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Identification and characterization of a novel nanobody against human placental growth factor to modulate angiogenesis

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ABSTRACT

Placental growth factor (PlGF), a member of vascular endothelial growth factors (VEGF) family, is considered as an important antigen associated with pathological conditions such as cancer cell growth, and metastasis. PlGF-targeting via nanobody (Nb) therefore could be beneficial to modulate these pathologies. In this work, phage-display and computational approach was employed to develop a high affinity PlGF-specific Nb. An Nb library was constructed against human recombinant PlGF (rPlGF). After panning on immobilized rPlGF the periplasmic-extract (PE) of individual colonies were screened by ELISA (PE-ELISA). The 3D structures of selected Nbs were then homology modeled and energy minimized using the AMBER force field. Binding score calculations were also assessed to reveal possible Nb-PlGF interactions. Via ELISA-based affinity/specificity determinations, the best-qualified Nb was further evaluated by proliferation, migration, 3D capillary formation, invasion assays and on Chick chorioallantoic membrane (CAM) model. An immune library of 1.5×10^7 individual Nb clones was constructed. By PE-ELISA 12 clones with strong signals were selected. Three out of 12 sequenced Nbs (Nb-C13, Nb-C18 and Nb-C62) showed high binding scores ranging between −378.7 and −461 kcal/mol. Compared to a control Nb, Nb-C18 significantly inhibited proliferation, migration and the 3D-capillary formation of HUVEC cells ($p < 0.05$) with an EC_{50} of 35 nM, 42 nM and 24 nM and invasion of MDA-MB231was significantly suppressed ($p < 0.05$) with an EC_{50} of57 nM. The result of the CAM assay shows that Nb-C18 could inhibit the vascular formation in the chicken chorioallantoic membrane. This Nb can be used as anti-angiogenesis agent in future.

1. Introduction

Angiogenesis is a strongly regulated multi-step process defined as the formation of new blood vessels from pre-existing vasculature. The vascular endothelial growth factors (VEGFs) are key molecules in angiogenesis. The VEGF family comprises VEGF-A, −B, −C, −D, −E, −F and placental growth factors (PlGFs). Unlike VEGF-A, which is essential in health and developmental conditions, PlGF is considered as an important factor in pathological situations such as tissue ischemia, malignancy, inflammation, and in a wide variety of other disorders (Carmeliet, 2003). Four isoforms of PlGF (PlGF 1-4) are known in human of which type PlGF-1 binds only to VEGF receptor1 (VEGFR1) (Dewerchin and Carmeliet, 2012). Although, VEGFR1 is known as a negative regulator of angiogenesis during

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early development, it is involved in tumor angiogenesis and metastasis (Kanno et al., 2000). The activation of VEGFR1 via PlGF also triggers the VEGFR-2 signaling pathway by the crosstalk between cytoplasmic domains of these receptors, and leading to progressive angiogenesis in tumor tissues (Autiero et al., 2003).

The PlGF enhances proliferation, migration, and survival of cancerous cells, which is detectable by standard molecular methods (Schmidt et al., 2011; Fischer et al., 2007; Bellik et al., 2005; Luttun et al., 2002). In addition, it has been demonstrated that the knockdown of PLGF by gene inactivation, antibody neutralization or small molecules (Carmeliet et al., 2001) impairs successfully the tumor growth and cancer metastasis. Therefore, targeting of PlGF might be a promising strategy to inhibit pathologic angiogenesis. However, all such PlGF knock-down approaches encounter their own practical obstacles such as a low penetration into the solid tissue or a low target-specificity and affinity.

Nanobodies (Nbs), the single domain antigen-binding fragments obtained from functional heavy chain antibodies (HCAbs) in camelids, have numerous advantages for medical applications. They are generally well expressed recombinantly at low cost in microbial systems. Nbs are highly stable and soluble in aqueous solutions even at elevated concentration, and share a close homology to human VH sequences(9).Several Nbs have been identified and reported to target angiogenic or pro-angiogenic factors, including Nbs against CD105 (Ahmadvand et al., 2008), anti-VEGFR2 (Behdani et al., 2011) anti-VEGF (Farajpour et al., 2014; Kazemi-Lomedasht et al., 2015). Moreover, many groups are currently involved in programs to develop novel binders in this area of research and diagnostics applications.

In the current study,an immune Nb phage library was constructed, panned and screened against PlGF. For the retrieved antigen-specific Nbs, *in silico* conformational binding calculations based on modeled Nbs-PlGF complexes were used to select potential potent binders. ELISA-based affinity and specificity analysis confirmed this prediction. Furthermore, cell proliferation and migration tests as well as 3D capillary formation, an invasion assay on endothelial and cancerous cells and an *in vivo* CAM angiogenesis model were used to characterize the potency of our lead Nb.

