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Single chain antibody fragments with pH dependent binding to FcRn enabled prolonged circulation of therapeutic peptide in vivo

Yangsheng Qiu, Wei Lv, Min Xu, Yuhong Xu *

School of Pharmacy, Shanghai Jiao Tong University, 800 Dongchuan Rd, Shanghai 200240, PR China

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ABSTRACT

The neonatal Fc receptor for IgG (FcRn) is considered critical for the regulation of endogenous IgG and serum albumin (SA) and their circulation half-life in vivo. Both IgG and SA can bind to FcRn tightly at acidic pH but not so much at neutral pH. Here we reported a few novel single chain antibody fragments (scFv) obtained based on screening of a phage library. FnAb-8 and FnAb-12 can bind to human FcRn with higher affinities than IgG at acidic pH but similar or lower affinities than IgG at pH 7.4. Fusion proteins consisted of the therapeutic peptide, GLP-1 (Glucagon-like peptide-1) connected to the N-terminus of FnAb-8 and FnAb-12, named as G8 and G12, were shown to retain the pH-dependent binding capabilities to FcRn while also bound to the GLP-1 receptor. In vivo efficacy studies in diet induced diabetes mice confirmed the GLP-1 receptor (GLP-1R) agonist activities and sustained blood sugar lowering effect. In vivo pharmacokinetics (PK) studies were performed in nonhuman primates and FnAb-8 was found to have circulation half-life several folds longer than what have been reported for scFvs. G8 may be developed into long acting GLP-1R agonists with great potentials in clinical applications.
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1. Introduction

The neonatal Fc receptor (FcRn) is well known for having the functions of transporting IgGs across fetal and neonatal tissue barriers and regulating the rate of IgG and SA degradation throughout life [1–3]. It was firstly identified from the neonatal rodent gut which led to its designation as the neonatal Fc receptor (FcRn) [4,5]. The expression of FcRn was originally believed to be restricted to those sites involved in the transport of maternal IgG from mother to fetus or neonatal [6–8]. However, the presence of FcRn have now been extensively reported in many tissues and cell types including the endothelial cells, epithelial cells, the majority of hematopoietic cells, and some specialized cells such as podocytes, keratinocytes, the blood brain barrier endothelia, and the ocular cells [9–14].

Human FcRn was characterized as a heterodimer of one β2-microglobulin (14 kDa) light chain and one α heavy chain (46 kDa), structurally homologous to the MHC class I molecule [15,16]. The binding of both IgG and SA to FcRn were found to be highly pH dependent, with high affinity at acidic pH and low affinity at neutral pH [17–20]. They were protected from degradation based on a similar mechanism although through different binding sites [21–23]. Specifically, serum proteins including IgG and SA in circulation are taken up by myeloid cells or endothelial cells without FcRn binding near the cell surface when the pH is close to neutral. Entry of IgG and SA into cells is followed by accumulation in early endosomes where the acidic pH is permissive for FcRn binding. Then the early endosomes containing complex of FcRn and IgG/SA are sorted to recycle back to cell surface and IgG/SA released by exocytic processes close to the physiological pH [24–28].

Many studies had used the FcRn binding domains from SA or IgG and made fusion proteins containing these domains to achieve recycling and circulation extension [29–32], there had been also some efforts made to improve drugs' circulation properties by engineering or developing new peptide or protein binding domains of FcRn [33–43]. But most of these attempts including Fc-fusion proteins could not match the endogenous IgGs' long circulation half-life. Higher binding affinity at endosomal pH may not always translate to half-life extension [44,45]. Other factors including the binding sites, pH dependency, and possibly affinity thresholds at different pHs may exist that governed the FcRn mediated recycling process [46].

In this study, we seek to explore whether it is possible to generate small affinity antibody fragments, i.e., single chain variable fragment (scFv Abs), that can interact directly with human FcRn in a pH-

* Corresponding author.
E-mail address: yhxu@sjtu.edu.cn (Y. Xu).

