Vaccine against arteriosclerosis: an update

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Abstract: Substantial data from experimental and clinical investigation support the role of immune-mediated mechanisms in atherogenesis, with immune systems responding to many endogenous and exogenous antigens that play either proatherogenic or atheroprotective roles. An active immunization strategy against many of these antigens could potentially alter the natural history of atherosclerosis. This review mainly focuses on the important studies on the search for antigens that have been tested in vaccine formulations to reduce atherosclerosis in preclinical models. It will also address the opportunities and challenges associated with potential clinical application of this novel therapeutic paradigm.

Keywords: apolipoprotein B-100, atherosclerosis, immunization, low-density lipoprotein

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Background

Atherosclerosis is currently viewed as an immune-mediated inflammatory disease of the arterial wall, with both the innate and adaptive immune systems responding to many endogenous and exogenous antigens. Cells of both innate and adaptive immune systems such as macrophages, dendritic cells, B and T-lymphocytes, and mast cells are involved in atherogenesis, as are the immune-inflammatory mediators such as pathogen-associated and danger-associated molecular pattern molecules, immunoglobulins, cytokines, chemokines, and complement proteins. It is beyond the scope of this review to describe the complex roles of these immune cells and mediators in detail and many excellent reviews are available for the interested readers.[1–5]

Given that atherosclerosis is an immune-mediated disease of the arterial wall, it is tempting to consider specific strategies to modulate the immune responses to favorably affect the natural history of atherosclerosis. The challenge of this approach is to identify specific antigens relevant to atherogenesis so that they can be used to activate an antigen-specific atheroprotective immune response. Most studies in search of potential antigens are in preclinical experimental stages; hence data discussed in this review refer to data from animal models of atherosclerosis unless otherwise stated.

From low-density lipoprotein to apoB-100-derived peptides as antigens: our experience

Because low-density lipoprotein (LDL) and other apoB-100-containing lipoproteins are the primary culprits with the strongest causative link with atherosclerosis, investigators have been asking if immunizing hypercholesterolemic animals with LDL or apoB-100-derived peptide-antigens would modulate atherosclerosis. When homologous whole native or modified LDL was used as an antigen in a vaccine formulation, immunizing experimental animals with these vaccine formulations demonstrated atheroprotective effects.[6–14]

Because LDL is a large, heterogeneous molecule containing apolipoproteins, cholesteryl esters, triglycerides, and phospholipids, it would be impractical to use whole homologous LDL as an antigen in a clinically usable vaccine formulation. To identify the potential atheroprotective antigenic epitopes in LDL, our laboratory in collaboration with Dr Jan Nilsson's laboratory at Lund University in Sweden, generated a library of 302 peptides spanning the entire 4536-amino acid sequence in human apoB-100 protein and selected 102 peptides based on the humoral immune response detected in pooled human plasma as potential candidates for the next round of screening.[15] Among these 102 peptides, certain peptide sequences, labeled here as p2, p143, and p210 resulted in a 40–70% decrease

in atherosclerosis and reduction in plaque inflammation when used in a vaccine formulation in hypercholesterolemic mice.[16,17] Knowing the defined epitope makes more definitive immunologic studies possible; our teams have been using p210 as a prototype antigen in vaccine formulations due to its consistent atheroprotective effects.[18–20]

Mechanisms of action of p210 vaccine

Immunization with the p210 vaccine resulted in a significant reduction of aortic atherosclerosis compared with controls in murine model of atherosclerosis.[18] Given that immunization activates both B and T-cells,[18] it is important to delineate the subset(s) of lymphocytes which mediate the p210 vaccine's atheroprotective effect. Currently existing experimental evidence does not support a strong role for the humoral response in mediating the protective effect of active immunization using the p210 vaccine. This is based on the observations that (1) apoB-100 peptide immunization reduced atherosclerosis without an increase in peptide-specific immunoglobulin (Ig)G,[19] and the induced antibody titers did not correlate with the lesion size;[20] (2) Adoptive transfer of B-cells from p210-immunized mice to nonimmunized recipient mice did not confer atheroprotective effect.[18] However, immunization with p210 peptide did alter the natural history of p210 antibody levels in apoE$^{-/-}$ mice. In control mice, p210 IgG titer remained low between baseline and 25 weeks; whereas mice receiving adjuvant only or p210 vaccine developed high p210 IgG titer at 25 weeks. Interestingly IgG titer at 25 weeks from p210-immunized mice was lower when compared with that of mice receiving adjuvant only.[18] p210 IgM has a different profile; low level before immunization but titers increased over time, regardless of whether or not mice were immunized with p210 vaccine. This observation suggests that: (1) an endogenous IgM immune response against p210 exists, (2) induction of p210 IgG may serve as a marker of vaccination effect but not a marker of atheroprotective efficacy of the p210 vaccine.

