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Article *in* Laboratory Medicine · July 2010

DOI: 10.1309/LMMH2WYRLP7B3HJN

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Submitted 1.26.10 | Revision Received 2.22.10 | Accepted 2.26.10

Inhibition of Angiogenesis by Recombinant VEGF Receptor Fragments

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Abstract

Background: Blocking vascular endothelial growth factor receptor (VEGFR) is a successful approach for inhibiting vascular endothelial growth factor (VEGF) signaling. Small molecules impairing the interaction of VEGF with VEGF receptors have been synthesized and evaluated in this research.

Methods: In this study, we amplified and cloned the cDNA of VEGFR fragments. After expression of the fragments in *Escherichia coli* (*E. coli*), they were purified by immobilized metal affinity chromatography (IMAC). The biological activity of recombinant KDR fragments was evaluated by human umbilical vein endothelial cells (HUVEC) proliferation assay.

Results: The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PSGE) and immune-blotting results confirmed the production and purification of recombinant VEGFR fragments. The purified VEGFR2-III domain, VEGFR2-II-III domains, and VEGFR1-II domain showed 24%, 36%, and 27% inhibition effect in HUVEC proliferation assay, respectively. The recombinant VEGFR2-II-III domains strongly inhibited formation of capillary-like structures (CLS).

Conclusions: The produced recombinant proteins will serve as small soluble molecules with an inhibitory effect against VEGF in anti-angiogenesis researches.

Keywords: angiogenesis, VEGFR fragments, expression

Angiogenesis is a multi-step process including endothelial cell migration and proliferation, organization of cell clusters into tubular structures that eventually join each other, and maturation of tubular assemblies into stable vessels. Normal tissue growth, such as in embryonic development, wound healing, and the menstrual cycle, is characterized by dependence on new vessel formation for the supply of oxygen and nutrients as well as the removal of waste products.[1] Angiogenesis is an important biological process in physiological conditions and some pathological conditions such as cancer, diabetic retinopathy, and rheumatoid arthritis.[2,3] Angiogenesis is crucial for tumor development. The capillary and vascular network allows tumors to metastasize and spread to other sites in the body. Vascular endothelial growth factor (VEGF)-expression in cancer cells is induced during tumor formation by environmental stimuli such as hypoxia.[4,5]

The VEGF ligands bind to and activate 3 structurally similar type III receptor tyrosine kinases, designated vascular endothelial growth factor receptor 1 (VEGFR1) (fms-like tyrosine kinase [Flt-1]), VEGFR2 (kinase insert domain-containing receptor [KDR] in humans and fetal liver kinase receptor 1 [Flk-1] in mice) and VEGFR3 (also known as FLT4).[6,7] Expression of VEGFR2 is up-regulated in the tumor vasculature compared with normal vasculature.[6]

In mammals, the VEGF family consists of 5 members; VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PLGF). VEGF-A is the major player in angiogenesis among the VEGF family and activates 2 high-affinity tyrosine kinase receptors, VEGFR1 and VEGFR2.[8] VEGFR1 has a very tight ligand binding domain for VEGF-A, whereas VEGFR2 has strong tyrosine kinase activity. These 2 receptors play different roles in angiogenesis.[8] The KDR/Flk-1 appears to be the major transducer of VEGF signals in endothelial cells which result in cell proliferation, migration, differentiation, tube formation, an increase of vascular permeability, and maintenance of vascular integrity. Both VEGFR1 and VEGFR2 consist of 1338 and 1356 amino

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Abbreviations

VEGFR, vascular endothelial growth factor receptor; VEGF, vascular endothelial growth factor; E. coli, Escherichia coli; IMAC, immobilized metal affinity chromatography; HUVEC, human umbilical vein endothelial cells; SDS-PSGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CLS, capillary-like structures; Flt-1, fms like tyrosine kinase; KDR, kinase insert domain-containing receptor; Flk-1, fetal liver kinase receptor 1; PLGF, placenta growth factor; Ig, immunoglobulin; HRP, horseradish peroxidase; FBS, fetal bovine serum; Ni+-NTA, nickel nitrilo-triacetic acid; HBSS, Hank's buffer saline solution

