Cholesteryl Ester Transfer Protein Activity Enhances Plasma Cholesteryl Ester Formation

Studies in CETP Transgenic Mice and Human Genetic CETP Deficiency

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Abstract The plasma cholesteryl ester transfer protein (CETP) promotes the removal of HDL cholesteryl esters and is thought to stimulate reverse cholesterol transport (RCT). However, mechanisms by which CETP may stimulate RCT are poorly understood. Thus, we examined the relationship between plasma CETP expression and plasma cholesteryl ester formation in CETP transgenic (Tg) mice, hamsters, and human subjects with genetic CETP deficiency. Incubation of CETP Tg mouse plasma showed a 20% to 40% increase in plasma cholesterol esterification rate (CER, $P<.05$) compared with control mice. Injection of a neutralizing CETP monoclonal antibody (MAb) (TP2) into natural flanking region CETP Tg mice resulted in an increase in plasma free cholesterol (FC) concentration, FC/CE ratio, FC/phosphatidylcholine ratio, and hepatic CETP mRNA. In hamsters, CETP inhibition also resulted in an increase in plasma FC/phosphatidylcholine ratio and increased CETP mRNA in adipose tissue. In humans with two common CETP gene mutations (an intron 14 splicing defect and a D442G missense mutation), mean plasma CERs were 39 and 60, respectively, compared with 89 nmol × mL^{-1} × h^{-1} in normal subjects. By contrast, lecithin:cholesterol acyltransferase (LCAT) mass was normal in CETP-deficient subjects. MAb neutralization of CETP activity in incubated human plasma did not alter the LCAT reaction, even after supplementation with discoidal HDL and VLDL. Thus, genetic alterations in CETP levels lead to secondary changes in the plasma LCAT reaction, possibly because of remodeling of HDL by CETP acting in concert with other factors in vivo. In human genetic CETP deficiency, a moderate impairment in the plasma LCAT reaction may contribute to a defect in RCT, providing a potential mechanism to explain the recently observed excess of coronary heart disease in these subjects. (***Arterioscler Thromb Vasc Biol.* 1997;17:1045-1052.**)

Key Words • cholesteryl ester transfer protein • transgenic mice • reverse cholesterol transport • cholesterol esterification rate

The plasma CETP is a hydrophobic, M_r 70 000 glycoprotein that stimulates the exchange of HDL CE with triglycerides of VLDL and chylomicrons.[1] CETP is thought to participate in RCT, ie, the transfer of cholesterol from the periphery back to the liver via the HDL fraction.[2] CETP is known to promote the net transfer of HDL CE from plasma into the liver, probably by transferring CE to triglyceride-rich lipoproteins, which are cleared relatively rapidly by this organ.[3-5] However, this process would not lead to an overall stimulation of RCT unless the plasma lipid transfer process resulted in a net increase in the transfer of cholesterol from peripheral tissues into HDL. One way this could occur would be if the action of CETP led to stimulation of the LCAT reaction, which would then drive the net efflux of cellular cholesterol into HDL.[6,7]

There is conflicting information on the effect of CETP activity on the LCAT reaction. Chajek et al[8] initially suggested that CETP could relieve CE product inhibition of the LCAT reaction in nascent HDL particles. In subsequent studies, however, inhibition of CETP in incubated plasma with a CETP MAb, TP2, showed no effect on the initial or subsequent rate of CE formation, suggesting that plasma CETP is not subject to product inhibition.[9] More recently, human CETP Tg mice were shown to have decreased HDL CE levels and a marked depletion of FC in VLDL, LDL, and HDL, suggesting that CE removal from HDL in vivo leads to a stimulation of the plasma cholesterol esterification.[10] Humans with genetic CETP deficiency have increased HDL levels, but reports on the rate of CE formation in plasma from these subjects are conflicting.[11,12]

Heterozygous genetic CETP deficiency is present in 5% to 7% of the general Japanese population,[13] and there also appear to be common, functionally significant genetic variants at the CETP locus in European populations.[14] Lower CETP levels are associated with increased HDL, a factor usually associated with protection from CHD.[15] However, we recently found an increased prevalence of CHD in humans with genetic CETP deficiency despite increased HDL levels[16] and a decrease in early atherosclerotic lesions in hypertriglyceridemic CETP Tg mice.[17] One possible explanation for the effects of CETP expression on atherosclerosis is related to its putative stimulation of the LCAT reaction in vivo. Thus, we have used genetic alterations in CETP or CETP inhibition by neutralizing MAbs to examine the relationship between CETP gene expression and the plasma LCAT reaction.

