Abstract—Oxidation of low density lipoproteins (LDL) obviously plays an important role in the pathogenesis of atherosclerosis. The purpose of the study was to determine whether antibodies against oxidized LDL are associated with coronary artery disease (CAD). We determined the serum levels of antibodies against copper-oxidized LDL by enzyme-linked immunoassorbent assay in 58 patients with angiographically verified CAD and 34 controls without CAD. The mean antibody level, expressed in optical density units, was significantly higher in patients than in controls (0.150±0.088 versus 0.094±0.054, respectively; \( P = 0.0089 \)). In logistic regression analysis, high antibody level against oxidized LDL was associated significantly with CAD (\( P = 0.0114 \)), independent of age (\( P = 0.00137 \)), gender (\( P = 0.0021 \)), body mass index (\( P = 0.5947 \)), triglyceride concentration (\( P = 0.9813 \)), and total cholesterol−high density lipoprotein (HDL) cholesterol (\( P = 0.0080 \)) group. Similar analysis in nondiabetic subjects (\( n = 79 \)) and in men only (\( n = 75 \)) showed analogous results, with only minor changes in \( P \) values. The antibody level against oxidized LDL differed significantly between nonsmokers and smokers in CAD patients (\( P < 0.00197 \)) but not in controls (\( P \) NS). In addition, the antibody level against oxidized LDL differed significantly between nonsmokers and smokers in subjects with low HDL cholesterol (≤0.9 mmol/L) but not in subjects with high HDL cholesterol (>0.9 mmol/L). In conclusion, elevated levels of antibodies against oxidized LDL were associated with CAD. The data suggest that oxidized LDL plays a role in the pathogenesis of atherosclerosis and suggest a protective function for HDL against LDL oxidation. (Arterioscler Thromb Vasc Biol. 1999;19:23-27.)

Key Words: autoantibodies ■ coronary artery disease ■ low density lipoproteins ■ oxidized lipoproteins

Oxidized low density lipoproteins (LDLs) are believed to play an important role in the progression of atherosclerosis.\(^1\) Oxidative modification of LDL is a prerequisite for rapid accumulation of LDL in macrophages and for the formation of foam cells. LDL isolated from atherosclerotic lesions, but not from normal arteries, resembles oxidized LDL in its physical, chemical, and immunological properties.\(^2\) Epitopes characteristic of oxidized LDL can be found in atherosclerotic lesions by immunocytochemical techniques.\(^3\),\(^4\) and atherosclerotic lesions contain immunoglobulins that recognize oxidized LDL.\(^5\),\(^6\) In addition, antioxidant therapy reduces atherogenesis in animal models.\(^5\),\(^6\) Antibodies against malondialdehyde (MDA)-modified LDL, detected by radioimmunoassay, have been reported to be predictive of the progression of carotid atherosclerosis,\(^7\) coronary artery disease (CAD),\(^8\) and myocardial infarction.\(^9\) Furthermore, recent results from Heitzer et al\(^10\) and Raitakari et al\(^11\) indicate that antibodies against copper-oxidized LDL are associated with impaired endothelial function.

The purpose of this work was to determine whether antibodies against copper-oxidized LDL are associated with angiographically verified CAD and whether the autoantibody levels are associated with high density lipoprotein (HDL) cholesterol concentrations.

Methods

Subjects

The subjects were selected from patients referred from 1988 to 1990 to the Tampere University Hospital for coronary angiography because of chest pain or otherwise clinically suspected CAD. The study group of 58 patients (54 men and 4 women) consisted of subjects whose plasma samples were available for determination of antibodies against oxidized LDL. All had angiographically verified 3-vessel CAD. The control group consisted of 34 patients (21 men and 13 women) without angiographically significant CAD (stenosis <50%).

The average age, body mass index (BMI), and levels of major CAD risk factors of the patient and the control groups are seen in Table 1. Each patient was questioned by a doctor regarding smoking habits, hypertension, diabetes mellitus, and medication usage. The

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study protocol was approved by the Ethical Committee of the Tampere University Hospital.

**Coronary Angiography and Blood Samples**

CAD was confirmed by a cardiologist using standard Judkins technique.15 A transmural narrowing of ≥50% was defined as significant. Fasting blood samples were collected into EDTA tubes from 92 participants. Plasma was separated by centrifugation (2000 rpm, 20 minutes) and frozen (−20°C) for up to 2 years until analyzed.