acids in humans, respectively. They consist of the following 4 regions: the extracellular ligand-binding domain, a short transmembrane domain, tyrosine kinase domain, and downstream carboxy-terminal region. The extracellular domains of VEGFR1 and VEGFR2 are composed of 7 immunoglobulin-like (Ig-like) domains.[9] The binding site for VEGF-A is located in the second Ig-like domain (II domain) in VEGFR1 and the second/third Ig-like domains (II-III domains) in VEGFR2. The downstream structure from the fourth to seventh Ig-like domains in these receptors plays a major role in receptor dimerization and activation.[10,11] VEGF binds to the II-III domains of VEGFR2, and these 2 domains are sufficient for high affinity binding of VEGF. Deletion of Ig-like III domain caused more than 1000 fold reduction in binding affinity.[12,13]

Soluble VEGFR2 fragment, extracellular Ig-like domains, can theoretically bind to VEGF but cannot work to the receptor tyrosine transphosphorylation and activation of downstream signal transduction to induce endothelial proliferation, because of the lack of the tyrosine kinase domain. Soluble VEGFR2 forms a heterodimer with wild VEGFR2 or dominant-negative to block the activity of the entire VEGFR2 by competitive suppression. Therefore, soluble VEGFR2 is a potent endogenous inhibitor of VEGF-mediated angiogenesis.[14]

Since VEGF/KDR plays an essential role in new blood vessel growth, inhibition of angiogenesis could be achieved by functional blocking of VEGF/KDR interaction. This could be accomplished by producing antagonistic agents such as antibodies against VEGF or VEGFR, VEGF-containing immunotoxins, soluble receptor, and small molecular kinase inhibitors.[15-17] In this research work, the VEGFR fragments from human umbilical vein endothelial cells (HUVEC) have been cloned in an expression vector, and the functional activity of these molecules was evaluated.

Materials and Methods

Materials

Monoclonal anti-His-tag antibody and rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents used in this study were of analytical grade and purchased from Sigma-Aldrich.

Cell Culture

The HUVEC were obtained from Pasture cell bank (Iran Pasture Institute, Tehran, Iran) and cultivated in DMEM (Sigma-Aldrich) and Hams F12 (Sigma-Aldrich) (1:1) media containing 20% FCS and DMEM (Sigma-Aldrich) supplemented by 10% fetal bovine serum (FBS), respectively. HUVEC at passage 2 to 3 were used in this research. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

RNA Extraction and cDNA Synthesis

The cells were harvested by centrifugation at 800 rpm for 10 minutes. Total RNA was extracted by Qiagen RNA purification kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using M-MuLV reverse transcriptase (MBI, Fermentas, St. Leon-Rot, Germany) and oligo-dT primers.

Preparation of Constructs

The cDNA encoding the second and second/third Ig-like domains (domains III and II-III) in VEGFR2 and the second Ig-like domain in VEGFR1 were amplified by specific primers containing *Bpu*AI and *Bam*HI restriction enzymes sites (**Table 1**). The amplification protocol consisted of denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 55°C-58°C for 30 seconds and 72°C for 50 seconds. The amplified genes were gel purified from agarose by a high pure PCR product purification kit (Roche, Mannheim, Germany). The purified genes and vector, pSJ, were digested by *Bpu*AI and *Bam*HI, ligated and electroporated into Rosetta gami2 *E. coli* electrocompetent cell (Pharmacia, Uppsala, Sweden). The cells were allowed to recover for 1 hour at 37°C at 250 rpm. Transformants were plated on LB plates containing 100 µg/mL ampicillin and grown at 37°C. Selected clones were confirmed by colony-PCR and sequencing.[18]

Cultivation Conditions

The transformants were cultured in 15 mL of LB medium containing 150 µg /mL ampicillin and grown overnight at 37°C. These pre-inocula were then transferred to 200 mL of TB medium containing ampicillin at the same concentration .The cultures were grown at 37°C and 250 rpm until OD_{600} of 0.8 was achieved. These cultures were aliquoted (5mL) in sterile 45 mL culture tubes. The tubes were induced with different concentrations of IPTG (0.5, 1, and 2 mM) in various conditions of temperature (28°C, 30°C, and 37°C) and induction time (2 hours, 4 hours, and 8 hours).[19]