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dependent way. The scFv fragments should have higher binding affinity at pH 6.0 towards FcRn than those of hIgG or SA, but almost no binding at pH 7.4. A specific scFv screening scheme was devised and successfully implemented. The detailed characterizations of the screen results indicated they may have excellent recycling properties and could be used as a long circulation carrier for therapeutical proteins and peptides.

2. Materials and methods

2.1. Materials

Human serum albumin (SA) was purchased from Abcam. Human IgG and HLA-A2 proteins, Lipofectamine2000, 293fectine and AF647 (AlexaFluor647) antibody labeling kit were from Invitrogen. Mouse anti-M13 phage antibody, anti-Poly-histidine tag antibody and GLP-1 were purchased from Genscript. GLP-1R-Fc, β2-microglobulin (β2M) protein, β2M expression vector, FcRn and HLA-A2 cDNAs were obtained from Sinobiological. Anti-hFcRn and anti-HLA-A2 antibodies were from Santa-Cruz. Strepavidin-AF647, anti-mouse IgG-AF647, anti-mouse-IgG-HRP were from Jackson Immunology research. Streptavidin-coupled Dynabeads was from ThermoFisher. Biotin labeling kit was from SoluLink. The luciferase assay system was from Promega. Cell culture medium and reagents were all purchased from GIBCO. Anti-FLAG magnetic beads and other chemicals were from Sigma-Aldrich.

2.2. Protein expression

Soluble extracellular domains of human, cynomolgus and mouse FcRn were cloned, expressed, and purified based on a previous study [47]. Briefly, the cDNA encoding extracellular domains (residues 1 to 297) of hFcRn, cynoFcRn and mFcRn with a c-terminal poly-histidine tag were cloned respectively into pCDNA3.1 vectors. Then the vectors were co-transfected with a β2M expression vector into HEK293 6E cells using 293fectine. After 72 h of incubation with the transfection complex, the supernatants were harvested, and secreted proteins were purified using a HisTrap FF column on AKTA explorer (GE Healthcare). The fractions containing FcRn&β2M heterodimer were pooled and buffer-changed to PBS.

For the production of scFv antibody fragments based on selected phage clone sequences, the genes were obtained by PCR using different sets of primers encompassing parts of the scFv gene and the restriction site EcoRI at the N-terminal or HindIII at the C-terminal. The PCR products were cloned into an expression vector containing a C-terminal FLAG and a poly-histidine tag. Then the vectors were transformed into HEK293 6E cells using 293fectin reagent. scFv fragments were purified from the supernatants using HisTrap FF column followed by affinity purification using anti-FLAG beads. The quality of the scFvs was determined by BCA and SDS-PAGE analysis.

Fusion proteins consisted of the modified GLP-1 sequence (no. 7-36 AA with mutations at A8G, G22E, R36G) connected through a $(G4S)_3$ linker with the selected scFv sequences (FnAb8 or FnAb12) or an unrelated scFv sequence (scFv-n) were synthesized (Genscript) and cloned into the same expression vector as described above. The production and purification of the three fusion proteins were also similar. There were designated as G8, G12, and Gn respectively. The quality of the fusion proteins was determined by BCA and SDS-PAGE analysis.

