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EVALUATION OF THE BINDING AND ANTI-ANGIOGENIC CAPACITY OF ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 2 NANOBODY (ANTI-VEGFR2 NANOBODY)

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ABSTRACT

The use of antibody in targeted therapy has become the credible option in the treatment of cancers due to its specificity and the fact that it is associated with relatively lower toxicity compared to the other treatment options like chemotherapy and radiotherapy. However, antibody targeting could be associated with certain functional limitations due to their large molecular sizes (150KD) and the presence of the Fc fragment. Generating antibody fragments of smaller molecular sizes devoid of Fc fragment may be the way forward in curbing these functional limitations. In this study, five nanobodies 4N, 5N, 32N, 71N and 91N targeting vascular endothelial growth factor receptor 2 (VEGFR2) were generated from camel. The nanobodies were then screened by ELISA, immunoblotting and surface plasmon resonance (SPR) to select the nanobodies with high binding affinities. The nanobodies 5N and 32N demonstrated the highest binding affinities and therefore were selected for further studies. This was further confirmed by flow cytometry assay. Additionally, the selected nanobodies 5N and 32N demonstrated significant anti-angiogenic and anti-neoplastic abilities by restraining the proliferation of VEGFR2 expressing human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner. These nanobodies are therefore potential anti-angiogenic agents which could possibly be used in cancer therapy.

KEYWORDS: Nanobody, vascular endothial growth factor receptor 2, targeted therapy, antibody.

INTRODUCTION

Vascular endothelial growth factor (VEGF) and the associated receptors, especially vascular endothelial growth receptor 2 (VEGFR2) have been implicated in tumor cells proliferation and survival.[1-2] Binding of VEGF to VEGFR2 results in the homodimerization of VEGFR-2 which leads to a strong autophosphorylation of VEGFR-2 on tyrosine residues.[3] Autophosphorylated VEGFR-2 initiates MAP-kinase and DNA synthesis by the phospholipase-Cgprotein kinase-C pathway, leading to pathological angiogenesis.[4] Other VEGFR-2 dependent pathways which have been studied and reported include PI3K-PKBAKT focal adhesion kinase, Src kinase, Rho family of GTPases, and other multifunctional docking proteins and adaptors such as TSAd, Shb, Gab1 and Gab2, Crk and Nck.[5-6] VEGFR2/VEGF has therefore become therapeutic target for cancer treatment. Targeting VEGFR2/ VEGF with potent therapeutic agent can inhibit the VEGFR2-VEGF signaling and for that matter inhibit tumor proliferation. There are about thirteen approved anti-angiogenesis agents targeting VEGFR2-VEGF signaling pathway which are being used for cancer therapy.[7] These anti-angiogenesis agents work by inhibiting crucial cell signaling pathways that promote cancer cells proliferation. These agents are mostly monoclonal antibodies, small molecule tyrosine kinase inhibitors (TKIs) and inhibitors of mammalian target of rapamycin (mTOR).[7-8] The naturally occurring antibody of heavy chain variable fragment (V_{HH}) devoid of light chain variable fragment (V_L) and CH1 domain have been described in camilidae (camel-like) family as the second category of antibodies next to the conventional antibody repertoire.[9] This heavy chain variable fragment consists of a single domain called V_{HH} or nanobody. The nanobody exhibits characteristics such as heat resistance, high solubility, low immunogenenicity in addition to its smaller molecular size (15KD) making it suitable candidate in clinical use.[10] In this study, the binding capacity of five nanobodies (V_{HH}) namely 4N, 5N, 32N, 71N and 91N generated from camel which target VEGFR2 were evaluated to determine their binding and therapeutic potentials. Nanobodies 5N and 32N had higher affinities than the other three and therefore were selected for further studies.

MATERIALS AND METHODS

Cell culture

The adherent human umbilical vein endothelial cells (HUVECs) were cultured in endothelial culture medium (ECM) supplemented with 5% (v/v) FBS and 1% (v/v) endothelial cell growth supplement. Human embryonic kidney cell line HEK293 preserved in our lab was

cultured in DMEM medium (high glucose), supplemented with 10% (v/v) fetal bovine serum (FBS).

