Antibodies to Oxidized LDL in Relation to Intima-Media Thickness in Carotid and Femoral Arteries in 58-Year-Old Subjectively Clinically Healthy Men

Johannes Hulthe, Lena Bokemark, Björn Fagerberg

Abstract—Antibody (Ab) titers to oxidized low density lipoprotein (OxLDL) have been found to be independent predictors of the progression of carotid atherosclerosis. Ab titers against OxLDL may be related to the entire burden of atherosclerosis in the vascular tree or, more specifically, to the disease process in different arterial regions. Therefore, the aim of the present study was to investigate the relationship between IgG and IgM titers to modified LDL and intima-media thickness (IMT) in the carotid and femoral arteries in subjectively clinically healthy 58-year-old men. IMT was measured by ultrasound, and Ab titers to modified LDL were measured by ELISA. The results showed that the common carotid artery IMT was associated with elevated titers of IgG-OxLDL Ab and independently with systolic blood pressure, smoking, and body mass index. The femoral artery IMT showed a negative correlation to IgM-OxLDL Ab and independent associations with smoking, systolic blood pressure, and total cholesterol. To summarize, in 58-year-old subjectively clinically healthy men recruited from the general population, there was a positive association between IgG-OxLDL Ab and IMT in the common carotid artery and a negative association between IgM-OxLDL Ab and IMT in the common femoral artery. However, these associations were not independent of other risk factors. (Arterioscler Thromb Vasc Biol. 2001;21:101-107.)

Key Words: antibody ■ oxidized LDL ■ intima-media thickness

Immune mechanisms have been suggested to play a key role in the development of atherosclerosis.1 Several lines of evidence support the possibility that oxidized LDL (OxLDL) may be a key antigen in atherosclerosis. T-cell clones responsive to OxLDL have been isolated from human lesions.2 Immune responses to OxLDL have also been observed in apoE knockout mice, an animal model for the development of atherosclerosis.3 Furthermore, antibodies (Abs) against epitopes of OxLDL have been found in several studies in human4,5 and rabbit 4–6 plasma and in atherosclerotic lesions. So far, it has not been established whether the immune responses to OxLDL are proatherogenic or antiatherogenic in vivo.7,8

There are case-control studies suggesting an elevated Ab titer against OxLDL in patients with various manifestations of atherosclerotic disease.9–13 High titers of Abs have also been found to be independent predictors of the progression of carotid atherosclerosis.14 In other recent studies, however, no such relationships have been found between atherosclerotic disease and Ab titers.15–17 In 2 recent studies, a reduction of OxLDL Abs was reported in patients with ischemic stroke18 and in patients with acute myocardial infarction.19 Measurements of IMT are used in pathophysiological studies of the atherosclerotic process, eg, in studies of the factors regulating the early development of atherosclerosis in the carotid and femoral arteries. An increased IMT is also used as a marker of generalized atherosclerosis, including coronary atherosclerosis.20,21 and carotid artery IMT has also been shown to be associated with coronary atherosclerosis, as measured by coronary angiography in several studies.22,23 However, the mechanisms involved in the atherosclerotic process may not be identical in different vascular territories. Thus, myocardial infarction, stroke, and peripheral artery disease have slightly different cardiovascular risk factor profiles; eg, cholesterol is a strong risk factor for myocardial infarction, whereas the association with stroke and intermittent claudication is weak or inconsistent.24 Hypothetically, the level of circulating Abs against OxLDL may be related to the entire burden of atherosclerosis in the vascular tree or, more specifically, to the disease process in specific arterial regions.

Therefore, the aim of the present study was to investigate the relationship between Ab titers (IgG and IgM) to modified LDL and IMT in the carotid and femoral arteries in 58-year-old healthy subjects recruited from the general population.

Methods

Study Subjects

The inclusion criteria were age 58 years, male sex, and Swedish ancestry. Exclusion criteria were cardiovascular or other clinically
overt disease (eg, malignancy or psychiatric disease), continuous medication with cardiovascular drugs (ie, antidiabetic, lipid-lowering, antihypertensive, or heart failure–related drugs or drugs for angina pectoris) that might disturb the measurements performed in the study, or unwillingness to participate. The subjects were randomly selected among men in the County Council register and were invited to a screening examination.25

A power calculation indicated that it was necessary to recruit at least 300 men in the study, with the main objective being to examine the relationship between insulin sensitivity and ultrasound- assessed atherosclerosis. The present report is a substudy of that project.

