Influence of Plasma Cholesteryl Ester Transfer Activity on the LDL and HDL Distribution Profiles in Normolipidemic Subjects

Laurent Lagrost, Hassan Gandjini, Anne Athias, Valérie Guyard-Dangremont, Christian Lallemant, and Philippe Gambert

The relations of cholesteryl ester transfer protein (CETP) activity to the distribution of low density lipoproteins (LDLs) and high density lipoproteins (HDLs) were investigated in fasting plasma samples from 27 normolipidemic subjects. LDL and HDL subtractions were separated by electrophoresis on 20-160 g/L and 40-300 g/L polyacrylamide gradient gels, respectively. Subjects were subdivided into two groups according to their LDL pattern. Monodisperse patterns were characterized by the presence of a single LDL band, whereas polydisperse patterns were characterized by the presence of several LDL bands of different sizes. To investigate the influence of lipid transfers on LDL patterns, total plasma was incubated at 37°C in the absence of lecithin: cholesterol acyltransferase (LCAT) activity. The incubation induced a progressive transformation of polydisperse patterns into monodisperse patterns. Under the same conditions, initially monodisperse patterns remained unchanged. Measurements of the rate of radiolabeled cholesteryl esters transferred from HDL to very low density lipoproteins (VLDLs) and LDLs revealed that subjects with a monodisperse LDL pattern presented a significantly higher plasma CETP activity than subjects with a polydisperse LDL pattern (301±85%/hr per milliliter versus 216±47%/hr per milliliter, respectively; p<0.02). In addition, when total plasma was incubated for 24 hours at 37°C in the absence of LCAT activity, the relative mass of cholesteryl esters transferred from HDLs to apolipoprotein B-containing lipoproteins was greater in plasma with monodisperse LDL than in plasma with polydisperse LDL (0.23±0.06 versus 0.17±0.06, respectively; p<0.02). These results indicated that in normolipidemic plasma, CETP could play an important role in determining the size distribution of LDL particles. The analysis of lipoprotein cholesterol distribution in the two groups of subjects sustained this hypothesis. Indeed, HDL cholesterol levels, the HDL:VLDL+LDL cholesterol ratio, and the esterified cholesterol: triglyceride ratio in HDL were significantly lower in plasma with the monodisperse LDL pattern than in plasma with the polydisperse LDL pattern (p<0.01, p<0.01, and p<0.02, respectively). Plasma LCAT activity did not differ in the two groups. Plasma CETP activity correlated positively with the level of HDL (r=0.542, p<0.01) in the entire study population. Whereas plasma LCAT activity correlated negatively with the level of HDL (r=-0.455, p<0.05) and positively with the levels of HDL (r=0.475, p<0.05) and HDL* (r=0.485, p<0.05), no significant relation was observed with the level of HDL. These results suggest that both monodisperse LDL patterns and high levels of HDL particles are specific markers of an elevation of CETP activity in normolipidemic plasma.

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KEY WORDS • LDL • HDL • cholesteryl ester transfer protein • lecithin: cholesterol acyltransferase • polyacrylamide gradient gel electrophoresis

In human plasma, cholesteryl ester transfer protein (CETP) plays a determinant role in the metabolism of cholesterol by promoting the transfer of cholesteryl esters from high density lipoproteins (HDLs) to very low density lipoproteins (VLDLs) and low density lipoproteins (LDLs). As high LDL cholesterol (LDL-C) concentrations and low HDL cholesterol (HDL-C) concentrations are associated with the development of atherosclerosis,2,3 CETP could be regarded as a potentially atherogenic factor. Recently, plasma CETP concentrations in monkeys have been shown to correlate positively with the extent of coronary artery atherosclerosis.4 In addition, an increased net mass transfer of HDL cholesteryl esters to apolipoprotein (apo) B-containing lipoproteins has been reported in patients with peripheral vascular disease.5 These results sustain the concept that in vivo CETP could play a determinant role in redistributing cholesterol from some tissues to LDL and then into the coronary arteries.6 In fact, the potentially atherogenic role of CETP could relate to its ability to bypass in vivo the reverse cholesterol transport pathway, i.e., the transport of cholesterol from peripheral tissues back to the liver. In
human plasma, a number of factors can potentially influence the cholesterol ester transfer activity, including the concentration, structure, and composition of donor as well as acceptor lipoprotein substrates. In consequence, CETP mass may not exactly reflect the cholesterol ester transfer activity in total plasma. This raises interest in identifying reliable markers of CETP activity in human plasma. Since CETP can modify the structure and composition of LDL and HDL, the size distribution of lipoproteins might be one of these markers.

Analyses of LDL size distribution in normal subjects’ plasma as well as in dyslipidemic subjects’ plasma have revealed that LDLs can be distributed either as monodisperse or polydisperse populations of particles. Polysize LDL patterns are frequently associated with hypertriglyceridemia, and among hypertriglyceridemic subjects, LDL heterogeneity increases with increasing LDL concentrations. However, individuals with multiple LDL subfractions have also been shown to be spread throughout a normal population without relation to lipid levels. These data indicate that the distribution profile of LDL particles could result from a complex process that depends on several factors. Among them, lecithin:cholesterol acyltransferase (LCAT) and lipoprotein and hepatic lipases can potentially affect LDL pattern. More recently, it has been suggested that CETP could also influence the LDL distribution profile. Indeed, native gradient gel electrophoresis revealed an abnormal speciation of LDL subclasses in CETP-deficient patients compared with normal control subjects. In vitro studies confirmed that lipid transfers from HDL to LDL might be a process of intravascular LDL remodeling.