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Selected Abbreviations and Acronyms

apo = apolipoprotein
CE = cholesteryl ester
CER = cholesterol esterification rate
CETP = cholesteryl ester transfer protein
CHD = coronary heart disease
FC = free cholesterol
LCAT = lecithin:cholesterol acyltransferase
MAb = monoclonal antibody
PCR = polymerase chain reaction
Tg = transgenic

Methods

Human Subjects

Plasma samples were obtained from subjects with genetic CETP deficiency. Five subjects harboring the CETP intron 14 G to A mutation and 5 with the CETP exon 15 D442G mutation were compared with 20 healthy control subjects. The intron 14 mutant group included 2 homozygotes and 3 heterozygotes, all men; average age, 62.4±9.1 years. The exon 15 mutant group included 5 heterozygotes, 4 men and 1 woman; average age, 41.8±11.4 years. In the control group, all subjects were male, with an average age of 41.1±9.6 years. None of the subjects were receiving lipid-lowering drugs. CETP genotype was determined by polymerase chain reaction–based methods on extracted DNA as previously described.[18,19]

Animal Studies

The Tg animals used in this study were human NFR´CETP Tg mice originally described by Jiang et al[20] and NFR´CETP Tg mice crossbred with apo C-III Tg line 3707 originally described by Walsh et al.[21] Mice of both sexes, 2 to 4 months old, were housed in a room with a 12-hour light-dark cycle and maintained on water and rodent chow diet (Purina Chow 5001, Ralston Purina Co) ad libitum. For CETP inhibition studies, the animals were studied on chow or after 1 week of Western-type diet containing 20% fat and 0.15% cholesterol by weight (Research Diets). A dose of 25 mg/kg body wt of human CETP MAb TP2[22] or control mouse IgG (Sigma Chemical Co) was injected subcutaneously between 10 and 11 AM. Before the injection, the antibodies were filtered through an endotoxin-removing gel (Detoxi-Gel, Pierce Chemical Co). Blood samples (50 to 100 μL) were obtained at time 0 (just before the injection) and at 24, 48, and 72 hours after the antibody administration by retro-orbital bleeding under methoxyflurane anesthesia. At day 3, the mice were exsanguinated, and the liver was excised, frozen in liquid nitrogen, and stored at -70°C for RNA analysis. Male Syrian hamsters (150 to 180 g) from Charles River Laboratory (Wilmington, Mass) on a high-cholesterol diet containing 20% fat and 1% cholesterol by weight (Research Diets) for 2 weeks were also injected subcutaneously with TP2 or mouse IgG (12.5 mg/kg body wt) and bled from the retro-orbital plexus at time 0 and 72 hours after injection. Adipose tissue was obtained for RNA analysis.

Cholesterol Esterification Assays

Endogenous CER

Endogenous CE formation was determined after incubation of plasma at 37°C for 1 hour (mouse plasma) or 4 hours (human plasma) by measurement of the decrease in FC levels with an enzymatic kit (Wako Bioproducts). Results are expressed as nanomoles of cholesterol esterified per hour per milliliter of plasma.

Apo AI Discoidal Substrate Assay

LCAT-mediated CE formation was also measured with disks containing phosphatidylcholine, [^{3}H]cholesterol, and apo A-I (molar ratio, 250:12.5:0.8) prepared by the cholate dialysis technique according to Chen and Albers.[24] Briefly, a lipid mixture containing 7.7 mg phosphatidylcholine, 0.12 mg unlabeled FC, and 10 μCi [^{3}H]cholesterol (DuPont–New England Nuclear) was dried under a stream of nitrogen at room temperature. Tris-HCl buffer solution (2.5 mL) (in mmol/L: Tris-HCl 10, NaCl 140, and EDTA 1, pH 7.4), 0.8 mL 1.1 mg/mL human apo A-I, and 0.3 mL 725 mmol/L sodium cholate solution were added to the dried lipids, mixed on a vortex mixer for 1 minute, and incubated for 20 minutes at 24°C. The mixture was extensively dialyzed against Tris-HCl buffer for 48 hours at 4°C to remove the cholate. The dialysate was adjusted to 4 mL with Tris-HCl buffer and filtered through a 0.22-μm Millipore filter. The LCAT assay consisted of preincubation of 120 μL Tris-HCl buffer, 40 μL apo A-I discoidal substrate, and 4.5 μL 20% BSA (essentially fatty acid free) for 20 minutes at 37°C. Nine microliters of 100 mmol/L b-mercaptoethanol and 6 μL plasma were added and incubated for different times at 37°C. The enzymatic reaction was stopped by Bligh-Dyer lipid extraction.[25] Free and esterified cholesterols were separated by thin-layer chromatography in silica gel^7 developed in hexane/ethyl ether/acetic acid (70:30:1, vol/vol/vol). CE radioactivity was determined by liquid scintillation spectrometry. The mass of CE formed was obtained from the original cholesterol specific activity in the apo A-I discoidal substrate. Results are expressed as nanomoles per hour per milliliter.

