Enhanced Susceptibility to In Vitro Oxidation of the Dense Low Density Lipoprotein Subfraction in Healthy Subjects

Jacqueline de Graaf, Heidi L.M. Hak-Lemmers, Magda P.C. Hectors, Pierre N.M. Demacker, Jan C.M. Hendriks, and Anton F.H. Stalenhoef

Oxidative modification of low density lipoprotein (LDL) has been implicated as a factor in the generation of macrophage-derived foam cells, the hallmark of atherosclerotic plaques. Because LDL consists of discrete subfractions with different physicochemical characteristics, the question arises as to whether these LDL subfractions differ in their susceptibility to oxidative modification. To answer this question, three LDL subfractions, LDL₁, LDL₂, and LDL₃, were isolated from the plasmas of 11 healthy volunteers by density gradient ultracentrifugation. The LDL subfractions were oxidatively modified by incubation with copper ions. Differences in the subfractions' susceptibilities to lipid peroxidation were studied by measuring the formation of the 234-nm-absorbing oxidation products every 3 minutes on an ultraviolet spectrophotometer. A significant inverse linear relation was found between LDL subfractions and lag time (regression coefficient = \(-8.50\), \(p<0.001\)), indicating that both the dense LDL₃ and the light LDL₁ were less well protected against oxidative modification than the very light LDL₂. The LDL subfractions showed a positive linear relation with the rate of oxidation (regression coefficient = \(0.46\), \(p<0.001\)) and the amount of conjugated dienes formed in the LDL subfractions after 4 hours of oxidation (regression coefficient = \(9.24\), \(p<0.001\)). Thus, both LDL₁ and LDL₂ were more extensively modified in time than LDL₃, which may be explained by the significantly higher concentration of polyunsaturated fatty acids in LDL₃ (micromoles per gram LDL cholesterol) compared with LDL₁ (Tukey's test, \(p<0.05\)). These results indicate that the more dense LDL subfractions, that is, LDL₁ and LDL₂, are more susceptible to oxidative modification and therefore may contribute more to foam cell formation than the less dense LDL subfraction LDL₃.

Plasma levels of low density lipoproteins (LDLs) are positively correlated with the incidence of coronary artery disease (CAD).¹ In the last decade, evidence has accumulated that human plasma LDL comprises discrete subfractions, varying in size, density, and lipid content.²⁻⁶ Two to three LDL subfractions can be detected and isolated from normolipidemic plasma by density gradient ultracentrifugation.⁷ These LDL subfractions have been found to differ in chemical composition and molecular size.⁷ Several lines of evidence suggest that the heavy LDL₃ subfraction in particular predisposes to CAD.⁸⁻¹² However, the mechanism for the suggested higher atherogenicity of LDL₃ has not yet been elucidated. An early event in the development of atherosclerosis is the accumulation of lipid-loaded "foam cells" in the subendothelial space of the vessel wall. Recent studies have demonstrated that most of the foam cells are derived from monocyte macrophages.¹³⁻¹⁵ Native LDL is taken up by the LDL receptor (apolipoprotein [apo] B/E receptor) at a rather low rate and without a marked accumulation of cholesterol.¹⁶ However, cultured macrophages accumulate large amounts of cholesterol lipids and develop a foam cell-like appearance when incubated with chemically or biologically modified LDL.¹⁷⁻¹⁹ The uptake of these modified forms of LDL occurs through the so-called scavenger receptor, which is not downregulated by the amount of internalized cholesterol.¹⁶,²⁰ Therefore, modification of native LDL is necessary to transform macrophages into foam cells. Steinbrecher

From the Division of General Internal Medicine (J.d.G., H.L.M.H.-L., M.P.C.H., P.N.M.D., A.F.H.S.), Department of Medicine, and Department of Statistical Consultation (J.C.M.H.), University Hospital Nijmegen, Nijmegen, The Netherlands.

Supported by a grant from the Netherlands Heart Association (NHS 88.100). A.F.H.S is a Clinical Investigator of the Netherlands Heart Foundation.

Address for reprints: Jacqueline de Graaf, MD, Department of General Internal Medicine, University Hospital Nijmegen, PO Box 9101, 6500 HB, Nijmegen, The Netherlands.