The subjects received written and oral information before they gave their consent to participate. The study was approved by the ethics committee at Sahlgrenska University Hospital.

**Measurements**

All measurements were performed in the morning. Venous blood samples were drawn after a fasting period of 10 to 12 hours, and serum was separated and frozen within 4 hours at −70°C. Body weight, height, waist, and hip circumference were measured, and body mass index (BMI) and the waist-to-hip ratio (WHR) were calculated.

Information on general health and smoking habits was obtained by a self-administered questionnaire. The total number of years of smoking was multiplied by the number of cigarettes smoked daily. The product was called “cigarette-years.”

**Ab Titers Against Modified Lipoproteins**

**Lipoprotein Preparation**

LDL (1.019 to 1.063 g/mL) was prepared from pooled plasma from 2 healthy human donors by sequential ultracentrifugation in the presence of 0.2% Na2-EDTA. The isolated lipoprotein was extensively dialysed against PBS (0.14 mol/L NaCl/0.01 mol/L PBS) containing 0.1 mmol/L Na2-EDTA, 2.5 μL 0.4 mol/L AEBSF, and 5 mL penicillin/streptomycin per liter (pH 7.4). LDL was steriley filtered, and the protein content was determined by the method of Lowry et al.26 Malondialdehyde (MDA)-treated LDL (MDA-LDL) was prepared as described by Palinski et al.27 OxLDL was prepared by oxidation of LDL in the presence of 5 mmol/L CuSO4 for 13 hours at 37°C. As a routine procedure, modifications were checked by controlling the electrophoretic mobility in agarose gel of the modified lipoproteins.

**Determination of Ab Titers Against Modified Lipoproteins**

Ab titers were determined with a solid-phase ELISA, as earlier described.20 Ab titer was defined as follows: titer=absorbance (patient serum–postcoat)/(internal Ab titer standard serum–postcoat).

For IgG, the postcoated wells gave no absorbance; therefore, this correction was made only for IgM.

**Internal Ab Titer Standard Used**

On each plate, 2 different internal standard serum samples were repeatedly performed. The absorbances for these 2 samples, named internal control sample (ICS) and internal standard sample (ISS), were used to calculate the ratio of ICS to ISS, which was used as the internal Ab titer standard. When earlier described predefined criteria for reanalyzing Ab titers were used, the variability in used was shown to be satisfactory.20 SDs for the mean value of the ratio of ICS to ISS (ie, internal Ab titer standard used) from all plates were 0.07 and 0.03 for IgG titers against OxLDL (IgG-OxLDL Ab) and MDA-LDL (IgG-MDA-LDL Ab), respectively, and 0.06 and 0.05 for IgM titers against OxLDL (IgM-OxLDL Ab) and MDA-LDL (IgM-MDL-LDL Ab), respectively, when the predefined criteria were used.

**Ultrasoundography: IMT**

Examination was performed by use of an ultrasound scanner (Acuson 128) with a 7-MHz linear transducer aperture of 38 mm. The ECG signal (lead II) was simultaneously recorded to synchronize the image capture of the top of the R wave to minimize variability during the cardiac cycle. The left and right carotid arteries were scanned at the level of the bifurcation, and images for IMT measurements were recorded from the far wall in the common carotid artery and the carotid artery bulb and from the right femoral artery. The software program gives the average thickness of the intima-media complex (ie, the IMT). Measurements in the common femoral artery were made in a manner similar to that for the carotid artery but along a 15-mm-long section proximal to the bifurcation.30 IMT was defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface of the far wall. At the position of the thickest part of the wall (visually judged), a frozen longitudinal image was captured and recorded on videotape. A short sequence of real-time images was also recorded on videotape to assist in the interpretation of the frozen images. The images were measured in an automated analyzing system,31 which was based on automatic detection of the echo structures in the ultrasound image but with the option of making manual corrections by the operator. The interobserver variations for IMT have been shown to be satisfactory.32

The present analysis used the average of measurements of IMT from the left and right common carotid arteries (common carotid artery and carotid bulb).

