Detection of Two Species of Low Density Lipoprotein Particles in Cholesteryl Ester Transfer Protein Deficiency

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By equilibrium density gradient ultracentrifugation, we analyzed the chemical composition and particle size of low density lipoproteins (LDLs) in 16 subtractions separated from the LDL fractions (1.019<d<1.063 g/ml) of two hyperalphalipoproteinemic patients who had a deficiency of cholesteryl ester transfer protein (CETP). The LDLs of these patients comprised a group of heterogeneous lipoprotein particles distributed almost equally in a wide density range from d=1.025 g/ml to d=1.053 g/ml, whereas LDLs from normal controls were a homogeneous group of lipoprotein particles distributed in a narrow density range from d=1.030 g/ml to d=1.046 g/ml. The LDL in each subfraction derived from the patients' plasma samples was poor in cholesteryl ester and rich in triglycerides and apolipoproteins. Each subfraction of normal control LDL contained only one species of homogeneous LDL particles, which progressively decreased in size with an increase in the density of the fraction. In contrast, each subfraction of patient LDL contained two species of LDL particles: smaller LDLs existed, in addition to those that were found to be identical to the normal control LDL particles observed in the corresponding subfractions. The intermediate density lipoproteins of the two patients were also composed of two species of lipoproteins. From these results, we speculate that two metabolic pathways may exist in the LDL formation process. In this process, the transfer of cholesteryl ester from high density lipoproteins by CETP may convert the smaller lipoprotein particles to the larger ones, forming the homogeneous LDL species. (Arteriosclerosis and Thrombosis 1991;11:71-79)

Several hyperalphalipoproteinemic patients with a deficiency of cholesteryl ester transfer protein (CETP) activity have been identified in Japan, including those reported from our institution.1-3 We have recently reported that the deficiency of CETP activity is attributable not to the presence of an inhibitor but to the lack of the protein itself.4 More recently, Brown et al5 have reported one hyperalphalipoproteinemic family with a point mutation (G-A substitution in the 5'-splice donor site of intron 14 of the CETP gene). Although the negative correlation between serum high density lipoprotein (HDL) cholesterol levels and the incidence of atherosclerosis is widely accepted,6-8 the question remains to be resolved as to whether hyperalphalipoproteinemia caused by CETP deficiency is antiatherogenic or not. In this connection, it is important to analyze the lipoprotein metabolism in patients with CETP deficiency to clarify the physiological function of CETP. We have previously reported that a deficiency of CETP activity causes the accumulation of cholesteryl ester in HDLs, forming HDL-like particles.9,10 Abnormalities of lipoprotein metabolism existed not only for HDL but also with respect to low density lipoprotein (LDL); LDL showed marked polydispersity, and the mean particle size was small.3 In this study, we analyzed the abnormal LDL from patients with CETP deficiency more precisely by the method of equilibrium density gradient ultracentrifugation. We offer some speculations on the role of CETP in the process of LDL formation, and we propose a new concept of the process of LDL formation.

Methods

Subjects

Two hyperalphalipoproteinemic patients, a 45-year-old Japanese man (case 1) and a 54-year-old Japanese woman (case 2), were investigated in this study. Case 1 has been previously described as case 2.3 Their serum HDL cholesterol concentrations were 231 and 175 mg/dl, respectively. CETP activity

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was measured according to the method of Albers et al and was completely deficient in both patients. Their clinical profiles and the concentrations of serum lipids and apolipoproteins (apos), as well as the lipid concentrations in each lipoprotein fraction, have been described in detail elsewhere. Informed consent was obtained from each patient.

Four healthy, normolipidemic, male volunteers were also investigated. The age of these control subjects was 33±2 years (mean±SD; range, 31–35 years). The analyses were performed for all controls, and the gel representative of these controls is shown in the “Results” section.

