LDL Immunization Induces T-Cell–Dependent Antibody Formation and Protection Against Atherosclerosis

Xinghua Zhou, Giuseppina Caligiuri, Anders Hamsten, Ann Kari Lefvert, Göran K. Hansson

Abstract—Atherosclerosis is an inflammatory disease, and the involvement of immune mechanisms in disease progression is increasingly recognized. Immunization with oxidized low density lipoprotein (LDL) decreases atherosclerosis in several animal models. To explore humoral and cellular immune reactions involved in this protection, we immunized apolipoprotein E knockout mice with either homologous plaque homogenates or homologous malondialdehyde (MDA)-LDL. Immunization with both these antigen preparations reduced lesion development. The plaques contained immunogen(s) sharing epitopes on MDA-LDL, MDA–very low density lipoprotein, and oxidized cardiolipin. This shows that a T-cell–dependent antibody response was associated with protection against atherosclerosis. The protection was associated with specific T-cell–dependent elevation of IgG antibodies against MDA-LDL and oxidized phospholipids, and the increased titers of IgG antibodies were correlated with decreased lesion formation and lower serum cholesterol levels. (Arterioscler Thromb Vasc Biol. 2001;21:108-114.)

Key Words: atherosclerosis ■ immunization ■ low density lipoproteins ■ antibodies ■ T cells

Histopathologic analysis of atherosclerotic plaques suggests that lesion formation represents an inflammatory-proliferative response to lipid metabolic disturbances in regions of the vasculature exposed to hemodynamic strain.1,2 The involvement of immune mechanisms in this process is increasingly recognized.1,3–7 Thus, atherosclerotic plaques contain significant amounts of T cells, many of which are in an activated state8–14; major histocompatibility complex class II molecules are expressed on endothelial and smooth muscle cells in the vicinity of activated T cells15; and B cells, immunoglobulins, and C5b-9 complement complexes are also present in the plaques.16–19 All these findings support the notion that atherosclerosis may be an immune-modulated disease.

Lipoprotein oxidation seems to play a critical role in the development of atherosclerosis.2,20 Highly reactive products from lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxynonenal, bind to free amino groups of lysines and other charged amino acid side chains of apoB.2 The phospholipids of LDL can also be oxidized,21 and cardiolipin is recognized by some autoantibodies reactive against oxidized LDL (oxLDL). Circulating autoantibodies to epitopes of oxLDL have been detected in the plasma of patients and experimental animals with atherosclerosis.13,22–29 It has recently been shown that the titer of autoantibodies to oxLDL is correlated with the extent of atherosclerotic lesions in LDL receptor–deficient mice.30 OxLDL present in atherosclerotic lesions31,32,34,35 is highly immunogenic11,23,34 and can stimulate the recruitment of immune cells.35–37 Antibodies isolated from atherosclerotic lesions recognize epitopes of oxLDL, and some of them form immune complexes with oxLDL.38 Approximately 10% of the T cells cloned from human atherosclerotic lesions respond specifically to oxLDL.11 Taken together, these findings suggest that humoral and cellular immune responses to oxLDL affect the atherosclerotic process. In addition, heat shock protein 65, Chlamydia pneumoniae, herpes simplex type I, and cytomegalovirus have also been suggested as possible immunogens in the plaque.6,39,40 Therefore, it seems that the atherosclerotic plaque might contain various antigens that are targeted by the immune system.

Recently, several groups have studied the effect of immunization on atherosclerosis. Palinski et al41 and Ameli et al42 showed that immunization of hypercholesterolemic rabbits with MDA-LDL41 or Cu²⁺-oxidized LDL42 reduced lesion formation. Similarly, immunization of LDL receptor knockout mice fed a Western diet43 and apoE-deficient (E₀) mice fed normal chow inhibited the disease process.44 However, the mechanism of the protective effect is currently unknown.

To explore humoral and cellular immune reactions involved in this protection, we immunized E₀ mice with either homologous plaque homogenates or homologous MDA-LDL. Immunization with either preparation reduced lesion development in proportion to rises in the titers of T-cell–dependent antibodies to oxLDL and oxidized phospholipids. This suggests an important role for antibodies and T-cell–B-cell interactions in the protection conveyed by immunization.
Methods

Mice

Male E0 mice were backcrossed 10 times onto the C57BL/6J background (strain C57BL/6J-ApoE(-/-)); these mice were obtained from M&B Breeding and Research Center, Ry, Denmark. Eight-week-old mice were fed a Western diet containing 0.15% cholesterol.12,13 Animal care was in accordance with national guidelines, and all experiments were approved by the local ethics committee.

