A Novel Peptide Isolated from a Phage Display Library Inhibits Tumor Growth and Metastasis by Blocking the Binding of Vascular Endothelial Growth Factor to Its Kinase Domain Receptor*

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Vascular endothelial growth factor (VEGF), one of the most important angiogenic factors, plays an essential role in both physiological and pathological angiogenesis. The VEGF receptor KDR/Flk-1 (a kinase domain receptor) mediates various biological activities of VEGF related to proliferation, differentiation, and migration of endothelial cells. Here we present a novel peptide designated K237-(HTMYYHHYQHHL), which was isolated from a phage-displayed peptide library, binding to KDR with high affinity and specificity. By interfering with the VEGF-KDR interaction, the peptide K237 inhibited proliferation of cultured primary human umbilical vein endothelial cells induced by recombinant human VEGF165 in a dose-dependent and cell type-specific manner. The peptide also exerted an anti-angiogenesis activity in vivo as revealed using the chick embryo chorioallantoic membrane angiogenesis assay. Moreover, the peptide K237 significantly inhibited the growth of solid tumors implanted beneath the breasts and their metastases to lungs in severe combined immunodeficient mice. Taken together, these findings suggest that the peptide K237 can functionally disrupt the interaction between VEGF and the KDR receptor and cause potent biological effects that include the inhibition of angiogenesis and tumor growth. As a consequence, this peptide (and its future derivatives) may have use as a potential cancer therapy.

Neovascularization is critical for supporting the rapid growth of solid tumors beyond 1–2 mm in diameter (1) and for tumor metastasis (2). The generation of new capillaries involves a multistep process involving dissolution of the membrane of the originating vessel, endothelial cell migration and proliferation, and formation of a new vascular tube (3–5). Suppression of any one of these steps would inhibit the formation of new vessels and would therefore affect tumor growth and metastasis.

Tumor angiogenesis appears to be achieved by the expression of angiogenic agents within solid tumors that stimulate host vascular endothelial cell mitogenesis and possibly chemotaxis. So far, several angiogenic factors have been identified (6) including the particularly potent vascular endothelial growth factor (VEGF),

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A Peptide Blocking VEGF Binding to KDR Inhibits Tumor Growth

growth and metastasis in severe combined immunodeficient (SCID) mice.

EXPERIMENTAL PROCEDURES

Materials—Primers for amplifying KDR cDNA coding the immunoglobulin (Ig) similar to domains I–IV (sense, 5′-AAGGATCTCCTCCCAG-GCTACACATAA-3′, and antisense, 5′-GGGGAATCGGGGGAGGACA-TACACAAAC-3′) were synthesized by the Sangon Company (Shanghai, China). Human umbilical cord was obtained from Feking Hospital of Obstetrics and Gynecology (Beijing, China). Type II collagenase, TrizOL reagent, reverse transcription kit, fetal calf serum (FCS), RPMI 1640 medium, and [125I]VEGF were obtained from Invitrogen. The 12-mer phage-displayed library was purchased from New England BioLabs (Beverly, MA). Recombinant human VEGF165 (rh-VEGF165) and horseradish peroxidase-conjugated anti-M13 phage antibody were obtained from Sigma. Proteins R237-ITMYHYYQQHHL and control random peptide-(WHDPFTPWSSWE) were synthesized by Bio-Scientific Inc. (Xian, China). [3H]H was purchased from the Institute of Atomic Energy, Chinese Academy (Beijing, China). SCID mice were provided by the Animal Center of the Chinese Academy of Medical Science (Beijing, China).

