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Production of Novel VHH Nanobody Inhibiting Angiogenesis by Targeting Binding Site of VEGF

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Abstract Vascular endothelial growth factor (VEGF) is one of the key players in angiogenesis and is considered as one of the major targets in cancer therapy. VEGF is secreted by the cancerous cells to form new vessels that carry oxygen and nutrients to the tumor, allowing it to grow beyond 1–2 mm. Cancerous cells spread using these veins and cause malignancy. Therefore, neutralization of VEGF could prevent tumor growth and malignancy, and nowadays, antibodies are widely used for such purpose. Among antibody fragments, nanobodies possess unique characteristics which make them appropriate tools for cancer therapy. In this study, the receptor-binding region of VEGF was used to produce a nanobody using phage display technology. A camel was immunized with the recombinant VEGF, and VHH fragments were amplified using nested PCR on lymphocyte complementary DNA (cDNA). The highest binding affinity was achieved after three rounds of panning. Twenty-four clones were tested by monoclonal phage ELISA, and the clone with the highest affinity (VA12) was selected for soluble expression of nanobody. VA12 was tested under various physicochemical conditions and showed considerable stability in extreme temperatures, pH, and various urea concentrations. Stability of VA12 under such conditions is considered as an advantage over the prevailing antibodies.

Keywords Angiogenesis · Cancer · VEGF · VHH nanobody · Phage display

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Introduction

Vascular endothelial growth factor (VEGF) is a highly specific angiogenic factor with crucial role in angiogenesis of tumors [1]. Angiogenesis is described as the formation of new blood vessels from preexisting ones. VEGF also has an important role in the formation of new blood vessels under normal physiological conditions. In such conditions, angiogenesis is strongly regulated by positive effectors and natural inhibitors [2]. Excessive angiogenesis occurs in several diseases such as diabetic, retinopathy, and cancer [3, 4]. In a tumor, the newly formed vessels carry oxygen and nutrients to cancerous cells and allow the tumor mass to grow beyond 1–2 mm in diameter enabling a small cluster of mutant cells to travel across the body and form malignant tumors [5]. This will allow cancerous cells to spread to other organs and eventually leads to metastatic cancer [6, 7]. Thus, angiogenesis has a major role in the growth of tumors and metastasis. Blocking angiogenesis pathway can eliminate formation of new vessels and consequently cause starvation of tumor cells. Therefore, anti-angiogenic agents are considered valuable products for anti-cancer therapy [8, 9].

Angiogenesis is regulated by specific factors including VEGF which is secreted from the tumor cells. Binding of VEGF to its receptors induces a downstream signaling and ultimately causes vein formation [10]. Therefore, if the receptor-binding site of VEGF is blocked by an agent, angiogenesis process is ceased and tumor progression and metastasis can be prevented [11].

It was demonstrated that VEGF is upregulated in many cancers, including glioblastoma, colorectal, non-small-cell lung, renal cell, pancreatic, ovarian cancers, and other malignancies [9]. It has also been demonstrated that the receptor-binding domain of VEGF is located within the first 110 residues of the protein [12, 13].

Camels and other species of the camelidae family have developed a unique type of antibodies called heavy chain antibodies that lack the light chain of the conventional antibodies [14, 15]. The antigen-binding domain in heavy chain antibodies (HCAbs) has specific merits compared to the conventional ones. It has a considerable small size, and the CDR3 is significantly longer [15]. Also, in the framework regions (FRs), there are some amino acid substitutions. These new amino acid residues result in high solubility of the HCAbs [15]. In HCAbs, the antigen-binding fragment is only composed of one variable domain termed "VHH." VHH (or nanobody) is the smallest natural intact fragment with the capability of binding to an antigen [15]. It weighs about 15 kDa, with 2.5 nm in diameter and 4 nm in height [14]. In addition to low molecular mass, nanobodies possess many inherent favorable characteristics, such as high physiochemical stability, better tissue penetration, rapid clearance, and high expression in yeasts and bacteria [16]. Furthermore, the elongated CDR3 allows recognition of hidden epitopes of antigens that are normally not accessible by the conventional antibodies [17]. Overall, the unique characteristics of nanobodies have made them very useful tools for biotechnological and therapeutic applications.