SDS-PAGE and Immunoblotting

Electrophoresis of the proteins was performed as described by Laemmli[20], using 12% acrylamide gels followed by staining with Coomassie brilliant blue or blotting. For immunoblot analysis, after induction by IPTG, both transformed and non-transformed Rosetta gami2 cells were harvested by centrifugation, boiled at 100°C for 10 minutes, subjected to SDS-PAGE, and transferred from the gels onto nitrocellulose membranes. The membranes were blocked for 1 hour at room temperature in MPBST (0.137M NaCl, 2.7mM KCl, 8.0mM Na2HPO4, 1.8mM KH2PO4, 0.1% Tween 20, and 5% skimmed milk powder). Subsequently, the membranes were incubated for 90 minutes with monoclonal mouse anti-His-tag antibody (1:4000). Then HRP-conjugated rabbit anti-mouse were used at 1:2000 dilution

Table 1_The Sequences of Specific Primers Used for VEGFR Fragment Amplification

Primer Name	Sequence
For-VE-2-III	5′-TAATGGGATCCAGGTTTTTCATG-3′
Rev-VE-2-III	5′-TAATTCGAAGACTTTATTGATGTGGTTC-3′
For-VE-2-II/III	5′-CGCGGATCCGCT GAG AAC AAA AAC AAA ACT G -3′
Rev-VE-2-II/III	5′-CCTGAAGACAGGACCCTGACAAATGTGCTG-3′
For-VE-1-II	5′-TAATGGGATCCATGTGCAATCTATATATTTATTAG-3′
Rev-VE-1-II	5′-TAATTCGAAGACTTATGATTGTATTGGTTTGTCG-3′

to detect recombinant fragments. The immunoblot was visualized with DAB (3-3′-diaminobenzidine) substrate.[21]

Purification of Recombinant VEGFR Fragments

Large-scale production was conducted in 500 mL of M-9 medium. Selected clones were cultured in 50 mL of LB-medium at 30°C for 16 hours and inoculated in 500 mL M-9 containing casamino acid and ampicillin. After 24 hours the cultures were induced by adding 1mM IPTG and 50 mL of concentrated TB nutrients 10× and incubated an additional 24 hours at 30°C. Induced cultures were centrifuged at 10000×g for 30 minutes. After pelleting the cells, the cytoplasmic proteins were extracted as described previously.[22] The recombinant protein present in these extracts was purified by immobilized metal affinity chromatography with 5 mL of nickel nitrilo-triacetic acid (Ni+-NTA) resin (Qiagen). The products were dialyzed against a phosphate buffer (10 mM, pH 7.4), and the proteins were quantified by Bradford assay.[23] Purification using Ni-NTA agarose was confirmed by SDS-PAGE and immunoblot analysis.

Human Endothelial Cells Proliferation Inhibition Assay

Human endothelial cells under subconfluent conditions (10^4 cells/well) were plated onto 96-well culture plates (Corning, Corning, NY) in 200 µL of DMEM and Hams F12 (1:1) media containing 20% FCS, and incubated at 37°C in a CO_2 incubator overnight. Subsequently, HUVEC media was replaced with DMEM and Hams F12 (1:1) media containing 1% FCS for another 24 hour culture. Then human VEGFR2 (3H Biomedical AB, Uppsala, Sweden, 10, 100, 500 ng/mL), purified VEGFR2-III domain (10, 100, 500 ng/mL), purified VEGFR2-II-III domains (10, 100, 500 ng/mL), and purified VEGFR1-II domain (10, 100, 500 ng/mL) were added to wells. Additionally, wells without human VEGFR2/VEGFR2-III domain / VEGFR2-II-III domains / VEGFR1-II domain were used as a blank control. All assays were carried out in triplicate. Culture plates were kept in the incubator for 48 hours. During the incubation time, culture media were replaced with basal medium, containing not human VEGFR2/VEGFR2-III domain / VEGFR2-II-III domains / VEGFR1-II domain every 24 hours. Cell proliferation was assayed using the following methods.

(A) The cells were detached from the wells and counted using a cell counter (Coulter ZM).

