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Identification of a Neutralizing scFv Binding to Human Vascular Endothelial Growth Factor 165 (VEGF165) Using a Phage Display Antibody Library

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Abstract Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that plays a major role in angiogenesis. Alternative splicing causes the production of several different isoforms (VEGF121, 145, 165, 183, 189, 206). VEGF is essential for tumor angiogenesis, and several studies have correlated elevated VEGF levels with tumor stage, metastases, and progression. We now report the isolation by phage display of human single-chain antibody fragment (scFv) anti-VEGF165. After four rounds of panning against VEGF165, 40 out of 90 phage clones displayed VEGF165-binding activity. One of the positive clones, designated B8, bound to VEGF165 with relatively high affinity and neutralized VEGF165 bioactivity in vitro. The B8 clone was expressed in the soluble form in *Escherichia coli* HB2151 and purified by immobilized metal affinity chromatography. The purified scFv recognized VEGF165 with the K_D of 1.80×10^{-8} M without cross-reaction to VEGF121. In addition to binding, the purified scFv could does-dependently inhibit VEGF165-induced human umbilical vein-derived endothelial cells proliferation. Together with its fully human mature, B8 scFv may have therapeutic implications in therapy of angiogenesis-dependent diseases.

Keywords Phage display · VEGF165 · Single-chain antibody fragment (scFv) · Angiogenesis-dependent diseases

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Introduction

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 the dissolution of the membrane of the originating vessel, endothelial cell migration and proliferation, and formation of a new vascular tube [3]. Suppression of any one of these steps would inhibit the formation of new vessels and would therefore affect tumor growth and metastasis.

Expression of angiogenic agents within solid tumors stimulate host vascular endothelial cell mitogenesis and possibly chemotaxis. So far, several angiogenic factors have been identified [4] including the particularly potent vascular endothelial growth factor (VEGF), which acts as an endothelial cell-specific mitogen and plays important roles during tumor angiogenesis [5–7]. Six forms of human VEGF messenger ribonucleic acid encoding VEGF proteins of 121, 145, 165, 183, 189, and 206 amino acids are produced from a single gene as a result of alternative splicing [8–10]. These isoforms, combined with post-translational modifications including dimerization and plasmin digestion, lead to VEGF species with varying heparin-binding capabilities, tissue distributions, receptor binding abilities, and biological activities [11]. The 165-amino acid isoform (VEGF165) is the major gene product found in human tissue and is the most effective angiogenic factor in the VEGF family [12–14]. As a result, antibody antagonism of the effects of VEGF165 therefore has a potentially important therapeutic value in the context of cancer therapy.

However, the development of antibodies requires the use of animals, specialized cell culturing facilities, and an extensive commitment of time and labor. Advances in the field of recombinant antibody technology provide an alternative means to engineer low-cost antibodies with desirable affinity and specificity by enabling one to manipulate the basic domain structure of the immunoglobulin molecule. One of the most successful approaches is to display the single-chain antibody fragment (scFv) on filamentous phage [15–17]. ScFv is an antigen-binding protein, composed of an immunoglobulin heavy-chain variable domain (VH) and a light-chain variable domain (VL) joined together by a flexible peptide linker. When expressed with phage protein p3 (fd g3 protein of phage VCSM13) as a fusion protein, a high affinity scFv-producing phage clone can be enriched by a procedure called panning [18, 19]. In this report, we describe how novel antibodies, specific to VEGF165 (the 15 amino acids encoded by exon 7 that are not present in VEGF121), were isolated using phage antibody display technology and describe the possible use of these antibodies as high specificities and sensitivities to VEGF165.

Materials and Methods

Phage Libraries, Bacterial Strains, and Recombinant Human VEGF165 Protein

The Griffin.1 library is composed of human scFvs containing highly diverse CDR3s in both the VH and VL domains. This library was derived by recloning VH and VL from human synthetic Fab lox library vectors [20] into the phagemid vector pHEN2 [21]. ScFvs can be displayed on the surface of the bacteriophage when expressed in suppressor *Escherichia coli* strains TG1 or as soluble fragments that also contain the c-*myc* tag and carboxy terminus His-tag in nonsuppressor *E. coli* strains HB2151.

