

Vaccination against atherosclerosis? Induction of atheroprotective immunity

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Abstract Atherosclerosis involves the formation of inflammatory arterial lesions and is one of the most common causes of death globally. It has been evident for more than 20 years that adaptive immunity regulates the magnitude of the atherogenic proinflammatory response. T cells may also influence the stability of the atherosclerotic lesion and thus the propensity for thrombus formation and the clinical outcome of disease. Immunization of hypercholesterolemic animals with low-density lipoprotein preparations reduces atherosclerosis, suggesting that vaccination may represent a useful strategy for disease prevention or modulation. This review summarizes our current understanding of the role immunity in atherosclerosis and outlines strategies for antigen-specific prevention of this disease.

Introduction

The important role of inflammation in atherosclerosis is now established from a series of histopathological, clinical, and epidemiological studies [1]. Its mechanisms have been dissected in experimental models, including gene-targeted mice. We now know that low-density lipoprotein (LDL) accumulation in the artery wall triggers innate as well as adaptive immune responses that cause inflammation. The

smouldering inflammatory process in the arterial intima, in combination with continued LDL infiltration and cholesterol accumulation, gives rise to the characteristic pathology of the atherosclerotic lesion.

After years or decades, the silent lesion is converted to a clinically manifest one when a thrombus is formed on the plaque surface and causes ischemia in the downstream tissue, either locally or after embolization. Inflammatory cells and mediators have been identified as triggers of pathology also in this situation: An activation of the smouldering inflammatory process in the lesion is thought to elicit destabilization of the lesion, plaque rupture, and thrombosis. However, the molecular mechanisms involved in this phase of the course of atherosclerosis are less well known than those contributing to the initiation and early growth of lesions. This is largely due to lack of suitable experimental models. Fortunately, efforts in many laboratories are aimed at developing models of plaque activation and atherothrombosis.

The understanding that immune mechanisms play a decisive role in atherosclerosis has focused our attention on the immune system as a possible novel target in prevention and treatment of cardiovascular disease [2, 3]. In this chapter, we will discuss some possible targets for atheroprotective immunization and their development toward clinical use. First, we will outline briefly the types of immune activity that are associated with atherosclerosis.

Innate immunity

Innate immune activity is found in atherosclerosis from the initiation of disease and throughout the evolution of the atherosclerotic plaque [4]. The vascular endothelium expresses signaling as well as endocytosing pattern recog-

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niton receptors, and infiltrating macrophages contribute profoundly to innate immune activity in the artery wall.

In the lesion, differentiated macrophages express a large group of pattern recognition receptors involved in recognition and phagocytic clearance, also called scavenger receptors. Among them, SR-A and CD36 have been regarded as the most important scavenger receptors in the uptake of oxidized low-density lipoprotein by macrophages [5]. In addition, a broad repertoire of Toll-like receptors (TLRs) is expressed by endothelial cells and macrophages of the lesion [6]. Studies of gene-targeted mice point to pathogenetic roles for several types of TLRs, both on endothelial cells and macrophages [7–9]. However, human data are less clear, with conflicting results on the impact on myocardial infarction from different studies of genetic variants in TLR-4 [10–12].

Mast cells are present in lesions, although they are less frequent than macrophages. Similar to macrophages and T cells, they accumulate at sites of plaque rupture, suggesting that they may play an important role for plaque rupture and precipitation of acute coronary syndromes [13]. Experiments using mast cell-deficient mice suggest that these cells are important contributors also to earlier stages of atherosclerosis [14].

Adaptive immunity

T cells are present throughout the life of a plaque [3, 15]. The cells are localized to predilection sites for atherosclerosis, i.e., areas subjected to hemodynamic stress, even before any lipid deposition is detected. The infiltration, retention, and oxidation of low-density lipoprotein (LDL) initiate the formation of fatty streaks, consisting mostly of T cells, macrophages, and foam cells, which have developed from scavenger receptor-mediated uptake of oxidized LDL in macrophages [16]. When complex lesions start to develop with infiltrating smooth muscle cells forming the fibrous cap of a fibrofatty lesion, foam cells are primarily detected in the lipid core, whereas T cells are found in clusters within the fibrous cap and in the shoulder regions of the lesions [16]. Both CD4⁺ and CD8⁺ T cells are found in human lesions but CD4⁺ cells generally dominate. Most cells are TCR α/β , although TCR γ/δ -positive T cells are also present.

