Plasma Concentrations of Cholesteryl Ester Transfer Protein in Hyperlipoproteinemia

Relation to Cholesteryl Ester Transfer Protein Activity and Other Lipoprotein Variables

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Cholesteryl ester transfer protein (CETP) mediates an important pathway for reverse cholesterol transport. Concentrations of CETP in fasting plasma were measured by radioimmunoassay in two different groups of hyperlipoproteinemic subjects. Plasma CETP concentrations measured by radioimmunoassay correlated closely with cholesterol ester transfer activity in normal plasma ($r=0.86$). In the first group of 58 patients, plasma CETP concentrations were significantly increased, as compared with those in 79 normal subjects and in hypercholesterolemic (+26%) and combined hyperlipoproteinemic (+25%) subjects but were not altered in moderately hypertriglyceridemic subjects. Marked elevations in plasma CETP levels were documented in patients with dysbetalipoproteinemia (+68%) and severe chylomicronemia (+85%). Similar results were obtained in a second population of 50 hyperlipoproteinemic subjects. Significant correlations were found between plasma CETP levels and total cholesterol ($r=0.52$), very low density lipoprotein (VLDL) cholesterol ($r=0.63$), and apolipoprotein E concentration ($r=0.40$). Correction of the lipoprotein phenotype by dietary means resulted in significant reductions in plasma CETP concentrations in patients with chylomicronemia and dysbetalipoproteinemia. In these subjects, plasma high density lipoprotein cholesterol concentrations increased as CETP decreased. These studies indicate that CETP levels increase in association with enhanced peripheral cholesterol transport via low density lipoprotein, $\beta$-VLDL, or chylomicron remnants. (Arteriosclerosis and Thrombosis 1991;11:797-804)

Early studies by Rehnborg and Nichols and Nichols and Smith demonstrated that on incubation of normolipemic plasma, cholesteryl ester (CE) increased in very low density lipoprotein (VLDL), whereas triglyceride (TG) concentration decreased in VLDL but increased in high density lipoprotein (HDL) and low density lipoprotein (LDL). These experiments were the first to document CE transfer between lipoproteins, a phenomenon that was further clarified by the demonstration by Zilversmit et al that the $d>1.21$ g/ml fraction contained a protein that catalyzed CE transfer between lipoproteins, a protein that is now identified as cholesteryl ester transfer protein (CETP). More recently, two research groups have purified and characterized CETP, which is a very hydrophobic protein with a molecular size of $M$, $74,000$, in agreement with the data derived from the cloning and sequencing of the cDNA encoding for CETP.

While CETP mediates only the exchange of CEs between HDL and LDL, it does promote net transfer of CEs from HDL to VLDL and chylomicrons. This specificity of the transfer reaction makes it a major pathway for reverse cholesterol transport in many mammalian species. However, the significance of plasma levels of CETP in atherosclerosis must be considered in terms of the two facets of the CETP-mediated system—factors facilitating the accumulation of HDL CE (secretion of nascent HDL, its interaction with peripheral cells, and the activity of lecithin:cholesterol acyltransferase) and factors promoting the clearance of apolipoprotein (apo)B-containing lipoproteins, the recipient particles for CE transfer.
Using a sensitive and specific immunoassay, we have recently reported that plasma CETP concentrations vary fourfold in normolipemic individuals and are correlated with both apo A-I and apo E levels. In the present study, we have determined CETP concentrations in five common hyperlipoproteinemic phenotypes. Based on the results obtained, we suggest that CETP concentrations are increased under conditions of enhanced peripheral cholesterol transport by LDL and, particularly, by β-VLDL or chylomicron remnants.