Given that immunization with p210 vaccine activated CD8$^+$ T-cells,[18] we tested whether CD8$^+$ T-cells could be the immune cells that mediate the vaccine's atheroprotective effect by adoptively transferring CD8$^+$ T-cells from p210 immunized mice into nonimmunized mice. Such transfer recapitulated the atheroprotective effect of active immunization, confirming that CD8$^+$ T-cells mediate the atheroprotective effect of p210 vaccine.[18] Additionally we demonstrated reduction of CD11c+ cells in the plaques, injection site, and draining lymph nodes of p210 immunized mice and effector CD8$^+$ T-cells from p210-immunized mice developed a preferentially higher cytolytic response against p210-loaded dendritic cells *in vitro* indicating antigen-specific modulation of dendritic cells by p210 vaccine.[18] This may explain the observed reduction in dendritic cells in the immunization sites and in atherosclerotic plaques.

Immunization with p210 peptide also elicited a CD4$^+$ T-cell response. The reduction of atherosclerosis by p210 immunization was associated with a CD4$^+$CD25$^+$ T-cell response. Administration of antibodies against CD25 reduced CD4$^+$CD25$^+$ T-cells and abrogated the atheroprotective effect of p210 immunization.[21] Immunization of female apoE$^{-/-}$ mice intranasally with a recombinant protein consisting of p210 fused with the cholera toxin B (CTB) subunit (p210-CTB) reduced atherosclerosis in aortic sinuses of mice when compared with control mice. The rationale of using CTB conjugated with p210 was based on (1) CTB promotes the uptake of the antigen *via* the nasal mucosa to elicit protective immunity; (2) CTB-based vaccines have now been tested in a first human phase II trial in Behcet's disease. The investigators also observed that splenic CD4$^+$ T-cells from p210-CTB-immunized mice contained a higher percentage of the interleukin (IL)-10$^+$ subset, which were able to suppress effector CD4$^+$ T-cells functionally, without any differences between p210-CTB and controls in FoxP3, IL-10, or transforming growth factor beta (TGF-β) mRNA expression in the aorta.[20] Furthermore, there was no difference in the numbers of FoxP3$^+$ cells in aortic lesions or CD4$^+$FoxP3$^+$ T-cells in lung mucosa.[20] When p210 was delivered subcutaneously *via* an implanted mini-osmotic pump as a part of mixture of apoB-100 peptides (p210, malondialdehyde-modified-p210 and p240) or alone for 2 weeks, such treatment reduced atherosclerotic lesions in aortic sinuses and also retarded the progression of established atherosclerotic lesions in old female mice.[22] Subcutaneous peptide delivery was associated with reduced activation of CD4$^+$ T-cells and increased the CD4$^+$CD25$^+$FoxP3$^+$ subset of T-cells in lymph nodes. Ablation of CD25$^+$ T-cells by CD25-depleting antibody abrogated the atheroprotective effects of subcutaneous infusion of apoB-100 peptides, similar to the study by Wigren and colleagues.[21] Taking these reports

together, immunization with p210 clearly elicited a $CD4^+$ T-cell response (be it induction of $CD4^+CD25^+$ or $CD4^+IL\text{-}10^+$ T-cells). How such $CD4^+$ T-cell response is elicited or whether these $CD4^+$ T-cells directly mediate the atheroprotective effect of p210 immunization remains unknown.

