Library; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; AR, arginine-rich.
proliferation of human umbilical vein endothelial (HUVE) cells by direct interaction with VEGF165. The inhibition is concentration-dependent, and the peptides do not have any cytotoxic effect on HUVE cells. Furthermore, the basic peptides show significant inhibition of angiogenesis induced by VEGF in the chorioallantoic membrane (CAM) and the rabbit cornea neovascularization assays. They also inhibit growth and metastasis of VEGF-secreting HM7 human colon carcinoma cells in nude mice without cytotoxicity against the cells. These studies show that the peptides can be used for the treatments of various angiogenesis-dependent diseases including growth and metastasis of human tumors. The peptides will also serve as lead compounds for development of more effective anti-angiogenic molecules.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fetal bovine serum, bovine calf serum, antibiotics, medium 199, and Dulbecco's modified Eagle's medium were from Life Technologies, Inc. Tissue culture dishes and plastic-wares were obtained from Falcon Plastics. Tissue culture trays (24-well) were from Costar. Recombinant human VEGF was from R & D Systems or purified from Escherichia coli. Bovine basic fibroblast growth factor (bFGF) was from Roche Molecular Biochemicals. 125I-VEGF165 (2057 Ci/mmol), 125I-bFGF (1200 Ci/mmol), Na235I, and methyl-[2H]-thymidine (76 Ci/mmole) were obtained from Amersham Pharmacia Biotech. Fertilized chicken eggs (day 0) were from Pullmuone Co. Thermanox coverslip (13 mm diameter) were from Nunc. All the other reagents and chemicals were from Sigma. All the materials used in this report were verified as analytical grade.

**Positional Scanning-Synthetic Peptide Combinatorial Library**—The peptide library (PS-SPCL) was synthesized at the Peptide Library Support Facility of Pohang University of Science and Technology in Korea, according to the method suggested by Finilla et al. (28).

**Cell Culture**—HUVE cells (Clonetics) were cultured as described previously (29) with some modifications. Briefly, HUVE cells were grown and maintained in gelatin-coated dishes in medium 199 supplemented with 20% bovine calf serum, 5 μg/ml of heparin, 6 μg/ml of endothelial cell growth supplement, 5 mg/ml of bFGF, and antibiotics. HM7 human colon carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal bovine serum. All cultures were grown at 37 °C in a humidified atmosphere of 5% CO2/95% O2, passages every 2–3 days (28), and experiments with HUVE cells were carried out between passages 5 and 9.

**Binding of 125I-VEGFs and 125I-bFGF to Their Receptors on the Surface of HUVE Cells**—HUVE cells expressing VEGF and bFGF receptors were seeded at a density of 5 × 104 cells/well in gelatin-coated 24-well plates, incubated at 37 °C for 24 h, washed with warm binding buffer containing 25 mM HEPES, pH 7.4, 0.1% bovine serum albumin (BSA) in serum-free medium 199 (180 mM of salt), and incubated at 37 °C for 2–3 h. The cultures were then transferred to 4 °C on an oscillating platform set at 1 cycles/s. Various concentrations of radiolabeled VEGF or bFGF were added in 200 μl of binding buffer, and the binding was allowed to proceed at 4 °C for 3 h. Nonspecific binding was determined by binding experiments in the presence of 100-fold excesses of nonlabeled VEGF or bFGF. At the end of the incubation, the cells were washed twice with cold binding buffer followed by washing in cold PBS containing 0.1% BSA. Subsequently, the cells were solubilized by the addition of 0.5 ml of 20 mM Tris-HCl, pH 7.4, containing 1% Triton X-100 at room temperature for 20 min on an oscillating platform set at 2 cycles/s, and the receptor-bound radioactivity was determined in a γ counter. All experiments were carried out in duplicate and at least twice. The variation between duplicate determinations in an experiment was less than 10%.

**Screening of Peptide Libraries**—For screening of peptide libraries, the procedures described for the binding of 125I-VEGF165 to HUVE cells were followed. After washing HUVE cells in 24-well plates with warm binding buffer, 0.2 ng of 125I-VEGF165 (Amersham Pharmacia Biotech) and various concentrations of peptide pools or peptides were mixed in 200 μl of binding buffer with or without an excess of nonlabeled VEGF165 (20 ng/well) and preincubated at 4 °C for 30 min. The binding was allowed to proceed at 4 °C for 3 h. The peptide pools or peptides that showed inhibitory effect on the binding of 125I-VEGF165 to its receptors on the surface of HUVE cells were determined.