Methods

Subjects

Study 1. A total of 58 hyperlipidemic patients at the Royal Victoria Hospital Lipid Clinic in Montreal were studied before initiation of dietary or pharmaceutical therapy. No patient had previously been treated with a lipid-lowering drug, and use of other medications did not vary between groups. After a 14-hour fast, blood was drawn for determination of plasma lipoprotein levels; apo A-I, A-II, B, and E concentrations; and CETP levels. Certain patients were restudied after they had undergone intensive dietary treatment. In addition, CETP concentrations before and after heparin infusion (100 units/kg) were determined in nine hypertriglyceridemic patients. Patients were classified according to the Lipid Research Clinics prevalence data for age and sex. Seventeen patients were classified as hypercholesterolemic (LDL cholesterol >95th percentile, TGs <95th percentile); 14 as combined hyperlipidemic (LDL cholesterol >95th percentile, TGs >95th percentile); 10 as dysbetalipoproteinemic (total cholesterol >95th percentile, TGs >95th percentile, β-VLDL present on agarose gel electrophoresis, and apo E 2/2 phenotype by isoelectric focusing); 12 as hypertriglyceridemic (TGs >95th percentile, total cholesterol <90th percentile); and five as chylomicronemic (chylomicrons present in fasting serum, TGs >14 mmol/l). A group of 79 normolipemic subjects were used as controls. The control group has been described previously and consisted of 45 male and 34 female subjects in good health between the ages of 24 and 74 with cholesterol and TG levels below the 90th percentile; 10 as dysbetalipoproteinemic (total cholesterol >95th percentile, TGs >95th percentile, β-VLDL present on agarose gel electrophoresis, and apo E 2/2 phenotype by isoelectric focusing); 12 as hypertriglyceridemic (TGs >95th percentile, total cholesterol <90th percentile); and five as chylomicronemic (chylomicrons present in fasting serum, TGs >14 mmol/l).

Using a sensitive and specific immunoassay, we have recently reported that plasma CETP concentrations vary fourfold in normolipemic individuals and are correlated with both apo A-I and apo E levels. In the present study, we have determined CETP concentrations in five common hyperlipoproteinemic phenotypes. Based on the results obtained, we suggest that CETP concentrations are increased under conditions of enhanced peripheral cholesterol transport by LDL and, particularly, by β-VLDL or chylomicron remnants.

Study 2. Fifty subjects with primary hyperlipidemia were studied after they had received dietary therapy at the Leeds General Infirmary Lipid Clinic, Leeds, UK. Patients were classified by the previously mentioned criteria (20 hypercholesterolemic, 18 combined hyperlipidemic, four dysbetalipoproteinemic, and eight hypertriglyceridemic without chylomicronemia). The group of normolipemic subjects described above were used as controls. Lipoproteins, apo A-I, apo B, and CETP mass and activity were determined in fasting plasma as described below.

A larger population of 146 hyperlipidemic subjects, with a wide range of cholesterol and TG concentrations, was also studied to compare CETP activity with CETP mass. This population included the 50 subjects described above (study 2).

Determination of Plasma and Lipoprotein Lipids

Plasma cholesterol and TGs, HDL cholesterol, and LDL cholesterol were determined by the methods of the Lipid Research Clinics protocol. VLDL cholesterol was measured in the d<1.006 g/ml supernatant after ultracentrifugation. Total HDL cholesterol was measured after heparin–manganese precipitation of the apo B–containing lipoproteins in whole plasma.

Apolipoprotein E Phenotype

Apo E phenotype was determined by isoelectric focusing of apo VLDL.

Imunoasays

Apo A-I, apo E, and apo B in study 1 were measured by radioimmunoassay as described previously. In study 2, apo A-I and apo B were measured immunoturbidimetrically with the use of kits from Orion Diagnostica, London, UK. Plasma CETP was measured by solid-phase competitive radioimmunoassay in the presence of 0.5% Triton and a specific monoclonal antibody that was the generous gift of Linda Curtiss (Scripps Institute, La Jolla, Calif.). Plasma CETP was measured by solid-phase competitive radioimmunoassay in the presence of 0.5% Triton and a specific monoclonal antibody, TP-2, that has been previously characterized. In brief, plastic wells were coated with partially purified CETP in Na₂CO₃ buffer, saturated with bovine serum albumin (BSA), and washed. To the coated wells, equal volumes of the unknown antigen (diluted and preincubated in phosphate-buffered saline [PBS]–BSA buffer containing 1% Triton) and a limiting dilution of purified iodine-125–labeled monoclonal antibody TP-2 (diluted with immunoglobulin G protein in PBS with BSA) were added. The wells were incubated for 90 minutes at 20°C and then washed, and the bound radioactivity was determined.

All immunoasays were performed in a single run at the end of each study using plasma stored at −70°C.

