Electron Microscopic Structure of Serum Lipoproteins from Patients with Fish Eye Disease

Trudy M. Forte and Lars A. Carlson

The structure and composition of lipoprotein fractions from two patients with fish eye disease were examined. The composition of very low density lipoproteins (VLDL) was normal, although total mass was greatly elevated. The mean particle sizes of the VLDL were 44.6 ± 22.2 and 42.8 ± 19.8 nm for Patients 1 and 2, respectively. Intermediate density lipoprotein (IDL) concentrations in patients were elevated and contained increased triglyceride content; particle sizes for Patients 1 and 2 were 29.4 ± 3.5 nm and 28.0 ± 4.1 nm, respectively. In both patients, the triglyceride/cholesterol ester ratio in LDL was approximately tenfold higher than in normal individuals; however, the LDL particles were somewhat smaller in diameter (23.5 ± 3.0 nm for Patient 1 and 23.3 ± 3.8 nm for Patient 2) than those of controls (25.8 ± 3.0 nm and 24.9 ± 3.4 nm). In both patients, large vesicular structures were occasionally encountered in the LDL region. The high density lipoprotein (HDL) fraction of fish eye disease patients showed the greatest abnormalities. Not only was the total HDL concentration extremely low (approximately 10% of control levels), but unesterified cholesterol was increased relative to cholesteryl ester. Particle morphology was heterogeneous; the major HDL species was a small spherical particle with a diameter of 7.6 nm. Discoidal particles with a thickness of 4.4 nm and diameters between 17.4 and 20.8 nm were also present, together with large (40–90 nm) vesicles. Since lecithin:cholesterol acyltransferase activity levels appear normal in fish eye disease patients, the accumulation of these unusual structures must be due to other metabolic defects such as low total apolipoprotein A-I content and/or association of apolipoprotein E with unesterified cholesterol-rich particles that inhibit their esterification.

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**Methods**

**Subjects**

The two FED patients (FED 1 and FED 2) in the present study have been extensively described. Two normal adults (C 1 female and C 2 male) were used as controls. All subjects gave informed consent in accordance with institutional guidelines. All subjects fasted overnight, and blood was drawn in the morning. The blood was allowed to clot at room temperature for 1 to 2 hours and serum was separated after low speed centrifugation for 10 minutes. EDTA and merthiolate (1.4 and 0.25 mmol/l, respectively) were added to the serum.

**Preparation of Fractions**

The major lipoprotein fractions including VLDL (d < 1.006 g/ml), IDL (d = 1.006-1.019 g/ml), LDL (d = 1.019-1.063 g/ml), and HDL (d = 1.063-1.21 g/ml) were isolated by sequential ultracentrifugation as previously described. Densities were adjusted by the addition of NaCl/NaBr and checked in a Paar densitometer (Graz, Austria). The lipid and protein composition of fractions were determined as described elsewhere. The fractions in their respective salt backgrounds were packed on wet ice and air-shipped to Berkeley, California. Shipping required less than 24 hours.

**Electron Microscopy**

Upon arrival in Berkeley, the samples were immediately dialyzed against 0.13 M ammonium acetate buffer (pH 7.4) containing 345 μM EDTA for electron microscopy. The samples were mixed with 2% sodium phosphotungstate (pH 7.4) and after removal of excess fluid were immediately examined in a JEM 100C (JEOL, Tokyo, Japan) electron microscope. Particle size distribution was obtained on approximately 200 free-standing particles per sample with a sonic digitizer (Science Accessories Corporation Graf-Pen, Southport, Connecticut) as previously described. VLDL size was adjusted for flattening effects according to the procedure described by Glomset et al.

**Gradient Gel Electrophoresis**

The size distribution of the HDL samples were analyzed on precast 4-30% gels (PAA 4/30, Pharmacia, Piscataway, New Jersey) according to the procedure of Blanche et al. For all samples, 6 to 8 μg protein were applied to the gels. The reference proteins used to determine particle diameter included thyroglobulin, apoferritin, lactate dehydrogenase, and bovine serum albumin. The gels were stained with Coomassie G-250. Densitometric scans of gels were obtained with a Transidyne RFT densitometer (Transidyne Corporation, Ann Arbor, Michigan).