**Lipid Analyses**

Serum cholesterol and triglycerides were determined by enzymatic methods (Nycoest, Nycomed AS) using Monarch 2000 analyzer and SeroNorm Lipid (Nycomed AS) as standard. HDL cholesterol was measured with primary cholesterol reagent.13 The HDL assay was calibrated with primary cholesterol standard, 2.5 mmol/L (Orion No. 530). The interassay coefficients of variation were 2.8% for cholesterol, 3.6% for triglycerides, and 6.3% for HDL cholesterol, when frozen (20°C) pooled human serum was analyzed daily for 3 to 5 months. LDL cholesterol concentration was calculated with Friedewald’s formula.14 Lipid levels were measured with the same enzymatic method after precipitation of LDL and very low density lipoprotein with polyethylene glycol 6000 (Polysciences). Triglycerides were determined with a modified enzymatic method (Nycotest, Nycomed AS) using Monarch 2000 analyzer and the Triglyceride assay was calibrated with primary triglyceride standard, 1.27 mmol/L (Orion No. 540). The intra-assay coefficient of variation for the antibodies against oxidized LDL was 8.5%.

As an additional check, we run some of the sera in a separate assay in which 0.27 mmol/L PBS, 20 µmol/L BHT for 2 hours at 4°C. After removal of the unbound antigen and washing of the wells, the remaining nonspecific binding sites were saturated using 2% human serum albumin in PBS and 20 µmol/L BHT for 2 hours at 4°C. After incubation, the wells were aspirated and washed 6 times before an IgG-peroxidase–conjugated rabbit anti-human monoclonal antibody (No. 55220 Cappel, Organon), diluted 1:4000 (vol/vol) in buffer (0.27 mmol/L PBS, 20 µmol/L EDTA, 1% BHT, 0.05% Tween HSA), was added to each well for 4 hours at 4°C. After incubation and washing, 50 µL of the serum samples (diluted 1:20) were added to wells coated with native and oxidized LDL and incubated overnight at 4°C. After incubation, the wells were aspirated and washed 6 times before an IgG-peroxidase–conjugated rabbit anti-human monoclonal antibody (No. 55220 Cappel, Organon), diluted 1:4000 (vol/vol) in buffer (0.27 mmol/L PBS, 20 µmol/L EDTA, 1% BHT, 0.05% Tween HSA), was added to each well for 4 hours at 4°C. After incubation and washing, 50 µL of freshly made substrate (0.4 mg/mL o-phenylenediamine [Sigma] and 0.045% H₂O₂ in 100 mmol/L acetate buffer, pH 5.0) was added and incubated for exactly 5 minutes at room temperature. The enzyme reaction was terminated by adding 50 µL of 2 M H₂SO₄. The optical density (OD) was measured at 492 nm using a microplate reader (Multiskan MCC/330, Labsystems GmbH).

All measurements were blinded and done on coded serum samples. The results were expressed as the mean OD values from duplicate determinations, and level of autoantibody reactivity against oxidized LDL was calculated by subtracting the binding of antibodies to native LDL from that to copper-oxidized LDL. This approach reduces the possibility of getting false-positive values due to cross-reactivity with both LDL epitopes. The intra-assay coefficient of variation for the antibodies against oxidized LDL was 8.5%.

**Statistical Methods**

The results are expressed as mean±SD, unless otherwise stated. In Table 1, Mann-Whitney U test was used in group mean comparisons, and risk factor frequencies between patients and controls were compared by χ² test. To study associations between antibody reactivity levels and classic risk factors, Pearson correlation coefficients were calculated. The effect of different groups on oxidized LDL autoantibody reactivity against oxidized LDL was calculated by subtracting the binding of antibodies to native LDL from that to copper-oxidized LDL. This approach reduces the possibility of getting false-positive values due to cross-reactivity with both LDL epitopes. The intra-assay coefficient of variation for the antibodies against oxidized LDL was 8.5%.

