Antibodies to Oxidized LDL in Relation to Carotid Atherosclerosis, Cell Adhesion Molecules, and Phospholipase A₂

Johannes Hulthe, Olov Wiklund, Eva Hurt-Camejo, Göran Bondjers

Abstract—The role of the humoral immune response to oxidized low density lipoprotein (Ox-LDL) in atherogenesis is unclear and available studies are contradictory. The aims of the present study were (1) to compare antibody titers to modified LDL in a group of patients with hypercholesterolemia (n = 102) with those in matched controls (n = 102), (2) to analyze whether these titers were related to atherosclerosis development as measured by ultrasound, and (3) to analyze whether these titers were related to soluble cell adhesion molecules and secretory type II phospholipase A₂ in plasma. The results showed that male patients with hypercholesterolemia had lower immunoglobulin G (IgG) titers compared with those in healthy controls. In the control group, there was an inverse correlation between intima-media thickness of the carotid artery bulb and IgM titers against Ox-LDL and malondialdehyde-LDL (r = −0.35, P = 0.001; and r = −0.31, P = 0.003, respectively). In the patient group, however, only weak associations were seen. IgG titers were positively associated with soluble intercellular adhesion molecule-1, soluble E-selectin, and secretory type II phospholipase A₂. Taken together, the results of this study support the concept that the humoral immune response against Ox-LDL may be protective in early atherosclerosis. The pattern, however, is complex, and the role of the immune response may differ in different patient groups as well as at different stages of the disease. (Arterioscler Thromb Vasc Biol. 2001;21:269-274.)

Key Words: Ox-LDL ■ antibodies ■ atherosclerosis ■ intima-media thickness ■ inflammation

Immune mechanisms have been suggested to play a key role in atherosclerosis development. Several lines of evidence support the concept that oxidized (Ox)-LDL may be a key antigen in this process. T-cell clones responsive to Ox-LDL have been isolated from human lesions. Furthermore, in several studies, antibodies against epitopes of Ox-LDL have been found in both human and rabbit plasma and in atherosclerotic lesions. A relationship between circulating antibodies against Ox-LDL and atherosclerotic disease has, however, not been unequivocally shown. There are case-control studies suggesting an elevated antibody titer against Ox-LDL in patients with various manifestations of atherosclerotic disease. High titers of antibodies have also been found to be independent predictors of the progression of carotid atherosclerosis. In other recent studies, however, no such relationships have been found between atherosclerotic disease and antibody titers.

Soluble forms of cell adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1], intercellular adhesion molecule-1 [ICAM-1], and E-selectin) can be detected in serum, and these molecules have also recently been detected in several components of human atheroma; in addition, cross-sectional and prospective data suggest that soluble forms of these proteins are elevated among patients with diverse manifestations of atherosclerosis. Another possible marker for atherosclerosis development is the secreted group IIa phospholipase A₂ (snpPLA₂), which has been described in association with local and systemic inflammation. snpPLA₂ has been shown to stimulate oxidation of LDL by lipoxygenase. snpPLA₂ has been found in human atherosclerotic lesions and has also recently been found to be correlated with coronary artery disease and to predict coronary events.

Development of the B-mode ultrasound technique has made it possible to noninvasively study the atherosclerotic process. Intima-media thickness (IMT) of the carotid artery has been used as a noninvasive indicator for the atherosclerotic process in the coronary arteries. The aims of the present study were (1) to compare antibody titers to modified LDL in a group of patients with hypercholesterolemia (n = 102) with those in matched controls (n = 102); (2) to analyze whether these titers were related to atherosclerosis development, as measured by ultrasound; and (3) to analyze whether these titers were related to other inflammatory markers of possible interest in atherosclerosis development, eg, soluble cell adhesion molecules and snpPLA₂, in plasma.

Methods

Study Groups

Study Group With Primary Hypercholesterolemia
Subjects with primary hypercholesterolemia (n = 105) were recruited from the screening of subjects from the general population performed at the Wallenberg Laboratory (n = 50) and also from patients...
at the Lipid Clinic of the Sahlgrenska University Hospital (n=55). Inclusion criteria were willingness to participate, 20 to 70 years of age, serum cholesterol >6.5 mmol/L, LDL cholesterol >5.0 mmol/L, and serum triglycerides <4.5 mmol/L. Furthermore, the patients had to fulfill at least 1 of the following ultrasound criteria: (1) a maximum IMT in the common carotid artery >1.0 mm or (2) a measurable plaque in the carotid artery, arbitrarily defined as a 50% increase in IMT compared with that of neighboring sites. Lipids and ultrasound variables were measured twice before inclusion, and subjects had to fulfill inclusion criteria at both occasions. Because of technical reasons, 3 patients had no serum samples available; thus, the total number of patients included for the present report was 102. The study group has previously been well described.20