**Results**

The composition of the major lipoprotein fractions from the FED and two controls are shown in Table 1. It is evident that the triglyceride concentration in FED VLDL is five- to tenfold higher than in control sub-

### Table 1. Composition of Serum Lipoprotein Fractions from Fish Eye Disease Patients and Control Subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Protein (mg/ml serum)</th>
<th>TG (μmol/ml serum)</th>
<th>CE (μmol/ml serum)</th>
<th>UC (μmol/ml serum)</th>
<th>PL (μmol/ml serum)</th>
<th>Mole ratio (TG/CE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL FED 1</td>
<td>0.45</td>
<td>2.89</td>
<td>0.89</td>
<td>0.85</td>
<td>1.22</td>
<td>3.25</td>
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<tr>
<td>FED 2</td>
<td>0.48</td>
<td>3.47</td>
<td>0.86</td>
<td>0.95</td>
<td>1.28</td>
<td>4.03</td>
</tr>
<tr>
<td>C 1</td>
<td>0.14</td>
<td>0.59</td>
<td>0.17</td>
<td>0.14</td>
<td>0.26</td>
<td>3.47</td>
</tr>
<tr>
<td>C 2</td>
<td>0.09</td>
<td>0.28</td>
<td>0.08</td>
<td>0.07</td>
<td>0.13</td>
<td>3.50</td>
</tr>
<tr>
<td>IDL FED 1</td>
<td>0.26</td>
<td>0.33</td>
<td>0.31</td>
<td>0.26</td>
<td>0.31</td>
<td>1.06</td>
</tr>
<tr>
<td>FED 2</td>
<td>0.27</td>
<td>0.34</td>
<td>0.31</td>
<td>0.27</td>
<td>0.33</td>
<td>1.10</td>
</tr>
<tr>
<td>C 1</td>
<td>0.08</td>
<td>0.05</td>
<td>0.11</td>
<td>0.06</td>
<td>0.06</td>
<td>0.45</td>
</tr>
<tr>
<td>C 2</td>
<td>0.11</td>
<td>0.06</td>
<td>0.12</td>
<td>0.07</td>
<td>0.07</td>
<td>0.50</td>
</tr>
<tr>
<td>LDL FED 1</td>
<td>1.43</td>
<td>1.80</td>
<td>2.47</td>
<td>1.61</td>
<td>2.19</td>
<td>0.73</td>
</tr>
<tr>
<td>FED 2</td>
<td>1.41</td>
<td>1.64</td>
<td>2.67</td>
<td>1.70</td>
<td>2.23</td>
<td>0.61</td>
</tr>
<tr>
<td>C 1</td>
<td>0.82</td>
<td>0.21</td>
<td>2.80</td>
<td>1.14</td>
<td>1.29</td>
<td>0.08</td>
</tr>
<tr>
<td>C 2</td>
<td>0.65</td>
<td>0.15</td>
<td>2.02</td>
<td>0.98</td>
<td>0.88</td>
<td>0.07</td>
</tr>
<tr>
<td>HDL FED 1</td>
<td>0.17</td>
<td>0.02</td>
<td>0.04</td>
<td>0.05</td>
<td>0.13</td>
<td>*</td>
</tr>
<tr>
<td>FED 2</td>
<td>0.23</td>
<td>0.02</td>
<td>0.05</td>
<td>0.06</td>
<td>0.18</td>
<td>*</td>
</tr>
<tr>
<td>C 1</td>
<td>1.47</td>
<td>0.06</td>
<td>1.00</td>
<td>0.23</td>
<td>0.98</td>
<td>*</td>
</tr>
<tr>
<td>C 2</td>
<td>1.58</td>
<td>0.06</td>
<td>1.15</td>
<td>0.34</td>
<td>1.26</td>
<td>*</td>
</tr>
</tbody>
</table>

TG = triglyceride; CE = cholesteryl ester; UC = unesterified cholesterol; PL = phospholipid; FED = fish eye disease; C = control.