### Table 1 Major Risk Factor Levels in Patients and Controls (Mean±SD)

<table>
<thead>
<tr>
<th>Major Risk Factor</th>
<th>Patients With CAD (n=58)</th>
<th>Controls (n=34)</th>
<th>All Subjects (n=92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>54/4</td>
<td>21/13</td>
<td>75/17</td>
</tr>
<tr>
<td>Age, y</td>
<td>59.0±6.18*</td>
<td>54.5±7.54</td>
<td>57.4±7.00</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.1±2.90*</td>
<td>25.4±4.44</td>
<td>26.5±3.61</td>
</tr>
<tr>
<td>Smoker, % (n)</td>
<td>50.0 (29)</td>
<td>58.8 (20)</td>
<td>53.2 (49)†</td>
</tr>
<tr>
<td>Hypertension, % (n)</td>
<td>53.4 (31)‡</td>
<td>30.3 (10)</td>
<td>45.1 (41)</td>
</tr>
<tr>
<td>Diabetes, % (n)</td>
<td>19.0 (11)</td>
<td>6.1 (2)</td>
<td>14.3 (13)</td>
</tr>
<tr>
<td>Hypercholesterolemia, % (n)</td>
<td>55.2 (32)‡</td>
<td>30.3 (10)</td>
<td>46.2 (42)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.50±1.33</td>
<td>5.92±1.16</td>
<td>6.29±1.30</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>2.35±1.86</td>
<td>1.81±0.90</td>
<td>2.16±1.60</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>0.86±0.24§</td>
<td>1.16±0.41</td>
<td>0.97±0.34</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.58±1.27</td>
<td>4.03±1.08</td>
<td>4.39±1.23</td>
</tr>
<tr>
<td>Ox-LDL ab, OD</td>
<td>0.244±0.105§</td>
<td>0.179±0.087</td>
<td>0.220±0.092</td>
</tr>
<tr>
<td>Nat-LDL ab, OD</td>
<td>0.095±0.053</td>
<td>0.088±0.053</td>
<td>0.092±0.052</td>
</tr>
<tr>
<td>Ox-LDL ab:nat-LDL ab ratio</td>
<td>3.12±3.13</td>
<td>2.25±1.28</td>
<td>2.63±0.129</td>
</tr>
</tbody>
</table>

Hypercholesterolemia = total cholesterol >6.5 mmol/L. Ox indicates oxidized; ab, autoantibodies; and nat, native.

Mann-Whitney U test for means and χ² test for frequencies.

*P<0.01, patients vs controls.
†Nine ex-smokers included.
‡P<0.05, patients vs controls.
§P<0.001, patients vs controls.

### Enzyme-Linked Immunosorbent Assay for Antibodies Against Oxidized LDL

Autoantibodies against oxidized LDL were determined as described earlier.15 In short, antigens for this assay included (1) native LDL prepared from the pooled plasma of 10 donors and protected against oxidation by 0.27 mmol/L EDTA and 20 µmol/L butylated hydroxytoluene (BHT) in PBS, and (2) oxidized LDL obtained after 24-hour oxidation of the native LDL with 2 µmol/L CuSO₄.

For enzyme-linked immunosorbent assay, half of the wells on a polystyrene plate (Nunc) were coated with 50 µL of native antigen, and the other half was coated with 50 µL copper-oxidized LDL antigen (both at a concentration of 5 µg/mL) in PBS for 16 hours at 4°C. After removal of the unbound antigen and washing of the wells, the remaining nonspecific binding sites were saturated using 2% human serum albumin in PBS and 20 µmol/L BHT for 2 hours at 4°C. After washing, 50 µL of the serum samples (diluted 1:20) were added to wells coated with native and oxidized LDL and incubated overnight at 4°C. After incubation, the wells were aspirated and washed 6 times before an IgG-peroxidase–conjugated rabbit anti-human monoclonal antibody (No. 55220 Cappel, Organon), diluted 1:4000 (vol/vol) in buffer (0.27 mmol/L PBS, 20 µmol/L EDTA, 1% BHT, 0.05% Tween HSA), was added to each well for 4 hours at 4°C. After incubation and washing, 50 µL of freshly made substrate (0.4 mg/mL o-phenylenediamine [Sigma] and 0.045% H₂O₂ in 100 mmol/L acetate buffer, pH 5.0) was added and incubated for exactly 5 minutes at room temperature. The enzyme reaction was terminated by adding 50 µL of 2 M H₂SO₄. The optical density (OD) was measured at 492 nm using a microplate reader (Multiskan MCC/330, Labsystems GmbH).

All measurements were blinded and done on coded serum samples. The results were expressed as the mean OD values from duplicate determinations, and level of autoantibody reactivity against oxidized LDL was calculated by subtracting the binding of antibodies to native LDL from that to copper-oxidized LDL. This approach reduces the possibility of getting false-positive values due to cross-reactivity with both LDL epitopes. The intra-assay coefficient of variation for the antibodies against oxidized LDL was 8.5%.
ing explanatory (independent) variables: age, total cholesterol, HDL cholesterol, triglycerides, BMI, gender, LDL autoantibody level, and total/HDL cholesterol group. The 4 groups of cholesterol/HDL cholesterol were formed using the following arbitrary cut-off points: HDL \( \leq 0.9 \) or >0.9 mmol/L and total cholesterol >6.5 mmol/L or \( \leq 6.5 \) mmol/L. All statistical analyses were made using a microcomputer with the STATISTICA/Win program package (Statsoft, Inc). Statistical significance was \( P<0.05 \).