**Study Group of Control Subjects**

For each patient, 1 control subject was recruited from the same screening examination as mentioned above. Control subjects had no symptoms of or treatment for cardiovascular disease. All control subjects had serum cholesterol levels <6.5 mmol/L at the initial screening examination. Control subjects were matched for sex, age, height, weight, body mass index (BMI), and smoking habits.20 The protocol was approved by the ethics committee of Göteborg University, and informed consent was obtained from all subjects.

**Anthropometric Data, Serum Lipids, Blood Pressure, IMT, and Plaque Occurrence in the Patient and Control Groups**

Patients and controls were well matched according to age, body height, body weight, and BMI. As expected, patients had higher serum cholesterol, LDL cholesterol, and serum triglyceride levels compared with controls (Table 1). IMT in the carotid artery bulb was significantly thicker in male patients compared with controls: 1.22±0.33 mm (95% confidence interval [CI], 1.13 to 1.31) and 1.03±0.34 mm (95% CI, 0.93 to 1.12), respectively, as well as in female patients compared with controls: 1.37±0.46 mm (95% CI, 1.24 to 1.51) and 0.91±0.20 mm (95% CI, 0.85 to 0.97), respectively. The frequencies of moderate to large plaques in male patients and controls were 44.2% and 15.4%, respectively, and in female patients and controls, 52% and 2%, respectively.20

**Antibody Titers Against Modified Lipoproteins**

**Determination of Antibody Titers Against Modified Lipoproteins**

Antibody titers were determined with a solid-phase ELISA, as earlier described.21 Antibody titer was defined as absorbance=(patient serum–postcoat)/(internal antibody titer standard serum–postcoat). For IgG, the postcoated wells gave no absorbance; therefore, this correction was made only for IgM.

**Internal Antibody Titer Standard Used**

On each plate, 2 different internal standard serum samples were repeatedly tested. The absorbances for these 2 samples, named the internal control sample (ICS) and the internal standard sample (ISS), were used to calculate their ratio (ie, ICS/ISS), which was used as the internal antibody titer, the variability has previously been shown to be satisfactory.21 Standard deviations (SDs) for the mean value of the ratio ICS/ISS (ie, the internal antibody titer standard used) from all plates were 0.06 and 0.05 for IgG titers against Ox-LDL and malondialdehyde (MDA)-LDL, respectively, and 0.05 and 0.08 for IgM titers against Ox-LDL and MDA-LDL, respectively, when earlier predefined criteria were used.21

**Ultrasoundography**

Examination was performed with an ultrasound scanner (Acuson 128) with a 7-MHz linear transducer aperture of 38 mm, as described earlier.22,23 The right carotid artery was scanned at the level of the bifurcation, and images for IMT measurements were recorded from the far wall of the common carotid artery and the carotid artery bulb. The images were measured in an automated analyzing system.24 As described before, a subjective semiquantitative scale was used to grade the size of plaques into grades 1, 2, and 3, where grade 1 corresponds to 1 or more small plaques (less than ~10 mm2) and grade 3 corresponds to large plaques causing a hemodynamic change in blood flow.23,24 Reproducibility studies of blinded rereading of plaque occurrence in 53 male subjects showed that plaque size was assessed in the same way on both occasions in 95% of the cases.

**Quantification of Type II snpPLA2 by ELISA**

Levels of snpPLA2 in human sera were measured by enzyme-linked capture antibody immunoassay.25 The antibodies used were a monoclonal antibody against human snpPLA2 (1 µg/µL) from Cayman Chemicals and a polyclonal antibody (IgG fraction) against human recombinant snpPLA2 that was produced in our laboratory and demonstrated no cross-reactivity with type V PLA2 (Edward A. Dennis, personal communication) or actin.26 ELISA plates were read at 405 nm (Spectra MAX Plus microplate spectrophotometer system, Molecular Devices). Purified human recombinant snpPLA2 was used to generate a standard curve (62.5 to 2000 pg/50 µL per well). The between-assy variation was determined by comparing the same blood samples (n=15) on 3 different days. The coefficient of variation was found to range between 8.6% and 9.6%.

