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Permalink
https://escholarship.org/uc/item/9nm2h93r

Journal
Arteriosclerosis Thrombosis and Vascular Biology, 15(10)

ISSN
1079-5642

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Publication Date
1995-10-01

DOI
10.1161/01.atv.15.10.1569

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Increased Autoantibody Titers Against Epitopes of Oxidized LDL in LDL Receptor–Deficient Mice With Increased Atherosclerosis

Wulf Palinski, Rajendra K. Tangirala, Elizabeth Miller, Stephen G. Young, Joseph L. Witztum

Abstract Increasing evidence indicates that immune processes modulate atherogenesis. Oxidized LDL (Ox-LDL) is immunogenic, and autoantibodies recognizing epitopes of Ox-LDL have been described in plasma and in atherosclerotic lesions of several species. To determine whether the titer of such autoantibodies correlates with the extent of atherosclerosis, we followed the development of antibodies against malondialdehyde-lysine, an epitope of Ox-LDL, in two groups of LDL receptor–deficient mice for 6 months. One group was fed an atherogenic diet (21% fat and 0.15% cholesterol) that resulted in marked hypercholesterolemia and extensive aortic atherosclerosis; the other group was fed regular rodent chow (4% fat) that did not alter plasma cholesterol levels and induced minimal atherosclerosis. Autoantibody titers significantly increased over time in the group on the atherogenic diet, whereas they remained constant in the chow-fed group. When data from both groups were pooled, a significant correlation was found between the autoantibody titers and the extent of atherosclerosis (r=.61, P<.01). Autoantibody titers also correlated with plasma cholesterol levels (r=.48, P<.05). These results suggest that the rise in autoantibody titers to an epitope of Ox-LDL in this murine model is partially determined by the extent of atherosclerosis but could also be influenced by the degree of hypercholesterolemia or other factors that may influence lipid peroxidation. (***Arterioscler Thromb Vasc Biol.* 1995;15:1569-1576.**)

Key Words • oxidized lipoproteins • autoantibodies • atherosclerosis • immune system • mice

Oxidized lipoproteins are thought to contribute to atherogenesis by a number of mechanisms (for review, see References 1 through 3). During oxidative modification, reactive products resulting from lipid peroxidation form adducts with free amino groups of lysines and other amino acid residues of apoB. Even minor modifications of apoB render it highly immunogenic,[4] and in vitro modified LDL can be utilized to generate antibodies against various epitopes of oxidized LDL (Ox-LDL).[5-8] These antibodies have been used to demonstrate the occurrence of Ox-LDL in atherosclerotic lesions.[5,7-11] Ox-LDL present in vivo also appears to induce an immune response. Indeed, autoantibodies to epitopes of Ox-LDL have been demonstrated in the plasma of several species,[7,10,12,13] and immunoglobulins extracted from atherosclerotic lesions contain antibodies recognizing epitopes of Ox-LDL.[14] Some of these antibodies are present in lesions as part of immune complexes with Ox-LDL.[14] In addition, T cells isolated from human atherosclerotic lesions specifically respond to Ox-LDL.[15]

It is increasingly recognized that the immune system can modulate the atherogenic process and that both humoral and cell-mediated responses may be involved. Xu et al[16] have shown that an inflammatory type of lesion can be induced in the aorta of normocholesterolemic rabbits by immunization with heat-shock protein 65. Hyperimmunization of LDL receptor–deficient rabbits with malondialdehyde (MDA)-modified homologous LDL results in very high titers of antibodies with specificities similar to those of naturally occurring autoantibodies, and this intervention significantly reduces the progression of atherosclerosis.[17] This suggests that under certain conditions the activation of the humoral or cellular immune system may play a beneficial role in atherogenesis.

The oxidative modification of LDL may lead to the formation of a variety of immunogenic structures.[18,19] We have termed these "oxidation-specific" epitopes.[6,7] However, to date only a few model epitopes of Ox-LDL have been demonstrated in vivo.[6-10] Similarly, screening for autoantibodies has been performed with only a small number of antigens. The predominant population of autoantibodies to epitopes of Ox-LDL described to date is specific for MDA-lysine. As MDA may also be formed by processes unrelated to lipoprotein modification, eg, as a by-product of prostaglandin and leukotriene formation, or other processes in which lipid peroxidation occurs,[18] a priori it cannot be assumed that Ox-LDL is the only or even the predominant immunogen inducing the formation of MDA-lysine–specific autoantibodies in vivo. The assumption that Ox-LDL is the source of the MDA-lysine epitope would be strengthened if a correlation between the extent of atherosclerotic lesions and the autoantibody titer to MDA-LDL could be established.

Several studies of human subjects describe higher titers of circulating autoantibodies to MDA-LDL and other epitopes of Ox-LDL in patients with increased carotid atherosclerosis, coronary artery disease, diabetes, and peripheral vascular disease.[12,20-26] However, it is

Received February 19, 1995; revision accepted July 11, 1995.

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not clear whether the increased antibody titer reflects larger amounts of antigen in more extensive atherosclerotic lesions, proinflammatory conditions leading to the oxidative modification of LDL, or hypercholesterolemia itself, which may cause enhanced lipid peroxidation.[27] ApoE-deficient mice have autoantibody titers against MDA-lysine and other epitopes of Ox-LDL that are severalfold higher than those found in humans, rabbits, or chow-fed C57BL/6 mice.[10] These apoE-deficient mice have very high plasma cholesterol values and spontaneously develop extensive aortic atherosclerosis even on a low-fat, low-cholesterol diet. In contrast, the degree of hypercholesterolemia is relatively mild in LDL receptor–deficient mice fed regular rodent chow, and extensive atherosclerosis of the aorta is seen only in animals fed a high-fat, cholesterol-rich diet.[28,29] To test the hypothesis that increased lesion formation raises the titers of autoantibodies by increasing the exposure to the immunogen (Ox-LDL), we therefore determined the autoantibody titers in LDL receptor–deficient mice fed diets designed to induce either minimal or very extensive atherosclerosis and correlated the autoantibody titers with the extent of atherosclerosis.