2.3. Cell lines

For the characterization of antibody fragments binding to FcRn on cell membrane, we constructed an EGFP-hFcRn stable expression cell line $293T^{EGFP\text{-}hFcRn}$ and a control cell line $293T^{EGFP\text{-}HLA\text{-}A2}$ with stable expression of EGFP-HLA-A2 based on previously published protocols [48]. Specifically, to generate N-terminal EGFP-tagged construct, the pEGFP-N1 (Clontech, 6085–1) vector was modified by inserting the IL2 signal peptide sequence into the downstream of the CMV-IE promotor, between NheI and AgeI; to generate the hFcRn construct, the 5′-coding sequence (966 bp, from AA24 to AA325) was PCR-amplified from hFcRn cDNA using forward (CCT GTA CAA GGC AGA AAG CCA CCT CTC CCT C, BsrGI site underlined) and reverse (TCT AGA CTA CTA CCT CAT CCT TCT CCA, XbaI site underlined) primers. This PCR product was digested with BsrGI and XbaI, and inserted into the downstream of CMV-IE promoter, between BsrGI and XbaI restriction sites and in-frame with the EGFP coding sequence. The HLA-A construct was PCR-amplified from HLA-A2 cDNA using forward (GAG CTG TAC AAG GGC TCT CAC TCC ATG AGG TAT TT, BsrGI site underlined) and reverse (GTC TAG ACT ATC ACA CTT TAC AAG CTG TGA GAG ACA C, XbaI site underlined) primers. The 1030 bp PCR product was digested with BsrGI and XbaI and inserted into the pEGFP-N1 vector as described above. The EGFP-hFcRn and EGFP-HLA-A2 encoding vectors were respectively transfected into 293 T cells together with theβ2M expressing plasmid using Lipofectamine2000. Stably transfected 293 T cells were selected using 400 μg/ml G418 and 2.5 μg/ml Puromycin in 10% FBS-supplemented DMEM. The expression of hFcRn&β2M and HLA-A2&β2M heterodimers in their respective $293T^{EGFP\text{-}hFcRn}$ and $293T^{EGFP\text{-}HLA\text{-}A2}$ cell lines were confirmed using confocal microscopy and FACS analysis with specific antibodies.

The CRE-Luc/GLP-1R HEK293 cell line was obtained from HD Biosciences. HEK293 cells were co-transfected with GLP-1R expression plasmid and plasmid containing cAMP response element (CRE) fused with a Luciferase gene. The colonies expressing both human GLP-1 receptor and CRE-luciferase were selected using a luciferase assay system following treatment with 1 μM of GLP-1 for 24 h in 96-well plates.

2.4. Phage display library screening

The purified hFcRn&β2M heterodimer and β2M monomer were biotin labeled using a biotin protein labeling kit and attached to streptavidin-coupled Dynabeads. A naïve human scFv phage-display library (CP Co. Ltd., Shanghai) was used for subtractive panning against β2M and then to hFcRn&β2M heterodimer at pH 6.0. Briefly, the phage pool solution was adjusted to pH 6.0 and incubated firstly with β2M coated Dynabeads for 1 h at room temperature. These β2M beads were removed and hFcRn&β2M heterodimer Dynabeads were added for panning at pH 6.0 for another 1 h at room temperature. The resulted phage bound beads were then washed extensively with phosphate-buffered saline (PBS; pH 6.0) containing 0.05% Tween-20 and finally PBS (pH 7.4) to collect the released phage pool. The phage pool was amplified by infecting TG1 cells along with helper phage M13KO7 as described previously [49] and the panning was continued for 3 round. The phage clones selected were isolated and tested for binding and characterized by sequencing.

2.5. ELISA analysis of pH dependent binding of selected phages to FcRn

The various phage clones selected by screening were characterized for their pH dependent binding capabilities to FcRn using ELISA. Biotin labeled hFcRn&β2M orβ2M were captured by streptavidin pre-coated ELISA plates and incubated at 37 °C for 1 h. The plates were then washed with pH 6.0 PBST (0.05% tween-20 in PBS) for three times, then 100 μl of phage samples (pH adjusted to pH 6.0) were added, and incubated for 1 h at 37 °C. The plates were washed again with PBST at pH 6.0 for 5 times. Then 100 μl of mouse anti-M13 phage antibody in PBS at pH 6.0 was added to each well, and incubated for 1 h at room temperature, followed by 100 μl HRP-conjugated anti-mouse antibodies with washes between the steps, and detected with 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Pierce) after 5 min development and 2 M HCl stop. All antibodies and buffers were adjusted to pH 6.0. The similar series of protocols were repeated using pH 7.4 buffers to determine the pH dependent binding properties of the various phage clones. The optical absorbance at 450 nm were measured and reported.

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