Identification of Multiple Dense LDL Subfractions With Enhanced Susceptibility to In Vitro Oxidation Among Hypertriglyceridemic Subjects Normalization After Clofibrate Treatment

Jacqueline de Graaf, Jan C.M. Hendriks, Pierre N.M. Demacker, and Anton F.H. Stalenhoef

The influence of different plasma triglyceride concentrations on the heterogeneity of low density lipoprotein (LDL) and on the susceptibility of LDL to copper oxidation was investigated. By density gradient ultracentrifugation, LDL subfractions were isolated from the plasma of 10 normolipidemic control subjects and 12 hypertriglyceridemic patients both before and after clofibrate treatment. In the plasma of control subjects three LDL subfractions were present: LDL1 (d=1.030-1.033 g/mL), LDL2 (d=1.033-1.040 g/mL), and LDL3 (d=1.040-1.045 g/mL). In the plasma of nine moderately hypertriglyceridemic subjects up to five LDL subfractions could be detected: LDL1-LDL3, LDL4 (d=1.045-1.049 g/mL), and LDL5 (d=1.049-1.054 g/mL). This polydispersity of LDL was replaced by monodispersity with increasing plasma triglyceride concentrations in three subjects with chylomicronemia, in whom LDL was concentrated in the narrow LDL5 density range. Clofibrate treatment resulted in a lighter LDL subfraction pattern (LDL1-LDL4). In both the control and the moderately hypertriglyceridemic subjects, the small dense LDL subfractions appeared more prone to oxidative modification in vitro than the light LDL subfractions, as measured by the decreased lag time preceding the onset of lipid peroxidation. Furthermore, the dense LDL subfractions were more extensively modified over time, as shown by an increased oxidation rate and a greater number of dienes formed after 6 hours of oxidation. These results suggest an enhanced atherogenic potential of the small, dense LDL subfractions within each LDL subfraction profile. The hypertriglyceridemic LDL subfractions before therapy (LDL3-LDL5) were less resistant to in vitro oxidation than the light, control LDL subfractions (LDL1-LDL3). This lower resistance was probably related to the decrease in the vitamin E content from LDL1 to LDL5. After clofibrate treatment both the vitamin E content of the LDL subfractions and the lag time increased, indicating an enhanced resistance against oxidation. (Arteriosclerosis and Thrombosis 1993;13:712-719)

KEY WORDS • LDL subfractions • lipid peroxidation • conjugated dienes • vitamin E • fatty acids • atherosclerosis

Variation in the composition and size of plasma low density lipoprotein (LDL) particles has been well established. In patients with hypertriglyceridemia, LDL has been described as “polydisperse,” defined by the presence of more than one species of LDL over a broad density range (d=1.019-1.063 g/mL). However, the presence of only one major band of small-sized particles has also been described in hypertriglyceridemic sera with the use of gradient gel electrophoresis. To address this apparent controversy, we applied a density gradient ultracentrifugation method to identify and isolate LDL subfractions among subjects with varying degrees of hypertriglyceridemia. Their physicochemical characteristics were compared with LDL subfractions isolated from the plasma of matched normolipidemic control subjects.

Previously, we demonstrated that LDL subfractions in plasma from normolipidemic subjects differ in their tendency to undergo oxidative modification in vitro. In the present study, we investigated the susceptibility of the hypertriglyceridemic LDL subfractions to oxidation and related this to the content of antioxidants as well as the fatty acid pattern of LDL, with the goal of identifying a possible mechanism involved in the suggested increased atherogenicity of these lipoproteins. In addition, the influence of plasma triglyceride (TG) reduction by clofibrate treatment of the hypertriglyceridemic subjects, on both the physicochemical properties and the susceptibility to oxidation of the isolated LDL subfractions, was determined.