Recombinant VEGF165 and VEGF121 were cloned and expressed in *E. coli*, and the resulting insoluble bodies were separated from cellular debris by centrifugation and

solubilized with 8 M urea. A rapid and simple on-column refolding procedure was developed. It was applied, and then the refolded VEGF165 was purified by anion exchange. The purified final product was greater than 95% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue R-250. The correct refolding of the recombinant protein was verified in the recovery of its secondary and tertiary structures as assessed by circular dichroism and fluorescence emission spectra. The final purified material was biologically active in a validated induced human umbilical vein-derived endothelial cells (HUVECs) proliferation bioassay.

Panning for Anti-VEGF165 Phage-displayed scFv

Affinity selection for VEGF165-binding recombinant phages was performed by panning as described [20]. Briefly, Immunotubes (Maxisorp, Roskilde, Nunc, Denmark) were coated with VEGF165 diluted in coating buffer (50 mM $NaHCO_3$, pH 9.6)for 12 h at room temperature (RT), and phages were eluted with 100 mM triethylamine and quickly neutralized with 0.5 ml 1 M Tris, pH 7.4. Four rounds of selection were carried out using stringency conditions, which consisted in reducing progressively the VEGF165 concentration (80, 40, 20, and 10 μg/ml, respectively) for coating and increasing the washing steps from 10 to 20. The titer of the scFv-phage was estimated as follows: *E. coli* TG1 was grown in 2× yeast extract–tryptone (YT) media at 37 °C until optical density at 600 nm (OD_{600})= 0.5, and 50 μl of the culture was infected with 10 μl of serial dilution of the eluted or amplified scFv-phages, incubated for 30 min at 37 °C without shaking and plated on YT agar with 1% glucose and 100 μg/ml ampicillin. The titer of the scFv-phages was calculated by counting the number of grown colonies.

Each of the four phage populations obtained was tested for specificity to VEGF165 by polyclonal phage enzyme-linked immunosorbent assay (ELISA), whereas individual bacterial clones expressing VEGF165-reacting phages were selected by monoclonal phage ELISA.

Phage ELISA

Microtiter plates (High binding, Corning, USA) were coated with 10 μg/ml of bovine serum albumin (Sigma, USA), recombinant VEGF165 or VEGF121 in coating buffer overnight at 4 °C, and blocked with 2% skim milk in phosphate-buffered saline (MPBS) for 2 h at 37 °C. One hundred microliters (about 1×10^{12} pfu) of amplified phages after each round of panning were added into each well and incubated at RT for 90 min. The microtiter plate was washed with PBS–Tween three times, and anti-M13 antibody conjugated with horse radish peroxidase (HRP; Amersham Pharmacia biotech, USA) was added into each well. The BM Blue POD Substrate (Roche, USA) was used to visualize the signal. The plate was analyzed at 450 nm with ELISA reader (Bio-Rad Molecular Bioscience Group, USA).

DNA Sequencing Analysis

Phagemid deoxyribonucleic acid (DNA) was isolated from 3-ml overnight cultures of VEGF165-specific clones using a minipreparation kit according to the manufacturer's protocol (Wizard Minipreps DNA Purification System, Promega, USA). The nucleotide sequence of the VH and VL genes of selected clones was determined using the primers FOR_LinkSeq (GCCACCTCCGCCTGAACC) and pHEN-SEQ (CTATGCGGCCC CATTCA). The variable region sequences obtained were analyzed using the Ig-BLAST

(http://www.ncbi.nlm.nih.gov/blast/index.html) and V-BASE (http://www.mrc-cpe.cam.ac.uk/imt-doc/pub/INTRO html).