Regardless of which cellular immune responses were elicited or how and which form of p210 vaccine was delivered, the observed consistent reduction of atherosclerosis after p210 immunization strongly suggests that p210 is a promising candidate antigen for vaccine formulation optimization for potential future human testing.

Other apoB-100-related antigens

Many investigators have also tested additional apoB-100-derived peptides as potential antigens for vaccine formulation. Immunization with an apoB-100 peptide (amino acid residues 688–707) incorporated into a multiantigenic construct with peptidic epitopes from *Chlamydophila pneumoniae* and HSP60 reduced atherosclerosis accompanied by a reduction of macrophage infiltration and an increase of $CD4^+$FoxP3 T-cells in the plaques.[23]

Dr Klaus Ley's group surveyed the murine apoB-100 protein for peptide fragments that were predicted to bind to the mouse MHC-II molecule I-Ab by modeling algorithms. Overall, two peptide fragments, $ApoB_{3501–3516}$ and $ApoB_{978–993}$, were identified and were able to reduce atherosclerosis when used to immunize $apoE^{-/-}$ mice, possibly *via* an IL-10-dependent mechanism.[24]

Using T-cell hybridomas generated from human apoB-100 transgenic mice immunized with human oxidized LDL (oxLDL), Dr Goran Hansson's group was able to identify major histocompatibility class (MHC) class II-restricted, ApoB-100-responding $CD4^+$ T-cell hybridomas expressing a single T-cell receptor V beta chain, TRBV31. Immunizing with a TRBV31-derived peptide induced TRBV31 antibodies that blocked T-cell recognition of apoB-100 and significantly reduced atherosclerosis.[25] This innovative approach identified a potentially pathogenic $CD4^+$ T-cell population and used antigen-specific humoral immunity to block a proatherogenic cellular immune response, hence confirming the pathogenic role of $CD4^+$ T-cells in atherosclerosis.

Other lipid-related antigens

The complexity of atherosclerotic vascular disease presents the opportunity to target other potential sources of antigens. The search for suitable antigens for use in vaccines to modulate atherosclerosis has expanded to molecules other than LDL or apoB-100.

A natural IgM antibody recognizing the epitopes in oxLDL[26,27] and phosphorylcholine (PC) headgroups on the surface of apoptotic cells, and which inhibits uptake of oxLDL and apoptotic cells by macrophages, has been extensively studied.[27–29] Protection against infection from *Streptococcus pneumonia* is attributed to anti-PC antibodies.[30,31] Active immunization with *S. pneumoniae* in $LDL\text{-}R^{-/-}$ mice to induce anti-PC antibodies resulted in increased oxLDL antibodies, primarily of the IgM isotype, and reduced atherosclerosis.[32] The increase in oxLDL-specific IgM is attributed to the cross-reactivity of the phosphorylcholine moiety on oxLDL with *S. pneumonia*-induced antibodies, suggesting molecular mimicry between *S. pneumoniae* and oxLDL. This molecular mimicry was investigated further in the context of a vaccine using PC, the reported mimotope. Immunization of $apoE^{-/-}$ mice with PC-keyhole limpet hemocyanin (KLH)-conjugate coupled to unmethylated cytosine-guanine dinucleotides (CpG) oligonucleotides as adjuvant significantly increased IgG and IgM levels against PC and oxLDL, with reduced macrophage oxLDL uptake, and reduced atherosclerosis.[33] However, using myocardial infarction or stroke as endpoints, observational cohort studies in humans did not show consistent protective effects of pneumococcal vaccines,[34–37] leaving the question of whether active immunization against PC will reduce atherosclerosis in humans unanswered.