Plasma CETP and LCAT Mass

The concentration of CETP in plasma was determined by solid-phase competition radioimmunoassay using the CETP MAb TP2.[26] LCAT concentration was quantified by radioimmunoassay using a polyclonal antibody raised against human LCAT.[27] As quality control, four fresh-frozen samples with LCAT concentrations varying from low to high were analyzed twice at the beginning and at the end of each assay. The intra-assay and interassay variations were 5% and 7%, respectively.

Plasma CETP Activity and Lipid Analysis

CETP activity was determined in diluted plasma with [^{3}H]CE-HDL as CE donor and a mixture of VLDL and LDL as CE acceptor as described previously.[28] Plasma total cholesterol and FC and phospholipid levels were assayed by enzymatic methods (Wako Bioproducts). CE was derived from the difference between total cholesterol and FC and multiplied by 1.7. HDL cholesterol was determined in the plasma supernatant after precipitation of apo B–containing lipoproteins.

RNA Analysis

Total RNA was isolated from liver (mice) and adipose tissue (hamster) by the guanidinium thiocyanate method[29] with RNAzolTB (Cinna/Biotecx). The integrity of the RNA samples was evaluated by borate-agarose gel electrophoresis and ethidium bromide staining. LCAT mRNAs were determined by Northern blot analysis. Total liver RNA (10 μg) was subjected to electrophoresis in 1% formaldehyde-agarose gels,[30] transferred to Zeta-Probe GT blotting membranes (Bio-Rad), and cross-linked at 80°C for 1 hour under vacuum. Blots were prehybridized, hybridized, and washed according to the manufacturer's instructions. Blots were probed with 1×10^6 cpm/mL mouse LCAT cDNA kindly provided by Dr A.J. Lusis,[31] labeled with [^{32}P]dCTP by random oligonucleotide priming using the Prime-It II Kit (Stratagene), and exposed to autoradiography films. Subsequently, the LCAT probe was stripped and the membranes were reprobed with human gamma actin cDNA probe to normalize the amount of RNA per lane. CETP mRNA was determined by solution hybridization–ribonuclease protection assay as previously described.[32] Briefly, a human CETP antisense cRNA probe was prepared from the human cDNA fragment that included part of the exon 16 and 3′-untranslated region. A second riboprobe prepared from a mouse β-actin cDNA fragment (Ambion) was included in the assay, and all

values were normalized for the recovery of β-actin. Hamster adipose tissue samples were hybridized with a cRNA prepared from the hamster cDNA fragment spanning codons 143 to 181.[33] All riboprobes were prepared with the Stratagene RNA in vitro transcription kit. Total RNA (50 μg) was hybridized with 1×10^5 cpm of uridine [^{32}P]CETP riboprobe and 2.5×10^4 cpm of ^{32}P-labeled β-actin riboprobe in 30 μL of 80% formamide, 40 mmol/L PIPES, 0.4 mol/L NaCl, and 1 mmol/L EDTA, pH 6.4. After denaturation at 85°C for 5 minutes, the mix was hybridized at 48°C for 16 hours. Then, samples were digested with RNAse T2 (85 U/mL) in 350 μL of buffer containing 50 mmol/L sodium acetate, 0.1 mol/L NaCl, and 2 mmol/L EDTA, pH 5.0, for 2 hours at 37°C. After phenol/chloroform extraction and ethanol precipitation, the precipitate was analyzed on a 5% polyacrylamide-urea sequencing gel. The protected fragments were visualized by gel exposure to autoradiography films at -70°C with intensifying screen and quantified by film scanning using a laser densitometer (Molecular Dynamics Inc).

Statistical Analysis

Results are expressed as mean±SEM. The statistical significance of the differences between the groups was estimated by Student's *t* test (two-tailed) or one-way ANOVA when appropriate. Statview SE+ software (Abacus Concepts Inc) was used for all analyses.

Results

Enhanced Cholesterol Esterification in Plasma of CETP Tg Mice

In earlier studies, we observed a decrease in the FC/CE ratio in plasma and all lipoprotein fractions of CETP Tg mice, suggesting that CETP expression results in an enhanced plasma LCAT reaction in these animals.[10] To further investigate this finding, we measured plasma CER in incubated plasma of CETP Tg mice (Table 1). In two different lines of CETP Tg mice, CER was increased by 20% to 33% (both values, $P<.05$). We also measured CER in plasma of hypertriglyceridemic apo C-III/CETP Tg mice. These mice showed an ≈40% increase in CER, but the difference was not significant owing to greater variability in the CER in apo C-III Tg mice (Table 1).