Methods

Subjects

Twelve patients (11 men, one woman) with various degrees of primary hypertriglyceridemia (TG levels,
LDL Subfractionation performed by density gradient ultracentrifugation as described previously. After ultracentrifugation the LDL subfractions were visible as distinct bands in the middle of the tube. Depending on the degree of hypertriglyceridemia, one to five LDL subfractions could be detected, concentrated in the following density ranges: LDL1 (1.030-1.033 g/mL), LDL2 (1.033-1.040 g/mL), LDL3 (1.040-1.045 g/mL), LDL4 (1.045-1.049 g/mL), and LDL5 (1.049-1.054 g/mL). The ultracentrifugation tube containing the LDL subfractions stained with Coomassie Brilliant Blue R (reference tube) of each subject was placed in a specially designed rack and photographed. Accurate documentation of the different LDL subfraction patterns was obtained by scanning the slides in triplicate on an LKB 2202 ultrasonic laser densitometer. The mean peak heights (h1–h5) of the LDL subfractions (LDL1–LDL5) on the three scans were used to calculate the relative distribution of each LDL subfraction over total LDL (total LDL [%]=h1+h2+h3+h4+h5). By using the reference tube for each subject, LDL subfractions were accurately isolated by aspiration with a rubber bulb pipette from the tubes to which no stain had been added. The nonstained LDL subfractions of each subject were pooled and concentrated by a second ultracentrifugation run. The EDTA/BHT-containing LDL subfractions were dialyzed separately in the dark for 48 hours at 4°C against 3 L 0.01 M phosphate buffer (pH 7.4) containing 0.16 M NaCl and 0.1 μg/mL chloramphenicol. The buffer was made oxygen-free by vacuum degassing followed by purging with nitrogen; the buffer was changed after 24 hours. The EDTA/BHT-free LDL subfractions were filtered through a 0.45-μm filter and used for the oxidation study immediately. The density of the LDL subfractions was determined as described previously.

Oxidation of the LDL Subfractions

The oxidation experiments were performed essentially as described previously, with all subfractions of the same subject run in parallel. The EDTA/BHT-free dialyzed LDL subfractions were diluted with the dialysis buffer to a final concentration of 0.25 mg LDL protein

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**TABLE 1. Plasma Lipid and Lipoprotein Levels in 10 Normolipidemic Subjects and Nine Moderately Hypertriglyceridemic and Three Chylomicronemic Subjects Before and After Clofibrate Treatment**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Moderately HTG</th>
<th>Chylomicronemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>53.9 (47.2, 61.0)</td>
<td>57.1 (52.0, 62.2)</td>
<td>51.7 (38.0, 65.0)</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>23.6 (21.8, 25.4)*</td>
<td>25.9 (23.9, 28.0)§</td>
<td>25.8 (23.6, 28.1)</td>
</tr>
<tr>
<td><strong>TC</strong></td>
<td>5.40 (4.75, 6.14)*</td>
<td>6.93 (6.05, 7.93)§</td>
<td>6.56 (5.85, 7.35)</td>
</tr>
<tr>
<td><strong>TG</strong></td>
<td>1.26 (1.00, 1.59)¶</td>
<td>4.74 (3.71, 6.05)§</td>
<td>2.09 (1.64, 2.66)¶</td>
</tr>
<tr>
<td><strong>VLDL-C</strong></td>
<td>0.43 (0.29, 0.63)*</td>
<td>2.35 (1.56, 3.54)§</td>
<td>0.83 (0.56, 1.23)¶</td>
</tr>
<tr>
<td><strong>VLDL-TG</strong></td>
<td>0.71 (0.52, 0.98)‡</td>
<td>3.72 (2.66, 5.21)§</td>
<td>1.25 (0.90, 1.74)¶</td>
</tr>
<tr>
<td><strong>HDL-C</strong></td>
<td>1.14 (0.94, 1.37)</td>
<td>0.79 (0.65, 0.96)§</td>
<td>0.96 (0.89, 1.05)#</td>
</tr>
<tr>
<td><strong>LDL-C</strong></td>
<td>3.74 (3.12, 4.47)*</td>
<td>3.63 (3.01, 4.39)</td>
<td>4.65 (3.73, 5.80)**</td>
</tr>
</tbody>
</table>

**HTG, hypertriglyceridemic; BMI, body mass index; TC, total cholesterol; TG, triglycerides; VLDL-C, very low density lipoprotein cholesterol; VLDL-TG, very low density lipoprotein triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol. Lipid and lipoprotein values are expressed in millimoles per liter. Results are presented as median with 95% confidence interval in parentheses. For the chylomicronemic subjects only, results are indicated as mean values with ranges in parentheses.**