In this study, the binding domain of VEGF-A protein was chosen for production of high-affinity nanobodies via M13 phage display technology [18]. The characteristic of the nanobody was then analyzed in a series of in vitro assays.

Materials and Methods

Cloning, Expression, and Purification of Recombinant VEGF-A

The nucleotide sequence which encodes the first 110 residues of VEGF-A protein was optimized according to *Escherichia coli* codon usage and synthesized by Shine Gene Inc

(China). The recombinant *VEGF-A* gene was subcloned into pET22b expression vector. The new construct was then transferred into *E. coli* BL21 (DE3). The transformed bacteria were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at the optical density of 0.6 at 600 nm and incubated at 37 °C/250 rpm. The cells were collected after 4 h and resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris base, 8 M urea, pH 8). Following sonication, the recombinant VEGF (rVEGF) was analyzed on 14 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then gel purified. Purification of the rVEGF was verified by Western blotting. The rVEGF was refolded by sequential dialysis with gradual reduction of urea concentration. Concentration of the protein was determined by Bradford method [19].

Immunization of Camel

A male Arabian camel (*Camelus dromedarius*) was immunized as explained before [20, 21]. Blood samples were collected prior to each injection, and the sera were isolated and used for monitoring the immunization process.

Lymphocyte Isolation and VHH Cloning

Blood was collected 1 week after the fourth booster, and EDTA was added at 2 mg/ml as an anti-coagulant. Peripheral blood lymphocytes were isolated using Ficoll (Baharafshan Company, Tehran, Iran) gradient centrifugation and then washed with sterile phosphate-buffered saline (PBS) (0.8 % NaCl, 0.02 % KCl, 0.29 % Na_2HPO_4, and 0.02 % KH_2PO_4, pH 7.2). The lymphocytes were stored in RNase free tubes at −80 °C. Total RNA was isolated from 10^7 lymphocytes using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from RNA with Revert Aid™ first-strand cDNA synthesis kit (Fermentas). For RT-PCR reaction, the RNA was used at 2.2 μg concentration in a 20 μl reaction mixture using oligo dT as the primer. For amplification of VHH genes, nested PCR was performed using cDNA as the template in the first PCR. The VHH-related fragments were purified by gel purification kit (Bioneer) and used as the template in the second PCR. *sfi*I (Vivantis, South Korea) digested pComb3X phagemid and gel-purified VHH fragments were ligated together using T4 DNA ligase (Vivantis, South Korea). All primers and thermocycling conditions were the same as our previous studies [22, 23].

Library Construction

The recombinant constructs were transferred into *E. coli* TG1 cells via electroporation (Gene Pulser Xcell, Bio-Rad, USA). Library size was calculated by culturing 10 μl of the transformed cells on Lauria-Bertani (LB) agar plates containing 70 μg/ml ampicillin. Colony PCR was performed on randomly selected colonies to confirm the cloning process.

Library was constructed by culturing transformed bacteria in 30 ml Super Broth (SB) medium (3 % tryptone, 2 % yeast extract, and 1 % MOPS) containing 50 μg/ml ampicillin and then incubating at 37 °C/250 rpm until the optical density at 600 nm (OD_{600}) reached 0.6. The culture was inoculated with 10^{12} M13K07 helper phage (Amersham, USA) and incubated for 30 min/37 °C without shaking. To ensure expression of antibiotic resistance genes, the culture was incubated for another 30 min at 37 °C/250 rpm followed by increasing the culture volume

to 200 ml with fresh SB medium. Ampicillin and kanamycin were added to reach concentrations of 70 and 50 μg/ml, respectively, and the culture was incubated for 16 h at 37 °C/250 rpm.

The nanobody expressing phage particles were purified by centrifugation at 5000 rpm at 4 °C for 20 min. The supernatant was mixed with 20 % (*v*/*v*) PEG/NaCl solution (16.7 % PEG and 19.49 % NaCl) and incubated for 2 h on ice. The phage particles were sedimented at 15,000× *g* at 4 °C/20 min and resuspended in 2 ml of Tris-buffered saline (TBS) (150 mM NaCl and 50 mM Tris base, pH 7.2) containing 1 % bovine serum albumin (BSA) (*w*/*v*) followed by centrifugation at 12,000× *g* for 5 min at 4 °C to remove bacterial debris. The supernatant was passed through 0.2 μm filter (Whatman, USA) and kept at 4 °C until further use.