*The TG/CE mole ratio was not included for HDL since in FED patients this fraction is extremely heterogeneous with respect to particle morphologies.
Figure 1. Electron micrographs of negatively stained fractions from FED Patient 1. A. VLDL. Particles are extremely heterogeneous in size. B. IDL. C. LDL. Round particles are fairly homogeneous. Occasional large, lucent structures (arrowhead) are seen in this density range. D. HDL. Several morphological entities are present. The small, round structures in the background are the major morphological species. Discoidal structures that form rouleaux are also present together with an occasional, very large electron lucent particle (arrowhead); the latter are seen more clearly in the inset. Bars = 100 nm.
Figure 2. Electron micrographs of negatively stained fractions from a control subject. A. VLDL. Heterogeneous round structures are smaller than those seen in FED 1. B. IDL. Round, lucent particles. The irregularly shaped, small, light areas in the background are artifacts caused by uneven spreading of the stain due to hydrophobicity of the grid surface. C. LDL. D. HDL. Fraction consists of heterogeneous, small, round particles where the majority have diameters between 8.0–11.0 nm. Larger particles (arrowhead) which have diameters between 20.0–28.0 nm may be contaminating LDL. Bars = 100 nm.
jects. Similar elevations of triglyceride are also apparent in both the IDL and LDL fractions. Comparison of the mole ratio of triglyceride to cholesteryl ester indicates the core composition of FED VLDL is comparable to that of the controls. However, the triglyceride/cholesteryl ester ratio for both IDL and LDL from the FED patients is quite abnormal and suggests that the cores of both these particles are highly enriched in triglyceride. Table 1 also indicates that the total mass of FED HDL is approximately only 10% that of controls. Additionally, unesterified cholesterol is increased relative to cholesteryl ester in patients’ HDL in which unesterified cholesterol accounts for 55% and 56% of the total cholesterol, compared to only 19% to 23% in control HDL.

The electron microscopic structures of VLDL, IDL, LDL and HDL from FED Patient 1 are shown in Figure 1. For comparison, similar fractions isolated from a control subject are shown in Figure 2. The mean particle sizes of the lipoprotein fractions from both patients and two control subjects are summarized in Table 2. FED VLDL, like normal VLDL, are very heterogeneous in size as indicated in Figure 1 A, but have a larger mean particle diameter than comparable fractions from the controls (Table 2). A much broader range of particle size in FED patients (24.0 to 102.0 nm) compared to that of controls (24.0 to 74.0 nm) accounts for the larger mean particle size in the patients and shows that the patients possess larger particles not present in the controls. The IDL from FED patients are round structures (Figure 1 B) similar to those of the control subjects (Table 2). The LDL from FED patients (Figure 1 C) are fairly homogeneous round particles that have smaller mean diameters (23.5 ± 3.0 nm and 23.3 ± 3.8 nm for Patients 1 and 2, respectively, Table 2) than control LDL (25.8 ± 3.0 nm and 24.9 ± 3 nm for Control 1 and 2, respectively, Table 2). Although FED LDL have an overall smaller mean diameter, they have the same size range (18.0 to 32.0 nm) as control LDL. Occasionally, a few larger translucent structures (50 to 85 nm diameter) are seen among the patients’ LDL particles. As suggested by the representative micrograph of the control LDL (Figure 2 C), large translucent structures are absent from the normal LDL. The HDL fractions of FED patients, as exemplified by Figure 1 D, contain several particle morphologies. The two major components in Figure 1 D are small round particles which are 7.6 nm in diameter (range 6 to 10 nm) and discoidal particles which form short rouleaux. The long axis of the stacked discs is approximately 17.4 ± 4.7 nm, while the thickness is 4.4 ± 0.3 nm. In addition to small molecular weight HDL and discoidal particles, larger particles, which often appear electron-translucent, are also present in FED HDL (Figure 1 D). In FED Patient 2 these larger structures were particularly obvious in more highly concentrated electron microscopic fields (Figure 3). The large particles which range from 40 to 90 nm in diameter (mean diameter 70.3 ± 13.5 nm) appear to be flattened vesicles.

The spherical HDL particles of FED, as determined by electron microscopy, are smaller than those of controls (Table 2) which is consistent with the gradient gel electrophoresis patterns shown in Figure 4. These patterns indicate that FED HDL size distributions are shifted to smaller particles compared to the control. As reported previously,2,12 FED HDL are unusual in that they possess a pronounced gradient gel electrophoresis band at approximately 115,000 daltons and trace amounts of material with larger molecular weights. In the present study, scans of the electrophoretic pattern of FED HDL (Figure 4) indicate that the small molecular weight particle has a size of approximately 7.6 nm which is in the (HDL(3c)apo) region according to the nomenclature of Blanche et al.11 Scant material is found in this region in normal individuals where (HDL(3c)apo) is the prominent component. The present gradient gel electrophoretic analysis reveals that larger components extending into the (HDL(2i)apo) region are also present. This observation is consistent with electron microscopic data which indicates that more than one population of particles is present in FED HDL.

