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Isolation of human anti-serum albumin Fab antibodies with an extended serum-half life

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ABSTRACT

The serum albumin (SA) has been exploited to generate long-acting biotherapeutics by taking advantage of the FcRn-mediated recycling mechanism in a direct or an indirect way. Since Fab fragments have been proven to be clinically safe for human usage, we assumed that human anti-SA Fab antibodies could have a great potential as a carrier molecule to extend the serum half-life of therapeutic proteins. We, herein, had attempted to isolate anti-SA Fab antibodies from HuDVFab-8L antibody library *via* a phage display technology, and identified eight discrete human Fab antibodies. One of the Fab antibodies, SL335, showed the strongest binding reactivity to human SA with nM range of affinity at both pH 6 and pH 7.4, and cross-reacted to SAs from various species including rat, mouse, canine and monkey. The *in vivo* pharmacokinetic assay using a rat model indicated that SL335 has approximately 10 fold longer serum half-life and 26 to 44-fold increase in $AUC_{0\rightarrow\infty}$ compared to the negative control Fab molecule in both intravenous and subcutaneous administrations. Knowing that Fabs have proven to be safe in clinics for a long time, SL335 seems to have a great potential in generating long-acting protein drugs by tagging effector molecules with either chemical conjugation or genetic fusion.

1. Introduction

Phage display technology is frequently utilized to develop therapeutic antibodies because of its capability to obtain fully human antibodies [1], which is evidenced by the reports fact that numerous human antibodies created by this technology are currently in clinical trials for a wide range of human diseases including cancer, autoimmune disorders, graft rejection, and infectious diseases [2–4]. For generating therapeutic antibody candidates, in particular, a human naive immunoglobulin (Ig) gene repertoire would be a desired source since they may not be more immunogenic than those obtained from synthetic Ig sources for human usage, although this statement is rather circumstantial. Unfortunately, however, it seems difficult to isolate high affinity antibodies at nanomolar ranges from a naive Ig repertoire [5].

Various forms of an antibody fragment, such as Fabs, scFvs, and domain antibodies (dAb), with desired antigen-binding specificity can be routinely obtained from *in vitro* immunoglobulin (Ig) repertoires, and the production of functional antibody fragments in the periplasm of *Escherichia coli* where the oxidizing environment and the presence of chaperone proteins for correct folding are provided has prompted a major breakthrough in the field of antibody engineering [6,7]. These antibody fragments have been exploited to engineer "customized" therapeutics, with pharmacologic properties optimized for specific applications. For instance, the conjugation or genetic linkage of exogenous effector moieties such as cellular toxins, cytokines and enzymes to antibody fragments is an important approach because the antibody fragments provide an opportunity to more precisely and effectively deliver the functionality of therapeutic proteins to the desired targets [8–14].

Albumin and the IgG class of antibodies have a serum half-life of 3 weeks in humans, and both bind to the neonatal Fc receptor

Abbreviations: HSA, human serum albumin; RSA, rat serum albumin; MSA, mouse serum albumin; Fab, fragment antigen binding; hGH, human growth hormone; *E. coli*, *Escherichia coli*; FcRn, neonatal Fc receptor; mAb, monoclonal antibody; pAb, polyclonal antibody; HRP, horse radish peroxidase; AP, alkaline phosphatase; CH, heavy chain constant region; CL, light chain constant region; VH, heavy chain variable region; VL, light chain variable region; IPTG, isopropyl-β-D-1-thiogalactopyranoside; dAb, domain antibody; scFv, single chain variable fragment.

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(FcRn), which extends their half-life [15,16]. An antibody fragment specific for serum albumin (SA) is of particular interest since the fusion of various therapeutic proteins to albumin has also been shown to extend a serum half-lives of therapeutic proteins through the FcRn-mediated recycling mechanism [17,18]. Indeed, albumin-binding moieties, such as small peptides or domain antibodies (dAb), are able to prolong the half-lives of therapeutic proteins, which has a profound implication for the development of long-acting protein therapeutics [19,20]. Additionally, a rabbit anti-rat SA Fab′ had been reported to prolong the serum half-life and area under the curve [$AUC_{(0\rightarrow\infty)}$] of an anti-tumor necrosis factor (TNF) Fab as in a form of chemically conjugated bispecific anti-RSA-anti-TNF $F(ab')_2$ heterodimer [21], yet human anti-SA Fabs have not been reported in the literature so far.

Among various antibody fragments, Fab molecules are particularly attractive for being used in therapeutics due to their excellent thermostability [22] and clinical safety in human usage [13]. In addition, we presumed that a Fab molecule could be more apt for the genetic linkage of exogenous effector moieties than an Fv, scFv or dAb form because of the presence of C_{H1} and C_L domains which may provide enough space between the SA-binding domain and an effector molecule, resulting in the alleviation of steric hindrance to both region. It is also notable that Fab molecules, unlike scFv, Fv or dsFv, can be produced with ease up to 1–2 g/L as a soluble form in the periplasm of *E. coli* [23–25], or even in *Pseudomonas fluorescens* [26]. Therefore, we assumed that human anti-SA Fab antibodies may have a great potential as a carrier molecule to extend the serum half-life of therapeutic proteins. In this study, we attempted isolation of human anti-SA Fabs from a human naïve Ig repertoire *via* phage display technology and analyzed the feasibility of the Fabs in developing long-acting protein therapeutics.

