Circulating Autoantibodies to Oxidized LDL Correlate With Arterial Accumulation and Depletion of Oxidized LDL in LDL Receptor–Deficient Mice

Sotirios Tsimikas, Wulf Palinski, Joseph L. Witztum

Abstract—Autoantibodies to oxidized low density lipoprotein (OxLDL) are elevated in some human populations with increased risk of atherosclerosis. To determine whether autoantibody levels to epitopes of OxLDL reflect the extent of aortic atherosclerosis and the content of OxLDL, we measured IgG and IgM autoantibody titers to malondialdehyde (MDA)-LDL and copper-oxidized LDL (Cu-OxLDL) in 43 LDL receptor–deficient mice consuming atherogenic and regression diets. Antibody titers were correlated to percent atherosclerotic surface area, aortic weight, and aortic OxLDL content, measured as the in vivo uptake of 125I-MDA2, a monoclonal antibody to MDA-LDL. All mice were fed an atherogenic diet for 6 months, and 1 group was euthanized. The other 3 groups were fed an atherogenic diet (fat/CHOL group), normal mouse chow (chow group), or mouse chow supplemented with vitamins E and C (chow VIT group) for an additional 6 months. After dietary intervention, compared with their own baseline, autoantibody titers to MDA-LDL and Cu-OxLDL increased significantly in the fat/CHOL group, whereas they did not change or decreased significantly in the chow and chow VIT groups. Aortic weight and surface area showed significant progression in the fat/CHOL group, mild progression in the chow group, and no progression in the chow VIT group (P<0.001), whereas OxLDL content actually decreased in the latter 2 groups (P<0.001). Significant correlations were seen with MDA-LDL autoantibody titers and OxLDL content (IgM, R=0.64 and P=0.0009; IgG, R=0.52 and P=0.009), as well as with percent surface area and aortic weight. These data support the hypothesis that autoantibody titers to OxLDL reflect changes in OxLDL content in atherosclerotic lesions of LDL receptor–deficient mice. Whether autoantibody titers to OxLDL will provide similar valuable insights into the extent of human atherosclerosis, particularly anatomic measurements of plaque burden and OxLDL content, remains to be determined. (Arterioscler Thromb Vasc Biol. 2001; 21:95-100.)

Key Words: regression ■ progression ■ atherosclerosis ■ lipoproteins ■ autoantibodies

It is generally accepted that oxidative modification of LDL is intimately involved in the initiation and progression of atherosclerotic lesions.1 Human atherosclerotic lesions have been shown to contain abundant quantities of oxidized lipoproteins within foam cells and in the extracellular space.2–6 In patients with myocardial infarction, atherosclerotic lesions undergoing plaque rupture, erosion, and thrombosis often contain large lipid pools with significant amounts of extracellular oxidized lipids and oxidized LDL (OxLDL).7,8 Indeed, recent evidence suggests that plasma levels of malondialdehyde (MDA)-LDL, an epitope of OxLDL derived from lipid peroxidation, is elevated in patients with coronary artery disease and is a strong predictor of acute coronary syndromes.9,10

The immune system plays a significant role in modulating the development of atherosclerosis.11,12 Our laboratory originally demonstrated that OxLDL is highly immunogenic and that autoantibodies to various epitopes of OxLDL are prevalent in humans and animals with atherosclerosis.2,13,14 Because LDL undergoes oxidative modification in vivo in atherosclerotic lesions, it is therefore likely that OxLDL within atherosclerotic tissues may induce the generation of circulating autoantibodies.2,3,14–18 This is also supported by the observation that a surprisingly high percentage of T lymphocytes isolated from human atherosclerotic lesions specifically recognizes OxLDL, suggesting that they are involved in immune activation and inflammation of atherosclerotic plaques.19 A corollary of these observations is that the autoantibody titers may reflect the atherogenic process. Indeed, previous studies have shown that autoantibody titers to OxLDL are elevated in humans and animals with atherosclerosis.11,20 However, the relationship between the extent of atherosclerosis and the humoral immune response is complex. Most of the human studies have been hampered by the lack of a precise cumulative assessment of atherosclerosis, and the autoantibody correlations have, in large part, been based on...
clinical surrogates. In addition, only very limited data from animal models are available on the relationship between autoantibody titers and the overall extent of atherosclerosis, and no published studies exist addressing the relationship between autoantibody titers and the progression or regression of atherosclerosis.

