review

Plasma cholesteryl ester transfer protein

Alan R. Tall

Division of Molecular Medicine, Department of Medicine, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032

INTRODUCTION

The plasma lipoproteins are continuously remodeled during their transit through the plasma compartment, owing to the action of lipid metabolizing enzymes and lipid transfer processes. These activities have a major effect on the composition, size, and concentration of the lipoproteins. Lipid transfer processes involving neutral lipids and phospholipids are mediated by specialized plasma proteins, called lipid transfer proteins. The plasma lipid transfer proteins include the cholesteryl ester transfer protein (CETP) with specificity for both neutral lipids and phospholipids, as well as a phospholipid transfer protein (PTP). The plasma lipid transfer proteins are analogous to the cellular lipid transfer proteins, which mediate the transfer of lipids between organelles (1) or from sites of intracellular synthesis into nascent lipoproteins (2). The importance of lipid transfer proteins in lipoprotein metabolism and cellular processes has recently been confirmed by the discovery of genetic deficiency states causing remarkable phenotypes (3–5). Lipid transfer particles, which mediate the movement of diglycerides between lipophorins, are also present in the hemolymph of insects (6). This review will focus on the plasma cholesteryl ester transfer protein. There have been several recent excellent reviews on plasma lipid transfer activities (7, 8).

Since this topic was last reviewed in these pages (9), there has been considerable progress in understanding the biochemistry and properties of the plasma CETP. The CETP has been purified and cloned and the gene structure has been determined. The role of CETP in lipoprotein physiology has been illuminated by the availability of CETP neutralizing antibodies and the elucidation of human genetic CETP deficiency. CETP monoclonal antibodies have also been used to probe the structure–function relationships of CETP, although much remains to be learned in this area. Other areas for future investigation will include: *1)* the complex relationship of CETP to reverse cholesterol transport and atherogenesis; *2)* the apparently pleiotropic functions of CETP in different cells and tissues, such as adipocytes, macrophages and lymphocytes; *3)* the mechanisms of regulation of CETP gene expression by cholesterol and other factors; *4)* the function of CETP-like genes in species without plasma CETP activity; and *5)* the biochemical characterization and function of plasma phospholipid transfer proteins and CETP protein inhibitors.

MOLECULAR CHARACTERIZATION OF PLASMA CHOLESTERYL ESTER TRANSFER PROTEIN (CETP)

Plasma cholesteryl ester transfer protein (CETP) is a hydrophobic glycoprotein which appears as a broad band of M_r approximately 66,000 to 74,000 in SDS polyacrylamide gels (10–12). CETP purified from human plasma was found to be a single polypeptide species, with a unique N-terminal sequence and an unusually high content of hydrophobic amino acids (about 44%), compared to other water soluble proteins or apolipoproteins (apo) (11). The cloning of the cDNA of CETP revealed a novel 476 amino acid sequence, with a high content of hydrophobic amino acid residues scattered throughout the sequence and four potential N-linked glycosylation sites (13). The CETP displays cholesteryl ester, triglyceride, retinyl ester, and phospholipid transfer activities, and is responsible for all of the neutral lipid transfer activity of human plasma but only part of the phospholipid transfer activity (11, 14). Thus, patients with complete deficiency of CETP, caused by a CETP gene-splicing defect, have no measurable plasma cholesteryl ester or

Abbreviations: CETP, cholesteryl ester transfer protein; SDS, sodium dodecyl sulfate; PTP, phospholipid transfer protein; BPI, bactericidal permeability increasing protein; LBP, lipopolysaccharide binding protein; apo, apolipoprotein; LRP, LDL receptor-related protein; LPL, lipoprotein lipase; LC, low cholesterol; HC, high cholesterol; TC, total cholesterol; C, cholesterol; ChRE, cholesterol response element; HDL, high density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; CE, cholesteryl ester; TG, triglyceride; PC, phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; ACAT, acyl-CoA:cholesterol acyltransferase; NMR, nuclear magnetic resonance; RFLP, restriction fragment length polymorphism; NS, nephrotic syndrome; ER, endoplasmic reticulum; CETA, cholesteryl ester transferase activity; mAb, monoclonal antibody; RCT, reverse cholesterol transport.

triglyceride transfer activities, but have plasma phospholipid transfer activity at about 50% of the normal level (15).