2. Materials and methods

2.1. Camel immunization and monitoring of the immune response

A dromedary (*Camelus dromedarius*) was immunized with recombinant PlGF (rPlGF) to raise an immune response in their HCA bisotypes as described previously(Arezumand et al., 2014). Briefly, we used six subcutaneous injections, at weekly intervals. The first injection was with 100 μg antigen, mixed with 2 ml complete Freund's adjuvant. All subsequent injections were with antigen and incomplete Freund's adjuvant. Dromedary serum was harvested at weeks 4 and 7 from the day of the first injection. The project was found to be in accordance to the ethical principle and the national norm and standards for conducting Medical Research on human and animal in Iran in Pasteur institute of Iran (IR.PII.REC.1395.10).

The immune response within these sera of was monitored by ELISA. Diluted serum samples were added to wells of microtiter plates, which were coated with rPlGF(100 μl at 5 μg/ml). The residual protein binding sites on the plastic were saturated subsequently by incubation with 200 μl 4% skimmed-milk protein solution. The camel antibodies bound to the rPlGF were detected with a 1/3000 polyclonal rabbit anti-camel IgG (homemade), and finally followed by 1/3000 Horse Radish Peroxidase (HRP)-conjugated anti-rabbit-IgG (GE healthcare, USA). The peroxidase activity was detected by adding 3, 3′, 5, 5′-tetra methyl benzidine (TMB).

2.2. Construction of a Nb library in phage display vector

Three days after the last immunization, 250 ml blood was collected from the jugular vein. Lymphocytes were isolated from this anti-coagulated blood on lympholyte CL-5020 (Cederline). Total RNA was purified from 1×10^7 blood lymphocytes using RNX-plus^{TM}(Cinagen, Iran) and used as template in a reverse transcription reaction (Fermentas, Lithuania) for cDNA synthesis. We then used the cDNA as a template in a two-step PCR to amplify the DNA sequences encoding the VHH gene fragments. In a first PCR, we amplified the sequence between the CH2 region and the leader sequence with primers CALL001 (5′-GTC CTG GCTGCT CTT CTA CAA GG-3′) and CALL002 (5′-GGT ACG TGCTGT TGA ACT GTT CC-3′), respectively (Fu, 2013; Pardon et al., 2014). Two PCR products are obtained that are separated by agarose gel electrophoresis. The shorter PCR fragment (750 bp) corresponds to the VHH-hinge-CH2 region of the HCAbs. This shorter PCR product was extracted from agarose gels and amplified by nested PCR with primers A6E (5′-GAT GTG CAG CTG CAG GAG TCT GGR GGA GG-3′) and 38 (5′-GGA CTA GTG CGG CCG CTG GAG ACG GTG ACC TGG GT-3′) with *PstI* and *NotI* restriction enzyme sites underlined (Movahedi et al., 2012). The resulting VHH-PCR product was digested with *PstI* and *NotI* restriction enzymes and 8.4 μg of PCR fragment was ligated to 4.4 μg pHEN4 phagemid vector (cut with the same restriction enzymes), and transformed into *E. coli* TG1 electro-competent cells (homemade). The transformed cells were plated on LB agar plates containing glucose (1%) and ampicillin (100 μg/ml). After overnight growth, the cells were scraped from the plates and after washing in LB medium they were stored in glycerol (final concentration of 20% in LB) at 1.5×10^7 cells/ml. Assessment of the percentage of clones with a phagemid containing the correctly sized VHH gene was performed by colony PCR with GIII and RP primers on thirty, randomly chosen colonies. The GIII primer (5′-CCA CAG ACA GCC CTC ATA G-3′) and universal sequencing RP primer (5′-TCA CAC AGG AAA CAG CTA TGA C-3′) anneal within the vector sequences abutting the cloned VHH insert.

2.3. Phage display of Nbs and biopanning

An aliquot of the cells (330 μl) containing the Nb library was grown in 300 ml 2 × TY with 4% glucose and 100 μg/ml ampicillin until an $OD_{600\,nm}$ of 0.6–0.9 was reached. The bacteria were mixed with 10^{12} VCSM13 helper phages and left at room temperature for 30 min to infect bacteria. The cells were harvested by low speed centrifugation (2700 rpm for 10 min) and the cell pellet was resuspended in fresh 2 × TY supplemented with ampicillin (100 μg/ml) and kanamycin (70 μg/ml) to produce virions displaying the Nbs of the library. Virions were purified from the culture supernatant by polyethylene glycol/NaCl precipitation and resuspended in a total volume of 1 ml PBS (Lee et al., 2007).

For bio-panning, 100 μl of rPlGF (10 μg/ml) was added to a well of a microtiter plate (a well without rPlGF was used as negative control) and residual protein binding sites were blocked with 4% skimmed-milk proteins. Phage particles (3×10^{11} plaque forming units) were added to the wells and incubated for 1 h at room temperature. After removing non-adsorbed phages (lacking PlGF-specific Nbs) by multiple washings with PBS containing 0.05% Tween-20, we eluted the PlGF-bound phage particles with 100 mM triethylamine (pH 11.0) for 5 min. Next, the solution was transferred to a fresh tube and neutralized with 1.0 M Tris-HCl (pH 7.4). The eluted phages were used to infect exponentially growing *E. coli* TG1 cells and taken to the next round of panning (Lee et al., 2007). The panning was performed for 4 consecutive rounds of selection.