In the vicinity of the T cells, MHC class II expressing macrophages and dendritic cells can be detected, indicating communication between T cells and antigen-presenting cells [16, 17]. The density of T cells increases with the severity of disease.

Both LDLR^{-/-} and apoE^{-/-} mice develop lesions in the aortic root and throughout the arterial tree. In lesions from both mouse strains, the dominant T cell subset is CD4⁺, although CD8⁺ T cells are also present [18]. The density of

T cells appears to decrease with maturation of the lesion, possibly reflecting a transition from inflammatory fatty streaks and early atheroma to stable fibrofatty plaques. In both strains, the T cells form clusters or are spread throughout fatty streak lesions, but in more advanced lesions, T cells localize predominantly in the fibrous cap, in shoulder regions, or subendothelially.

Most T cells found in human atherosclerotic plaques are effector/memory T cells [3, 15]. The cells display several activation markers, and the proportion of activated T cells increases with severity of coronary syndrome. TCR $\alpha\beta$ ⁺ T cells dominate over TCR $\gamma\delta$ ⁺ cells in atherosclerotic lesions and may therefore play a more significant role in lesion development. Indeed, TCR β -deficient apoE^{-/-} mice displayed reduced atherosclerosis, whereas mice deficient in TCR $\gamma\delta$ ⁺ cells were only marginally affected [19].

A global deficiency of adaptive immunity leads to reduced atherosclerosis in apoE^{-/-} and LDLR^{-/-} mice, although the effect of the immune deficiency is less pronounced at extreme cholesterol levels [20–22]. Reconstitution of immune deficient *scid/scid* mice with CD4⁺ T cells accelerates disease, pointing to an important proatherogenic role of this T cell subset [21, 23].

B cells and dendritic cells—other contributors to adaptive immunity

The dendritic cell (DC), a specialized cell type in the myeloid lineage, is a professional antigen-presenting cell with a unique capacity to activate naive T cells. DC patrol tissues in the search for antigen have an exceptional endocytotic capacity and target internalized antigens for binding to MHC class II molecules through the endosomal pathway. When they mature, upon stimulation of pattern recognition receptors and cytokine receptors, they “freeze” MHC–antigen complexes at high density on their cell surfaces. Such structures provide strong stimulation for antigen-specific T cells. Together with expression of costimulatory factors, this high-density stimulation of clonotypic T cell receptors is sufficient to activate the naive T cell. After a few rounds of division, it differentiates into an effector/memory phenotype that has a lower threshold for reactivation.

DC are found in atherosclerotic plaques [17] and probably travel through vascular tissues in search of antigen [24]. However, antigen presentation to naive T cells likely occurs in regional lymph nodes. The effector/memory T cells generated through this process are prone to enter plaques since they express adhesion molecules that can tether counter receptors on the activated endothelium and respond to chemokines produced in the plaque [15]. Since oligoclonal T cell clusters are observed in plaques, it is likely that reactivation of effector T cells can occur here [25]. Indeed, T

cell clones recognizing epitopes on LDL have been isolated from human atherosclerotic plaques [26].

B cells representing the humoral arm of adaptive immunity are sparse in plaques [16]. In spite of this, atherosclerosis is associated with production of a set of autoantibodies, including IgG antibodies, to oxidatively modified LDL particles (e.g. malondialdehyde-lysine epitopes) and to heat shock protein-60, as well as IgM antibodies to phosphocholine and other lipid components of oxidized LDL [27–29]. While B cells producing the latter type of antibodies do not require T cell help, hence the term “natural antibodies”, those making IgG antibodies depend on stimulation by activated T cells [30]. Therefore, T cells probably travel from plaques to regional lymph nodes, where they provide help for B cells producing disease-associated antibodies.

In advanced atherosclerosis, the artery is surrounded by a highly cellular, sometimes fibrotic periadventitial infiltrate, sometimes termed retroperitoneal fibrosis. This tissue contains abundant B cells, including germinal centers with centroblasts and other stages of B-cell dedifferentiation and plasma cell formation [31]. T cells and follicular dendritic cells are also found here, and the entire scenario needed for B-cell activation, plasma cell development, and IgG antibody production is therefore in place. Recent studies show that macromolecules may penetrate from lesions, through conduit structures in the media, to these germinal centers [32]. It is likely that a significant proportion of disease-associated autoantibodies are formed in these adventitial tertiary lymphoid organs, at least in advanced cases of atherosclerosis.