The CER depends both on the amount of LCAT and on the nature and amount of its substrate lipoproteins in plasma. To quantify the amount of active enzyme in mouse plasma, we determined the initial rate of the reaction, using apo A-I/FC/lecithin discoidal substrates (also called proteoliposomes[24]). For both CETP and apo C-III/CETP Tg mice, the rate of CE formation during the first hour of incubation was similar to that in control mouse plasma (Fig 1A and 1B, respectively). With longer incubations, however, the curves diverged because of a higher rate of CE formation in the mouse plasma that contained CETP. Similar results were obtained in several separate experiments (n=5). These

TABLE 1. CER in CETP Tg Mice

Group	Control, nmol · mL^{-1} · h^{-1}	CETP Tg, nmol · mL^{-1} · h^{-1}
CETP (line 1) vs non-Tg	154±10 (6)	183±8* (8)
CETP (line 2) vs non-Tg	118±14 (7)	156±9* (6)
CETP (line 1)/apo C-III vs apo C-III	91±22 (9)	127±4 (8)

CER was calculated as the difference between FC levels before (0°C) and after 1 hour of incubation of fresh plasma at 37°C. Values are mean±SEM (n).
*$P<.05$ (Student's *t* test).

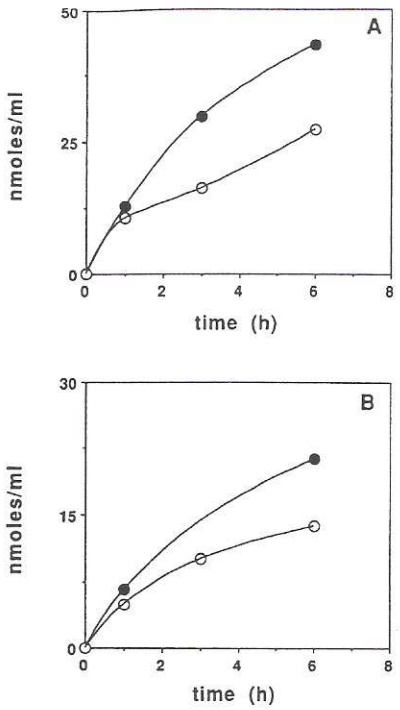

FIG 1. Measurement of plasma LCAT activity in wild-type and CETP Tg mouse plasma using exogenous apo A-I discoidal substrate containing phosphatidylcholine, [^{3}H]cholesterol, and human apo A-I (molar ratio, 250:12.5:0.8). Incubations contained 8 μg apo A-I discoidal substrate, 0.5% BSA, 5 mmol/L β-mercaptoethanol, and 6 μL plasma in a final volume of 200 μL. [^{3}H]CE formed was determined after Bligh-Dyer lipid extraction and TLC. A, Plasma of CETP Tg mice (●) and non-Tg mice (○). B, Plasma of CETP/apo C-III double Tg mice (●) and apo C-III Tg mice (○). Results are average of triplicates of pooled plasma samples from three animals, representative of three independent experiments (A) and two independent experiments (B).

results suggest that there is no difference in LCAT levels in CETP Tg mouse plasma. However, CETP was able to enhance the saturation level of the esterification reaction, possibly by maintaining the LCAT substrate in an optimal form.

CETP Inhibition Increases Plasma FC Concentration and CETP mRNA in Tissues of CETP Tg Mice and Hamsters

The CETP Tg mice have a mixed genetic background (CBA/C57BL6), which raises the possibility that differences in CER were related to uncontrolled genetic variation. To confirm that CETP is the main factor responsible for increased CER in the plasma of CETP Tg mice, in vivo neutralization of plasma CETP activity was performed. Thus, CETP Tg mice were injected with MAb TP2 or with control mouse IgG. Injection of TP2 resulted in a sustained neutralization of plasma CE transfer activity (Fig 2A), paralleled by a rise in plasma FC concentration (Fig 2B) and an increase in FC/CE ratio (Fig 2C) to levels similar to those in non-Tg littermates. Also, the plasma FC/phosphatidylcholine ratio was significantly increased (+21%, $P<.02$) in mice injected with TP2 compared with control IgG. Similar data were obtained in mice fed chow or Western diets (not shown). There was a marked decrease in CER in plasma from

