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Characterization of nanobodies binding human fibrinogen selected by *E. coli* display

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ABSTRACT

Abnormal levels of fibrinogen (Fib) in blood plasma are associated with several pathological conditions and hence methods for its detection in blood and body fluids are essential. Nanobodies (Nbs) or (VHHs) are single domain antibodies derived from camelids with excellent biophysical and antigen-binding properties, showing great promise in diagnostics and therapy. In this work, we select and characterize high affinity Nbs binding human Fib employing an *E. coli* cell surface display system based on the fusion of an immune library of VHH domains with the β-domain of Intimin. Bacteria displaying high-affinity Nbs against Fib were selected using magnetic cell sorting (MACS). Specific binding of the selected clones to Fib was confirmed by flow cytometry of *E. coli* bacteria, as well as by enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) with the purified Nbs. *E. coli* display also provided an excellent estimation of the affinity of the selected Nbs by flow cytometry analysis under equilibrium conditions, with equilibrium constant (K_D) values very similar to those obtained by SPR analysis. Finally, pairwise epitope-scouting studies revealed that the selected Nbs bound distinct epitopes on Fib. The selected Nbs are promising diagnostic tools for determination of human Fib levels.

1. Introduction

Fibrinogen (Fib) is the third most abundant blood plasma protein (after albumin and the immunoglobulins) and is present at a circulating concentration of 1.5–5.5 mg/ml under normal physiological conditions. It is a large glycoprotein of 340 kDa, which is synthesized in the liver and plays key roles in the coagulation cascade (Pacella et al., 1983), in artherogenesis and during inflammation, where plasma levels of Fib increase rapidly in response to pro-inflammatory agents such as Interleukin-6 and other cytokines (Gruys et al., 2005). Abnormal plasma levels of Fib have been associated with a series of pathological conditions such as cardiovascular diseases (Tatli et al., 2009), atherosclerosis (Sabeti et al., 2005), cancer (Hefler-Frischmuth et al., 2015; Troppan et al., 2016) and rheumatoid arthritis (Cilia La Corte et al., 2011; Raijmakers et al., 2012). Thus, Fib is routinely used as an early phase prognostic disease marker and development of diagnostic reagents that facilitate its detection are of great interest.

Antibodies (Ab) represent the largest and fastest growing sector of the biopharmaceutical industry, and are used for an increasingly broad and steadily expanding spectrum of applications, from human therapy to *in vitro* diagnostics. Combinatorial libraries and phage display have revolutionized the way Abs are isolated (Beck et al., 2010; Holliger and Hudson, 2005; Hoogenboom, 2005; Winter et al., 1994; Zhao et al., 2016) and have paved the way for the development of novel Ab formats with distinct functional properties (e.g. reduced size for better tumor penetration, multivalent or multi-specific antigen binding, reduced half-life for immunotherapy or *in vivo* imaging, stabilization agents in crystallography, etc.) (Boder and Jiang, 2011; Freise and Wu, 2015; Griffin and Lawson, 2011; Holliger and Hudson, 2005). One class of such naturally occurring Ab formats is the single domain VHHs from camelid heavy-chain-only Abs, also referred to as nanobodies (Nbs). Nbs

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Table 1
E. coli strains and plasmids.

Name	Genotype and relevant properties	Reference
E. coli strains		
DH10B-T1^R	F$^-$ *mcrA* Δ*mrr-hsdRMS-mcrBC ϕ80lacZDM15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK rpsL (StrR) nupG tonA* λ$^-$	Invitrogen
EcM1	MG1655Δ*fim*A-H	(Blomfield et al., 1999)
HM140	F$^-$ Δ*lacX74 galE galK thi rpsL(StrR)* Δ*phoA (PvuII) degP ptr ompT eda tsp rpoH15*	(Meerman and Georgiou, 1994)
Plasmids		
pAK-Not	(Cm^R), lacI^q-P*lac* promoter, pBR322 ori	(Veiga et al., 2004)
pNeae2	pNeae-derivative; for fusions to Neae-myc [Intimin$_{EHEC}$ (1-654)-E-His-myc tag]	(Salema et al., 2013)
pNVFibn	pNeae-myc-derivative; NVFIB (n clone) fusion [Intimin$_{EHEC}$ (1-654)-E-VFIBn-myc tag]	This work
pMAL1-VFibn	pMAL-p2E derivative, for fusion of Fib VHH (n clone) to MBP [MBP-VFIBn-His-myc tag]	This work

have a number of outstanding properties that make them of great biotechnological interest, including their small size (ca. 14 kDa), simple structure, high-affinity and specificity, long CDRs that can target cryptic epitopes, high stability to chemical and physical denaturation, and low immunogenicity (Muyldermans, 2013).

In previous work, we have reported a Nb selection system based on its display on the *E. coli* cell surface fused to the β-domain of Intimin (Salema et al., 2013), thus not requiring the use of bacteriophages for the process of display and selection. In this study, we used the *E. coli* display system to select high affinity Nbs from an immune library generated against human Fib. *E. coli* bacteria displaying the anti-Fib immune Nb library were screened for antigen binding clones by magnetic cell sorting (MACS). Specific binding of the selected clones to Fib was demonstrated by flow cytometry of *E. coli* cells displaying the Nbs, as well as by enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) with the purified Nbs. Finally, we demonstrated that the selected Nbs bind distinct epitopes on Fib and that their K_Ds, estimated by *E. coli* display using flow cytometry analysis at equilibrium conditions, are comparable to those determined by SPR.