Quantitative enzyme linked immunosorbent assay (ELISA)

Two 96-well plates were coated at 100µl/well with 10000nM VEGFR2 diluted in plating buffer (0.05M $NaHCO_3$, pH9.6) and incubated overnight at 4^oC. The plating buffer was subsequently removed and the wells blocked with PBS containing 5% skim milk (200µl /well containing 5% skim milk). It was then incubated for 2h at 37^oC. The plates were then washed thrice with PBST (PBS containing 0.05%Tween-20) and PBS, respectively. They were then incubated with serial dilutions of each nanobody (0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000nM) for 1.5h at RT. The plates were again washed with PBST and PBS and then incubated with anti-His (1:2000) diluted in 3% skim milk at room temperature for 1.5h. The plates were then washed as described previously. HRP conjugated Goat Anti-Mouse IgG (1:5000, Millipore) was then added and incubated for 1.5h at RT. The solution was discarded and wells washed as described above. Finally, TMB peroxidase substrate (BBI) was added followed by 50µl of 2.5M sulfuric acid to stop the enzyme action. The absorbance was measured at OD_{450}-OD_{630}. VEGFR2 was expressed in our laboratory using the expression system *E. coli BL21(DE3)*/pET22b-KDR3 preserved in our laboratory.

Immunoblotting

VEGFR2/MICA (Major Histocompatibility Complex class I-related chain molecules A) of concentration 100µg/ml was loaded into five wells of 15% (w/v) SDS-PAGE for electrophoresis. The protein samples were transferred onto polyvinyllidenedifluoride (PVDF) membrane (Millipore). This was carried out for 1.5h in a blotting apparatus (Bio-Rad) under a constant voltage of 100V. The blotted membrane was placed in blocking buffer TBS (20mM Tris–HCl, pH 7.4, 150mM NaCl) with 5% (w/v) skim milk at 37^oC for 2h. The membrane was then washed three times with TBS and incubated with 0.35mg/ml each of the five nanobodies at 4^oC overnight. The membrane was then washed three times with TBST (TBS containing 0.05% Tween-20) and TBS followed by incubation with Anti-His at 37^oC for 2h. It was then washed with PBS. This was followed by incubating with HRP conjugated Goat Anti-Mouse IgG (1:5000, Millipore) at 37°C for 1.5h. After successive washing as described previously, the blots were treated with enhanced chemiluminescence (ECL) solution and exposed in gel imaging system (Bio-Rad).

Binding kinetics of fusion protein

The binding kinetics of the five nanobodies against VEGFR2 was measured with a Biacore X100(GE) instrument (Biacore X100, GE Healthcare, Sweden) at 25^{o}C. The running buffer (10mM HEPES, 150mM NaCl, 0.005% Surfactant polysorbate20, 50µM EDTA at pH7.4) and the dispensor buffer (10mM HEPES, 150mM NaCl, 0.005% Surfactant polysorbate20, 3mM EDTA) were filtered and degassed prior to their usage. Firstly, the five nanobodies were diluted in the running buffer and immobilized on Sensor Chip CM5 (GE Healthcare, BR-1000-12) with target resonance unit (RU) density of 2000. Different concentrations of VEGFR2 (250, 125, 62.5, 31.5, 15.6, 7.8nM) were then injected. The capture was done at a constant flow rate of 40µl/min. The kinetic constants association (k_a), dissociation (k_d), and equilibrium constant K_D were calculated with 1:1 binding model.

Flow cytometry assay

Flow cytometry assays were done with HUVECs and HEK293. 5x10^{5} of these cells per sample were suspended in PBS containing 5% BSA and incubated with 2000nM nanobodies (5N and 32N) at 4^{o}C for 1h. The cells were then incubated with His-probe (H-15) rabbit (sc-803) or goat (sc-803-G) poly-clonal affinity purified antibody (Santa Cruz Biotechnology). The cells were washed afterwards and binding assays performed with a BD FACS flow cytometer. MICA, scFv and PBS were used as controls.