Manipulating the immune system to treat disease

There are two major ways of manipulating the immune system: immunosuppressive drugs and immunization. Immunization can either be active, in which an immune response is induced through exposure to an antigen, or passive, in which preformed antibodies are administered directly.

The immunosuppressive drugs available today include (a) anti-inflammatory corticosteroids, (b) cytotoxic drugs such as azathioprine and cyclophosphamide, and (c) fungal and bacterial derivatives inhibiting T-cell activation such as cyclosporine A and rapamycin. Immunosuppressive drugs are used to prevent acute rejection after organ transplantation and to treat certain autoimmune diseases. Because aggressive arteriosclerosis (transplant vascular sclerosis [TVS]) in arteries of the transplanted organ is the major cause of late rejection, it would be beneficial if immunosuppressive drugs also inhibited TVS. Although several animal studies have suggested that cyclosporine A and rapamycin may have an inhibitory effect, data suggest that the cytotoxicity of immunosuppressive drugs causes vascular injury, thus

contributing to TVS progression. Most immunosuppressive drugs also have adverse effects on cardiovascular risk factors such as dyslipidemia, hypertension, and diabetes.

The immunosuppressive compounds cyclosporin A and rapamycin not only act on T cells but can also inhibit smooth muscle proliferation and the response to vascular injury [33, 34]. Coating of stents with rapamycin may therefore help prevent restenosis. Corticosteroids have also been shown to reduce atherosclerosis in experimental animals. However, cytotoxicity and other adverse side effects associated with the immunosuppressive drugs available at present make them less suitable for wider use in prevention and treatment of nontransplant atherosclerosis.

Immunization strategies

Passive immunization denotes transfer of preformed antibodies and includes treatment of certain infections with hyperimmune sera. Recombinant antibodies could also be considered examples of passive immunization. The remarkable effects of anti-tumor necrosis factor antibodies in rheumatoid arthritis and of antibodies to platelet glycoprotein IIb/IIIa in unstable angina and restenosis prevention may be viewed as examples of how passive immunization can be used to treat noninfectious disease in humans.

Active immunization uses antigen preparations, vaccines, to induce a protective immune response. Vaccines are likely to be the most important medical contribution to public health during the last 100 years. They have dramatically reduced death from infectious disease and resulted in the global eradication of smallpox. Modern vaccines are cheap, highly specific, and have generally few adverse side effects. In recent years, attempts have been made to fight noninfectious chronic diseases by using immunization approaches. The recent introduction of a vaccine against carcinogenic strains of human papilloma virus is likely to substantially reduce the incidence of cervical cancer. Different types of cancer vaccines based on tumor cells taken from patients and made immunogenic by addition of adjuvant or by transfection of genes encoding costimulatory molecules are also being clinically tested, as are vaccines against Alzheimer's disease and diabetes.

When considering the possibility of developing vaccines against atherosclerosis, it will be important to learn from the experience of developing immunotherapeutic approaches to other chronic inflammatory diseases such as rheumatoid arthritis, type I diabetes, and Alzheimer's disease.

Developing a vaccine against atherosclerosis

As the importance of immunity in atherosclerosis has been revealed, it has become interesting to clarify if this

knowledge can be used to develop new treatments for cardiovascular disease. The first evidence that this could be possible came from studies in which hypercholesterolemic rabbits were immunized with oxidized LDL [35, 36]. The initial aim of these studies was to test if activation of immunity to oxidized LDL was associated with a more aggressive progression of disease but it was found, surprisingly, that oxidized LDL-immunized animals developed a partial protection against atherosclerosis.

This observation was subsequently confirmed in a number of different animal models of atherosclerosis and suggested the fascinating possibility that a vaccine could be developed for atherosclerosis. However, since oxidized LDL is a complex particle with an antigen composition that is difficult to standardize and since it potentially also may contain harmful antigens, it is in itself not an ideal vaccine component. Over the last few years, considerable efforts have therefore been made to characterize the precise antigens and antigenic epitopes in oxidized LDL that induce atheroprotective immunity.

The first antigens in oxidized LDL to be identified were oxidized phospholipids. Palinski et al. established a panel of B cell hybridomas from apoE^{-/-} mice and found that several clones produced antibodies specifically binding to modified phospholipids in oxidized LDL [37]. Oxidized phospholipid antigens are not only present exclusively in oxidized LDL but are also found on apoptotic cells and on some microorganisms such as *Streptococcus pneumoniae* [38]. These antigens are recognized by a subclass of IgM referred to as natural antibodies. They are usually defined as antibodies produced in complete absence of exogenous antigenic stimulation and are produced primarily by B-1 cells in the peritoneal cavity and spleen [39]. Natural antibodies provide a first line of defense against invading micro-organisms, but react also with self-antigens associated with senescent cells and cellular debris.

