Circulating Oxidized Low-Density Lipoprotein and Its Association With Carotid Intima-Media Thickness in Asymptomatic Members of Familial Combined Hyperlipidemia Families

Ming-Lin Liu, Kati Ylitalo, Riitta Salonen, Jukka T. Salonen, Marja-Riitta Taskinen

Objective—Oxidized low-density lipoprotein (Ox-LDL) is implicated in the pathogenesis of atherosclerosis. Circulating oxidation-specific epitopes on plasma Ox-LDL has been linked with coronary artery disease, but its determinants and its association with early development of atherosclerosis in familial combined hyperlipidemia (FCHL) has not been very well studied. This study aimed to investigate the determinants of the circulating Ox-LDL and the association between Ox-LDL and carotid intima-media thickness (IMT) in asymptomatic members of FCHL families.

Methods and Results—Ox-LDL, susceptibility of LDL to oxidation in vitro, plasma 8-isoprostane and antioxidants, lipids and lipoproteins, LDL particle size, and carotid IMT were measured in 150 asymptomatic FCHL family members. Affected FCHL family members had reduced LDL particle size and lag time for LDL oxidation, increased plasma levels of Ox-LDL, increased plasma urate and α-tocopherol, and a trend for the increase of 8-isoprostane as compared with nonaffected FCHL. Ox-LDL was independently associated with serum LDL cholesterol, apoB, and 8-isoprostane in multivariate analysis but only univariately correlated with LDL particle size and lag time for LDL oxidation. In addition, Ox-LDL was significantly associated with carotid mean IMT independently of other clinical and biochemical variables in a multivariate model.

Conclusion—Serum LDL cholesterol, apoB levels, and 8-isoprostane were the most important determinants of Ox-LDL. Ox-LDL is independently associated with carotid IMT in asymptomatic FCHL family members and can be used as a marker of early atherosclerosis in FCHL. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: carotid arteries hyperlipoproteinemia familial combined lipoproteins low-denisty lipoprotein oxygen radical ultrasonography

There is substantial evidence that oxidized low-density lipoprotein (Ox-LDL) is present in vivo within atherosclerotic lesions of arteries. Under the oxidative stress, oxidative modification of LDL may take place in the subendothelial space of the arterial wall, and a small amount of Ox-LDL may also be released into the circulation. When “fully oxidized LDL” enters the circulation in minor quantities, it will be rapidly cleared by the reticuloendothelial system, particularly in the liver, or it will be removed by the preexisting circulating autoantibodies to Ox-LDL. In contrast, the “minimally modified LDL,” in which oxidative modification has not been sufficient to cause changes recognized by scavenger receptors, can be found in circulation. Other studies have defined the presence of oxidation-specific epitopes on plasma LDL or baseline levels of conjugated dienes in lipids extracted from LDL (LDL-BDC) as measures of LDL oxidation in vivo. Recently, several groups have developed several specific methods to measure circulating Ox-LDL using different anti-Ox-LDL antibodies. As a sensitive biochemical marker, Ox-LDL has been related to coronary artery disease (CAD) in several clinical studies. Plasma Ox-LDL has also been associated with subclinical atherosclerosis in clinically healthy population. Interestingly, high plasma and plaque levels of Ox-LDL are associated with the vulnerability of the plaques. However, the origin of plasma Ox-LDL as well as its determinants are unknown.

Familial combined hyperlipidemia (FCHL) is characterized with elevated levels of plasma cholesterol and/or triglyceride. FCHL associates with early atherosclerosis and is responsible for >10% of premature CAD. Experimental studies have observed the enhanced generation of free radi-
carboxylic acids in leukocytes from hypercholesterolemic and hypertriglyceridemic patients. Previous studies also reported the increased formation of isoprostanes, markers of in vivo oxidative stress, in hyperlipidemic patients. Interestingly, our data have shown an adaptive increase of circulating antioxidants with increased oxidative stress in asymptomatic FCHL patients. In addition, LDL from FCHL patients is characterized by a predominance of small dense LDL and an increased susceptibility to oxidative modification. All these factors may influence the metabolism of circulating Ox-LDL in FCHL.

