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Biochemical and Biophysical Research Communications 346 (2006) 896–903
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Construction and characterization of a pseudo-immune human antibody library using yeast surface display

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Received 22 May 2006
Available online 9 June 2006

Abstract

Lymphocytes from eight individuals out of 60 healthy donors, whose plasmas showed relatively higher antibody titer for a target antigen of death receptor 5 (DR5), were selected for the source of antibody genes to construct so called an anti-DR5 pseudo-immune human single-chain fragment variable (scFv) library on the yeast cell surface ($\sim 2 \times 10^6$ diversity). Compared with a large nonimmune human scFv library ($\sim 1 \times 10^9$ diversity), the repertoire of the pseudo-immune scFv library was significantly biased toward the target antigen, which facilitated rapid enrichments of the target-specific high affinity scFvs during selections by fluorescence activated cell sortings. Isolated scFvs, HW5 and HW6, from the pseudo-immune library showed much higher specificity and affinity for the targeted antigen than those from the nonimmune library. Our results suggest that a pseudo-immune antibody library is very efficient to isolate target-specific high affinity antibody from a relatively small sized library.

Keywords: Human antibody; Pseudo-immune scFv library; Nonimmune scFv library; Yeast surface display; Death receptor 5

Recombinant antibody libraries have been constructed by cloning antibody heavy- or light-chain variable genes directly from lymphocytes of animals or human and then expressing as a single-chain fragment variable (scFv) or as an antigen-binding fragment (Fab) using various display technologies (reviewed in Ref. [1]). The recombinant antibody technology, an alternative to traditional immunization of animals requiring immunization of animals, facilitates to isolate target specific high affinity monoclonal antibodies without immunization by virtue of combination with high throughput screening techniques. Phage and ribosome display platform technologies have been extensively used to construct a large scFv or Fab library [2,3]. Recently human scFv and Fab libraries have been generated on the yeast surface, which can be propagated without the growth-mediated clonal diversity loss observed with phage antibody library [4–6].

Strategy of creating of a combinatorial antibody library is very important to isolate high specificity and affinity antibodies against targeted antigens. There have been mainly two approaches to generate antibody library in terms of antibody library sources, i.e., nonimmune and immune library [1]. Nonimmune, or naïve, antibody library is constructed from B lymphocytes derived from healthy donors. The most important parameter in the nonimmune antibody library is the library diversity, i.e., library size, in an aspect that, in general, the larger the library, the higher the likelihood is to isolate high affinity binders to a particular antigen [2,3]. Typically, approximately 10^9–10^{11} library diversity has been reported to generate specific high affinity binders in the 1–1000 nM affinity range [2,3,7]. However, creating a large antibody library is time consuming and

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.05.202

does not always guarantee to isolate high affinity binders to any given antigens. Alternatively, immune antibody library constructed from B lymphocytes of immunized donors or patients with high antibody titer to particular antigens has shown efficient isolation of specific high affinity binders from relatively small to medium sized library ($\sim 10^7$–10^8) [8,9]. However, the construction of immune library is not always available due to difficulty in obtaining antigen-related B lymphocytes source.

In this study, we describe an alternative strategy of construction of antibody library to increase likelihood of isolating target-specific high affinity antibodies from a small sized library. We prescreened 60 plasma of healthy donors to select eight samples showing relatively higher antibody titer for a model antigen of death receptor 5 (DR5) and then constructed so called an "anti-DR5 pseudo-immune human scFv library" from lymphocytes of the eight selected individuals on the yeast cell surface. Then the pseudo-immune scFv library with a very small diversity ($\sim 2 \times 10^6$) was compared with a large nonimmune human scFv library ($\sim 1 \times 10^9$ diversity) for any repertoire biases of library prior to selection and antibody enrichment efficiency during screenings, using the targeted and its homologous antigens. To our knowledge, the construction of a pseudo-immune scFv library to a targeted antigen and subsequent direct comparisons with a large nonimmune library have not yet been performed.

Materials and methods

Materials. Restriction enzymes, T4 DNA ligase, and high fidelity DNA polymerase were purchased from New England Biolabs (MA, USA). All other chemicals and solvents used were of analytical grade. Anti-c-myc monoclonal antibody (mAb) 9E10 from Ig Therapy (Chunchun, Korea), FITC-labeled anti-mouse mAb and anti-biotin-FITC mAb from Sigma, and streptavidin–*R*-phycoerythrin conjugate (SA-PE) from Molecular Probes (Eugene, USA) were used. A Miltenyi MidiMacs system with LS columns with anti-biotin or streptavidin microbeads was used for magnetic activated cell sorting (MACS) for the nonimmune scFv library. Death receptor 5 (DR5), DR4, death decoy receptor 1 (DcR1), and DcR2 were expressed and purified from bacteria, essentially as previously described [10]. For MACS and flow cytometry, proteins were biotinylated using an EZ-LINK™ Sulfo-NHS-LC-Biotinylation kit (Pierce Inc., USA) according to the manufacturer's instructions. The oligodeoxynucleotides were synthesized from Bionics Co. (Seoul, Korea). Yeast strain of *Saccharomyces cerevisiae* EBY100 and the yeast surface display plasmid, pCTCON, were previously described in detail [11–13].

Preparation of human plasma and peripheral blood mononuclear cells (PBMCs). Whole blood samples were obtained from 60 healthy donors (10 ml per individual) under permit of the Ethical Committee of the Medical Center of the Ajou University. PBMCs were isolated from blood by Ficoll-Hypaque (Sigma) density centrifugation (400 g for 25 min). Plasma fractions of upper phase were stored at −70 °C until used for antibody titration. Interfacial mononuclear cells were collected and washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline.