**Cell Adhesion Molecules**

Circulating soluble VCAM-1, E-selectin, and ICAM-1 levels were determined by commercially available ELISA kits and standards (R&D Systems Europe Ltd). The between-assy variation was determined by comparing the same blood samples (n=41) on 2 different plates. The coefficient of variation was found to be 3.3%, 5.1%, and 4.8% for VCAM-1, E-selectin, and ICAM-1 levels, respectively.
Biochemical Analysis

Blood samples for serum cholesterol, serum triglycerides, and lipoprotein fractions were drawn after a fasting period of 10 to 12 hours. Cholesterol and triglyceride levels were determined by fully enzymatic techniques.\textsuperscript{27,28} HDL was determined after precipitation of apolipoprotein (apo) B–containing lipoproteins with MnCl\textsubscript{2} and dextran sulfate. LDL cholesterol was calculated as described by Friedewald et al.\textsuperscript{29} All lipid analyses were performed at the Wallenberg Laboratory.

Statistical Analysis

All statistical analyses were performed with SPSS 8.0 for Windows. The Mann-Whitney test was used when comparing mean values of antibody titers in patients and controls. Furthermore, a $t$-distributed variable was used to calculate 95% CIs for differences. Comparisons between groups for anthropometric and ultrasound data were not formally tested for significance. Only 95% CIs for mean values are presented because patients and controls had been selected and matched on the basis of anthropometric and ultrasound variables. Mantel’s test for linear association was used to test the relationship between antibody titers and Ox-LDL and plaque occurrence and size in the carotid artery.

Partial correlation (adjusted for sex) coefficients were used to investigate the relationships between antibody titers against Ox-LDL, IMT, cell adhesion molecules, and snpPLA\textsubscript{2} in the patient and control groups.

Probability values <0.05 (2-sided) were regarded as statistically significant.

Results

Antibody Titers Against Modified Lipoproteins in Patients With Hypercholesterolemia and Controls

Male patients with hypercholesterolemia had significantly lower IgG titers against Ox-LDL and MDA-LDL compared with control subjects. No other significant differences were observed (Table 2). When patients and controls were analyzed together, women had significantly higher mean values for IgM titers against Ox-LDL and MDA-LDL compared with men ($P=0.016$ and 0.010, respectively).

Table 2: Antibody Titers Against Ox-LDL and MDA-LDL in Patients and Controls

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Control Group</th>
<th>Patients</th>
<th>Mean Difference</th>
<th>95% CI for Difference</th>
<th>$P$ Value for Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males/females, n/n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>52/50</td>
<td>52/50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ox-LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.01±0.27</td>
<td>0.86±0.24</td>
<td>0.15</td>
<td>0.05 to 0.25</td>
<td>0.005</td>
</tr>
<tr>
<td>Female</td>
<td>0.92±0.25</td>
<td>0.92±0.25</td>
<td>0.00</td>
<td>−0.10 to 0.10</td>
<td>&gt;0.300</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.01±0.12</td>
<td>0.94±0.13</td>
<td>0.07</td>
<td>0.02 to 0.12</td>
<td>0.024</td>
</tr>
<tr>
<td>Female</td>
<td>0.96±0.13</td>
<td>0.97±0.13</td>
<td>−0.01</td>
<td>−0.07 to 0.04</td>
<td>&gt;0.300</td>
</tr>
<tr>
<td>IgM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ox-LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.17±0.30</td>
<td>1.25±0.28</td>
<td>−0.08</td>
<td>−0.19 to 0.04</td>
<td>&gt;0.212</td>
</tr>
<tr>
<td>Female</td>
<td>1.32±0.27</td>
<td>1.32±0.32</td>
<td>0.00</td>
<td>−0.12 to 0.12</td>
<td>&gt;0.300</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.26±0.36</td>
<td>1.33±0.28</td>
<td>−0.07</td>
<td>−0.19 to 0.06</td>
<td>&gt;0.265</td>
</tr>
<tr>
<td>Female</td>
<td>1.42±0.36</td>
<td>1.44±0.38</td>
<td>−0.03</td>
<td>−0.16 to 0.13</td>
<td>&gt;0.300</td>
</tr>
</tbody>
</table>

Values shown for titers are mean±SD.