Expression and Purification of KDR—The cDNA coding the Ig-like domain of the VEGF receptor KDR (22, 23) was obtained from human umbilical vein endothelial cells (HUVECs) by reverse transcription-PCR according to manufacturers’ instructions (Invitrogen). The amplified cDNA was then cloned into glutathione S-transferase (GST) expression vector pGEX-2T (Amersham Biosciences) and sequenced. Escherichia coli JM109 competent cells were transformed with pGEX-2T-KDR, cultured in Luria-Bertani (LB) medium supplemented with 60 µg/ml ampicillin, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C to allow expression of fusion protein GST-KDR. The pellets from 200 ml of induced bacteria were ultrasonicated on ice in 2 ml of TE buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) and then centrifuged at 12,000 rpm for 10 min at 4 °C. The resulting precipitate was resuspended in 2 ml of 2% sodium deoxycholate and incubated for 30 min at room temperature followed by centrifugation at 10,000 rpm for 1 h. The supernatant was then applied to 10 ml diethiothreitol, 50 mM Tris-HCl, pH 8.5, 5 mM urea) and shaken gently for 3 h at room temperature. Soluble GST-KDR (sKDR) was obtained by gradually dialuting the denatured protein with 8–10 ml of refolding buffer (5 mM reduced glutathione, 2 mM oxidized glutathione, 5 mM EDTA, 50 mM Tris-HCl, pH 8.5) and shaking for 4 h at room temperature. The supernatant containing the sKDR was analyzed with 12% SDS-PAGE and shown to correspond to a single detectable protein band. sKDR was immobilized on 96-well plates and blocked as described above. Seven different phage clones, which were selected by the above phage ELISA, were then incubated with the sKDR-coated plates for 1 h at room temperature at a concentration of 1012 pfu/ml. After this preincubation, [125I]VEGF (2 µg/liter, 50 µM) was added directly to the wells without the removal of the phage. An additional 2-h incubation at room temperature was then performed followed by thorough washing. Bound radioactivity was then counted using a liquid scintillation counter (Wallac Co., Turku, Finland). Two of the phage clones, which exhibited the most promising results, synthetic peptides were generated that corresponded to the encoded displayed peptide motifs. These peptides were then used in the competition assay with [125I]VEGF (see above).

HUVEC Proliferation Assay—HUVECs were seeded into 24-well plates at a density of 3 × 104 cells/well in RPMI 1640 medium supplemented with 20% FCS (NIH 3T3 cells were seeded at a density of 1 × 104 cells/well in Dulbecco's modified Eagle's medium supplemented with 10% FCS). After 24 h, the medium was replaced with RPMI 1640 containing 4% FCS (for NIH 3T3 cells, the medium was replaced with Dulbecco's modified Eagle's medium containing 0.4% FCS). Cells were cultured for another 24 h and then incubated in the presence of rhVEGF165 (2 ng/ml) together with peptide K237 or control peptide at various concentrations (0, 50, 100, 200, and 300 µM). 48 h later, [3H]thymidine was added to the cells, and after a 6-h incubation at 37 °C, the cultures were harvested and analyzed for their incorporation of [3H]thymidine using a scintillation counter. NIH 3T3 cells were used as the control culture.

Angiogenesis Assay—The CAM angiogenesis assay was performed as described previously with some modifications (24, 25). After the fertilized eggs had been incubated for 3 days and then opened, the embryos were incubated in Petri dishes (100-mm-diameter) at 37 °C with 100% humidity. After 3 days, round glass cellulose filters (3-mm-diameter) soaked with either 10 µl of rhVEGF165 (2 ng/ml) alone or with 10 µl of rhVEGF165 (2 ng/ml) plus 20 µl of peptide K237 or control peptide at various concentrations (0.1, 0.5, 1.0, and 5 µM) were placed on the surface of CAM. Filters were replaced daily with new filters with the fresh rhVEGF165 and peptide until angiogenic response peaked. CAMs were examined and documented by photomicroscope.