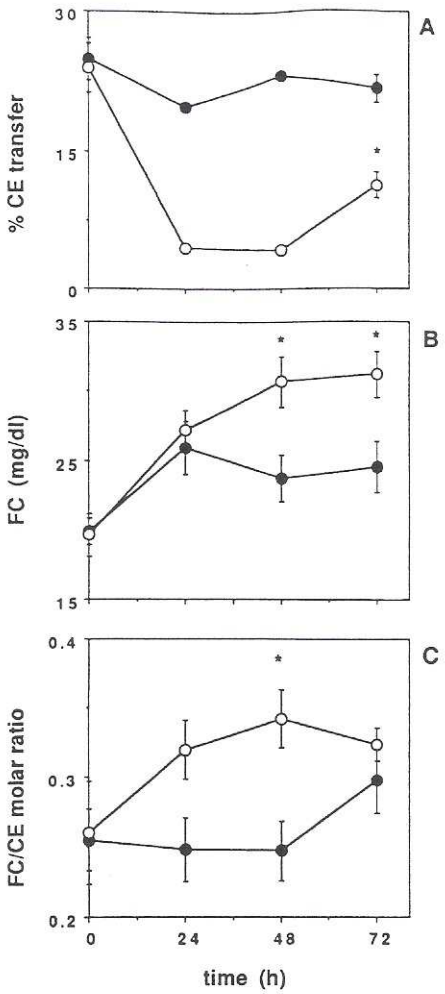

FIG 2. Plasma CETP activity (A), FC levels (B), and FC/CE molar ratio (C) in CETP Tg mice after a subcutaneous injection (25 mg/kg body wt) of CETP MAb TP2 (○) (n=9) or control mouse IgG (●) (n=5). Bars represent SEM. *$P<.03$ or better.

TP2 versus control IgG-injected mice fed either diet (Table 2). These results confirm that the depletion of plasma FC and the enhanced plasma CER in CETP Tg mice are a direct consequence of CETP expression.

It is well known that CETP gene expression is stimulated by high plasma cholesterol levels.[1] Therefore, we tested the hypothesis that CETP inhibition, by increasing plasma FC concentration, might lead to an increase in hepatic CETP mRNA. Hepatic CETP mRNA was, in fact, significantly increased at the end of the experiments shown in Fig 2, ie, after 3 days of CETP inhibition (Table 3). By contrast, hepatic LCAT

TABLE 2. Plasma CER in CETP Tg Mice After CETP Neutralization by MAb (nmol · mL^{-1} · h^{-1})

Diet	Control (IgG Treated), nmol · mL^{-1} · h^{-1}	CETP Inhibited (TP2 Treated), nmol · mL^{-1} · h^{-1}
Chow	193±61 (5)	90±9* (9)
Western	228±11 (5)	152±28* (6)

Data obtained 3 days after a subcutaneous injection of 25 mg/kg body wt of control IgG or CETP MAb TP2. CER was calculated as the difference between FC levels before and after 1 hour of incubation at 37°C. Values are mean±SEM (n).

*$P<.05$ (Student's t test).

TABLE 3. Hepatic CETP and LCAT mRNA in CETP Tg Mice 3 Days After Treatment With CETP-Neutralizing MAb

mRNA	Control (IgG Treated)	CETP Inhibited (TP2 Treated)
CETP	100±10 (n=10)	164±20* (n=15)
LCAT	100±10 (n=10)	120±53 (n=15)

CETP and LCAT mRNA were determined in the liver of animals 3 days after a subcutaneous injection of 25 mg/kg body wt of control IgG or CETP MAb TP2. CETP mRNA was determined by solution hybridization RNase protection assay and LCAT mRNA by Northern blot analysis. Results are corrected by actin mRNA expression and are expressed relative to the control animal levels (100%). Pooled results from animals fed chow or Western-type diet. Values are mean±SEM.

*$P<.02$ (Student's t test).

mRNA was not significantly changed after CETP inhibition (Table 3).

To see whether CETP also drives plasma cholesterol esterification in an animal that naturally expresses CETP, we carried out similar experiments in hamsters. Hamsters have high levels of plasma CETP activity, and CETP mRNA is abundant in adipose tissue.[33] We used a protocol for inhibiting hamster CETP similar to that previously described.[23] Plasma total, free, and esterified cholesterol increased about 2-fold above baseline 3 days after TP2 injection. The plasma FC/phosphatidylcholine ratio was increased by 40% ($P<.001$) 3 days after TP2 administration, whereas it did not change significantly in control IgG-treated animals. Adipose tissue CETP mRNA was increased 3.3-fold in the TP2-treated group compared with IgG-treated controls (n=3 in each group) at day 3 of the experiment. Thus, the inhibition of CETP resulted in analogous responses of plasma cholesterol and CETP mRNA levels in hamsters and CETP Tg mice.

