Biochemical and Biophysical Research Communications 446 (2014) 132–136

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A nanobody directed to a functional epitope on VEGF, as a novel strategy for cancer treatment

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ARTICLE INFO

Article history:
Received 13 February 2014
Available online 22 February 2014

Keywords:
VEGF
Nanobody
Phage display
Epitope
HUVEC

ABSTRACT

Compelling evidence suggests that vascular endothelial growth factor (VEGF), due to its essential role in angiogenesis, is a critical target for cancer treatment. Neutralizing monoclonal antibodies against VEGF are important class of drugs used in cancer therapy. However, the cost of production, large size, and immunogenicity are main drawbacks of conventional monoclonal therapy. Nanobodies are the smallest antigen-binding antibody fragments, which occur naturally in camelidae. Because of their remarkable features, we decided to use an immune library of nanobody to direct phage display to recognition of novel functional epitopes on VEGF. Four rounds of selection were performed and six phage-displayed nanobodies were obtained from an immune phage library. The most reactive clone in whole-cell ELISA experiments, was purified and assessed in proliferation inhibition assay. Purified ZFR-5 not only blocked interaction of VEGF with its receptor in cell ELISA experiments, but also was able to significantly inhibit proliferation response of human umbilical vein endothelial cells to VEGF in a dose-dependent manner. Taken together, our study demonstrates that by using whole-cell ELISA experiments, nanobodies against antigenic regions included in interaction of VEGF with its receptors can be directed. Because of unique and intrinsic properties of a nanobody and the ability of selected nanobody for blocking the epitope that is important for biological function of VEGF, it represents novel potential drug candidate.

1. Introduction

It has been well established that angiogenesis is critical for the tumor growth and metastasis [1]. Moreover, it is proposed that pro-angiogenic factors (e.g., vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF)) are upregulated in tumor cells [2]. Among these, VEGF acts as an endothelial cell-specific mitogen and plays important roles during tumor angiogenesis [3]. Its function is mediated by binding to two specific tyrosine-kinase receptors: VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk1), located on vascular endothelial cells [4]. It has also been hypothesized that VEGF exhibits potent pro-survival and anti-apoptotic effects in endothelial cells, besides their pro-angiogenic effects [2]. In addition, VEGF exhibits the chemo- and radio-protective effects through interaction with several different pathways [5]. Therefore, VEGF is an attractive target for antiangiogenic therapeutic intervention and consequently, treatment of cancer.

Several strategies have been used to target interaction of VEGF with its receptors. Tyrosine kinase inhibitors [6] and antibodies against either VEGF or its receptors are the most common approaches used as VEGF signaling inhibitors [7]. Monoclonal antibodies (mabs) have been demonstrated to be important class of new therapeutic molecules. Bevacizumab, a humanized mab, is the first FDA-approved anti-VEGF antibody used as a standard first-line therapy for metastatic colorectal cancer [8]. In spite of the clinical success of bevacizumab, as a conventional mab, there are still important drawbacks. They include the cost of manufacturing and purification process of mabs [9,10], large size and immunogenicity [11]. Moreover, bevacizumab inhibits VEGF binding to its receptors by steric hindrance mechanism [12]. It has also been reported that VEGF has distinct binding epitopes for bevacizumab and for receptors [12]. Therefore, new neutralizing antibody fragments targeting different epitopes on VEGF has been searched in several studies [13,14] to find efficient molecules for VEGF blocking purposes.

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http://dx.doi.org/10.1016/j.bbrc.2014.02.069
0006-291X/

The serum of camels and llamas contains a unique kind of antibody that is devoid of light chains. The antigen-binding domain of these antibodies consists of one single domain, referred to as nanobody (Nb) [15]. Nbs are the smallest known antigen-binding antibody fragments [16]. Their specific biophysical and biochemical properties and their potential of targeting novel epitopes [17], render them to potent drug candidates.

In this study, we decided to direct an immune phage display library towards recognition of epitopes that are involved in interaction of VEGF with its receptor.

2. Materials and methods

2.1. Materials

Recombinant human VEGF was obtained from BioVision (Milpitas, CA). The nickel nitrilotriacetic acid (Ni^+-NTA) resin was supplied by Qiagen (Hilden, Germany). The 6-well plates and MaxiSorp 96-well plates were from Nunc (Roskilde, Denmark).

2.2. Bacterial strains

Escherichia coli TG1 was obtained from Pharmacia (Uppsala, Sweden). *E. coli* Rosetta-gami 2 was purchased from Novagen (Madison, WI).