PLASMA PHOSPHOLIPID TRANSFER PROTEIN

A plasma phospholipid transfer protein (PTP) has been separated from the plasma CETP, partially purified, and characterized (10, 16, 17). Distinctive functions of the CETP and PTP are suggested by the finding that only the PTP would mediate net transfer of phospholipids from vesicles into HDL (16). CETP was not active in this assay but did mediate exchange of phospholipids between HDL and LDL. Albers et al. (10) have designated the plasma CETP as LTP-I and the phospholipid transfer protein as LTP-II. Proteins of different M_r have been identified in the most purified fraction containing phospholipid transfer activity. In a recent report LTP-II was described as a protein of M_r 70,000 with a pI of 5.0 (17), which is remarkably similar to the M_r and pI of CETP. One of the functions of the phospholipid transfer protein could be to facilitate the net transfer of phospholipid from triglyceride-rich lipoproteins into HDL during lipolysis (18). Phospholipid-enriched HDL is a better substrate for CETP (9), indicating that there could be a synergistic interaction between the two lipid transfer proteins.

POST-TRANSLATIONAL MODIFICATIONS OF CETP

The difference in molecular weight between the polypeptide encoded by the CETP cDNA (53,000) and CETP purified from plasma (74,000) indicates significant post-translational modification. The existence of a closely spaced protein doublet in high resolution gels of purified plasma CETP also indicates micro-heterogeneity (19); a similar doublet exists in recombinant CETP, suggesting that the micro-heterogeneity reflects post-translational modification (20). Several isoforms of CETP, due to variable sialation, can be shown by isoelectric focussing. A plasma neuraminidase activity that co-purifies with CETP may produce heterogeneity during storage (21); however, variable sialation does not account for the two different M_r forms of CETP (20).

Recent mutagenesis experiments have elucidated the nature of the two distinctive M_r forms of plasma and recombinant CETP (20). Metabolic labeling and site-directed mutagenesis studies show that each of the four potential N-linked sites of CETP is occupied by carbohydrate, but N-linked sugar at the third site from the amino terminus [at asparagine 341 (13)] is relatively resistant to removal by glycohydrolase. This glycosylation site is variably used in recombinant and plasma CETP, producing two forms of CETP which either do or do not contain sugar at this site. The variable use of the this glycosylation site accounts for the doublet present in plasma and recombinant CETP (20). Both forms of CETP are active in cholesteryl ester transfer but the form without carbohydrate at this site has about 40% higher specific activity. The different glycosylation of the two forms could result in subtle differences in metabolic activity or in turnover rate of plasma CETP. However, in normolipidemic male and female subjects, the ratio of the two M_r forms of CETP appears to be invariant.

HUMAN CETP cDNA AND GENE STRUCTURES; HOMOLOGY BETWEEN SPECIES AND TO OTHER cDNAs

The CETP cDNA has been cloned from human and rabbit (13, 22) and a partial cDNA from hamster (23). Within the protein encoding region, the rabbit and hamster cDNAs each show about 80% homology to each other and to the human cDNA on a nucleotide and amino acid level, but the nucleotide sequences are divergent within the 3' untranslated region. The sequence of the CETP cDNA showed no general relationship to the coding sequences of the plasma apolipoproteins, enzymes of lipid metabolism or to cellular lipid transfer proteins (13).

The human CETP gene exists in a single copy, comprising 16 exons and encompassing about 25 kbp of genomic DNA (24). The CETP gene is situated on the long arm of chromosome 16 (16 q 12–21 region), near the LCAT gene locus (25). Although the CETP cDNA sequence shows no general homology to the apolipoprotein cDNAs, there is a conserved pentapeptide (val-leu-thr-leu-ala) within the signal sequences of human CETP, lipoprotein lipase, apolipoprotein A-I, and apolipoprotein A-IV (24). This homology is particularly striking since these proteins do not show homology elsewhere in their sequences, and since this sequence is not found in signal peptides of other proteins. The conservation of the pentapeptide within the signal sequence suggests that this sequence may have a common conserved function perhaps related to translational or post-translational regulation of expression.