**Binding of 125I-VEGF165 to Immobilized Peptides—Hexapeptide (100 ng/well) in 20% acetic acid was added to each well of enzyme-linked immunosorbent assay plate and dried overnight. The wells were washed and blocked three times with PBS containing 0.1% BSA at room temperature. To each well was added 125I-VEGF165 (0.2 ng/well) in 50 μl of PBS containing 0.1% BSA with or without an excess of nonlabeled VEGF165 (500 ng/well) or various amounts of incubation for 1 h at 37 °C, each well was washed with PBS containing 0.1% BSA at room temperature four times, and the bound 125I-VEGF165 was solubilized with 150 μl of 0.1 N NaOH. The bound radioactivity was determined in a γ counter.

**Binding of Peptide to Heparin**—To investigate the interaction of Receptor, Physiological salt concentration was determined in a liquid scintillation counter. All experiments were carried out in triplicate and at least twice.

**Cell Proliferation Assay**—Growth assay of HUVE cells was carried out according to the procedure described by Kondo et al. (31) with some modifications. HUVE cells were seeded in a gelatin-coated 48-well culture plate at a density of 104 cells/well in 300 μl of the growth medium, and incubated at 37 °C for 2 days. The plate was washed three times with warm, serum-free medium 199, and incubated in 200 μl of serum-free medium 199 containing 10 ng/ml of VEGF and various concentrations of hexapeptide for a day. Thereafter, 10 μl of methyl-[2H]-thymidine (0.5 μCi) was added to each well, and the cells were incubated for a day. Cells were washed four times with PBS containing 0.1% BSA, solubilized with 150 μl of 0.4 N NaOH at room temperature for 20 min, and then neutralized with 30 μl of 2.0 N HCl. The radioactivity was determined in a liquid scintillation counter. All experiments were carried out in triplicate and at least twice.

**CAM Assay**—The CAM assay has been extensively used to study the effect of angiogenic and antiangiogenic compounds in vivo (32). Fertilized chicken eggs were incubated at 37 °C in a humidified air and designated as day 0. Ovalbumin (2 ml) was removed from each egg on
Tumor cells were grown to confluency in 75-cm² tissue culture flasks, the hepatic portal system was tested in a splenic injection model (34). Tides on colonization of colon cancer cells in the liver after entry into the bloodstream were considered statistically significant.

Confluent HM7 human colon cancer cell cultures were harvested by brief trypsinization (0.05% trypsin and 0.02% EDTA in Hank's balanced salt solution without calcium and magnesium), washed three times with calcium and magnesium-free phosphate-buffered saline solution, and resuspended at a final concentration of 5 × 10⁶ cells/ml in serum-free Dulbecco's modified Eagle's medium. The presence of single-cell suspensions was confirmed by a phase-contrast microscopy, and cell viability was determined by Trypan blue exclusion, and only single-cell suspensions of ≥90% viability were used. Equal volumes of normal physiologic saline or each of the two peptides (EEFDFA or RRKKRR) at a concentration of 0.5 μg/ml were mixed with the cell suspensions. 5 × 10⁶ viable tumor cells containing normal saline or each of the peptides in 100 μl were injected subcutaneously over the right scapular region of pathogen-free, 4-week-old male athymic nude mice (BALB/c nu/nu). From the following day, mice received subcutaneous injection of each of the peptides (50 μg/ml) until 15 days after tumor cell implantation. Mice were surveyed regularly, and tumors were measured with a caliper, and volumes were determined using the following formula: volume = 0.5 × (width)² × length. Each experimental group consisted of six to seven animals, and a p of <0.05 was considered statistically significant.