Cholesterol Ester Transfer Activity Assay

Transfer activity was measured as described previously. Briefly, plasma (<6 nmol HDL cholesterol) was mixed with tritium-labeled CE-containing HDL₃ (24 nmol CE) and an excess of acceptor LDL (300 nmol CE) in a final volume of 100 μl 50 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl, 2 mM EDTA buffer, pH 7.4. Samples and blanks containing no plasma were incubated for 3 hours at 37°C. The transfer of CE was determined as loss of radioactivity after removal of the apo B–containing lipoproteins by heparin–manganese precipitation.

Statistical Methods

Statistical analyses were performed using Student's t test with correction for unequal variances and linear and stepwise regression using the SAS statistical package.
Results

Plasma Cholesteryl Ester Transfer Protein Concentrations

Study 1. Plasma lipoprotein, apolipoprotein, and CETP levels for 79 normal and 58 hyperlipidemic subjects from study 1 are shown in Table 1. The mean age of the normal subjects was lower than that of the hyperlipidemic subjects, but we have previously reported that CETP levels do not vary with age. As compared with those of normal subjects, CETP concentrations were significantly increased in patients with hypercholesterolemia (+36%) and combined hyperlipidemia (+40%), with no difference in CETP between normal and hypertriglyceridemic subjects. Again, CETP concentrations were observed to be significantly increased (+95%) in patients with dysbetalipoproteinemia.

Relation Between Cholesteryl Ester Transfer Protein and Other Lipoprotein Variables

The Pearson correlation coefficients for CETP versus other lipoprotein variables are summarized in Table 2.
Table 3. Among the 58 hyperlipidemic subjects of study 1, significant relations were observed between CETP and total cholesterol \((r=0.52)\), CETP and TGs \(r=0.53\), CETP and VLDL cholesterol \((r=0.63)\), and CETP and apo E \((r=0.40)\) (Figure 1). The relation between CETP and TG disappeared when chylomicronemic subjects were excluded from the correlation analysis. No significant correlations were observed between CETP and HDL cholesterol, apo A-I, apo A-II, or apo B in the group of hyperlipidemic subjects.

A strong relation between CETP and total cholesterol was also observed in study 2 (Table 3). No relation was observed between CETP and TG or between CETP and VLDL cholesterol in this study population, which contained no chylomicronemic patients and had fewer patients with dysbetalipoproteinemia than did study 1. Further analysis of the second study also revealed a correlation between CETP and LDL CE for this group of hyperlipidemic subjects \((r=0.37, p<0.05)\).

**Effect of Dietary Treatment on Cholesteryl Ester Transfer Protein Concentrations**

Three chylomicronemic patients and two patients with dysbetalipoproteinemia were studied before and after dietary treatment. In each case, CETP levels declined as the lipoprotein profile was normalized (Table 4). HDL cholesterol levels in chylomicronemic subjects increased markedly as CETP levels decreased.

**Short-term Effects of Heparin Infusion on Plasma Levels of Cholesteryl Ester Transfer Protein**

Eight patients with hypertriglyceridemia (four with severe chylomicronemia) were studied before and 30 minutes after heparin infusion (100 units/kg). CETP levels did not change significantly after heparin-induced lipolysis (2.21 \(\mu\)g/ml before versus 2.34 \(\mu\)g/ml after heparin).

**Correlation Between Cholesteryl Ester Transfer Protein Mass and Cholesteryl Ester Transfer Protein**

Analysis of 146 hyperlipidemic subjects, regardless of treatment, revealed a strong positive correlation between mass and activity of CETP \((r=0.72, p<0.001)\). A similar relation existed for a group of nine normal and 50 primary hyperlipidemic subjects not on any lipid-lowering therapy \((r=0.71, p<0.001)\) (Figure 2). This relation was also observed in normolipemics \((r=0.86, p<0.005, n=9)\), hypercholesteroleemics \((r=0.72, p<0.001, n=20)\), and hypertriglyceridemics \((r=0.94, p<0.001, n=8)\). These results show that the concentration of CETP in plasma is the major factor determining total lipid transfer activity, as assayed in diluted plasma in the presence of excess substrate.