Methods

Mice

Sixteen 2- to 3-month-old homozygous LDL receptor–deficient mice (hybrids between C57BL/6J and 129Sv strains; Jackson Laboratory)[28] were divided into two groups of 8 animals each and matched for gender, body weight, and total plasma cholesterol and triglyceride levels. One group was fed an atherogenic high-fat diet containing 21.2% fat and 0.15% cholesterol but no cholic acid (TD 88137; Harlan Teklad); the other group was fed a control diet containing 4% fat (regular rodent diet W860; Harlan Teklad). Dietary intervention lasted for 6 months, during which plasma cholesterol and triglyceride levels and autoantibody titers were monitored at monthly intervals by testing 100-μL blood samples obtained from the retro-orbital plexus and collected in heparinized hematocrit tubes. At the end of the experiment, 1 to 1.5 mL of blood was obtained by cardiac puncture; 1000 U/mL heparin was added to each sample. Total plasma cholesterol and triglyceride levels were determined by using an automated enzymatic technique (Boehringer Mannheim). Aliquots of plasma for the determination of autoantibody titers were stored at −70°C.

Determination of Antibody Titers

The titer of autoantibodies in all plasma samples from the same experimental group was determined in a single assay by using solid-phase radioimmunoassay techniques.[6] Human LDL modified in vitro with MDA or native LDL was used as antigen, and the amount of autoantibody bound was detected with ^{125}I-labeled goat anti-mouse IgG, IgM (Sigma), or IgA (Zymed). We defined a titer as the ratio of antibody binding to MDA-LDL (or native LDL) divided by the binding to "postcoat."[20] The postcoat, a 2% bovine serum albumin solution, blocked any adsorptive sites on the plastic wells after the plating of the antigen.[6,20] Mouse sera were used at varying dilutions as indicated in the legends to figures.

Morphometric Determination of Atherosclerosis

The extent of atherosclerosis was determined in the entire aorta of the LDL receptor–deficient mice.[10,30] Mice were killed, and the aorta was perfusion-fixed via a cannula inserted into the left ventricle; unrestricted efflux was allowed from an incision in the right atrium. Blood was removed by perfusion with phosphate-buffered saline containing 20 μmol/L butylated hydroxytoluene and 2 mmol/L EDTA, pH 7.4. The perfusion was continued for 20 minutes with formal-sucrose (4% paraformaldehyde, 5% sucrose, 20 μmol/L butylated hydroxytoluene, and 2 mmol/L EDTA, pH 7.4) to obtain an initial fixation. The aorta was dissected from the aortic valve to the iliac bifurcation, opened longitudinally, and pinned flat on a black wax surface. After overnight fixation with formal-sucrose and a 12-hour rinse in phosphate-buffered saline, the aortas were stained with Sudan IV.[10] Images of three segments per aorta were captured with a Sony DXC-960MD three-chip CCD color video camera. Image analysis was performed on 24-bit color images by using OPTIMAS 4.0 (Bioscan) image-analysis software, an Oculus TCX true-color frame grabber with 4 Mb of frame buffer memory (Coreco), and a separate VGA image monitor.[30] A threshold was selected for the three basic colors so that the shape of the highlighted threshold area on the processed image corresponded as closely as possible to that of the actual lesions. The stained arteries, viewed through a stereo microscope, served as a reference during the imaging. The size of the lesion areas was then determined by the software. The surface area of the aortic segment itself was determined by using an auto-tracing feature that follows the contrast between the vessel and the black background. Results were expressed as percent of the aorta covered by atherosclerotic lesions.

Immunocytochemistry

Paraffin-embedded sections of the aortic root of LDL receptor–deficient mice were immunostained with the avidin-biotin-alkaline phosphatase method[10] by using two guinea pig antisera against epitopes generated during the oxidative modification of LDL, MAL-2 (specific for MDA-lysine) and HNE-7 (specific for 4-hydroxynonenal-lysine),[6] as well as a rabbit antiserum to murine macrophages (AIA31240; Accurate Chemical and Scientific Corp). Primary antibodies bound to the tissue were detected by using biotinylated anti–guinea pig or anti-rabbit immunoglobulins (Vector Labs). Endogenous mouse immunoglobulins present in lesions were detected with biotinylated antibodies against murine IgG and IgM (Vector Labs). Control slides were incubated without primary antibody.

Statistical Analysis

Data on total plasma cholesterol levels and extent of atherosclerosis were compared by using Student's unpaired t test. Correlations between amount of autoantibodies, extent of lesions, and plasma cholesterol levels were determined by using linear regression. Multivariate ANOVA for repeated measurements was used to compare parameters in experiments with multiple groups or time-course experiments.

Results

To determine if autoantibody titers would correlate with the extent of atherosclerosis, we measured the autoantibody titers in LDL receptor–deficient mice fed either a control (4% fat) or a high-fat (21.2% fat and 0.15% cholesterol) diet over a 6-month period. As expected, consumption of the high-fat diet led to a five- to sixfold increase in total plasma cholesterol levels that was achieved within 1 month of initiation of the diet. The plasma cholesterol levels subsequently remained steady during the following 5 months of the experiment (Fig 1). By contrast, plasma cholesterol levels in mice fed the control diet remained stable throughout the experiment. After 6 months, 6 of the 8 animals fed the control diet failed to display any lesions in the aorta (Fig 2A); 2 animals had minimal lesions that involved less than 0.5% of the surface area. In sharp contrast, all animals fed the high-fat diet showed extensive atherosclerosis throughout the aorta (18.7±2.4% of the aortic surface; Fig 2B) at sites with a typical predilection for lesions (eg, the arch, the branch sites of the mesenteric and renal

