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Selection and characterization of human PCSK9 antibody from phage displayed antibody library

Yan Cao [1], Huan Yang [1], Xiaolong Zhou [1], Huiwen Mao, Tingting Gao, Zhigang Hu, Lingfeng He, Feiyan Pan, Zhigang Guo*

Jiangsu Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University, 1 WenYuan Road, Nanjing 210046 Nanjing, China

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9), which involves in low-density lipoprotein cholesterol (LDL-C) metabolism by interacting with the LDL receptor, is considered as a potent therapeutic target for treating hypercholesterolemia. Here, a fab antibody phage display library was constructed and employed for bio-panning against recombinant PCSK9. A Fab fragment (designated PA4) bound with high affinity to PCSK9 was isolated after four rounds of panning. The fully human antibody IgG1-PA4 bound specifically to PCSK9 with nanomolar affinity. In vitro, IgG1-PA4 inhibited PCSK9 binding to LDLR and attenuated PCSK9-mediated degradation of LDLR on the HepG2 cell surface. In C57BL/6 mice, administration of IgG1-PA4 at 30 mg/kg increased hepatic LDLR protein levels by as much as 3 fold when compared with control. Taken together, these results suggested that the IgG1-PA4 can be served as a potential candidate for the treatment of hypercholesterolemia by inhibiting PCSK9-mediated degradation of cell surface LDLRs.

1. Introduction

Hypercholesterolemia is a major risk factor for cardiovascular disease. Accumulating exprimental and clinical studies have implicated lowering levels of low density lipoprotein cholesterol (LDL-C) as a major contributor in reduced cardiovascular risk and improved clinical outcomes [1]. Statins have been the cornerstone of lipid therapy for the last two decades, owing to side effects, a significant percentage of patients cannot tolerate any statin dose or a high enough statin dose to reach their recommended LDL cholesterol goals [2]. Therefore, it is necessary to explore novel drug treatments to lower LDL-C in these special patient populations.

Proprotein convertase subtilisin kexin 9 (PCSK9), a member of the subtilisin serine protease family, has been demonstrated to be able to raise LDL cholesterol levels by binding to low density lipoprotein cholesterol receptor (LDLR) by both extracellular and intracellular pathways and targeting the receptor to lysosome for degradation [3–6]. In human, mechanistic studies of PCSK9 have shown that gain-of-function mutations cause a form of familial hypercholesterolemia [3–5], whereas loss-of-function mutations result in significantly decreased LDL-C level and cardiovascular risk [7]. In mice, the deletion of PCSK9 led to increased LDLR levels, accelerated the clearance of circulating lipoproteins, and descreased plasma cholesterol levels [8]. Several different approaches have been explored as means to inhibit or reduce PCSK9, including antisense oligonucleotides, lipidoid nanoparticle (LNP) formulated short interfering RNA (siRNAs) directed against the PCSK9 messenger RNA (mRNA) [9], antibodies directed against circulating PCSK9 protein [10–13] and small peptides that blocked the PCSK9/LDLR interaction [14]. Taken together, these findings indicate that PCSK9 represents an excellet target for hypercholesterolemia.

Monoclonal antibodies (mAb), because of their high specificity toward a given target, represent a unique class of novel therapeutics as PCSK9 inhibitors. Here we reported a novel PCSK9 antibody generated from phage displayed antibody library. This antibody was able to compeletively block the binding of PCSK9 to LDLR and inhibit the PCSK9-mediated reduction of LDLR in HepG2 cells and mice model. This antibody has great potential for use in the treatment of hypercholesterolemia.

* Corresponding author.
E-mail address: guozgang@gmail.com (Z. Guo).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Cell culture and reagents

Chinese hamster ovary cells (CHO–K1) and HepG2 cell lines were obtained from ATCC. CHO–S cells were purchased from Invitrogen (Carlsband, CA). All the reagents used in this study are described in the Supplementary materials and methods.