Expression and Western Blotting

The phage clone that was sequenced was selected for induction of soluble scFv production. Briefly, plasmid minipreparations were made and transformed into the nonsuppressor *E. coli* strain HB2151. One colony with an apparently intact scFv insert (checked by polymerase chain reaction [PCR]) from each set was selected and inoculated into 3 ml 2× YT (including 1% glucose and 100 μg/ml ampicillin), followed by shaking at 37 °C until OD_{600}=0.9. Isopropyl-β-D-thiogalactogalactopyranoside (IPTG; final concentration 1 mM) was added into the culture, and shaking was continued after 0, 2, 4, and 6 h of adding IPTG. The bacteria samples obtained were boiled with loading buffer and subjected to electrophoresis in 13% SDS-PAGE. After that, the whole bacteria protein was transferred to nitrocellulose membrane (Amersham Pharmacia biotech) from the gel 1.5 h at 150 mA in 50 mM Tris–HCl (pH 8.0), 150 mM glycine, and 20% methanol. Next, the membrane was blocked with 5% MPBS for 1 h before washing with PBS. Then, 5-ml 1:2,500 mouse mAb 9E10 (Santa Cruz Biotechnology, USA) was incubated with the membrane at 30 °C for 2 h. Bound anti-c-*myc* mouse antibody 9E10 was detected with 1:1,000 HRP-conjugated goat anti-mouse IgG mAb (Santa Cruz Biotechnology). Finally, the membrane was washed, and 3,3′,5,5′-tetramethyl benzidine chemiluminescence (Promega) as a staining substrate was added for color development.

ScFv Production and Purification

To obtain a large quantity of purified scFv, 5 ml of overnight activated *E. coli* HB2151 (transformed by positive colony) culture was transferred to 500 ml YT (including 1% glucose and 100 μg/ml ampicillin) and shaken at 37 °C until OD_{600}=0.9. IPTG (final concentration 1 mM) was added into the culture, and shaking was continued at 30 °C for 4 h. Then, the bacteria were collected and lysed for scFv purification. The scFv was purified from the bacterial lysates by immobilized metal affinity chromatography using Ni^{2+} isotope dilution analysis (IDA) His-bind resin (Novagen, USA) according to the instructions of the manufacture. Purified scFv was evaluated by 13% SDS-PAGE. The proteins were visualized by staining the gel with Coomassie Brilliant Blue R-250. The concentration of the purified scFv was determined by measuring the absorbance value at 280 nm.

Antigen-Binding Specificity Analysis

The binding specificity of the purified scFv against VEGF165 was evaluated by Western blotting. The aliquots of VEGF165 and VEGF121 proteins (500 ng) were subjected to 13% SDS-PAGE and transferred to nitrocellulose membrane. The membrane blocked with 3% MPBS was incubated with 10 μg/ml purified scFv for 2 h at 37 °C, and the bound scFv was detected by mouse mAb 9E10 and HRP-conjugated goat anti-mouse IgG Conjugate as described as above.

Determination of Binding Parameters by Surface Plasmon Resonance

Binding constants of the interaction between VEGF165 and scFv were determined by surface plasmon resonance with a BIAcore X instrument (Biacore, Uppsala, Sweden).

Purified scFv was coupled to a carboxymethyl dextran chip using the *N*-hydroxysuccinimide/dimethylaminopropyl ethylcarbodimide hydrochloride kit. Different concentrations of VEGF165 diluted in the hydroxyethyl-piperazineethanesulfonic acid buffer containing 0.001% detergent P-20 was injected onto the sensor surface. The real time curves were recorded automatically and analyzed using the BIA evaluation software 3.0.

Endothelial Cell Proliferation Assay

HUVECs were plated in 96-well plates at 5×10^3 cells/well and incubated overnight in 100 μl of growth medium containing heparin and supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, USA). The medium was changed after cell adhesion to endothelial serum-free media and incubated for 24 h. HUVECs were then exposed to VEGF165 (2 μg/ml final concentration), and varing amounts of scFv-phage or scFv as indicated were added to wells. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for the time indicated in the results of each experiment. Cell proliferation was measured by the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide method using a Bio-Rad model 550 microplate reader [13].

Results

Panning of VEGF165-specific Binding Clones from the Phage Antibody Library

Four rounds of selection and amplification of the bound phage were performed. In each round, the input phage titers were kept uniformly at 1×10^{13} pfu. After each round of panning, the titer of the eluted phage was measured to monitor the efficiency of the selection process (Fig. 1). An almost 200-times increase in phage recovery after the third round in comparison to the second round of selection and a minimal increase after the fourth round in comparison to the third indicated that the library was already enriched in VEGF165-specific binders.