Bio-Panning

VEGF was coated in the wells of a 96-well ELISA plate (Nunclon, Denmark) at 10 μg concentration in 100 mM carbonate/bicarbonate coating buffer and incubated at 4 °C for 16 h. Wells were washed with TBS containing 0.2 % Tween-20 (*v*/*v*) (TBS-T) and blocked at 37 °C for 45 min with 200 μl of TBS containing 5 % BSA (*w*/*v*). After washing the wells with TBS-T, 100 μl of the phage library was added and the plate was incubated at 37 °C for 2 h. Wells were washed several times with TBS-T. Attached phage particles were eluted with 50 μl of elution buffer (0.1 M HCl–glycine, pH 2.2) and then neutralized with 10 μl of neutralization buffer (1 M Tris base, pH 9). Eluted phage particles were used to infect *E. coli* TG1 bacteria at the OD_{600} of 0.4. VHH encoding phage particles were propagated as described under "*Library construction*." Phage particles obtained from each round of panning was used in the next round. To allow isolation of nanobodies with high affinity, the amount of Tween-20 was increased by 0.1 % after each round of bio-panning.

Panning process was monitored by polyclonal phage ELISA. For each panning round, two wells were coated with rVEGF. ELISA was carried out as described above. Instead of eluting phage particles, 100 μl of anti-M13 horseradish peroxidase (HRP)-conjugated antibody (Amersham, USA) was added to each well and the plate was incubated at 37 °C for 45 min. Wells were washed with TBS-T, and 100 μl of TMB substrate (Sigma) was added. The reaction was stopped with addition of 100 μl 3 N H_2SO_4 after 15 min. Absorbance was read at 450 nm.

Nanobody Selection

Monoclonal nanobodies with the highest absorbance were selected from the third round of panning.

Colonies were randomly selected and cultured in SB medium. Nanobody-encoding phage particles were propagated as described under Library construction. Phage particles obtained from 24 clones were analyzed by monoclonal phage ELISA. Phage ELISA was performed as described under "*Bio-panning*."

Expression of Anti-VEGF VHH

The phage with the highest absorbance in monoclonal phage ELISA was named VA12 and selected for soluble expression of VHH. After phagemid extraction, the VHH was amplified via PCR using Fr4-*Eco*RI and Fr1-*Hin*dIII primers [22]. The amplified VHH gene was cloned

into pET28a expression vectors. The construct was transferred into *E. coli* BL21 (DE3). The cloning process was confirmed by digestion of recombinant plasmids with *Eco*RI and *Hin*dIII enzymes. Transformed bacteria were cultured in LB medium containing 70 μg/ml kanamycin and incubated at 37 °C/250 rpm. Expression was induced with 1 mM IPTG at OD_{600} of 0.6. Six hours after induction, cells were collected by centrifugation at 5000 rpm for 20 min. Cells were resuspended in lysis buffer and lysed by sonication. After overnight incubation at 37 °C, bacterial debris were removed by centrifugation at 13,000 rpm at 4 °C for 20 min. Expression was studied on 14 % SDS-PAGE and confirmed by Western blotting using anti-His tag antibodies (Qiagen). For Western blotting, proteins were transferred onto nitrocellulose paper by Bio-Rad mini-protein tetra system for 90 min at 75 V. The nitrocellulose paper was washed with PBS and blocked with PBS containing 5 % skim milk (*w*/*v*) at 4 °C for 16 h. After washing the nitrocellulose paper with PBT containing 0.5 % Tween-20 (PBS-T (*v*/*v*)) for 20 min, anti-His tag antibodies conjugated with HRP (Qiagen) were added at a final concentration of 1/10,000. The paper was incubated at 37 °C for 45 min, then washed twice with PBS-T and submerged in substrate solution (50 mM Tris, 0.6 mg/ml diaminobenzoic acid and 1 μl/ml H_2O_2) until the bands were visualized. The reaction was stopped with addition of PBS.

Anti-VEGF VHH was purified using Ni-NTA chromatography (Qiagen).

Affinity Determination and Characterization of Anti-VEGF VHH

Affinity of VA12 was estimated using a method described by Beatty et al. [24].