Binder and coworkers established a pathobiological role of anti-phospholipid antibodies in atherosclerosis by immunizing LDL receptor knockout mice with *S. pneumoniae* [38]. This treatment resulted in the induction of high levels of oxidized LDL-specific IgM and a modest reduction of atherosclerosis. Fario-Neto et al. subsequently showed that treatment with anti-phosphorylcholine IgM isolated from a T15 idiotype hybridoma reduced vein graft atherosclerosis in apoE^{-/-} mice [40]. Finally, Caligiuri et al. [41] found that immunization with phosphorylcholine coupled to a carrier protein was associated both with a three-fold increase in specific antibodies and a 40% reduction of atherosclerosis at the aortic root in the same type of mice. Taken together, these observations suggest that stimulation of the immune response against oxidized phospholipids represents one possible approach for development of an immunomodulatory therapy for atherosclerosis.

The major challenges associated with this approach are as follows: (1) We need better understanding of how the expression of natural antibodies is regulated; (2) the effect of cross-reactivity with antigens expressed on other structures than oxidized LDL must be clarified; (3) the relation to the anti-phospholipid antibodies associated with thrombotic disease must be determined.

The other major class of antigens in oxidized LDL is the peptide fragments generated as a result of proteolytic degradation of apoB-100, the only protein permanently associated with LDL. These antigens have the advantage of being specific for oxidized LDL because they have a unique amino acid sequence. Fredrikson et al. [28, 42] used ELISAs based on a library of 20-amino acid-long polypeptides covering the complete apoB-100 sequence to identify a number of apoB-associated antigens recognized by autoantibodies present in human plasma. While some of these antibodies recognize aldehyde-modified peptides, others bind to native apoB peptides. Interestingly, titers of IgG antibodies to a native apoB peptide are inversely correlated to myocardial infarction [43].

T cell epitopes are less well characterized than B cell epitopes. Studies of T cell specificity have used human, plaque-derived T cell clones as well as mouse hybridomas obtained after immunization [26]. The emerging picture is one of MHC class II restricted T cell epitopes detected by CD4⁺ T cells through their TCR $\alpha\beta$ receptors [15]. The antigens presented by MHC class II molecules are generally 13–17 amino acids long, which make the chance for cross-reactivity with other peptide sequences minimal. Several different T-cell epitopes are present within the large apoB-100 protein but their fine specificity remains a topic of investigation.

Immunizations of apoE^{-/-} mice with certain apoB-100 peptides reduces atherosclerosis by up to 70% [44]. It decreases macrophages and expression of genes induced by proinflammatory cytokines while increasing the collagen content of remaining plaques [[44] and data not shown]. Therefore, immunization likely reduces plaque inflammation. Immunizations resulted in a marked increase in specific IgG, but had only marginal effects on IgM levels. IgG expression also changed from IgG2a to IgG1, suggesting activation of a Th2 response.

The high specificity as well the possibility to produce standardized vaccine preparations are some advantages with apoB peptide-based vaccines, and human vaccines are presently in preclinical development. The disadvantages with this approach include that it may turn out to be necessary to perform HLA genotyping of patients before treatment since vaccines may need to be individualized depending on HLA type.

A limitation with both phosphorylcholine- and apoB peptide-based vaccines is that the mechanism of action is

poorly understood. In mice immunized with oxidized LDL, a more effective inhibition of atherosclerosis is seen in the mice with the highest antibody response, suggesting that the protection may be mediated by antibodies [30]. Several other observations also favor this possibility. Treatment with polyclonal IgG significantly reduces the development of atherosclerosis in apoE^{-/-} mice [45], while splenectomy aggravates disease [46]. Importantly, B-cell reconstitution rescues apoE^{-/-} mice from the enhanced development of atherosclerosis caused by splenectomy [46].

Schiopu et al. [47] produced human recombinant IgG against an aldehyde-modified form of the apoB p45 peptide and demonstrated that four treatments with this antibody reduced atherosclerosis by almost 50% over a 5-week period. An even more dramatic effect of p45 IgG treatment was observed in mice carrying the human gene for apoB-100 [48]. Although these studies collectively provide strong support for the notion that the protective effect of immunization depends on generation of specific antibodies other mechanisms may also be involved.