*0.01≤p≤0.05; †0.001≤p≤0.01 vs. HTG subjects before and after therapy (t test).‡0.01≤p≤0.05; §0.001≤p≤0.01; #p<0.01 vs. control subjects (t test). ¶p<0.001; ¥0.001≤p≤0.01; **0.01≤p≤0.05 vs. HTG subjects before and after therapy (paired t test).
per milliliter. Oxidation was initiated by addition of freshly prepared 1.66 μM CuCl₂ solution. The kinetics of the oxidation process of the LDL subfractions were determined by monitoring the change in the 234-nm absorbance at room temperature on a Perkin-Elmer Lambda 5 UV spectrophotometer equipped with a six-position automatic sample changer. The absorbance curves of the LDL subfractions from each subject were determined in parallel. The baseline values of the absorbance at 234 nm ranged between 0.1 and 0.3, indicating that the LDL subfractions were not oxidized in vivo or during the isolation procedure. After the initial absorbance at 234 nm was set to zero, the increase in absorbance was recorded every 3 minutes for approximately 6 hours. The change in absorbance at 234 nm versus time was divided into three consecutive phases: a lag phase, a propagation phase, and a decomposition phase. From this absorbance curve the lag time (in minutes), the maximal rate of oxidation (nanomoles of dienes formed per minute per milligram of LDL protein subfraction), and the maximal number of conjugated dienes formed per milligram of LDL protein subfraction were calculated. In four additional subjects (serum TG, 0.74–5.65 mmol/L), LDL subfractions were isolated without the use of BHT, and oxidation measurements were performed with a modified method as described previously. 12

**Assay of Fatty Acids and Vitamin E in Native LDL Subfractions**

Fatty acids and vitamin E concentrations in the native LDL subfractions of the moderately hypertriglyceridemic and control subjects were measured on a high-performance liquid chromatograph (model 8800, Spectra Physics, Eindhoven, The Netherlands) with spectrophotometric and fluorescence detection, respectively, essentially as described previously. 7 The native LDL subfractions were supplemented with BHT (4.4 μg/mL) and EDTA (1 mg/mL) to prevent oxidation during storage at −80°C for up to 4 weeks. The measurements of the vitamin E and fatty acid concentrations appeared reproducible on repeated analysis; the coefficient of variation was <5% (n=20) for the concentrations of both vitamin E and the various fatty acids.

**Analytical Methods**

Very low density lipoprotein (VLDL)+intermediate density lipoprotein (IDL) (d<1.019 g/mL) was isolated by ultracentrifugation for 16 hours at 40,000 rpm in a fixed-angle rotor. 13 High density lipoprotein (HDL) cholesterol was measured in whole plasma by the polyethylene glycol 6000 method. 13 LDL cholesterol was calculated by subtracting VLDL+IDL cholesterol and HDL cholesterol from total plasma cholesterol. Total cholesterol, unesterified (free) cholesterol, phospholipids, and TGs were determined by enzymatic, commercially available reagents (Boehringer Mannheim, Mannheim, FRG, catalogue No. 237574, 310328, 691844, and No. 6639, Sera Pak, Miles, Italy, respectively). The protein content of the LDL subfractions was measured by the method of Lowry et al. 14

For comparison of the relative size of the various isolated LDL subfractions, gradient gel electrophoresis was performed using 2–16% polyacrylamide gels (Pharmaclia, Uppsala, Sweden, catalogue No. 19-1264-01). 5 Agarose gel electrophoresis was performed at pH 8.6 in a barbital buffer using the Beckman Paragon System. 15 The possible degradation of apoprotein B-100 in the LDL subfractions was studied by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 3% or 4% discontinuous polyacrylamide disc gels. 16

**Statistical Analysis**

Before statistical analysis the data were logarithmically transformed to homogenize the variances. Due to their small number, the subjects with chylomicronemia (n=3) were excluded from statistical analysis. Differences in the mean values of age, body mass index, and plasma lipid and lipoprotein levels between the various groups were tested for significance by t test procedures. Paired t test procedures were used to assess the effect of clofibrate treatment on plasma lipid and lipoprotein levels in the moderately hypertriglyceridemic subjects.