2.3. Phage display library

An immune phage display (10^9 clones) library derived from *Camelus dromedaries* immunized with homogenized human cancerous tissues was used for phage selection [18].

2.4. Biopanning of the nanobody phage display library

VEGF-reactive phages were captured by subtractive biopanning against Nb phage display library containing phagemid vector pComb3X grown to logarithmic phase as described previously [19]. Briefly, wells of 6-well plate were coated overnight with 0.5 μg/mL VEGF as a target antigen or the same amount of bovine serum albumin (BSA) as an irrelevant antigen. Four rounds of selection were performed. The library containing 10^9 transducing phages was applied to each round and rescued by M13KO7 helper phage. After amplification, phage particles were precipitated in 20% (w/v) polyethylene glycol 6000/2.5 M NaCl and centrifuged for 20 min at 19,000*g* at 4 °C. The pellet was resuspended in 4% skimmed milk in phosphate-buffered saline (MPBS) and used for subsequent rounds.

2.5. Enrichment assessment

The enrichment of VEGF-specific phages was monitored by titration of phages eluted at each round of the process. Briefly, eluted phages (100 μL) from the each round of selection were used to infect *E. coli* TG1 (OD600 = 0.6) grown at 37 °C. Infected cells were plated on a 2XYT plate containing ampicillin (100 μg/mL). plaque-forming unit (pfu) on TG1 bacteria eluted at each stage of the selection, was counted and the yield of each round was identified.

2.6. Phage ELISA

Individual TG1 clones were picked, grown at 37 °C, and rescued with M13KO7 helper phage as described above. The amplified phage preparation, blocked in 4% MPBS, was added to VEGF (0.5 μg/mL) coated MaxiSorp 96-well microtiter plates. The same amount of BSA was used as a control for nonspecific binding. After incubation for 1 h at 37 °C, the plates were washed four times with PBS containing Tween 20 (PBST) and incubated with a 2500-fold diluted solution of mouse anti-M13 phage-horseradish peroxidase (HRP) conjugate (Mannheim, Germany). The plates were washed with PBST and PBS, respectively and developed with tetramethylbenzidine (TMB) substrate (100 μL/well). The absorbance was read at 450 nm using an ELISA plate reader (STAT FAX 2100 Awareness Technology). Clones that exhibited at least two times stronger ELISA signals on antigen-coated plates in comparison to signals on BSA-coated plates were scored as positive.

2.7. Production of soluble nanobody

Plasmids of individual clones shown to be positive in phage ELISA against VEGF were used to transform *E. coli* Rosetta-gami 2 to obtain soluble Nbs. Expression of the Nbs was induced by addition of 1 mM IPTG (Fermentas) for 16 h at 30 °C. Bacterial supernatants were tested by ELISA to confirm specificity of soluble binders for binding to VEGF.

2.8. Whole-cell ELISA with endothelial cells

To assess the blocking effect of selected Nbs, two parallel experiments (A and B) were performed using whole-cell ELISA method with human umbilical vein endothelial cells (HUVECs). Whole-cell ELISA technique was adapted from the procedures described by Erdag et al. [20] and Hoogenboom et al. [21] except some differences described below.

HUVECs were plated in 96-well plates (5000 cells/well) in DMEM supplemented with 20% FBS (Gibco/Invitrogen) and allowed to attach at 37 °C in a 5% CO_2 humidified incubator. After reaching to 90–95% confluency, cells were fixed with ice-cold methanol (100%) for 10 min at RT and blocked with 5% MPBS for 1 h on ice. In experiment A, VEGF (0.5 μg/mL) solution was added into the each well and incubated for another 1 h at RT on ice. The wells were then washed twice with PBS containing 1% BSA (BSA/PBS). Subsequently, bacterial supernatants were added to the wells, incubated for 1 h at RT and again washed. In parallel experiment (B), plates were coated as in experiment A. VEGF (0.5 μg/mL) preincubated with Nb in a final volume of 200 μL/well was added to each well containing HUVECs and incubated as described for experiment A. The wells were washed, incubated with a 1:5000 dilution of HRP conjugated anti-HA (Roche) for 1 h and the absorbance was read at 450 nm as described above. Controls included HUVECs incubated with VEGF in the absence of Nb, or Nb in the absence of VEGF.

2.9. Nanobody purification

Expression of ZFR-5 in *E. coli* Rosetta-gami 2 cells was induced by culturing the cells in 2XYT medium containing 1 mM IPTG. The bacterial suspension was prepared and subjected to sonication for 10 min at 50% pulse (Dr. Hielscher, GmbH). After centrifugation for 20 min at 16,000*g*, the soluble Nb was purified from the supernatant by immobilized metal affinity chromatography with 5 mL Ni^+-NTA resin and the purity of the preparation was examined using SDS–PAGE.