2.2. Cloning, expression and purification of human recombinant PCSK9 protein

Human PCSK9 (Genbank AX207686) was amplied from cDNA library HepG2 cells using primers listed in Supplementary materials and methods. The obtained PCSK9 gene was cloned into pIRESneo2 with a 6XHis tag. The resulted plasmid was transfected into CHO–K1 cells. PCSK9 proteins expressed in CHO–K1 cells was purified by using PrepEase His-tagged protein purification resin (USB, USA).

2.3. Construction of large naive Fab libraries

Human Fab library was generated according to the published protocols with minor modification [15]. Detailed description of this process can be found in the Supplementary materials and methods.

2.4. Biopanning of the Fab phage library

The human Fab phage library with a repertoire size of $>10^{10}$ clones was biopanned against recombinant PCSK9 according to standard methods [16]. Detailed description of this process can be found in the Supplementary materials and methods.

2.5. Phage ELISA

100 ul of phages were added to PCSK9-coated ELISA plates and incubated at 37 °C for 1 h. Following washing, a rabbit anti-M13 HRP conjugate secondary antibody (GE) was added (1:5000 in PBS) and incubated at 37 °C for 1 h. Colorimetric detection was performed using as substrate TMB. Reaction were stopped by the addition of 2M H_2SO_4 and absorbance was read at 450 nm.

2.6. Expression of the soluble Fab fragments

The soluble Fab was purified from periplasmic fraction of bacteria as described previously [17]. Briefly, the Fab gene was cloned into the expression vector plasmid in frame with a 6XHis tag. The resulting plasmid was transformed into E. coli TG1. The transformed TG1 was grown in the 2 YT medium and induced by 1 mM IPTG. Induced bacteria were collected and the Fab-PA4 was released from the periplasmic fractions, followed by purification with NTA-column.

2.7. Binding assay of PCSK9 with PA4 antibody by ELISA

PCSK9 was biotinatyed and immobilized on avidin coated microtiter plate, followed by addtion of various amounts of soluble Fab. The plate-bound Fab were detected by HRP-conjugated goat anti-human antibody.

2.8. Expression and purification of human IgG1-PA4

To generate full antibody, Fab-PA4 fragment was fused with the Fc fragement of human IgG1. The full length of IgG1-PA4 was cloned into eukaryotic expression vector pAS-puro (Millipore) and transfected into CHO–S cells. The detailed procedure for generating IgG1-PA4 stable cell line and the expression of IgG1-PA4 antibody was described in Supplementary materials and methods.

2.9. Binding-affinity measurements

The binding kinetics of IgG1-PA4 to PCSK9 were measured by bio-layer interferometry on a BLItz instrument (ForteBio) as described in Ref. [18]. In short, Protein A biosensors were loaded with 50 μg/ml solutions of purified IgG1-PA4. The loaded sensors were washed with HBS and association and dissociation measurements for PCSK9 at concentrations from 4.3 nM to 147.7 nM were carried out for 2 min each. Kinetic parameters (k_{on} and k_{off}) and affinities (K_D) were calculated from a non-linear fit of the Blitz instrument data using the Blitz software.

2.10. ELISA for measuring the inhibition of PCSK9 binding to LDLR

Biotinylated PCSK9 mixed with different amount of IgG1-PA4 antibody was applied to microtitre plate coated with rLDLR (recombinant human LDLR extracellular domain) (Sino Biological Inc, Beijing, China). Bound biotinylated PCSK9 was detected by avidin-HRP and TMB. Reaction were terminated by the addition of 2M H_2SO_4 and absorbance was read at 450 nm.

2.11. Inhibition of PCSK9 activity by PA4 in HepG2 cell assay

HepG2 cells were seeded into 6-well plates and cultured overnight. Then the medium was changed to DMEM containing 10% lipoprotein deficient serum (LPDS). After 24 h, 15 μg/ml PCSK9, 20 μg/ml IgG1-PA4 or a combination of both was added to the cells and incubated for 24 h. Cells were lysed in 500 μl of RIPA buffer. The amount of LDLR in HepG2 was detemined by western bloting.