(B) In different conditions of cultures, proliferation was assessed by MTT Cell Viability Assay Kit (Biotium, Hayward, CA). After incubation, MTT reagent (10 µL) was added to the wells and incubated for 3 hours at 37°C. At the end of the incubation time, the medium was removed and 100 µL DMSO was added into each well. In order to dissolve the formazan crystals, the supernatant was pipetted up and down several times. Absorbance on an ELISA plate reader at a wavelength of 540 nm was measured.[24]

In vitro Angiogenesis Inhibition

Human endothelial cells were grown in 199 medium supplemented with 20% heat-inactivated FBS. One mL of fibrinogen solution (4 mg/mL) were mixed with thrombin (0.2 units/mL) in Ca/Mg free Hank's buffer saline solution (HBSS), in 3.5 cm diameter petri dishes, in order to obtain fibrin gels of 1 mm thickness. Fibrin was allowed to polymerize overnight at 37°C.

For the assays, 200000 cells suspended in 2 mL of 199 medium containing human VEGFR2 (500 ng/mL), purified VEGFR2-III domain (500 ng/mL), purified VEGFR2-II-III domains (500 ng/mL), and purified VEGFR1-II domain (500 ng/mL) were seeded on fibrin gels. Under these conditions capillary-like structures (CLS) formed within 3–4 days in a control condition (without VEGFR2 or VEGFR fragments). Endothelial tube formation was digitally photographed under an inverted light microscope (Diaphot Nikon, Melville, NY).

Results

Preparation of Constructs

Cultured HUVEC were used for mRNA extraction and cDNA synthesis. PCR products were assessed by 1% agarose gel electrophoresis. **Image 1** shows the result of PCR-amplified genes from HUVEC cells cDNA. From the VEGFR2-III domain, VEGFR2-II-III domains, and VEGFR1-II domain gene amplification, 349, 620, and 330 bp bands were obtained, respectively. In order to enhance expression of VEGRFR fragments, VEGFR2-III domain, VEGFR2-II-III domains, and VEGFR1-II domain encoding sequences were cloned in the pSJ expression vector. This plasmid is designed for expression of recombinant protein fused to a Cmyc-tag and 6 amino acids His-tag downstream of the cloning site. The resulting constructs, pS-VEGFR2-III, pS-VEGFR2-II-III, and pS-VEGFR1-II, were transformed into electrocompetent Rosetta gami2 cells. After cloning the genes, their sequences and orderings were confirmed by PCR, digestion using restriction enzymes (*Bpu*AI and *Bam*HI) and sequencing.

Comparison of Cultivation Conditions

IPTG dose dependence, temperature, and time course studies of the induction of the recombinant protein expression of pS-VEGFR2-III, pS-VEGFR2-II-III, and pS-VEGFR1-II containing clones analyzed by SDS-PAGE led to an IPTG concentration of 1 mM and induction time of 20 hours at 30°C.

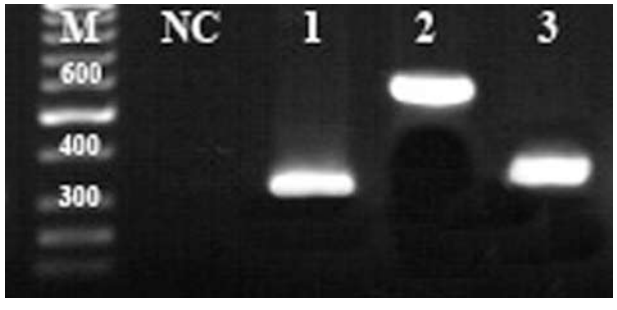

Image 1_Agarose gel electrophoresis of PCR products; M: size marker, NC: negative control, lane 1: VEGFR2-domain III, lane 2: VEGFR2-domains II-III, and lane 3:VEGFR1-domain II.

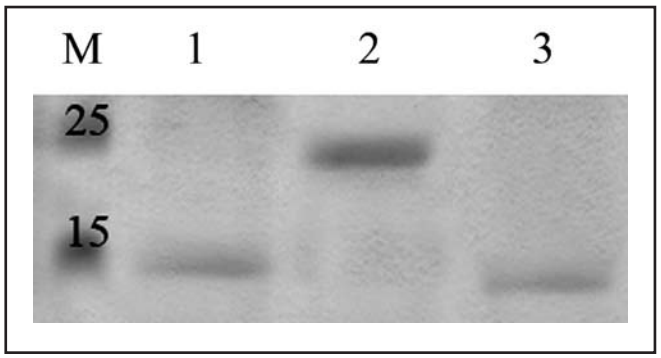

Image 2_Immunoblotting analysis; M: molecular weight marker, lane 1: VEGFR2-domain III, lane 2: VEGFR2-domains II-III, and lane 3: VEGFR1-domain II.