Received June 8, 1990; revision accepted November 12, 1990.
et al recognized a clear connection between lipid peroxidation and modification of LDL. This biologically modified form of LDL can be generated in vitro by incubation of LDL with endothelial cells, smooth muscle cells, and macrophages. These cells can oxidize LDL by a lipid peroxidation process. The cell-induced oxidative modification can be mimicked by simply incubating LDL in serum-free medium in the presence of copper or iron. Hence, oxidized LDL is now considered a candidate for naturally occurring modified LDL. The lipid peroxidation process starts, after consumption of the antioxidants present in LDL, with the peroxidation of polyunsaturated fatty acids (PUFAs) in LDL lipids and their degradation to a complex variety of products as conjugated dienes and aldehydes. The kinetics of the lipid peroxidation process can be followed by continuously measuring the increase of the 234-nm absorbance on an ultraviolet (UV) spectrophotometer. This absorption develops in LDL during oxidation by the conversion of PUFAs into fatty acid hydroperoxides with conjugated double bonds and by the formation of other oxidation products absorbing in this region. The aim of our study was to compare the susceptibility of three LDL subfractions to in vitro lipid peroxidation as a model of biologic modification.

Methods

Experimental Protocol

LDL subfractions were isolated from the plasmas of 11 healthy volunteers. The susceptibility to oxidation of the three LDL subfractions from each subject was determined in parallel by measuring the changes in the 234-nm absorption on a UV spectrophotometer every 3 minutes during the incubation of the subfractions with copper ions.

Plasma

Blood samples were obtained from 11 healthy volunteers, six men and five women aged 20-45 years, after an overnight fast. All subjects gave their informed consent. The blood was collected into EDTA/BHT-containing tubes. Plasma was isolated immediately and supplemented with butylated hydroxytoluene (BHT, 4.4 μg/ml). All subjects were normolipidemic and did not use any medication. Concentrations of serum triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol, and LDL cholesterol in the subjects studied ranged from 0.64 to 1.83 mmol/l, 3.6 to 6.0 mmol/l, 0.95 to 2.36 mmol/l, and 1.52 to 4.81 mmol/l, respectively.

Low Density Lipoprotein Subfractionation

Isolation of LDL subfractions was performed by density gradient ultracentrifugation, essentially as described previously, with some slight modifications to increase the distance between the LDL subfractions to facilitate the isolation of the various subfractions by aspiration. From each subject, 3.0 ml of freshly isolated plasma containing EDTA (1 mg/ml) and BHT (4.4 μg/ml) was pipetted into six polycarbonate centrifuge tubes. The density of the plasma was raised to 1.10 g/ml by dissolving 0.42 g KBr. As a reference for the position of the LDL subfractions, 20 μl freshly prepared 15 g/l aqueous solution of Coomassie Brilliant Blue R was added to one of the tubes. The plasma was then successively overlayed by four solutions of decreasing density (2 ml of d = 1.065 g/ml, 3.0 ml of d = 1.035 g/ml, 3.0 ml of d = 1.020 g/ml, and 1.5 ml of d = 1.006 g/ml solution). For optimal staining, the density solutions used in the reference tube were adjusted to pH 4.5–5.0 with 1 M HCl. After ultracentrifugation at 37,000 rpm at 20°C for 19.5 hours in a Beckman SW 40 rotor in Beckman L7-55 ultracentrifuges (Beckman Instruments, Palo Alto, Calif.), three LDL subfractions (LDL1, d = 1.030–1.033 g/ml, LDL2, d = 1.033–1.040 g/ml, LDL3, d = 1.040–1.045 g/ml) were separated by four solutions of decreasing density (2 ml of d = 1.006 g/ml solution).

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The EDTA/BHT-containing LDL subfractions from each person 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 from the plasma of each subject were filtered through a 0.45-μm filter and then used for oxidation study immediately.

Oxidation of Low Density Lipoprotein Subfractions

The oxidation experiments were performed essentially as described by Esterbauer et al. The EDTA/BHT-free dialyzed LDL subfractions were diluted with the dialysis buffer to a final concentration of 0.25 mg/ml. Oxidation was initiated by addition of freshly prepared 1.66 μM CuCl2 solution. The kinetics of the oxidation of LDL subfractions was determined by monitoring the change in the 234-nm absorbance at 37°C on a Beckman Model 25 UV spectrophotometer equipped with a four-position automatic sample changer. The absorbance curves of the three LDL subfractions from each subject were determined in parallel. The initial absorbance at 234 nm was set to zero, and the increase in absorbance was then recorded every 3 minutes for 4 hours. The change in absorbance at 234 nm versus time was divided into three consecutive phases, that is, a lag phase, a propagation phase, and a decomposition phase (Figure 1).

