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Inhibition of angiogenesis in human endothelial cell using VEGF specific nanobody

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ABSTRACT

Angiogenesis is an important step in tumor development and metastasis. Vascular endothelial growth factor (VEGF) plays an important role in progression of angiogenesis. $VEGF_{121}$ and $VEGF_{165}$ are the most relative forms of VEGF family which contain the full biological activity. Nanobodies derived from *camelidae* are the smallest biding site of antigen. Unique characteristic of nanobodies make them as a useful candidate for research. In this report, we describe the isolation of VEGF specific nanobodies from *dromedaries* immunized with purified VEGF antigen using phage display. Four clones that showed the highest signal value in ELISA experiment were selected and expressed as a His-tagged fusion protein. Four selected nanobodies were reacted strongly to VEGF in cross-reactivity assay. The binding affinity of selected nanobodies named Nb22, Nb23, Nb35 and Nb42 were differed from 0.1 to 60 nM. The nanobodies inhibited endothelial cell proliferation or tube formation in response of VEGF in a dose-dependent manner. These results indicate the potential of nanobodies in inhibition of VEGF and represent a promising candidate for cancer research and therapeutics.

1. Introduction

Angiogenesis is one of the highly regulated processes which refer to the formation of new blood vessel. Angiogenesis plays important role in physiological condition such as embryogenesis, wound healing and adults reproductive cycle. In pathological condition, angiogenesis resulted in the growth, invasion and metastasis of cancer (Folkman, 2007; Papetti and Herman, 2002). Inhibition of angiogenesis can be an attractive target for treatment of cancer. This idea was proposed in 1980 by Folkman and colleagues. The vascular endothelial growth factor (VEGF-A) is the most important factor of angiogenesis and its over expression has been investigated in most solid tumors (Ferrara, 2004). VEGF-A function via its receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR) in endothelial cells (Ferrara, 2004). The VEGF-A gene contains 8 exons that under alternative splicing fall into 5 main isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$). $VEGF_{121}$ and $VEGF_{165}$ are the most common isoforms, which consist of 121 and 165 amino acids, respectively (Dulak et al., 2000; Kim et al., 2007; Yue and Tomanek, 2001). $VEGF_{121}$ and $VEGF_{165}$ are detected in the majority of cells expressing the VEGF gene. The low molecular weight of $VEGF_{121}$ makes it soluble and freely diffusible (Liu et al., 2003). Many studies have been demonstrated that $VEGF_{121}$ contain the full biological activity as the larger isoforms (Liu et al., 2003). Blocking of the VEGF pathway has been used as a major target of angiogenesis (Jain, 2008). Recent studies have focused on the development of antibodies, especially monoclonal antibodies (mAbs), and small molecules that target the tumor-associated endothelial cells (Youssoufian et al., 2007; Zhang et al., 2009). So during recent years inhibitors of the VEGF-A have been developed. Bevacizumab (Avastin®, Genentech) is a recombinant anti-VEGF that binds and inactivates all isoforms of human VEGF-A. Thus bevacizumab inhibits angiogenesis and limits tumor growth (Ferrara et al., 2005). Bevacizumab in 2004 approved by the Food and Drug Administration (FDA) for metastatic colorectal cancer treatment (Hurwitz et al., 2004). However, bevacizumab in some case show some side-effects such as bleeding, phlebitis and embolism, that leads to the stopping of treatment (Ranpura et al., 2011). Ranibizumab (Lucentis; Genentech) is an antibody fragment which developed from bevacizumab and approved by FDA in 2006 for treatment of AMD (Campochiaro,

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2007). Ranibizumab is highly effective and considerably expensive than bevacizumab. So bevacizumab instead of ranibizumab are used for treatment of AMD (Klettner and Roider, 2008). However monoclonal antibodies have some advantage because of their specificity to target, but there are some concerns which restrict their use: stimulation of immune responses, unable to reach hinder region of solid tumors, demanding production process and large size (Kolkman and Law, 2010).

Identification of nanobodies or VHH opened new field in antibody technology that contain advantage of small molecules and mAbs (Kolkman and Law, 2010). In fact, Nanobody™ (Nb) is a single domain antibody that derived from the variable region of heavy chain antibodies (HCAbs) (Muyldermans et al., 2009). Heavy chain antibodies are in serum of *camelidae* (camel and lama) and do not have light chain and CH1 domain and was identified by Hamers-Casterman in 1993. The molecular weight of nanobodies is approximately 15 kDa and their size is 2.5 nm in diameter and 4 nm in height (Rahbarizadeh et al., 2011). Nanobodies have many advantageous properties: low immunogenicity (because of high homology of nanobodies to humanVH3 domains), high affinity, high solubility and stability, binding to hinder epitope and high yield expression in *Escherichia coli* or yeast (Behdani et al., 2012). Ideal biophysical and pharmacological properties such as small size, single domain, antigen binding affinity in nanomolar rang and drug format, make nanobodies attractive candidates for cancer therapy (Kolkman and Law, 2010). Nanobodies have been reported against wide range of antigens such as immunogenic proteins, enzymes, toxins and haptens (Rahbarizadeh et al., 2011). According to the importance of angiogenesis in research for treatment of angiogenesis-dependent disease such as cancer, this study aimed to generate, identify and purify several nanobodies against human VEGF (one of the most important angiogenesis factor), with unique characteristics.