2.12. Mouse model of liver LDLR degradation

All experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Normal University. Eight-week old male C57BL/6J mice were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). Mice were randomly divided into three groups (three mice/group) and given IgG1-PA4 or PBS (vehicle/control) at the indicated dose through the intravenous route. After 24 h, mice were dosed intravenously with 30 μg/mouse of PCSK9 or PBS. After 1 h, livers were harvested and snap frozen for further Western blot analysis.

2.13. Western blot analysis

Crude protein extracts were prepared from mouse liver or HepG2 using RIPA buffer. A total of 50 μg of proteins per lane were loaded in 10% SDS-PAGE gels. LDLR protein was detected using rabbit anti-LDLR antibody. β-Tubulin protein was detected with anti-Tubulin antibody to normalize protein loading. Bands were quantified by densitometry using ImageJ.

3. Results

3.1. Preparation of recombinant hPCSK9 protein

To prepare PCSK9 proteins for antibody generation, the full human PCSK9 cDNA was amplified from HepG2 cDNA library. HepG2 cells were chosed since PCSK9 has been reported to be up-regulated in human and rat hepatic cells [19,20]. The 2096 bp DNA encoding hPCSK9 gene was subcloned into the vector pIRES-neo2 (with His tag fusion), and expressed in CHO–K1 cells. PCSK9

purified from supernant of cell culture were separated by SDS-PAGE (Fig. 1A). Two bands were observed: a major band of 70 kDa corresponding to the catalytic and C-terminal domains and a minor band of 17 kDa corresponding to the prodomain, which were consistent with previously reports [21].

3.2. Generation of human Fab antibody against PCSK9 from phage displayed antibody library

A large human Fab phage library containing 3×10^{10} clones was constructed in our laboratiory using pMID21 vector (Fig. 1B) and used to isolate PCSK9 antibody. A total 4 rounds of selection were performed on immobilized PCSK9, with varying protein concentrations and number of washings. After each round selection, 96 phage clones were randomly picked and assayed for PCSK9 binding activity by phage ELISA. As shown in Fig. 1C, the clones which were positve in binding for PCSK9 increased significantly after the third selection and reached to 87% after the fourth selection, suggesting a remarkable enrichment through each round of panning. Nineteen colonies recovered from the fourth round were analyzed by DNA sequencing. As shown in Fig. 1D, five clones showed high binding ability to PCSK9 (clones 4, 5, 6, 9, 10) shared identical DNA sequence. Clone 4 was designated as PA4 and chosen for further study. The phage gene III stump was then removed from Fab-PA4. Soluble Fab-PA4 was then epxressed in TG1 and purified from bacteria periplasmic fractions (Fig. 1E). The binding affinity of soluble Fab-PA4 to PCSK9 was confirmed by the direct binding ELISA and western blot, as shown in Fig. 1F and G.

3.3. Construction and characterization of full IgG1-PA4 antibody

To generate full antibody, Fab-PA4 fragment was fused with the Fc fragement of human IgG1, followed by transfecting CHO−S for stable cell line screening (Fig. 2A). IgG1-PA4 was expressed in CHO−S cells and purified by Protein A affinity chromatography. The yield of IgG1-PA4 was approximately 60 mg/L culture. SDS-PAGE analysis showed that purified product consist of two monomers (H-chain and L-chain) under reducing condition (Fig. 2B) and form a homodimer under non-reducing condition (Fig. 2C), which was consistent with normal human IgG.