Carotid artery intima-media thickness (IMT), measured noninvasively by high-resolution B-mode ultrasonography, has been associated with the risk of CAD, stroke, and myocardial infarction, and it predicts the progression of CAD. The present study was conducted to investigate the determinants of plasma Ox-LDL and the association between Ox-LDL and carotid IMT in asymptomatic FCHL family members.

Methods

Study Subjects

The study subjects were recruited according to the study protocol as reported previously. All subjects gave their informed consent to the study protocol, which was approved by the ethical committees. Briefly, the FCHL probands were required to be 30 to 60 years of age, have verified CAD, and have serum total cholesterol (TC) and/or triglycerides (TG) age- and sex-specific levels in the 90th Finnish population percentiles. The TC and TG percentiles used in the present study were derived from the results of the surveys based on the Finnish population. Families with ≥2 affected family members presenting different lipid phenotypes were classified as FCHL. Family members who had diabetes or history of CAD or stroke and those with lipid medication were excluded. As described previously, altogether 150 FCHL (75 affected and 75 nonaffected defined according to the aforementioned lipid criteria) family members from 38 well-defined Finnish FCHL families participated in the present study.

Venous blood samples were collected after overnight fasting for the biochemical measurements. EDTA plasma was separated by centrifugation and stored at −80°C until analyzed. Pulse pressure was calculated as the difference between the systolic and diastolic blood pressures. Pack-years were calculated by multiplying duration of smoking by the number of cigarettes smoked per day divided by 20.

Measurement of Plasma Ox-LDL and LDL Oxidation In Vitro

Plasma levels of Ox-LDL were measured by a competitive enzyme-linked immunosorbent assay using a specific marine monoclonal antibody mAb-4E6 (Mercodia, Uppsala, Sweden). The coefficient of variation for the assay was 7.4% to 8.3%. The mAb-4E6 is directed against a conformational epitope in the apolipoprotein B-100 (apoB-100) moiety of LDL that is generated as a consequence of substitution of at least 60 lysine residues of apoB-100 with aldehydes. This number of substituted lysines corresponds to the minimal number of substituted lysines required for scavenger-mediated uptake of oxLDL. Substituting aldehydes can be produced by peroxidation of lipids of LDL. LDL particle size was determined using gradient gel electrophoresis. All lipid and lipoprotein measurements were performed as described previously. Briefly, serum TC and TG concentrations were determined enzymatically, serum high-density lipoprotein (HDL) cholesterol by precipitation procedures, and serum apolipoprotein B (apoB) concentration by an immunoturbidimetric assay. LDL was separated by sequential flotation as described.

Ultrasound Examinations

Carotid IMT was determined as described previously. Briefly, longitudinal images from 3 projections (anterolateral, lateral, and posterolateral) were measured by Hewlett-Packard Image Point M2410A ultrasound system for the common carotid artery, carotid bulb, and internal carotid artery. Measurements were performed at a total of 28 sites, both the far wall and the near wall of the arterial segments, right and left distal 1 cm of common carotid artery, carotid bulb, and proximal 1 cm of and internal carotid artery. All 3 projections in common carotid artery and carotid bulb, and a single angle in and internal carotid artery with the best visibility were used. The mean, maximum, and minimum IMT were derived from each measurement. The average of all mean IMT measurements over 28 (or fewer) sites was chosen as the outcome variable. The coefficient of variation for mean IMT measurements was 2.4%. Carotid IMT examination was performed at the same visit as blood sampling or within a difference of few weeks.

Statistical Analysis

Values are given as means ± SE. Variables with nonnormal distribution were log10-transformed. Differences in means between affected and nonaffected family members or among the different Ox-LDL tertile groups were tested by 2-way ANOVA (Figure A). The frequency distribution of the categorical variables between 2 groups was compared by the χ² test. Univariate association was tested by Pearson correlation analysis. The predictors for the subsequent multivariate analysis were selected on the basis of the correlation analysis (P < 0.20). Backward multivariate analyses were performed to assess the predictors of Ox-LDL and carotid mean IMT. Both in the ANOVA and the backward multivariate analyses, family number (which indicates belonging to a certain family) was used to correct for the dependence of the study subjects.