A weak but general homology of CETP to proteins that bind lipopolysaccharides was recently discovered (26, 27). Two lipopolysaccharide binding proteins, the bactericidal permeability increasing protein (BPI) found in leukocyte granules and the plasma lipopolysaccharide binding protein (LBP), show homology to CETP throughout their sequences. Sequence alignments of CETP and LBP indicate that about 23% of the amino acid residues are identical and another 23% of the residues are conservative substitutions. LBP and BPI belong to an ancient family of proteins that offer protection against gram negative organisms by binding the lipid A moiety of lipopolysaccharide (28). This family also includes the limulus proteins of the horseshoe crab. CETP, BPI, and

LBP have a similar disposition of charged amino acid residues within their sequences, with a predominance of basic residues in the N-terminal half and of acidic residues in the C-terminal half (26). In BPI the basic residues of the N-terminal portion are thought to bind the negatively charged lipid A phosphate groups, while the C-terminal, more hydrophobic portion, anchors BPI to the membrane of neutrophil granules. These structure–function features suggest an analogy with CETP. The binding of CETP to lipoproteins is thought to involve ionic interactions between a subset of positively charged residues on CETP and the negative charges of phosphate groups of the lipoprotein phospholipids (29). Also, CETP has a hydrophobic C-terminal domain containing a neutral lipid binding site (30). Thus, the lipid transfer properties of CETP may have evolved from an ancestral lipopolysaccharide binding protein.

EXPRESSION OF CETP IN DIFFERENT TISSUES AND CELL TYPES

In humans the most abundant sources of the CETP mRNA are liver, spleen, and adipose tissue, with lower levels of expression in small intestine, adrenal, kidney, and heart. The tissue showing the most conserved expression in different species is probably adipose (23). In some species adipose tissue is the most plentiful source of CETP mRNA. For example, hamsters have very low levels of CETP mRNA in liver but abundant CETP mRNA in adipose tissue.

A wide variety of cell types have been reported to secrete cholesteryl ester transfer activity, or protein, or to contain the CETP mRNA (13, 23, 31–36). Appreciable levels of cholesteryl ester transfer activity are present in seminal fluid and in cerebrospinal fluid (37). These findings strongly suggest that CETP influences lipid transfer processes in plasma, interstitial fluids, and within tissues. CETP activity has been found to influence the movement of cholesteryl esters between cells (HepG2 cells and macrophages) and lipoproteins (38, 39). In the case of HepG2 cells, this apparent transfer is due to CETP-mediated transfer of CE from high density lipoprotein (HDL) to low density lipoprotein (LDL) and subsequent uptake by the cell (40). In seminal fluid CETP can enhance sperm capacitation, suggesting that it modulates membrane composition.

Although a variety of cell types have the capacity to secrete CETP or contain CETP mRNA, the physiological significance of some of these observations is uncertain. HepG2 cells, CaCo2 cells, macrophages, adipocytes, and neuroblastoma cells have all been reported to secrete cholesteryl ester transfer activity (13, 23, 31–36). However, in each case the levels of activity secreted are extremely low. HepG2 cells and CaCo2 cells contain authentic CETP mRNA, as determined by RNAse protection assay, but the abundance is less than 1/10th of that of the native tissue (Quinet, E., and A. Tall, unpublished results). In human and hamster spleen the major source of the CETP mRNA appears to be not macrophages but lymphocytes, particularly B-cells (Quinet, E., and A. Tall, unpublished results). However, peripheral blood lymphocytes contain no detectable CETP mRNA. Although adipocytes contain the CETP mRNA in adipose tissue (23), transformed lines of human or murine adipocytes do not contain detectable CETP mRNA.

The low levels of secreted activity or mRNA suggest that transformed cell lines or long-term primary cell cultures (of adipocytes, lymphocytes, or hepatocytes) have much lower levels of CETP expression than the same cells in native organs. This finding may, in part, be explained by the recent discovery that the CETP gene promoter contains a binding site for the transcription factor C/EBP; this factor binds specifically to site A in the proximal CETP gene promoter and transactivates the CETP gene (41). C/EBP is abundant in fully differentiated tissues such as hepatocytes of liver lobules but is present at very low levels in transformed cells (such as HepG2 cells), and disappears rapidly after cell isolation.