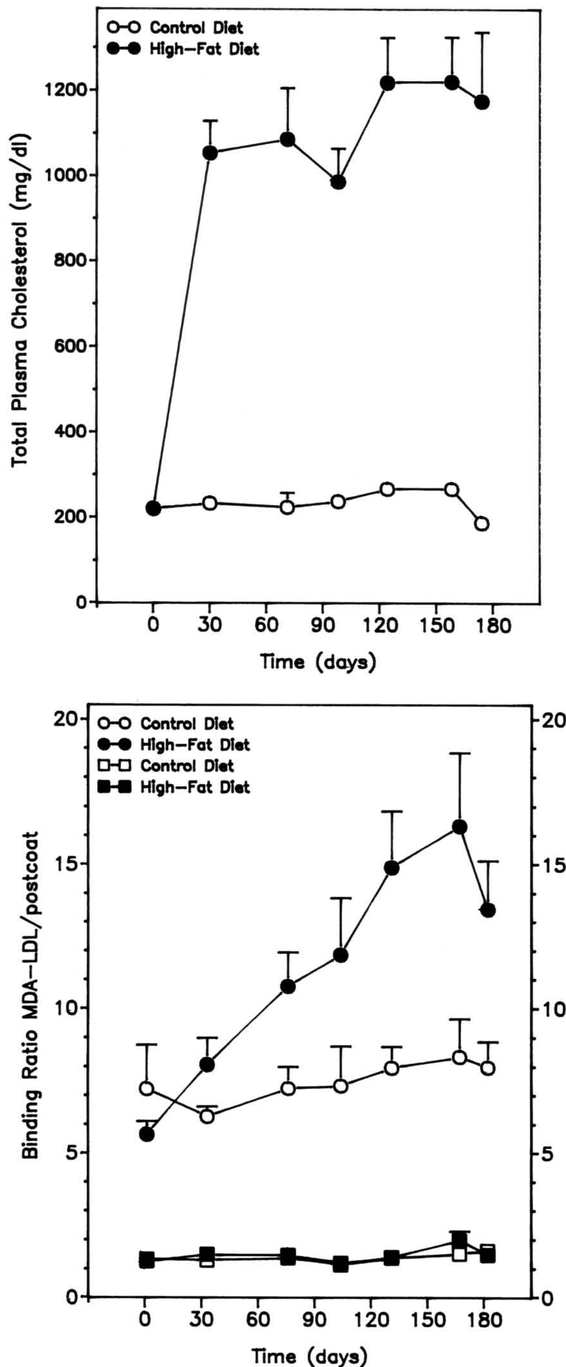


FIG 1. Top, Total plasma cholesterol levels in LDL receptor–deficient mice fed the low-fat control or high-fat diet containing 0.15% cholesterol during the 6-month study (mean±SEM, n=8 in each group). Bottom, Binding of autoantibodies in a 1:256 dilution of mouse plasma to human malondialdehyde (MDA)-LDL (circles, left scale) or to native (unmodified) human LDL (squares, right scale) over the 6-month dietary intervention. Antigen was plated at 5 μg/mL in this solid-phase radioimmunoassay, and bound antibodies were detected with ^{125}I-labeled goat anti-mouse IgG as described in "Methods." Results are reported as the ratio of antibody binding to MDA-LDL (or native LDL) divided by binding to postcoat (mean±SEM, n=8, where the data for each animal represented the mean of duplicate determinations). Antibody binding to MDA-LDL was significantly higher in the high-fat–diet group than in the control-diet group (multiway ANOVA for repeated measurements, $P<.0005$). The low, constant binding to native LDL in animals fed both diets indicates the lack of autoantibodies specific for native LDL. Error bars smaller than the symbol size are not shown in the figure.

arteries, and the iliac bifurcation), and both the distribution and extent of the lesions were remarkably constant in all 8 animals of this group (Fig 2C). Examination of cross-sections of the heart at the level of the aortic valve revealed atherosclerotic lesions of various stages in both groups, but lesions were far more extensive in the high-fat–diet group. As we have recently shown, the extent of atherosclerosis in the aortic origin correlates with the extent of lesions formed in the entire aorta in LDL receptor–deficient mice.[30]

Immunocytochemistry revealed that immunoglobulins of the IgM and IgG class were quite prevalent in these lesions (Fig 2D and 2E, respectively). Immunostaining with guinea pig antisera against two model epitopes of Ox-LDL, 4-HNE–lysine and MDA-lysine,[6] demonstrated the occurrence of these oxidation-specific epitopes in atherosclerotic lesions of LDL receptor–deficient mice (Fig 2G and 2H, respectively), often in the same distribution as that of macrophage/foam cells (Fig 2F). The distribution of these epitopes within lesions resembled that observed in apoE-deficient mice and other animal models of atherosclerosis.