The lag time was defined as the interval between the intercept of the tangent of the slope of the curve with the time-scale axis, expressed in minutes. As...
shown in Figure 1, the lag time for LDL1 in this subject was 76.5 minutes. The maximal rate of oxidation, calculated from the slope of the absorbance curve during the propagation phase, was expressed in absorbance/min. As lipid hydroperoxides rapidly accumulate during the propagation phase indicating that their rate of formation is much higher than their rate of degradation to aldehydes and other 234-nm-absorbing oxidation products,31 the molar absorptivity of conjugated dienes (\( \varepsilon = 234 = 29,500 \) 1/mol/cm) was used to express the maximal rate of oxidation as nanomoles of dienes formed per minute per milligram of LDL protein. The maximal increase in absorbance was read from the absorbance curve as the difference in absorbance found at the beginning of the decomposition phase minus the absorbance found at the start of the lag phase. With this value, the total amount of conjugated dienes (in nanomoles) formed per milligram of LDL protein could be calculated in the same way as described above for the maximal rate of oxidation. The absorbance profiles of the LDL subfractions appeared reproducible on repeated analysis of freshly isolated plasma, obtained from two different subjects, with an interval of 2 months (coefficient of variation <5% for the three parameters derived from the absorbance curves).

**Agarose Gel Electrophoresis and Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis**

Agarose gel electrophoresis was performed at pH 8.6 in a barbital buffer using the Beckman Paragon system.32 The eventual degradation of apo B-100 in the LDL subfractions was studied by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 3%/4% discontinuous polyacrylamide disc gels as described earlier.33

**Vitamin E Measurement**

In native and oxidized LDL subfractions, vitamin E concentrations were measured on a high-performance liquid chromatography (HPLC) Spectra Physics Model 8800 (Spectra Physics, Eindhoven, The Netherlands) with fluorescence detection essentially as described previously.34 BHT (4.4 \( \mu \)g/ml) and EDTA (1 mg/ml) were added to the native samples to prevent oxidation. Samples were stored at \(-20^\circ\)C for less than 3 weeks. For extraction of vitamin E, 0.2 ml LDL subfraction was vortex mixed with 2 ml acetone and 2 ml petroleum ether (40–60°C). The organic phase was then removed by pipet, followed by two extractions of the aqueous phase with 2 ml petroleum ether. The pooled organic phase was evaporated to dryness under a stream of nitrogen. The samples were dissolved in 0.4 ml methanol and injected into the chromatograph. A ChromSep column, packed with Spherisorb ODS 5 (chrompack, Middelburg, The Netherlands), was used. The flow rate was 0.4 ml/min with 98% methanol as the mobile phase. Fluorescence was measured with a fluorescence spectrophotometer (Applied Biosystems, Maarsen, The Netherlands). The excitation wavelength was set at 288 nm, and the emitted fluorescence was measured at 340 nm. The chromatogram results were quantified by peak heights using an external standard for vitamin E.

**Assay of Fatty Acids in Low Density Lipoprotein Subfractions**

LDL subfractions, stored for 6 weeks at \(-20^\circ\)C in the presence of BHT (4.4 \( \mu \)g/ml) and EDTA (1 mg/ml), were saponified by mixing 0.4 ml of each LDL subfraction with 1.6 ml 0.3 M NaOH in 90% ethanol (vol/vol), followed by incubation at 37°C for 1 hour. To 0.4 ml of the mixture, diluted to 0.8 ml with Millipore-water (Millipore Corp., Ettenleuver, The Netherlands), 50 \( \mu \)l 37% HCl was added. Fatty acids were extracted twice with vortex mixing with 2 ml n-hexane. The pooled organic phase was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 \( \mu \)l ethanol containing 400 \( \mu \)mol/l heptadecanoic acid as an internal standard. Derivatization to 4-nitrophenyldihydrazides was performed as described.35 Briefly, to 100 \( \mu \)l of the ethanolic mixture of LDL subfraction sample, 200 \( \mu \)l of a 20 mM solution of 4-nitrophenyldihydrazine hydrochloride in 25% ethanol and 400 \( \mu \)l of an ethanol solution containing 125 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 1.5% pyridine were added, and the mixture was heated at 60°C for 20 minutes. To the resulting mixture of hydrazides, 0.2 ml 0.5 M Tris (pH 10.0) and 2 ml n-hexane were added. After vortexing for 30 seconds and centrifugation at 1,500g for 5 minutes, the n-hexane layer was taken and evaporated under a stream of nitrogen at room temperature. This procedure was repeated twice. The residue was dissolved in 800 \( \mu \)l 100% methanol, and a 2-\( \mu \)l aliquot was injected into the chromatograph. Fatty acids were measured on an HPLC Spectra Physics Model 8800 with spectrophotometric detection. A ChromSep column was used, which was packed with Chromspher C8 (2×10-cm,
Analytical Methods