Experiments of Solid Tumor Growth and Metastasis in Mice—A SCID mouse model was used to further investigate the effect of K237 on tumor growth and metastasis. For this purpose, BICR-H1 cells (1 × 105) were injected beneath the breasts of SCID mice (BICR-H1 cells correspond to a breast carcinoma cell line, which has the ability to generate spontaneous lung metastasis). On the next day, the peptide K237 and control peptide were injected (60 µl, 0.5 mm, respectively), around tumor sites. Peptide injections continued every 2 days for a period of 3 weeks, and tumor size was measured every 3 days with a vernier caliper. Tumor volume was calculated by the following formula: V/6 · (long diameter) × (short diameter)2. On the 48th day of post-cell injection, mice were sacrificed. Nodules formed by implanted tumors at injection sites were isolated and then weighed. The lungs were also removed, fixed with 10% formalin, embedded in paraffin, cross-sectioned, stained with hematoxylin and eosin, and analyzed microscopically for the presence of tumor metastasis. Statistical significance between test and control groups was determined using the Student’s t test (28).
quantified using a temperature and washed extensively, and bound radioactivity was then the binding of sKDR became saturated when [125I]VEGF was using a solid-phase binding assay. This analysis revealed that were coated with sKDR as described in (GST as control) was absorbed onto the surface of 96-well plates (50 µl/well, 2 µg/ml), and various amounts of [125I]VEGF were then added to the wells (as indicated). The plates were incubated for 3 h at room temperature and washed extensively, and bound radioactivity was then quantified using a γ-counter. The data presented are the means of three independent experiments, and the error bars indicate mean ± S.D. B, competition binding assay using [125I]VEGF and free sKDR. Plates were coated with sKDR as described in A, and [125I]VEGF (8 ng/ml) was then mixed with various concentrations of sKDR prior to addition to wells and incubation for 3 h at room temperature. After extensive washing, the bound radioactivity was then measured. The data represent the mean ± S.D. of three independent assays.

RESULTS

Binding of sKDR to [125I]VEGF—Total RNA was extracted from HUVECs, and a cDNA fragment encoding the extracellular Ig-like domains I–IV of KDR was amplified by reverse transcription-PCR and cloned into vector pGEX-2T. After sequence analysis of the inserted cDNA, the GST-KDR fusion protein was expressed in E. coli JM109. After denaturation and refolding, the purity of the prepared sKDR was estimated to be 85–90% as determined by SDS-PAGE (data not shown).

sKDR was then examined for its ability to bind to [125I]VEGF using a solid-phase binding assay. This analysis revealed that the binding of sKDR became saturated when [125I]VEGF was at the concentration of 8 ng/ml, indicating a specific interaction between the soluble receptor and the ligand (Fig. 1A). The binding of [125I]VEGF to immobilized sKDR was reduced when free sKDR was added in competition binding experiments (Fig. 1B).

The Isolated Clones Binding to KDR—The sKDR was used to develop receptor antagonists. To do this, a random 12-mer peptide library composed of 2 × 10⁹ independent phage clones was screened through biopanning against plate-bound sKDR. The sKDR-binding phage population became enriched over the course of four cycles of biopannings, and after the fourth round of selection, roughly 40% of the phage clones analyzed exhibited sKDR binding activity (125 of 300 clones that were analyzed; data not shown). Of the 125 sKDR-binding clones that were identified, eight clones were able to bind specifically to plate-immobilized sKDR and HUVECs (Fig. 2A). Sequences of the displayed peptides that were encoded by these phage clones were determined through sequencing of the phage DNA (the DNA sequence of peptide K93 was not available because of the quality of the sequencing gel) (Table I). A multiple alignment analysis on all selected sequences was performed using the DNAStar sequence analysis package (DNAStar, Inc. Madison, WI) to identify possible consensus motifs, which might be responsible for binding to KDR. No such consensus sequences could be identified, but it was noted that all of the selected peptides contained one or multiple histidines and that all of the peptides were rather hydrophobic.