Plasma HDLs have also been shown to consist of several subfractions. By using native polyacrylamide gradient gel electrophoresis, at least five distinct subfractions of HDLs have been identified. Hepatic lipase, lipoprotein lipase, LCAT, and CETP have been implicated in the size redistribution of HDL subpopulations. In particular, recent in vitro studies have demonstrated that CETP alone, in the absence of other enzyme activities known to affect the lipoprotein structure, induces the redistribution of HDL toward particles of both larger and smaller size.

In the present study, we have investigated whether a relation could exist between CETP activity and the distribution of both LDLs and HDLs in human plasma. For that purpose, CETP activity and lipoprotein patterns have been analyzed in total plasma from 27 normolipidemic subjects. In addition, because LCAT, a key enzyme of the reverse cholesterol transport pathway, has been shown to influence the size distribution of lipoprotein particles, cholesterol esterification activity has also been evaluated.

Methods

Subjects

Twenty-seven normolipidemic subjects (18 women and nine men; age range, 25–53 years; plasma cholesterol and triglyceride concentrations <230 mg/dL and <85 mg/dL, respectively) were selected for the study. They did not take drugs with the exception of five women who took oral contraceptives.

Blood Samples

Venous blood from all subjects was collected on the same day after a 12-hour overnight fast. Blood samples were drawn into EDTA-containing glass tubes, and plasma was promptly separated by a 3-minute centrifugation at 3,000g. Plasma was kept at 4°C and analyses were started within a few hours after sample collection.

Lipoprotein Preparation

Lipoprotein fractions were isolated from total plasma by sequential ultracentrifugation at a speed of 100,000 rpm (250,000g) in a TLA-100.2 rotor in a Beckman TL-100 ultracentrifuge (Beckman, Palo Alto, Calif.). Densities were adjusted by the addition of potassium bromide and checked with a DMA 35 digital densitometer (Paar, Graz, Austria). Total lipoproteins and HDLs were isolated as the plasma fractions d<1.21 and 1.13<d<1.21 g/mL, respectively. Centrifugations were performed at 5°C for 5 hours at d=1.13 g/mL and for 7 hours at d=1.21 g/mL. Lipoproteins were recovered by tube slicing.

Electrophoretic Separation of Lipoprotein Subfractions

Plasma lipoproteins were separated by electrophoresis on nondenaturing polyacrylamide gradient gels according to the procedure previously described. To improve the separation of both LDL and HDL subfractions, aliquots of the d<1.21 g/mL plasma fraction were subjected to electrophoresis in a 20–160 g/L polyacrylamide gradient gel (PAA 2/16, Pharmacia, Uppsala, Sweden) and in a 40–300 g/L polyacrylamide gradient gel (PAA 4/30), respectively. The migration buffer solution was 14 mmol/L tris(hydroxymethyl)aminomethane (Tris) and 110 mmol/L glycine, pH 8.3. After a 1-hour preelectrophoresis at 200 V, 10-µL aliquots of each sample (5–10 µg protein) were applied to the gels in 3-mm-large slots. The electrophoresis was performed at 4°C for 26 hours: 2 hours at 30 V, 12 hours at 50 V, and 12 hours at 150 V. At the end of the electrophoresis, gels were fixed, stained with Coomassie brilliant blue G, and destained as previously described. The distribution profile of lipoprotein subfractions was finally obtained by densitometric scanning of the gels at 633 nm with a 2202 Ultrascan laser densitometer (LKB, Bromma, Sweden) attached to a 2220 integrator (LKB).

The apparent diameters of the separated lipoprotein subfractions were determined by comparison with proteins (Pharmacia High Molecular Weight Protein Calibration kit) and carboxylated latex beads (Duke Scientific, Palo Alto, Calif.) subjected to electrophoresis together with the samples. The mean apparent diameters of LDL subfractions were determined by comparison with a calibration curve constructed with ferritin (12.20 nm), thyroglobulin (17.00 nm), and latex beads (38.00 nm). The mean apparent diameters of HDL subfractions were determined by comparison with a calibration curve constructed with albumin (7.10 nm), lactate dehydrogenase (8.16 nm), ferritin (12.20 nm), and thyroglobulin (17.00 nm).

The relative proportions of HDL subfractions were obtained by determining the relative areas under the scan curve for each subclass according to the classification of Blanche et al and by relating them to the total area corresponding to the entire HDL distribution.
Incubations
Six hundred microliters of total plasma containing 1.5 mmol/L iodoacetate was placed in stoppered plastic tubes and incubated for up to 24 hours at 37°C. Nonincubated controls were stored at 4°C.