Cell proliferation assay

The assay was done by seeding 4x10^{3} HUVEC or HEK293 cells into 96-well plates and incubating at 37^{o}C for 24h. Different concentrations of the nanobodies 5N and 32N (0, 1.95, 3.9nM, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500nM) were added and incubated at 37°C for 48h. The untreated groups were used as vehicle of control. Following the 48h incubation, cell viability was quantified by MTT assay and the inhibitory rates expressed as percentages of the vehicle control (100%).

Statistical analysis

The data of the study were analyzed using Excel and SPSS 17.0 software. Results are presented as the mean ± SD from at least three independent experiments. The t-test was used to compare the inhibitory rates of different samples in the MTT assay (cell proliferation assay). A p value < 0.05 was considered statistically significant.

RESULTS

Immunoblotting and ELISA

Immunoblotting was done to determine whether the five nanobodies could bind to the antigen VEGFR2. As presented in fig. 2, all the five nanobodies exhibited specific binding to the VEGFR2. This was further confirmed by ELISA test. All the nanobodies 4N, 5N, 32N, 71N and 91N demonstrated dose-dependent binding to VEGFR2 (fig. 3). Additionally, it was done to compare the binding capacities of the five nanobodies. As it turned out, nanobodies 5N and 32N comparatively exhibited the highest binding capacity to the antigen VEGFR2 (fig. 2).

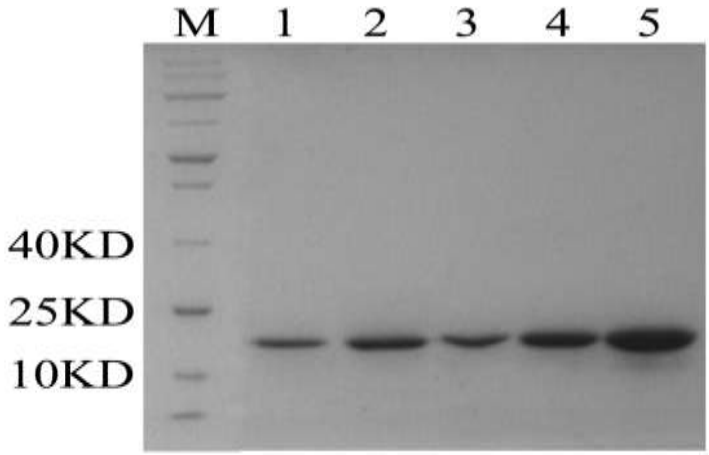

Figure 1. Coomassie-stained SDS-PAGE analysis of the purity of five nanobodies with molecular size (15KD).Lane M: Marker, Lane 1: N4, Lane 2: 71N, Lane 3: 91N, Lane 4: 5N, Lane 5: 32N.

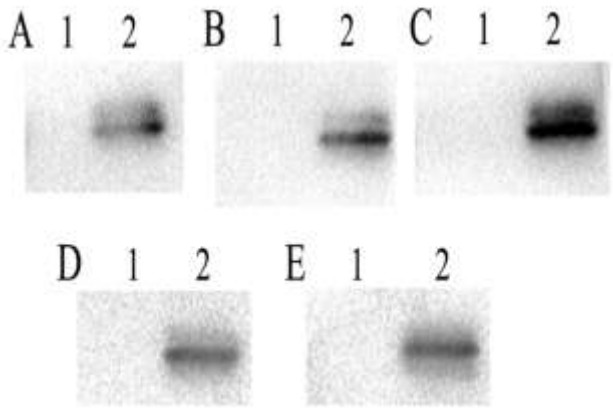

Figure 2. Western blotting analysis of expression of the five nanobodies. (A) Lane 1: MICA (negative), Lane: N4, (B) Lane 1: MICA, Lane 2: N5, (C) Lane 1: MICA, Lane 2: N32, (D) Lane 1: MICA, Lane 2: N71, (E) Lane 1: MICA, Lane 2: N91.