Results

Ox-LDL and Other Study Variables of the FCHL Family Members

Table 1 summarizes clinical and other study variables of the subjects. By definition, TC, TG, LDL cholesterol, and apoB were significantly higher in affected than in nonaffected family members. Affected family members had significantly smaller LDL size, shorter lag time for LDL oxidation, and higher plasma Ox-LDL as compared with those in nonaffected members. Plasma 8-isoprostane levels tended to be higher in affected than in nonaffected family members, but the difference did not reach statistical significance. Plasma α-tocopherol and urate were significantly increased in affected FCHL compared with those in nonaffected family members as reported in our previous study. Other plasma antioxidants (vitamin C, thiol groups, β-carotene, retinol) were comparable between the 2 groups (data not shown).

As reported previously, we did not see significant difference in carotid mean IMT between affected and nonaffected family members.
mean IMT in highest Ox-LDL tertile group was significantly thicker than that in lowest Ox-LDL tertile (0.78±0.02 versus 0.69±0.02 mm, P=0.003). In addition, there were more affected subjects in the highest Ox-LDL tertile group (40/50) than in the middle (23/50) and in the lowest (12/50) Ox-LDL tertile groups (P<0.001, ANOVA; Figure A). Plasma Ox-LDL correlated with carotid mean IMT in affected and nonaffected family members as well as in the combined group including all family members (Figure B).

Carotid mean IMT correlated significantly with age, body mass index, smoking pack-years, pulse pressure, log TG, LDL cholesterol, apoB, LDL size, lag time for LDL oxidation, plasma urate, and α-tocopherol, as well as Ox-LDL. However, we did not observe any correlation between 8-isoprostane and mean IMT in univariate analysis. In the multivariate analysis, only age (β=0.745, P<0.001), pulse pressure (β=0.158, P=0.004), LDL size (β = −0.169, P=0.022), and Ox-LDL (β=0.178, P=0.038) were independent predictors for the variation of carotid mean IMT. The association between Ox-LDL and mean IMT persisted even after adjustment for logTG, LDL cholesterol, and apoB. These results demonstrate that Ox-LDL is associated with mean IMT in FCHL family members independently of clinical and lipid variables.

Discussion
The present study shows that LDL cholesterol, apoB, and 8-isoprostane were independent determinants of plasma Ox-LDL. The Ox-LDL was associated with carotid mean IMT independently of other variables in asymptomatic FCHL family members. Therefore, the Ox-LDL is a useful marker of early atherosclerosis in FCHL.

Circulating Ox-LDL, 8-Isoprostane, and Antioxidants
There is increased oxidative stress in dyslipidemia. In this study, the plasma Ox-LDL was significantly higher in affected FCHL family members as compared with nonaffected subjects. Likewise, there was a trend for an increase of the plasma 8-isoprostane in affected FCHL family members. The present data confirmed our previous results showing an adaptive increase of plasma antioxidants (α-tocopherol and urate) in the presence of the increased oxidative stress in FCHL. Recent data suggested that supplementation of α-tocopherol cannot prevent lipoprotein oxidation in the vessel wall with the increased levels of α-tocopherol in the circulation and in the arterial wall. A population study showed that α-tocopherol supplementation in healthy individuals increases plasma levels of α-tocopherol and reduces LDL oxidative susceptibility and circulating oxidized LDL. In contrast, several other clinical studies have reported that supplementation with α-tocopherol has no effect on autoantibodies against Ox-LDL in hyperlipidemic patients, in patients with chronic renal failure, or in chronic smokers. In agreement with the latter results, the plasma levels of Ox-LDL were increased in affected FCHL family members, despite the elevation of plasma α-tocopherol. In addition, the low-fat, high-vegetable diet, which increased plasma concentrations of vitamin C, β-carotene, and lycopene, failed to
decrease plasma levels of Ox-LDL measured with mAb-EO6. The positive correlation between Ox-LDL and plasma antioxidants (Table 2) in FCHL family members may reflect the fact that adaptive increase in plasma antioxidants cannot

TABLE 1. Clinical and Biochemical Characteristics and Other Study Variables in FCHL Family Members