Anti-VEGF Peptides Inhibit Tumor Growth and Metastasis

To determine if anti-VEGF peptides inhibited tumor growth and metastasis, male New Zealand White rabbits weighing 3 kg were used for corneal neovascularization experiment. Intramuscular ketamine anesthesia (44 mg/kg) was supplemented by retrobulbar infiltration with 2% lidocaine. The eye was moved forward and secured in position by fingers. With a Bard-Parker #11 blade, a superficial incision 3 mm long was made on the corneal dome to one side of its center. The incision was then continued down into, but not through, the cornea. A #15 blade was inserted and an oblong pocket was fashioned within the corneal stroma, and then the peripheral pockets ended 3 mm from the limbus. Recombinant human VEGF₁₆₅ (10 ng, R & D Systems) with or without 1 μg of EEFDFA or RRKKRR was added on the sterile 3.0-mm-diameter thermoxan disc, and it was dried overnight in the clean bench. The disc was deposited in the center of each pocket, which is then sealed spontaneously. All procedures were performed under sterile condition. Sixteen days later, when the neovascularization was prominent, the corneal vessels were photographed with photo slit lamp (Nikon, FS-2, Japan). Six animals were used for each group.

Tumor Growth Inhibition Assay—Confluent HM7 human colon cancer cell cultures were harvested by brief trypsinization (0.05% trypsin and 0.02% EDTA in Hank's balanced salt solution without calcium and magnesium), washed three times with calcium and magnesium-free phosphate-buffered saline solution, and resuspended at a final concentration of 5 × 10⁶ cells/ml in serum-free Dulbecco's modified Eagle's medium. The presence of single-cell suspensions was confirmed by a phase-contrast microscopy, and cell viability was determined by Trypan blue exclusion, and only single-cell suspensions of ≥90% viability were used. Equal volumes of normal physiologic saline or each of the two peptides (EEFDFA or RRKKRR) at a concentration of 0.5 μg/ml were mixed with the cell suspensions. 5 × 10⁶ viable tumor cells containing normal saline or each of the peptides in 100 μl were injected subcutaneously over the right scapular region of pathogen-free, 4-week-old male athymic nude mice (BALB/c nu/nu). From the following day, mice received subcutaneous injection of each of the peptides (50 μg/ml) until 15 days after tumor cell implantation. Mice were surveyed regularly, and tumors were measured with a caliper, and volumes were determined using the following formula: volume = 0.5 × (width)² × length. Each experimental group consisted of six to seven animals, and a p of <0.05 was considered statistically significant.

Results

In this study we attempted to identify the peptide sequences that inhibit binding of VEGF to the cell surface receptor from mixtures of random sequences of peptide (peptide library). The identified peptides would then be investigated for their effect on the VEGF-induced proliferation of endothelial cells and angiogenesis. The peptide library used in this study was PS-SPCL originally developed by Houghten and his group (28). 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. By assaying 120 pools, the amino acids important at each position of active peptides can be determined. Based on this information, reiterative synthesis of peptides can be conducted to identify the sequences of most active peptides.

Establishment of the Assay for Binding of VEGF₁₆₅ to Endothelial Cells—With the conditions described under “Experimental Analysis—Values of results are expressed as the means and standard deviation of the mean, and significance is established by Student's t test. In all analyses, the level of statistical significance was more than 95% confidence level (p < 0.05).

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Anti-VEGF Peptides Inhibit Tumor Growth and Metastasis

The most effective candidate amino acids at different positions are shown. The amino acids with decreasing activity are written from the top of each column. Synthesis of 12 hexapeptides with defined sequences were based on all of the screening results of original library and subpeptide library pools. The effect of each pool is shown as 50% inhibitory concentration (IC50).

| Amino acids at each position | Peptide number |
|----------------------------|---------------|
|                            | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1 (N) | R | R | R | R | R | R | R | R | R | R | R | R |
| 2 | R | K | R | R | R | R | K | R | R | R | R | R |
| 5 | K | R | K | R | K | R | K | R | R | R | R | R |
| 6 | H | R | R | R | R | R | R | R | R | R | R | R |
| IC50 (µM) | 3.4 | 6.0 | >20 | 8.5 | 4.5 | >20 | 6.5 | 2.0 | >20 | 7.8 | 3.8 | >20 |

The peptides composed of arginines and lysines showed no inhibitory activity for the binding of 125I-VEGF165 to HUVE cells grown in a 24-well plate. There are two types of binding sites, one with dissociation constant (KD) of 3 pM (~2000 sites/cell) and the other with KD of 50 pM (~6000 sites/cell). The results are in agreement with those reported by others (30, 35–38). The binding of 125I-VEGF165 is saturable and abolished by the presence of an excess amount of nonlabeled VEGF. Nonspecific binding was less than 5%.

