Cholesteryl Ester Transfer Activity in Plasma of Patients with Familial High-Density Lipoprotein Deficiency

D. L. Sparks, J. Frohlich, and P. H. Pritchard

We determined cholesteryl ester transfer activity in whole plasma and in lipoprotein-depleted plasma of normolipidemic subjects and of patients with severe high-density lipoprotein (HDL) deficiency: Tangier disease, lecithin:cholesterol acyltransferase (LCAT) deficiency, and "fish-eye" disease. Transfer rates in plasma were positively correlated ($r = 0.950$) with rates measured in the absence of the endogenous lipoproteins. This suggests that lipoprotein composition and content may not affect total cholesteryl ester transfer activity in normolipidemic and the HDL-deficient subjects. Cholesteryl ester transfer from solid-phase-bound HDL to plasma lipoproteins was decreased by 39% in fish-eye disease and 33% in LCAT deficiency but increased by 57% in Tangier disease, as compared with normal values. Changes were similar for lipoprotein-depleted plasma from the same individuals. Transfer to plasma HDL was significantly decreased in all HDL-deficient patients, whereas transfer to very-low- and low-density lipoproteins was increased only in Tangier disease. Differences in transfer rates between the patients studied appeared to reflect the LCAT activity and the need to transport cholesteryl ester rather than the HDL cholesteryl concentration. Thus, the concentration of HDL in plasma does not directly affect total cholesteryl ester transfer activity in HDL deficiency.

Additional Keyphrases: heritable disorders - cholesterol - lecithin:cholesterol acyltransferase - transfer proteins

Atherosclerotic risk is positively correlated with low concentrations of high-density lipoproteins (HDL) in plasma (1). In some cases however, individuals presenting with severe HDL deficiencies appear to be at only moderate risk for premature coronary heart disease (2). For example, Tangier disease, lecithin:cholesterol acyltransferase (LCAT) deficiency, and "fish-eye" disease are all disorders associated with low HDL concentrations in plasma without markedly increased risk for atherosclerosis (2). Because the antiatherogenic activity of HDL is thought to be associated with its central role in reverse cholesterol transport—i.e., the net movement of free cholesterol out of cells, its esterification, and transport to hepatic degradation sites (1)—some other process must be acting in these individuals to maintain reverse cholesterol transport.

Cholesteryl ester transfer proteins (CETP) promote the transfer and exchange of cholesteryl esters between lipopro-

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2 Address correspondence to this author at: Dept. of Pathol., The Research Center, 950 West 28th Ave., Vancouver, BC, Canada V5Z 4H4.
3 Nonstandard abbreviations: HDL, LDL, VLDL, high-, low-, and very-low-density lipoproteins, respectively; CETP, cholesteryl ester transfer proteins; LCAT, lecithin:cholesterol acyltransferase (phosphatidylcholine-sterol acyltransferase, EC 2.3.1.43).
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Materials and Methods

Materials

Cholesteryl (3H)oleate (specific activity 72 kCi/mol) and (3H)cholesterol (specific activity, 9 kCi/mol) were from NEN Research Products, Lachine, Que., Canada. Cyanogen bromide-activated Sepharose-4B was from Sigma Chemical Co., St. Louis, MO. Total and free cholesterol determination reagent kits were from Boehringer, Mannheim, F.R.G. All other chemicals were of analytical grade, from BDH Chemicals Canada Ltd., Vancouver, BC.

Patients and Controls

Patients with the homozygous forms of LCAT deficiency, fish-eye disease, and Tangier disease were recruited from the Shaughnessy Hospital Lipid Clinic. The clinical and laboratory findings for these individuals have been previously reported (6–8). Control plasma samples were obtained from 16 healthy normolipidemic volunteers.

Procedures

Isolation of lipoproteins. Blood from normal and HDL-deficient subjects who had fasted for 16 h was collected into EDTA-containing tubes. Plasma was removed by centrifugation at 1750 × g for 10 min and promptly analyzed. Substrate and acceptor HDL, LDL, and VLDL were isolated from normal plasma by ultracentrifugation at d 1.210–1.063, 1.063–1.006, and 1.006, respectively (9), and centrifuged at the same density to remove any remaining plasma proteins. The washed lipoproteins were dialyzed four times against 100-fold volumes of buffer A (per liter, 150 mmol of NaCl, 10 mmol of Tris HCl, 0.3 mmol of EDTA, and 4.6
mmol of Na\(_2\)B, pH 7.4), then characterized by electrophoresis on agarose (9).