Impaired CE Formation in Plasma of CETP-Deficient Humans

We also determined the CER in stored frozen plasma of subjects with a genetic deficiency of CETP and related the findings to measurements of CETP and LCAT concentration as determined by radioimmunoassay. Subjects with both the intron 14 splicing defect and exon 15 missense mutation showed a marked and significant decrease in plasma CER compared with controls (Table 4). The defect was more pronounced in the intron 14 mutation group, which included several homozygotes and was associated with lower CETP levels in plasma. In

TABLE 4. CER, CETP, and LCAT Levels in the Plasma of CETP-Deficient and Control Subjects (Frozen Plasma)

	Intron 14 Mutation (n=5)	Exon 15 Mutation (n=5)	All Mutations (n=10)	Control (n=20)
CER, nmol · mL^{-1} · h^{-1}	3.2±2*	32.3±6.8†	17.8±5.9*	96.0±8.7
CETP activity, %CE transfer	6.6±2.7*	23.6±3.6	15.1±3.6*	28.6±1.3
CETP mass, μg/mL	0.59±0.2*	1.71±0.3*	1.15±0.3‡	2.31±0.1
LCAT activity, nmol · mL^{-1} · h^{-1}	66.0±1.6	75.0±2.2	70.6±1.9	74.0±2.2
LCAT mass, μg/mL	6.2±0.4	6.0±0.6	6.1±0.4	5.6±0.2

CETP-deficient subjects with the intron 14 (G to A) splicing defect included 2 homozygotes and 3 heterozygotes, and exon 15 (D442G) missense mutation included 5 heterozygotes. Values are mean±SEM.

*$P<.0005$, †$P<.002$, ‡$P<.015$: CER determined by the endogenous cholesterol esterification assay and LCAT activity determined by the apo A-I discoidal substrate assay. CETP and LCAT mass were determined by radioimmunoassay.

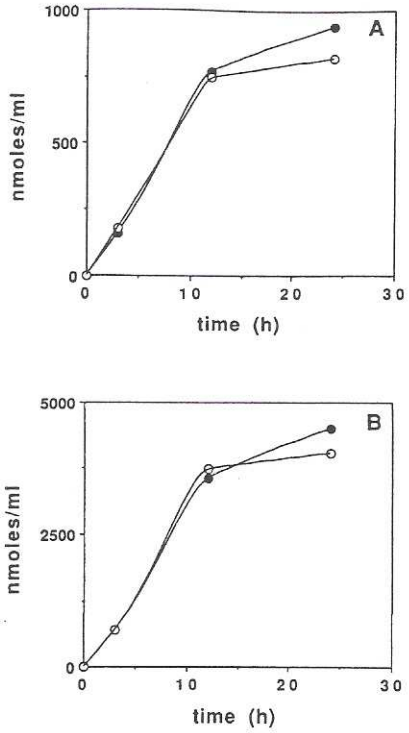

FIG 3. Plasma LCAT activity measured with exogenous apo A-I discoidal substrate labeled with [^{3}H]cholesterol in human plasma in the presence (○) or absence (●) of CETP MAb TP2 (20 μg/mL). CETP activity was 100% inhibited by this amount of +P2 assay, as in legend of Fig 1. A, Plasma+apo A-I discoidal substrate; representative of four independent experiments. B, Plasma+apo A-I discoidal substrate+FC/lecithin liposome (molar ratio, 2; 100 μg/mL)+VLDL (300 mg/dL triglycerides). Average of triplicates.

contrast, LCAT concentration and activity did not vary between the different groups. To confirm these findings, plasma CER was also determined in fresh nonfrozen plasma samples. This revealed a more moderate but still significant defect in CER for both intron 14 and exon 15 mutations (Table 5).

In earlier studies, Yen et al[9] found that MAb inhibition of CETP did not influence CER in normolipidemic human plasma. In view of the marked impairment of CER in plasma of subjects with genetic CETP deficiency, we reinvestigated this issue by incubating human plasma containing optimal discoidal LCAT substrates, with or without CETP inhibition by TP2. CETP inhibition had no effect on the initial or subsequent rates of the LCAT reaction in normolipidemic plasma (Fig 3A) or in plasma supplemented with VLDL to enhance the CE transfer reaction[34] (Fig 3B). Thus, CETP inhibition in incubated human plasma does not affect the rate of the LCAT reaction, suggesting that CETP does not directly influence the CER by releasing the LCAT reaction from end-product inhibition.