Cholesteryl ester transfer protein (CETP) is a key enzyme in the high-density lipoprotein (HDL) metabolic pathway. Immunization of rabbits against CETP-induced neutralizing antibodies and markedly increased High density lipoprotein-cholesterol (HDL-C) levels concomitant with reduced atherosclerosis.[38–40] Nasal immunization of rabbits with a vaccine targeting both CETP and heat shock protein-65 (HSP65) has also been shown to reduce aortic atherosclerosis.[41] However, a phase I human trial did not show consistent induction of CETP antibody nor significant changes in CETP function or HDL levels with CETP immunization.[42]

Rider and colleagues have eluted peptides from murine MHC-II molecules and found these

peptides are predominantly fragments of self-proteins. Among these eluted peptides is Ep1 (237–252), an apoE-derived peptide.[43] As a part of Ep1, Ep1.B (239–252) was able to reduce early atherosclerosis when administered intravenously.[44] The atheroprotective effect of Ep1.B is thought to be due to the induction of plasmacytoid dendritic cells to generate regulatory T-cells, hence the induction of peripheral tolerance in adaptive immune responses toward atherogenesis.[45]

Vaccines targeting heat shock protein

Heat shock proteins (HSPs) are stress proteins that are highly conserved in all organisms and can be expressed at high levels when cells are exposed to stresses, such as altered pH or oxygen deprivation. HSPs have also been implicated in atherogenesis.[46–49] However, the effect of immunization with HSPs on atherosclerosis has been inconsistent. Several groups reported that immunization with HSP65 induces atherosclerotic lesions,[50–52] whereas others reported reduced atherosclerotic lesions.[53–55] The difference in outcomes could be attributed to the difference in the adjuvant used or the mode of antigen delivery.

HSP-based vaccines delivered *via* mucosal approach can elicit a down-modulation of immune responses to specific antigens. Intranasal vaccinations using either plasmid DNA encoding HSP65 or whole protein HSP65, or both in phosphate buffered saline (PBS) in rabbits induced HSP65 IgG responses, increased serum IL-10, and reduced interferon (IFN)-γ, and reduced atherosclerosis accompanied by decreased cholesterol levels.[55] Sublingual immunization of hypercholesterolemic mice with a recombinant HSP60 from *Porphyromonas gingivalis* prevented *P. gingivalis*-accelerated atherosclerosis by inducing an increase of IFN-γ^+ or IL-10^+Foxp3^+ cells in draining lymph nodes and reduction of serum levels of C-reactive protein (CRP), monocyte chemotactic protein 1 (MCP-1) and oxLDL.[56] Another group of investigators reported nasal immunization with HSP60 attenuated atherosclerosis in aortic root with the induction of $CD4^+GARP^+$ T-regulatory cells (Tregs), Type 1 regulatory T cell (Tr1) cells and $CD4^+CD25^+FoxP3^+$ Tregs.[57]

Vaccines against host cell surface, extracellular matrix proteins or plasma proteins

The involvement of certain inflammatory cells in atherosclerotic plaque formation suggested that specific cell surface markers could be potential antigens for immunization. Oral DNA vaccines targeting cell surface proteins thought to contribute to atherosclerosis have been tested experimentally with success. By delivering the antigen *via* an expression plasmid that encodes the antigen, this strategy transfers the genetic material from the carrier to host phagocytes in the gastrointestinal tract. The phagocytes then express the antigen *de novo* in the cytosol, and present it on MHC molecules.[58] In the reported studies using this approach, constructs were designed to encode CD99[59] or vascular endothelial growth factor receptor 2 (VEGFR2)[60] carried by live attenuated *S. typhimurium* and delivered orally. The expressed antigens were presented by MHC-I, which elicited a $CD8^+$ cytolytic T-cell response targeting cells that expressed VEGFR2 or CD99, resulting in reduced atherosclerosis.

LDL retention in the arterial wall by extracellular matrix (ECM) is an early step in the development of atherosclerotic lesions. Fibronectin is an ECM protein found in plaques. Immunization with fibronectin formulated with alum as the adjuvant significantly reduced atherosclerosis in $apoE^{-/-}$ mice, and was associated with increased Th2-type antibody production and increased regulatory T-cells.[61] Interestingly, plasma cholesterol was significantly reduced in the immunized mice, suggesting an interaction between immune responses to ECM proteins and cholesterol metabolism.