**Biochemical Analysis**

Cholesterol and triglyceride levels were determined by fully enzymatic techniques.33,34 HDL was determined after precipitation of apoB-containing lipoproteins with Mn-chloride and dextran sulphate. LDL cholesterol was calculated as described by Friedewald et al.35 Blood glucose was measured with the glucose oxidase technique. Plasma insulin was determined in all subjects with a radioimmunoassay (Pharmacia Insulin RIA, Pharmacia Diagnostics). All lipid analyses were performed at the Wallenberg Laboratory.

**Statistical Analysis**

All statistics were analyzed by using SPSS for Windows 8.0. A nonparametric Spearman rank correlation test was used in the correlation analysis, with the relationship illustrated by the Pearson correlation coefficient (r). The Mantel test for linear association was used to test the relationship between tertiles of common carotid IMT and the variables displayed in Tables 1 and 2. The Mantel test and the Mann-Whitney test were used when investigating the relationship between tertiles of Ab titers against OxLDL and IMT in the common carotid and common femoral arteries. A stepwise multiple regression model was used to study the determinants of IMT in the carotid and femoral arteries. Triglyceride was logarithmically transformed for statistical testing to improve skewness. A value of P<0.05 (2-sided) was regarded as statistically significant, with the exception of the correlations between IMT and cardiovascular risk factors. Because of the large number of correlations performed, the significance level for these analyses was set at P≤0.01. For anatomic reasons, there were missing data for common carotid and femoral artery IMT measurements (n=3 and n=18, respectively). For technical reasons, there were also missing data for Ab titers in 8 cases.

**Results**

**Characteristics of Subjects by Tertiles of Common Carotid Artery IMT**

No subject had clinically recognized diabetes mellitus. Blood glucose ≥6.1 mmol/L was found in 22 subjects. When the subjects were divided into tertiles on the basis of IMT in the common carotid artery, there were positive trends between IMT and BMI, WHR, systolic and diastolic blood pressure, total cholesterol, LDL cholesterol, triglycerides, blood glucose, and smoking (Table 1). There was a significant positive trend between IMT and IgG-OxLDL Ab.

**Characteristics of Subjects by Tertiles of Common Femoral Artery IMT**

When the subjects were divided into tertiles on the basis of IMT in the common femoral artery, there were positive trends between IMT and BMI, WHR, systolic and diastolic blood pressure, total cholesterol, LDL cholesterol, triglycerides, blood glucose, and smoking (Table 1). There was a significant positive trend between IMT and IgG-OxLDL Ab.
pressure, heart rate, total cholesterol, LDL, triglycerides, and smoking (Table 2). There was a negative trend between IMT and IgM-OxLDL Ab (P = 0.050).

The common carotid artery IMT was associated with the femoral artery IMT (r = 0.22, P < 0.001).

Covariates to IMT in the Common Carotid and Common Femoral Arteries

In the correlation analyses, IgG-OxLDL Abs were significantly associated with IMT in the common carotid artery (r = 0.13, P < 0.01). IgM-MDA-LDL Abs were negatively associated with IMT in the in the common femoral artery (r = −0.10, P < 0.05).

When the subjects were divided on the basis of IgG-OxLDL Ab titer, there was a positive relationship between IMT in the common carotid artery and increasing Ab titer (Figure 1). In the femoral artery, there was a negative relationship between IMT and tertiles on the basis of IgM-OxLDL Ab titer (Figure 2).

The Ab titers showed high intercorrelations for IgG (IgG-OxLDL Ab versus IgM-MDA-LDL Ab, r = 0.75 and P < 0.001) and for IgM titers (IgM-OxLDL Ab versus IgM-MDA-LDL Ab, r = 0.91 and P < 0.001). The IgG-OxLDL Ab and IgM-MDA-LDL Ab titers were significantly and negatively correlated (r = −0.10, P < 0.05).

As shown in Table 3, BMI, WHR, systolic and diastolic blood pressure, triglycerides, cigarette-years, and IgG-OxLDL Abs were significantly associated with IMT in the common carotid artery (P < 0.01). Correspondingly, WHR, systolic and diastolic blood pressure, heart rate, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, and cigarette-years were significantly associated with IMT in the common femoral artery (P < 0.01).