To test the affinity of IgG1-PA4 for PCSK9, we performed western blot and the direct binding ELISA. Western blot indicated that

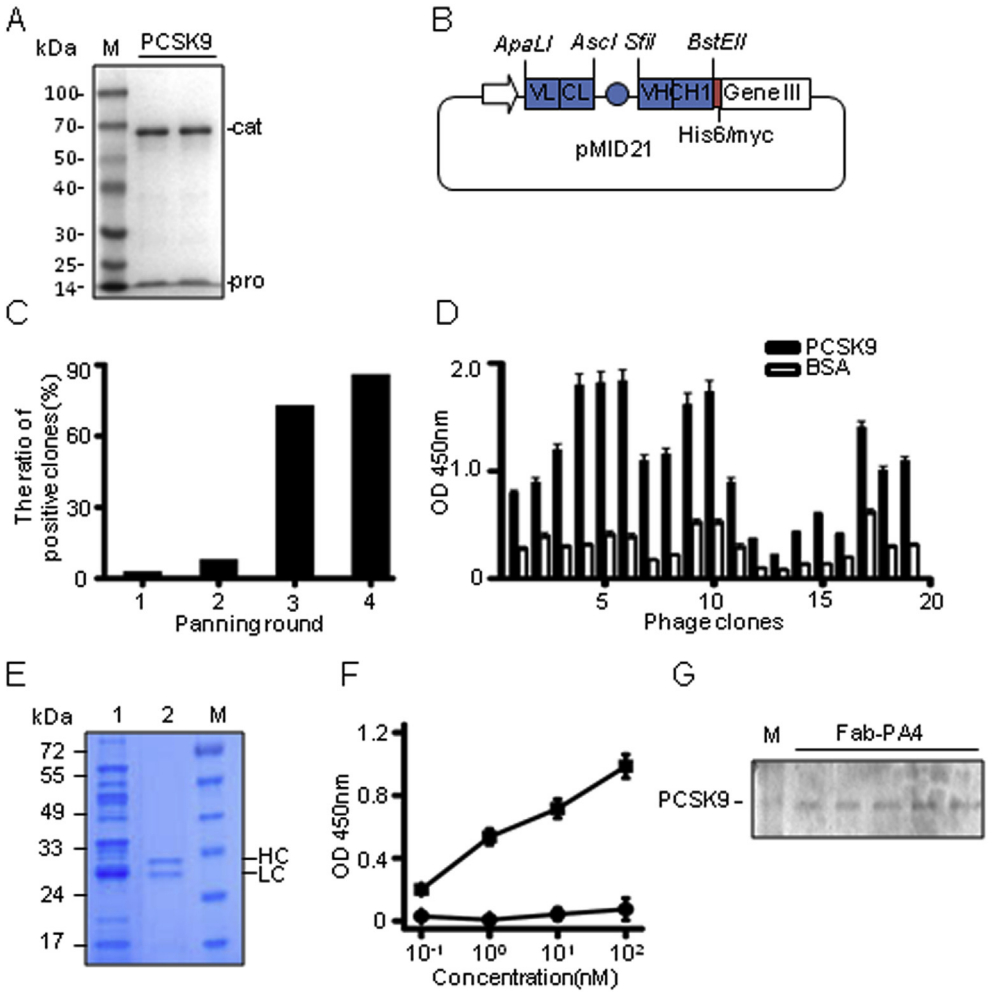

Fig. 1. Selection of anti-human PCSK9 Fab antibodies by phage display. (A) SDS-PAGE analysis of purified PCSK9 under reducing conditions. *Cat* and *Pro* indicate the mature fragments of PCSK9 including the catalytic and cysteine-rich C-terminal domains and the prodomain of PCSK9, respectively. (B) The phagemid vector pMID21 used for display of antibody Fab fragments. (C) Specific enrichment of recovered phages. (D) Identification of the binding selectivity of nineteen clones by phage ELISA. Phage clones binding to PCSK9 (black bars) and BSA (grey bars) were detected by the HRP-conjugated anti-M13 phage antibody. Triplicate determinations were done at each data point, and average OD450 nm of two types of proteins are shown. (E) SDS-PAGE analysis of purified soluble Fab-PA4 under reducing conditions. M, molecular weight marker; Lane 1: the lysate of TG1; Lane 2: purified Fab-PA4. (F) Dose-dependent binding of the Fab-PA4 to immobilized PCSK9.Various amounts of purified Fab were added to 96-well plates coated with PCSK9 or BSA (1 μg/ml), BSA was used as negative control. ■ PCSK9; ● BSA. (G) Fab-PA4 bound to PCSK9, as assessed by Western blot analysis.