The most commonly used measure of atherosclerosis in animal models is percent atherosclerotic surface area of the aorta. In a previous study in LDL receptor–deficient (LDLR−/−) mice, we have shown that OxLDL content may be measured by intravenous injection of radiolabeled oxidation-specific antibodies (125I-MDA2). In that study, we induced lesion progression and regression and measured 3 parameters of atherosclerotic burden, ie, percent atherosclerotic surface area, aortic weight, and aortic uptake of 125I-MDA2. Focusing on the relationship between these parameters, we showed that in progressing atherosclerosis induced by a high-fat high-cholesterol diet, plaque uptake of 125I-MDA2 was correlated exceedingly well with the percent atherosclerotic surface area and the aortic weight (which reflects plaque volume). After prolonged exposure of mice with preexisting lesions to “regression diets” consisting of regular chow and of regular chow supplemented with antioxidants (1.0% vitamin E added to the chow and 0.05% vitamin C added to the drinking water), respectively. Group IV (fat/CHOL group, n=10) was continued on the high-fat/cholesterol diet for 6 months. Atherosclerosis was measured in terms of percent Sudan-positive aortic surface area, aortic weight, and uptake of 125I-MDA2, as previously described. Throughout the study, blood samples were obtained from the retro-orbital plexus and placed in EDTA tubes, and the plasma was isolated and stored at −70°C. These studies were approved by the Animal Subjects Committee of the University of California, San Diego.

**Determination of Antibody Binding to MDA-LDL and Cu-OxLDL**

The levels of IgM and IgG autoantibodies binding to MDA-LDL (anti-MDA-LDL) and Cu-OxLDL (anti-Cu-OxLDL) were determined in murine sera by use of a chemiluminescence ELISA. Autoantibody levels were determined in individual samples from all 4 groups of mice after the initial 6 months of the atherogenic diet and in the chow, chow+VIT, and fat/CHOL groups after 6 more months of dietary intervention. Antigens for the ELISA, MDA-LDL or Cu-OxLDL, were generated as described. In this assay, 5 μg/mL of the antigen in 50 mmol/L Tris-buffered saline (TBS), pH 7.5, containing 0.27 mmol/L EDTA, 0.02% azide, and 20 μmol/L butylated hydroxytoluene (dilution buffer), was added to each well of a 96-well white round-bottomed MicroFluor microtitration plate (Dynatech Laboratories, Inc) and incubated overnight at 4°C. Plates were washed 4 times with washing buffer (TBS containing 0.27 mmol/L EDTA, 20 μmol/L butylated hydroxytoluene, 0.02% NaN3, and 0.001% aprotinin) with the use of an automated plate washer. Murine sera were diluted 1:500 in dilution buffer containing 1% BSA, and 50 μL was added to each well and incubated for 1 hour at room temperature. After 4 washes, plates were incubated with 50 μL per well of an alkaline phosphatase–labeled goat anti-mouse IgG (γ-chain specific) or phosphatase-labeled goat anti-mouse IgM (μ-chain specific, both from Sigma) for 1 hour at room temperature. These antibodies were diluted in 1% BSA/TBS according to the supplier’s specifications. After the plates were washed, 25 μL of a 50% solution of Lumi-Phos 530 (Lumigen) was added to each well, and the plates were incubated for 1 to 2 hours at room temperature in the dark, and luminescence was determined. Antibody binding was measured as relative light units (RLU) in 100 milliseconds. Triplicate determinations were performed for each plasma sample. Measurement of antibody binding to a given antigen was performed in a single assay. A high and a low standard serum was included on each plate of a given assay to detect potential variations between microtitration plates. The intra-assay coefficients of variation for these assays were 6% to 10%.