Antigen Preparation

Homologous Lipoprotein Isolation

Blood was obtained by heart puncture from 6- to 8-week-old anesthetized male E0 mice and pooled into vacuum tubes containing Na2-EDTA. VLDL and LDL were isolated from plasma by ultracentrifugation through a discontinuous NaCl gradient of 1.006 to 1.065 mg/mL for 20 hours at 4°C in a Beckman L8-80 ultracentrifuge with a 50.3-Ti Beckman fixed-angle rotor. The lipoprotein preparation with added Na2-EDTA (1 mg/mL) was sterilely filtered, kept at 4°C under N2, and used within 2 weeks. The modification of MDA-LDL and MDA-VLDL was performed as described.13 Human LDL was prepared from venous blood obtained from healthy donors after an overnight fast, pooled into vacuum tubes containing Na2-EDTA (1 mg/mL), and treated in the same way as mouse LDL.

Plaque Homogenate

The heart and proximal aorta of aged male E0 mice were briefly perfused with PBS, dissected out, and put in ice-cold preservatives, which included 1 mg/mL Na2-EDTA, 2 mmol/L benzamidine,1 mol/L phenylmethylsulfonyl fluoride, 0.01% aprotinin, and 0.008% gentamycin in PBS. The atherosclerotic plaques from the root of the aorta were isolated under a dissection microscope within 1 hour in ice-cold preservatives without EDTA and benzamidine and were frozen immediately in liquid nitrogen. Plaques were then homogenized in a Dismembrator (B. Braun Melsungen AG) and suspended in PBS. The protein content was determined by the Lowry method.

Immunization Protocol

At 6 weeks of age, male E0 mice were randomly divided into 3 groups (n = 5 or 6 per group). They were injected with homologous plaque homogenate (100 µg protein per mouse), homologous MDA-LDL (100 µg protein per mouse), or PBS in the foot pads, which was boosted 4 times at 2-week intervals (ratio of antigen [or PBS] to adjuvant 3:2). The antigens used in the first injection were emulsified with complete Freund’s adjuvant. Incomplete Freund’s adjuvant was used in booster injections. All mice were euthanized at 18 weeks of age after 10 weeks on the Western diet.12

Quantification of Plaque Size and Cellular Components

Because the correlation is strong between the extent of atherosclerosis in the aortic root and in the entire aortic tree in murine atherosclerosis models,47 we measured lesions in the aortic root by use or the method described by Paigen.48 In brief, the mice were euthanized in a CO2 chamber and perfused transcardially with PBS. The heart was dissected out. The tissue segment from the sinus aorticus to the lower tips of the right and left atria was isolated and snap-frozen in liquid nitrogen and embedded with OCT compound (Miles Laboratories). The tissue block was sectioned with a thick-snap-frozen in liquid nitrogen and embedded with OCT compound.

Flow Cytometry

Cells were prepared from freshly isolated inguinal lymph nodes. A fraction of the cells was used to check the proportion of T and B cells. They were stained with FITC-conjugated anti-CD3, phycoerythrin-conjugated anti-CD19, and Cy-Chrome–conjugated anti-CD45 (PharMingen). The remaining cells were challenged with MDA-LDL for 6 hours to activate antigen-specific T cells. The very early activation marker, CD69, was detected on T-cell subsets by staining for 30 minutes at 4°C with FITC-conjugated anti-CD69, phycoerythrin-conjugated anti-CD8, and Cy-Chrome–conjugated anti-CD4 antibodies (all from PharMingen). The cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson).

Serum Cholesterol and Triglyceride Analysis

Cholesterol and triglyceride concentrations in mice sera were determined by use of enzymatic methods (Unimate 5 Chol, Hoffman-La Roche; triglycerides/6B, Boehringer-Mannheim) and a Cobas Mira System.

Statistical Analysis

Results are expressed as mean±SEM. Data were analyzed by the Wilcoxon nonparametric test. The significance level was set at P<0.05. Correlations were estimated by use of the Spearman rank correlation test.
Results

Lesion Development in Immunized E₀ Mice

At 18 weeks of age, E₀ mice fed a Western diet showed fibrofatty plaques that were filled with macrophage-derived foam cells, smooth muscle cells, and CD4⁺ T cells as well as CD22⁺ B cells.¹²,¹⁶,⁵² In the present study, E₀ mice were immunized with homologous MDA-LDL, homologous plaque homogenates, or PBS injections (controls). At 18 weeks of age, after 10 weeks on a Western diet, all mice had developed fibrofatty plaques with lipid-rich core regions covered by fibrous caps. Mice immunized with either plaque homogenate or homologous MDA-LDL showed a 39% and 46% reduction in lesion size, respectively, compared with the PBS-treated controls (Figure 1). This is in agreement with the previous finding that oxLDL immunization alleviates atherosclerosis.⁴¹–⁴⁴ It is interesting that plaque homogenate immunization also had a protective effect.