Stability of VA12 VHH was assessed at various pHs, urea concentrations, and temperatures. In different reactions, VA12 VHH was incubated at 90, 70, 50, 37, 15, and 4 °C for 2 h and then allowed to reach room temperature (RT) for 20 min. For evaluating the VHH stability at various pHs, VA12 was incubated in PBS with pHs of 10, 8, 7, 5, and 3 for 2 h in separate reactions. VHH resistance to denaturation was studied by incubating VA12 in PBS containing 8, 4, and 2 M concentrations of urea. Immunoreactivity of VA12 nanobody incubated at various conditions was analyzed by ELISA on rVEGF. In all tests, 10 μg of VA12 was used per well. For each incubation condition, two wells were coated with 10 μg of rVEGF in 100 mM carbonate/bicarbonate buffer. Wells were washed and blocked with PBS containing 5 % BSA at 37 °C for 45 min. After washing the wells with PBT-T, VA12 nanobodies were added to their corresponding wells and the ELISA plate was incubated at 37 °C for 2 h. Wells were washed with PBS-T, and 100 μl of anti-His tag antibody conjugated with HRP (Qiagen Company) at the dilution of 1/10,000 was added to each well. The ELISA plate was incubated at 37 °C for 45 min, then washed, and 100 μl of TMB substrate (Sigma) was added to each well. The immunoreactions were stopped by addition of 100 μl 3 N H_2SO_4 after 15 min.

In Vivo Assay

For analysis of anti-angiogenesis effects of the produced nanobody, chorioallantoic membrane assay (CAM assay) was performed. Fertilized hen eggs were obtained from Morghak Company (Karaj, Iran). Eggs were incubated in an egg incubator at 37 °C and 75 % humidity. On incubation day three approximately 3 ml of albumin were removed using sterile syringe to create false air sac. Eggs were kept incubated under the same conditions. On incubation day 9, small hatch was opened and 20 μg of VA12 nanobody was applied on chorioallantoic membrane of the test samples and same volume of PBS on the control ones. The results were monitored after 24 h. All procedures were performed under sterile conditions.

Results

Preparation of Recombinant VEGF-A and Immunization of Camel

Cloning of the recombinant VEGF gene into pET22b vector was confirmed with digestion of the recombinant construct. Expression of the protein was confirmed with the observation of a 17 kDa band on SDS-PAGE. After purification of the protein, a single band of 17 kDa was observed on the SDS-PAGE (Fig. 1a).

Induction of immune response was evaluated by ELISA using sera obtained after each injection. Serial dilution of immune and non-immune sera was tested in an indirect ELISA, and considerable rise in an anti-VEGF-A antibody was observed after the fourth booster.

Production of VHH and Library Construction

In the first PCR, regions between CH2 and framework 1 were amplified in both VH and VHH genes of conventional and heavy chain antibodies. The second pair of primers anneal to the FR1 and FR4 regions of the VHH gene. The first PCR resulted