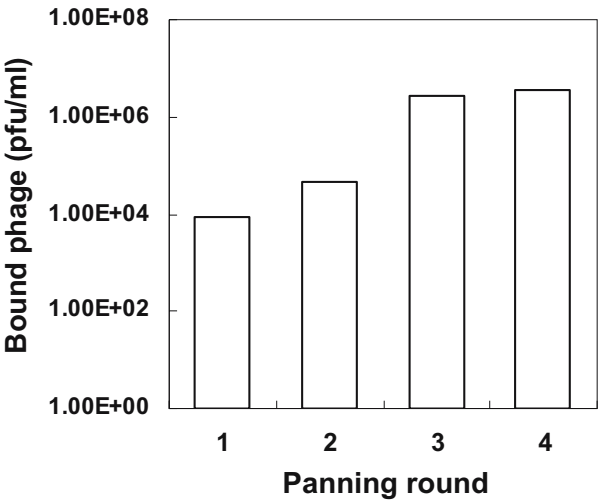

Fig. 1 Selection of scFv-phages binding to VEGF165. The number of phages bound to immunotube coated with VEGF165 after each panning round is indicated. The bound phages were eluted from the immunotubes by incubation with 100 mM triethylamine as explained in "Materials and Methods." After recovery, the titers of these phage were determined on *E. coli* TG1 cells and selected for ampicillin resistance. In each panning round, the number of input phage was kept constant at 1×10^{13} pfu and the phage that did not bind VEGF165 were removed by washing with PBS

The increasing number of the specific binders in the total phage population was confirmed with polyclonal phage ELISA, which was done with equivalent amounts of amplified phages (about 1×10^{12} pfu) after each round of selection. Polyclonal phage ELISA after each round of panning gave higher values of absorbance than in the previous round (Fig. 2), verifying the continuous increase in the number of specific phage binders during selection.

The polyclonal character of the phage ELISA does not give a clear image of the antibody specificity because of different growth rates and expression levels between individual clones. Therefore, the eluted scFv-phages after the fourth round of selection were used for infection of *E. coli* TG1, which were then plated to form single colonies. Next, 90 colonies selected at random from the fourth round of selection were picked and tested for binding to VEGF165 in monoclonal phage ELISA. From the fourth selection, 40 clones were found to be specific VEGF165 binders. The reaction was considered positive if the threshold value (mean of the background plus three times the standard deviation) was exceeded. For further binding analysis, nine representative colonies, A5, B8, B9, C2, F3, F8, E6, H8, and F1, were selected (Fig. 3). PCR analysis confirmed that the selected clones contained an insert corresponding to the size of an scFv fragment (data not shown).

Selection and DNA Sequence Analysis of Phage Clone Inhibiting VEGF165 Bioactivity

HUVECs proliferation assay was used to select the phage clone that can neutralize VEGF165 bioactivity. As shown in Fig. 4, clone B8 could inhibit VEGF165-induced HUVECs proliferation strongly, compared to the other eight clones.

The double-stranded phagemid DNA was isolated from the *E. coli* cells of the B8 clone, and the DNA sequence of its scFv genes was determined. The deduced amino acid sequence of B8 is shown in Fig. 5, and the predicated molecular weight of the whole soluble scFv is about 32 kDa.

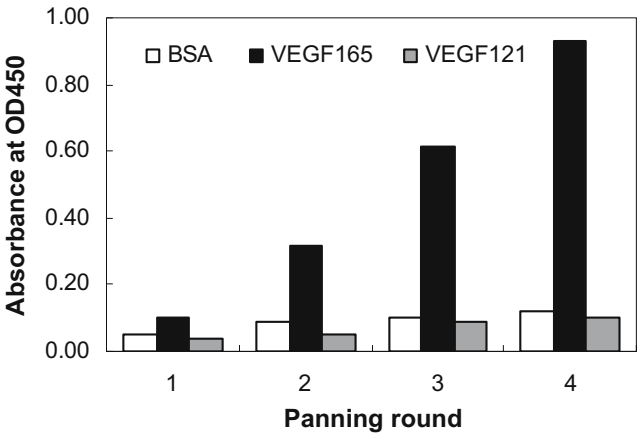

Fig. 2 Determination of antigen-specific selection after panning by polyclonal phage ELISA. A microtiter plate was coated with 10 μg/ml of bovine serum albumin (*BSA*), recombinant vascular endothelial growth factor 165 (*VEGF165*) or vascular endothelial growth factor 121 (*VEGF121*) protein at room temperature overnight. After washing the plate, amplified phage particles (about 1×10^{12} pfu) after each panning were added into each well. Anti-M13 antibody conjugated with horse radish peroxidase was used as a secondary antibody, and the BM Blue POD substrate was used as a substrate. Absorbance was measured at OD 450 nm. The experiment was done in triplicate, and the average absorbance value is shown in the figure