Partial Correlations Between Antibody Titers, Cell Adhesion Molecules, and snpPLA\textsubscript{2} in Patients With Hypercholesterolemia and Controls

When adjusted for sex, IgG titers against both Ox-LDL and MDA-LDL in the patient group were significantly associated with ICAM, E-selectin, and snpPLA\textsubscript{2}. In addition, IgG titers against MDA-LDL were also associated with VCAM in the patient group (Table 3). Otherwise there were no significant relationships observed in the patient and control groups (Table 3).

IMT in Relation to Antibody Titers in the Patient and Control Groups

When adjusted for sex, no significant relationships were observed in the patient group between IMT of the common carotid artery or the carotid artery bulb and antibodies (both IgG and IgM) against Ox-LDL and MDA-LDL, respectively.

Table 3: Partial Correlation Coefficients, Adjusted for Sex, Regarding the Relations Between Antibody Titers Against Ox-LDL, Cell Adhesion Molecules, and snpPLA\textsubscript{2}

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox-LDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>ICAM</td>
<td>0.30*</td>
</tr>
<tr>
<td>r</td>
<td>VCAM</td>
<td>0.17</td>
</tr>
<tr>
<td>r</td>
<td>E-selectin</td>
<td>0.27*</td>
</tr>
<tr>
<td>r</td>
<td>snpPLA\textsubscript{2}</td>
<td>0.27*</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>0.17</td>
<td>0.27*</td>
</tr>
<tr>
<td>0.22†</td>
<td>0.08</td>
<td>−0.05</td>
</tr>
<tr>
<td>0.28*</td>
<td>−0.15</td>
<td>−0.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls (n=102)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM 0.15</td>
</tr>
<tr>
<td>VCAM 0.07</td>
</tr>
<tr>
<td>E-selectin 0.14</td>
</tr>
<tr>
<td>snpPLA\textsubscript{2} 0.07</td>
</tr>
</tbody>
</table>

*P<0.01, †P<0.05.
The main finding in the present study was the association of antibody titers against Ox-LDL and MDA-LDL and IMT of the carotid artery (Table 4). In the patient group, common carotid artery IMT tended to be negatively associated with IgM titers against Ox-LDL (r = -0.19, P = 0.055). Furthermore, IMT of the carotid artery bulb was significantly and negatively associated with IgM titers against both Ox-LDL and MDA-LDL (r = -0.35, P = 0.001; and r = -0.31, P = 0.003, respectively). IgG titers in the control group were not significantly associated with IMT (Table 4).

Plaque Occurrence and Size in Relation to Antibody Titers Against Modified Lipoproteins in the Patient and Control Groups

For men in the patient group, decreasing IgG titers against MDA-LDL were significantly associated with plaque occurrence and size in the carotid artery (P = 0.041). For women in the patient group, increasing IgG titers against Ox-LDL were significantly associated with plaque occurrence and size in the carotid artery (P = 0.045). Otherwise no significant relationships were found between antibody titers and plaque occurrence and size in the patient group (Table 5). In the control group, there were no significant relationships between IgG titers and plaque occurrence and size (Table 5). However, decreasing IgM titers against Ox-LDL were significantly associated with plaque occurrence and size for men in the control group (the Figure). Decreasing IgM titers against MDA-LDL were also significantly associated with plaque occurrence and size in both men and women in the control group (P = 0.003 and P = 0.010, respectively; Table 5).

Discussion

The main finding in the present study was the association between subclinical atherosclerosis and low antibody titers against Ox-LDL or MDA-LDL. Male patients with hypercholesterolemia and signs of early atherosclerosis had lower IgG titers compared with those in healthy controls. Furthermore, in the control group, there was a significant inverse correlation between IMT of the carotid artery bulb and IgM titers against Ox-LDL and MDA-LDL (r = -0.35, P = 0.001; and r = -0.31, P = 0.003, respectively). There was also a highly significant inverse correlation between plaque occurrence and size and IgM titers in the control group. In the patient group, only weak associations were seen. IgG titers against both Ox-LDL and MDA-LDL were significantly and positively associated with ICAM, E-selectin, and snpPLA2 levels in the patient group but not in the control group.

The above-mentioned observations between subclinical atherosclerosis and antibody titers are confusing but interesting. So far, the general hypothesis has been that high antibody titers to Ox-LDL predispose to atherosclerosis.6–8 On the other hand, there are recent reports indicating that immunization of experimental animals with Ox-LDL, leading to dramatically increased IgG levels, may inhibit the progression of atherosclerosis.30–32 In this context, the role of humoral immune reactions in atherogenesis is not clear.33 The protective role of the humoral immune response is supported by a recent study by Nicoletti et al.34 In that study, a spleen-associated immune response could protect against atherosclerosis in apoE-deficient mice. B cells can produce IgM without T-cell help, and the high titer of IgM to Ox-LDL in the present study may reflect T-cell–independent B-cell activation.