Isolation of Lipoproteins

Plasma was obtained after all subjects underwent an overnight fast. Blood was drawn from an antecubital vein into tubes containing Na2EDTA at a final concentration of 1 mg/ml. Plasma was immediately separated by low-speed centrifugation at 3,000 rpm for 20 minutes. A stock solution of aprotonin, gentamicin, and Na2EDTA was added soon after plasma separation to final concentrations of 10,000 IU/ml, 0.0005% (wt/vol), and 1 mM, respectively, to inhibit microbial growth and degradation of lipoproteins. Very low density lipoprotein (VLDL, d<1.006 g/ml), intermediate density lipoprotein (IDL, 1.006<d<1.019 g/ml), and low density lipoprotein (LDL, 1.019<d<1.063 g/ml) were isolated by sequential preparative ultracentrifugation of plasma. The nonprotein solvent density of LDL samples was adjusted to 1.040 g/ml. All procedures were done at 4°C.

Density Gradient Fractionation of Low Density Lipoprotein

Density gradient ultracentrifugation analysis was performed according to the method of Chapman et al with some modifications. Discontinuous density gradients were constructed in centrifuge tubes (Polyallomer, 3% in ×4 in., Beckman, Palo Alto, Calif.) by underlaying 2.6 ml of d=1.019 g/ml solution, 2.6 ml of d=1.024 g/ml solution, 4.6 ml of an LDL sample at d=1.040 g/ml, and 6 ml of d=1.054 g/ml solution. The tubes were then centrifuged to equilibrium at 25,000 rpm for 97 hours at 20°C with a Beckman SW28.1 rotor and a Beckman LS-55M ultracentrifuge. At the end of the centrifugation, the gradients were fractionated by manual aspiration with Pasteur pipettes into 16 tubes (1 ml in each tube). The salt density of the subfractions in a background tube was measured with a digital precision density meter (model DMA 60, Anton Paar, Graz, Austria) equilibrated at 20°C. The standard deviation of average density in each subfraction within four separate runs was less than 0.001 g/ml, and that within four separate tubes in the same ultracentrifugal run was also less than 0.001 g/ml.

Determination of Lipoprotein Composition

Total cholesterol, free cholesterol, triglycerides, and phospholipids were determined by enzymatic methods (Kyowa Medex Co., Tokyo, Japan). Cholesterol ester concentrations were calculated as (total cholesterol—free cholesterol)×1.68. Protein concentrations were determined by the method of Lowry et al using bovine serum albumin (BSA) as the standard.

Nondenaturing Polyacrylamide Gradient Gel Electrophoresis of Lipoproteins

Nondenaturing polyacrylamide gradient gel electrophoresis of total LDL (1.019<d<1.063 g/ml) and each subfraction was performed in an electrophoresis apparatus (model GE-2/4, Pharmacia, Uppsala, Sweden) with 2–16% gradient gels (PAAG 2/16, Pharmacia). Electrophoresis was performed at 125 V for 24 hours at 4°C in a Tris/borate buffer (0.09 M Tris/0.08 M boric acid/0.003 M Na2EDTA, pH 8.3). Gels were prerun at 125 V for 30 minutes before sample application. Three micrograms protein for a normal control and 6 µg protein for the patients was applied to each lane in a sample volume of 1–20 µl. Since the electrophoresis of LDL from the patients formed several bands on this gel as described below, we needed more protein for the patients’ samples to visualize each band clearly. Electrophoresis was started at 20 V for 120 minutes; then, the voltage was raised to 40 V for 20 minutes, to 80 V for 40 minutes, and finally to 125 V for 24 hours. After electrophoresis, the gels were fixed and stained in 50% methanol/9% acetic acid/0.1% (wt/vol) Coomassie Brilliant Blue R250 for 30 minutes and then destained with 20% methanol/9% acetic acid. Densitometric scanning of the gels was performed at 555 nm with a Model CS-9000 scanner from Shimadzu Corp., Kyoto, Japan. A calibration curve was generated from the migration distances of standards of known diameter, such as latex beads (380 Å), thyroglobulin (170 Å), and ferritin (122 Å). The Stokes’ radius corresponding to each peak of LDL was calculated from the calibration curve.