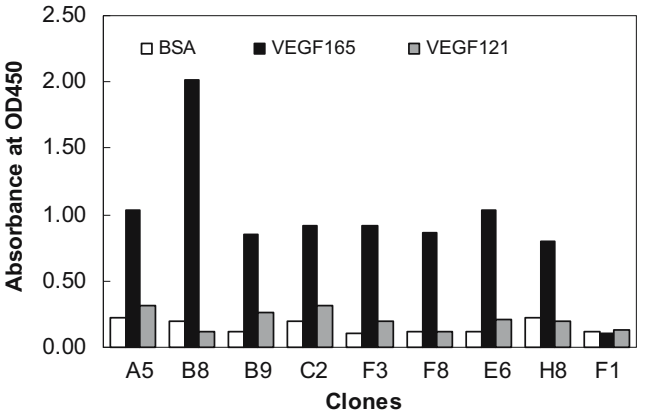

Fig. 3 Identification of VEGF165-specific phage clones by monoclonal phage ELISA. After the fourth round of panning, the eluted phages were used to infect E. coli strains HB2151 and grown in LB/amp plates overnight at 37 °C; *E. coli* clones were randomly selected and grown in LB/amp. Each amplified phages (about 1×10^{12} pfu) were added into the microtiter plate coated with 10 μg/ml of BSA, VEGF165, or VEGF121 protein. Anti-M13 antibody conjugated with horse radish peroxidase (HRP) was used as a secondary antibody, and the BM Blue POD substrate was used as a substrate Absorbance was measured at OD 450 nm. The experiment was done in triplicate, and the average absorbance value is shown in the figure

Expression and Purification of Soluble scFv

In the nonsuppressor strain *E. coli* HB2151, the amber stop codon between c-*myc* tag and p3 in scFv clones is recognized as a stop codon, and soluble scFv fusion protein is produced as a consequence. Clone B8 was chosen for expression analysis, and the samples aspirated according to induced time were subjected to 13% SDS-PAGE gel. Then, the

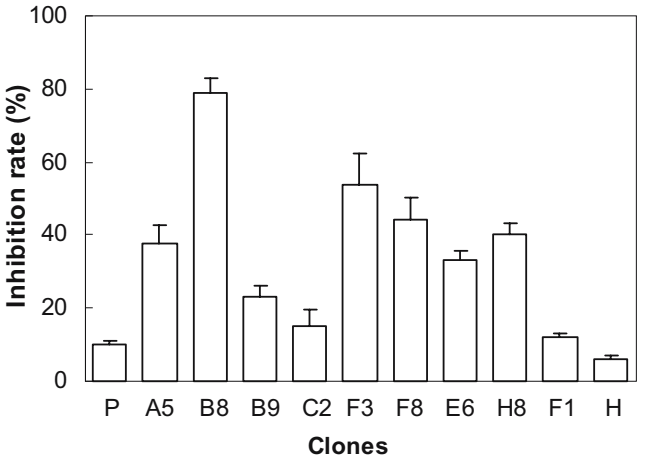

Fig. 4 Selection of phage clone inhibiting VEGF165 bioactivity. About 10^5 Human umbilical vein-derived endothelial cells (HUVECs) were cultured in triplicate in 96-well flat-bottom plates and stimulated with 2 μg/ml VEGF165 and 1×10^{10} phage particles. A5, B8, B9, C2, F3, F8, E6, H8, and F1 are monoclonal clones that give a positive signal in monoclonal phage ELISA. *P* (primary library), *H* (helper phage VCSM13), and irrelevant phage clone F1 were used as the negative controls. Cell growth was then evaluated by the MTT assay. The experiment was done in triplicate, and the average value is shown in the figure