Total cholesterol (TC), unesterified (free) cholesterol (FC), phospholipids (PLs), and triglycerides (TGs) were measured by commercially available enzymatic reagents (Boehringer-Mannheim, Mannheim, F.R.G., Nos. 237574, 310328, and 691844; and No. 6639, Sera Pak, Miles, Italy, respectively). The protein content of the LDL subfractions was determined by the method of Lowry et al. From these data, the mean ratio of cholesterol (FC+cholesterol moiety of cholesteryl ester [CE] \(=0.59 \times \text{weight of CE}\)) to protein was calculated. HDL cholesterol was determined in whole plasma by the polyethylene glycol 6000 method. LDL cholesterol was calculated with the Friedewald formula.

Statistical Analysis

The influence of the LDL subfractions on lag time was analyzed with a two-way analysis of variance (ANOVA). The dependent variable was the lag time, and the independent variables were subjects and LDL subfractions. The same model was used to analyze the influence of the LDL subfractions on 1) the maximal rate of oxidation, 2) the amount of conjugated dienes, 3) the vitamin E concentration, 4) the fatty acid concentrations, and 5) the relative amounts of FC, CE, PLs, TGs, and protein in the LDL subfractions. Differences in the mean value of the lag time, the maximal rate of oxidation, the amount of conjugated dienes, the vitamin E concentration, the fatty acid concentrations, and the amounts of FC, CE, PLs, TGs, and protein among the LDL subfractions were tested for significance by Tukey's studentized range test. Therefore, Tukey's test was used as a contrast test additional to the ANOVA.

To investigate the relation of vitamin E (milligrams per gram of LDL protein) with lag time, the rate of oxidation, and the amount of conjugated dienes, this variable was added as a covariable to the above-mentioned models, together with all possible two-variable interaction terms (three-way analysis of covariance [ANCOVA]). The statistically insignificant interaction terms were deleted from the model. The same procedure was used to determine the relation of the chemical composition data (FC, CE, PL, TG, and protein) and the fatty acid concentrations of the three LDL subfractions with the lag time, the rate of oxidation, and the amount of conjugated dienes.

Pearson's correlation coefficients were computed to determine correlations between plasma lipoprotein subfractions, that is, TC, TGs, LDL cholesterol, HDL cholesterol, and the mean lag time, the mean rate of oxidation, the mean amount of conjugated dienes, and the mean vitamin E concentration for all three LDL subfractions. The statistical analyses were performed with procedures available in the Statistical Analysis System software package (SAS Institute Inc., Cary, N.C.).

Results

Low Density Lipoprotein Subfraction Pattern

Three LDL subfractions were observed, separated by a clear interface, after density gradient ultracentrifugation of the plasma from each subject: the very light LDL₁, the light LDL₂, and the heavy LDL₃ (LDL₁, \(d=1.030-1.033\text{ g/ml}\); LDL₂, \(d=1.033-1.040\text{ g/ml}\); and LDL₃, \(d=1.040-1.045\text{ g/ml}\)). The subfractions were isolated by aspiration and were used for the oxidation experiments.

Physicochemical Characteristics of Low Density Lipoprotein Subfractions

The analysis of the chemical composition data of the LDL subfractions revealed significant differences in FC (ANOVA, \(p<0.001\)), CE (ANOVA, \(p<0.01\)), PL (ANOVA, \(p<0.001\)), and protein (ANOVA, \(p<0.001\)) content among some of the LDL subfractions. The TG content did not differ significantly among the LDL subfractions. The relative content of FC, CEs, and PLs decreased and that of protein increased with increasing density, although not all differences were statistically significant (Table 1). Except for TGs, LDL₁ and LDL₃ differed significantly in all constituents. For CEs, PLs, and protein, LDL₂ was different from LDL₁. For FC, LDL₂ was different from LDL₁ (Tukey, \(p<0.05\)). Mean values are presented in Table 1. The mean ratio of cholesterol (i.e., FC plus cholesterol moiety of CE \(=0.59 \times \text{weight of CE}\)) to protein was 1.46±0.21, 1.31±0.20, and 0.99±0.11 for LDL₁, LDL₂, and LDL₃, respectively. This ratio was used to convert LDL protein concentration to LDL cholesterol concentration for each LDL subfraction in the vitamin E assay and in the calculation of the fatty acid concentrations of the LDL subfractions to correct for differences in cholesterol content among LDL subfractions.