**Results**

The levels and differences of major coronary risk factors in patients and controls are shown in Table 1. Antibodies to oxidized LDL were found in both subjects with CAD and controls, but the mean reactivity level, expressed in OD units, was significantly higher in patients with CAD than in controls (0.150±0.088 versus 0.094±0.054, respectively; \( P=0.00089 \); Figure 1). Similar analysis in nondiabetic subjects (n=79; \( P=0.00085 \)) and in men only (n=75; \( P=0.00180 \)) showed analogous results. In addition, we analyzed our data by using the calculation method proposed by Salonen et al,\(^7\) in which autoantibody reactivity is expressed as the ratio of antibody binding to copper-oxidized LDL divided by antibody binding to native LDL. However, in our experiment, the LDL autoantibody ratio between CAD patients and controls was not statistically significant (\( P=0.065 \); Table 1).

Factorial ANCOVA (\( 2 \times 2 \); dimensions, high/low total cholesterol and high/low HDL cholesterol) revealed a significant difference in plasma antibodies against oxidized LDL (\( P<0.0198 \)) between subjects with low and high HDL cholesterol levels but not between subjects with low (\( \leq 6.5 \) mmol/L) and high (>6.5 mmol/L) total cholesterol levels (\( P=0.6849 \)). Subjects with a combination of both risk factors, ie, high total cholesterol and low HDL cholesterol, tended to have a higher level of antibody reactivity against oxidized LDL (0.159±0.094) than other groups.

When smoking status was taken into consideration by using 2-way ANCOVA (dimensions, smoking/nonsmoking and controls/CAD patients), the analysis revealed a significant study group by smoking status interaction (\( P<0.0235 \)). The antibody reactivity level against oxidized LDL differed significantly between nonsmokers and smokers in subjects with low HDL (\( \leq 0.9 \) mmol/L) cholesterol but not in subjects with high HDL (>0.9 mmol/L) cholesterol (\( P=NS \); Figure 2B). Again, subjects with a combination of both risk factors, ie, smoking and low HDL cholesterol, showed a marked increase in the level of antibody reactivity against oxidized LDL (0.176±0.114; \( P<0.0277 \) for trend in 1-way ANCOVA) versus other groups (Figure 2B). All result were adjusted by age and BMI.

In logistic regression analysis, high antibody reactivity level against oxidized LDL was associated significantly with CAD (\( P=0.0114 \)), independent of age (\( P=0.0014 \)), gender (\( P=0.0021 \)), BMI (\( P=0.5947 \)), triglyceride concentration (\( P=0.9813 \)), and total cholesterol/HDL cholesterol group (\( P=0.0080 \)). Similar analysis in nondiabetic subjects (n=79) and in men only (n=75) showed analogous results, with only minor changes in \( P \) values.

There was a significant negative correlation (\( r=−0.42; P<0.05 \)) between HDL cholesterol and antibody reactivity.
levels against oxidized LDL in patients with both CAD and total cholesterol >6.5 mmol/L but no correlation (r=0.05; P=NS) in patients with total cholesterol ≤6.5 mmol/L.

Discussion

Our study group of 58 patients consisted of subjects referred to the Tampere University Hospital from 1988 to 1990 for coronary angiography because of chest pain or otherwise clinically suspected CAD. Unfortunately, there were only 4 women in our study group; this low number of women is a common problem in these kinds of studies, because the prevalence of CAD is higher in men than in women. Consequently, we could neither study whether antibodies against oxidized LDL are associated with CAD in women nor compare the antibody reactivity against oxidized LDL in men and women.