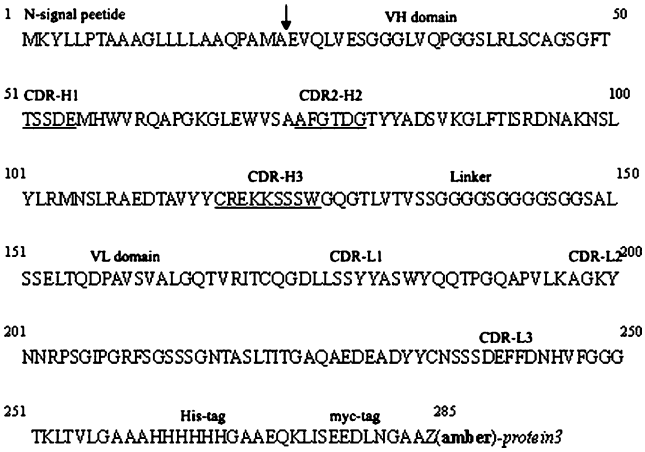

Fig. 5 Amino acid sequence of B8 scFv. The amino acid sequence of the B8 scFv protein encoded by the phagemid is shown. The positions of the N-terminal signal peptide, the VH domain, the linker peptide, the VL domain, and the c-*myc* tag are indicated. The complementarity-determining regions (CDR) of the VH and VL domains are labeled and *underlined*. The site of cleavage of the bacterial signal peptidase is marked below the sequence of B8 scFv. When produced in *E. coli* TG1 cells (*sup*E), these scFvs are also synthesized with p3 of M13. The location of the suppressed stop codon (*amber*), which is placed between the scFv and p3 coding sequences, is indicated

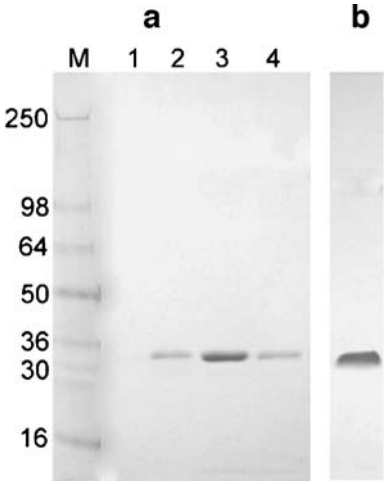

Fig. 6 Expression and purification analysis of periplasmic preparation of HB2151 containing B8 scFv inserts. *a*, analysis of the B8 scFv expression by detecting c-*myc* tag in Western blotting. The peiplasmic preparation was electrophoresed through a 13% SDS-PAGE gel and transferred into the NC membrane. The c-*myc* tag was detected using the monoclonal antibody 9E10. *Lanes 1–4*: total cell lysates from clone B8-transformed *E coli* HB2151 cells after 0, 2, 4, 6 h of being induced by 1 mM IPTG, respectively. Induced after 2, 4, and 6 h gave the positive signal. *b*, Coomasie Blue-stained 13% SDS-PAGE showing the purification of B8 scFv by IMAC. Approximately, the 30-kDa band corresponds to purified scFv

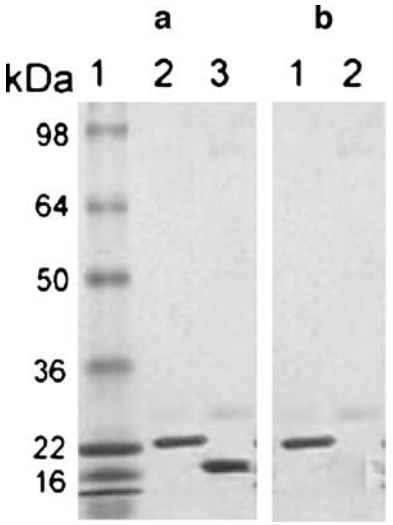

Fig. 7 Analysis of specificity of purified scFv to VEGF165 by western blotting. *a*, Proteins were subjected to 13% SDS-PAGE gel under reducing conditions and stained with Coomassie blue R-250. *Lane 1*: LMW marker of protein; *lane 2*: VEGF165 (500 ng/lane); *lane 3*: VEGF121 (500 ng/lane). *b*, Immunoblotting of VEGF165 and VEGF121 protein stained with purified scFv (10 μg/ml), and the bound scFv was detected by mAb 9E10 and HRP-conjugated goat anti-mouse IgG; the peroxidase activity was developed with TMB. *Lane 1*: VEGF165 (500 ng/lane); *lane 2*: VEGF121 (500 ng/lane)

proteins on the gel were transferred to nitrocellulose membrane and used for Western blotting analysis (Fig. 6a) according to the former described method. The result showed that scFv was expressed because the c-*myc* tag was detected in Western blotting.