Discussion

In this study, we have examined the relationship between CETP activity and the plasma LCAT reaction. Results in three different species suggest that CETP activity drives plasma CE formation in vivo. However, because CETP inhibition in human plasma does not alter CE formation, we conclude that the action of CETP is indirectly mediated and that CETP may interact with other factors in vivo to maintain the LCAT substrate (HDL) in an optimal form. In human genetic CETP deficiency, there is a moderate impairment of the plasma LCAT reaction. This implies a defect in RCT, which may be a mechanism that contributes to increased CHD.

The effect of CETP on the plasma CER could result from changes in LCAT levels or efficiency. The first possibility can be excluded because plasma LCAT levels were not different in CETP-deficient and control subjects and appeared to be the same in control and CETP Tg mouse plasma. The efficiency of the LCAT reaction could be improved by CETP-mediated removal of CE, relieving end-product inhibition of the LCAT reaction.[8] or by an effect of CETP to modify the LCAT substrate (HDL) into an optimal form. In an earlier study, Yen et al[9] showed that MAb inhibition of CETP in human plasma did not result in changes in CER. These findings were confirmed and extended in the present investigation when it was shown that LCAT reaction was not affected by CETP inhibition even when plasma was supplemented with discoidal substrates to drive the LCAT reaction or with VLDL to stimulate the CE transfer reaction. Thus, it appears that the LCAT enzyme is not subject to direct end-product inhibition. We conclude that CETP enhances the LCAT reaction by helping to form an optimal substrate. Because this effect is seen in ex vivo plasma samples but not in CETP-inhibited plasma, it is likely that CETP interacts with other factors in vivo to remodel HDL into an optimal form for the LCAT reaction.

A clue to the other factors that interact with CETP to influence the plasma LCAT reaction is provided by the observation that in murine plasma, the presence of CETP activity results in a higher rate of CE formation after the initial hour of incubation, whereas rates remain identical in human plasma with or without CETP activity (Figs 1 and 3). An important difference between mouse and human plasma is that the former contains a high level of hepatic lipase activity.[35] CE that accumulates in discoidal HDL particles may be removed by CETP in exchange for triglyceride: in mouse plasma, the subsequent action of hepatic lipase would be expected to regenerate discoidal particles. Thus, hepatic lipase may act in conjunction with CETP to maintain the presence of the optimal discoidal HDL substrate in mouse but not in human plasma. Another factor contributing to a secondary state of LCAT deficiency after TP2 injection in vivo could be enhanced clearance of HDL particles

TABLE 5. CER in Plasma CETP-Deficient and Control Subjects (Fresh Plasma)

	Intron 14 Homozygotes (n=6)	Exon 15 Heterozygotes (n=7)	Control Subjects (n=63)
CER, nmol · mL^{-1} · h^{-1}	39±4* (31-59)	60±4* (51-74)	89±2 (55-124)

Determined in fresh nonfrozen plasma by the method of Nagasaki and Akanuma.[53]
*P<.01 vs control.

containing both LCAT and CETP molecules, because there is some evidence that these may reside on the same HDL particles.[36]

The plasma HDL fraction is heterogeneous, and CETP plays an important role in determining the size and structure of HDL.[1] Subjects with genetic CETP deficiency have markedly increased levels of large CE- and apo E–rich HDL particles and a relative deficiency of smaller HDL_3 particles.[37] Plasma lipoprotein analysis in hamsters after CETP inhibition by MAbs revealed a profile very similar to that reported for subjects with CETP deficiency.[23,38] HDL particles of different sizes and shapes have markedly different reactivities with LCAT. For example, the size of substrate particles correlates positively with the K_m of the LCAT reaction, and spherical HDLs are considerably less reactive with LCAT than with discoidal HDL particles.[39] Similarly, discoidal HDL particles have an affinity for CETP higher by an order of magnitude than spherical HDL.[40] Thus, in vivo CETP action may be targeted to nascent discoidal particles and, acting in concert with hepatic lipase, may maintain the LCAT substrate in optimal form.

Interestingly, there appears to be a feedback loop between plasma CETP activity and CETP mRNA in liver (CETP Tg mice) or adipose tissue (hamster), possibly mediated by changes in the plasma LCAT reaction. Although the changes in hepatic CETP mRNA were modest in magnitude (+64%, Table 3), in other settings in which plasma FC level is more markedly increased, there are much larger alterations in CETP gene expression.[41] The induction of CETP gene expression in the liver is independent of the presence of apo E or LDL receptors, indicating that the mechanism does not require classic receptor-mediated endocytosis of cholesterol-rich lipoproteins.[41] Consistent with the present results, Masucci-Magoulas et al[41] speculated that a gradient of FC between the plasma lipoproteins and the hepatocyte plasma membrane could lead to increased cholesterol content in the cellular cholesterol regulatory pool, causing increased CETP gene expression.