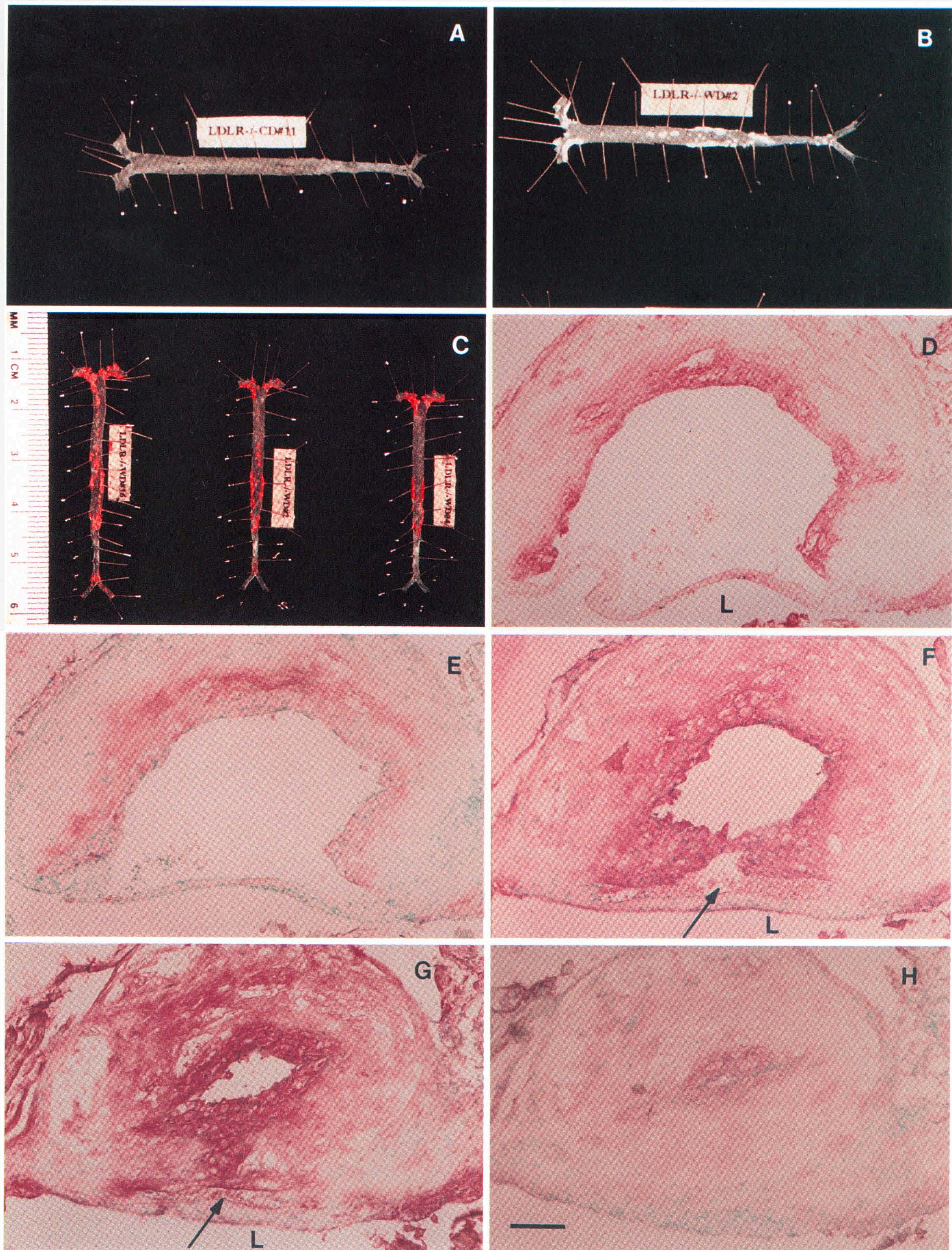
At the beginning of the experiment animals of both dietary groups had similar titers of IgG autoantibodies binding to MDA-LDL (Fig 1). Autoantibody titers, expressed as the ratio of antibody binding to MDA-LDL divided by that to postcoat (2% bovine serum albumin), remained constant throughout the 6 months of observation in the group fed the control diet. In contrast, antibody concentrations rose continuously in the mice on the high-fat diet ($P<.0005$ between the two groups). The ratio of the binding of IgG to LDL/binding to postcoat remained at a low and constant level in both groups, indicating the lack of high-affinity autoantibodies to native, unmodified LDL (Fig 1).

When data from all LDL receptor–deficient mice were analyzed together, the extent of aortic lesions correlated both with total plasma cholesterol levels ($r=.93$, $P<.0001$) and the titer of IgG autoantibodies ($r=.61$, $P<.01$) (Fig 3). There was a weaker but still significant correlation between the autoantibody titer and the total plasma cholesterol level ($r=.48$, $P<.05$) (Fig 3). However, it should be noted that whereas the plasma cholesterol levels rose promptly after initiation of the high-fat diet (Fig 1), the rise in the antibody titer to MDA-LDL occurred much more slowly and peaked only toward the end of the intervention period (Fig 1).

Autoantibodies of the IgM class followed the same pattern as the IgG antibodies, ie, they increased over time in the group on the high-fat diet, whereas they remained fairly constant in the group on the control diet. By contrast, no significant differences between experimental groups were detected in IgA autoantibodies. Fig 4 shows the individual binding ratios for IgG, IgM, and IgA autoantibodies in the plasma samples obtained after 167 days.

Discussion

Ox-LDL is highly immunogenic,[4] and autoantibodies against various epitopes of Ox-LDL are found in the



plasma and within atherosclerotic lesions.[7,10,14] We therefore hypothesized that oxidized lipoproteins constitute a major source of the immunogen inducing the formation of these autoantibodies. Because oxidation-specific epitopes are found primarily in atherosclerotic tissues in animal models of atherosclerosis,[9] we assume that a large proportion of the oxidized lipoproteins occurs in the vascular wall. If this is the case, the amount of Ox-LDL antigen present would be a function of the extent of atherosclerosis. In this study we determined the autoantibody titers against MDA-lysine, an epitope formed during the oxidative modification of lipoproteins, in LDL receptor–deficient mice fed two diets that induced vastly different degrees of hypercholesterolemia and atherosclerosis. In the animals fed the atherogenic diet but not in controls there was a prompt increase in