The soluble scFv (containing a ployhistidine tail) was purified from bacterial lysates by Ni^{2+}-IDA His-bind resin, and the purity of the obtained scFv was confirmed by 13% SDS-PAGE stained with Coomassie Brilliant Blue R-250 (Fig. 6b), where the purified scFv appeared as a single 32-kDa band at high purity (more than 95%, as estimated by

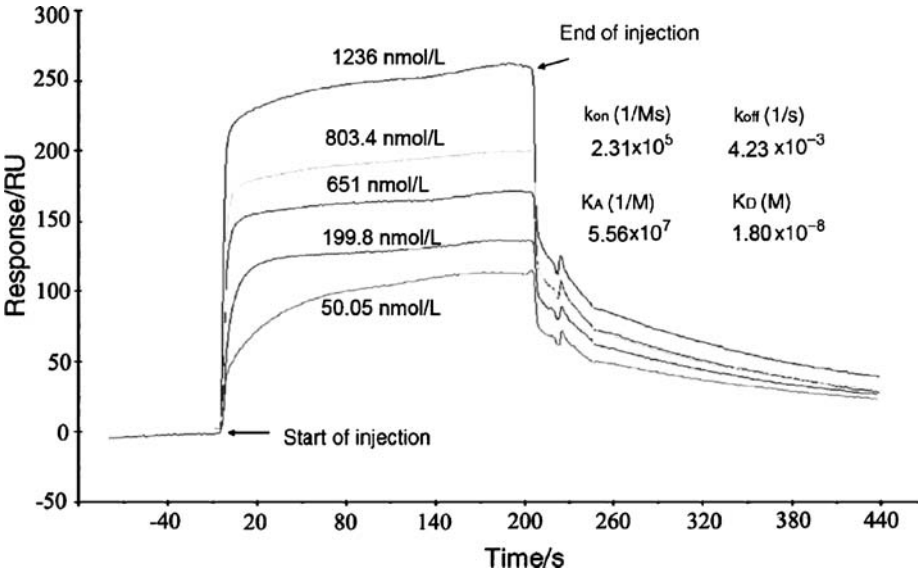

Fig. 8 Measurement of binding parameters of scFv to reteplase using SPR. scFv was immobilized on the surface of sensor chip CM5 and challenged with VEGF165 at varied concentrations. The *arrows* indicate the beginning or end of the injection

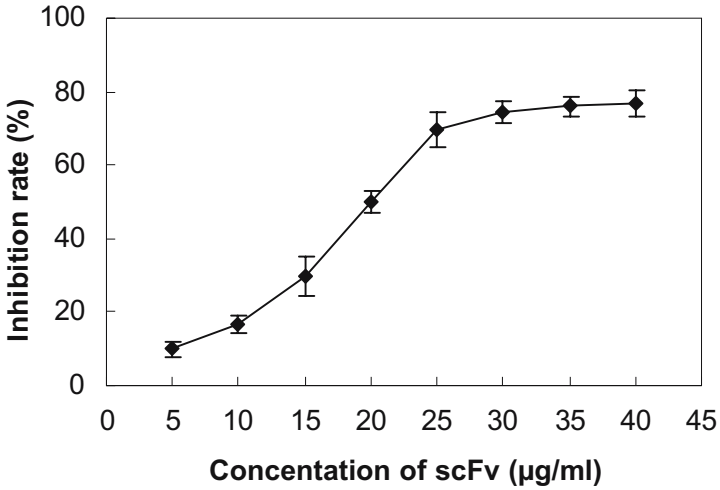

Fig. 9 Inhibition of purified B8 scFv to HUVECs proliferation assay induced by VEGF165. About 10^5 HUVECs were cultured in triplicate in 96-well flat-bottom plates and stimulated with 2 μg/ml VEGF165 and various concentrations of B8 scFv. Cell growth was then evaluated by the MTT assay. The experiment was done in triplicate, and the average value is shown in the figure

absorbance scanning). The purified scFv with a yield of around 2.5 mg/l of the production culture was quantified by absorbance at 280 nm of the purity solution.