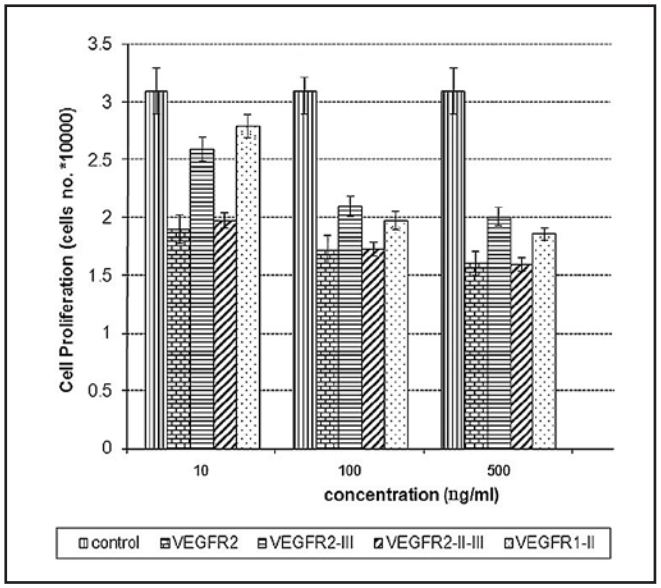

Figure 1_Human endothelial cells proliferation inhibition assay in various dilutions of control (cell culture medium without VEGFR2 and VEGFR fragments), VEGFR2-domain III, VEGFR2-domains II-III, and VEGFR1-domain II. All assays were performed in triplicate.

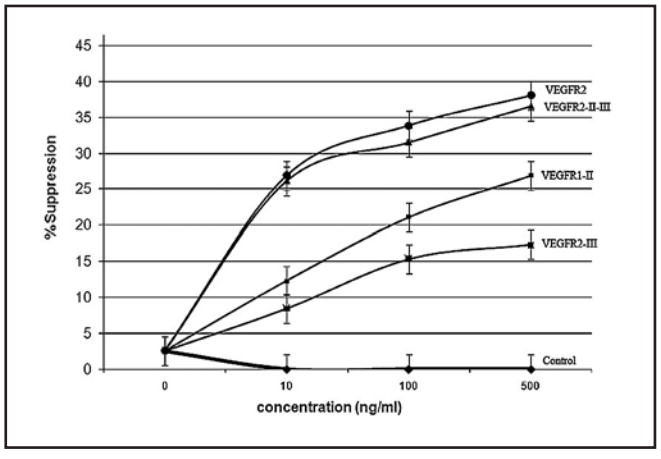

Figure 2_Dose-dependent suppression of endothelial cell growth in various dilutions of control (cell culture medium without VEGFR2 and VEGFR fragments), VEGFR2-domain III, VEGFR2-domains II-III, and VEGFR1-domain II. All assays were performed in triplicate.

SDS-PAGE and Blotting

Homogenized transformed and non-transformed *E. coli* cells were subjected to 12% SDS-PAGE and expressed proteins were transferred from the gels onto nitrocellulose membranes. The expressed bands were found to be at nearly 12, 25, and 12 KDa for VEGFR2-III domain, VEGFR2-II-III domains, and VEGFR1-II domain on SDS-PAGE gel, respectively. No specific bands were observed at this region for non-transformed bacteria. Immunoblot analysis indicated the expressed proteins could be recognized by monoclonal antibody against His-tag. This result showed that VEGFR fragments can be successfully expressed in a bacterial expression system.

Purification of Recombinants VEGFR Fragments

Purification on a Ni-NTA column with elution by imidazole was carried out. The eluted fractions containing the recombinant proteins were analyzed by a SDS-PAGE and immunoblotting (**Image 2**). The yield of expression of the fusion proteins containing the VEGFR fragments was considered 0.04, 0.38, and 0.05 g/L of pS-VEGFR2-III, pS-VEGFR2-II-III, and pS-VEGFR1-II bacterial extract, respectively.