2.10. Endothelial cell assay

The 96-well plates were cultured with HUVECs (5000 cells/well) and incubated at 37 °C for 24 h. The cells were then treated with a mixture of various amounts of Nb (0.5, 2.5, 5, and 10 μg/mL), preincubated with 5 ng/ml of VEGF, for an additional 72 h. At the end of the culture period, the MTT assay was

performed using the MTT Cell Viability Assay Kit (Biotium, Hayward, CA) according to the manufacturer's protocol. Absorbance was measured at a wavelength of 540 nm.

2.11. Statistical analysis

Statistical analyses of the data were performed using One-way ANOVA and *t* tests. Results are expressed as means ± standard deviations. Results are considered significant at a *p* value of <0.05.

3. Results and discussion

3.1. Selection and enrichment of VEGF-binding phages

An immune library containing approximately 10^9 phage clones was screened through biopanning against VEGF. Four rounds of panning were carried out to isolate specific anti-VEGF Nbs. The yield was calculated as the number of outputed phage/number of inputed phage at each round and compared to the previous round to assess enrichment of VEGF-specific phages during the panning process. Input and output phages were measured by counting of pfu on TG1 bacteria. As presented in Table 1, a 142-fold increase in the enrichment factor was observed after the third round of panning. Of the 60 individual clones from outputed phages of the round three, fifteen clones exhibited ability to bind specifically to VEGF coated plates by using monoclonal phage ELISA (Fig. 1).

3.2. Expression and analysis of soluble nanobodies

Among the selected target specific phages, six clones, ZFR-1, ZFR-2, ZFR-3, ZFR-4, ZFR-5, and ZFR-6, contained an insert corresponding to the size of a Nb, were utilized to produce soluble proteins. Results demonstrating the reactivity of soluble proteins with VEGF-coated plates are shown in Table 2.

3.3. Identification of a specific nanobody that block VEGF binding to HUVECs

Two parallel whole-cell ELISA experiments were performed to investigate the ability of selected Nbs to inhibit VEGF binding to its receptors on HUVECs. In the first experiment, Nbs were added after preincubation of HUVECs with VEGF. Due to the interaction of VEGF with its receptor, binding of Nb to corresponding epitope was prohibited and ELISA signal was decreased (Fig. 2A). In another experiment, Nbs were preincubated with VEGF, followed by adding to HUVECs. Since, a blocking Nb was able to inhibit binding of VEGF to its cognate epitope, again a low ELISA signal was achieved (Fig. 2B). Out of the six soluble Nbs, only ZFR5 exhibited low signal in both experiments. Other ELISA positive clones did not show a significant reduction in signal intensity in any experiment, indicating that these proteins did not share any epitope with VEGF. As shown in Fig. 2A and B, ELISA signal in control wells was also reduced. Taken together, the results of whole-cell ELISA experiments show that ZFR-5 is able to block interaction of VEGF with

Table 1
Enrichment of VEGF-binding phages during four rounds of panning.

Panning number	1	2	3	4
Input phage (pfu/ml)	7×10^{12}	1×10^{12}	8×10^{11}	2×10^{11}
Output phage (pfu/ml)	6×10^{4}	7×10^{5}	8×10^{6}	7×10^{6}
Yield	8.5×10^{-5}	7×10^{-5}	1×10^{-3}	3.5×10^{-3}
Enrichment[a] (yield *n*/yield *n*−1)	_	0.82	142	3.5

[a] The Enrichment in binders after third and fourth rounds of panning is achieved. The yield is expressed as follows: (The number of output phage divided by the number of input phage at each round) × 100. Input and output phages were measured by counting of plaque-forming unit (pfu) on TG1 bacteria. Enrichment is expressed as the yield of round *n* divided by the yield of round *n*−1.

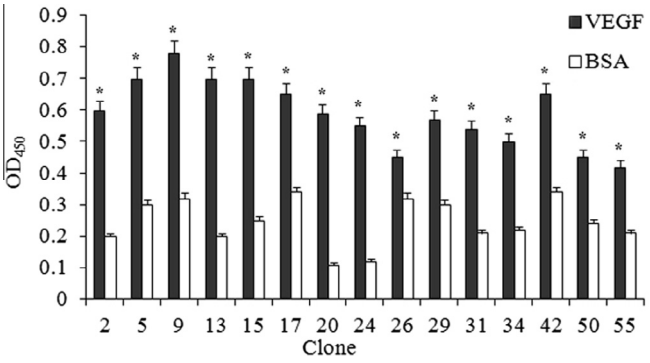

Fig. 1. Results of monoclonal Phage ELISA. Phage outputs from the third round of the panning process were used to infect exponentially growing *E. coli* TG1. After plating the infected cells, individual clones were picked and tested for binding to VEGF by phage ELISA. Detection was performed using a 2500-fold diluted solution of anti-M13-HRP and the absorbance was read at 450 nm. Phage ELISA data for fifteen positive clones are shown. Clones that exhibited at least two times stronger ELISA signals on VEGF-coated plates in comparison to signals on BSA-coated plates were scored as positive. The data are presented as mean ± SD from triplicate experiments.