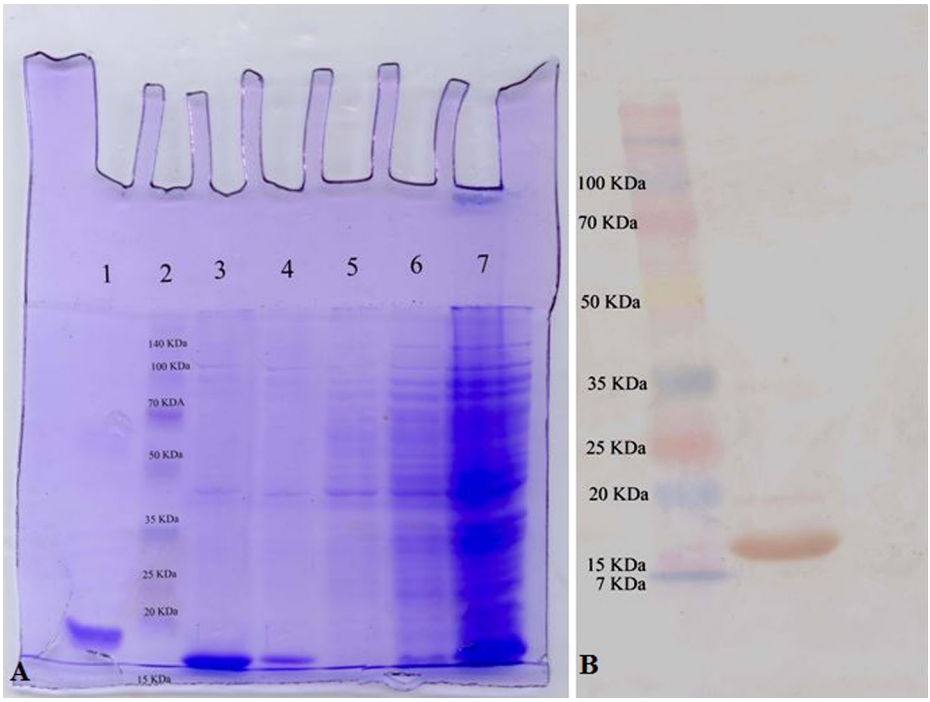

Fig. 1 **a** SDS-PAGE. *Lane 1*: rVEGF expression in pET22b and gel purification. *Lane 2*: standard protein molecular weight marker. *Lane 3*: VA12 nanobody expressed in pET28a and purified via Ni-NTA chromatography. *Lanes 4 to 6*: Ni-NTA column outcome after loading washing buffers D and C. *Lane 7*: Expression of VA12 nanobody via 1 mM IPTG after 6 h. **b** Western blotting of VA12 nanobody. Expression of the VA12 nanobody was confirmed via Western blotting. The 17 kDa band indicated the expression of VA12 nanobody

in 620, 690, and 900 bp bands and 620 and 690 bp bands were gel purified and used in the second PCR. A 400 bp VHH band was observed in the second PCR. The VHH gene was digested with *sfi*I and ligated to similarly digested pComb3X phagemid. Calculation of library size revealed 7.8×10^8 members.

Screening for Anti-VEGF VHH

Four rounds of panning were carried out, and colonies obtained from each round were confirmed by colony PCR for the presence of VHH gene. The panning process was monitored by polyclonal phage ELISA using eluted phages from each panning round (Fig. 2). Third round of panning showed the highest absorbance in polyclonal phage ELISA and was used for further analysis. Twenty-four colonies selected from the third round and were used for monoclonal phage ELISA. Four colonies named VA3, VA12, VA14, and VA15 had the highest immunoreactivity (Fig. 3). VA12 with the highest affinity was selected for soluble expression of the nanobody.

Expression in Soluble Form

The VHH gene was subcloned in pET28a expression vector. The cloning was confirmed by PCR and digestion of the phagemid with *Eco*RI and *Hind*III enzymes. The expression induced by 1 mM IPTG, resulted in 17 kDa band on the SDS-PAGE (Fig. 1a). Expression was confirmed by Western blotting (Fig. 1b). Purification with Ni-NTA chromatography resulted in a single band of 17 kDa (Fig. 1a).

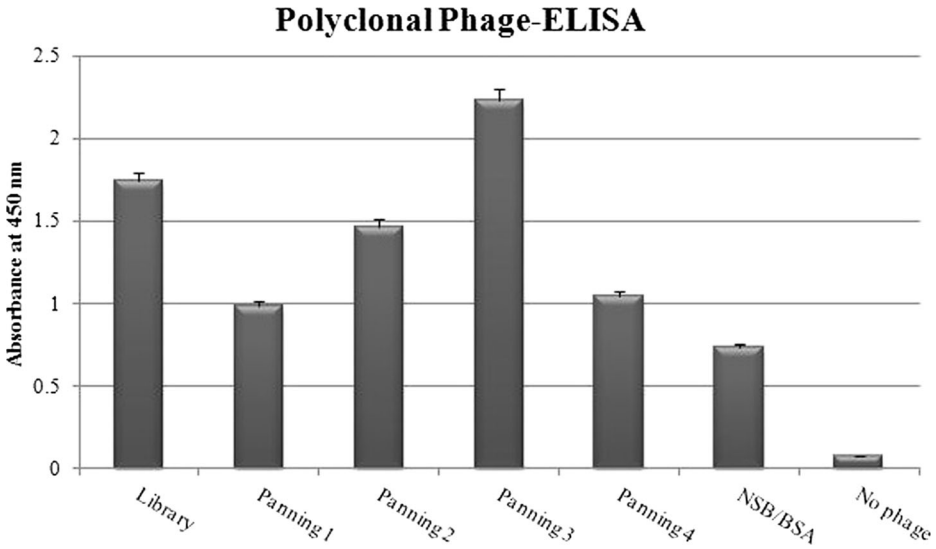

Fig. 2 Polyclonal phage ELISA. ELISA was performed after four rounds of panning. Third round showed the highest absorbance and was used for monoclonal phage ELISA. BSA was used to measure the non-specific binding (NSB). Binding of secondary antibody (anti-M13) to VEGF was also measured and showed no immune reaction