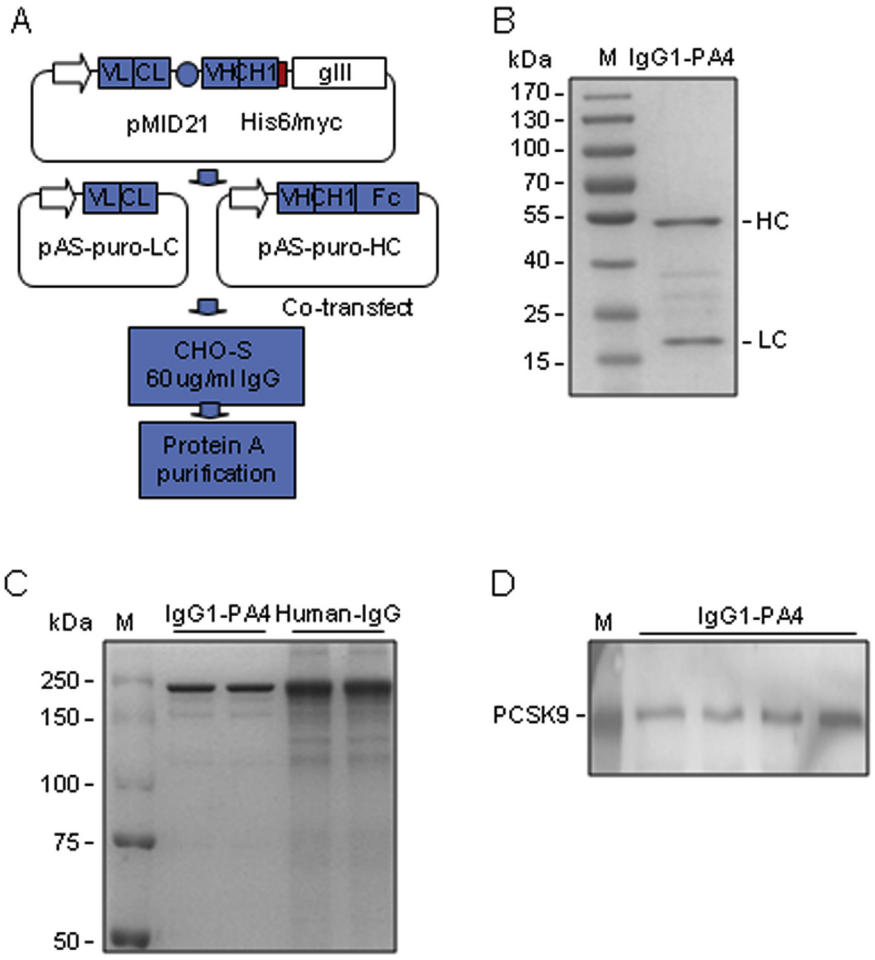

Fig. 2. Generation of full IgG1-PA4 antibody. (A) Flow diagram for the construction of expression plasmids. (B) SDS-PAGE analysis of purified IgG1-PA4 under reducing conditions. C) SDS-PAGE analysis of purified PA4-IgG1 under non-reducing conditions. M, molecular weight marker; Lane 1: purified IgG1-PA4; Lane 2: normal human IgG. D) IgG1-PA4 bound to PCSK9, as assessed by Western blot analysis.

IgG1-PA4 could bind to PCSK9 (Fig. 2D). Direct binding Elisa showed that IgG1-PA4 bound to immobilized PCSK9 in dose-dependent manner (Fig. 3A). Furthermore, the kinetics of IgG1-PA4 binding to PCSK9 was determined by bio-layer interferometry on a BLItz instrument (Fig. 3B). IgG1-PA4 bounds to PCSK9 with K_D of 1.05 nM. In the competition binding ELISA, IgG1-PA4 blocked PCSK9 from binding to LDLR, with an IC_{50} of approximately 7 nM, whereas normal human IgG control had no effect (Fig. 3C and D). All these data suggested that the full IgG1-PA4 is a functional antibody against PCSK9.