Identification of Inhibitory Peptides from Peptide Libraries—

Subconfluent HUVE cells were incubated with 125I-VEGF and various concentrations of each peptide pool or 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- and intra-cross-linking of peptides. Thus, the libraries (2.8 × 104 hexapeptides) are composed of 19 × 6 pools in which each pool contain about 2.5 × 106 diversities. Fig. 1 shows the general pattern of inhibition of the binding of VEGF to its receptor by PS-SPCL, and the amino acids at each position of the hexapeptides that show significant contribution to the inhibition are listed in Table I. Lysine and arginine showed dominant inhibitory activity at all six positions.

A subpeptide library was prepared with the amino acids listed in Table I, and each pool was tested for its effect on binding of VEGF to its receptor. Based on the results obtained from the screening of original library and subpeptide library pools, we found that the most important amino acid at the first, fourth, and sixth positions was arginine, and the sequences of amino acids at the second (arginine, lysine, and histidine), third (lysine and arginine), and fifth (lysine and arginine) positions still remained to be determined (Table I). Therefore, 12 defined sequences of hexapeptide (1 × 3 × 2 × 1 × 1 × 2) were synthesized (Table I) and purified with C18 reverse phase HPLC column. Amino acid sequence of each peptide was determined by amino acid composition analysis. Inhibitory effects of the 12 hexapeptides were tested at concentrations of 1, 4, 10, and 20 µM/hexapeptide (Fig. 2A). Three peptides RRKRRK, RKRRKR, and hexa-arginine were found to be most effective for inhibition of binding of 125I-VEGF165 to the receptor (boxed numbers in Fig. 2A). The IC50 values were 2 µM for RRKRRR, 3.4 µM for RKRRKR, and 3.8 µM for hexa-arginine (Table I). To investigate whether the results reflect requirement of positive charges rather than specific amino acids, we tested effect of hexa-lysine on the binding of labeled VEGF165 to endothelial cells. Hexa-lysine at a high concentration showed no inhibitory effect (10 µM in Fig. 2B and 100 µM; data not shown).
the three-dimensional structure of VEGF-RRKRRR peptide complex, we prepared VEGF_8–109. It was reported that the N- and C-terminal regions of VEGF interfere with the elucidation of crystal structure of VEGF, and the crystal structure of VEGF_8–109 was reported (41). However, the AR peptide does not inhibit binding of VEGF_8–109 to the cell surface receptor (Fig. 4E). The peptide also does not inhibit the binding of VEGF_8–121 (Fig. 4C) and VEGF_109 (Fig. 4D) to the receptor. The combined results suggest that the AR peptide inhibits the binding of VEGF to the cell surface receptor independent of the heparin-binding domain and may bind to a region of VEGF that involves interaction of both ends of VEGF_121 polypeptide.

The AR Hexapeptides Inhibit the Cell Proliferation Induced by VEGF_165—VEGF induces proliferation of HUVE cells (30, 31, 37), vascular permeability (12, 42), and neovascularization in the chick CAM assay (30, 42, 43). It was investigated whether the AR peptides inhibit the VEGF-induced DNA synthesis in HUVE cells. As shown in Fig. 5, the AR peptides inhibited the proliferation of HUVE cells induced by VEGF_165 in a concentration-dependent manner, and the IC₅₀ values were ~10–20 μM. To exclude the possibility that the inhibition might be caused by direct cytotoxicity of the peptides, HUVE cells were exposed to the peptides for 24 h in the absence of any peptides was 10,970 and 1,547 cpm, respectively. *, nonspecific binding determined by binding in the presence of excess of cold VEGF_165 or bFGF.
VEGF to cell surface receptor. Different forms (VEGF165, 121, 8–121, VEGF109; VEGF8–109) were prepared and labeled with 125I as described under "Experimental Procedures." Binding of the labeled VEGF to the cell surface receptor was determined in the presence of respective nonlabeled VEGF and RRKRRR. A, VEGF165; B, VEGF121; C, VEGF8–121; D, VEGF109; E, VEGF8–109.