CETP Activity
The cholesteryl ester transfer activity in total plasma was measured as the capacity of the sample to promote the transfer of radiolabeled cholesteryl esters from a tracer amount of labeled HDL₃ to the apo B-containing fraction of total plasma (d<1.068 g/mL plasma fraction). Radiolabeled HDL₃s were prepared as previously described.³⁶ Each incubation mixture contained a 25-μL aliquot of total plasma, radiolabeled HDL₁₋₃ (2.5 nmol of cholesterol), and iodoacetate (75 nmol) in a final volume of 50 μL. Incubations were conducted in triplicate for up to 6 hours at 37°C. A 45-μL volume of incubated mixtures was added to 1.95 mL of a d=1.07 g/mL potassium bromide solution in Beckman centrifugation tubes (2-mL Quick-Seal polyallomer tubes). The tubes were subjected to ultracentrifugation for 7 hours at 50,000 rpm (269,000g) in a 50.4 Ti rotor on an L7 ultracentrifuge (Beckman). One-milliliter volumes of d<1.068 and d>1.068 g/mL fractions were transferred to 6-mL counting vials. Two milliliters of scintillation fluid (OptiScint Hisafe 3, Pharmacia) was added to each vial, and the radioactivity was assayed for 5 minutes in a Wellac 1410 liquid scintillation counter (Pharmacia). The recovery of radioactivity after the fractionation of d<1.068 and d>1.068 g/mL plasma fractions was consistently >95%. In nonincubated controls, the radioactivity recovered in the fraction d<1.068 g/mL was <5% of the total.

Cholesteryl ester transfer activity was measured as the rate of total radiolabeled cholesteryl esters transferred from the HDL₃-labeled tracer to the d<1.068 g/mL plasma fraction during a 3-hour incubation at 37°C compared with control mixtures kept at 4°C. Results were expressed in percentage of radiolabeled cholesteryl esters transferred per hour per milliliter of plasma.

LCAT Activity
A [³H]cholesterol-albumin emulsion ([³H]cholesterol 6.7 mCi/L, albumin 50 g/L) was prepared as previously described.³⁷ A 5-μL aliquot of the emulsion was added to 50 μL of total plasma or 50 μL of Tris buffer (TBS control), pH 7.4. After a preincubation at 4°C for 1 hour to allow equilibration of the radiolabeled cholesterol with endogenous free cholesterol,³⁷ the mixtures were incubated for up to 6 hours at 37°C. Nonesterified cholesterol remaining in the mixture at the end of the incubation was precipitated by digitonin.³⁸ Five hundred microliters of an ethanol solution of digitonin (5 g/L) was added to the samples. Nonprecipitated controls received 500 μL ethanol. After a 10-minute incubation at room temperature, the precipitates were removed by centrifugation for 15 minutes at 10,000g in an Eppendorf centrifuge.

LCAT activity was measured as the rate of total radiolabeled cholesterol esterified during a 3-hour incubation at 37°C compared with TBS control. Results were expressed in percentage of radiolabeled cholesterol esterified per hour per milliliter of plasma.

Lipoprotein Component Assays
All chemical assays were performed on a Cobas-Fara Centrifugal Analyzer (Röche, Basel, Switzerland). Total cholesterol, unesterified cholesterol, and triglyceride concentrations were measured by enzymatic methods using Boehringer (Mannheim, FRG) reagents. Plasma HDL components were measured after selective precipitation of apo B-containing lipoproteins with Boehringer phosphotungstic acid/MgCl₂ reagent as recommended by the manufacturer. Concentrations of plasma apo B, apo A-I, and apo A-II were determined by immunoturbidimetry³⁹ with anti-apo B, anti-apo A-I, and anti-apo A-II antibodies purchased from Behring (Marburg, FRG). Apo B and apo A-I standards were purchased from Behringwerke AG (Marburg, FRG). Apo A-II standard was purchased from Immuno AG (Vienna, Austria).

Statistical Evaluations
Mann-Whitney tests were used to compare the significance of the difference between two means. Correlations between plasma parameters were analyzed by Spearman rank correlation analysis.

Results

Measurements of CETP and LCAT Activities in Total Plasma
CETP activity. CETP activity was evaluated in total plasma by measuring the rate of transfer of radiolabeled cholesteryl esters from HDL₃s to apo B-containing lipoproteins. To determine the incubation time required for the evaluation of CETP activity, mixtures of total plasma and HDL₃-radiolabeled tracer were incubated for up to 6 hours. The CETP assay was linear over a 6-hour period (Figure 1A).

To further check that CETP activity measurements were not artificially dependent on the relative dilution of the radiolabeled HDL₃ in the plasma HDL pool, CETP activity was determined in the same plasma sample supplemented with various amounts of the radiolabeled tracer. Cholesteryl ester transfer activity values did not differ, as the final cholesterol concentration of radiolabeled HDL₃ ranged from 0.012 to 0.096 mmol/L (Figure 2). These results indicated, therefore, that potential variations in CETP activity among various plasma samples were unlikely to relate artificially to variations in the relative dilution of radiolabeled HDL₃ in the plasma HDL pool.

In subsequent experiments, CETP assays were conducted by incubating total plasma in the presence of a tracer dose of radiolabeled HDL₁₋₃ (cholesterol concentration, 0.05 mmol/L) for 3 hours at 37°C. Results are expressed as percent per hour per milliliter.