Table 2. Size (nm ± sd) of Lipoproteins from Patients with Fish Eye Disease: Summary of Electron Microscopic Data

<table>
<thead>
<tr>
<th>Subject</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spherical</td>
<td>Long axis disc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FED 1</td>
<td>44.6±22.2</td>
<td>29.4±3.5</td>
<td>23.5±3.0</td>
<td>7.6±1.3</td>
</tr>
<tr>
<td>FED 2</td>
<td>42.8±19.8</td>
<td>28.0±4.1</td>
<td>23.3±3.8</td>
<td>7.8±1.3</td>
</tr>
<tr>
<td>C 1</td>
<td>39.5±11.4</td>
<td>31.1±3.3</td>
<td>25.8±3.0</td>
<td>8.7±1.4</td>
</tr>
<tr>
<td>C 2</td>
<td>38.4±12.5</td>
<td>30.1±3.3</td>
<td>24.9±3.4</td>
<td>8.1±1.6</td>
</tr>
</tbody>
</table>

FED = fish eye disease; C = control.

*Large vesicular structures were also present in this fraction in FED subjects. Disc thickness = 4.4 ± 0.3 nm.
LIPOPROTEIN STRUCTURE IN FISH EYE DISEASE  Forte and Carlson

Figure 3. HDL fraction from FED Patient 2. Micrograph shows morphology of the large, lucent particles in this fraction. When particles are crowded, they stack and appear to be flattened vesicles (arrows). Bar = 100 nm.

Figure 4. Densitometric scans of HDL applied to 4–30% gradient gels (approximately 8 μg of protein per sample). Gels were stained with Coomassie G-250 stain before scanning. A. FED Patient 1. B. FED Patient 2. C. Control. The (HDL)_{gge} subspecies as described by Blanche et al.\textsuperscript{11} are indicated. The numbers above the major peaks and shoulders indicate particle diameter in nm.

Discussion

FED is characterized by severe corneal opacity that results in visual impairment; moreover, this disease is also associated with pronounced dyslipoproteinemia. The latter is most apparent in patients’ LDL which have elevated levels of triglyceride and in HDL which are present at 10% the level of normal individuals.\textsuperscript{1,2} The concentration of total serum triglyceride is also elevated, which, as shown in the present study, is due to substantially elevated triglyceride levels in both VLDL and LDL. The morphology of FED VLDL is normal, although the mean diameter is larger than in normal subjects. The larger mean diameter of FED VLDL is consistent with the increased lipid/protein weight ratio which is apparent in Table 1. Hypertriglyceridemia with elevated triglyceride in VLDL and LDL is also a feature of familial LCAT deficiency. This latter disorder, however, contains VLDL with unusual morphologies in which the particle surfaces appear notched.\textsuperscript{10} However, similar profiles were not seen in FED VLDL.

Electron microscopy revealed that FED LDL are approximately 10% smaller in diameter than control LDL; this difference based on t-test analysis is significant (\(p < 0.0001, n = 400\)). LDL particles with diameters smaller than control values have been reported in Tangier disease\textsuperscript{13} and in LCAT deficiency.\textsuperscript{14} Triglyceride-laden LDL appear to be a phenomenon associated with disorders demonstrating abnormally
low levels of HDL\textsubscript{4}, \textsuperscript{13-16} which indicates that normal levels of HDL are required for clearance of triglyceride in less dense particles. Lipase levels are normal to 50% of normal in these various disorders, but if triglyceride for cholesteryl ester exchanges are depressed due to extremely reduced HDL levels, this mechanism is interrupted and triglyceride accumulates abnormally. In the case of FED, Calvert and Carlson\textsuperscript{17} have recently shown that the activities of cholesteryl ester and triglyceride exchange proteins are normal, thus supporting the hypothesis that in this disease severely reduced HDL mass is the most likely explanation for abnormal accumulation of triglyceride in LDL.

A preponderance of small, spherical (7.6 nm) HDL particles is the hallmark for FED HDL. Small molecular weight particles have previously been described in LCAT deficiency\textsuperscript{18, 19} and in Tangier disease.\textsuperscript{20} These latter two disorders are associated with abnormalities in apolipoprotein content; however, this is not the case in FED where no qualitative abnormalities in HDL apolipoproteins are apparent.\textsuperscript{12} The accumulation of large vesicular structures in FED HDL is unusual; however, they are morphologically similar to the large, flattened particles found in Tangier HDL after a fat meal.\textsuperscript{21} These particles may represent surface remnant structures generated during lipolysis. Low HDL concentration and the concomitant decrement of apolipoproteins required for complete metabolism of vesicular particles may lead to their accumulation in FED patients. The fact that similar structures are encountered in the LDL region suggests a precursor-product relationship in which surface remnants are originally generated in less dense regions by lipolysis.