There is controversy concerning the major cell type containing the CETP mRNA in liver. This has been attributed both to hepatocytes (36) or to the non-parenchymal cell fraction (41). The CETP gene promoter is active in HepG2 cells, when a source of C/EBP is provided. Studies of liver parenchymal cells show that the CETP mRNA is rapidly lost following cell isolation, in parallel with the disappearance of C/EBP mRNA (41). Recent studies carried out in human-CETP-transgenic mice show that hepatocytes account for the major part of the CETP mRNA in liver, but the non-parenchymal cell fraction also contains some CETP mRNA (Jiang, X., and A. Tall, unpublished results). In rabbits the opposite appears to be true, i.e., the non-parenchymal cell fraction (primarily macrophages) is the major source of CETP mRNA and the parenchymal cells are the lesser source. Thus, it appears that both parenchymal cells and non-parenchymal cells of liver express CETP mRNA, with major species variation in the relative importance of the different sources. The studies in HepG2 cells and in human-CETP-transgenic mice suggest that in humans hepatocytes are likely to be an important source of CETP.

Hepatic synthesis appears to be the major source of plasma CETP in primates. In monkeys receiving a high fat, high cholesterol diet, there is a strong correlation between plasma CETP mass and the abundance of hepatic CETP mRNA, or the output of CETP in liver perfusates (42). This result contrasts with results obtained for apoA-I, apoB, and apoE in similar studies, where variations in liver mRNA abundance do not appear to determine individual differences in plasma apolipoprotein levels. Adipose tissue mRNA abundance is also correlated with

plasma CETP levels, including results of multivariate analysis, suggesting that adipose tissue may also contribute to plasma CETP. Plasma CETP in hamsters also arises from nonhepatic sources, probably adipose tissue and muscle (23).

REGULATION OF CETP GENE EXPRESSION BY CHOLESTEROL, HORMONES, DRUGS, AND APOE GENOTYPE

Son and Zilversmit (43) discovered that rabbit plasma cholesteryl ester transfer activity is increased in response to a high cholesterol, high fat diet. The increased activity was found to be due to increased CETP mass and to be associated with an increased abundance of CETP mRNA in liver (44). The induction of hepatic CETP mRNA and plasma CETP by an atherogenic diet has been documented in rabbits (44), in cynomolgus and African Green monkeys (42, 45), and in transgenic mice expressing the human CETP gene with its natural flanking sequences (46). Detailed analysis of dietary variables shows that it is the cholesterol component of the diet which is largely responsible for the increase in CETP mRNA and protein.

The CETP mRNA in peripheral tissues is also increased in response to a high cholesterol diet. In hamsters the CETP mRNA present in adipose tissue, heart, and skeletal muscle was induced by increases in dietary cholesterol (23). Similarly, humans fed diets of fixed composition display increases in plasma CETP and in adipose tissue CETP mRNA in response to changes in dietary cholesterol (47). The function of induction of peripheral CETP mRNA by dietary cholesterol is unknown but it could help to recycle cholesterol deposited in adipose tissue and muscle during lipolysis of chylomicrons carrying a load of dietary cholesterol (23). Consistent with an active role of adipose tissue in the metabolism of chylomicron cholesterol, adipocytes synthesize apoE (48)and take up chylomicron remnants in an insulin-stimulated process mediated by the LDL receptor-related protein (LRP) (49). Thus, there appear to be regulated processes for the uptake and disposal of dietary cholesterol in adipose tissue.

Although CETP mRNA is found in many tissues containing lipoprotein lipase (LPL), CETP mRNA in adipose tissue and heart muscle is increased with 12 h fasting, compared to feeding a high carbohydrate diet (23). This regulation tends to be opposite to that of LPL, which shows increased activity with feeding and decreased activity with fasting. In human subjects there is an inverse correlation of plasma CETP concentration and plasma postheparin LPL activity (Brinton, E., and A. Tall, unpublished results), consistent with opposite regulation. Exercise conditioning is associated with decreased plasma CETP concentration in human subjects, which may reflect both decreased adiposity and more effective insulin action. Plasma CETP concentration is inversely related to parameters of effective insulin action (50). These findings suggest that CETP mRNA in adipose and muscle could be decreased during feeding as a result of insulin action. The increase in CETP mRNA during fasting could be linked to the mobilization of fatty acids from the periphery, resulting in lipoprotein synthesis in the liver. CETP induction during fasting could help to mobilize cholesterol from adipose tissue, for use in hepatic lipoprotein synthesis.