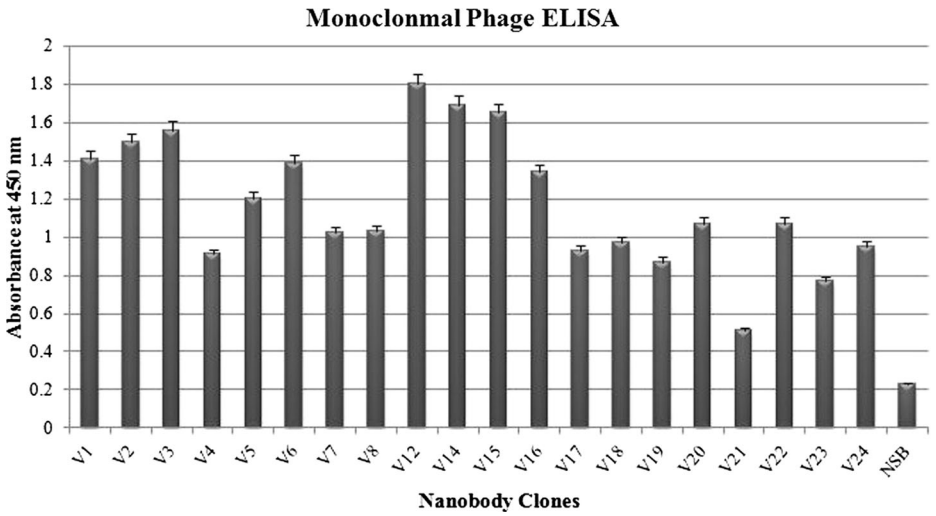

Fig. 3 Monoclonal phage ELISA. Twenty-four clones (20 are shown) were selected from the third round of panning, and their affinity toward VEGF was measured by monoclonal phage ELISA. Clone VA12 showed the highest affinity and was used for further analysis

VA12 Characterization and Affinity Estimation

VA12 nanobody showed significant binding stability after incubation at various conditions (Fig. 4). VA12 retained 85 % activity after incubation at 90 °C for 2 h, and 56 % activity was observed after incubation in 8 M urea for 2 h. VA12 nanobody also showed considerable stability at various pH values (Fig. 4). The binding activity of VA12 was reduced to 53 % after 2-h incubation at an acidic pH of 3. The affinity of VA12 nanobody was determined to be about 3×10^{-9} M.

In Vivo Assay

Comparing to the control samples, VA12 nanobody showed substantial anti-angiogenesis activity (Fig. 5).

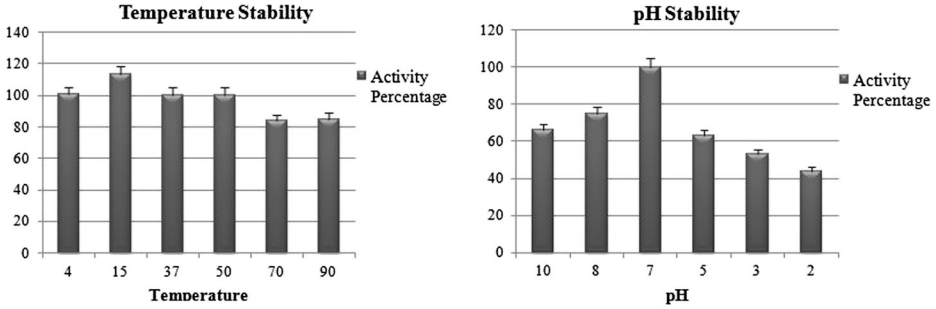

Fig. 4 Stability and binding activity of VA12 were analyzed in different conditions. Binding activity of VA12 nanobody toward VEGF was measured 85 % after 2-h incubation in 90 °C. VA12 nanobody also showed significant stability in various pH. After 2-h incubation at pH of 3, VA12 retained 53 % of its original activity