Table 2
Results of the reactivity of soluble Nbs in ELISA.

Soluble Nb	ZFR-1	ZFR-2	ZFR-3	ZFR-4	ZFR-5	ZFR-6
VEGF	3.2 ± 10	2.7 ± 15	3.10 ± 18	2.6 ± 19	2.1 ± 55	3.6 ± 08
BSA	0.7 ± 13	0.9 ± 15	1.15 ± 12	0.4 ± 22	0.8 ± 18	0.7 ± 10

The cytoplasmic extracts were tested individually in ELISA experiments for production of soluble Nbs. The data are presented as mean ± SD from triplicate experiments.

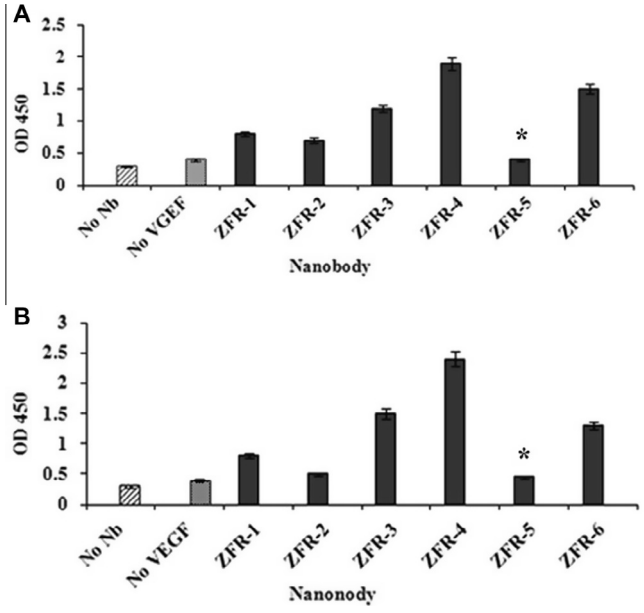

Fig. 2. Whole-cell ELISA. HUVECs were cultured in 96-well plates (5000 cells/well) in DMEM supplemented with 20% FBS. After reaching 90–95% confluency, cells were fixed with ice-cold methanol (100%) and blocked with 5% MPBS. Two parallel whole-cell ELISA experiments were performed: (A) Nbs were added to the wells preincubated with VEGF (0.5 μg/mL). (B) VEGF (0.5 μg/mL) preincubated with Nb (final volume of 200 μL/well) were added to HUVECs. The amount of bound Nbs was detected using anti-HA. The data are presented as mean ± SD from triplicates experiments. Wells without Nb or without VEGF were taken as controls.

its receptors, indicating that this antibody fragment targets epitope included in the antigenic region recognized by VEGF.

3.4. Purification of nanobody protein

We decided to purify ZFR-5 to investigate its potential for inhibition of VEGF-stimulated proliferation on HUVECs. To do this, *E. coli* Rosetta-gami 2 was used to produce soluble protein as described above. After IPTG induction, soluble ZFR-5 was subsequently purified by a Ni^{+}-NTA resin via its histidine tag. The purification was confirmed by SDS–PAGE analysis (Fig. 3A) and immunobloting (Fig. 3B). Results demonstrated in Fig. 3A also shows that the purified ZFR-5 has an electrophoretic mobility of approximately 17 kDa.

3.5. Functional inhibition assay

Since the VEGF stimulates the proliferation of endothelial cells through its cognate receptors, we decided to test whether ZFR-5 is able to suppress endothelial cell proliferation in response to VEGF. To do this, serial dilutions of ZFR-5 were added to a constant amount of VEGF and endothelial proliferation inhibition assay was performed using the MTT assay. As shown in Fig. 4, ZFR-5 significantly inhibited proliferation response of HUVECs to VEGF in a dose-dependent manner, while an equivalent concentration of control Nb (Anti MUC1) had no significant effect on the proliferation of endothelial cells.