2. Material and methods

2.1. Molecular cloning procedure

All of the DNA cloning experiments were performed according to standard procedures [27]. The oligonucleotides of sequencing grade were synthesized by Bioneer, Daejeon, South Korea. PCR amplification was performed using Pyrobest or Ex-*Taq* DNA polymerase (Takara, Ōtsu**,** Japan) under the condition of 25 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min unless otherwise noted. The restriction endonucleases, shrimp alkaline phosphatase (SIP) and T4 DNA ligase were also purchased from Takara. The *E. coli* MC1061 strain [*ara*D139 Del(*ara*A-*leu*) 7697 Del(*lac*)X74 *gal*K16 *gal*E15(GalS) lambda-e14-*mcr*A0 *rel*A1 *rps*L150(*str*R) *spo*T1 *mcr*B1 *hsd*R2] was used for cloning and recombinant protein expression. The *E. coli* TG1 strain {F′ [*tra*D36 *pro*AB$^+$ *lac*I^q *lac*ZΔM15]*sup*E thi-1 Δ(*lac-pro*AB) Δ(*mcr*B-*hsd*SM) 5, ($r_K^-m_K^-$)} was used for recombinant phage preparations.

2.2. Biopanning of the HuDVFab-8L antibody library

An enrichment of recombinant phages bound to target antigens from naïve human Fab repertoires was performed in a hierarchical manner using a set of two antibody libraries, HuDVFab-8L library and a target-biased L ($V_L + C_{Lκ}$) chain shuffling library [28,29]. HuDVFab-8L library serves for isolating low to medium affinity Fab clones as well as for identifying target-specific H [or Fd ($V_H + C_{H1}$)] chains. On the other hand, a target-biased L chain library serves for affinity maturation of target-specific H chains *via* L chain shuffling [30]. Both libraries utilize a dual vector system to comprise Fab diversities in that HuDVFab-8L library is consisted of pHf1g3A-2 phagemid encoding H chains and pLT-2 plasmid encoding L chains, and a L chain library is composed of pHg3A-3 plasmid encoding H chains and pLf1T-3 phagemid vector encoding L chains.

For screening HuDVFab-8L library, tosylated magnetic beads conjugated with human, rat or mouse serum albumin (HSA, RSA or MSA, respectively) (Sigma–Aldrich, St. Louis, MO, USA) were mixed with 10^{10} phages from the library under the pH 6 or pH 7.4 condition for 4 h at 4 °C, and washed three times with phosphate-buffered saline containing 0.02% Tween (PBST). The phage antibodies that were bound to the beads were eluted with elution buffer (0.1 M glycine, pH 2). The recombinant phages were then amplified using Ex-12 helper phage (AprilBio) for subsequent panning [28]. After the final panning, a monoclonal phage ELISA was performed to identify the positive phage clones. These recombinant phage progenies possess pHf1g3A-2 phagemid genome encoding SA-specific H chains. To construct SA-biased L chain libraries for L chain shuffling, the H genes were obtained from the positive phage clones and subcloned into the pHg3A-3 vector, followed by combining with ~10^8 human naïve κ L chain repertoire in pLf1T-3 phagemid vector, and three rounds of panning were performed against the same magnetic beads used beforehand to isolate SA-specific phage progenies [29]. These final recombinant phage progenies possess pLf1T-3 phagemid genome encoding SA-specific L chains.

2.3. DNA sequencing analysis

The pHf1g3A-2 phagemid housing SA-specific H chain genes and the pLf1T-3 plasmid carrying SA-specific L chain genes were isolated from *E. coli* cells producing anti-SA Fab molecules using the Wizard Plasmid Miniprep Kit (Promega, Madison, WI, USA). Two different sequencing primers (5′-gtgccgttctatagccatagcac-3′ and 5′-ggcactggctggtttcgctaccgtg-3′) that were complementary to pHf1g3A-2 or pLf1A-3 were used to read the V_H and V_L genes, respectively. The DNA sequencing was performed by SolGent, Daejeon, South Korea.

2.4. Phage enzyme-linked immunosorbent assay (ELISA)

For the monoclonal phage ELISA, the recombinant phage was obtained from positive *E. coli* clones by phage rescue, and ~10^8 CFU/well were added to MaxiSorb ELISA plates (Nunc, Roskilde, Denmark) that were coated with 5 μg/ml HSA, RSA, MSA or bovine SA (BSA). The phage was allowed to bind to the antigens either at pH 6 or at pH 7.4 for 1 h at 37 °C. A goat anti-human kappa L polyclonal antibody (pAb)-conjugated with horse radish peroxidase (HRP) (Sigma–Aldrich) was used as a secondary antibody. The binding signals were visualized with a TMB substrate (BD Science, San Jose, CA, USA), and the absorbance at 450 nm was measured using an ELISA reader (Bio-Rad, Hercules, CA, USA). The data represent the average of three experiments ± standard deviation.

2.5. Preparation of soluble Fab antibodies

The Fd and L chain gene fragments of the anti-SA Fab clones were assembled through linking PCR (sense primer 5′-gggaagcttattaacaagatttgggctcaactctcttgtcc-3′, and anti-sense primer 5′-gggggatccatgaaaaagacagctatcgcgattgcagtg-3′), and the resulting PCR product of ~1.4 kbp in size was excised from the agarose gel. Thereafter, the PCR product and the pHEKA plasmid (AprilBio) were restricted with *Bam*H I and *Hind* III, ligated together using T4 DNA ligase for 2 h at RT, and electroporated into *E. coli* MC1061 electrocompetent cells. The pHEKA plasmid is an in house expression vector with P_{tac} promoter that designed for periplasmic production of Fab molecules (unpublished). Soluble Fab antibodies were produced by growing the cells in 10 ml of 2× YT medium containing 50 μg/ml kanamycin at 37 °C until an