Biochemistry and Biophysics Reports 3 (2015) 169–174

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Selection and characterization of high affinity VEGFR1 antibodies from a novel human binary code scFv phage library

Xiaolei Wang [a,1], Hye-Yeong Kim [b,1], Brendon Wahlberg [a], W. Barry Edwards [a,*]

[a] Molecular Imaging Laboratory, Department of Radiology, University of Pittsburgh, Pittsburgh, PA 15219, United States
[b] Cardiovascular Molecular Imaging Laboratory, Section of Cardiovascular Medicine and Yale Cardiovascular Research Center, Yale University School of Medicine, New Haven, CT 06520, United States

ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form
21 July 2015
Accepted 5 August 2015
Available online 6 August 2015

Keywords:
scFv
Phage display
Phage library
Binary code
VEGFR1

ABSTRACT

VEGFR1 is a receptor tyrosine kinase that has been implicated in cancer pathogenesis. It is upregulated in angiogenic endothelial cells and expressed on human tumor cells as well. VEGFR1 positive hematopoietic progenitor cells home to sites of distant metastases prior to the arrival of the tumor cells thus establishing a pre-metastatic niche. To discover high affinity human antibodies selective for VEGFR1 molecular imaging or for molecularly targeted therapy, a novel phage display scFv library was assembled and characterized. The library was constructed from the humanized 4D5 framework that was mostly comprised tyrosine and serine residues in four complimentarity determining regions (CDRs). The library produced diverse and functional antibodies against a panel of proteins, some of which are of biomedical interest including, CD44, VEGFA, and VEGFR1. After panning, these antibodies had affinity strong enough for molecular imaging or targeted drug delivery without the need for affinity maturation. One of the anti-VEGFR1 scFvs recognized its cognate receptor and was selective for the VEGFR1.

1. Introduction

The human vascular endothelial growth factor receptor-1 (VEGFR1 or Flt-1) contributes to the pathogenesis of both neoplastic and inflammatory diseases [1]. In human cancer, VEGFR1 mediated signaling is responsible for angiogenesis. In animal models for example, inhibition of VEGFR1 signaling by peptides reduces angiogenesis of xenografted human tumors [2]. VEGFR-1 mediated activation of nonmalignant supporting cells such as tumor associated macrophages, are also likely important for cancer pathogenesis. The expression of VEGFR1 on these macrophages is associated with a more aggressive clinical phenotype of breast cancer [3]. Finally, in response to chemokine activity within the primary tumor, VEGFR1 positive hematopoietic progenitor cells preferentially localize to pre-metastatic sites [4]. Because of the involvement of VEGFR1 in cancer pathogenesis, our goal is to develop high affinity antibodies for molecular imaging or molecularly targeted therapy of cancer. To supply these antibodies, we assembled and characterized a phage displayed scFv library.

While most approaches in creating diversity through degenerate codons draw from all or most of the genetically encoded amino acids, previous work with a phage displayed Fab library has shown that restricting diversity to only two amino acids consisting of tyrosine (Tyr) and serine (Ser) can yield high affinity antibodies in a few rounds of panning without the need for further affinity maturation which is a highly prized characteristic for a phage displayed library [5,6]. Whereas Fab molecules are generally regarded as more stable than single chain variable fragment antibodies (scFvs), the scFv format offers some distinct advantages. Fab antibodies are heterodimers composed of a V_H–C_{H1} and a V_L–C_L domain while scFvs, comprised the V_H and V_L chains linked by a flexible peptide linker, are monomeric. As single molecules, scFvs have a greater efficiency of functional display on filamentous phage and they are amenable to fusion with other monomeric proteins [7–9]. Additionally, like Fabs, scFvs have demonstrated efficacy *in vivo*, as tumor imaging agents, when labeled with near infrared dyes or radioisotopes and as mediators of molecularly targeted gene delivery [10]. Further, as monomers, scFvs are readily converted to either minibodies or a diabodies for multivalent effects [11]. These bivalent formats offer superior properties for molecular imaging in experimental animal models, without concern for a loss of affinity upon conversion and are attractive for the development of high-affinity ligands for cell surface receptors [11].

Therefore, we have designed and constructed a scFv phage display library for selection of high affinity functional antibodies for molecular targeting of VEGRF1. The library (BCscFv library) was

* Corresponding author. Fax: +1 412 624 2598.
E-mail address: edwardsw@upmc.edu (W.B. Edwards).
[1] Both authors contributed equally to the manuscript.

http://dx.doi.org/10.1016/j.bbrep.2015.08.004

constructed with binary code mutations comprised Tyr and Ser residues in all of the CDRs of variable heavy chain (V_H) and in the CDR3 of variable light chain V_L [12]. The library was built upon the humanized and stable 4D5 framework [13]. After the new library was characterized, and the diversity was determined, the functionality of the library was tested by screening against a variety of antigens which included hemoglobin, ubiquitin, VEGFA, CD44, as well as our antigen of interest, VEGFR1.

2. Materials and methods

Phusion high-fidelity DNA polymerase, restriction endonucleases NcoI, DpnI and NotI, and T4 DNA ligase are purchased from New England BioLabs. TG1 phage display competent cells were purchased from Lucigen. Gel Extraction Kit, PCR Purification Kit, and plasmid Spin Miniprep Kit were purchased from Qiagen. Protein Prestained Standards are purchased from BioRad.

2.1. Phagemid construction

The pIT2 vector was used as a parent vector for a phagemid construction. Nucleotide sequences of scFvs of the humanized 4D5 antibody were spanned by restriction enzyme sites NcoI and NotI and synthesized for cloning into pUC57 vector from GenScript. The synthesized sequences were digested and purified using agarose gel electrophoresis, and ligated into NcoI and NotI digested phagemid pIT2 using T4 DNA ligase. After transformation, positives were selected by colony PCR screening and further confirmed by DNA sequencing (Genewiz) using purified phagemids.