The discoidal particles seen in FED HDL are similar in overall dimension to those described for LCAT deficiency.\textsuperscript{7} These discoidal particles, together with the flattened vesicular structures, may account for the larger particles seen in gradient gel electrophoresis. Apo A-I-phospholipid complexes with discoidal morphology have recently been shown by Nichols et al.\textsuperscript{22} to migrate in the (HDL\textsubscript{2a},apoE) and (HDL\textsubscript{3a},apoE) regions. A broad spectrum of HDL particle size, as determined by gradient gel electrophoresis, has also been reported for familial LCAT deficiency by Soutar et al.\textsuperscript{18} Since the major HDL structure in the latter disorder is discoidal, it strongly suggests that discoidal structures in both FED and LCAT deficiency can account for the large spread of particle size seen in the gradient gels.

The elevated relative concentration of unesterified cholesterol in FED HDL may be accounted for in part by the presence of both discoidal and vesicular particles. Compositional studies on discoidal HDL in LCAT deficiency have clearly shown that these particles are rich in unesterified cholesterol. Similarly, large vesicular particles found in the LDL region of LCAT deficiency are also rich in unesterified cholesterol. Although compositional and morphological similarities between LCAT deficiency and FED are somewhat striking, FED patients have normal levels of LCAT activity.\textsuperscript{2} The persistence of discoidal particles in conjunction with apparently normal levels of LCAT activity in FED patients is certainly anomalous. This is particularly true since the blood of our patients was allowed to clot at room temperature for 1 to 2 hours before serum was removed, conditions which would favor cholesterol esterification. In the present study, we cannot completely rule out that some changes in HDL size distribution and morphology have occurred during the processing of FED serum in the absence of specific LCAT inhibitors. Previous in vitro incubation studies by Norum et al.\textsuperscript{23} with LCAT-deficient plasma and purified LCAT enzyme that was partially inactivated by heat or p-chloromercuriphenylsulfonic acid, yielded particles intermediate in size between large and small molecular weight HDL components. More recently it was shown that untreated normal plasma incubated for either 6 hours\textsuperscript{24} or 24 hours\textsuperscript{25} at 37 °C resulted in a shift of smaller HDL particles (HDL\textsubscript{2a,apoE}) and (HDL\textsubscript{3a,apoE}) to larger (HDL\textsubscript{2a,apoA}) particles. Mercaptoethanol, which stimulates LCAT activity, produced a double shift in HDL size with a 6-hour incubation at 37 °C,\textsuperscript{26} a very pronounced peak appeared in the (HDL\textsubscript{2a,apoE}) region and a distinct peak appeared in the (HDL\textsubscript{3a,apoE}) region. This shift was not totally inhibited by the LCAT inhibitor, 5,5'-dithiobis-2-nitrobenzoic acid. Incubation studies with FED patients' plasma in the presence and absence of LCAT inhibitors are currently under investigation to obtain insights into the origin of the HDL species. A possible explanation for the presence of discoidal particles in FED patients is that the low total HDL apo A-I, a cofactor for LCAT, may be responsible for accumulation of unesterified cholesterol and discoidal particles in the face of normal enzyme levels. One can also not exclude the possibility that apo E, which appears to inhibit LCAT activity\textsuperscript{27} and which, according to Carlson and Holmquist,\textsuperscript{12} is abundant in FED HDL, may be associated with the unesterified cholesterol-rich particles, thus preventing esterification. It has recently been shown\textsuperscript{28} that discoidal HDL structures are present in patients with familial partial LCAT deficiency. In this disorder, where LCAT activity is 40% of normal, the discoidal particles are associated with apolipoproteins E, A-I and A-IV, giving further support to the possibility that the apolipoprotein composition of the particles may play a role in their interaction with LCAT. The basic metabolic defect(s) responsible, however, for abnormally low HDL levels in FED still remains to be elucidated. In addition, the relationship between aberrant HDL and accumulation of lipid in the cornea and nonassociation of reduced HDL levels with atherosclerosis are as yet unanswered questions.

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LIPOPROTEIN STRUCTURE IN FISH EYE DISEASE

Forte and Carlson

References


Index Terms: fish eye disease • electron microscopy • gradient gel electrophoresis • very low density lipoproteins • intermediate density lipoproteins • low density lipoproteins • high density lipoproteins
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