To corroborate the results obtained with CAM, we investigated the effect of one of the AR peptides (RRKRRR) on the angiogenesis induced by VEGF in rabbit cornea (Fig. 6A). RRKRRR (1 μg) completely inhibited the angiogenesis in rabbit cornea induced by VEGF165 (Fig. 6B). A peptide with negative charges (EEFDDA) showed no inhibitory activity (Fig. 6C). For each group, six rabbits were used, and all animals showed similar results.

**AR Peptides Inhibit Tumor Growth and Metastasis in Nude Mice**—The acquisition of an angiogenic phenotype is considered decisive for tumor progression (45). Neovascularization is crucial for sustained tumor growth, because it allows oxygenation and nutrient perfusion of the tumor as well as removal of waste products. We have investigated effect of peptide RRKRRR on growth on HM7 human colon carcinoma cells that was transplanted into nude mice. HM7 cells express VEGF (data not shown) and have potential to invade into liver (34, 46). RRKRRR at 50 μg/day inhibited growth of the HM7 cells by 28% of control after 15 days of injection, whereas EEFDDA showed no effect (Fig. 7). The inhibitory activity of RRKRRR is most likely due to the anti-VEGF activity.

Activation of angiogenesis is also responsible for the increased tumor cell entry into circulation and metastasis. Therefore, halting angiogenesis by blocking of VEGF signaling may suppress tumor metastasis (6). Here, we investigated whether RRKRRR also blocks metastasis of malignant human colon carcinoma cell line, HM7, from spleen to liver in mice. As shown in Fig. 8, RRKRRR at 50 μg/day reduced the number of metastatic nodules and liver weight by ~16 and ~33% of control, respectively. Hexa-lysine had much lower inhibitory effect at the same dose (~80% of control). Negatively charged EEFDDA had no effect. Therefore, these results suggest that the AR peptide inhibits growth and metastasis of malignant tumor cells by way of blocking VEGF signaling.

**DISCUSSION**

Without suitable angiogenesis, the tumor cannot grow and metastasize (47). Thus, down-regulation of secretion or production of angiogenic factor or blocking of the action of angiogenic factors will provide potential ways of therapeutic intervention for cancer treatment. Considering the various activities of VEGF essential in angiogenesis (12, 30, 31, 48), the localized expression of its receptors only in endothelial cells (15, 16), and the secretion of VEGF by many tumors (36, 49–51), VEGF signal transduction may represent a useful target for development of antiangiogenic drugs against angiogenesis-dependent diseases, especially cancer.

In this study, we identified hexapeptides from the screening of peptide libraries. The AR peptides RRKRRR, RKKRRK, and hexa-arginine can directly interact with VEGF165 (Kd = 5, 2, and 22 μM, respectively) and inhibit the binding of VEGF to its cell surface receptor in a concentration-dependent manner (IC50 = 2–4 μM). The AR peptides exert their inhibitory activities against the VEGF-induced endothelial cell proliferation in culture at relatively high concentration (~10 μM) with no apparent cytotoxicity. The action of these peptides appears to be specific for VEGF system because they have no apparent effect on binding of bFGF to its receptor on the surface of endothelial cells.

Heparin may regulate the VEGF binding ability of the VEGF receptor (40). Because of their positive charges, it is possible that the AR peptides may bind to the cell surface heparin and subsequently block the binding of VEGF165 to its receptors. Although the AR peptides bind to heparin at low ionic strength, they do not bind to heparin in the saline solution or cell culture medium (0.18 M NaCl; Fig. 3C). Therefore, the AR peptides most likely do not interact with heparin or heparan sulfate.
Anti-VEGF Peptides Inhibit Tumor Growth and Metastasis

**TABLE II**

**Antiangiogenic activity of the AR peptides in the chick CAM assay**

| Samples                  | Positive eggs/total eggs | Positive response | p<sup>a</sup> | Control values |
|--------------------------|--------------------------|------------------|--------------|----------------|
| Water                    | 3/28                     | 10.8 (1.4)<sup>b</sup> | 0.004        | 100            |
| VEGF (10 ng)             | 9/27                     | 33.6 (3.8)       | 0.245        | 21             |
| VEGF + RRKKRRR (1 µg)    | 4/26                     | 15.6 (5.1)       | 0.271        | 21             |
| VEGF + RRKKRRR (1 µg)    | 4/26                     | 15.6 (4.5)       | 0.245        | 21             |
| VEGF + RRKKRRR (1 µg)    | 4/26                     | 15.6 (4.5)       | 0.245        | 21             |
| VEGF + KKKKKK (1 µg)     | 8/25                     | 32.6 (12.2)      | 0.038        | 97             |
| VEGF + protamine (50 µg) | 5/26                     | 18.8 (4.1)       | 0.128        | 35             |

<sup>a</sup> p values were obtained from Student’s t-test by comparison of water used as negative control.