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total (n=150)</th>
<th>Affected (n=75)</th>
<th>Nonaffected (n=75)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>40.3 ± 0.9</td>
<td>40.4 ± 1.3</td>
<td>40.1 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>56/94</td>
<td>29/46</td>
<td>27/48</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking pack-years</td>
<td>6.6 ± 0.7</td>
<td>7.0 ± 1.1</td>
<td>6.2 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.6 ± 0.3</td>
<td>26.5 ± 0.5</td>
<td>24.7 ± 0.4</td>
<td>0.025</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
<td>47.6 ± 1.0</td>
<td>49.1 ± 1.6</td>
<td>46.1 ± 1.1</td>
<td>0.019</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>5.80 ± 0.09</td>
<td>6.40 ± 0.13</td>
<td>5.20 ± 0.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.65 ± 0.12</td>
<td>2.18 ± 0.21</td>
<td>1.13 ± 0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>3.57 ± 0.08</td>
<td>3.99 ± 0.12</td>
<td>3.13 ± 0.09</td>
<td>0.002</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.49 ± 0.04</td>
<td>1.41 ± 0.05</td>
<td>1.56 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>102.0 ± 2.6</td>
<td>119.3 ± 3.6</td>
<td>84.5 ± 2.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL particle size, nm</td>
<td>26.6 ± 0.1</td>
<td>26.0 ± 0.2</td>
<td>27.3 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL lag time, min</td>
<td>109.4 ± 0.9</td>
<td>106.3 ± 1.4</td>
<td>112.4 ± 1.2</td>
<td>0.010</td>
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<tr>
<td>Circulating oxidized LDL, U/L</td>
<td>89.5 ± 2.1</td>
<td>101.9 ± 2.8</td>
<td>77.0 ± 2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8-Isoprostane, pg/mL</td>
<td>315.9 ± 16.9</td>
<td>336.3 ± 25.0</td>
<td>295.5 ± 8</td>
<td>NS</td>
</tr>
<tr>
<td>Urate, mol/L</td>
<td>283.6 ± 6.3</td>
<td>307.7 ± 9.6</td>
<td>259.5 ± 7.2</td>
<td>0.019</td>
</tr>
<tr>
<td>α-Tocopherol, μmol/L</td>
<td>32.1 ± 0.8</td>
<td>35.3 ± 1.1</td>
<td>27.9 ± 0.8</td>
<td>0.001</td>
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<tr>
<td>Carotid mean IMT, mm</td>
<td>0.74 ± 0.01</td>
<td>0.75 ± 0.02</td>
<td>0.73 ± 0.02</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are means ± SE.
M/F indicates male/female; BMI, body mass index; U/L, unit/liter; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; NS, not significant.
Statistical comparisons between affected and nonaffected family members were performed by 2-way ANOVA. The frequency distribution of the categorical variables between the 2 groups was compared by the χ² test.

TABLE 2. Determinants of Circulating Oxidized LDL in FCHL Family Members

<table>
<thead>
<tr>
<th>N</th>
<th>Variable</th>
<th>r</th>
<th>P</th>
<th>β</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate Analysis</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>β</td>
<td></td>
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<td></td>
<td>β</td>
<td></td>
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<tr>
<td></td>
<td>Adjusted R²</td>
<td>0.695</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r indicates correlation coefficient; β, standardized coefficient; NE, does not enter the final model.
Family number was forced into the regression analysis model.

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Potential Mechanisms of Production of Circulating Ox-LDL

The characteristics of Ox-LDL isolated from plasma of CAD patients are comparable to those of Ox-LDL isolated from atherosclerotic lesions. Lysine-aldehyde adducts, which may be a marker of Ox-LDL, are detected in the macrophage-rich lesions of human and rabbit aorta. In the present study, Ox-LDL was measured by a specific enzyme-linked immunosorbent assay method in which mAb-4E6 is directed against a conformation epitope in the apoB-100 moiety of LDL with at least 60 aldehyde-substituted lysine residues of apoB-100. The mAb-4E6 is specific to Ox-LDL but, to some extent, it also detects circulating MDA-LDL. Several clinical studies have shown that plasma levels of circulating Ox-LDL measured by mAb-4E6 are associated with CAD and subclinical carotid atherosclerosis. The potential origin of circulating Ox-LDL may be a direct release of modified LDL from ruptured or permeable plaques, or ischemic injury. Generation of Ox-LDL in arterial wall is probably affected by susceptibility of LDL to oxidation, the particle size, and the number of LDL in the circulation, the composition of LDL, and local oxidative stress in the arterial wall. Thus, circulating Ox-LDL may reflect the combined effect of these factors via additive and synergistic actions.