In general, it may be hypothesized that the physiological role of antibodies against Ox-LDL and related compounds is...
to trigger removal of the compounds from the circulation and possibly also from the arterial wall. Accordingly, Shoji et al recently showed that there was a negative correlation between antibody titers to Ox-LDL and plasma levels of Ox-LDL. Also in clinical studies there are reports supporting an association between low antibody titers and increased atherosclerosis. We have reported earlier on patients with familial hypercholesterolemia that those with a previous myocardial infarction had lower IgM titers compared not only with patients without a previous myocardial infarction but also with controls. A low antibody titer has also been reported in subjects with borderline hypertension.

Another explanation for the inverse relationship between antibody titers to Ox-LDL and atherosclerosis could be the formation of soluble antigen-antibody complexes. Lopes-Virella et al recently showed that there was a significant negative correlation between free Ox-LDL antibodies and immune complexes in subjects with insulin-dependent diabetes mellitus. Hence, the possibility that immune complex formation may affect antibody titers, as determined in the present study, should be considered.

The above-mentioned explanations for the inverse relationship between atherosclerosis and antibody titers (eg, removal of antigen from the circulation and complex formation) do not explain why there was a relationship between IgM titers (but not IgG titers) and atherosclerosis in the control group (but not in the patient group). We find methodological inconsistencies less likely as an explanation for the varying results, since we in earlier studies have shown that the measurement of subclinical atherosclerosis as well as the determination of antibody titers have good reproducibility and specificity.

The discrepancies may instead indicate differences in the character of atherosclerotic disease between patients and controls. The patient group was characterized by hypercholesterolemia and a high prevalence of sub-clinical atherosclerosis. On the other hand the control group was normocholesterolemic and had a low prevalence of sub-clinical atherosclerosis. In this context it may be suggested that the humoral immune response play a different role in different stages of the development of atherosclerosis as well as in the presence of various other risk factors. In the above mentioned study by Shoji et al the subjects were all healthy. Also, the animals immunized with modified LDL were free from significant atherosclerosis at the time of immunization. Furthermore, Fukumoto et al recently showed a negative relationship between common carotid IMT and antibody titers to Ox-LDL in a healthy population. The results from the present study are in line with these latter findings.

Because the composition of immune-competent cells in atherosclerotic plaques is quite different between early and advanced lesions, the biological role of immune responses against Ox-LDL probably differs in various stages of atherogenesis. This might explain why we found an inverse correlation in the control group but not in the patient group.

Another difference between the patient and control groups that could have affected the results is that some of the patients had been on lipid-lowering therapy. The drugs were withdrawn before inclusion to the study, but one may hypothesize that treatment could have long-term effects on antibody titer formation. Furthermore, it is not known whether the withdrawal of lipid-lowering medication may lead to an acute increase in antigen (ie, LDL cholesterol) formation, which in the present study might have caused short-term changes in antibody titers.

In the control group, IgM–Ox-LDL was associated with subclinical atherosclerosis in the carotid bulb only but not with IMT in the common carotid artery. Atherosclerotic changes are first seen in the carotid bulb, and subclinical atherosclerosis in this region, as measured by ultrasound, has also previously been shown to be associated with coronary atherosclerosis, as measured by coronary angiography. It is therefore not surprisingly to find a relationship between atherosclerotic changes in the carotid bulb and antibody titers in this group of healthy subjects.

IgG titers against Ox-LDL and MDA-LDL were positively correlated with cell adhesion molecule levels and snpPLA2 levels in patients but not in controls. Again, this could be due to different responses in different stages of the atherosclerotic process. The role of these inflammation markers and whether they are regulated by the immune response or nonspecific inflammatory stimulation in atherosclerosis cannot be concluded from the present study. However, inflammation may trigger LDL oxidation. Furthermore, the degradation of LDL by snpPLA2 may make the lipoprotein particles more sensitive to oxidation. These factors may therefore participate in triggering the immune response against Ox-LDL.

Taken together, the findings of this study support the concept that the humoral immune response against Ox-LDL may be protective in early atherosclerosis. The pattern, however, is complex, and the role of the immune response may be different in different patient groups as well as at different stages of the disease.

Acknowledgments

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