In light of the ability of the selected peptides to bind sKDR, we examined the ability of these peptides to block the interaction between KDR and its ligand, VEGF. To do this, a fixed amount of phage particles (10¹³ pfu/ml) was added to sKDR-coated wells, and its ability to block the binding of [125I]VEGF was then examined. This analysis revealed that phage-K237 could inhibit the binding of [125I]VEGF to sKDR (Fig. 2B), whereas phage-K93 inhibited the interaction to a somewhat
Table I
Sequences of the peptides selected by binding to sKDR

| Phage clone | Encoded insert                  |
|------------|--------------------------------|
| K112       | MHNHHNHPPRSS                    |
| K57        | MHSMDHAVPSDI                    |
| K150       | HTPHPHAWMLSH                    |
| K237       | KMYIHYQYQHKL                    |
| K202       | LPNFTSPR8WVG                     |
| K212       | CDPKIXIHTHPK                     |
| K25        | NHPAVVEPAPSL                     |

lesser extent; the other clones did not interfere with $^{125}\text{I}\text{VEGF}$ binding to sKDR.

Peptide K237 Blocks the Interaction Between VEGF and KDR—Based on the results of the competition assay, phage clone K237 was chosen for further study. The phage-encoded peptide was synthesized and used in an in vitro competition assay as described above. This analysis revealed that the peptide K237 could antagonize the binding of $^{125}\text{I}\text{VEGF}$ to sKDR in a dose-dependent manner (Fig. 3).

Peptide K237 Specifically Inhibits the Proliferation of HUVEC—It is well known that VEGF stimulates the proliferation of endothelial cells through the KDR receptor. Because peptide K237 had the ability to block interaction between VEGF and KDR, we therefore decided to test whether peptide K237 might also suppress endothelial cell proliferation in response to rhVEGF$_{165}$. As shown in Fig. 4, peptide K237 significantly suppressed the mitogenic response of HUVEC to rhVEGF$_{165}$. Cellular proliferation was reduced by >90% when peptide K237 was present at a concentration of 300 μM, whereas an equivalent concentration of the control peptide had no significant effect on the cell growth (Fig. 4).

Using the same in vitro proliferation assay, we also examined the effect of K237 on NIH 3T3 cells to confirm the specificity of the growth inhibition that we observed in HUVECs treated with rhVEGF$_{165}$. This analysis revealed that the proliferation of NIH 3T3 cells was not modified by peptide K237 (data not shown).

Peptide K237 Inhibits Vascularization of CAM—The effect of peptide K237 on angiogenesis was tested by a CAM vascularization assay. For this experiment, a round glass cellulose filter was impregnated with rhVEGF$_{165}$ alone or with rhVEGF$_{165}$ plus either peptide K237 or the control peptide at varying concentrations. These filters were then directly applied to the CAM, and angiogenesis was measured 3 days later. This experiment revealed that vascularization around the treated sites was significantly reduced in CAMs that received rhVEGF$_{165}$ plus peptide K237 (5 μM) versus CAMs that received rhVEGF$_{165}$ plus the control group or rhVEGF$_{165}$ alone (Fig. 5).

Peptide K237 Suppressed Tumor Growth and Metastasis in Vivo—To determine the effect of peptide K237 on tumor growth and metastasis in vivo, SCID mice were injected subcutaneously with human breast carcinoma cells (BICR-H1 cell line). The tumor sites were subcutaneously subjected to injections with peptide K237 or control peptide every 2 days, and the growth of the tumors was then assessed over time. The results of this analysis showed that there was a significant (70%) reduction in the weight of implanted tumors ($p < 0.05$) that were injected with peptide K237 versus the control group whose five-tumor weight was 1.56 g as measured at sacrifice (the 48th day following implantation of the tumor cells) (Fig. 6, panels 1 and 2).