Immunohistochemistry showed that CD4⁺ T cells, CD8⁺ T cells, and CD22⁺ B cells were present in all sections (data not shown). CD22⁺ B cells and CD4⁺ cells were always more frequent than CD8⁺ T cells. For CD22⁺ and CD4⁺ cells, no differences were observed between groups. In contrast, CD8⁺ cells were significantly fewer in the MDA-LDL–immunized group (data not shown).

T-Cell–Dependent Antibody Response to MDA-LDL, Oxidized Phospholipids, and ApoB-100/48

To characterize the specific immune response induced by immunization, circulating IgM and IgG to MDA-LDL and oxidized cardiolipin were determined by ELISA. No increase in the titer of IgM against MDA-LDL could be detected after immunization (Figure 2A). However, the titer of T-cell–dependent IgG antibodies to MDA-LDL was elevated dramatically in mice immunized with plaque homogenate and with MDA-LDL (Figure 2A). The titer of circulating IgG to oxidized cardiolipin was also increased significantly in mice immunized with plaque homogenate or MDA-LDL (Figure 2B). Competitive inhibition assays indicated that IgG antibodies to either MDA-LDL or oxidized cardiolipin could be blocked by MDA-LDL (Figure 2C). This cross-reactivity could be due to the presence of oxidized cardiolipin on MDA-LDL particles or could represent antibodies to shared epitopes.²⁷ We also assessed another potential cross-reactivity, which is caused by β2-glycoprotein I binding to...
oxidized cardiolipin; therefore, some anti-phospholipid antibodies could react with β2-glycoprotein I rather than oxidized phospholipids. However, no anti-β2-glycoprotein I antibodies could be detected in any of the groups (data not shown).

The protein component in the plaque homogenates was analyzed by SDS-PAGE. A major band at 210 kDa was observed; it had the same apparent molecular mass as apoB-48 (Figure 3A). The circulating IgG antibodies from the plaque homogenate–immunized group recognized epitopes on a 500- and 210-kDa band from human and mouse MDA-LDL as well as mouse MDA-VLDL (Figure 3B), which indicated that a significant amount of MDA-LDL is present in plaques of E0 mice and implied that the MDA-LDL epitopes of B cells may be located within the sequence of apoB-48.

**T-Cell Response to MDA-LDL in Draining Lymph Nodes of E0 Mice**

Cells of draining lymph nodes were exposed to MDA-LDL to detect T cells reactive with this antigen. The expression of CD69, a T-cell marker for very early activation, was measured on the cells after a 6-hour culture with homologous MDA-LDL. This permitted an early detection of activated T cells before viability was reduced in the primary cultures.

CD4+ and CD8+ T cells of mice immunized with plaque homogenate or MDA-LDL were activated by homologous MDA-LDL, indicating the existence of cellular immune responses to the immunogens (Figure 4).

**Serum Lipids, Body Weight, Plaque Size, and Induced IgG Antibodies**

No significant differences in cholesterol or triglyceride levels or in body weight were found between the groups (data not shown). Serum cholesterol was correlated with body weight (Figure 5A) and plaque size (Figure 5B). Importantly, higher titers of IgG antibodies to MDA-LDL and oxidized cardiolipin were associated with lower serum cholesterol levels (Figure 5C and 5E) and with smaller plaques (Figure 5D and 5F). MDA-LDL immunization was associated with an increased proportion of CD19+CD45+ B cells in draining lymph nodes (54.5±1.2% in MDA-LDL–immunized mice versus 49.3±2.0% in PBS-injected controls) and a concomitant reduction in the proportion of CD3+CD45+ T cells (data not shown).

**Discussion**

The results of the present study show (1) that immunization with homologous plaque homogenate as well as homologous...
the sera of E0 mice immunized with plaque homogenate, and modified protein components of oxLDL were detected in bodies reactive with oxidized cardiolipin as well as MDA-antibodies reactive with oxidized lipoproteins after immunization. The development of oxidation as well as the inflammatory-immune hypothesis for lesterolemic animals. This provides strong support for the picking in vivo modified LDL reduce lesion size in hypercho-

Figure 5. Correlation between serum cholesterol level vs body weight (r = 0.637, P < 0.02; A); serum cholesterol level vs plaque size (r = 0.809, P < 0.002; B); titers of IgG against MDA-LDL vs serum cholesterol level (r = 0.530, P < 0.05; C); titers of IgG against MDA-LDL vs plaque size (r = 0.561, P < 0.05; D); serum cholesterol level vs ratio of IgG against ox-cardiolipin and cardi-

lipin (r = 0.743, P < 0.01; E); and ratio of IgG against ox-cardiolipin and cardiolipin vs plaque size (r = 0.843, P < 0.002; F).