In a multiple regression analysis with the common carotid artery IMT as a dependent variable and the variables significantly associated with IMT (Table 3), systolic blood pressure (F value 42.7), cigarette-years (F value 30.1), and BMI (F value 22.9) turned out to be independent predictors (adjusted R² = 0.15) of common carotid IMT. Correspondingly, the common femoral artery IMT was independently associated with cigarette-years (F value 78.9), systolic blood pressure (F value 45.0), and total cholesterol (F value 32.9). Adjusted R² for the model was 0.21.

Discussion

The results of the present study showed that the common carotid artery IMT was associated with elevated titers of IgG Abs to OxLDL and independently with systolic blood pressure, smoking, and BMI. The femoral artery IMT did not show any statistically significant associations with the IgG Abs but a negative correlation to the IgM Ab titer of OxLDL and independent associations with smoking, systolic blood pressure, and total cholesterol.

We studied serum titers of different types of Abs to modified LDL in venous blood drawn from an antecubital vein. It is reasonable to assume that the observed titers reflect an overall immune response, necessitating knowledge of atherosclerosis in several vascular sites. We assessed IMT in 2 of the most prevalent sites for the atherosclerotic process, ie, the carotid and femoral arteries. In concordance with previous reports, it was found that IMT, as a measure of atherosclerosis, was associated with many established risk

<p>| TABLE 1. Characteristics of Subjects When Divided Into Tertiles of Common Carotid IMT |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Tertiles of Common Carotid Artery IMT</th>
<th>Lowest (n=130)</th>
<th>Middle (n=129)</th>
<th>Highest (n=129)</th>
<th>P for Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI, kg/m²</td>
<td>25.2±4.1</td>
<td>26.5±4.3</td>
<td>27.4±4.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WHR</td>
<td>0.93±0.06</td>
<td>0.94±0.06</td>
<td>0.96±0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic, mm Hg</td>
<td>118±13</td>
<td>122±17</td>
<td>130±18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic, mm Hg</td>
<td>69±9</td>
<td>72±11</td>
<td>75±11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>60±9</td>
<td>60±8</td>
<td>61±8</td>
<td>0.276</td>
</tr>
<tr>
<td>Serum cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total, mmol/L</td>
<td>5.86±1.09</td>
<td>5.99±1.12</td>
<td>6.17±1.11</td>
<td>0.013</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.28±0.38</td>
<td>1.32±0.38</td>
<td>1.21±0.34</td>
<td>0.138</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>3.93±0.94</td>
<td>4.03±0.98</td>
<td>4.17±0.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>1.47±1.14</td>
<td>1.42±0.85</td>
<td>1.80±1.10</td>
<td>0.011</td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
<td>4.74±0.75</td>
<td>4.89±1.24</td>
<td>5.10±1.46</td>
<td>0.017</td>
</tr>
<tr>
<td>Cigarette-years</td>
<td>236±324</td>
<td>339±396</td>
<td>425±487</td>
<td>0.001</td>
</tr>
<tr>
<td>IgG Ab titers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OxLDL</td>
<td>1.07±0.43</td>
<td>1.18±0.41</td>
<td>1.19±0.48</td>
<td>0.032</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>1.07±0.14</td>
<td>1.09±0.17</td>
<td>1.09±0.16</td>
<td>0.211</td>
</tr>
<tr>
<td>IgM Ab titers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OxLDL</td>
<td>1.19±0.29</td>
<td>1.12±0.29</td>
<td>1.14±0.28</td>
<td>0.246</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>1.31±0.37</td>
<td>1.23±0.37</td>
<td>1.26±0.36</td>
<td>0.259</td>
</tr>
</tbody>
</table>

Values are mean±SD.
factors for cardiovascular disease, with smoking as the most important factor in the femoral artery and systolic blood pressure as the most important factor in the carotid artery.36,37 When the Ab titers to modified LDL were examined, the pattern that emerged was that elevated titers of IgG Abs to OxLDL were positively associated with IMT in the carotid artery, whereas the IgM Ab to OxLDL was inversely associated with IMT in the femoral artery. The data from the present cross-sectional study do not allow an interpretation of a difference in the immune response between the carotid and femoral arteries. However, the reverse relationship between the IgG and IgM Abs versus ultrasound-assessed atherosclerosis is also supported by the negative association between IgG and IgM Ab titers. Our interpretation of the results is that elevated IgG Abs to OxLDL accompanied the atherosclerotic process, whereas the IgM Abs showed a reversed response. These associations were not independent of established risk factors for atherosclerotic disease.