Characterization of the Purified scFv

VEGF121 has the most similar structure, as VEGF165 could be used to detect the specificity of the scFv. As seen in Fig. 7, the soluble scFv specifically recognized VEGF165 with no cross-reaction to VEGF121. The fact that the scFv bound to the antigen run on the gel under denaturing conditions shows that the epitope region that the scFv recognizes is linear.

Purified scFv was immobilized on the chip surface and challenged with VEGF165 at five different concentrations (Fig. 8). The K_D was 1.80×10^{-8} M, suggesting a sufficient affinity for VEGF165.

To investigate the functional potential of the selected scFv, HUVECs proliferation assay was used. As shown in Fig. 9, HUVECs proliferate in response to the costimulation by VEGF165 signaling. In contrast, a dose-dependent inhibition effect was obtained with B8 scFv.

Discussion

The aim of the study was the generation and characterization of scFv directed against VEGF165, which could have therapeutic applications in angiogenesis-dependent diseases. After four rounds of selection, 40 out of 90 phage clones displayed positive clones. Input/out ratio and affinity assays confirmed that all above improvements were effective. Phages

binding to VEGF165 specifically were riched. Koivumen et al. [22] found that some phages with high affinity could not be recovered by acid elution (1 M HCl, adjusted to pH 2.2 with glycine). In our study, it was observed that the retained phages after triethylamine elution instead of acid elution could be harvested by cytolysis. In this method, freshly diluted 100 mM triethylamine (pH 10.0) is incubated with the bound phage for 10 min. The pH of the eluted phage is then adjusted immediately with one-half volume 1 M Tris–HCl, pH 7.4. The phage can then be used to infect TG1 as normal. Care must be taken with this method because elution for longer than 10 min can damage the phage and affect infection. If there is still difficulty in eluting VEGF165-specific phages using this method, then it is possible to add the log-phase TG1 directly to the well (100 μl) or immunotube (2 ml with slow rotation) and incubating for 30 min at 37 °C. Amplification can then proceed as normal.

Western blotting analysis indicated that the purified B8 scFv exhibited the specificity to VEGF165. The K_D of B8 scFv for VEGF165 was determined by surface plasmon resonance experiments. The affinity of scFv was usually less than that of the scFv-phage, this might be caused by residues at both ends of scFv displayed on phages, which stabilize the structure of scFv and reinforce its binding to VEGF165. The scFv is an artificial monovalent molecule with a different conformation at the antigen-binding site. The reduced binding affinity of scFv can be improved by changing the amino acid sequence [23] or by constructing a divalent form of the diabody [24] with similar binding affinities to those of the whole antibody.

In contrast to other angiogenic growth factors, VEGF acts almost exclusively on endothelial cells, where its high-affinity tyrosine kinase receptors KDR/Flk-1and Flt-1 reside [5]. VEGF, besides being an angiogenic factor, is also a survival factor for newly formed blood vessels and is a highly potent direct mediator of microvascular permeability [7], a process that is essential for vessel sprouting. An important role for VEGF as a mediator of tumor angiogenesis is suggested by the observation that VEGF is abundantly expressed and secreted by several tumors [8]. The multifunctional role of VEGF in promoting tumor angiogenesis and metastases renders VEGF unique, and not all of its functions may be substituted for by other angiogenic factors. Therefore, VEGF is an attractive target for antiangiogenic therapeutic intervention. Specific antibodies against a diversity of antigen were developed to block the function of antigen or guide effective molecules to in applied research such as diagnosis and treatment of diseases because the foundation of antigen–antibody interaction [25]. Our functional assay also showed the purified scFv could dose-dependently inhibited VEGF165-induced HUVECs proliferation. Moreover, because of it single-gene structure, it will be very easy to conjugate this molecule with other effectors such as human Fc fragment and immunotoxin by fusing with their genes. The construction of the scFv–Fc fusion will offer a potential candidate molecule for angiogenesis-dependent diseases.

In conclusion, as to human VEGF165, this report takes advantages over previous work in two aspects at least. First, hybridoma technique was replaced with a more convenient phage display technique. Second, a human-originated phage display library was used to avoid the immunoreaction between species. Our data indicate that by designing a proper selection of antibodies from an antibody library, specific antibodies toward VEGF165 can be achieved. Further research work is in progress.

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