**Discussion**

There are three possible routes for the return of HDL-derived cholesterol to the liver. HDL particles rich in apo E may interact directly with a hepatic apo E receptor. This is likely to be the major pathway for reverse cholesterol transport in species such as rodents that are deficient in CETP. The selective uptake of HDL CE without particle endocytosis has been proposed as a second mechanism for disposal of HDL cholesterol. Finally, CETP transfers CE in exchange for TG from apo A-I-containing lipoproteins to apo B-containing lipoproteins, mainly VLDL and chylomicrons and their remnant particles. The latter are likely to represent the major pathway for reverse cholesterol transport in humans. It is of interest that the apo E-rich subfraction of HDL increases markedly in CETP-deficient subjects. This may represent, in response to a malfunctioning CETP pathway, either a compensatory increase in apo E or a decreased clearance of apo E-rich HDL. In any case, significant accumulation of CE in HDL is characteristic of the CETP-deficient state, suggesting that the apo E-mediated pathways do not have sufficient capacity to maintain optimal reverse cholesterol transport.

The present studies demonstrate, for the first time, that increased plasma concentrations of CETP occur in subjects with hypercholesterolemia, alone or in combination with hypertriglyceridemia. This pattern occurs irrespective of the underlying metabolic disorder (familial hypercholesterolemia, familial combined hypercholesterolemia, or polygenic/environmental hypercholesterolemia). On the other hand, CETP concentrations were not elevated in subjects with hypertriglyceridemia and normal LDL cholesterol levels. Similarly, we have recently demonstrated that significant increases in plasma concentrations of CETP develop as LDL cholesterol increases in response to cholesterol feeding in normal subjects. One interpretation of these findings is that CETP increases in situations where there is increased cholesterol flux via LDL or other apo B-containing lipoproteins.
CETP levels were also increased in patients with severe and chronic chylomicronemia and did not alter after heparin-induced lipolysis. Similarly, marked elevations of CETP were observed in patients with dysbetalipoproteinemia due to homozygosity for the E2 allele. Previous studies also demonstrated that CETP activity in lipoprotein-free plasma was increased in dysbetalipoproteinemia.\textsuperscript{16} In the present study, for both chylomicronemic and dysbetalipoproteinemic subjects, correction of the phenotypic profile by dietary means resulted in normalization of the CETP concentration, suggesting that the observed increases in plasma concentrations of CETP were, at least in part, secondary to the lipoprotein abnormality.

Both of these conditions (severe chylomicronemia and dysbetalipoproteinemia) also result in increased peripheral delivery of cholesterol via macrophage receptors.\textsuperscript{22,23} CETP concentrations were closely correlated with total cholesterol for hyperlipidemic subjects and for normal and hyperlipidemic subjects combined (stud-
TABLE 4. Effect of Dietary Treatment on Plasma Cholesteryl Ester Transfer Protein Concentrations

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Sex (M/F)</th>
<th>Disorder</th>
<th>Measurement relative to dietary treatment</th>
<th>TC (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>VLDL-C (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>CETP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.B.</td>
<td>37</td>
<td>F</td>
<td>CM</td>
<td>Pre</td>
<td>25.5</td>
<td>25.9</td>
<td>22.0</td>
<td>3.0</td>
<td>0.57</td>
<td>4.80</td>
</tr>
<tr>
<td>T.G.</td>
<td>40</td>
<td>M</td>
<td>CM</td>
<td>Pre</td>
<td>13.5</td>
<td>21.9</td>
<td>10.9</td>
<td>2.2</td>
<td>0.44</td>
<td>3.50</td>
</tr>
<tr>
<td>D.P.</td>
<td>55</td>
<td>F</td>
<td>CM</td>
<td>Pre</td>
<td>18.2</td>
<td>49.8</td>
<td>15.0</td>
<td>2.9</td>
<td>0.34</td>
<td>3.70</td>
</tr>
<tr>
<td>J.K.</td>
<td>65</td>
<td>M</td>
<td>DB</td>
<td>Pre</td>
<td>9.3</td>
<td>21.8</td>
<td>6.0</td>
<td>2.8</td>
<td>0.54</td>
<td>2.48</td>
</tr>
<tr>
<td>W.C.</td>
<td>30</td>
<td>M</td>
<td>DB</td>
<td>Pre</td>
<td>7.3</td>
<td>2.0</td>
<td>2.3</td>
<td>1.8</td>
<td>1.09</td>
<td>3.38</td>
</tr>
</tbody>
</table>