Based on the results of the proliferation assay and whole cell ELISA experiments, it can be understood that the inhibition of HUVEC proliferation in response to VEGF is due to direct binding of this Nb to an epitope included in the interaction of VEGF with its receptor. This mechanism is unlike the steric hindrance mechanism by which, bevacizumab blocks VEGF binding to its receptor [12]. These findings are consistent with previous data that demonstrate the ability of Nbs to simultaneously inhibit crucial growth factors and their receptors [16]. On the other hand, the ability of ZFR-5 in targeting VEGF binding site on its receptor, is more supported by previous study demonstrating the potential of Nbs in targeting novel epitopes that are not accessible by conventional antibodies [17].

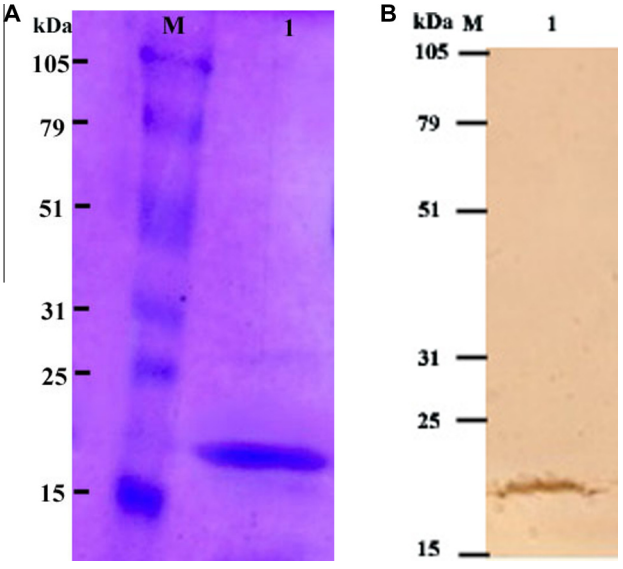

Fig. 3. Purification of selected Nb. ZFR-5 was expressed in *E. coli* Rosetta-gami 2 and purified by affinity chromatography. (A) SDS–PAGE analysis of ZFR-5. (B) immunobloting. M, molecular weight marker; Lane 1, purified Nb.

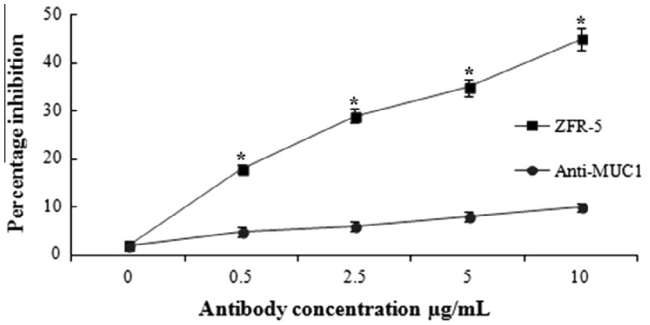

Fig. 4. Inhibition of VEGF-induced HUVEC proliferation. HUVECs were cultured in 96-well plates (5000 cells/well) in DMEM supplemented with 20% FBS. After reaching 90% confluency, various amounts (0.5, 2.5, 5, and 10 µg/mL) of ZFR-5 Nb or anti-MUC1 Nb (negative control) with a constant amount of VEGF (5 ng/mL final concentration) were added to the cells and incubated at 37 °C. After incubation for 72 h, the cells were subjected to the MTT assay and the absorbance was read at 540 nm. The data are presented as mean ± SD from triplicates experiments.

Several studies have been conducted to generate antibodies against VEGF [4,13,14]. It has also been demonstrated that different antibodies that recognize diverse epitopes on the same antigen may result in novel biological effects [7]. In addition, it has been reported that VEGF has distinct binding epitopes for bevacizumab and for the receptors and they are partially overlapping [12,22]. Important drawbacks including, the cost of generation of mabs, their large size, and immunogenicity are hindrances of conventional monoclonal therapy [11]. Nbs with their small size and unique biophysical and biochemical properties, such as simple purification, high solubility, high stability and binding to epitopes that are inaccessible for other antibodies, have been shown to be ideal approaches for tumor targeting purposes [23]. Taken together, our results demonstrated that ZFR-5 Nb was not only able to specifically bind to VEGF, but it also inhibited the VEGF-induced proliferation of HUVECs. Further *in vitro* and *in vivo* studies should be performed to elucidate the importance of this agent within tumors and as a potential drug candidate.

Acknowledgments

This work was supported by Shahid Beheshti University of Medical Sciences, Tehran, Iran and the Iran National Science Foundation (INSF) reference 90006943.

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