If autoimmune Th1 responses against oxidized LDL and other plaque-specific antigens are involved in the disease process, they should be susceptible to downregulation by antigen-specific Tregs. A frequently used approach to induce tolerogenic responses is by mucosal (oral or intra-nasal) immunization. Oral tolerance is an important physiological mechanism to avoid development of delayed-type hypersensitivity and other allergic reactions to food proteins. Van Puijvelde et al. recently reported that oral administration of oxidized LDL is associated with suppression of atherosclerosis and that this is associated with an induction of Tregs in peripheral lymphoid tissues [49].

Inhibition of atherosclerosis has also been observed following mucosal immunization with HSP 60/65 and β 2-glycoprotein I [50, 51], further supporting the notion that induction of tolerance against atherosclerosis-associated antigens by mucosal immunization represents one promising approach for future atherosclerosis vaccine development. However, experience from other autoimmune disease points to difficulties in developing oral tolerance-based immunotherapy for humans. Future studies will tell whether this approach will be successful for atherosclerosis.

The secret to success may be in the packaging of the antigen

The outcome of an immunization is determined not only by the antigen but also on the adjuvant in which it is administered and the route used for administration. Although immunologists have focused their efforts almost exclusively on identifying and designing antigens, it has long been known that adjuvants and route of administration play a decisive role for the response.

Immunologic adjuvants are agents that act to enhance, accelerate, modify, or prolong specific immune responses to vaccine antigens. They function through three basic mechanisms: (1) effects on antigen delivery and presentation, (2) induction of immunomodulatory cytokines, and (3) effects on antigen-presenting cells. The original adjuvants were based on aluminum salt-containing gels (alum) primarily functioning by complexing and retaining the antigens at the site of injection. These still remain the only adjuvants in US-licensed vaccine formulation. Subsequent development in adjuvant technology has shown that adjuvants work more effectively if they also interact directly with immune cells.

Most of these adjuvants are mixtures of oils, salts, killed bacteria, etc., that are empirically known to promote adaptive immune responses. For instance, parenteral administration using Freund's complete adjuvant usually induces strong Th1/delayed-type hypersensitivity (DTH) and antibody responses. This adjuvant contains mineral oil and heat-killed mycobacteria (with substantial amounts of heat shock proteins [Hsps]). Incomplete Freund's adjuvant, which lacks mycobacteria, induces antibody production but does not promote DTH reactions to the same extent as complete Freund's adjuvant.

Dendritic cells can be activated by coadministration of DNA sequences containing unmethylated cytosine guanine dinucleotides (CpG) motifs, which ligate TLRs; this increases antigen presentation, leading to enhanced immune responses to the antigen [52]. Several new types of adjuvants have been tested in preclinical and clinical trials, including liposomes, immunostimulatory complexes, and biodegradable polymer microspheres. Some of these may become relevant for testing in immunomodulation of atherosclerosis because they mimic the pattern through which, for example, oxidized LDL antigens are presented. Other adjuvant approaches introduced more recently include coadministration of cytokines such as interleukin-12 and interferon-gamma and genetic synthesis of fusion proteins containing peptide sequences of antigen and costimulatory molecules.

What adjuvant and route of administration should be used when immunizing against atherosclerosis? Identifying the key antigens responsible for activation of immune responses involved in atherosclerosis is a prerequisite for development of an immunization therapy. However, finding the most suitable adjuvant and route of administration represents an equally important challenge. Understanding the mechanisms through which each antigen contributes to the disease is also necessary for making the best combination of antigen and vehicle of administration.

There is accumulating evidence that activation of immunity in atherosclerosis primarily involves proinflammatory Th1 cells and that these promote disease development [53–55]. Accordingly, adjuvants that favor a shift

toward an anti-inflammatory Th2 response, such as alum and Freund's incomplete adjuvant, may be more effective than adjuvants favoring Th1 responses. Alternatively, it may be possible to inhibit activation of Th1-mediated immune responses through induction of tolerance by mucosal administration.

Conclusion

It is now evident that atherosclerosis is an inflammatory disease and involves autoimmune reactions against LDL lipoprotein particles accumulating in the artery wall. Some of the lipid and peptide epitopes of these antigens have been identified. Immunization of atherosclerosis-prone mice with LDL results in very substantial beneficial effects against atherosclerosis, with reduced lesion formation in the arteries. Recent data support a role for antibodies but also for cellular immunity in this atheroprotective immune response. Ongoing studies should identify major mechanisms and hopefully pave the way for clinical studies in this exciting area.

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