Only the main LDL subfractions present in the moderately hypertriglyceridemic group, i.e., LDL3, LDL4, and LDL5 before therapy and LDL1, LDL2, and LDL3 after therapy, were included in the analysis of the variables measured in the isolated LDL subfractions. A two-way analysis of variance (two-way ANOVA) was used to analyze the differences between the three main LDL subfractions of the moderately hypertriglyceridemic group before therapy. A three-way ANOVA was used to analyze simultaneously the differences between the control group and the moderately hypertriglyceridemic group after therapy as well as the differences between their three main LDL subfractions. For all analyses, the retransformed (antilog) values of the mean with 95% (symmetrical) confidence intervals are presented as the median with 95% (asymmetrical) confidence intervals. Statistical analyses were performed with procedures available in the STATISTICAL ANALYSIS SYSTEM software package (SAS Institute Inc., Cary, N.C.).

**Results**

**Effect of Clofibrate on Plasma Lipid and Lipoprotein Levels**

The lipid and lipoprotein profiles of the control subjects and the patients with hypertriglyceridemia before and after clofibrate treatment are summarized in Table 1.

**Effect of Plasma TG Concentration on the LDL Subfraction Pattern**

Clear differences in number, intensity, and density range of the distinct LDL subfractions, visible as colored bands along the density gradient, were present between the control subjects and the subjects with various degrees of hypertriglyceridemia. Analysis of the densitometric scans revealed that the composition of the LDL subfraction patterns differed for the control and hypertriglyceridemic subjects and changed after clofibrate therapy (Figure 1).

In the plasma of the normotriglyceridemic control subjects, mostly three LDL subfractions, LDL1, LDL2, and LDL3, were present in a relatively narrow density range, with LDL2 and LDL3 as the predominant fractions (Table 2). In the plasma of the moderately hyper-
triglyceridemic subjects, the LDL subfractions were more dense, namely LDL3, LDL4, and/or LDL5, but in some LDL1 and LDL2 were also present. The moderately hypertriglyceridemic LDL thus tended to be polydisperse, consisting of multiple subfractions over a broad density range, with the dense LDL subfractions (LDL3–LDL5) contributing most to total LDL (Table 2). Clofibrate treatment of the moderately hypertriglyceridemic subjects induced a lighter LDL subfraction pattern (LDL1–LDL4), with LDL1, LDL2, or LDL3 as the main subfraction (Table 2).

In the subjects with chylomicronemia, a very dense LDL subfraction pattern was found that contained only one LDL5 subfraction band of very low intensity and high density. After treatment the LDL subfraction pattern was composed of two dense LDL subfractions, LDL4 and LDL5, with LDL4 predominating (Table 2).

**Physicochemical Characteristics of the LDL Subfractions**

**Chemical composition and size.** The LDL subfractions were isolated as discrete bands and their physicochemical characteristics were analyzed. Table 3 gives the relative free cholesterol, cholesteryl ester, TG, phospholipid, and protein contents of the main LDL subfractions present in the control group and the moderately hypertriglyceridemic group before and after therapy. The relative content of free cholesterol, cholesteryl ester, and phospholipid was significantly less and the TG and protein content significantly greater in LDL5 than in both LDL3 and LDL4 (Scheffé test, \( p < 0.05 \)). The main differences in composition (i.e., the relative free cholesterol, TG, and protein contents) in LDL1, LDL2, and LDL3 were found between LDL1 and LDL3 (Scheffé test, \( p < 0.05 \)) in both the control group and the hypertriglyceridemic group after treatment, whereas the cholesteryl ester and phospholipid contents differed significantly in the control group only (Scheffé test, \( p < 0.05 \); Table 3).

Figure 2 shows that the migration distance of the LDL subfraction particles in gradient gel electrophoresis increased from LDL1 to LDL5, indicating a gradual decrease in particle size with increasing density of the LDL subfractions. The migration distance of the LDL subfractions in the hypertriglyceridemic group before therapy increased significantly from LDL3 to LDL5 (Scheffé test, \( p < 0.05 \)). Moreover, LDL3 moved significantly farther than LDL2, and LDL2 moved significantly farther than LDL1 (Scheffé test, \( p < 0.05 \)). The migration distance was similar for the control group and hypertriglyceridemic group after therapy.