FIG 2 (facing page). Photomicrographs showing atherosclerotic lesions of LDL receptor–deficient mice. A, Aorta of a mouse fed control chow. To obtain a flat preparation for imaging, the aorta was opened longitudinally, the arch was dissected along the major curvature, and half the arch was folded to the left. No lesions were detectable in this aorta, and only 2 of 8 animals showed any atherosclerosis in the entire aorta (less than 0.5% of the surface area). B, Aorta of an animal fed the high-fat diet. Lesions were found primarily in the arch and in the vicinity of the orifices of branching arteries. C, Sudan IV–stained aortas from 3 LDL receptor–deficient mice fed the high-fat diet. Lesion distribution was remarkably consistent in these mice and 18.7±2.4% of the aortic surface area was covered by lesions. Atherosclerotic lesions were found in cross-sections of the aortic origin of animals fed the high-fat diet (shown here) and in animals fed the control diet, although lesions were far less extensive in the latter group. D through H, Immunocytochemical staining of an advanced lesion in the aortic origin of an LDL receptor–deficient mouse. Sections of the proximal aorta were cut perpendicular to the aortic root and stained with the avidin-biotin–alkaline phosphatase method. Specific epitopes recognized by the primary antibody are indicated by red; nuclei were counterstained with methyl green. The lesion shown in these serial but nonconsecutive sections initially covered the entire aortic wall between the origins of the aortic valve leaflet (L) and progressively occluded most of the lumen. In addition to the central lumen, a small area adjacent to the leaflet containing red blood cells (arrow) is visible in some sections. Lesions contained IgM (D) and IgG (E) (both stained with 1:200 dilutions of biotinylated antisera against the respective murine immunoglobulin) and macrophages (F; stained with a 1:350 dilution of a rabbit antiserum against mouse macrophages). Immunostaining with HNE-7, a guinea-pig antiserum against 4-HNE–lysine (G; 1:400 dilution), and MAL-2, an antiserum against malondialdehyde-lysine (H; 1:250 dilution) demonstrates the presence of epitopes formed during the oxidative modification of LDL in these lesions. Staining with HNE-7 and MAL-2 was predominantly found in macrophage-rich areas (D through H, ×85; bar=100 μm).

plasma cholesterol levels that was sustained for the 6-month intervention. In these mice, the autoantibody titers gradually increased over time, and at the end of the intervention period achieved levels three- to fourfold above starting titers. A significant correlation was found between the autoantibody titers at the end of the study and the extent of atherosclerosis.

The titer of autoantibodies may be influenced not only by the amount of antigen, its immunogenic properties, and the tissue sites where it occurs, but also by complex regulatory mechanisms of the cellular and humoral immune system. Therefore, one could not assume, a priori, that there would be a direct correlation between the titer and the amount of antigen (indicated by the extent of lesion). Nevertheless, the results of our study in LDL receptor–deficient mice suggest that such a correlation exists in this model and that the autoantibody titers reflect the generation of oxidized lipoproteins that accumulate in atherosclerotic lesions.

Alternatively, the increase in antibody titer response could reflect the generation of antigen by hypercholesterolemia itself. Hypercholesterolemia may lead to enhanced generation of reactive oxygen species by endothelial cells,[27] which in turn could drive lipid peroxidation and formation of MDA-lysine adducts in lesions. Hypercholesterolemia could also lead to enhanced lipid peroxidation in nonarterial tissues. For example, Liao et al[31] have demonstrated that increased conjugated dienes occur in the liver of cholesterol-fed mice. Indeed, in our study the antibody titers at the end

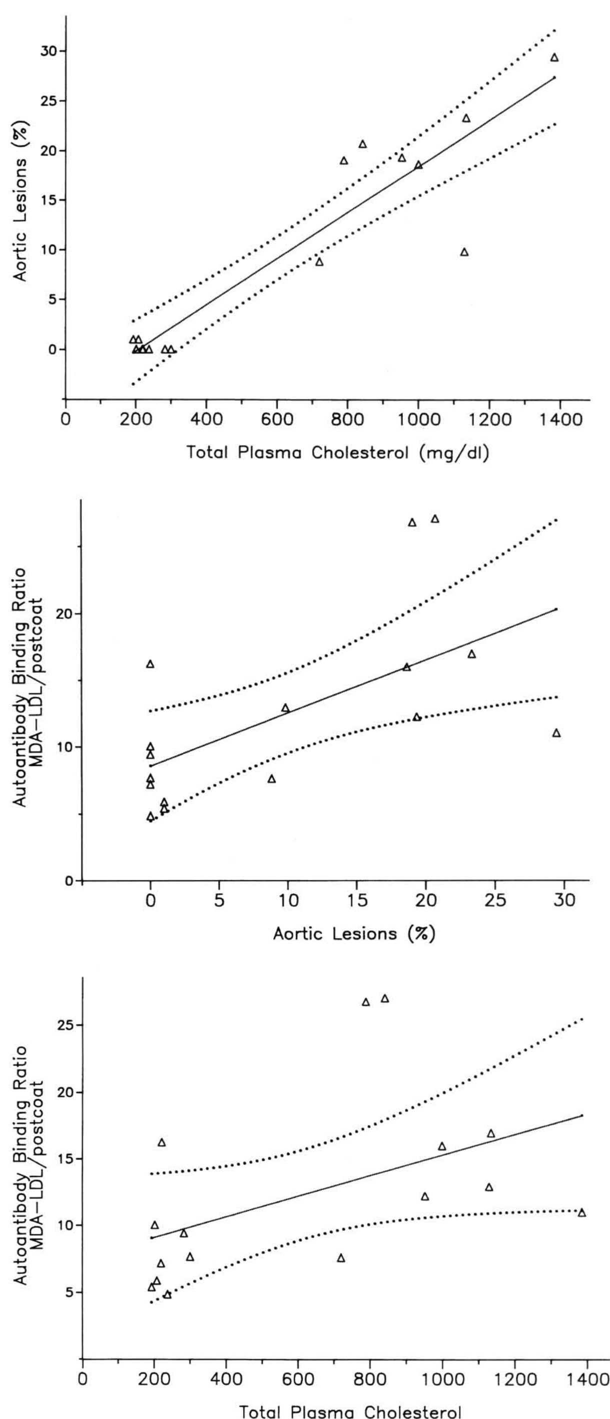


FIG 3. Correlation between average total plasma cholesterol during the 6 months of observation and the extent of atherosclerosis in the entire aorta (r=.93, P<.0001) (top). Correlation between the extent of atherosclerosis in the aorta and the titer of IgG autoantibodies to malondialdehyde (MDA)=LDL in the plasma samples obtained after 167 days (expressed as the MDA-LDL/postcoat binding ratio of antibodies in a 1:1024 dilution of murine plasma) (r=.61, P<.01) (middle). Correlation between average total plasma cholesterol and the autoantibody titer to MDA-LDL in the plasma sample obtained after 167 days (r=.48, P<.05) (bottom). The regression line and 95% confidence intervals were calculated from the pooled data from animals fed the high-fat and control diets.