Immunoblotting Analysis of Lipoproteins in Density Gradient Subfractions

After electrophoresis, the separated particles were blotted to nitrocellulose paper (Bio-Rad, Richmond, Calif.) at 100 mA for as long as 72 hours at 4°C. The papers were blocked in incubation with 2% BSA in phosphate-buffered saline (PBS) overnight. The papers were then incubated for 1 hour at ambient temperature with anti-human apo A-I rabbit immunoglobulin G (IgG), anti-human apo B goat IgG, or anti-human apo E goat IgG (Daiichi Pure Chemicals, Tokyo, Japan) diluted 1:1,000 in PBS. After extensive washing with 0.05% polysorbate 20 (Tween 20) in PBS, they were exposed to a 1:3,000 dilution (in PBS) of antibody–biotin conjugates specific for anti-rabbit IgG or anti-goat IgG (Sigma Chemical Co., St. Louis, Mo.) for 1 hour. The avidin/biotin peroxidase method was used to stain the blots (Vectastain ABC kit, Vector, Burlingame, Calif.).
Results

Polyacrylamide gradient gel electrophoresis (2–16%) of the ultracentrifugally separated LDL fractions (1.019<d<1.063 g/ml) from the patients and the representative control and the densitometric scans of the same gels are shown in Figure 1. The LDL fractions from the patients contained several species of LDL particles ranging in size from 230 Å to 290 Å, while LDL from the normal controls consisted of a species of lipoprotein particles distributed in a relatively narrow size range. Case 1 also had a lipoprotein species smaller than 230 Å, which was faintly detectable in this gel. We have previously confirmed that this smaller lipoprotein is rich in cholesteryl ester and contains apo A-I and apo E but not apo B, and it is thought to be an HDL-like particle.9,10

Figure 2 shows the distribution of cholesterol mass and total protein mass among the density gradient subfractions prepared by equilibrium density gradient ultracentrifugation. The profile of total protein (panel B) was similar to that of cholesterol mass (panel A). The control LDL cholesterol was distributed around a narrow density range from 1.030 g/ml to 1.046 g/ml, with a single sharp peak; about 75% of total LDL was recovered in subfractions 5–10. In contrast, a wide distribution without a prominent peak was observed over subfractions 1–13 (1.025<d<1.053 g/ml) for the patients’ LDL. Since insufficient material was available for accurate analysis of subfractions 14, 15, and 16, the following analyses were performed only for 13 fractions.

The weight percent chemical compositions of total LDL (1.019<d<1.063 g/ml) and the density gradient subfractions are presented in Table 1. In normal controls, the proportion of free cholesterol tended to decrease slightly, from 9.7% in subfraction 1 to 7.2% in subfraction 13, as the density increased. Phospholipids showed the same tendency as free cholesterol, with the proportion of the former being 21.7% of subfraction 1 and 18.2% of subfraction 13. The proportion of protein increased progressively with the elevation in density. The proportion of cholesteryl ester gradually increased from 38.9% in subfraction 1 to a maximum of 45.0% in subfraction 6, followed by a decrease to 34.9% in subfraction 13. In contrast, the proportion of triglycerides initially fell from 10.0% in subfraction 1 to 3.1% in subfraction 9, followed by a rise to 5.8% in subfraction 13.

In the patients, all the subfractions were rich in triglycerides and poor in cholesteryl ester. The proportion of cholesteryl ester tended to diminish with the increase in density of the subfractions. The proportion of protein gradually increased with the elevation in density of each subfraction in a manner similar to that of the controls, but the proportion was slightly higher in the patients than in the controls. Free cholesterol and phospholipids accounted for a relatively constant proportion of the subfractions as they did in the normal control.

Figure 1. Panel A: Polyacrylamide gradient gel electrophoresis (2–16%) of the low density lipoprotein (LDL) fraction (1.019<d<1.063 g/ml) from a normal control and two patients (cases 1 and 2) with cholesteryl ester transfer protein deficiency. Three micrograms protein for the control or 9 μg for the patients was applied to each lane. Panel B: Densitometric scans of the same gels at 555 nm.

Polyacrylamide gradient gel electrophoresis demonstrated that each control LDL subfraction contained relatively homogeneous lipoprotein particles, which became progressively smaller with the increase in
density as shown in Figure 3. The particle size ranged from 290 Å to 250 Å (Figure 4). In contrast, there were two species of LDL particles with different sizes in each LDL subfraction from the patients. In addition to LDL, a smaller lipoprotein was also faintly detectable in case 1. The large LDL particle species was observed well in the lower-density subfractions, diminished with the increase in density, and was absent in subfractions 11, 12, and 13.

