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A cholesterol-lowering VLP vaccine that targets PCSK9

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secretory protein that controls cholesterol homeostasis by enhancing endosomal and lysosomal degradation of the low-density lipoprotein receptor (LDL-R). Mutations that cause increased activity of PCSK9 are associated with hypercholesterolemia, atherosclerosis and early cardiovascular disease (CVD), whereas individuals with loss-of-function mutations in PCSK9 are apparently healthy but are hypocholesterolemic and have a dramatically decreased risk of CVD. In this study, we generated virus-like particle (VLP)-based vaccines targeting PCSK9. Mice and macaques vaccinated with bacteriophage VLPs displaying PCSK9-derived peptides developed high titer IgG antibodies that bound to circulating PCSK9. Vaccination was associated with significant reductions in total cholesterol, free cholesterol, phospholipids, and triglycerides. A vaccine targeting PCSK9 may, therefore, be an attractive alternative to monoclonal antibody-based therapies.

1. Introduction

Elevated low-density lipoprotein cholesterol (LDL-C) is associated with an increased risk of cardiovascular disease (CVD) [1]. Although lifestyle changes and medication can significantly reduce LDL-C, a substantial percentage of at-risk patients on lipid lowering therapy (>60%) still go on to have a cardiovascular event [2]. Currently, treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) is the standard of care for hypercholesterolemic patients. Intensive statin therapy has some risks [3] and ~20% of high-risk patients with hypercholesterolemia do not achieve adequate control of LDL-C with just statins [2].

LDL-C in plasma is primarily removed from circulation when it interacts with LDL receptors (LDL-R) that are abundantly expressed on hepatocytes. Upon LDL-R binding, LDL-C is endocytosed and undergoes lysosomal catabolism. Following this process, LDL-R is recycled back to the cell surface. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a hepatic secretory protein that acts as a negative regulator of LDL-R by blocking the recycling of the receptor to the cell surface. PCSK9 in plasma binds to the extracellular domain of LDL-R and mediates its internalization and degradation, thus increasing circulating levels of LDL-C by preventing its uptake [4,5]. Genetic studies have shown that mutations that modulate PCSK9 activity can have profound effects on LDL-C levels. Gain of function mutations in PCSK9 are associated with autosomal dominant hypercholesterolemia, a disease that is characterized by increased LDL-C levels (>300 mg/dL) and a corresponding increased risk of CVD [6]. In contrast, humans with loss-of-function PCSK9 mutations are hypocholesterolemic (15–25% decrease in LDL-C) and have approximately half the incidence of CVD, most likely because of a life-long reduction of LDL-C [7]. Strikingly, individuals with compound heterozygote loss-of-function mutations, have exceptionally low serum LDL-C (<20 mg/dL) and appear healthy despite having no detectible circulating PCSK9 [8].

Given the important role of PCSK9 in regulating LDL metabolism and the fact that loss-of-function mutations appear not to be associated with adverse effects, PCSK9 has emerged as an attractive therapeutic target. PCSK9-specific monoclonal antibodies (mAbs), including evolocumab (Amgen), bococizumab (Pfizer), and alirocumab (Aventis/Regeneron) work synergistically with statins, and markedly reduce LDL-C levels by about 60% and, in early stage clinical trials, have been shown to reduce the incidence of cardiovascular events [9–11]. Statin therapy alone increases circulating levels of PCSK9 by as much as 30% as compared to placebo, making them somewhat self-limiting in their ability to further reduce LDL-C [12–14]. This likely occurs because the transcription factor

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SREBP-2, that is indirectly upregulated by statins, activates the *Ldlr* and *Pcsk9* genes [15]. Indeed, statins are much more effective when there is a deficit of PCSK9 [16]. Therefore, PCSK9-targeted therapeutics may have value in preventing and treating CVD in combination with statins or in vulnerable populations that are either resistant to statin therapy or statin intolerant.

Induction of antibody responses against a self-antigen, such as PCSK9, are seemingly limited by the mechanisms of B cell tolerance, which eliminate, inactivate, or alter the specificity of potentially self-reactive B cells. Yet B cell tolerance is actually highly inefficient and anti-self antibody responses can be readily elicited by immunizing with vaccines that have features that provoke the efficient activation of self-reactive B cells. Vaccines that display self-antigens in a dense, repetitive array and provide a source of foreign T helper epitopes can induce particularly robust, high-titer autoantibody responses [17]. Display of self-antigens in a highly dense, repetitive format on the surface of virus-like particles (VLPs) is one approach for inducing strong antibody responses against self-antigens. VLP display has been successfully used to target self molecules that are involved in the pathogenesis of a variety of chronic diseases, including Alzheimer's Disease, hypertension, and certain cancers [18]. Many of these vaccines have shown clinical efficacy in animal models and several have been tested in human clinical trials. For example, clinical trials of a VLP-based vaccine targeting angiotensin II, a regulator of blood pressure, showed that this vaccine was highly immunogenic and significantly reduced blood pressure in hypertensive patients [19].

In this study, we used several different approaches to identify a bacteriophage VLP-based vaccine that elicits strong antibody responses against PCSK9. Using both mice and non-human primates, we show that vaccination with VLPs displaying an epitope derived from PCSK9 was associated with significant reductions in pro-atherogenic plasma lipids and lipoproteins.