Plasma screening by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well polystyrene microtiter plates (Nunc, Life Technologies Ltd., USA) were coated with 100 μl of antigens at 10 μg/ml in 50 mM Tris–Cl, pH 7.5, 50 mM NaCl ("TBS") for 1 h at 25 °C, washed (3×) with TBS containing 0.05% Tween 20 ("TBST"), and then blocked with TBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma). Then, plasma (100 μl of 1:100 diluted samples) was added and incubated. After washing the wells with TBST, the wells were incubated with goat anti-human IgG + IgM + IgA antibody (Pierce) and then anti-goat IgG conjugated to alkaline phosphatase (Pierce). Each incubation step was performed for 1 h at 25 °C, followed by washing (3×) with TBST. Finally, *p*-nitrophenyl phosphate (*p*-NPP) (Sigma) solution (1 mg/ml in 0.1 M glycine, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$, pH 10.3) was added to each well and absorbance was read at 405 nM in a microplate reader.

Construction of an anti-DR5 pseudo-immune scFv library. Individual total cellular RNA was isolated from 5×10^6 PBMCs using TRIzol reagent (Invitrogen) according to the recommended protocol. Isolation of mRNA from total RNA was carried out using Oligotex mRNA kits (Qiagen). The first strand cDNAs were synthesized by reverse transcription of mRNA pools (2 μg) with random hexamers as primers (Amersham Pharmacia Biosciences) using AccuPower RT PreMix (Bioneer, Korea). For the amplification of VH of IgG (γ) and IgM (μ), V_κ, and V_λ gene, a total of 71 reactions (28, 16, and 27 reactions for VH, V_κ, and V_λ gene, respectively) were carried out using each gene specific primer set. The primers used were from Little et al. [14] with the following slight modifications to generate a linker sequence between VH and VL gene, and to allow overlapping sequences for cloning into the yeast surface display vector, pCTCON. For the insertion of a $(G_4S)_3$ linker between VH and VL gene, the following sequence was added to the 5′ end of the VH reverse primer (5′-CGA GCC CCC GCC ACC CGA ACC GCC CCC ACC TCT-3′) and the 5′ end of the V_κ and V_λ forward primer (5′-GGT TCG GGT GGC GGG GGC TCG GGC GGG GGT GGC TCA GAT CT-3′). To allow homologous recombination of amplified scFv inserts with pCTCON by gap repair function [15], the following sequence was added to the 5′ end of the VH forward primer (5′-AGT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GGT GGT GGT GGT TCT GCT AGC-3′) and the 5′ end of the V_κ and V_λ reverse primer (5′-TCA GAT CTC GAG CTA TTA CAA GTC CTC TTC AGA AAT AAG CTT TTG TTC GGA TCC-3′). Then each amplified VH and VL family gene was purified from 1% agarose gels and quantified by absorbance at 260 nM. Equal amounts of VH and VL genes were mixed together to generate various combinatorial pools between VH and VL family genes, and then overlap extension PCR was carried out to create scFv gene repertoires. The assembled scFv gene products (~800–850 bp) were purified from 1% agarose gels and concentrated using Pellet Paint™ (Novagen) [12]. The scFv antibody gene libraries (10 μg) were mixed with linearized pCTCON (1 μg) by *Nhe*I/*Bam*HI digestion, and were transformed into the yeast EBY100 strain by homologous recombination using a Bio-Rad Gene Pulser electroporation apparatus [12,15]. A total of four transformations were performed in parallel. The transformants were pooled and propagated directly in liquid selective SD-CAA media (-ura, -trp), which contained 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids (Difco, USA), 5.4 g/L Na_2HPO_4, 8.6 g/L $NaH_2PO_4{\cdot}H_2O$, and 5 g/L casamino acids (Difco). Cell surface expression of scFv was induced in selective media of SG-CAA, which contained the same composition as SD-CAA, except glucose was replaced with galactose. Library size was determined by plating serial 10-fold dilutions of the transformed cell on the selective SD-CAA agar plates. The scFv antibody genes cloned into pCTCON were sequenced using a forward primer (5′-GTT CCA GAC TAC GCT CTG CAG G-3′) and a reverse primer (5′-GAT TTT GTT ACA TCT ACA CTG TTG-3′). The germline gene family usage of VH and VL was analyzed in the IMGT database (available at http://imgt.cines.fr:8104/textes/vquest/) [16].

Flow cytometric analyses and sortings. Yeast cell labelings for affinity analyses and sortings by flow cytometry were performed as described before [12,13]. Yeast cells were grown in SD-CAA media at 30 °C for 20 h to an OD_{600} of six, pelleted by centrifugation, and transferred into SG-CAA media and grown at 30 °C for 20 h to induce the surface expression human scFv library [4,12,13]. Approximately 10^7 cells from the induced yeast library were incubated with anti-c-myc 9E10 mAb (1:100 dilution) and 1 μM biotinylated antigens at 25 °C for 30 min in 0.2 ml PBSB (phosphate-buffered saline, pH 7.4, containing 1 mg/ml BSA). Cells were washed with ice cold PBSB, and labeled with FITC-labeled anti-mouse IgG (1:25 dilution) and streptavidin–*R*-phycoerythrin conjugate (SA-PE) (1:100 dilution) in a final volume of 0.2 ml for 20 min on ice with frequent