Vitamin E

Per gram of LDL protein, a significant influence of the native LDL subfractions was found on the vitamin E concentration (ANOVA, \(p<0.05\)). LDL₁ contained significantly less vitamin E (milligrams per gram of LDL protein) than did either LDL₁ or LDL₂ (Tukey, \(p<0.05\)). LDL₁ did not differ significantly from LDL₂ in the concentration of vitamin E (Table 1). When corrected for the differences in cholesterol content among LDL subfractions, no significant difference in vitamin E concentration (milligrams per gram of LDL cholesterol) among the three LDL
TABLE 1. Chemical Composition, Vitamin E Content, and Fatty Acid Content in Low Density Lipoprotein Subfractions Before Oxidation

<table>
<thead>
<tr>
<th>Physicochemical variable (N=11)</th>
<th>LDL₁</th>
<th>LDL₂</th>
<th>LDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density range (g/ml)</td>
<td>1.030-1.033</td>
<td>1.033-1.040</td>
<td>1.040-1.045</td>
</tr>
<tr>
<td>Cholesteryl ester (%)</td>
<td>39.61±2.90</td>
<td>39.49±3.82</td>
<td>35.03±4.21*</td>
</tr>
<tr>
<td>Triglycerides (%)</td>
<td>4.46±1.14</td>
<td>4.29±1.59</td>
<td>5.71±2.55</td>
</tr>
<tr>
<td>Free cholesterol (%)</td>
<td>10.80±1.17†</td>
<td>9.85±1.22</td>
<td>9.38±1.23</td>
</tr>
<tr>
<td>Phospholipids (%)</td>
<td>21.31±1.33</td>
<td>20.61±1.62</td>
<td>19.27±0.97*</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>23.82±2.98</td>
<td>25.76±2.74</td>
<td>30.62±1.85*</td>
</tr>
<tr>
<td>Ratio cholesterol/protein</td>
<td>1.46±0.21</td>
<td>1.31±0.20</td>
<td>0.99±0.11</td>
</tr>
<tr>
<td>Vitamin E (mg/g LDL protein)</td>
<td>4.53±0.95</td>
<td>4.66±0.65</td>
<td>3.73±0.98*</td>
</tr>
<tr>
<td>Vitamin E (mg/g LDL cholesterol)</td>
<td>3.06±0.39</td>
<td>3.46±0.69</td>
<td>3.81±1.02</td>
</tr>
<tr>
<td><strong>Fatty acid content (μmol/LDL cholesterol; n=8)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>76.18±23.30</td>
<td>83.93±34.63</td>
<td>110.02±39.72*</td>
</tr>
<tr>
<td>16:0</td>
<td>440.63±92.43</td>
<td>484.19±156.30</td>
<td>636.63±96.87*</td>
</tr>
<tr>
<td>18:0</td>
<td>139.35±33.84</td>
<td>155.76±54.43</td>
<td>204.45±37.36*</td>
</tr>
<tr>
<td>18:1</td>
<td>376.10±77.16</td>
<td>380.19±111.20</td>
<td>550.46±84.12*</td>
</tr>
<tr>
<td>18:2</td>
<td>782.69±157.54</td>
<td>897.86±230.27</td>
<td>1140.08±232.35*</td>
</tr>
<tr>
<td>20:4</td>
<td>282.72±61.59</td>
<td>272.02±59.90</td>
<td>363.80±75.25*</td>
</tr>
<tr>
<td><strong>Total FAs</strong></td>
<td>2,097.66±400.27</td>
<td>2,273.95±572.50</td>
<td>3,005.44±473.67*</td>
</tr>
<tr>
<td><strong>Total PUFAs</strong></td>
<td>1,065.41±210.83</td>
<td>1,169.88±278.57</td>
<td>1,503.88±288.71*</td>
</tr>
</tbody>
</table>