Immunoblotting analysis of electrophoresed lipoproteins from case 1 were performed with a polyclonal antibody specific for human apo A-I, apo B, or apo E (Figure 5). It was revealed that the two bands clearly stained by Coomassie Blue in the patients (Figure 3) had apo B, and both bands were confirmed to be metabolic LDL. It was also shown that the faintly detectable lipoprotein smaller than LDL contained apo A-I and apo E, suggesting it consisted of HDL₁- or HDL₂-like particles.

VLDL (d<1.006 g/ml) and IDL (1.006<d<1.019 g/ml) were also analyzed by gradient gel electrophoresis (Figure 6). The IDL of the patients contained two species of lipoproteins, both of which were larger than the respective LDL subspecies in the most buoyant subfraction. However, VLDL could not be clearly separated into two species of lipoproteins using this gel.

**Discussion**

In this study, we clarified that LDL from patients with a CETP deficiency was distributed almost equally over a wide density range from 1.025 to 1.053 g/ml, whereas the LDL in normal controls formed a homogeneous group of LDL particles in a narrow range from 1.030 to 1.046 g/ml. This confirmed our previous findings that LDL particles in hyperalphalipoproteinemic patients with CETP deficiency showed marked polydispersity.³

Fractionation of lipoprotein particles through equilibrium density gradient ultracentrifugation showed that each subfraction in normal controls contained only one species of homogeneous lipoprotein particle, which decreased in size with the increase of density. However, in CETP deficiency, each subfraction contained two species of LDL particles that were different in size. The size of the large LDL particles was almost identical to that of normal LDL in the corresponding subfraction, so that the appearance of a species of small LDL particles over a wide density range was the characteristic abnormality de-
### Percent Chemical Composition of Low Density Lipoprotein in Density Gradient Subfractions

<table>
<thead>
<tr>
<th>Subfraction number</th>
<th>Total LDL (d=1.019-1.063)</th>
<th>1 (1.025)</th>
<th>2 (1.026)</th>
<th>3 (1.027)</th>
<th>4 (1.029)</th>
<th>5 (1.031)</th>
<th>6 (1.033)</th>
<th>7 (1.035)</th>
<th>8 (1.038)</th>
<th>9 (1.040)</th>
<th>10 (1.043)</th>
<th>11 (1.046)</th>
<th>12 (1.050)</th>
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<tr>
<td>CE</td>
<td>43.6±1.4</td>
<td>38.9±2.1</td>
<td>40.2±1.7</td>
<td>41.4±2.0</td>
<td>44.0±2.9</td>
<td>44.1±3.5</td>
<td>45.0±3.3</td>
<td>44.7±2.6</td>
<td>44.4±2.2</td>
<td>42.8±3.0</td>
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<td>41.9±2.5</td>
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<td>FC</td>
<td>8.6±0.5</td>
<td>9.7±0.6</td>
<td>9.5±0.8</td>
<td>9.6±0.4</td>
<td>9.3±1.0</td>
<td>9.6±0.6</td>
<td>9.4±0.3</td>
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<td>TG</td>
<td>21.7+0.7</td>
<td>21.7+1.5</td>
<td>21.6±0.5</td>
<td>21.5+1.0</td>
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<td>30.2</td>
<td>31.3</td>
<td>30.0</td>
<td>29.5</td>
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<td>31.9</td>
<td>31.8</td>
<td>31.5</td>
<td>30.1</td>
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<td>Protein</td>
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<td>29.5</td>
<td>30.7</td>
<td>31.9</td>
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<td>30.1</td>
<td>28.2</td>
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<tr>
<td>Subfraction number</td>
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<td>35.4</td>
<td>37.4</td>
<td>37.3</td>
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<tr>
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<td>37.4</td>
<td>37.3</td>
<td>35.9</td>
<td>34.6</td>
<td>32.7</td>
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<td>30.6</td>
<td>30.6</td>
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<td>FC</td>
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<td>9.8±0.9</td>
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<td>9.7±1.2</td>
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<td>9.1±0.9</td>
<td>8.9±0.9</td>
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<tr>
<td>TG</td>
<td>21.7±1.5</td>
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<tr>
<td>Protein</td>
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**TABLE 1. Percent Chemical Composition of Low Density Lipoprotein in Density Gradient Subfractions**