2. Materials and methods

2.1. Cell lines and proteins

HUVECs primary cell (isolated form Umbilical cord in our lab) were cultured in endothelial cell basal medium (EBM-2) supplemented with the EGM-2 BulletKit (Lonza), and 2% FBS at 37 °C with humidified air containing 5% Co_2 incubator. HUVECs cultured in T-25 fask and after 4–5 days confluency reached to 90–100%. The cells were washed with PBS and detached by trypsin (Gibco) before experiment. In all experiment HUVECs up to passage 4 were used.

Recombinant human $VEGF_{121}$ was expressed in *E. coli*, purified with nickel affinity chromatography and refolded as described previously (Kazemi-Lomedasht and Behdani, 2014). Recombinant extracellular domain of VEGFR2, recombinant human $VEGF_{121}$ and $VEGF_{165}$ were purchased from R&D. Bevacizumab was purchased by Roche (Basel, Switzerland). pHEN-4 and pHEN-6C vectors gifted by Serge Muyldermans (Laboratory of Cellular and Molecular Immunology, Vrije University Brussels, Brussels, Belgium). pHEN-4 is a phagemid vector and contain the gene III of phage capsid protein and HA-tag which express nanobody-PIII as a HA-tag fusion. pHEN-6C is bacterial expression vector and contain His-tag which express nanobody as a C-terminal His-tag.

2.2. Camel immunization

A young male camel (*Camelus dromedarius*) was subcutaneously injected with purified $VEGF_{121}$ for six times at weekly intervals. First immunization was performed with 200 μg of $VEGF_{121}$ resuspended in 2 ml PBS and mixed with 2 ml of freunds complete adjuvant. Booster injection was performed with freunds incomplete adjuvant (equal volume of adjuvant and protein) (Behdani et al., 2012).

Before immunization and after each injection sera were collected for measurement of antibody level. The immune response was analyzed using ELISA on serum of camel. Camel antibodies that bounded to $VEGF_{121}$ coated plate, were detected by rabbit anti-camel antibody (previously developed in our lab by immunizing rabbits with camel IgGs). And then horseradish peroxides (HRP) conjugated anti-rabbit antibody (sigma) was used as secondary antibody. The reaction was developed by tetramethylbenzidine (TMB) and was then stopped with 2 N H_2SO_4 and optical density (OD) was measured at 450 nm by ELISA reader.

2.3. Library construction and selection of phage displayed VHH (nanobody)

A week after the last injection, about 150 ml of camel blood sample was taken in ethylene diamine tetra acetic acid (EDTA, sigma) coated tube and peripheral blood mononuclear lymphocytes (PBMCs) were isolated by ficoll standard protocol. Total RNA was extracted from PBMCs, and cDNA was synthesized by reverse transcriptase (MBI, Fermentase, Opelstr, Germany) using oligo dT primers. VHH gene was amplified by nested PCR. In first PCR, both VH and VHH genes were amplified using leader-specific primer CALL001 (5′-GTCCTGGCTGCT CTT CTACAAGG-3′) and CH2-specific primer CALL002 (5′-GGTACGTGCTGTTGAACTGTTCC-3′). The fragments of heavy chain antibody (about 600 and 700 bp) were extracted from agarose gel and then used as template for second PCR by nested primers, A6E (5′-GATGTGCAGCTGCAGGAGTCTGGRGGAGG-3′) and primer38 (5′-GGACTAGTGCGGCCGCTGGAGACGGTGACCTGGGT-3′). These primers were designed for frame work 1 and frame work 4 of VHH and contain PstI and NotI restriction sites. The VHH gene was purified from agarose gel and digested with PstI and NotI restriction enzymes (Fermentase, Germany). The digested VHHs cloned into pHEN-4 vector, which was digested with same restriction enzymes (PstI and NotI). The recombinant phagemids were transformed into *E. coli* TG1 (electro competent) cells. The transformed TG1 was sub-cultured on Luria–Bertani (LB) agar containing ampicillin. The VHH library was displayed on phage after infection of TG1 with 10^7 pfu/ml VCSM13 helper phage (Amersham-Pharmacia) (Behdani et al., 2012).

2.4. Biopanning of the nanobody library

Four consecutive rounds of biopanning were performed on purified recombinant $VEGF_{121}$ for selection of phages displaying VHHs against the $VEGF_{121}$. Therefore $VEGF_{121}$ specific phages were enriched by biopanning. For the first round of panning, microtiter well (Nunc, Roskilde, Denmark) was coated overnight at 4 °C with 100 μl of 10 μg/ml $VEGF_{121}$ in sodium bicarbonate buffer and 100 μl of sodium bicarbonate buffer as negative control. For subsequent rounds of panning, 1 μg/ml of $VEGF_{121}$ in sodium bicarbonate buffer were used. The wells were blocked with 4% skimmed milk in PBS (MPBS) at room temperature for 1 h. After blocking, 10^{12} cfu (colony forming unit) of phage particle was added to each well and incubated at room temperature for 1 h. The wells then washed 5 times with PBST (0.5% (V/V) Tween 20 in PBS). The washing steps throughout the panning process were more stringent using increased amount of Tween 20 in PBST (0.5%, 1%, 2%, and 4%). The bound phages were eluted using 100 μl of 100 mM triethylamine (TEA, pH 10.0), for 10 min. TEA was then neutralized with 1 M Tris–HCl (pH 8.0). 10 μl of eluted phages (output) (10 fold serially diluted) were used for infecting log phase *E. coli* TG1. Phage titration was performed by plating each dilution on TYE medium containing ampicillin. The enrichment of phage particles carrying