Because of technical errors, data from only eight subjects were available for the analysis of the fatty acid concentrations in the LDL subfractions. In all three LDL subfractions, approximately 50% of the total fatty acids consisted of PUFAs. The main PUFAs were linoleic acid (18:2, 70%) and arachidonic acid (20:4, 30%) (Table 1). Per gram of LDL protein, no significant differences in the concentrations of the fatty acids among LDL subfractions were found. However, when corrected for the differences in cholesterol content among the LDL subfractions, significant differences in total fatty acid concentration and total PUFAs concentration among the LDL subfractions were found (ANOVA, p<0.001). The total fatty acid and PUFAs concentrations (micromoles per gram of LDL cholesterol) were significantly higher in LDL₃ than in either LDL₁ or LDL₂ (Tukey, p<0.05). LDL₁ and LDL₂ did not differ significantly in the concentrations of total fatty acids and PUFAs (Table 1). ANOVA of each individual fatty acid (micromoles per gram of LDL cholesterol) revealed significant differences (p<0.01) in the concentrations of myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and arachidonic acid (20:4) among LDL subfractions. For all individual fatty acids, the concentration in LDL₃ (micromoles per gram of LDL cholesterol) was significantly higher than in either LDL₁ or LDL₂ (Tukey, p<0.05). No significant differences were found between LDL₁ and LDL₂ in the concentration of each individual fatty acid (Table 1). Furthermore, it was found that the total PUFAs concentration (micromoles per milligram of vitamin E) differed significantly among LDL subfractions (ANOVA, p<0.01). The total PUFAs concentration (micromoles per milligram of vitamin E) was significantly higher in LDL₃ than in LDL₁ (Tukey, p<0.05).
LDL1-LDL2 and LDL2-LDL3 values did not differ significantly. The higher PUFA concentration in LDL2 could be explained by the significantly higher concentration of linoleic acid (micromoles per milligram of vitamin E) in LDL2 compared with LDL1 (Tukey, \( p<0.05 \), Table 1). The total fatty acid concentration (micromoles per milligram of vitamin E) did not differ significantly among LDL subfractions (ANOVA, \( p>0.05 \), Table 1).

**Continuous Monitoring of Oxidation of Low Density Lipoprotein Subfractions**

Differences in absorbance curves among LDL1, LDL2, and LDL3 were found in all subjects. Representative absorbance curves of the three LDL subfractions for one subject are shown in Figure 1. The curve of LDL1 was situated below that for LDL3 in all subjects (\( N=11 \)). The curve of LDL2 was intermediate between that for LDL1 and LDL3 in most subjects (\( n=8 \)). In three subjects, the curves for LDL2 and LDL3 were similar. The distance between the pairwise absorbance curves of LDL1, LDL2, and LDL3 differed among subjects, indicating that each subject had his/her own characteristic LDL subfraction absorbance profile. The absorbance curves were divided into three consecutive phases, that is, a lag phase, a propagation phase, and a decomposition phase (Figure 1).

A highly significant difference in lag time among LDL subfractions was found (ANOVA, \( p<0.001 \)). The lag time of LDL1 was significantly longer than those for LDL2 and LDL3 (Tukey, \( p<0.05 \)). The difference in lag time between LDL1 and LDL2 did not reach statistical significance (Table 2). These results suggest a negative linear trend for the lag time with the LDL subfractions. To demonstrate this possible linear relation between lag time and LDL subfractions, the same models as mentioned in "Statistical Analysis" were used except that the variable LDL subfractions were defined as covariates instead of class variables. Indeed, a negative linear relation between the LDL subfractions and lag time was found (regression coefficient = \(-8.50\pm1.78 \) min per g/ml, \( p<0.001 \)).

Three-way ANCOVA models were applied to determine the influence of the vitamin E concentration, the total fatty acid and PUFA concentrations, and the relative amounts of FC, CEs, TGs, PLs, and protein in the LDL subfractions on lag time. Only the amount of FC (percent of LDL dry mass) contributed significantly to the explanation of the difference in lag time among LDL subfractions. The estimated regression coefficient for FC in the model was \( 4.94\pm2.31, p<0.05 \).

The maximal rate of oxidation among LDL subfractions differed significantly (ANOVA, \( p<0.001 \)). The maximal rate of oxidation of LDL1 was significantly higher than that for LDL3 (Tukey, \( p<0.05 \)). The maximal rate of oxidation for LDL2 was intermediate between those for LDL1 and LDL3. The differences between LDL1-LDL2 and LDL3- LDL4 did not reach statistical significance (Table 2). The suggested positive trend was confirmed by linear regression analysis, which showed a significant positive linear relation between the rate of oxidation and the LDL subfractions (regression coefficient = \( 0.46\pm0.06 \) nmol dienes/min/mg LDL per g/ml, \( p<0.001 \)).

Three-way ANCOVA models revealed that the chemical composition data for FC, CEs, TGs, and PLs in the LDL subfractions, the vitamin E concentration, and total fatty acid and PUFA concentrations in the LDL subfractions had no significant influence on the oxidation rate. Only the amount of protein (percent of LDL dry mass) in the LDL subfractions contributed significantly to the model (regression coefficient = \( 0.097\pm0.038, p<0.05 \)).