3.4. Inhibition of PCSK9-mediated LDLR degradation with IgG1-PA4

To test if the IgG1-PA4 antibody is functional in a cell based assay. The effects of IgG1-PA4 on PCSK9 mediated LDLR degradation were measured in HepG2 cells. In consistent with previous reports [22,23], Fig. 4A showed that incubation of HepG2 cells with recombinant PCSK9 protein reduced the LDLR level dramatically. However, co-incubation with IgG1-PA4 restored the PCSK9-induced LDLR degradation.

To determine IgG1-PA4 activity in vivo, we measured its effect on inhibiting PCSK9-mediated degradation of mouse liver LDLR. Mice were given a single i.v. injection of either PBS or IgG1-PA4 (10 mg/kg) followed by a bolus of recombinant human PCSK9 (30 μg/mouse), and livers were collected and analyzed 1 h later. As shown in Fig. 4B, treatment with PCSK9 reduced liver LDLR to <10% of normal levels. IgG1-PA4 restored liver LDLR to almost 90% of control levels. Together, these results and the in vitro PCSK9-LDLR binding data suggest that IgG1-PA4 exerts an inhibitory effect on the PCSK9-mediated degradation of LDLR.

4. Discussion

Hypercholesterolemia, a major risk factor for coronary heart disease, is increasing in incidence worldwide. Conventional lipid-lowering drug statins, which lower LDL-C in a dose-dependent manner, are often not sufficient to achieve target levels in patients with very high LDL-C at high risk for coronary heart disease [24].Therefore, it is necessary to search for novel hypocholesterolemic agents.

Previous studies have shown that plasma LDL-C is controlled through its uptake into cells upon binding the LDLR [25]. PCSK9, which binds to the LDLR and promotes its degradation, resulting in increased plasma LDL-C is regarded as a key regular of plasma LDL-cholesterol. Elimination or reduction of PCSK9 activity via genetic deletion or pharmaceutical intervention, such as antisense, RNA interference, or monoclonal antibodies, has been shown to cause an increase in hepatocyte LDLR and a subsequent reduction in serum LDL-C levels. Thus, blocking the interaction of PCSK9 and