In the present study, antibodies against oxidized LDL were measured using copper-oxidized LDL as the antigen. In some previous studies, MDA-modified LDL has been used as an antigen in similar assays.7,8,16,17 MDA-lysine epitopes in MDA-modified LDL represent 1 class of oxidation-derived epitopes generated during LDL oxidation, but there are also many others, such as hydroxynonenal (HNE) epitopes and other peroxidation-derived aldehyde adducts.1 Antibodies against copper-oxidized LDL was selected, because copper-modified LDL contains a collection of various epitopes typical for oxidation process and thus may mimic the situation in the arterial wall better than MDA-LDL or HNE-LDL. However, the density of each of the oxidation-derived epitopes in copper-oxidized LDL is likely to be much lower than in MDA-LDL or HNE-LDL, which rely on only 1 or a few epitopes generated during the reaction with aldehydes. Consequently, assays using copper-oxidized LDL as the antigen may be less sensitive than the assays using MDA-LDL or HNE-LDL but should reflect a more generalized immunoresponse against oxidized LDL. There are some previous studies showing increased oxidation susceptibility of LDL in coronary bypass and CAD patients, when formation of conjugated dienes is measured after exposure of LDL to CuSO4.18,19 These studies are in line with our results, but the measured parameters, ie, shorter lag phase and faster propagation rate in CAD patients, do not necessarily give information on the oxidation process that occurs in vivo.

Mironova et al20 demonstrated that the autoantibodies against oxidized LDL are predominantly of moderate to low affinity. In the present study, we determined autoantibody reactivity at only 1 dilution of the serum sample (1:50). Previously, we used 3 different plasma dilutions (1:20, 1:50, and 1:100), but results have been essentially similar with different dilutions.15 Also, we have found that immunoglobulins isolated from atherosclerotic lesions using protein G high-performance liquid chromatography method react in ELISA assay quite reproducibly irrespective of the dilution of the sample.2 In some previous studies, autoantibody reactivity is expressed as the ratio of antibody binding to copper-oxidized LDL divided by antibody binding to native LDL as originally proposed by Salonen et al.7 In our study, the level of the autoantibody reactivity against oxidized LDL was calculated by subtracting the binding of antibodies to native LDL from that to copper-oxidized LDL. Improved autoantibody assay has allowed us to use OD readings as final results. Similar analysis has been used previously and been found useful to report the results.10

In the present study, the antibody reactivity level against oxidized LDL was found to be significantly higher in subjects with CAD than in controls. Oxidative modification of LDL is thought to be a key process in the development of endothelial dysfunction10,11,21 and atherosclerosis.1,12,23 Because of the high antioxidant levels in plasma,21 LDL oxidation is suggested to occur mainly in subendothelial space of the arterial wall, where the concomitant presence of large amounts of reactive oxygen species generated by endothelial cells, activated leukocytes, and transition metals, such as copper,24 would be a sufficient stimulus to initiate the peroxidation of LDL lipids, leading to oxidized LDL found in atherosclerotic lesions.4

We found that the antibody reactivity levels against oxidized LDL were significantly higher in subjects with low HDL cholesterol together with hypercholesterolemia or smoking than in subjects with high HDL cholesterol concentration together with these other risk factors. High levels of HDL cholesterol are associated with decreased risk of atherosclerosis. Because HDL is able to pass through the vascular endothelium and reach the subendothelial space of the intima, there might be an interaction between HDL and LDL. In previous studies, it has been shown that HDL prevents the cytotoxicity and atherogenic properties of LDL25,26 and that HDL inhibits the oxidation of LDL.27,28 HDL (or some of its components, eg, apolipoprotein E) may inhibit the immunogenic response against oxidized LDL and thus diminish the formation of antibodies against oxidized LDL.29,30 In apolipoprotein E–deficient mice, there are high levels of antibodies against epitopes of MDA-, 4-HNE–, and copper-modified LDL, and there is also an extensive deposition of IgG-, IgM-, and oxidation-specific epitopes in their atherosclerotic lesions.29,30 Our results might reflect the protective effect of HDL against LDL oxidation in vivo.

Our results are in agreement with the previous results, indicating that antibodies against MDA-modified LDL are associated with progression of atherosclerosis,7 severity of CAD,8 and myocardial infarction.9 Some of the autoantibodies are found to cross-react with MDA-modified LDL and cardiolipin.31,32 On the other hand, anti-cardiolipin antibodies also predict the risk of myocardial infarction.33 Because of this cross-reactivity of the autoantibodies determined in ELISA, further studies are needed to evaluate the usefulness of different oxidized LDL autoantibody assays in predicting the progression of atherosclerotic vascular diseases. Our results suggest that oxidized LDL plays a role in the pathogenesis of atherosclerosis and may indicate the protective role of HDL against LDL oxidation.

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Autoantibodies Against Oxidized Low Density Lipoprotein in Patients With Angiographically Verified Coronary Artery Disease

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