Lipoprotein-depleted plasma was prepared from control subjects and patients' plasma by adding solid NaBr to give a solution density of 1.21 kg/L, ultracentrifuging at 114,000 × g for 48 h, removing the lipoproteins by tube slicing, and dialyzing the infranates against buffer A. Lipoprotein-depleted plasma was prepared as described previously (11). Because a significant amount of the transfer activity in plasma is recovered between \(d = 1.21\) and \(d = 1.25\), and would be removed in the lipoprotein supernate at \(d < 1.25\).

**Preparation of assay substrates.** Solid-phase-phase-bound HDL was prepared as previously described (11). We labeled freshly isolated HDL with \(^{3}H\)cholesterol oleate and then covalently linked this to CNBr-activated Sepharose 4B.

LDL was labeled with \(^{3}H\)cholesterol ester as described by Hough and Zilversmit (12). \(^{3}H\)Cholesterol oleate/vesicular phospholipids was prepared by sonication, then incubated with fresh plasma for 24 h at 37 °C; radiolabeled LDL was isolated at \(d = 1.019-1.063\).

**Assay of cholesteryl ester transfer.** Two different assay methods were used to measure cholesteryl ester transfer activity in plasma and in lipoprotein-depleted plasma.

Cholesteryl ester transfer activity from solid-phase-bound HDL to plasma lipoproteins was measured as previously described (11) except that the final specific radioactivity was 4200 dpm per microgram of HDL protein and the bound HDL was washed extensively to deplete the nonspecific transfer. After incubating 600 μL of fresh plasma with 200 μg of solid-phase-bound HDL protein for 1 h at 37 °C, we centrifuged the samples to pellet the HDL-bound Sepharose, immediately removed 600 μL of supernatant plasma from each tube, and counted the radioactivity of 200 μL of this to determine the total \(^{3}H\)cholesterol ester transferred to plasma. The amount of \(^{3}H\)cholesterol ester transferred to HDL was determined in the remaining supernatant fluid by precipitating the VLDL and LDL with heparin/MnCl\(_2\) and counting the unprecipitated radioactivity. Transfer to the precipitated lipoproteins (VLDL and LDL) was calculated as the difference between the values for the total and nonprecipitated radioactivity.

Cholesteryl ester transfer activity in lipoprotein-depleted plasma was measured by a modification of the method of Morton and Zilversmit (13). Purified HDL (30 μg of cholesterol) was incubated for 1.5 h at 37 °C with \(^{3}H\)LDL (30 μg of cholesterol), the \(d = 1.21\) fraction of each plasma sample (300 μL), and buffer A (total volume 700 μL). Total \(^{3}H\)cholesteryl ester transferred to HDL was determined by precipitation of LDL with heparin/MnCl\(_2\), followed by the determination of radioactivity in the supernate. For controls, we repeated the CETP incubations but omitted the \(d = 1.21\) fraction, and subtracted the rate of transfer of radioactivity from that measured for the test incubations.

**Determination of LCAT activity.** LCAT activity was measured by using single bilayer vesicles prepared by the method of Batzri and Korn (14). Each assay contained 4.65 mmol of unesterified \(^{3}H\)cholesterol and 15 μg of apolipoprotein A-I. The molar ratio of cholesterol to egg-yolk phosphatidylcholine was 1:4. Esterification rates for 15 μL of plasma sample were measured for 30 min at 37 °C. Less than 10% of the cholesterol in the substrates was esterified under these conditions.

**Other procedures.** Free and total cholesterol and triglycerides were determined enzymatically (15, 16) with a commercial kit. Protein was determined by the Lowry method as modified for plasma lipoproteins (9).