**Methods**

**Animal Models and Dietary Intervention**

The design of the intervention study has been described in detail previously and is summarized in Figure 1. In brief, 6-month-old male LDLR−/− mice (n=43) were placed on an atherogenic diet containing 21% milk fat and 1.25% cholesterol for 6 months to induce atherosclerosis. The mice were then divided into 4 groups. Group I (n=13) was euthanized as the baseline control group. For an additional 6 months, groups II (chow group, n=10) and III (chow+VIT group, n=10) were placed on hypocholesterolemic “regression diets” consisting of regular chow and of regular chow supplemented with antioxidants (1.0% vitamin E added to the chow and 0.05% vitamin C added to the drinking water), respectively. Group IV (fat/CHOL group, n=10) was continued on the high-fat/cholesterol diet for 6 months. Atherosclerosis was measured in terms of percent Sudan-positive aortic surface area, aortic weight, and uptake of 125I-MDA2, as previously described. Throughout the study, blood samples were obtained from the retro-orbital plexus and placed in EDTA tubes, and the plasma was isolated and stored at −70°C. These studies were approved by the Animal Subjects Committee of the University of California, San Diego.
Results

Autoantibody Determinations

The mean autoantibody levels in the entire cohort of 43 mice after 6 months of the atherogenic diet were as follows: anti–MDA-LDL IgG, 2525 ± 1228 RLU; anti–MDA-LDL IgM, 15182 ± 6412 RLU; anti–Cu-OxLDL IgG, 1285 ± 587 RLU; and anti–Cu-OxLDL IgM, 3562 ± 1777 RLU. These levels are generally much higher than found in chow-fed C57B6 mice.14,24 At the end of the initial 6 months on a high-fat diet, mice were divided into the 4 experimental groups. There were no significant differences in the autoantibody titers among the groups except for the anti–MDA-LDL IgM titers, which were higher in the mice assigned to the chow and chow + VIT groups (P = 0.02, data not shown). Note that the assignment of animals to the groups was aimed at matching cholesterol levels (and thus, presumably, atherosclerosis) at baseline, whereas no matching for antibody levels was performed as part of the original design.22 The baseline control group was euthanized at this time, whereas the other 3 groups were continued under the study protocol (Figure 1) for another 6 months. At the end of the study, levels of anti–MDA-LDL and anti–Cu-OxLDL autoantibody titers were consistently higher (1.2- to 1.5-fold) in the fat/CHOL group compared with the other groups (Table). Because this comparison uses mice at different time points (the baseline control group was 6 months younger than the other 3 groups at the terminal autoantibody measurement) and because starting levels of autoantibodies were not equal, for all subsequent analyses, we compared the changes in autoantibody levels for each individual animal within each group, ie, before and after the dietary intervention. Figure 2A summarizes the mean changes in OxLDL autoantibody levels in the 3 intervention groups. When changes were compared within each group, in the fat/CHOL group, there was a significant increase in all autoantibodies to OxLDL, with anti–MDA-LDL IgG showing the greatest increase (81%, P < 0.001) compared with the preintervention values in the same group of mice. In contrast, in the chow and chow + VIT groups, the IgG autoantibody titers for anti–MDA-LDL and anti–Cu-OxLDL did not change, whereas the IgM titers actually decreased significantly after the regression/antioxidant diet. The corresponding changes in the cumulative measures of atherosclerosis and plaque OxLDL content are shown in Figure 2B. Because only 1 measurement was possible in these parameters (at the time of euthanasia), the atherosclerosis indices were compared with those in the baseline control group (P < 0.001). There was reduced progression of atherosclerosis measured by surface area (P < 0.001) but not aortic weight in the chow group. No significant changes in surface area or aortic weight were seen in the chow + VIT group. In contrast, significant reductions were noted in the OxLDL content, as reflected by 125I-MDA2 aortic uptake, in the chow and chow + VIT groups (P < 0.001 for both). Therefore, MDA-LDL and Cu-OxLDL autoanti-

### Table 1

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>Baseline Control</th>
<th>Fat/CHOL</th>
<th>Chow</th>
<th>Chow + VIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-LDL IgG</td>
<td>2336±1321</td>
<td>3734±1210</td>
<td>3208±1370</td>
<td>2866±1108</td>
</tr>
<tr>
<td>MDA-LDL IgM</td>
<td>12,021±5199</td>
<td>18,120±5858</td>
<td>15,085±7608</td>
<td>13,449±6234</td>
</tr>
<tr>
<td>Cu-OxLDL IgG</td>
<td>1120±457</td>
<td>1530±498</td>
<td>1462±754</td>
<td>1067±677</td>
</tr>
<tr>
<td>Cu-OxLDL IgM</td>
<td>3082±1541</td>
<td>5051±2304</td>
<td>4233±3504</td>
<td>2993±1505</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

Autoantibody titers are expressed as RLU/100 milliliters.
body levels qualitatively reflected the changes seen in the atherosclerosis indices and OxLDL content.