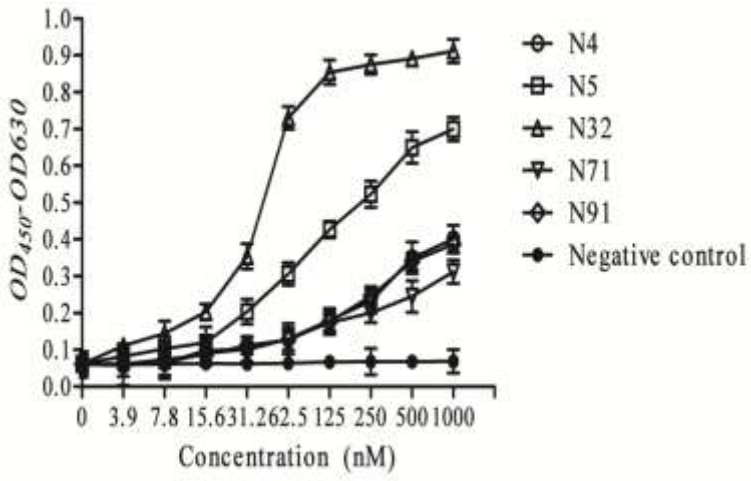

Figure 3. Evaluation of the binding capacity of the nanobodies to VEGFR2 by ELISA. Nanobodies N5 and N32 demonstrated the highest binding affinities.

Binding kinetics of fusion protein

This was done to determine the binding kinetics between the five nanobodies and the antigen VEGFR2. As shown in table 1, the binding kinetics between the VEGFR2 and all the five nanobodies were high. The KD values 3.75E-09, 9.14E-13, 3.87E-10, 1.83E-09 and 3.41E-08M were recorded for 4N, 5N, 32N, 71N and 91N nanobodies respectively. The data demonstrated that, the association rate increased with increasing concentration of the VEGFR2. The kinetic process can therefore be described as quick association and slow dissociation.

Table 1. The binding Kinetics of the five nanobodies and VEGFR2.

Nanobody	ka (1/Ms)	kd (1/s)	KD (M)
N4	1.95E+10	73.13	3.75E-09
N5	6.77E+06	6.18E-06	9.14E-13
N32	6.74E+11	261	3.87E-10
N71	3.71E+10	67.77	1.83E-09
N91	8.78E+09	299.4	3.41E-08

Flow cytometry assay

The ability of the nanobodies 5N and 32N to bind to native VEGFR2 was determined using flow cytometry assay. The two were chosen because of their high binding capacity. As presented in fig. 4, the two nanobodies demonstrated significant binding to VEGFR2 expressing HUVECs but not the control cells HEK 293, suggesting that the nanobodies can specifically bind to the antigen VEGFR2.

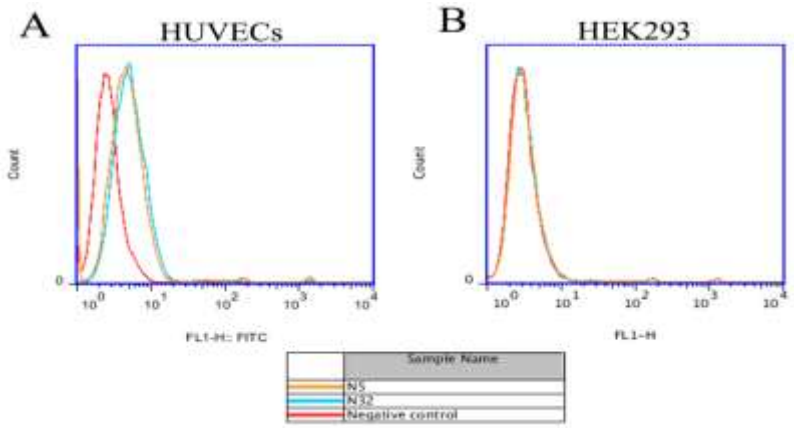

Figure 4. Evaluation of the ability of the nanobodies 5N and 32N to bind to VEGFR2 expressing HUVECs. (A) Nanobodies 5N and 32N demonstrated significant binding affinity VEGFR2 expressing HUVECs. (B) Nanobodies 5N and 32N showed no binding affinity to negative control cell line HEK 293, demonstrating that the nanobodies specifically bind to VEGFR2.

Cell proliferation assay

The ability of the nanobodies 5N and 32N to inhibit the proliferation of VEGFR2 expressing HUVECs was determined by cell proliferation assay. As presented in fig. 5, the nanobodies dose-dependently restrained the proliferation of the HUVECs but not the control cell line HEK 293 which is without the antigen VEGFR2. The data demonstrated that the nanobodies specifically inhibited the proliferation of the HUVECs by blocking the VEGFR2-VEGF signaling pathway. It can therefore be inferred that the nanobodies 5N and 32N are potential anti-angiogenic agents.