β2-glycoprotein I (β2-GPI) is a glycosylated plasma protein that has been implicated to play a role in atherogenesis. Immunization with β2-GPI attenuated the development of early atherosclerosis, presumably by inducing tolerance against β2-GPI.[62] Immunization with peptides from the N-terminus of the C5a receptor also reduced early atherosclerotic lesion formation possibly *via* induction of regulatory T-cell response.[63] Pro-inflammatory cytokine IL-1α is another potential target for vaccine development. Immunization with full-length, native IL-1α chemically conjugated to virus-like particles reduced inflammatory response in the plaques and atherosclerosis progression in aorta and aortic root.[64]

PCSK9 vaccines

Proprotein convertase subtilisin/kexin type 9 (PCSK9) secreted by the liver negatively regulates the LDL-receptor (LDL-R) by binding to LDL-R and blocking the recycling of the receptor to the

Table 1. Summary of published reports utilizing LDL, modified LDL or apoB-100 related peptides as antigens for immunization.

LDL or its modified form					
Animal	**Antigens**	**Adjuvant**	**Immunization route**	**Effect on atherosclerosis**	**Reference**
LDL-R$^{-/-}$ rabbits	MDA-LDL	Freund's complete followed by incomplete adjuvant	Subcutaneous followed by intramuscular	Reduced (aorta)	Palinski *et al.*[10]
NZW rabbits on high cholesterol diet	Native LDL or Cuox-LDL	AdjuPrime (carbohydrate polymer)	Subcutaneous	Reduced (aorta)	Ameli *et al.*[6]
LDL-R$^{-/-}$ mice	Native LDL or MDA-LDL	Freund's complete followed by incomplete adjuvant	Subcutaneous followed by intraperitoneal	Reduced (aortic sinus)	Freigang *et al.*[8]
ApoE$^{-/-}$ mice	MDA-LDL	Freund's complete followed by incomplete adjuvant	Subcutaneous	Reduced (aortic sinus)	George *et al.*[9]
ApoE$^{-/-}$ mice	Plaque homogenate or MDA-LDL	Freund's complete followed by incomplete adjuvant	Foot pad injection	Reduced (aortic sinus)	Zhou *et al.*[12]
ApoE$^{-/-}$ mice	Native LDL	IL-12	Subcutaneous	Reduced (aortic sinus)	Chyu *et al.*[7]
ApoE$^{-/-}$ or apoE/ CD4 double knockout mice	MDA-LDL	Freund's complete followed by incomplete adjuvant	Subcutaneous	Reduced (aortic sinus)	Zhou *et al.*[13]
LDL-R$^{-/-}$ mice	Cuox-LDL	Dendritic cells	Intravenous delivery of oxLDL pulsed dendritic cells	Reduced (accelerated carotid atherosclerosis induced by pericarotid collar)	Habets, 2010[70]
ApoE$^{-/-}$ mice	Cuox-LDL	None	Nasal delivery	Reduced (aortic sinus and aorta)	Zhang *et al.*[52]
LDL-R$^{-/-}$ and apoE$^{-/-}$ mice	Cuox-LDL or AGE-LDL	Alum (Pierce)	Subcutaneous	Reduced (aortic sinus and aorta)	Zhu *et al.*[14]
apoB-100 peptide					
Animal	**Antigens**	**Adjuvant**	**Immunization route**	**Effect on atherosclerosis**	**Reference**
ApoE$^{-/-}$ mice	Mixture of p143 and p210	Alum (Pierce)	NA	Reduced (descending aorta)	Fredrikson, 2003b
ApoE$^{-/-}$ mice	MDA-modified p45 or p74	Alum (Pierce)	NA	Reduced (descending aorta)	Fredrikson, 2005[71]
ApoE$^{-/-}$ mice	p2	Alum (Pierce)	Subcutaneous followed by intraperitoneal	Reduced (aorta)	Chyu *et al.*[16]
LDL-R$^{(-/-)}$/ human apoB-100 transgenic mice	p210	Alum (Pierce)	NA	Reduced (descending aorta)	Fredrikson *et al.*[19]
ApoE$^{-/-}$ mice	p210	CTB (p210-CTB fusion protein)	Intranasal	Reduced (aortic sinus)	Klingenberg *et al.*[20]
ApoE$^{-/-}$ mice	p210	Alum (Pierce)	Subcutaneous	Reduced (descending aorta)	Wigren *et al.*[21]
ApoE$^{-/-}$ mice	Mixture of p210, MDA-p210 and p240 or p210 only	No adjuvant	Continuous subcutaneously delivery	Reduced (aortic sinus)	Herbin *et al.*[22]
ApoE$^{-/-}$ mice	p210	Alum (Pierce)	Subcutaneous	Reduced (aorta)	Chyu *et al.*[18]
ApoE$^{-/-}$ mice	$ApoB_{3501–3516}$ or $ApoB_{978–993}$	Freund's complete followed by incomplete adjuvant	Subcutaneous followed by intraperitoneal	Reduced (aortic sinus and aorta)	Tse *et al.*[24]