Correlations Between Autoantibody Levels and Atherosclerosis Indices

Correlations were carried out between atherosclerosis indices and the percent change in OxLDL autoantibody levels. Figure 3 shows the correlations between MDA-LDL autoantibody titers and the percent surface area, aortic weight, and uptake of 125I-MDA2. Highly significant correlations were seen with atherosclerosis parameters and anti–MDA-LDL autoantibodies, particularly with aortic uptake of 125I-MDA2 (for IgM, \( R = 0.64 \) and \( P = 0.0009 \); for IgG, \( R = 0.52 \) and \( P = 0.009 \)).

The correlations with anti–Cu-OxLDL IgG were more modest (for 125I-MDA2, \( R = 0.42 \) and \( P = 0.044 \); for aortic weight, \( R = 0.45 \) and \( P = 0.033 \); and for percent surface area, \( R = 0.37 \) and \( P = 0.075 \)). A trend was noted for the correlation between anti–Cu-OxLDL IgM and 125I-MDA2 uptake \( (R=0.37, \ P=0.09) \), but there was no correlation with lesion area or weight.

Discussion

In the present study, we demonstrate that autoantibody titers to OxLDL are significantly influenced by diet and antioxidant supplementation in atherosclerotic LDLR-/- mice, and we provide evidence that they indicate lesion progression as well as depletion of OxLDL during dietary regression. Plasma autoantibody levels to OxLDL increased significantly in mice continually fed an atherogenic diet but did not change or actually decreased in mice that were switched to a regression diet consisting of normal mouse chow or chow supplemented with vitamins E and C. A good correlation with traditional measures of atherosclerosis, such as percent atherosclerotic surface area or aortic weight, was noted for anti–MDA-LDL IgG and IgM autoantibodies. An even stronger correlation was noted with the in vivo aortic uptake of 125I-MDA2, which reflects the in vivo OxLDL content of lesions. These data provide evidence that in vivo 125I-MDA2 aortic uptake accurately reflects the presence of MDA-LDL and malondialdehyde-lysine epitopes within the plaque in atherosclerosis progression and “regression”, ie, depletion of MDA-LDL and malondialdehyde-lysine epitopes.

In humans, elevated titers of OxLDL autoantibodies have been documented in patients with myocardial infarction and angiographically documented coronary artery disease, peripheral vascular disease, and accelerated progression of carotid atherosclerosis. Elevated titers also seem to predict impaired endothelial function by demonstrating an inverse correlation with forearm blood flow in hypercholesterolemic smokers and with reduced coronary flow reserve assessed by positron emission tomographic scanning. In animal models, apoE-deficient and LDLR-/- mice have been shown to have elevated titers of autoantibodies to multiple epitopes of OxLDL. Freigang et al have also shown that immunization of LDLR-/- mice with homologous MDA-LDL results in markedly increased antibody titers to OxLDL, which were associated with a protective role against atherosclerosis. More recently, Cyrus et al showed that disruption of the 12/15-lipoxygenase gene in apoE-deficient mice resulted in reduced aortic root lesions and markedly diminished autoantibody titers to MDA-LDL and Cu-OxLDL. These human and animal studies clearly demonstrate that the humoral responses to OxLDL reflect changes within atherosclerotic plaques.
The potential role of autoantibodies to OxLDL as indicators of the regression of atherosclerosis has not been previously studied. Our data demonstrate that autoantibody titers to epitopes of OxLDL are well correlated with changes in atherosclerotic lesions, particularly their content of OxLDL. We have previously shown that in vivo aortic uptake of 125I-MDA2 is a sensitive marker of the progression and regression of atherosclerosis. The fact that the uptake of 125I-MDA2 decreased as a result of regression diets clearly suggests that the lesion content of OxLDL decreases, and this was confirmed by immunohistochemistry. Although antigen formation may also occur elsewhere in lipid-rich tissues and may equally be affected by the regression diets, our results support the assumption that the decrease in antibody titers is at least in part the result of the reduced presence of OxLDL in the aorta. The analogy in the changes in autoantibody titers and atherosclerosis (Figure 2) and the correlations between the these parameters (Figure 3) also support this conclusion.

The autoantibody titers were qualitatively similar in the chow and chow + VIT groups, but the surface area and aortic weight were different, although only the surface area was statistically significant between the 2 groups. This apparent discrepancy likely reflects the fact that the autoantibody titers more accurately indicate the content of OxLDL within the lesions, which were similarly reduced in both groups to the same extent, rather than other lesion components that contribute to plaque mass.