Human Endothelial Cells Proliferation Inhibition Assay

The effect of VEGFR fragments on the proliferation of human endothelial cells was investigated by cell counter and MTT assay. Our results showed that treatment of HUVEC with VEGFR2-III domain, VEGFR2-II-III domains, and VEGFR1-II domain, in contrast to a blank control, resulted in the significant repression of endothelial cells grown in a dose-dependent manner. The VEGFR2-II-III domains suppressed human endothelial cells proliferation nearly similar to human VEGFR (38% and 36%, respectively) and stronger than VEGFR2-III domain and VEGFR1-II domain (24% and 27%, respectively) in this assay. The suppressive effect of the VEGFR2-III domain was nearly similar to the VEGFR--II domain (**Figure 1** and **Figure 2**).

In Vitro Angiogenesis Inhibition

After 3–4 days, endothelial cells formed CLS on a fibrin gel without human VEGFR2 and purified VEGFR2-II-III domains. While in presence of these proteins, CLS formation was inhibited. The Purified VEGFR2-III domain and VEGFR1-II domain moderately inhibited the CLS formation (**Figure 3**).

Discussion

VEGF represents a central molecular target for anti-angiogenic intervention because of its integral involvement in endothelial cell proliferation and migration. There are currently more than 20 different VEGF-targeted agents in clinical trials.[25-29] Biological activity of VEGF is mediated by 3 receptor tyrosine kinases: VEGFR1, VEGFR2, and VEGFR3. VEGFR1 is expressed on the vasculature and several other types of cells. VEGFR1 has a tenfold higher binding affinity to VEGF but exerts less activation of intracellular signaling intermediates than VEGFR2. VEGFR1 can function as