of the experiment also correlated with the plasma cholesterol levels, albeit to a lesser degree than with the

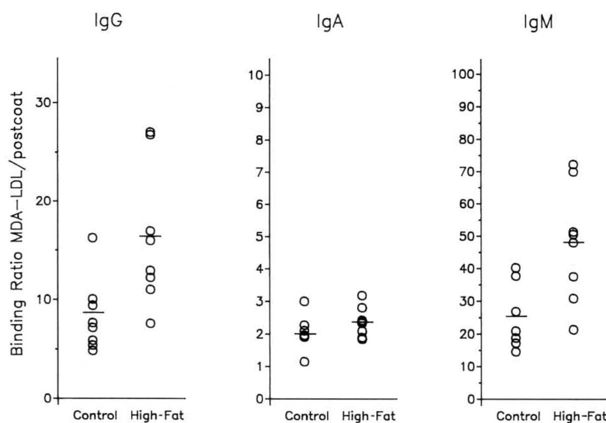


FIG 4. Autoantibodies of the IgG, IgA, and IgM type in the plasma of LDL receptor–deficient mice fed the high-fat (n=8) or low-fact control (n=8) diet for 167 days. Data are presented as the ratio of the antibody binding to MDA-LDL divided by the binding to postcoat (BSA). Serum dilution was 1:1024 for IgG and 1:258 for IgM and IgA type antibodies. Differences between animals on the high-fat diet and control diets were significant for IgG and IgM ($P<.0005$) but not significant for IgA.

extent of atherosclerosis ($r=.48$, $P<.05$). However, the fact that plasma cholesterol levels of 1000 mg/dL were achieved within a month in mice on the atherogenic diet (Fig 1), whereas the autoantibody titer levels rose gradually during the intervention period, supports the hypothesis that antigens accumulating in atherosclerotic lesions were the primary immunogens responsible for the increase in autoantibody titers, although similar antigens could also be generated and accumulated elsewhere, eg, in the liver.

LDL receptor–deficient mice provide an excellent model with which to study the correlation between the humoral immune response and the extent of atherosclerosis because great differences in the extent of atherogenesis can be induced by dietary intervention in otherwise perfectly matched groups of genetically uniform animals. However, for the same reasons, the degree of correlation found in this study may not represent that found in other animal models of atherosclerosis and humans.

To illustrate this point, we also measured autoantibody titers in mice that overexpressed human apoB (both transgenic mice and their nontransgenic littermates, 87.5% C57BL/6 and 12.5% SJL, respectively). These mice had been fed either a high-fat diet containing 1.25% cholesterol and cholate or a control diet (breeder chow containing 9% fat and no cholesterol) for 18 weeks in Dr Young's laboratory.[32] In both transgenic and nontransgenic animals the degree of hypercholesterolemia and the extent of atherosclerosis in the aortic root were significantly greater in animals fed the high-fat diet. When we determined the titers of autoantibodies in serum samples from a subset of these animals, we found that the increase in antibody titers over the 18 weeks of intervention was significantly greater in animals on the atherogenic diet than in the respective animals on the control diet (W.P., E.M., S.Y., J.W., unpublished data, 1995). Thus, in both the transgenic and nontransgenic mice the rise in autoantibody titers was significantly greater in the mice that developed the highest cholesterol levels and most atherosclerosis, as was observed in the LDL receptor–deficient mice. However, it is important to point out that the rise in autoantibody titer was nearly as great as that observed in LDL receptor–deficient mice on an atherogenic diet, despite the fact that the absolute extent of their lesions was much less than that in LDL receptor–deficient animals. This demonstrates that there is not a simple one-to-one relation between antigen burden and antibody response. Furthermore, there was also a rise in titers (albeit significantly smaller) in the control mice, which received a 9% fat diet, even though these mice did not develop increased cholesterol levels nor significant atherosclerosis. Obviously, the humoral immune response (as measured by the titer of plasma autoantibodies) may be different for a given stimulus and does not reflect only antigen burden. In addition to different genetic background, which could independently influence immune responses, other factors may be involved, such as the rate of antibody production, catabolism, or complexing with antigens in atherosclerotic lesions. Finally, differences in genetic background may directly influence the amount of antigen formed. For example, different strains of mice develop different degrees of lipid peroxidation in the liver in response to the same dietary fat challenge.[31,33]

Atherosclerotic lesions contain large numbers of antigen-presenting cells, mainly macrophages, and immunocytochemical evidence suggests that oxidation-specific epitopes are taken up by macrophages.[7,9,10] Uptake of oxidized lipoproteins via scavenger receptors has been extensively documented, and uptake of immune complexes between Ox-LDL and specific autoantibodies via Fc receptors is also likely to occur.[34] Atherosclerotic lesions also contain several subsets of T lymphocytes,[35-37] and numerous observations indicate that the immune-competent cells in the intima are activated[38-40] (for review, see reference 41). For example, a significant percentage of intimal cells in atherosclerotic lesions express interleukin-2 receptors,[17,42] and major histocompatibility class II molecules are expressed on vascular smooth muscle cells in the vicinity of T lymphocytes.[41] Finally, the presence of terminal C5b-9 complement complexes and the expression of complement receptors by vascular cells indicate activation of the humoral immune systems.[43-46]

Evidence for the involvement of oxidized lipoproteins in the activation of the immune system is provided by Stemme et al,[15] who report that a surprisingly large percentage of $CD4^+$ cells cloned from human atherosclerotic plaques proliferate in response to Ox-LDL in an HLA-specific manner. Furthermore, hyperimmunization of LDL receptor–deficient rabbits with homologous MDA-LDL significantly reduces the extent of atherosclerosis in the entire aorta.[17] Several other studies showing increased atherogenesis in immune-impaired animals also support the idea that the immune system may play a protective role in atherogenesis under some circumstances. Hansson and colleagues[47] have demonstrated that the elimination of T lymphocytes with monoclonal antibodies results in larger proliferative lesions in balloon-catheterized rat aortas, possibly because of loss of interferon gamma, which inhibits smooth muscle proliferation. Fyfe et al[48] have shown that class I major histocompatibility–deficient C57BL/6 mice, which lack cytolytic T cells and have impaired natural killer cell

activity, develop a threefold increase in lesions in the aortic-valve region when fed a high-fat diet.