LCAT activity. LCAT activity was evaluated by measuring the rate of esterification of exogenous radiolabeled cholesterol. To determine the incubation time required for the evaluation of LCAT activity, mixtures of total plasma and radiolabeled cholesterol tracer were incubated for up to 6 hours. The LCAT assay was linear during the first 3 hours of the incubation, and the curve reached a plateau at incubation times >6 hours (Figure 1B).
FIGURE 1. Line graphs showing time course of the cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT) assays in plasma from normolipidemic subjects. CETP activity (panel A) was measured as the rate of transfer of radiolabeled cholesteryl esters from an HDL-labeled tracer to plasma apolipoprotein B-containing lipoproteins during incubation of total plasma for up to 6 hours at 37°C. LCAT activity (panel B) was measured as the rate of radiolabeled cholesterol esterified during incubation of total plasma for up to 6 hours at 37°C. HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

The complete equilibration of radiolabeled cholesterol with plasma free cholesterol was not reached after a 1-hour preincubation period at 4°C and required, at this temperature, preincubation times of about 48 hours (results not shown). Consequently, to validate the 1-hour, 4°C preincubation step proposed by Channon et al., we compared LCAT activity values obtained after either a 1-hour or a 48-hour preincubation period. A good correlation between the two sets of values was observed (Figure 3). These results confirmed that, as previously proposed, a 1-hour preincubation period allows a reliable evaluation of the relative rates of cholesteryl esterification in human total plasma.

In subsequent experiments, LCAT assays were conducted by preincubating total plasma with the radiolabeled cholesterol-albumin emulsion for 1 hour at 4°C, followed by the incubation of plasma mixtures for 3 hours at 37°C. Results are expressed in percent per hour per milliliter.

CETP Activity, LCAT Activity, and Plasma Lipid Parameters

The correlations of CETP and LCAT activities with various lipid and lipoprotein parameters in the normolipidemic population are summarized in Table 1.

Significant negative correlations were observed between CETP activity and HDL-C levels, the HDL:C:VLDL+LDL-C ratio, and the HDL esterified cholesterol:triglyceride ratio. The negative correlation between plasma CETP activity and HDL-C levels suggested that, as previously proposed, plasma HDL could specifically decrease cholesteryl ester transfer activity. This hypothesis was confirmed by progressively increasing the HDL-C content in total plasma. Indeed, increasing HDL-C concentrations from 55 to 95 mg/dL in a single plasma sample progressively reduced the cholesteryl ester transfer activity (Figure 4).

LCAT activity had a significant negative correlation with total cholesterol, HDL-C, and VLDL+LDL-C levels (Table 1). No significant correlation was observed...
TABLE 1. Correlations of Plasma Lipid Parameters With CETP and LCAT Activities in Plasma From 27 Normolipidemic Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CETP activity</th>
<th>LCAT activity</th>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>0.000</td>
<td>-0.7422</td>
</tr>
<tr>
<td>VLDL+LDL-C</td>
<td>0.150</td>
<td>-0.616†</td>
</tr>
<tr>
<td>HDL-C</td>
<td>-0.569†</td>
<td>-0.440*</td>
</tr>
<tr>
<td>HDL-C:VLDL+LDL-C</td>
<td>-0.584†</td>
<td>0.000</td>
</tr>
<tr>
<td>HDL esterified cholesterol: HDL esterified cholesterol: triglycerides</td>
<td>-0.604†</td>
<td>-0.211</td>
</tr>
</tbody>
</table>

CETP, cholesteryl ester transfer protein; LCAT, lecithin: cholesterol acyltransferase; VLDL, very low density lipoprotein; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

Values are Spearman rank coefficients of correlation. *p<0.05; †p<0.01; ‡p<0.001.

between LCAT activity and the HDL-C:VLDL+LDL-C ratio or between LCAT activity and the HDL esterified cholesterol: triglyceride ratio.

**LDL Patterns and CETP Activity**

**Determination of LDL patterns.** The LDL patterns were determined after electrophoresis of the d<1.21 g/mL plasma fraction in a nondenaturing polyacrylamide gradient gel ranging from 20 to 160 g/L. Patterns obtained after analysis of 27 normolipidemic subjects' plasma samples were divided into two types, monodisperse and polydisperse, according to the number of main bands observed in the LDL range. The monodisperse patterns (type 1) (Figure 5A), which were present in 33% of the plasma studied, were characterized by the presence of a single, well-individualized LDL band with an apparent diameter varying from 23.0 to 25.7 nm. By contrast, the polydisperse patterns (Figures 5B and 5C) were characterized by the presence of two (type 2) or three or four (type 3) distinct major bands in the 21.6–22.6-nm range (Table 2). No statistical differences were observed between the mean sizes of the LDL bands in monodisperse and polydisperse patterns (Table 2). In polydisperse subjects, the major band, corresponding to the highest LDL peak, was usually the largest band, except for one individual. The male:female ratio was not significantly different between types 1, 2, and 3.