2. Materials and methods

2.1. Construction of PCSK9-displaying VLPs

Qβ VLPs were produced in *Escherichia coli* using methods that we have previously described for the production of MS2 bacteriophage VLPs [20]. Peptides representing huPCSK9 amino acids 68–76, 153–163, and 207–223 were synthesized (GenScript) and modified to include a C-terminal cysteine residue preceded by a 2-glycine-spacer sequence. Peptides were conjugated to VLPs using the bifunctional cross-linker succinimidyl 6-[(β-maleimidopropionamido)hexanoate] (SMPH; ThermoScientific) [21]. Efficiency of conjugation was measured using denaturing polyacrylamide gel electrophoresis.

Recombinant PCSK9-VLP expression vectors were constructed by genetically inserting huPCSK9 sequences (amino acids 153–163, 188–200, 208–222, and 368–381) by PCR at the N-terminus of a single-chain dimer version of the MS2 bacteriophage coat protein [20]. All constructs were sequenced to verify correct location and sequence of PCSK9 insert. Recombinant MS2 VLPs were expressed and purified as described [20].

2.2. Immunizations

All animal studies were performed in accordance with guidelines of the University of New Mexico and NHLBI Animal Care and Use Committees (protocols 12-100827-HSC and H-0059R3). Mouse immunization experiments were performed using 4–6-week old male Balb/c mice. Mice were immunized with 5 μg of VLPs three times at 2-week intervals. Vaccines were formulated with incomplete Freund's adjuvant (Sigma Aldrich) at a 1:1 (v:v) ratio in a total volume of 100 μl. Blood plasma was collected prior to the first immunization and 2-weeks following the third immunization.

Macaque studies were performed using nine 9–17 year old rhesus macaques (seven females and two males) that were divided into three experimental groups of three animals each. Groups were vaccinated three times at 2-week intervals with either 50 μg of (i) Qβ-PCSK9$_{207–223}$ without exogenous adjuvant, or (ii) Qβ-PCSK9$_{207–223}$ formulated with 2% Alhydrogel adjuvant (Invivogen) at a 1:4 (v:v) ratio or, as a control, (iii) wild-type Qβ VLPs plus Alhydrogel. Plasma was obtained prior to immunization and 2 weeks following each immunization. Approximately 6 months after the initial set of immunizations, groups i and ii were re-boosted with Qβ-PCSK9$_{207–223}$ formulated with 2% Alhydrogel adjuvant and then treated with simvastatin (at 30 mg/kg/day) for 2 weeks and these two group were combined for subsequent analyses. Group iii (control group) was reboosted with wild-type Qβ VLPs plus Alhydrogel and treated with simvastatin (at 30 mg/kg/day) for 2 weeks.

2.3. Characterization of antibody responses

PCSK9-specific IgG titers were determined by end-point dilution ELISA, using either PCSK9 peptide or recombinant huPCSK9 protein (R&D Systems) as the antigen. For peptide ELISAs, Immulon 2 plates (Thermo Scientific) were incubated with 500 ng streptavidin (Invitrogen) in pH 7.4 phosphate-buffered saline (PBS) for 2 h at 37 °C. Following washing, SMPH was added to wells at 1 μg/well and incubated for 2 h at room temperature. Individual peptides corresponding to the Qβ-conjugated peptides were added to the wells at 1 μg/well and incubated overnight at 4 °C. For PSCK9 ELISAs, plates were incubated with recombinant human PCSK9 protein (R&D Systems) at a concentration of 500 ng/well in PBS overnight at 4 °C. In all ELISAs, plates were blocked with 0.5% milk in PBS for 2 h, and 4-fold dilutions of plasma were added to each well and incubated for 2.5 h. The wells were probed with horseradish peroxidase (HRP)-conjugated secondary antibody [goat anti-mouse-IgG (Jackson ImmunoResearch; 1:5000) or goat anti-monkey IgG (Fitzgerald Industries; 1:4000) for 1 h. The reaction was developed using TMB (ThermoScientific) and stopped using 1% HCl. Reactivity of sera for the target antigen was determined by measuring optical density at 450 nm (OD_{450}). Wells with twice the OD_{450} value of background were considered to be positive and the highest dilution with a positive value was considered the end-point dilution titer.

2.4. Plasma lipid and lipoprotein quantification

Plasma lipids were measured enzymatically using a ChemWell instrument and Roche reagents. ApoB was measured nephelometrically on a Dimension analyzer (Siemens). LDL-C was calculated by the Friedewald equation. HDL and LDL particle counts were measured with a Vantera NMR analyzer (LipoScience).

2.5. Plasma PCSK9 quantification

Plasma PCSK9 levels were quantitated by a mouse PCSK9 ELISA kit (R&D Systems) by comparing experimental sera samples diluted 200-fold to an internal standard curve. PCSK9 was quantified in this way both before and after removal of immunoglobulin using Protein G-coated magnetic beads (Life Technologies). Briefly, plasma samples diluted 1:200 were split into equivalent volumes, then either (a) incubated for 10 min with magnetic Protein G beads or (b) set aside at room temperature. The Protein G beads were isolated using a magnet, and the Ig-cleared supernatant was then used

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