- Total LDL: Low density lipoprotein; CE, cholesteryl ester; FC, free cholesterol; TG, triglycerides; PL, phospholipids.
- Values are mean±SD of four normolipidemic controls.
Figure 3. Photograph of polyacrylamide gradient gel electrophoresis (2–16%) of LDL in density gradient subfractions (Fr.) 1–13. Three micrograms protein for the normal control or 6 μg for the patients with CETP deficiency was applied to each lane in a sample volume of 1–10 μL LDL, low density lipoprotein; CETP, cholesteryl ester transfer protein.

From these results, we speculate that two metabolic pathways may exist for LDL formation. We detected in these patients. Both of these two species of LDL particles decreased in particle size successively with an increase in the subfraction density, as observed in the normal controls. The IDL in the patients with CETP deficiency was also composed of two species of lipoprotein particles, each of which was larger than the corresponding LDL particle in the most buoyant density subfraction.

Figure 4. Line plots of LDL particle size (Å) in density gradient subfractions 1–13. Shaded area represents mean±SD of four separate LDL subfractions from normal controls. Large and small LDL particle sizes are present in both patients (○—○, case 1; △—△, case 2). LDL, low density lipoprotein.
propose the following hypothesis. VLDL is secreted by the liver as two species of lipoprotein particles differing in size, and each type of VLDL is successively metabolized to LDL through IDL by a separate pathway (Figure 6). Various modulations might be involved in producing cholesterol-rich LDL, which possesses a high affinity for LDL receptors. The hydrolysis of triglycerides by lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) and the transfer of cholesteryl ester from HDL to apo B-containing lipoproteins by CETP may be important in this process. In fact, the progressive reduction in the size of LDL observed in both the normal controls and the patients might be caused by the actions of LPL and HTGL. The present results suggest that CETP may play an important role in converting small LDL particles to large and homogeneous LDL particles by transferring cholesteryl esters from HDL. This transfer process is probably accomplished in an early phase of LDL formation, such as the VLDL or IDL phase, so that we cannot confirm the presence of the pathway for the smaller LDL in normal controls. Krauss and Musliner et al have reported multiple precursor-product relations involving individual VLDL, IDL, and LDL subpopulations and have suggested the existence of dual pathways for the production of IDL and LDL subspecies. They demonstrated the existence of two sizes of subpopulations in both VLDL and IDL, but only one in LDL, of normal human plasma. The occurrence of the early transfer of cholesteryl ester from HDL to apo B-containing lipoproteins could be supported by their findings. In this study, we could not clearly separate two species of VLDL particles. This difficulty may be attributable to the inability of this gel to separate particles as large as VLDL. The previous findings by discontinuous nonequilibrium density gradient ultracentrifugation of the < 1.019 g/ml lipoproteins, by electron microscopy, and by kinetic study of VLDL have shown the possibility for the presence of two species of VLDL particles in plasma. From these findings, we suppose that two distinct size species of VLDL may be secreted by the liver. However, VLDL may enter into each metabolic pathway after secretion as one species.
Recently, examination of conformational changes has revealed that LDL rich in cholesteryl ester is rapidly taken up into cells via LDL receptors. 24–27 It has also been reported that the small and dense LDL subclass pattern is significantly associated with a risk of myocardial infarction, independent of age, sex, and relative body weight. 28–30 The change in cholesteryl ester content and LDL size induced by CETP, in addition to LPL and HTGL, may facilitate the uptake of LDL particles by cells. It has been suggested that CETP deficiency might be antiatherogenic, since HDL cholesterol levels were increased. However, the abnormality of LDL particles observed in CETP deficiency may not be a favorable change in lipoprotein metabolism. 31 Further characterization of the LDL involved in the small-particle pathway is required, and this may shed light on the problem of whether CETP acts antiatherogenically. 32,33

References

KEY WORDS • reverse cholesterol transport • cholesteryl ester transfer protein • intermediate density lipoproteins • low density lipoproteins • atherosclerosis • high density lipoproteins • hyperalphalipoproteinemia
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