**Plasma lipoprotein parameters.** Lipoprotein parameters in plasma with monodisperse and polydisperse LDLs were compared (Table 3). No significant differences were observed in total cholesterol, esterified cholesterol, apo B, or apo A-II concentrations between the two groups. In contrast, HDL-C and apo A-I concentrations were significantly higher in plasma with polydisperse LDL. Among the polydisperse group, HDL-C was slightly higher in plasma with type 3 LDL patterns than in
plasma with type 2 LDL patterns (69.4±11.8 versus 64.5±13.1 mg/dL). However, differences between the two groups did not reach significance.

The composition analysis of HDL revealed further that the significant increase in the cholesterol content of HDL from plasma with polydisperse LDL was explained by an increase in both esterified and free cholesterol concentrations (Table 4). Subsequently, as the triglyceride concentrations in HDL of both groups did not differ, the esterified cholesterol:triglyceride ratio was significantly higher in HDL from plasma with polydisperse LDL than in HDL from plasma with monodisperse LDL (Table 4).

**Plasma CETP and LCAT activities.** CETP activity and LCAT activity in both monodisperse and polydisperse LDL groups were compared. CETP activity, measured as transfer of radiolabeled cholesteryl esters from HDLs to the d<1.21 g/mL fraction, was significantly lower in plasma with polydisperse LDL patterns (type 2+type 3) than in plasma with a monodisperse LDL pattern (type 1) (Table 5). In contrast, plasma LCAT activity was identical in both groups. Among the population with polydisperse LDL, CETP activity was lower in subjects with type 3 pattern than in subjects with type 2 pattern (n=13) (193±28 versus 225±49%/hr per milliliter, respectively). However, this difference did not reach significance.

**Effect of incubation of total plasma on LDL patterns.** To investigate a potential influence of lipid transfers on the LDL distribution profile, total plasma with monodisperse or polydisperse LDL was incubated at 37°C for 6 and 24 hours in the presence of an LCAT inhibitor (iodoacetate, 1.5 mmol/L). At the end of the incubation, the LDL pattern was determined by gradient gel electrophoresis of the d<1.21 g/mL plasma fraction.

Incubation of plasma induced some alterations in the distribution profile of polydisperse LDL (Figure 6). These changes were characterized by a progressive disappearance of the minor LDL bands. Indeed, after a 24-hour incubation, LDL with an initial polydisperse pattern exhibited a monodisperse profile. Under the same conditions, LDL with a monodisperse profile was not significantly affected by the incubation. Thus, after a 24-hour incubation at 37°C, all LDL profiles showed a monodisperse pattern.

**Effect of incubation of total plasma on the cholesteryl ester content of HDL.** Total plasma was incubated at 37°C for 24 hours in the presence of an LCAT inhibitor (iodoacetate, 1.5 mmol/L). At the end of the incubation, apo B-containing lipoproteins were selectively precipitated, and HDL cholesterol ester concentrations were measured in the resulting supernatants. Incubation induced a significant decrease in the cholesteryl ester content of HDL in plasma with both monodisperse and polydisperse LDL, indicating that a significant proportion of HDL cholesteryl esters was transferred to apo B-containing lipoproteins during the incubation of total plasma at 37°C. The absolute mass transfer of cholesteryl esters from HDL to apo B-containing lipoproteins was slightly higher in plasma with monodisperse LDL than in plasma with polydisperse LDL (Table 6). The differences between the two groups did not reach significance, but the relative mass transfer of cholesteryl esters from HDLs to apo B-containing lipoproteins was significantly higher in plasma with monodisperse LDL than in plasma with polydisperse LDL (Table 6).

**Table 2.** Particle Size of Monodisperse and Polydisperse LDL From Plasma of Normolipidemic Subjects

<table>
<thead>
<tr>
<th>Monodisperse LDL (n=9)</th>
<th>Polydisperse LDL (n=18)</th>
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<tbody>
<tr>
<td>Single band</td>
<td>Major band</td>
</tr>
<tr>
<td>Particle size (nm, mean±SD)</td>
<td>24.2±1.2</td>
</tr>
<tr>
<td>Range (nm)</td>
<td>23.0-25.7</td>
</tr>
</tbody>
</table>

**Table 3.** Lipoprotein Parameters in Plasma With Monodisperse and Polydisperse LDL

<table>
<thead>
<tr>
<th>Monodisperse LDL (n=9)</th>
<th>Polydisperse LDL (n=18)</th>
</tr>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>186±25</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>64±18</td>
</tr>
<tr>
<td>VLDL+LDL-C</td>
<td>135±26</td>
</tr>
<tr>
<td>HDL-C</td>
<td>51±5</td>
</tr>
<tr>
<td>HDL-C: VLDL+LDL-C</td>
<td>0.39±0.09</td>
</tr>
<tr>
<td>Apo B</td>
<td>114±19</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>156±15</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>30±3</td>
</tr>
</tbody>
</table>

**Table 4.** Composition of HDL From Plasma With Monodisperse and Polydisperse LDL

<table>
<thead>
<tr>
<th>Monodisperse LDL (n=9)</th>
<th>Polydisperse LDL (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esterified cholesterol</td>
<td>33±4</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>18±3</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>31±8</td>
</tr>
<tr>
<td>Esterified cholesterol:triglycerides</td>
<td>1.04±0.31</td>
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</tbody>
</table>

**Table 5.** CETP and LCAT Activities in Plasma With Monodisperse and Polydisperse LDL

<table>
<thead>
<tr>
<th>Monodisperse LDL (n=9)</th>
<th>Polydisperse LDL (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CETP activity</td>
<td>301±85</td>
</tr>
<tr>
<td>LCAT activity</td>
<td>71±25</td>
</tr>
</tbody>
</table>

CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein. CETP and LCAT activities are given in percent per hour per milliliter.