CTB, cholera toxin B; Cuox, copper oxidized; IL, interleukin; LDL, low-density lipoprotein; LDL-R, LDL receptor; MDA, malondialdehyde; NA, not applicable; NZW, New Zealand White; oxLDL, oxidized LDL; SQ, subcutaneously.

cell surface, thus decreasing the uptake of LDL particles and increasing the circulating level of LDL-C. The loss of function of PCSK9 mutation in humans results in life-long hypocholesterolemia and lower incidence of cardiovascular events.[65] Antibodies against PCSK9 have been developed and successfully reduced LDL-C levels in conjunction with use of statin in clinical trials.[66,67] Evolocumab (Amgen, USA), alirocumab (Sanofi/Regeneron, France/USA) and bococizumab (Pfizer/Rinat, USA) are now undergoing phase III clinical trial testing to determine their clinical efficacy to reduce cardiovascular events. However, the biggest disadvantage of using a PCSK9 antibody as a treatment strategy is the need for repeated injections and its associated high cost, potentially limiting the wide clinical use of the PCSK9 antibody. Thus investigators have been testing the notion of active immunization against PCSK9 to achieve similar but long-term biological effects to the use of PCSK9 antibody. In preclinical experiments, PCSK9 peptide-based vaccines have been shown to elicit a PCSK9-specific antibody with a significant reduction of LDL-C.[68,69] If these vaccines can be further demonstrated to be well tolerated and effective for clinical application, active immunization against PCSK9 will be an attractive alternative to the PCSK9 antibody.

Implications and clinical perspectives

The idea of developing vaccination strategies to modulate the highly prevalent atherosclerotic cardiovascular disease is exciting and daunting but still in its infancy. In this review, we discussed the experimental evidence and efficacy of reducing atherosclerosis by vaccination using many different antigens tested in preclinical models. Before translating the aforementioned promising preclinical observations into the clinical arena, we need to answer many challenging questions while designing proper clinical studies. We believe there is currently no lack of suitable antigens to be tested in vaccine formulation for clinical testing. Numerous clinical trials have established the causative role of LDL in atherogenesis, which makes LDL and its apoB-100 reasonable and logical initial targets for vaccination. This is also supported by preclinical studies using LDL or apoB-100 peptides as candidate antigens in vaccine formulation (Table 1). The challenges reside in the following areas: choice of formulation and route of delivery, vaccine safety and stability, schedule and durability of immunization, proper selection of patient populations for testing, and determination and monitoring of efficacy endpoints in clinical studies. Potential side effects of immunization such as undesirable immune activation, whether they are specifically related to atherogenesis or not, are the additional challenges that need to be addressed in early safety trials. This is going to be a costly and long journey. We have the vision of moving this idea of vaccination to reduce atherosclerosis into clinical testing, but this goal cannot be achieved without academic investigators, professional societies, government agencies, funding organizations, and the pharmaceutical industries working together to initiate this long journey. With all of these challenges in mind, we are cautiously optimistic about the potential for future clinical application.

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Conflict of interest statement

Dr Chyu and Dr Shah are co-inventors of the apoB-100-based peptide vaccine. Patent rights are assigned to Cedars-Sinai Medical Center, CA, USA.

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