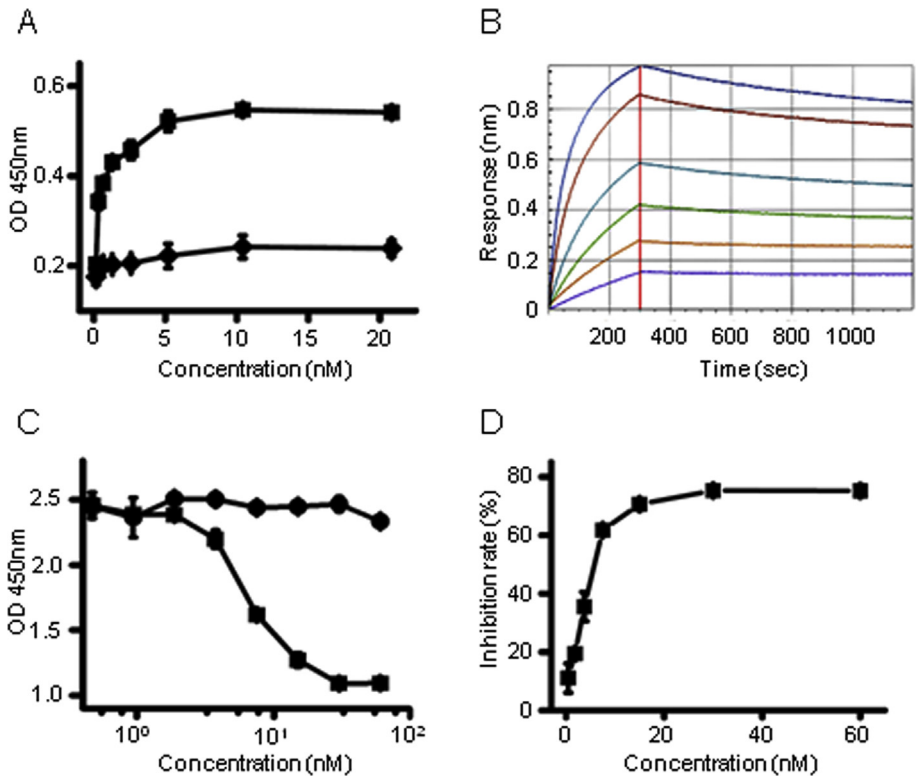

Fig. 3. Characterizati of full IgG1-PA4 antibody. A) Dose-dependent binding of the IgG1-PA4 to immobilized PCSK9.Various amounts of mab were added to 96-well plates coated with PCSK9 (1 μg/ml), normal human IgG was used as control. ■ IgG1-PA4; ● normal human IgG. (B) Binding kinetics of IgG1-PA4 to PCSK9 was measured using Biolayer Interferometry on a BLItz instrument. The determined K_D for PA4-IgG1 was 1.05 nM (k_{on} 1.57×10^5 M^{-1} s^{-1}; k_{off} 1.66×10^{-4} s^{-1}). (C) IgG1-PA4 competes with LDL receptor binding to PCSK9. Serial dilutions of IgG1-PA4 were mixed with 4 μg/mL biotinylated PCSK9 and added to plates coated with rLDLR (2 μg/ml). Bound biotinylated PCSK9 was detected by avidin-HRP. ■ IgG1-PA4; ● normal human IgG. (D) Concentration-dependent inhibition of IgG1-PA4 to PCSK9.