*p<0.05; **p<0.01.
**FIGURE 6.** Densitometric scans showing effect of the incubation of total plasma on the low density lipoprotein (LDL) distribution pattern. Total plasma from normolipidemic subjects was incubated for 6 or 24 hours at 37°C. Controls were maintained at 4°C. At the end of incubation, the density of each sample was adjusted to \( d=1.21 \) g/mL with potassium bromide, and the total lipoprotein fraction was recovered by ultracentrifugation and subjected to electrophoresis on 20–160 g/L polyacrylamide gradient gels (see "Methods"). 

**CASE 1**
- Control 4°C
- 37°C, 6h
- 37°C, 24h

**CASE 2**
- Control 4°C
- 37°C, 6h
- 37°C, 24h

**CASE 3**
- Control 4°C
- 37°C, 6h
- 37°C, 24h

**PARTICLE DIAMETER (nm)**

<table>
<thead>
<tr>
<th>CASE 1</th>
<th>CASE 2</th>
<th>CASE 3</th>
</tr>
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<tbody>
<tr>
<td>23.2</td>
<td>22.8</td>
<td>21.8</td>
</tr>
<tr>
<td>25.8</td>
<td>25.3</td>
<td>23.2</td>
</tr>
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</table>

**HDL Distribution and CETP Activity**

**Determination of the HDL distribution profiles.** To investigate a potential relation between the HDL distribution pattern and CETP and LCAT activities, HDL subpopulations were separated by polyacrylamide gradient gel electrophoresis. A semiquantitative measurement of the distinct HDL subfractions was obtained by a graphic analysis of the densitometric profile. The densitometric scans of HDL separated on gradient gels ranging from 40 to 300 g/L of polyacrylamide showed several distinct fractions. According to the classification of Blanche et al., five subpopulations with different mean apparent diameters were defined: HDL-2a (9.71–12.90 nm), HDL-2b (8.77–9.71 nm), HDL-3a (8.17–8.77 nm), HDL-3b (7.76–8.17 nm), and HDL-4a (7.21–7.76 nm) (Figure 7).

**Relations between HDL subfractions and plasma LCAT and CETP activities.** Spearman rank correlation analysis revealed that the relative abundance of distinct HDL subpopulations could relate to both CETP and LCAT activities (Table 7). The level of HDL-2a correlated positively with plasma CETP activity. Plasma LCAT activity correlated positively with the levels of HDL-2a and HDL-3a but negatively with the level of HDL-3b. LCAT activity did not correlate with the level of HDL-3b.

**Discussion**

The aim of the present study was to determine whether the distribution profiles of LDL and HDL in plasma from normolipidemic subjects were related to CETP activity. The results revealed that plasma CETP activity was significantly higher in subjects with monodisperse LDL than in subjects with polydisperse LDL. In addition, a significant positive correlation was observed between CETP activity and the relative abundance of HDL-2a particles. Conversely, in the same normolipidemic population, plasma LCAT activity related neither to the LDL pattern nor to the HDL-3b fraction.

Under the present experimental conditions, CETP and LCAT activities were measured in total plasma, and values reflect enzyme activities as modulated by plasma parameters such as concentration and composition of...
HDL cholesterol 59 mg/dL, CETP activity 275%/hr per milliliter. HDL-C, total cholesterol 157 mg/dL, triglycerides 47 mg/dL, HDL-C mg/dL, cholesteryl ester transfer protein (CETP) activity 169%/hr per milliliter. Lower panel Female, 35 years old, amide gradient gels stained for proteins with Coomassie blue.

TABLE 6. Effect of the Incubation of Total Plasma With Monodisperse and Polydisperse LDL on the Cholesteryl Ester Content of HDL

<table>
<thead>
<tr>
<th></th>
<th>Monodisperse LDL (n=9)</th>
<th>Polydisperse LDL (n=18)</th>
</tr>
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<tbody>
<tr>
<td>HDL esterified cholesterol (4°C)</td>
<td>33.2±3.8</td>
<td>41.3±7.9</td>
</tr>
<tr>
<td>HDL esterified cholesterol (37°C)</td>
<td>25.5±3.2*</td>
<td>34.4±6.5†</td>
</tr>
<tr>
<td>Absolute mass transfer (mg/dL)</td>
<td>7.7±2.2</td>
<td>6.9±3.1</td>
</tr>
<tr>
<td>Relative mass transfer*</td>
<td>0.23±0.06</td>
<td>0.17±0.06‡</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; HDL, high density lipoprotein. Plasma was supplemented with a lecithin: cholesterol acyltransferase inhibitor (iodoacetate, 1.5 mmol/L) and was either maintained at 4°C or incubated for 24 hours at 37°C. At the end of the incubation, apolipoprotein B-containing lipoproteins were selectively precipitated (see "Methods") and supernatants containing HDL were assayed for esterified cholesterol. Concentrations are expressed in milligrams per deciliter of total plasma.