**Results**

**Characterization of patients.** Lipid values for HDL-deficient patients and normal subjects are shown in Table 1. For all three patients HDL cholesterol was <10% of normal values; the patients with Tangier and fish-eye disease had moderate hypertriglyceridemia. Total cholesterol values were more than 2 SD lower than for normals in both Tangier disease and LCAT deficiency, but were significantly increased in fish-eye disease. LCAT activity was not detectable in LCAT deficiency, whereas in fish-eye disease it was 6% and in Tangier disease 58% of that for normal subjects (Table 1). These results agree with previously reported data (6–8).

**Characterization of cholesteryl ester transfer activity in lipoprotein-depleted plasma.** The standard curve was linear to 3 h with up to 0.5 mL of lipoprotein-depleted plasma per assay (Figure 1, A and B). In addition, lipid transfer depended on the concentration of LDL substrate (Figure 2A), but transfer rates were independent of HDL concentrations greater than 5 μg of total cholesterol per assay (Figure 2B). Thus, any very-high-density lipoproteins in the lipoprotein-depleted preparation would negligibly affect transfer rates.

**Cholesteryl ester transfer activity in untreated and lipoprotein-depleted plasma.** Total cholesteryl ester transfer activity measured in normolipidemic plasma correlated positively (\(r = 0.950\)) with the transfer rate measured in the lipoprotein-depleted plasma from the same individuals (Figure 3). This suggests that lipoprotein composition or content in normolipidemic subjects does not affect total cholesteryl ester transfer and that the transfer we have measured is associated with the activity of CETP in plasma.

| Table 1. Lipid and Lipoprotein Composition of Plasma in Severe HDL Deficiency |

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Control*</th>
<th>Tangier disease</th>
<th>LCAT deficiency</th>
<th>Fish-eye disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl ester, mg/L</td>
<td>1600 ± 250</td>
<td>600b</td>
<td>120b</td>
<td>2050</td>
</tr>
<tr>
<td>Free cholesterol, mg/L</td>
<td>480 ± 80</td>
<td>402</td>
<td>820b</td>
<td>1340b</td>
</tr>
<tr>
<td>Triglycerides, mg/L</td>
<td>850 ± 240</td>
<td>3200b</td>
<td>750</td>
<td>2700b</td>
</tr>
<tr>
<td>HDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesteryl ester, mg/L</td>
<td>430 ± 100</td>
<td>20b</td>
<td>30b</td>
<td>30b</td>
</tr>
<tr>
<td>Free cholesterol, mg/L</td>
<td>150 ± 50</td>
<td>30b</td>
<td>20b</td>
<td>40b</td>
</tr>
<tr>
<td>Triglycerides, mg/L</td>
<td>280 ± 40</td>
<td>110b</td>
<td>90b</td>
<td>100b</td>
</tr>
<tr>
<td>LCAT act. (mmol/mL per hour estd.)</td>
<td>31.6 ± 4.6</td>
<td>18.3b</td>
<td>0.0b</td>
<td>1.8b</td>
</tr>
</tbody>
</table>

*Mean ± SD, n = 16. **Diffs from the mean for the control subjects by >2 SD.
Fig. 1. Characterization of the transfer of cholesteryl ester from LDL to HDL in lipoprotein-depleted plasma (LPDP).

A. We incubated 300 μL of LPDP with [3H]LDL and unlabeled HDL (30 μg of total cholesteryl each) and buffer A (total volume 700 μL) at 37°C for 0-180 min. The percentage of [3H]cholesteryl ester transferred to HDL was calculated from the amount of radioactivity remaining after precipitation of LDL with heparin/MnCl₂. Control incubations that lacked a source of cholesteryl ester transfer activity were subtracted to determine CETP-dependent lipid transfer. Values are the average of duplicate determinations.

B. We incubated 0-600 μL of LPDP with [3H]LDL and unlabeled HDL for 90 min as described above. Transfer rates were determined as the average of duplicate determinations.

Fig. 2. Effect of HDL concentration on cholesteryl ester transfer from LDL in lipoprotein-depleted plasma.

We incubated 300 μL of lipoprotein-depleted plasma with the indicated amount of [3H]LDL (A), or HDL (B) at 37°C for 60 min. Transfer rates were determined as in Fig. 1. Each data point represents the average of duplicate determinations.