The amount of conjugated dienes formed per milligram of LDL protein subfraction differed significantly among LDL subfractions (ANOVA, \( p<0.001 \)). After 4 hours of oxidation, the amount of conjugated dienes formed per milligram protein of LDL1 was significantly lower than in either LDL2 or LDL3 (Tukey, \( p<0.05 \)). The concentration of conjugated dienes in LDL2 and LDL3 did not differ significantly (Table 2). Linear regression analysis showed a significant positive relation between LDL subfractions and the amount of dienes formed per milligram of LDL protein after 4 hours of oxidation (regression coefficient = \( 9.24\pm1.42 \) nmol dienes/mg LDL per g/ml, \( p<0.001 \)).

Of the chemical composition data (FC, CE, TG, PL, and protein), the vitamin E concentration, and the total fatty acid and PUFA concentrations, only the amount of protein (percent of LDL dry mass) in the LDL subfractions related significantly to the amount of dienes formed in the LDL subfractions (three-way ANCOVA, regression coefficient = \( 2.94\pm0.70, p<0.001 \)).
No significant correlations between mean lag time, mean rate of oxidation, mean amount of conjugated dienes over all three LDL subfractions, and TC, TGs, LDL cholesterol, and HDL cholesterol were found (Pearson).

Discussion

By means of single-spin density gradient ultracentrifugation, three LDL subfractions, very light LDL1, light LDL2, and heavy LDL3, were detected and isolated from normolipidemic plasma. Each subject (N=11) had his/her own LDL subfraction pattern that was characterized by the distribution of LDL among the three LDL subfractions. These LDL subfractions differed in chemical composition as shown in Table 1. The relative content of FC, CE, and PLs decreased and that of protein increased with increasing density. A preponderance of small, dense LDL particles has been associated with a threefold increased risk of myocardial infarction, independent of age, sex, and relative weight.12 Also in patients with hyperapobetalipoproteinemia, there is an increase of the heavy LDL3 subfraction in plasma and an increased risk of atherosclerosis.10,11,39 However, the reason for the suggested higher atherogenicity of LDL3 is not yet clear.

The biologic modification of LDL seems to play an important role in the development of atherosclerosis (for review, see Reference 40). In vivo, the most likely process responsible for this modification of LDL is lipid peroxidation.41,42 In our study, we showed that the three LDL subfractions differed in their susceptibility to lipid peroxidation in vitro; the heavy LDL3 was less well protected against oxidative modification than the very light LDL1. Once oxidation started, LDL3 was more extensively modified in time than was LDL1. The oxidation profile of LDL2 was intermediate between those of LDL1 and LDL3 but did not differ significantly from the oxidation profile of LDL3 (Table 2). These findings provide a hypothetical but intriguing explanation for the suggested higher atherogenicity of the more dense LDL subfractions, that is, LDL2 and LDL3; they are more susceptible to oxidative modification and, therefore, contribute more to foam cell formation during atherogenesis than does the less dense LDL subfraction (LDL1).

In our study, the kinetics of the lipid peroxidation process was determined by measuring the formation of 234-nm-absorbing oxidation products every 3 minutes on a UV spectrophotometer.30,31 This method requires only a small sample size and provides the opportunity to follow the lipid peroxidation process more continuously than does the measurement of malondialdehyde as a thiobarbituric acid–reactive substance.43

Several studies have shown that the initiation of lipid peroxidation in LDL is intimately linked with its antioxidant content.29,31,44 However, in our study the concentration of vitamin E (milligrams per gram LDL protein) in the LDL subfractions did not contribute to the explanation in the variation of lag time among LDL subfractions (Table 2). Apparently, variable concentrations of additional antioxidants (lycopene, retinoids, carotenoids) in the different LDL subfractions may be responsible for their differences in lag time, in agreement with the findings of others.45,46 In this study, it was found that the concentration of PUFAs per milligram of vitamin E was significantly higher in LDL3 than in LDL1 (Table 1), suggesting a relative deficiency of vitamin E in LDL3. This could help to explain the shorter lag time of LDL3 compared with that of LDL1.