The present studies show that, in general, increased autoantibody titers occur in parallel with increased atherosclerosis but that the response may also be influenced by other factors as well as by the genetic background. The role played by the humoral response in the pathogenesis of atherosclerosis in LDL receptor–deficient mice is unknown. If the immune response is beneficial, one may ask why the mice that developed increasing antibody titers developed extensive atherosclerosis. It is most likely that under the experimental conditions used, the rise in titers simply reflects a response to the generation of Ox-LDL and that this response is insufficient to significantly affect lesion formation. In the LDL receptor–deficient rabbit experiment[17] the autoantibody titers achieved by exogenous immunization were in excess of 100 000. In contrast, the titers in the mice described here were much lower, developed very slowly, and may have been insufficient to influence the disease process. However, it is quite possible that atherogenesis would have been worse without this response. Of course, it is also possible that the antibody response was proatherogenic in this murine model. Experiments in which LDL receptor–deficient mice are similarly hyperimmunized as the LDL receptor–deficient rabbits will be needed to determine if the immune system in general and the autoantibodies in particular are beneficial in this murine model.

Acknowledgments

These studies were supported by National Heart, Lung, and Blood Institute grant HL14197 to the La Jolla Specialized Center of Research in Arteriosclerosis. We thank Florencia Casanada, Mercedes Silvestre, Joe Juliano, and Frank Peralta for excellent technical assistance.

References

1. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: modifications of low density lipoprotein that increase its atherogenicity. *N Engl J Med*. 1989;320:915-924.
2. Witztum JL. The oxidation hypothesis of atherosclerosis. *Lancet*. 1994;344:793-795.
3. Palinski W, Witztum JL. Oxidative stress and diabetes mellitus. In: Born GVR, Schwartz CD, eds. *New Horizons in Diabetes Mellitus and Coronary Heart Disease*. London, England: Current Science; 1995:111-123.
4. Witztum JL, Steinbrecher UP, Fisher M, Kesaniemi A. Nonenzymatic glucosylation of homologous LDL and albumin render them immunogenic in the guinea-pig. *Proc Natl Acad Sci U S A*. 1983;80:2757-2761.
5. Haberland ME, Fong D, Cheng L. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science*. 1988;241:215-241.
6. Palinski W, Ylä-Herttuala S, Rosenfeld ME, Butler S, Socher SA, Parthasarathy S, Curtiss LK, Witztum JL. Antisera and monoclonal antibodies specific for epitopes generated during the oxidative modification of low density lipoprotein. *Arteriosclerosis*. 1990;10:325-335.
7. Palinski W, Rosenfeld ME, Ylä-Herttuala S, Gurtner GC, Socher SA, Butler S, Parthasarathy S, Carew TE, Steinberg D, Witztum JL. Low density lipoprotein undergoes oxidative modification in vivo. *Proc Natl Acad Sci U S A*. 1989;86:1372-1376.
8. Boyd HC, Gown AM, Wolfbauer G, Chait A. Direct evidence for a protein recognized by a monoclonal antibody against oxidatively modified LDL in atherosclerotic lesions from a Watanabe heritable hyperlipidemic rabbit. *Am J Pathol*. 1989;135:815-826.
9. Rosenfeld ME, Palinski W, Ylä-Herttuala S, Butler S, Witztum JL. Distribution of oxidation-specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis*. 1990;10:336-349.
10. Palinski W, Ord V, Plump AS, Breslow JL, Steinberg D, Witztum JL. Apoprotein E–deficient mice are a model of lipoprotein oxidation in atherogenesis: demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. *Arterioscler Thromb*. 1994;14:605-616.
11. Jürgens G, Chen Q, Esterbauer H, Mair S, Ledinski G, Dinges HP. Immunostaining of human autopsy aortas with antibodies to modified apolipoprotein B and apoprotein(a). *Arterioscler Thromb*. 1993;13:1689-1699.
12. Parums DV, Brown DL, Mitchinson MJ. Serum antibodies to oxidized low-density lipoprotein and ceroid in chronic periaortitis. *Arch Pathol Lab Med*. 1990;114:383-387.
13. Orekhov AN, Tertov VV, Kabakov AE, Adamova IYU, Pokrovsky SN, Smirnov VN. Autoantibodies against modified low-density lipoprotein: nonlipid factor of blood plasma that stimulates foam cell formation. *Arterioscler Thromb*. 1991;11:316-326.
14. Ylä-Herttuala S, Palinski W, Butler S, Picard S, Steinberg D, Witztum JL. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized low-density lipoprotein. *Arterioscler Thromb*. 1994;14:32-40.
15. Stemme S, Faber B, Holm J, Bondjers G, Witztum JL, Hansson GK. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc Natl Acad Sci U S A*. 1995;92:3893-3897.
16. Xu Q, Dietrich H, Steiner HJ, Gown AM, Mikuz G, Kaufmann SHE, Wick G. Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat-shock protein 65. *Arterioscler Thromb*. 1992;12:789-799.
17. Palinski W, Miller E, Witztum JL. Immunization of LDL receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci U S A*. 1995;92:821-825.
18. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. *Free Radic Biol Chem*. 1991;11:81-128.
19. Esterbauer H, Quehenberger O, Jürgens G. Oxidation of human low density lipoprotein with special attention to aldehydic lipid peroxidation products. In: Rice-Evans C, Halliwell B, eds. *Free Radicals: Methodology and Concepts*. London, England: Richelieu Press; 1988:243-268.
20. Salónen JT, Ylä-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssönen K, Palinski W, Witztum JL. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet*. 1992;339:883-887.
21. Branch DW, Mitchell MD, Miller E, Palinski W, Witztum JL. Preeclampsia and serum antibodies to oxidised low density lipoprotein. *Lancet*. 1994;343:645-646.
22. Maggi E, Finardi G, Poli M, Bollati P, Filipponi M, Stefano PL, Paolini G, Grossi A, Clot P, Albano E, Bellomo G. Specificity of autoantibodies against oxidized LDL as an additional marker for atherosclerotic risk. *Coron Artery Dis*. 1993;4:1119-1122.
23. Armstrong VW, Wieland E, Diedrich F, Renner A, Rath W, Kreuzer H, Kuhn W, Oellerich M. Serum antibodies to oxidised low-density lipoprotein in pre-eclampsia and coronary heart disease. *Lancet*. 1994;343:1570. Letter.
24. Maggi E, Chiesa R, Melissano G, Castellano R, Astore D, Grossi A, Finardi G, Bellomo G. LDL oxidation in patients with severe carotid atherosclerosis: a study of in vitro and in vivo oxidation markers. *Arterioscler Thromb*. 1994;14:1892-1899.
25. Bellomo G, Maggi E, Poli M, Agosta FG, Bollati P, Finardi G. Autoantibodies against oxidatively modified low-density lipoproteins in NIDDM. *Diabetes*. 1995;44:60-66.
26. Bergmark C, Wu R, de Faire U, Lefvert AK, Swedenborg J. Patients with early-onset peripheral vascular disease have increased levels of autoantibodies against oxidized LDL. *Arterioscler Thromb Vasc Biol*. 1995;15:441-445.
27. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest*. 1993;91:2546-2551.
28. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest*. 1993;92:883-893.
29. Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK. Massive xanthomatosis and atherosclerosis in cholesterol-fed LDL receptor-negative mice. *J Clin Invest*. 1994;93:1885-1893.
30. Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apoprotein E-deficient mice. *J Lipid Res*. In press.