The presumed etiology of the bulk of the “oxidation-specific” antigens responsible for the generation of these autoantibodies is thought to be the vessel wall. However, it is possible that the marked hypercholesterolemia could have induced increased oxidation-specific epitopes elsewhere as well. In addition, it has been shown that oxidized cholesterol and oxidized lipids in the diet accelerate the development of atherosclerosis in mice and rabbits. In those studies, the dietary levels of oxidation byproducts were significantly increased by heating vitamin E–depleted corn oil or cholesterol to 100°C for several hours. In contrast, in the present study, all diets were refrigerated until time of use and were not vitamin E–depleted. Although we did not measure the levels of dietary peroxides in the present study, the plasma vitamin E levels (corrected to total plasma cholesterol) were higher in the fat/CHOL group than in the chow group, which may have provided some antioxidant protection.

It is not surprising that the autoantibody titers to MDA-LDL showed the best correlation with the in vivo aortic uptake of 125I-MDA2, because of the identity of the antigens. However, the observation that antibodies to Cu-OxLDL are also correlated to some extent with 125I-MDA2 aortic uptake amplifies the fact that the uptake of 125I-MDA2 provides a representative measure of OxLDL in the artery wall, not just a measure of its content in MDA-lysine epitopes. Indeed, although MDA-LDL is only 1 of many epitopes of OxLDL, it is clearly ubiquitous in atherosclerotic lesions. The correlations with lesion size were also much better for MDA-LDL than for Cu-OxLDL autoantibodies. Besides MDA-lysine epitopes, copper-induced oxidation of LDL also generates many other epitopes, such as oxidized phospholipids. Therefore, some differences between these autoantibody measurements would be expected. Additionally, the measured plasma autoantibody levels reflect an equilibrium between rates of formation and removal as well as immune complex formation (which we did not measure in the present study), which may more accurately reflect the development or regression of lesions.

Measurements of autoantibody titers to OxLDL have not yet been reported in human studies of dietary or pharmacological regression. Whether these measurements will be useful in the clinical arena remains to be seen. The cholesterol levels in the LDLR−/− mice after a Western-type diet are very high and are not usually seen in human subjects. The dietary regimens also resulted in drastic reductions in total cholesterol levels. The lesions induced, however, do reflect human lesions in many respects; therefore, we believe that the relevance of this model in humans is valid in principle. It is likely, however, that changes in humans will be much more modest. Nevertheless, depletion in the content of OxLDL may occur in patients treated with hypolipidemic agents and antioxidants over longer periods of time; this depletion will, in turn, be reflected in reduced autoantibody titers.

Significant effort has gone into developing techniques that provide clinically useful information on atherosclerotic lesions in patients. Unfortunately, most of the current gold-standard techniques are invasive, and we lack noninvasive techniques to either image the vessel wall or to ascertain plaque composition. The present data suggest that measurement of autoantibody titers to OxLDL can provide at least a cumulative measure of plaque OxLDL content. Whether measurement of autoantibody titers to OxLDL will be useful in assessing the regression of human atherosclerotic lesions needs to be evaluated in studies using quantitative techniques (such as intracoronary ultrasound) that completely assess the atherosclerotic plaque burden.

Acknowledgments

This investigation was supported by an National Heart, Lung, and Blood Institute (NHLBI) Mentored Clinical Scientist Development Award (HL-07444 to S.T.), by a New Investigator Award from the California Tobacco-Related Diseases Research Project (HT-0106 to S.T.), and by an NHLBI grant (HL-56989, La Jolla Specialized Center of Research in Molecular Medicine and Atherosclerosis to W.P. and J.L.W.). We would like to thank Jennifer Pattison, Florencia Casanada, Mercedes Silvestre, Elizabeth Miller, and Joseph Juliano for excellent technical assistance.

References


Circulating Autoantibodies to Oxidized LDL Correlate With Arterial Accumulation and Depletion of Oxidized LDL in LDL Receptor–Deficient Mice
Sotirios Tsimikas, Wulf Palinski and Joseph L. Witztum

_Arterioscler Thromb Vasc Biol._ 2001;21:95-100
doi: 10.1161/01.ATV.21.1.95

_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

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/21/1/95