2.2. Site-directed mutation by PCR and TG1 electroporation

To introduce mutations, primers containing binary code of Tyr and Ser were designed as previously described and synthesized from Integrated DNA technology with 5′ phosphate modification (Table S1) [12]. Site-directed mutagenic PCRs were prepared using Phusion polymerase (2 U/reaction), with a reaction system containing 80 ng of template plasmid, 1 × phusion HF buffer, 7% DMSO (v/v), 55 pmol of forward and backward primers, 10 pmol of dNTPs, and H_2O added to final volume as 50 μL. PCR reactions were performed using a BioRad T100 Thermal Cycler programmed as follows: 2 min at 98 °C; 35 cycles of 40 s at 98 °C, 1 min at 68 °C, 3 min at 72 °C; 10 min at 72 °C; hold at 4 °C. PCR products were incubated with 1 unit endonuclease DpnI overnight at 37 °C to digest the template plasmid. The next day, DpnI was inactivated by heating for 10 min at 80 °C. PCR products were resolved on a 1% agarose gel. The target DNA fragment was excised and purified with a Gel Purification Kit. Self-ligation was accomplished with T4 ligase for 72 h at 16 °C. Ligation reactions were heat-inactivated for 15 min at 70 °C.

The ligated plasmids were purified from the ligation reactions with a PCR Purification Kit, and were electroporated into TG1 electrocompetent cells (Lucigen) as described in the manufacture's protocol. Briefly, 1 μL of ligated plasmid was mixed with 25 μL of ice-thawed TG1 and transferred into an ice-cooled 0.1 cm gap cuvette (BioRad). Electroporation was carried out with a BioRad Gene Pulser using the following conditions: 10 μF, 600 Ω, and 1800 V. Within 10 s of the pulse, 975 μL of warm kit-provided recovery medium was added to resuspend the cells, and they were transferred to a culture tube. After cells were recovered for 1 h at 37 °C, 10 μL of the cells were used for titration with serial dilutions. The rest was cultured for phage library preparation.

2.3. Screening the BCscFv library

BCscFv library was constructed (Fig. S1, Table S2) and characterized (Fig. S2).

The antigens, ubiquitin (R&D Systems), hemoglobin (Sigma), VEGFA (Sino Biological), VEGFR1 (R&D Systems), and CD44 (R&D Systems), were incubated in 96-well Nunc Maxisorp plates (Thermo Scientific) at 4 °C for 16–20 h for immobilization. For stringent phage selections, the amount of antigen for immobilization was gradually decreased: first round: 5 μg/mL, second round: 2.5 μg/mL, and third round: 1.25 μg/mL in PBS (100 μL). After each well was rinsed with PBST (PBS containing 0.05% Tween-20), non-specific binding sites were blocked with alternating proteins (300 μL) to prevent the emergence of phage that bind the blocking protein for 2 h (first round: 0.5% BSA in PBS, second round: 0.5% ovalbumin in PBS, and third round: 0.5% casein in PBS). The plate was rinsed with PBST, and phages in blocking buffer (100 μL, 10^{13} CFU/mL) were added to the plates for affinity selection and incubated for 2 h at room temperature. The wells were rinsed three times with PBST in the first and second rounds of screening and ten times in the third round of screening. The remaining scFv-expressing phages were eluted by a treatment with trypsin (100 μL/well, 1 mg/mL in PBS) for 10 min. The eluted phage were collected and stored at 4 °C for titration or amplification.

2.4. Phage ELISA

96-well plates were coated with antigens or BSA overnight (0.5 μg in 100 μL of PBS) and washed three times with PBST. The non-specific binding was blocked with a blocking solution (5% non-fat milk in PBS, 300 μL) for 2 h followed by washing (3 ×, PBST). Blank wells received only the blocking solution ($n=96$). The phages in 5% non-fat milk in PBS (100 μL, 10^{13} CFU/mL) were added to the antigen- or BSA-immobilized wells and incubated for 1 h followed by rinsing with PBST (10 ×, 300 μL). Each well was incubated with an HRP-anti-M13 antibody (1:5000, 100 μL; GE Healthcare Life Sciences, 27-9421-01) for 1 h. The wells were washed (8 ×, PBST), and HRP enzyme substrate solution (100 μL/well; BioRad, 172-1064) was added into the wells. The enzymatic reaction was stopped using H_2SO_4 (2 N, 100 μL), and the plate was read at OD 450 nm (SynergyH4 Hybrid Reader, BioTek). Means of the BSA-controls ($n=96$) plus three times the standard deviation (SD) are set as cut-off for positives [14].

2.5. Confocal microscopy of VEGFR1 expressing cell lines

The cell lines SK-BR3, U87 was seeded for 48 h and fixed with Methanol/Acetone (V:V=1:1) for 10 min at −20 °C, following by modified protocol descripted in supplemental data. Fluorescence microscopic images were taken with a Zeiss Observer.Z1/Apotome 2 Microscope (Carl Zeiss) equipped with an EMCCD camera (Evolve 512 Delta, Photometrics, Tuscon, AZ).

3. Results

3.1. Characterization of the library with phage display against five human antigens

To assess the functionality of the BCscFv phage library, biopanning was performed with five human antigens: hemoglobin, VEGFA, ubiquitin, CD44 and VEGFR1. The antigen amounts were decreased during panning to increase stringency. In some cases the enrichment values decreased for the third round of panning (hemoglobin and ubiquitin) (Table S3). To verify antigen mediated