**Fatty acid composition.** The major fatty acids in the main LDL subfractions were linoleic acid (18:2), palmitic acid (16:0), and oleic acid (18:1), followed by arachidonic acid (20:4) and stearic acid (18:0). Between LDL3, LDL4, and LDL5 no significant difference in the concentration (micromoles per gram LDL protein) of each of these individual fatty acids was found. The concentration of each individual fatty acid decreased among the subfractions with increasing density, mainly due to significant differences between LDL1 and LDL3 (Scheffé test, \( p < 0.05 \)). Consequently, the total fatty acid and polyunsaturated fatty acid (PUFA) content decreased significantly from LDL1 to LDL3.

**Table 2.** Characteristics of LDL Subfraction Profiles Among 10 Normolipidemic, Nine Moderately Hypertriglyceridemic, and Three Chylomicronemic Subjects Before and After Clofibrate Treatment

<table>
<thead>
<tr>
<th>Controls</th>
<th>Relative distribution</th>
<th>Moderately HTG</th>
<th>Chylomicronemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before therapy</td>
<td>After therapy</td>
<td>Before therapy</td>
</tr>
<tr>
<td>LDL1</td>
<td>0.13±0.16</td>
<td>0.02±0.05</td>
<td>0.12±0.12</td>
</tr>
<tr>
<td>LDL2</td>
<td>0.41±0.12</td>
<td>0.04±0.09</td>
<td>0.25±0.19</td>
</tr>
<tr>
<td>LDL3</td>
<td>0.45±0.19</td>
<td>0.24±0.33</td>
<td>0.52±0.24</td>
</tr>
<tr>
<td>LDL4</td>
<td>...</td>
<td>0.59±0.39</td>
<td>0.11±0.30</td>
</tr>
<tr>
<td>LDL5</td>
<td>...</td>
<td>0.12±0.21</td>
<td>...</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; HTG, hypertriglyceridemic; LDL1, \( d=1.030–1.033 \) g/mL; LDL2, \( d=1.033–1.040 \) g/mL; LDL3, \( d=1.040–1.045 \) g/mL; LDL4, \( d=1.045–1.049 \) g/mL; LDL5, \( d=1.049–1.054 \) g/mL.
TABLE 3. Chemical Composition and Vitamin E Concentration of the Main LDL Subfractions in 10
Normolipidemic and Nine Moderately Hypertriglyceridemic Subjects Before and After Clofibrate Treatment