2. Materials and methods

2.1. Bacterial strains, bacteriophages, growth and induction conditions

The *E. coli* strains used in this work are listed in Table 1. Bacteria carrying plasmids with VHH were grown at 30 °C in Luria Bertani (LB) liquid medium or on agar plates with the appropriate antibiotic for plasmid selection: chloramphenicol (Cm, 30 μg/ml) for pNeae-derivatives, and ampicillin (Ap, 100 μg/ml) for pMAL1-derivatives. LB plates and pre-inoculum media prior to induction contained 2% (w/v) glucose (Glu) for repression of the *lac* promoter. The preinocula cultures were started from individual colonies (for single clones) or from a mixture of clones (in case of libraries), freshly grown and harvested from plates from LB-Glu-Cm, diluted to an initial OD_{600} of 0.5, and grown overnight (o/n) under static conditions. For induction, bacteria (corresponding to an OD_{600} of 0.5) were harvested by centrifugation (4000 × *g*, 5 min), and grown in LB-Cm media with 0.05 mM isopropylthio-β-D-galactoside (IPTG), for 3 h with agitation (160 rpm), unless indicated otherwise. For over-expression of soluble Nb in the periplasm, *E. coli* HM140 cells with the corresponding pMAL1-VHH plasmid were induced in LB-Ap with 0.3 mM IPTG for 3 h at 30 °C.

2.2. Plasmids, DNA constructs and oligonucleotides

Plasmids used in this study are summarized in Table 1. DNA manipulation, ligation, transformation and plasmid preparation were performed following standard techniques. Oligonucleotides were synthesized by Sigma Genosys, except those used for VHH amplification, which were from Scandinavian Gene Synthesis (SGS). All DNA constructs were sequenced by Secugen SL. PCR reactions for cloning were performed with proof-reading Vent DNA polymerase (New England Biolabs) or Taq DNA polymerase (Roche). The plasmid pNeae2 (Cm^R) (Salema et al., 2013) encodes N-terminal Intimin residues 1-654 (from enterohemorrhagic *E. coli* EHEC O157:H7 strain EDL933*stx*-), called Neae (Bodelón et al., 2009), followed by the E-tag (GAPVPYPDLEPA), the hexa-histidine (His6) epitope, and a C-terminal myc-tag (EQKLISEED). Cloning of VHH domains by *Sfi*I-*Not*I digestion replaces His6 epitope and generates Intimin-E-VHH-myc fusions (called NVHH).

2.3. Immunization and VHH library construction

The camel immunization protocol followed the animal experimentation guidelines published by the Canary Islands Regional Government (Spain) and was approved by the Ethics Commission of the Department of Animal Medicine and Surgery, University of "Las Palmas de Gran Canaria" (Spain). About 0.2 mg of human fibrinogen (described below) in 2 ml sterile water was mixed with the same volume of adjuvant (Veterinary Vaccine Adjuvant, GERBU) and injected subcutaneously in one male dromedary camel (*Camelus dromedarius*) once in every week during 5 weeks. The total volume (4 ml) was injected in three distinct sites in the camel. Pre-immune serum was prepared from a small blood sample (5 ml) before the first injection. Seven days after the last injection, immune serum was prepared similarly and an additional 50 ml of blood was collected from jugular vein in tubes containing EDTA as anticoagulant using Venoject system. Serial dilutions of pre-immune and immune sera were used in ELISA to confirm Ab immune response against human Fib with Protein-A conjugated to peroxidase (POD) as secondary. For lymphocyte isolation, uncoagulated blood sample was mixed with the same volume of RPMI-1640 medium (Sigma), divided in 4 aliquots of 25 ml and each aliquot added on top of a 25 ml of Ficoll-Paque Plus (StemCell Technologies) in 50 ml sterile Falcon tubes (BD Biosciences). After centrifugation (800 × *g*, 30 min, RT), lymphocytes were recovered from the interphase, washed twice in RPMI-1640 by centrifugation (800 × *g*, 10 min), resuspended in 5 ml of RPMI-1640, and the number of cells determined in a Neubauer hematocytometer (Hausser Scientific). About 2×10^7 cells were lysed with 2 ml of TRIzol (Invitrogen) for RNA extraction following manufacturer's instructions. The poly-A+ mRNA was purified from total RNA using an oligo-dT resin (Oligotex mRNA Minikit, Qiagen) and directly employed as template for first-strand cDNA synthesis reactions with random hexamers and oligo-dT primers (iScript cDNA Synthesis, Bio-Rad). Aproximately 1 μl of each cDNA synthesis was used as template in 50 μl PCR reactions with oligonucleotides CALL001 (5′-GTC CTG GCT CTC TTC TAC AAG G-3′) and CALL002 (5′-GGTACGTGCTGTTGAACTGTTCC-3′). The amplified fragments of ~0.6 kb, corresponding to VHH-CH2 domains, and ~0.9 kb, corresponding to conventional VH-CH1-CH2 domains, were separated in 1.2% (w/v) low melting agarose gel and the ~0.6 kb band was purified (QIAEX II Gel Extraction kit, Qiagen). This fragment was used as template in a second PCR reaction with oligonucleotides VHH-Sfi2 (5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCT CAG GTG CAG CTG GTG GA-3′) and VHH-Not2 (5′-