Once the lipid peroxidation process started (propagation phase, Figure 1), the rate of oxidation for LDL3 was higher than that for LDL1 (Table 2), indicating that the breakdown of PUFAs in dense LDL was faster than that in very light LDL1. The main PUFAs in the LDL subfractions were linoleic acid (70%) and arachidonic acid (30%) (Table 1). These PUFAs differ in their susceptibility to oxidation; the more unsaturated the fatty acids, the more susceptible they are to oxidation.44,47 The concentration of arachidonic acid (micromoles per gram of LDL cholesterol) in LDL3 was significantly higher than in LDL1 (Table 1), which may explain the higher rate of oxidation of LDL3 compared with that of LDL1. After 4 hours of oxidation, the amount of conjugated dienes formed in LDL3 was higher than in LDL1 (Table 2). As the concentration of PUFAs (micromoles per gram of LDL cholesterol and micromoles per milligram of vitamin E) in LDL3 was higher than in LDL1 (Table 1), presumably more PUFAs will be degraded to conjugated dienes and other oxidation products during the oxidation process of LDL3 than of LDL1. However, no significant influence of the fatty acid and PUFAs concentrations on the rate of oxidation or the amount of conjugated dienes in the LDL subfractions was found. This may be explained by the small number of subjects (n=8) and the relatively large variation in the concentration of the fatty acids among the subjects, together with the fact that the total fatty acid and PUFAs concentrations were calculated by adding the individual fatty acids.

In trying to explain the differences in response to oxidation among LDL subfractions, we also considered the different physiochemical properties of the LDL subfractions. The LDL subfractions differ in chemical composition as shown in Table 1. Several studies have indicated that differences in chemical composition can lead to conformational changes of the LDL particle.48,49 This could theoretically result in a different exposure of the antioxidants and PUFAs in the LDL subfractions to attack by free radicals during the lipid peroxidation process. A possible association between structural differences and susceptibility to oxidation was studied by investigating the influence of the chemical composition data on the parameters of the oxidation profile of the three LDL subfractions. The shorter lag time of LDL3 may result from enhanced consumption of antioxidants due to the structure of the LDL3.
particle. Statistical analysis revealed that only the FC content of the LDL subfractions helped to explain the difference in lag time among the LDL subfractions. The biologic relevance of this finding remains unclear. Also, we found that the amount of protein in the LDL subfractions helped to explain the difference in oxidation rate and the amount of conjugated dienes formed after 4 hours of oxidation among LDL subfractions. Recently, new information on the formation of apo B and on structural differences among human LDL subfractions has been reported, which could result in a more obvious exposure of arachidonic acid and other PUFAs in LDL to free radicals formed during the lipid peroxidation process, thus explaining the higher rate of oxidation of LDL and the higher concentration of conjugated dienes found in LDL after 4 hours of oxidation, respectively.

In addition to conjugated dienes, presumably other reactive aldehydes may also be present in LDL than in LDL and LDL. The aldehydic lipid peroxidation products react with the positively charged ε-amino groups of lysine residues of apo B, resulting in a progressive increase of the negative charge of the modified LDL. Also, apo B-100 will be degraded to a certain extent during the oxidation process. Our results have shown that the oxidized LDL subfractions had an increased electrophoretic mobility on agarose gel and extensive degradation of apo B-100. These results confirm that, indeed, we were studying the phenomenon known as biologic modification of LDL.

In summary, the differences in response to oxidation among LDL subfractions in one subject and among different subjects can be explained by the large variability in the fatty acid and antioxidant contents in the LDL subfractions and in the subjects. It is reasonable to assume that this reflects to some extent the different dietary conditions of the subjects studied. Also, specific different physicochemical and structural properties of the LDL subfractions can contribute to the differences in oxidizability among LDL subfractions.

As reviewed by Steinberg et al, oxidative modification is not likely to occur in the circulation. However, it may take place in the vascular wall, which is devoid of well-equipped antioxidant mechanisms. The small quantitative differences in the susceptibility to oxidation among LDL subfractions in vitro, as shown in this report, may have implications for the in vivo situation. In conditions like hyperapobetalipoproteinemia, the relatively faster oxidative modification of heavy LDL in the vessel wall, combined with the elevated plasma LDL concentration, may result in excessive uptake of the modified LDL by the scavenger receptor, converting macrophages into foam cells, the hallmark of atherosclerotic plaques. This hypothe-
sis may explain the higher incidence of CAD in patients with hyperapobetalipoproteinemia.

References


**KEY WORDS** • low density lipoprotein subfractions • lipid peroxidation • conjugated dienes • vitamin E • fatty acids • atherosclerosis
Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects.

J de Graaf, H L Hak-Lemmers, M P Hectors, P N Demacker, J C Hendriks and A F Stalenhoef

doi: 10.1161/01.ATV.11.2.298

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