*Relative mass transfer represents the ratio of the difference between cholesteryl ester content of HDL in plasma maintained at 4°C and incubated at 37°C to the cholesteryl ester content of HDL in plasma maintained at 4°C.

†p<0.002, ‡p<0.02 different from mixtures maintained at 4°C.

§p<0.02 different from plasma with monodisperse LDL.

endogenous lipoprotein substrates. We observed significant negative correlations between CETP activity and both HDL-C levels and the HDL:VLDL+LDL-C ratio. Whereas earlier studies demonstrated that CETP mass correlated positively with HDL-C levels in normolipidemic healthy subjects, recent studies showed different results. For example, CETP showed no relation to HDL in hyperlipoproteinemic patients. Moreover, in monkeys fed high-fat, high-cholesterol diets, plasma CETP concentration had a strong inverse correlation with HDL-C concentration but was positively correlated with LDL-C concentration. In fact, similar results were observed in humans. Recently, inverse correlations were noted between the HDL-C concentration and both CETP mass and activity in healthy, normolipidemic subjects. Conversely, plasma CETP concentration correlated positively with the VLDL+LDL-C:HDL-C ratio. These results agreed with the data of the present report. In addition, our measurements showed a significant negative correlation between CETP activity and the esterified cholesterol:triglyceride ratio in HDL. This was in good agreement with the characteristic increase of the esterified cholesterol: triglyceride ratio observed in the HDL from subjects with low CETP activity. In the present study, LCAT activity correlated negatively with total cholesterol, HDL-C, and VLDL+LDL-C concentrations. Whereas these observations confirmed some of the data previously published, they disagreed with others. For example, it has been shown that plasma LCAT concentration correlated positively with plasma cholesterol, HDL-C, and non-HDL-C levels. These conflicting data could be explained by the influence of various lipoproteins and apolipoproteins on the cholesterol esterification activity, as observed in vitro. Indeed, HDL, especially HDL2, can markedly inhibit LCAT activity. As HDL concentrations in the plasma we studied were relatively high, they could have reduced LCAT activity. This could explain, at least in part, the negative correlation we and others observed between plasma LCAT activity and HDL-C levels.

By using ultracentrifugation and nondenaturing gradient gel electrophoresis, multiple LDL subfractions have been identified in normolipidemic and dyslipidemic plasma, even within individuals. Among various populations of subjects, different LDL patterns have been determined. Recently, three main LDL patterns have been defined in normolipidemic sera: type 1, characterized by the presence of only one major band; type 2, characterized by the presence of two major bands; and type 3, characterized by the presence of at least three distinct bands. In the present study,
the separation of LDL subfractions by gradient gel electrophoresis confirmed that, in normolipidemic plasma, LDLs are distributed in either one or several subpopulations. As a limited number of samples could be processed simultaneously, we chose to subdivide all of the normolipidemic plasma samples into only two groups. The group with monodisperse LDL corresponded to the type 1 pattern, and the group with polydisperse LDL corresponded to both types 2 and 3 patterns as previously defined.22 Monodisperse, homogeneous patterns were characterized by one focused major band, whereas polydisperse, heterogeneous patterns were composed of at least two populations of LDLs (Figure 2). As previously observed, the analysis of plasma lipoprotein parameters revealed that subjects with the polydisperse pattern presented higher HDL-C levels and a higher HDL-C: VLDL-C ratio than subjects with the monodisperse pattern.22 In addition, the present results revealed that the polydisperse pattern was accompanied by a significant increase in apo A-I levels. Another characteristic feature of HDL from plasma with polydisperse LDL compared with HDL from plasma with monodisperse LDL was a higher cholesteryl ester: triglyceride ratio. By promoting the transfer of cholesteryl esters from HDLs to apo B-containing particles in exchange for a reciprocal transfer of triglycerides in the reverse direction, human CETP has been shown to alter both the LDL pattern and the HDL cholesteryl ester: triglyceride ratio.4 Thus, lipid transfers in vivo could affect not only the composition but also the size distribution of lipoprotein particles. This hypothesis was evaluated by incubating several samples of normolipidemic plasma with typical monodisperse and polydisperse LDL patterns at 37°C in the absence of LCAT activity. During incubation, polydisperse patterns were progressively transformed into monodisperse patterns. Under the same conditions, initially monodisperse patterns remained virtually unchanged. These results indicate that CETP, by transferring lipids between lipoprotein subclasses, could promote the transformation of polydisperse LDL into a homogeneous, monodisperse population of particles. This transformation could further sustain this hypothesis. Indeed, subjects with polydisperse LDL showed a significantly lower cholesteryl ester transfer activity than subjects with monodisperse LDL. By contrast, cholesterol esterification activity did not differ in the two groups, indicating that differences in LDL patterns as well as in the HDL esterified cholesterol: triglyceride ratio were unlikely to result from variations in plasma LCAT activity. When total plasma was incubated for 24 hours at 37°C in the absence of LCAT activity, the relative mass of cholesteryl esters transferred from HDLs to apo B-containing lipoproteins was significantly higher in plasma with monodisperse LDL than in plasma with polydisperse LDL. Thus, taken together, the data of the present study indicate that CETP-mediated lipid transfers, particularly transfers of cholesteryl esters from HDL to LDL, could be major factors in determining the LDL pattern, as previously suggested.22 Lipid exchanges between LDL and VLDL could also constitute some important reactions influencing the size distribution of LDL, as it has been previously demonstrated that LDL heterogeneity increases with increasing VLDL concentrations.13 However, the normolipidemic subjects examined in the present study had very low plasma triglyceride levels. Thus, under these particular conditions, triglyceride-rich lipoproteins were unlikely to play a major role in the plasma LDL distribution. This was shown by the absence of significant differences in the triglyceride content of plasma with either monodisperse or polydisperse LDL patterns. We postulate that, in the low triglyceride plasma we studied, the heterogeneity of LDL would primarily result in a relatively reduced lipid transfer between HDL and LDL, and that this reduced transfer would not be high enough to allow a complete focusing of polydisperse LDL. In a previous study, incubations of LDLs and HDLs in the presence of purified CETP induced a shift of isolated LDLs toward particles of a larger size, but the transformation of polydisperse into monodisperse LDL patterns was not observed.22 This partial discrepancy between the two studies could be related to alterations that may have been induced in the earlier study during the processes of lipoprotein isolation and CETP purification.22 On the other hand, the results of the present study are consistent with observations made in plasma from CETP-deficient patients.19-21 Indeed, native gradient gel electrophoresis of LDL from homozygotes with virtually no CETP activity revealed an abnormally polydisperse LDL pattern, with some bands larger and others smaller than normal LDL.20 The origin of the heterogeneity of LDL is still not fully understood. As recently suggested, LDL subpopulations of distinct size could be formed subsequent to the actions of lipoprotein lipase and hepatic lipase on VLDL particles.21 According to the present study, CETP, by transferring cholesteryl esters from HDL, could therefore play an important role in converting polydisperse LDL to homogeneous particles. The size redistribution of LDL particles could be accompanied by an increase in the cholesteryl ester: triglyceride ratio in HDL. Indeed, monodisperse LDLs have been shown to contain a higher esterified cholesterol: triglyceride ratio compared with polydisperse LDLs.13 In addition, LDLs from CETP-deficient subjects present a reduced esterified cholesterol: triglyceride ratio compared with these subjects' own HDL or to LDL from normal subjects.20