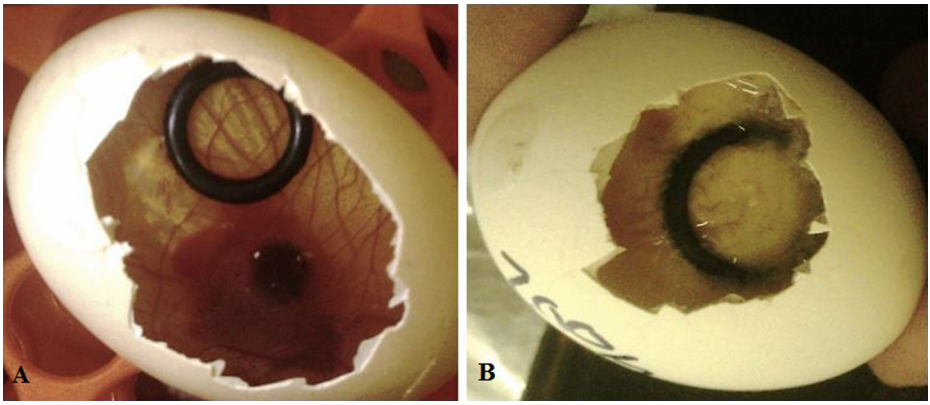

Fig. 5 CAM assay. Anti-angiogenesis potential of VA12 nanobody was evaluated by applying 20 μg of the nanobody on the chorioallantoic membrane of a fertilized egg (**b**). Control samples were received same volume of PBS (**a**)

Discussion

Conventional monoclonal antibodies have several disadvantages as pharmaceutical drugs compared to small molecules, for instance, their large size, instability, difficulty in production, and high manufacturing expenses. This is the main reason for emergence of nanobodies. Similar to conventional antibodies, nanobodies have high specificity and affinity toward a wide range of antigens [25].

Anti-VEGF antibodies have always played a significant role in cancer therapy with high specificity and low toxicity compared to chemical therapy [26–28].

Bevacizumab is the first anti-angiogenic agent which was approved by the United States Food and Drug Administration in 2004 and is proven to be effective in several malignancies, such as metastatic colorectal, lung, and breast cancers [29, 30]. Bevacizumab is a VEGF-specific monoclonal antibody which binds to VEGF and blocks its interaction with VEGF receptors (VEGFR-1 and VEGFR-2), inhibiting endothelial cell proliferation and angiogenesis [31]. Sorafenib and sunitinib, two tyrosine kinase inhibitors, are approved for use in metastatic renal cell carcinoma [32]. Sunitinib is approved for use in gastrointestinal stromal tumor patients [33]. In addition to inhibiting receptors and soluble kinases in tumor cells, they also block VEGF receptors in endothelial cells [34].

Here, we describe, for the first time, isolation and characterization of an anti-VEGF-A nanobody. The isolated nanobody was tested in different conditions and showed significant stability. The VA12 nanobody showed considerable pH stability and retained 63 and 53 % of its activity in pHs of 5 and 3, respectively. The pH resistance and stability of VA12 nanobody are important because of the microenvironment of tumor and high metabolism rate of cancerous cells which results in production high amounts of CO_2 and lactic acid thus decreases the pH of tumor microenvironment [35]. The binding of antibody to antigen is mostly aided by the electrostatic and hydrogen bonds, which essentially depends on the protein surface charge [36]. The fact that VA12 nanobody is able to maintain the binding activity even at low pH conditions is a considerable advantage over conventional anti-VEGF antibodies.

VA12 also demonstrated substantial thermostability, and the binding activity of 85 % at 90 °C was observed as compared to optimum binding activity at 37 °C. The thermostability of antibodies raises their half-life in the environment and increases the shelf life [37].

In conclusion, the VA12 nanobody showed high affinity and stability at various physico-chemical conditions and significant anti-angiogenesis potential in CAM assay. These merits give VA12 nanobody advantage over the prevailing antibodies. Additionally, the small size of nanobodies allows more tissue penetration and facilitates diffusion through the tissues which is important in solid tumors and increases the efficiency of anti-cancer agents.

Conclusion

Although clinical application of VA12 requires more in vivo testing, nevertheless, the features of this novel anti-VEGF promise emergence of an efficient anti-cancer antibody.

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