<table>
<thead>
<tr>
<th>Before clofibrate</th>
<th>LDL3 (n=8)</th>
<th>LDL4 (n=7)</th>
<th>LDL5 (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol</td>
<td>7.9 (7.0, 8.9)</td>
<td>7.1 (6.3, 8.1)</td>
<td>5.8 (5.1, 6.6)†</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>34.1 (29.7, 39.2)</td>
<td>38.5 (33.1, 44.6)</td>
<td>24.1 (20.7, 27.9)†</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>6.9 (5.1, 9.2)</td>
<td>6.3 (4.6, 8.5)</td>
<td>9.9 (7.2, 13.5)†</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>18.7 (17.0, 20.7)</td>
<td>18.8 (16.9, 20.8)</td>
<td>15.0 (13.5, 16.7)†</td>
</tr>
<tr>
<td>Protein</td>
<td>31.3 (26.5, 36.8)</td>
<td>27.6 (23.2, 32.9)</td>
<td>43.0 (36.1, 51.2)†</td>
</tr>
<tr>
<td>PUFAs</td>
<td>44.7 (41.8, 47.7)</td>
<td>45.8 (42.6, 49.1)</td>
<td>47.5 (43.7, 51.7)†</td>
</tr>
<tr>
<td>Vitamin E (µmol/g LDL protein)</td>
<td>10.2 (8.9, 11.6)</td>
<td>10.4 (9.1, 12.0)</td>
<td>7.5 (6.4, 8.7)†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>After clofibrate</th>
<th>LDL1 (n=8)</th>
<th>LDL2 (n=9)</th>
<th>LDL3 (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol</td>
<td>10.4 (9.3, 11.7)</td>
<td>9.6 (8.7, 10.6)</td>
<td>8.7 (7.9, 9.7)‡</td>
</tr>
<tr>
<td>Cholesteryl ester*</td>
<td>36.7 (33.0, 40.7)</td>
<td>37.2 (33.9, 40.8)</td>
<td>38.3 (34.9, 42.0)</td>
</tr>
<tr>
<td>Triglycerides*</td>
<td>8.2 (5.6, 12.1)</td>
<td>6.7 (4.8, 9.5)</td>
<td>5.5 (3.9, 7.1)‡</td>
</tr>
<tr>
<td>Phospholipids*</td>
<td>21.3 (19.8, 22.9)</td>
<td>21.8 (20.5, 23.2)</td>
<td>20.9 (19.7, 22.3)</td>
</tr>
<tr>
<td>Protein</td>
<td>22.7 (21.1, 24.4)</td>
<td>24.0 (22.4, 25.6)</td>
<td>26.2 (24.5, 28.00)</td>
</tr>
<tr>
<td>PUFAs</td>
<td>41.6 (34.9, 49.5)</td>
<td>40.5 (35.5, 46.2)</td>
<td>39.9 (35.0, 45.5)</td>
</tr>
<tr>
<td>Vitamin E (µmol/g LDL protein)*</td>
<td>15.7 (11.5, 21.5)</td>
<td>14.7 (11.6, 18.5)</td>
<td>13.6 (10.7, 17.1)†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control subjects</th>
<th>LDL1 (n=9)</th>
<th>LDL2 (n=10)</th>
<th>LDL3 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol</td>
<td>10.8 (9.8, 11.9)</td>
<td>9.7 (8.8, 10.6)</td>
<td>9.1 (8.3, 10.0)‡</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>39.3 (36.4, 42.4)</td>
<td>41.7 (38.8, 44.9)</td>
<td>37.4 (34.8, 40.2)#</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>5.7 (4.0, 7.9)</td>
<td>4.8 (3.5, 6.6)</td>
<td>4.5 (3.3, 6.2)‡</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>21.2 (20.1, 22.2)</td>
<td>20.8 (19.9, 21.8)</td>
<td>19.1 (18.2, 20.0)</td>
</tr>
<tr>
<td>Protein</td>
<td>22.4 (20.2, 24.8)</td>
<td>22.5 (20.4, 24.8)</td>
<td>29.4 (26.7, 32.4)</td>
</tr>
<tr>
<td>PUFAs</td>
<td>46.8 (44.0, 49.7)</td>
<td>46.8 (44.3, 49.3)</td>
<td>47.7 (45.2, 50.4)</td>
</tr>
<tr>
<td>Vitamin E (µmol/g LDL protein)</td>
<td>13.3 (10.3, 17.1)</td>
<td>11.5 (9.3, 14.2)</td>
<td>10.4 (8.4, 12.9)¶</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; PUFAs, polyunsaturated fatty acids. Chemical compositions of the LDL subfractions are given as percent of dry mass. Values are presented as median with 95% confidence interval in parentheses.

*Hypertriglyceridemic group after therapy and the control group differ significantly for the presented variable (three-way analysis of variance, p<0.05).

Symbols indicate differences between LDL subfractions within each group (Scheffe test, p<0.05): LDL5 vs. LDL4 and LDL3; #LDL1 vs. LDL2 and LDL2 vs. LDL3 and LDL3 vs. LDL1; ¶LDL1 vs. LDL2 and LDL2 vs. LDL3; ||LDL3 vs. LDL1 and LDL2; ¶¶LDL3 vs. LDL1; #LDL2 vs. LDL3.

(Scheffé test, p<0.05), whereas the percentage of PUFAs did not differ significantly (Table 3).

After clofibrate treatment the total fatty acid concentration increased considerably; in the LDL subfractions of the treated hypertriglyceridemic group, the concentration of the fatty acids was significantly greater than in the control group (three-way ANOVA, p<0.05) except for linoleic acid; the concentration of this fatty acid was significantly lower (three-way ANOVA, p<0.05).