To our knowledge, this is the first study that has examined the relationship between different Abs to modified LDL and different vascular territories in subjectively clinically healthy subjects recruited from the general population. In a previous study using ultrasound examinations of the carotid and femoral arteries of hypertensive subjects, a positive association was found between autoantibodies against OxLDL and MDL-LDL and carotid IMT in patients without atherosclerotic plaques.38

We studied 58-year-old, white, untreated men of Swedish ancestry who were chosen to minimize the effect of confounding factors such as race, ethnicity, sex, age, and treatment with different drugs for cardiovascular disease. We believe that eliminating these factors enhanced the possibility of studying the relationship between IMT and Ab titers and modified LDL. The limitation is that women were not examined and that there was a stratified selection related to the degree of estimated insulin sensitivity. However, the relationship between cardiovascular risk factors and measured IMT followed the expected pattern.

OxLDL is believed to act as a driving antigen in the complicated immune response to atherosclerosis.7 It has been hypothesized that Ab titers to OxLDL may reflect the LDL Ab level and therefore confer important information. Some studies have reported results supporting such a concept.9–12 Circulating Abs against OxLDL have also been suggested to be an independent predictor for IMT progression in the carotid artery.14 The data from the present study showing a positive relationship between IMT in the common carotid artery and IgG-OxLDL Abs corroborate these earlier studies (eg, that the IgG response to modified LDL is proatherogenic).

However, it may also be postulated that the physiological function of Abs to OxLDL and related compounds is to participate in the removal of these agents from the artery wall and to have a protective role (eg, an antiatherogenic role of the humoral immune system), similar to the defense mechanisms in infectious disease. In support of the concept that the immune response might be antiatherogenic rather than proatherogenic are recent reports that have indicated that immunization of experimental animals with OxLDL leads to dramatically enhanced IgG levels and inhibits the progression of atherosclerosis.39–41 A published study indicated that low Ab levels may be associated with more pronounced athero-
sclerotic disease. In a previous study in our laboratory, we showed that subjects with familial hypercholesterolemia and previous myocardial infarction had significantly lower IgM titers compared with titers in subjects with familial hypercholesterolemia without previous myocardial infarction but also compared with titers in control subjects.

We observed that contrary to IgG-OxLDL Abs, IgM-OxLDL Abs were lower in subjects with a thick intima-media complex in the common femoral artery. The discrepancy relating to the above-mentioned findings could be due to different Ab responses in different stages of the atherosclerotic process or to different responses in different segments of the vessel wall. The present study does not give any information on underlying mechanisms. However, on basis of earlier studies, one might also speculate that the immune system has a partly protective role. As suggested by Hansson, the adaptive immune system appears to inhibit certain aspects of atherosclerosis, eg, smooth muscle cell proliferation and scavenger receptor expression, whereas other important components are enhanced, such as macrophage activation and protease secretion. A protective role of the humoral immune response is suggested by the animal studies mentioned above and in 1 recent study, in which Nicoletti et al found that 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 OxLDL in the present study may reflect T-cell-independent B-cell activation.

To summarize, in 58-year-old subjectively clinically healthy men recruited from the general population, there was a positive association between IgG-OxLDL Ab and IMT in the common carotid artery and a negative association between IgM-OxLDL Ab and IMT in the common femoral artery. However, these associations were not independent of other risk factors, such as metabolic variables and smoking.
TABLE 3. Covariates to IMT of Common Carotid Artery and Femoral Artery

<table>
<thead>
<tr>
<th>Variable</th>
<th>Femoral Artery</th>
<th>Common Carotid Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>WHR</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Systolic</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Heart rate</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Total</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>LDL</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>HDL</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cigarette-years</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>IgG-OxLDL Ab</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*p < 0.01.

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References


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