The defect in CER in plasma from subjects with genetic CETP deficiency provides a mechanism that could help to explain the recent observations of an excess of CHD in these individuals. In a recent report, subjects with absent plasma CETP activity were found to have low rates of CER.[12] Our findings extend these observations by demonstrating an inverse relationship between plasma CETP levels and the CER over a range of CETP levels, independent of LCAT mass (Tables 4 and 5). Furthermore, we studied this relationship in genotypically defined groups of subjects and showed a significant impairment of the LCAT reaction in subjects heterozygotic for the exon 15 missense mutation, who have smaller decreases in CETP levels than subjects with the intron 14 splicing defect.[19] This is important because our recent finding of increased CHD in men with genetic CETP deficiency is based largely on data obtained in subjects heterozygotic for the exon 15 mutation.[16] Moreover, the apparent mechanism underlying the defect in CER, involving remodeling of HDL by CETP acting in conjunction with hepatic lipase, is consistent with a recent finding that among subjects with CETP deficiency, those with low hepatic lipase activity have a higher prevalence of CHD.[42]

The defect in plasma CER in genetic CETP deficiency is associated with a modestly increased content of FC in HDL. In a study of subjects with the intron 14 splicing defect,[43] the HDL cholesterol/phospholipid molar ratios were 0.33 (homozygotes), 0.20 (heterozygotes), 0.20 (unaffected family members), and 0.17 (normal control subjects). The corresponding FC/CE ratios were 0.28, 0.21, 0.23, and 0.18. Compared with the defect in CER in incubated plasma (Table 5), the accumulation of FC seems mild. In part, this is because CETP deficiency causes accumulation of CE and phospholipid in HDL, so that the ratio of FC to these components is not markedly increased. It is also possible that the in vitro assay of CER overestimates the defect in vivo. It is of interest to note that this activity appeared to be more labile in stored frozen plasma samples of CETP-deficient subjects than in control subjects (Tables 4 and 5). It is possible that a small, relatively labile pool of HDL represents the optimal LCAT substrate pool and that this is rapidly consumed in vitro. Nonetheless, a 60% decrease in FC to CE conversion in vivo has been reported in CETP-deficient subjects.[44]

The secondary defect in LCAT activity in genetic CETP deficiency probably impairs RCT by producing a defect in the ability of HDL to promote peripheral cellular cholesterol efflux. The ability of LCAT to drive cellular cholesterol efflux has been demonstrated in several studies.[6,7,45,46] The impact of the LCAT reaction on net cellular cholesterol efflux is largely due to a decrease in cholesterol influx into cells.[6,7,45] Lipoprotein A-I and A-I/A-II isolated from subjects with CETP deficiency were shown to have a partial defect in associated LCAT activity with a parallel impairment in ability to promote net cholesterol efflux from cholesterol-loaded macrophages.[12] We conclude that genetic CETP deficiency results in a secondary impairment of the plasma LCAT reaction, and this is a plausible factor contributing to excess CHD in genetic CETP deficiency.

Recently, we showed a decrease in early atherosclerotic lesions in hypertriglyceridemic CETP Tg mice.[17] However, mice expressing CETP alone had an increase in such lesions.[47] Although the stimulation of CER probably represents an antiatherogenic mechanism in mice, we did not find that this parameter was markedly increased in apo C-III/CETP versus CETP Tg mice (Table 1). Thus, there could be other antiatherogenic mechanisms that are accentuated in hypertriglyceridemic mice. There is a striking synergistic interaction between CETP expression and hypertriglyceridemia to reduce HDL particle size.[48] Apart from interactions with LCAT, such smaller and pre–b-HDL particles may have enhanced ability to promote cholesterol efflux from macrophage foam cells[49]; they may also show enhanced penetration into the complex interstices of atheroma.[50,51]

It is likely that CETP may influence RCT and/or cholesterol removal from cells in atheroma in several different and opposing ways. On the one hand, CETP action reduces HDL concentration; on the other hand, CETP remodels HDL to produce particles that are optimal LCAT substrates and that have superior cellular cholesterol efflux properties.[36,52] For subjects with HDL cholesterol >60 mg/dL, those with and without CETP gene mutations have a similar low prevalence of CHD,[16] suggesting that direct antiatherogenic effects of high

HDL are predominant. Such effects could be related to RCT or other mechanisms. For individuals with CETP gene mutations and HDL cholesterol <60 mg/dL, subtle defects in RCT, such as those elucidated here, may be predominant, leading to an excess of CHD.

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