Vitamin E concentration. In the untreated hypertriglyceridemic group the vitamin E content (micromoles per gram LDL protein) was significantly less in LDL5 than in both LDL3 and LDL4 (Scheffé test, p<0.05; Table 3). In LDL1, LDL2, and LDL3 the vitamin E content also decreased with increasing density, due to significant differences between LDL1 and LDL3 (Scheffé test, p<0.05). The vitamin E content of the LDL subfractions in the treated hypertriglyceridemic group was significantly greater than in the control group, despite the similar LDL subfraction pattern (three-way ANOVA, p<0.05; Table 3). No detectable amounts of vitamin E were present in the LDL subfractions after the oxidation experiments.

Oxidizability of LDL Subfractions

The lag time of the LDL subfractions tended to decrease with increasing density (Figure 3). Among the subfractions of the hypertriglyceridemic group before therapy, the lag time of LDL5 was significantly shorter than that of LDL3 (Scheffé test, p<0.05) and that of LDL3 was significantly shorter than that of LDL1 (Scheffé test, p<0.05). The lag time of the LDL subfractions in the treated hypertriglyceridemic group did not differ significantly from that in the control group (three-way ANOVA, p>0.05).

The maximal rate of oxidation and the maximal number of dienes formed per milligram of LDL protein tended to increase from LDL3 to LDL5 (Figure 3); the values of both parameters were significantly greater in LDL5 than in LDL3 or LDL4 (Scheffé test, p<0.05). A similar trend was found from LDL1 to LDL3, with significantly greater values for oxidation rate and amount of maximal diene formation in LDL3 than in both LDL1 and LDL2 (Scheffé test, p<0.05). Among the LDL subfractions of the treated hypertriglyceridemic group, both the oxidation rate and the maximal number of dienes formed were significantly less than in...
Migration distance [mm]

FIGURE 2. Line graph shows migration distance in millimeters in gradient gel electrophoresis of the main low density lipoprotein (LDL) subfractions present in 10 normolipidemic subjects (—) and nine moderately hypertriglyceridemic subjects before (— —) and after (— — —) clofibrate treatment. Each point represents the median value with 95% confidence interval. The symbols indicate differences between LDL subfractions within each group by Scheffe test (p<0.05): *LDL1 vs. LDL2; **LDL2 vs. LDL3; §LDL3 vs. LDL1; ¶LDL3 vs. LDL4; ¶¶LDL4 vs. LDL5; **LDL5 vs. LDL3.

the control group, despite the similar LDL subfraction pattern (three-way ANOVA, p<0.05).

In the experiments in which no BHT was used during the isolation of the LDL subfractions, the same qualitative differences were obtained as described here (results not shown).

Discussion

The density distribution of LDL in our moderately hypertriglyceridemic patients covered a wide density range (1.030-1.054 g/mL) concentrated in five discrete LDL subfractions, LDL1-LDL5. The main LDL subfractions were abnormally small and dense (LDL3-LDL5), whereas for the normolipidemic subjects three distinct LDL subfractions, LDL1, LDL2, and LDL3, in a relatively narrow, light density range (1.030-1.045 g/mL)
could be detected. In the subjects with chylomicronemia (TG levels, >11 mmol/L) this polydispersity of LDL was absent, and only one LDL subfraction (LDL5) in a narrow density range (1.049-1.054 g/mL), defined as monodisperse LDL, was observed. So, in contrast with Fisher and colleagues, we found that polydispersity of LDL is not a simple function of plasma TGs. Our data indicate that both the number and the relative distribution of the LDL subfractions over total LDL are dependent on the extent of hypertriglyceridemia.

Subjects with moderate hypertriglyceridemia are considered to be at increased risk for CHD, especially men over 50 years with low HDL cholesterol levels.

An enhanced atherogenic potential of the hypertriglyceridemic LDL subfractions could contribute to this situation. Reportedly, alterations in the composition of LDL particles were associated with changes in LDL metabolism in cultured cells, which may render the hypertriglyceridemic LDL particle more atherogenic.

Another potential mechanism that may increase the atherogenicity of LDL particles includes the oxidative modification of LDL. This oxidative modification converts LDL to a form recognized by the scavenger receptor, and uptake of oxidized LDL induces foam cell formation in vitro, the hallmark of atherosclerotic plaques.