31. Liao F, Andalibi A, deBeer FC, Fogelman AM, Lusis AJ. Genetic control of inflammatory gene induction and NF-kappa B-like transcription factor activation in response to an atherogenic diet in mice. *J Clin Invest*. 1993;91:2572-2579.
32. Purcell-Huynh DA, Farese RV, Johnson DF, Flynn LM, Pierotti V, Newland DL, Linton MRF, Sanan DA, Young SG. Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet. *J Clin Invest*. 1995;95:2246-2257.
33. Liao F, Andalibi A, Qiao JH, Allayee H, Fogelman AM, Lusis AJ. Genetic evidence for a common pathway mediating oxidative stress, inflammatory gene induction, and aortic fatty streak formation in mice. *J Clin Invest*. 1994;94:877-884.
34. Khoo JC, Miller E, Pio F, Steinberg D, Witztum JL. Monoclonal antibodies against LDL further enhance macrophage uptake of LDL aggregates. *Arterioscler Thromb*. 1992;12:1258-1266.
35. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis*. 1986;6:131-138.
36. Emeson EE, Robertson AL Jr. T lymphocytes in aortic and coronary intimas: their potential role in atherogenesis. *Am J Pathol*. 1988;130:369-376.
37. Hansson GK, Jonasson L, Lojsthed B, Stemme S, Kocher O, Gabbiani G. Localization of T lymphocytes and macrophages in fibrous and complicated human atherosclerotic plaques. *Atherosclerosis*. 1988;72:135-141.
38. Jonasson L, Holm J, Skalli O, Gabbiani G, Hansson GK. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. *J Clin Invest*. 1985;76:125-131.
39. Xu Q, Oberhuber G, Gruschwitz M, Wick G. Immunology of atherosclerosis: cellular composition and major histocompatibility complex class II antigen expression in aortic intima, fatty streaks, and atherosclerotic plaques in young and aged human specimens. *Clin Immunol Immunopathol*. 1990;56:344-359.
40. Stemme S, Holm J, Hansson GK. T lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and the integrin VLA-1. *Arterioscler Thromb*. 1992;12:206-211.
41. Libby P, Hansson GK. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab Invest*. 1991;64:5-15.
42. Kishikawa H, Shimokama T, Watanabe T. Localization of T lymphocytes and macrophages expressing IL-1, IL-2 receptor, IL-6 and TNF in human aortic intima: role of cell-mediated immunity in human atherogenesis. *Virchows Arch A Pathol Anat Histopathol*. 1993;423:433-442.
43. Vlaicu R, Niculescu F, Rus HG, Cristea A. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis*. 1985;57:163-177.
44. Rus HG, Niculescu F, Vlaicu R. The relationship between macrophages and C5b-9 complement complexes in human atherosclerosis. *Clin Immunol Immunopathol*. 1988;48:307-316.
45. Seifert PS, Hansson GK. Complement receptors and regulatory proteins in human atherosclerotic lesions. *Arteriosclerosis*. 1989;9:802-811.
46. Seifert PS, Hugo F, Hansson GK, Bhakdi S. Prelesional complement activation in experimental atherosclerosis. *Lab Invest*. 1989;60:747-754.
47. Hansson GK, Holm J, Holm S, Fotev Z, Hedric HJ, Fingerle J. T lymphocytes inhibit the vascular response to injury. *Proc Natl Acad Sci U S A*. 1991;88:10530-10534.
48. Fyfe AI, Qiao JH, Lusis AJ. Immune deficient mice develop typical atherosclerotic fatty streaks when fed an atherogenic diet. *J Clin Invest*. 1994;94:2516-2520.