We also investigated whether the distribution of HDL subpopulations in plasma from normolipidemic subjects could be related to variations in CETP and LCAT activities. Some recent studies have demonstrated that both the severity and progression of coronary artery disease are related to the plasma concentration of individual HDL subclasses.34-35 Thus, the heterogeneity of HDL could have important implications, and it appears to be of great interest to study the factors that can affect the HDL distribution in human plasma. CETP could be a key factor in determining the size distribution of plasma HDL in vivo. In the present study, plasma CETP activity was correlated positively with the level of HDL-C. Whereas plasma LCAT activity correlated negatively with the level of HDL-C and positively with the levels of HDL-C and HDL-C, no significant relation was observed with the level of HDL-C. These results indicate that a specific increase in the HDL-C fraction would be a good marker of increased CETP activity. These results are in good agreement with previous in vitro observations that demon-
strated that CETP alone can promote the redistribution of HDL₃ particles toward smaller HDL₂ particles. This could account for the significant positive correlation observed in normolipidemic plasma between CETP activity and the HDL₂ fraction. In the present study, we also observed a significant positive correlation between the HDL₂ level and LCAT activity. This is consistent with the results of Nichols et al, who suggested that LCAT could induce a specific increase in HDL₃, but not HDL₂ particles. The significant negative correlation between the relative abundance of HDL₂ and LCAT activity is of great interest because, in normotriglyceridemic subjects, strong inverse correlations were found between the plasma levels of HDL₂ and both the severity and the rate of progression of coronary lesions. However, the mechanism of formation of HDL₂ is unclear, and by now it seems hazardous to try to connect these observations with previous in vitro studies. In fact, it is possible that the level of HDL₂ particles is dependent on other factors that can potentially affect the distribution of HDL. For example, in vitro studies have suggested that the transfer of phospholipids from VLDL to HDL can promote the formation of large HDL particles. The present analysis of HDL distribution suggests that an abundant population of HDL₂ would be a good marker of an elevation of CETP activity, whereas elevated HDL₃ and HDL₄ levels would relate specifically to an elevation of LCAT activity, a key enzyme in the reverse cholesterol transport pathway.

In conclusion, the results of the present study point to an increased HDL₂ particle level and a monodisperse LDL profile as two specific markers of relatively high CETP activity in normolipidemic subjects. As CETP can potentially influence the atherogeneity of the lipoprotein profile in plasma, the determination of LDL and HDL distribution patterns could constitute an effective means of evaluating a global risk for atherosclerosis. Additional studies are needed to determine whether the relation between lipoprotein distribution patterns and CETP activity is also true in patients in whom cholesteryl ester transfer is pathologically accelerated.

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Influence of plasma cholesteryl ester transfer activity on the LDL and HDL distribution profiles in normolipidemic subjects.

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