The transfer of cholesteryl ester to VLDL/LDL in Tangier-disease plasma was substantially (>2 SD) increased over controls, by 145% (Table 2). However, transfer to VLDL/LDL in LCAT deficiency and fish-eye disease was normal. In LCAT deficiency and fish-eye disease transfer to HDL was approximately 25% of normal values, and was 8.6% of normal in Tangier disease (Table 2). In addition, the increased transfer of cholesteryl ester to endogenous lipoproteins in Tangier disease was associated with an increased transfer rate in lipoprotein-depleted plasma (Figure 3). A similar relationship was observed for the low transfer rates in both plasma and lipoprotein-depleted plasma from the fish-eye disease and LCAT-deficient subjects. Linear regression analysis of all data (controls and HDL-deficient plasma) also gave a positive correlation with r = 0.950.

Table 2. Cholesteryl Ester Transfer Activities in Plasma

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Control</th>
<th>Tangier disease</th>
<th>LCAT deficiency</th>
<th>Fish-eye disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL</td>
<td>5.8 ± 1.1 *</td>
<td>0.5 b</td>
<td>1.2 b</td>
<td>1.4 b</td>
</tr>
<tr>
<td>VLDL/LDL</td>
<td>5.5 ± 0.9</td>
<td>13.3 b</td>
<td>7.1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

*Values are mean ± SD, n = 16. b Differs from the mean for the control subjects by >2 SD.

Discussion

The positive correlation between assays of cholesteryl ester transfer in the native and lipoprotein-depleted plasma indicated that differences seen between control and the HDL-deficient plasma were not solely attributable to changes in lipoprotein composition and (or) concentration. The results also indicate that the transfer of [3H]cholesteryl ester from solid-phase-bound LDL to plasma lipoproteins may be used to estimate the rate of cholesteryl ester transport in unmodified plasma samples. However, any attempt to correlate these activities with CETP mass would be difficult, because net neutral lipid transfer activity has been shown to be a result of both the activity of CETP and the effect of specific inhibitor proteins (13).

Our study shows that cholesteryl ester transfer activity may differ among patients with severe HDL-deficiency. Decreased activity in LCAT deficiency and fish-eye disease and the increased activity in Tangier disease indicate that the HDL concentration does not directly affect the cholesteryl ester transfer rate in these individuals. There may be a relation between LCAT activity and cholesteryl ester transfer, because patients with fish-eye disease and LCAT deficiency also have a markedly subnormal LCAT activity. This may result in a decreased requirement for the transport of cholesteryl esters and a subsequent decreased transfer activity. There is one report of normal cholesteryl ester transfer activity in two patients with fish-eye disease (17).
However, this study cannot be directly compared to our investigation, because the plasma fraction >1.25 kg/L was used as a source of cholesteryl ester transfer activity in that study. It has been well demonstrated that more than half of the plasma CETP is associated with the lipoproteins in the density range 1.21–1.25 kg/L. Thus, it seems likely that the transfer rates reported by Calvert and Carlson (17) were not representative of the total activity in their patients’ plasma.

We have also studied cholesteryl ester synthesis and transport in whole LCAT-deficient plasma to which purified LCAT was added (8). We determined that the net transfer of newly synthesized cholesteryl ester was impaired and esters accumulated in HDL, suggesting that HDL in LCAT deficiency may be unable to release cholesteryl ester to other lipoproteins. This indicates that the reduced transfer activity found in LCAT-deficient plasma may also have been due to changes in HDL composition. However, concomitant reductions in transfer activity determined in lipoprotein-depleted plasma suggest that protein mass may indeed be decreased.

Tangier disease, on the other hand, is associated with only slightly decreased LCAT activity despite the HDL deficiency. Thus, the increased transfer activity may reflect the need to transport newly synthesized cholesteryl ester to LDL and HDL, because there is no pool of HDL to act as an acceptor. It is also possible that the increased activity may play a more central role in the HDL deficiency itself. Some studies have indicated that low rates of transfer result in hyperalphalipoproteinemia (4). Increased lipid transfer activity plays a role in the hypoalphalipoproteinemia of Tangier disease, but a mechanism by which this is brought about is not elucidated by the present study. It is also possible that the changes in CETP activity in severe hyperalphalipoproteinemias are only adaptive mechanisms, developed to maintain effective reverse cholesterol transport.

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References