To the best of our knowledge, this is the first study analyzing systematically the potential determinants of circulating Ox-LDL in FCHL family members without clinical CAD (Table 2). LDL particle size, as well as plasma triglycerides, and apoB levels correlated positively with Ox-LDL, whereas HDL cholesterol was negatively related to Ox-LDL. Small LDL particles penetrate into the subendothelial space more easily and have high binding affinity with proteoglycans. This process promotes LDL modification in the arterial wall. In keeping with our results, other studies have shown that small dense LDL is associated with high levels of circulating MDA-LDL or autoantibodies against MDA-LDL. The negative correlation between circulating Ox-LDL and the lag time of LDL oxidation in vitro suggests that LDL particles susceptible to oxidation in vitro may also be easily oxidized in vivo. The antioxidant actions of HDL may explain the negative correlation between circulating Ox-LDL and HDL. In the multivariate analysis, the concentrations of serum LDL cholesterol, apoB, and plasma 8-isoprostane remained the independent determinants of Ox-LDL, thus being the most important factors contributing to the generation of circulating Ox-LDL (Table 2). In agreement, a recent study demonstrated that the levels of circulating Ox-LDL in subjects with impaired glucose tolerance were associated with components of dyslipidemia, but not with the antioxidant parameters. Several other studies have shown that LDL and total cholesterol concentrations are related to Ox-LDL measured by different antibodies. Previous studies have suggested that 8-isoprostane is formed during the in vitro oxidation of LDL. In this context, the increased plasma levels of LDL cholesterol and apoB in FCHL indicate the increased number of LDL particles available to penetrate and consequently enhance invasion of LDL into the subendothelium space, where the increased oxidative stress as measured by 8-isoprostane will promote the oxidative modification of LDL.

Association Between Ox-LDL and Carotid IMT

Oxidative modification of LDL is believed to play an important role in the development of atherosclerosis. Susceptibility of LDL to oxidation in vitro and autoantibodies against Ox-LDL have been related with atherosclerotic diseases in some, but not all, clinical studies. Recently, circulating Ox-LDL measured by different antibodies has been associated with cardiovascular diseases. In the present study, the carotid mean IMT increased by tertiles of Ox-LDL in the combined group (Figure A). In addition, the relative numbers of affected subjects were significantly increased in highest tertile group as compared with those in the middle and in the lowest tertile groups. These data suggest that the subjects with an increased level of Ox-LDL have increased carotid IMT, particularly in affected family members. Furthermore, carotid mean IMT was independently associated with age, pulse pressure, LDL size, and Ox-LDL in the univariate and multivariate analyses. The association between Ox-LDL and mean IMT persisted even after adjustment for logTG, LDL cholesterol, and apoB. The data indicate that Ox-LDL is a useful marker of the early stage of atherosclerosis in FCHL family members without clinical CAD. These results are in keeping with previous studies in which Ox-LDL was associated with the extent of CAD. Likewise, Ox-LDL was univariately correlated with IMT in carotid or femoral arteries and independently associated with subclinical plaque occurrence in carotid and femoral arteries in healthy population. However, no association between carotid IMT and plasma 8-isoprostane was observed in the present study, in line with the other studies. Pulse pressure, a pulsatile component of blood pressure determined by stiffness and elastic properties of arterial walls, is reported to be independently associated with carotid IMT in some studies. In agreement, we observed an independent association between carotid IMT and pulse pressure.

Conclusion

Serum levels of LDL cholesterol, apoB, and plasma 8-isoprostane are the most important determinants of Ox-LDL. Ox-LDL and LDL size were associated with carotid IMT independently of other clinical and lipid variables in the FCHL family members without clinical CAD. Therefore, our results suggest that the quantity of LDL particles and the oxidative stress in vivo determine the generation of circulating Ox-LDL, which may be a surrogate marker for CAD risk in the early stage of atherosclerosis in FCHL.

Acknowledgments

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