TC, total cholesterol; TG, triglycerides; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; CETP, cholesteryl ester transfer protein; CM, chylomicronemia; DB, dysbetalipoproteinemia.

ies 1 and 2). This may represent a relation between CETP and cholesterol flux. Alternatively, plasma CETP, by modifying VLDL composition, may enhance apo B transport into the LDL density range. There is evidence of decreased LDL cholesterol with CETP deficiency in contrast to increased LDL cholesterol in species with high CETP activity. In study 2, CETP levels were associated with LDL cholesterol ester concentrations, and this association could also be interpreted as an effect of CETP on LDL cholesterol ester production and composition, rather than or in addition to an effect of LDL cholesterol flux on CETP production.

We also observed a correlation between CETP and plasma TGs in study 1 that was significant only when chylomicronemic patients were included in the correlation analysis. This correlation was not observed in study 2, which did not include any subjects with fasting chylomicronemia. Although in normal fasting plasma CETP is primarily associated with the very high density lipoprotein plasma fraction, it is possible that CETP interaction with TG-rich particles occurs in chronic chylomicronemia.

In contrast to observations in normolipemic subjects, CETP showed no relation with apo A-I or any other HDL variable in hyperlipoproteinemic patients. This suggests that in situations where the flux of apo B-containing lipoproteins is enhanced, the availability of donor particles is not a major determinant of CETP mass.

We have confirmed that in hyperlipoproteinemic patients as in normolipemic subjects, CETP is significantly correlated with apo E (r=0.40). The highest levels of CETP were present in subjects with the greatest elevations in apo E (patients with dysbetalipoproteinemia and chylomicronemia). In other situations associated with increases in plasma concentrations of apo E, CETP is also increased. Cholesterol feeding results in significant increases in plasma levels of apo E and CETP. During probucol treatment, apo E and CETP increase, and the rise in apo E appears to precede the increase in CETP.

CETP and apo E are two important components of the reverse cholesterol transport process. CETP is essential for the transfer of HDL-derived cholesterol to TG-rich lipoproteins, and apo E markedly enhances hepatic clearance of these particles. Elevation of both CETP and apo E could represent a
compensatory response when cholesterol delivery to macrophages is enhanced via receptors for modified LDL,27 β-VLDL,22 or chylomicron remnants.23 In a rabbit model, cholesterol feeding increases hepatic mRNA for CETP. Hepatic mRNA for apo E does not change in the rabbit, but plasma levels of apo E increase,28 and in vitro incubation of macrophages with acetylated LDL results in enhanced macrophage secretion of apo E.29 Therefore, it would appear that while cholesterol transport regulates both CETP and apo E, the control of gene expression for these two proteins demonstrates different tissue specificities.

We have clearly demonstrated, using the methodology described, that there is a close relation between plasma concentrations of CETP and the in vitro CE transfer activity in normal plasma (r=0.86). This relation is only slightly less pronounced in hyperlipidemic plasma (r=0.72). It is important to emphasize that the in vitro activity assay used here for CETP has been developed to minimize the relative effects of donor and acceptor lipoproteins, as well as the effect of CETP inhibitor proteins on transfer activity. Therefore, the correlations noted here validate this assay method for CETP activity, which reflects mostly CETP concentration. However, none of these assays for mass or activity of CETP reflects the physiological CETP activity present in the blood at the time of sampling, and accordingly, we do not have direct evidence that plasma CETP concentration is the major determinant of net transfer activity in vivo.

CETP is an important component of the reverse cholesterol transport process and responds significantly to alterations in lipoprotein transport. Plasma concentrations of CETP appear to be increased in conditions where cholesterol flux is increased, via LDL or other lipoproteins, as in dietary and genetic hypercholesterolemia, dysbetalipoproteinemia, and chronic chylomycinemia. Furthermore, correction of the phenotypic abnormality by diet in dysbetalipoproteinemia and chylomycinemia results in normalization of plasma CETP levels. Further studies are in progress to investigate the control of CETP synthesis and catabolism in humans.

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