Recently, we showed that the more dense LDL subfractions in normolipidemic subjects, LDL2 and LDL3, were more susceptible to oxidative modification, as confirmed by others. The more dense LDL2 and LDL3 subfractions may therefore contribute more to foam cell formation per particle than the less dense LDL subfraction LDL1, thus explaining the association between dense LDL with an increased risk of CHD.

In addition, we now report that the dense LDL subfractions present in the hypertriglyceridemic subjects (LDL3-LDL5) also differ in their susceptibility to lipid peroxidation in vitro. The heavy LDL5 subfraction was less well protected against oxidative modification than LDL3, as measured by the significantly shorter lag time. Once the lipid peroxidation process started, LDL5 was more extensively modified over time than LDL3, as shown by the increased rate of oxidation and the greater number of dienes formed.
In trying to explain the differences in lag time among LDL subfractions, vitamin E appears to be an important factor, as suggested previously\(^{1,11,27-30}\); the decrease in lag time with increasing density of the LDL subfractions was associated with a decrease in vitamin E content (Table 3). When LDL is more or less depleted of antioxidants, the lipid peroxidation process starts with the peroxidation of the PUFAs in LDL lipids and their degradation to, among other products, conjugated dienes.\(^{27}\) The major PUFAs in LDL that can undergo lipid peroxidation are 18:2 and 20:4, which are mainly bound to phospholipids, cholesterol, and, to a lesser extent, to TGs.\(^{28}\) Our results indicate that in the more dense LDL subfractions within each group, more PUFAs were degraded during the oxidation process. However, the PUFA content did not differ significantly among LDL3–LDL5 in the hypertriglyceridemic group and even decreased from LDL1 to LDL3 among the normolipidemic subjects. Apparently other factors, including LDL particle size, conformational changes of apoprotein B\(^{13,18,19}\) and location of fatty acids (e.g., on surface phospholipids or in core TGs or cholesteryl esters), are also important in determining the rate of oxidation and diene formation.

It appeared that the rates of oxidation and diene formation with the LDL subfractions of the hypertriglyceridemic subjects were less than in the control subjects (Figure 3). These results do not appear to concur with the increased susceptibility to lipid peroxidation that was suggested above; we found, however, that after the lag phase had ended, extensive physicochemical alterations in all LDL subfractions were present on agarose and sodium dodecyl sulfate gels, suggesting biological modification of all LDL subfractions.\(^{30-32}\) Therefore, we suggest that more value should be set on the duration of the lag time.

After 2 months of clofibrate treatment the dense LDL subfraction pattern was replaced by a light LDL subfraction pattern (LDL1–LDL3); again, the most dense LDL subfraction (LDL3) showed an enhanced oxidizability compared with light LDL1 (Figure 3). The light LDL subfractions in the treated hypertriglyceridemic group (LDL1–LDL3) appeared more resistant to oxidation than before therapy (LDL3–LDL5) as measured by the increase in lag time to normal. This increase in lag time after clofibrate treatment was associated with an increase in vitamin E content from LDL5 to LDL1, stressing its role in retarding or preventing the oxidative modification of LDL subfractions, although other antioxidants may also contribute to the difference in lag time among LDL subfractions.\(^{28,33,34}\) Furthermore, the extent of oxidation, as determined by the oxidation rate and the number of dienes formed, did not change after treatment; this is consistent with the similar absolute linoleic acid and PUFA content of the LDL subfractions after clofibrate treatment, which agrees with results obtained with other fibrates.\(^{35-37}\) The LDL subfractions after clofibrate administration were still less extensively modified over time than the control LDL subfractions, a difference that was apparently related to the significantly lower concentration of PUFAs (per micromole vitamin E or per gram LDL protein) available for oxidation in the treated hypertriglyceridemic LDL subfractions.

In summary, our results show that LDL isolated from hypertriglyceridemic subjects is more prone to in vitro oxidative modification than LDL from control subjects. Clofibrate treatment reduced the susceptibility of LDL to oxidation. Hence, if the oxidative modification hypothesis in the development of atherosclerotic plaques is relevant,\(^{21}\) LDL in hypertriglyceridemic subjects has an enhanced atherogenic potential that reverts toward normal after treatment.

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