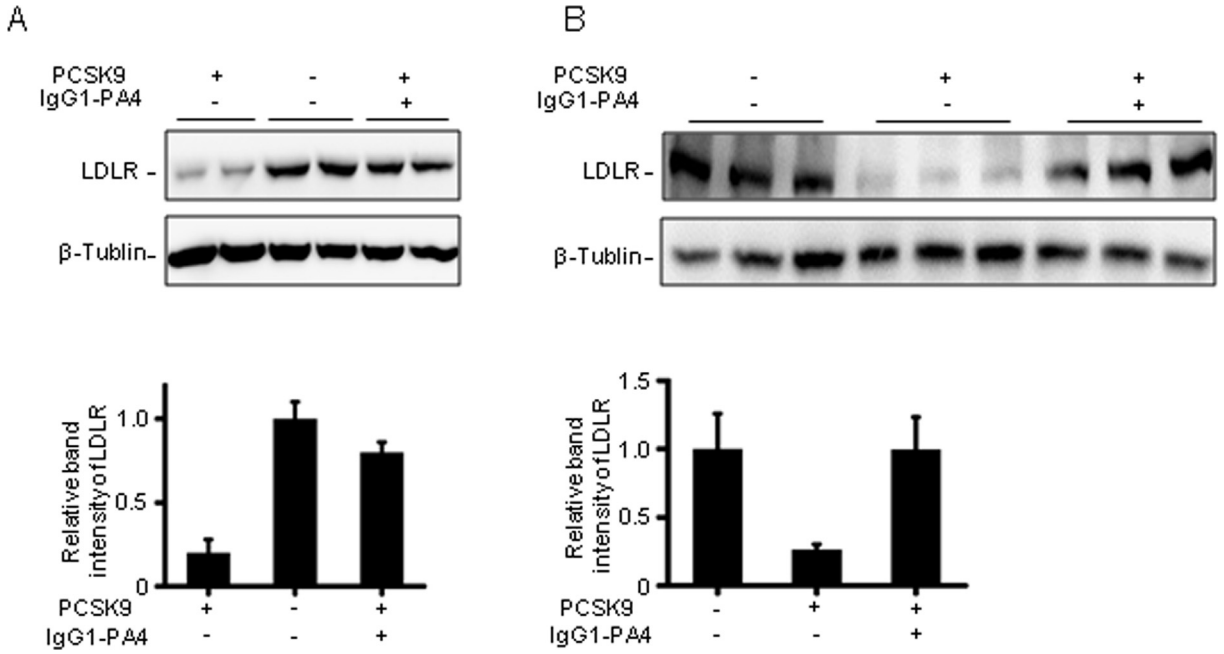

Fig. 4. Inhibition of PCSK9-mediated LDLR degradation by IgG1-PA4 in HepG2 and mice model. (A) HepG2 cells were treated for 24 h with 15 μg/ml PCSK9 with or without PA4-IgG4 (20 μg/ml), and LDLR was quantified by western blot analysis. (B) The effects of IgG1-PA4 to rescue liver LDLR level upon treatment of PCSK9 were tested in a mouse model. Mice were injected with PBS or IgG1-PA4 (10 mg/kg). A bolus injection of recombinant human PCSK9 (30 μg/mouse) were treated after 24 h. Livers were collected after 1 h, and LDLR was quantified by immunoblotting. The band intensities were quantified, normalized to β-Tublin.

the LDLR has emerged as a novel therapeutic target for hypercholesterolemia.

Phage display technology, a highly efficient tool for finding fully human therapeutic antibodies, has been investigated in recent years for the clinical diagnosis and treatment [26]. These antibodies with low immunogenicity, enhanced antigen binding and reduced cellular toxicity provide better clinical efficacy [27].

In the present study, we constructed a human naive phage display library to isolate PCSK9 antagonists. After four rounds of biopanning, the phage recovery rate increased gradually, and specific phage clones were effectively enriched. Five of the 19 selected candidate phage clones showed significantly positive signal according to phage ELISA. DNA sequence analysis revealed these five clones shared the same DNA sequence. Western blot and the direct binding ELISA further confirmed that soluble Fab fragment of clone PA4 was able to bind PCSK9 efficiency. Thus, it is reasonable to speculate that IgG1-PA4 may have the capability to bind PCSK9 and block the biology activity of PCSK9 by interrupting its interactions with LDLR.

The full-length human IgG1 mAb was generated based on the amino acid sequence of the Fab-PA4. Consistent with Fab-PA4, PCSK9 binding assays and affinity determination demonstrated a potent binding capacity of IgG1-PA4 to the recombinant PCSK9 with the nanomolar affinity. Compelling evidence demonstrate that PCSK9 binds to the LDLR and promotes its lysosomal degradation in cells and PCSK9 inhibitors are able to strongly block PCSK9/LDLR interaction, resulting in potent inhibition of PCSK9-mediated LDLR degradation. To confirm the biological function of IgG1-PA4, the effects of IgG1-PA4 on the PCSK9/LDLR interaction were determined. Competition ELISA revealed that IgG1-PA4 selectively blocked the PCSK9/LDLR binding, indicating IgG1-PA4 was able to disturb the interaction of PCSK9/LDLR in vitro. In both models employed, LDLR degradation of HepG2 cells and mouse liver, IgG1-PA4 showed superior antagonistic potency compared to the control. IgG1-PA4 was able to inhibit the degradation of LDLR, restore LDLR close to normal levels. These results imply that IgG1-PA4 can act as an antagonist of the LDL receptor-PCSK9 interaction.

In summary, we successfully isolated a high affinity anti-PCSK9 antibody, PA4 Fab fragment, and reassembled it as a human monoclonal antibody IgG1-PA4. This fully human Mab potently blocked PCSK9/LDLR interaction and inhibited PCSK9-mediated LDLR degradation. These data lend strong support to develop IgG1-PA4 as a novel therapeutic antibody for treating hypercholesterolemia.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.129.

Transparency document

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