<sup>b</sup> Values in parentheses are standard deviation.

**FIG. 6. Inhibition of VEGF<sub>165</sub>-induced angiogenesis by peptide RRKKRRR in rabbit cornea.** A, recombinant human VEGF<sub>165</sub> (10 ng) only; B, recombinant human VEGF<sub>165</sub> (10 ng) plus RRKKRRR (1 µg); C, recombinant human VEGF<sub>165</sub> (10 ng) plus EEFDDA (1 µg). Angiogenesis was induced with VEGF<sub>165</sub> or VEGF<sub>121</sub> plus peptide in rabbit cornea as described under “Experimental Procedures.” Six animals were used for each group, and the results were similar among animals.

**FIG. 7. Inhibition of HM7 tumor cell growth by RRKRRR.** HM7 human colon cancer cells (5 × 10<sup>6</sup> cells) were subcutaneously transplanted into 4-week-old nude mice. From the following day, mice received subcutaneous injection of each of the peptides (50 µg in 100 µl of saline) or saline solution (100 µl) until 15 days after tumor cell implantation. Tumor volumes were determined regularly as described under “Experimental Procedures.” Each experimental group consisted of six to seven animals, and *p < 0.05, when compared with saline solution or EEFDDA.

proteoglycan <i>in vivo</i>. Furthermore, we have demonstrated here that the AR peptides can prevent the binding of both VEGF<sub>165</sub> and VEGF<sub>121</sub> to their cell surface receptors on endothelial cells, but not VEGF<sub>8–121</sub>, 109, and 8–109 (Fig. 4). These observations imply that the anti-VEGF activity of the AR peptides results from the binding of the peptides to the main body of VEGF including the N- and C-terminal ends of VEGF<sub>121</sub> and is independent of the heparin-binding domain of VEGF.

The sequences of the AR hexapeptides suggest that basic charges may play an important role for the action of the peptides. Although it may be true, hexa-lysine showed little effect on the binding of labeled VEGF to endothelial cells even at a high concentration (100 µM) and minimal effect on the angiogenesis induced by VEGF. Therefore, repeating arginines or combinations of arginines and lysines appear to be important.

Protamine, a 4.3-kDa, arginine-rich DNA-binding protein found only in sperm, is an effective inhibitor for the interaction of VEGF and its receptor (IC<sub>50</sub> = 0.1 µM, determined in this study; data not shown) and also known as an antiangiogenic factor with an unknown mechanism (44). Thus, the protein has been studied as an attractive candidate of antiangiogenic drugs but excluded because of its extreme toxicity (IC<sub>50</sub> = −0.1 µM, determined in this study; data not shown). Considering the similar biological activities of the AR peptides and protamine and the presence of arginine-rich regions in protamine, the antiangiogenic action of protamine is most likely due to the arginine-rich region of protamine.

The AR peptides show their effect at micromole range, but the effective concentration of each peptide sequence was less than nm per sequence during the screening of the original hexapeptide library pools. As shown in Fig. 1 and summarized in Table I, there are several important amino acids at each position of hexapeptide library pools. As shown in Fig. 1 and summarized in Table I, there are several important amino acids at each position of hexapeptide library pools.

The AR peptides identified in this report also inhibit the VEGF-induced neovascularization <i>in vivo</i> in the chick CAM and the rabbit cornea. The peptides show no toxic effect on the proliferation of the HUVE cells. Therefore, the inhibition of the VEGF-induced angiogenesis by these peptides is not caused by cytotoxicity to endothelial cells of pre-existing blood vessels but...
is most likely caused by neutralization of VEGF added. These results also demonstrate that among the known angiogenic receptors for possible use for pharmaceutical purposes. Work is in progress to improve the activity of the AR peptides by modifying the structure of the peptides.

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