MDA-LDL inhibits plaque growth, suggesting that the plaque is an autoimmune target in the disease process; (2) that the immunogens of the plaque share epitopes with MDA-LDL, MDA-VLDL, and oxidized cardiolipin; (3) that antibody responses to oxidized lipoproteins and plaque antigens are T-cell dependent; and (4) that IgG antibody titers against MDA-LDL and oxidized cardiolipin correlate negatively with plaque size and serum cholesterol levels in immunized animals.

These data confirm and extend previous findings that immunization with MDA-LDL and other preparations mimicking in vivo modified LDL reduce lesion size in hypercholesterolemic animals. This provides strong support for the oxidation as well as the inflammatory-immune hypothesis for the pathogenesis of atherosclerosis. The development of antibodies reactive with oxidized lipoproteins after immunization with a plaque homogenate reveals immunologic cross-reactivities between oxLDL and plaque components. Antibodies reactive with oxidized cardiolipin as well as MDA-modified protein components of oxLDL were detected in the sera of E0 mice immunized with plaque homogenate, and the most likely explanation is that oxLDL was present in the plaque material. An alternative possibility could be that oxidative modification occurring in plaques generate B-cell epitopes similar to those present in oxLDL.

Immunizations were carried out by use of Freund’s adjuvant, which is strongly proinflammatory and promotes antigen processing by macrophages and dendritic cells. Such an activation of macrophages might, per se, affect atherosclerosis. Immunizations with Freund’s adjuvant can also induce immune responses to heat shock protein 65, which may be proatherogenic. However, the addition of immunogens (MDA-LDL and plaque homogenate) conferred protection when lesions were compared with those in mice injected with a PBS/Freund’s adjuvant emulsion. This fact and the finding that antibody titers correlated inversely with plaque size strongly support the notion that immunization with MDA-LDL or plaque material ameliorates atherosclerosis.

Antibodies were developed against protein and lipid components of oxLDL. In both cases, IgG antibodies were formed. This implies that T-cell help was provided for B-cell responses and resulted in immunoglobulin isotype switching. Although such a switch is the rule on booster immunization with protein antigens, antibodies to lipid antigens often develop in a non–T-cell–dependent manner. However, the present finding of IgG anti–oxidized cardiolipin suggests that T cells recognize oxidized cardiolipin or molecular structures associated with it. The present data emphasize the importance of molecular analyses to identify the epitopes and mechanisms involved in atheroprotective immunization.

High IgG titers against MDA-LDL and oxidized cardiolipin were correlated with a reduction in the size of atherosclerotic lesions. In fact, oxidized cardiolipin antibody titers showed a stronger (negative) correlation with lesion size than (the positive correlation of) serum cholesterol. This clearly suggests that B-cell responses mediate protection against atherosclerosis. Because the immunoglobulin isotype switch implied T-cell help, T-cell–B-cell cooperation during immune responses may play a role. However, the most obvious interpretation would be that antibodies to components of oxLDL confer protection against atherosclerosis. This might occur by Fc-dependent removal of oxLDL from the circulation or by neutralizing the effects of oxLDL systemically or locally.

Our conclusions differ from those of Freigang et al., who immunized LDL receptor–deficient mice with LDL preparations. Although these authors also observed a negative correlation between anti–MDA-LDL titers and atherosclerosis in MDA-LDL–immunized mice, they did not observe such a correlation in mice immunized with native LDL and therefore concluded that the antiatherogenic effect was probably not dependent on antibodies.

In the present study, the negative correlation between anti–MDA-LDL titers and lesions in mice immunized with plaque homogenate and in mice receiving MDA-LDL support the notion that protective antibodies may play a role. Our analysis of anti–oxidized cardiolipin antibodies renders further support to this hypothesis, inasmuch as the negative correlation between these titers and lesion size was even stronger than that for anti–MDA-LDL titers. Therefore, although cellular immune responses may also be important, our data suggest that humoral immune responses toward the
components of modified lipoproteins confer protection against atherosclerosis. It will now be important to evaluate whether transfer of specific antibodies and/or B cells can protect atherosclerosis-prone mice from disease.

Acknowledgments

Our work was supported by the Swedish Medical Research Council (project No. 6816), the Swedish Heart-Lung Foundation, the Johnson, Wallenberg, and Hedlund Foundations, King Gustaf V 80th Anniversary and King Gustaf V and Queen Viktoria Foundations, and the AFA Research Fund. We thank Karin Danell-Toverud for LDL preparations and Anita Larsson for measuring serum cholesterol and triglycerides.

References


LDL Immunization Induces T-Cell–Dependent Antibody Formation and Protection Against Atherosclerosis
Xinghua Zhou, Giuseppina Caligiuri, Anders Hamsten, Ann Kari Lefvert and Göran K. Hansson

doi: 10.1161/01.ATV.21.1.108

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/21/1/108

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/