The SCID mouse lungs were also examined at sacrifice by histological staining and microscopic inspection. The mean numbers of metastasized nodules per mouse were calculated from three inspected cross-lung sections. The results revealed that all animals injected with the control peptide developed intrapulmonary metastatic tumor nodules (identified as clusters of over five tumor cells), and there were 16 tumor nodules in total (Fig. 6, panel 3, C and D). In contrast, only three of the five animals injected with the peptide K237 developed detectable pulmonary nodules, and seven nodules could be observed in total (Fig. 6, panel 3, A and B). Based on the number of the pulmonary nodules per mouse in the two treatment groups, peptide K237 was determined to reduce the total number of metastasis by 53% ($p < 0.05$) as compared with the control peptide.

Discussion

Previous studies have demonstrated that antiangiogenic therapy is a promising approach for the treatment of cancer (1, 2), in part because solid tumor growth and metastasis are dependent on tumor vascularization. Furthermore, it has been shown that blocking the interaction between VEGF and its receptor can result in the regression of murine and human tumors (8). We report here the identification of a VEGF peptide
antagonist that we identified by screening a phage-displayed peptide library.

Direct screening of phage-displayed peptide libraries with soluble receptors is a relative new area of research, and there are only limited reports of such analysis in the literature. A major challenge is the removal of clones with nonspecific binding properties. In this study, we performed three rounds of negative selection to eliminate nonspecific binding clones using three recombinant fusion proteins (GST-p21, GST-VEGF, and GST-Ft1-1), which contained the GST moiety that was fused to our bait protein (GST-KDR). This resulted in the depletion of non-KDR-specific binding clones from the phage library. To isolate specific VEGF antagonists, the phage-displayed library was then subjected to four rounds of biopanning against plate-bound sKDR. 125 individual clones, which exhibited the ability to bind to sKDR, were then tested in a phage ELISA assay using HUVECs as targets. This resulted in the identification of eight individual phage clones, which could bind specifically both to recombinant sKDR and to primary HUVECs. Sequence analysis revealed that eight clones were histidine-rich and hydrophobic, but no clear conservative motif was identified. Furthermore, attempts to align the individual selected peptide sequences with the primary sequence of the VEGF did not identify any homology. However, it remains possible that one or more of the peptides might be a structural mimic of a discontinuous or conformational epitope with VEGF. This is potentially important because Muller et al. (27) have recently solved the crystal structure of VEGF and have mapped the region responsible for KDR binding to a series of discontinuous residues.

When the eight selected phage clones were analyzed for their ability to successfully compete with $^{125}$I-VEGF for binding to immobilized sKDR, only two clones showed detectable activity. The most efficient one of these, peptide K237 HTMYHYHQLQI, was selected for further analysis. This peptide was not only capable of specific binding to sKDR and to HUVECs, but it also inhibited the VEGF-induced proliferation of HUVECs. However, it did not modify the growth of a fibroblast cell line NIH 3T3, indicating that its inhibitory effect on HUVEC proliferation is not a result of nonspecific cellular cytotoxicity but an effect of K237 blocking the interaction between VEGF and its receptor KDR.

Finally, peptide K237 was able to inhibit the growth and metastasis of a breast carcinoma cell line in SCID mice. This suggests that this peptide as a VEGF antagonist might have potential application for the treatment of a variety of cancers. The small size of this peptide also offers the possibility of generating structurally similar molecules via standard organic synthesis. This could result in the production of inexpensive orally available drugs analogous to the RGD-peptidomimetic compound SCH221153, which binds to both $\alpha_v$/$\beta_3$ and $\alpha_v$/$\beta_5$ integrin receptor and inhibits angiogenesis and tumor growth (28).

In summary, our results demonstrate that the peptide K237...
is an effective VEGF antagonist. It functioned as an inhibitor of angiogenesis in vivo and reduced tumor growth and metastasis in a small animal model system. Thus, this molecule (or its derivatives) may have utility in clinical applications that might include cancer biotherapy, a treatment of diabetic retinopathy and other angiogenic or proliferative disorders that involve endothelial cells.

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