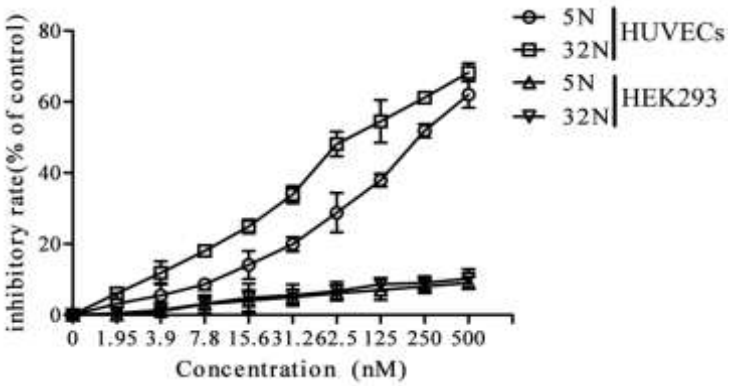

Figure 5. Evaluation of the anti-angiogenic ability of the nanobodies 5N and 32N on the proliferation of VEGFR2 expressing HUVECs. The nanobodies dose-dependently the inhibited the proliferation of VEGFR2 expressing VUVECs but not the control cell line HEK293, suggesting binding specificity.

DISCUSSION

Targeting VEGFR2 has become common and preferred strategy for the treatment of tumor cells. The use of antibodies in targeted therapy has become the credible option in the treatment of cancer due to its specificity and the fact that it is associated with relatively low toxicity compared to the other treatment options such as chemotherapy and radiotherapy. However, antibody targeting usually associated with functional limitations which can be attributed to the relatively large molecular size (150KD) and the presence of Fc fragment.[11-12] Antibodies usually have longer half-life because of their larger molecular sizes, which could result in higher toxic level. This is because the molecular sizes of antibodies (150KD) far exceed the renal clearance threshold (~70 kDa).[13] This prevents them from being eliminated through the kidney, which could possibly increase their toxicity. Additionally, large molecular size could reduce the absorption rate and for that matter, their efficacy. Moreover, there have been reports on Fc fragment-mediated toxicities.[11-12] Generating antibody fragments with smaller molecular size devoid of Fc fragment may be the way forward to curb the possible toxicities associated with larger molecular size and the presence of Fc fragment. In the current study, five nanobodies 4N, 5N, 32N, 71N and 91N targeting VEGFR2 were generated from camel and screened by ELISA, immunoblotting and SPR to select the nanobodies with high binding affinities. As shown in fig. 1 and 2, the nanobodies 5N and 32N demonstrated the highest binding affinity. The high binding affinities of the two nanobodies (5N and 32N) were further confirmed by flow cytomery assay, suggesting that they could be suitable candidates for cancer targeted therapy. Nanobody (V_{HH}) which is a single-domain antigen binding fragment derived from single variable heavy-chain of antibodies from the serum of camel has small molecular size (15KD) devoid of Fc fragment and single variable light-chain (V_L).[14] Additionally, nanobodies are relatively stable with high affinities and specificity. These suitable properties have made nanobodies, the preferred agents for cancer targeted therapy. The ability of the nanobodies 5N and 32N to restrain the proliferation of VEGFR2 expressing HUVECs was evaluated by cell proliferation assay. And as presented in fig. 5, the nanobodies significantly inhibited the proliferation of the target cell, suggesting that the selected nanobodies (5N and 32N) are potential anti-angiogenic agents.

In conclusion, nanobodies 5N and 32N generated from camel have demonstrated high binding affinity to VEGFR2 by ELISA, immunoblotting, SPR and flow cytometry assay. In addition, they demonstrated significant capacity to restrain the proliferation of VEGFR2 expressing HUVECs. This shows that the nanobodies specifically inhibited the VEGFR2-

VEGF signaling. The nanobodies are therefore potential anti-angiogenic and anti-neoplastic agents which can possibly be used in the treatment of cancer. Further studies are ongoing to improve its efficacy.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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