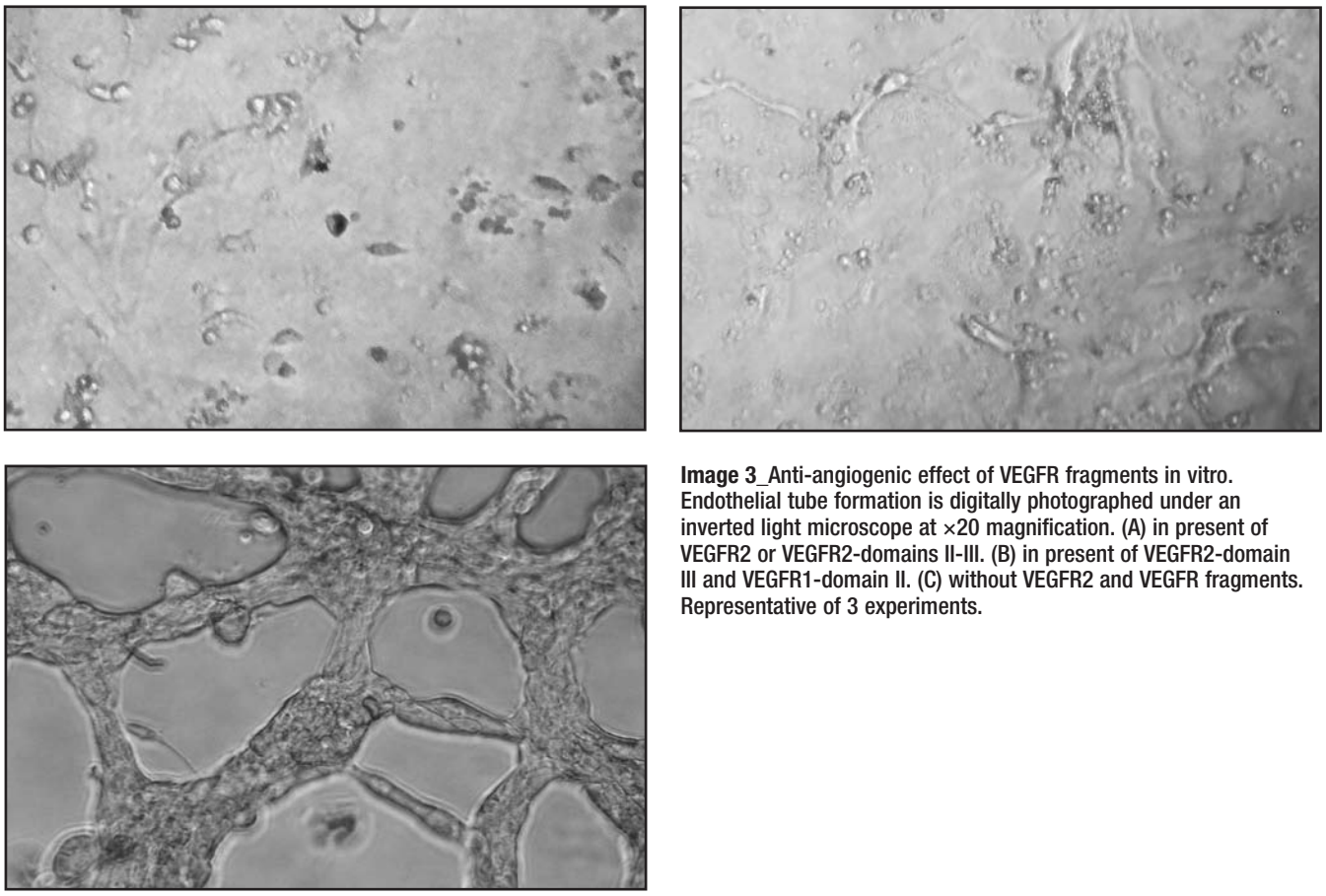

Image 3_Anti-angiogenic effect of VEGFR fragments in vitro. Endothelial tube formation is digitally photographed under an inverted light microscope at ×20 magnification. (A) in present of VEGFR2 or VEGFR2-domains II-III. (B) in present of VEGFR2-domain III and VEGFR1-domain II. (C) without VEGFR2 and VEGFR fragments. Representative of 3 experiments.

a negative regulator of angiogenesis, by binding VEGF and preventing its binding to VEGFR2. VEGFR2, which forms a high-affinity complex with VEGF, only exists in vascular endothelial cells and some tumor cells.

In 1971, Folkman proposed the theory that inhibition of angiogenesis may result in the arrest of tumor growth.[30,31] This inhibition can be obtained with some anti-angiogenic drugs. The anti-angiogenic drugs can be divided into the following 2 broad classes: the VEGF targeting agent and the agents designed to target the cell surface receptors.[32] The VEGF-targeted therapy has been shown effective as a single agent in renal cell carcinoma and hepatocellular carcinoma, whereas it is only of benefit when combined with chemotherapy for patients with metastatic colorectal, non-small-cell lung cancer, and metastatic breast cancer.[7,33]

Compelling evidence suggests that III domain of VEGFR2 (KDR) is a key factor for VEGF/KDR interaction. Thus in this research, we expressed and purified the VEGFR2-III domain, VEGFR2-II-III domains, and VEGFR--II domain, which are small molecules (116, 207, and 110 amino acids, respectively). We studied and compared the anti-angiogenesis functions of these small fragments. These recombinant VEGF targeting molecules with efficient solubility have an inhibitory effect on angiogenesis. Meanwhile, we found that these fragments, especially VEGFR2-II-III domains, combine to VEGF and inhibit the combination of VEGF and functional VEGFR resulting in suppressed in vitro capillary tube formation, a hallmark of angiogenesis.[7] When compared to blocking agents, such as anti-VEGFR antibodies, these molecules have an efficient clearance rate. Because of their small size and the fact that they originated from human cDNA, these agents have a low possibility for immunogenic reactions in clinical applications. Thus, these molecules could be useful for the generation of anti-angiogenic agents.

In summary, the approach applied in this study to express and examine small inhibitory molecules against VEGF can be viewed as a general route to obtain small binders with favorable characteristics. Taking into account all the above facts, it may be concluded that these small binders, which specifically block VEGF, could be useful to control the VEGF-VEGFR signaling. Thus they may open up a general and widely applicable approach for therapeutic applications. LM

Acknowledgements: This work was supported by: (1) Transplant Research Center, Shiraz University of Medical Sciences (Shiraz, Iran) and (2) Department of Biochemistry, School of Medicine, Tehran University of Medical Sciences (Tehran, Iran) (No. 87-01-30-6940).

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