Plasma CETP is also altered by steroid hormone and drug stimuli. Plasma CETP activity is decreased as plasma cholesterol declines during pregnancy in the rabbit; however, plasma CETP is increased during the third trimester of human pregnancy, suggesting an effect of sex hormones on CETP expression (51). Corticosteroid therapy is associated with a reduction of plasma CETP in normal subjects and in patients with nephrotic syndrome (52). Parallel studies in CETP transgenic mice show that corticosteroid therapy leads to a decrease in plasma CETP concentration and a marked reduction of CETP mRNA in liver (Moulin, P., L. Masucci, and A. Tall, unpublished results). Probucol therapy is associated with pronounced increases in plasma CETP (53–55). Since probucol is known to accumulate in adipose tissue, probucol might influence the CETP mRNA abundance in adipose tissue. Recent studies suggest that probucol may increase CETP mRNA in adipose tissue in hamsters, but only when the animals were fed a high cholesterol diet (55). Unexpectedly, probucol lowered CETP mRNA in human adipose tissue. The effect of probucol on CETP mRNA in cells may be conditioned by the cell's cholesterol pools.

Only limited information is available on the regulation of CETP expression in cultured cells, owing to the very low expression of CETP mRNA and protein in transformed cell lines. However, Faust and Albers (33) showed that oriented CaCo-2 cells (grown on filters) secreted CETP in a vectorial manner from the basolateral membrane. CaCo-2 enterocytes but not HepG2 cells showed a 3-fold increase in CETP secretion when treated with fatty acid complexed to albumin. Furthermore, differentiation of human monocytes into macrophages was associated with an increased secretion of cholesteryl ester transfer activity (35). Cholesterol loading of those cells, by exposure to acetylated LDL or free cholesterol, resulted in a pronounced increase in the secretion of cholesteryl ester transfer activity. Thus, several lines of evidence indicate that a variety of cell types respond to an increase in cholesterol load by increasing CETP secretion.

Martin et al. (47) have determined the effects of apoE genotype on the plasma lipoprotein and CETP responses to cholesterol feeding in young normal male subjects under rigorously controlled dietary conditions. Two diets, differing only in cholesterol content (low cholesterol (LC):80 mg cholesterol/1000 kcal and high cholesterol (HC):320 mg cholesterol/1000 kcal), were compared using

a random crossover design. At the end of the HC as compared to the LC period, total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and HDL_2-C increased by an average of 15%, 21%, 7%, and 23%, respectively, for the three genotype groups combined ($P < 0.001$ for each). The increase in HDL-C varied significantly according to the apoE genotype (E 3/2: 0 change, E 3/3: +4%, E 4/3: +12%; $P < 0.05$). The plasma cholesteryl ester transfer protein (CETP) response to cholesterol feeding also differed amongst the three apoE genotype groups (E 3/2: +37%, E 3/3: +18%, E 4/3: +9%) ($P < .05$). Thus, apoE genotype has significant and opposite effects on plasma CETP and HDL-C responses to dietary cholesterol in humans. One explanation for the effect of apoE genotype on CETP levels is that CETP might be catabolized in association with remnant particles.

REGULATION OF CETP GENE EXPRESSION—STUDIES IN TRANSGENIC MICE

Recent experiments have examined the regulation of CETP gene expression in transgenic mice (46). In these studies a human CETP minigene linked to the natural flanking sequences of the human CETP gene (3.2 kbp upstream and 2 kbp downstream) was used to prepare several lines of transgenic mice, and the expression of CETP mRNA in tissues was evaluated. All lines of transgenic mice expressed authentic human CETP in plasma. Most of the lines showed major expression of the CETP mRNA in liver, spleen, adipose tissue, and small intestine, suggesting that the natural flanking sequences or the minigene contain sufficient information to permit tissue selective expression similar to that of the native human gene. In response to a high cholesterol diet, there was a marked (4- to 10-fold) induction of hepatic CETP mRNA in the different lines of mice, an increased rate of transcription of the CETP gene, and a 3- to 4-fold increase in plasma CETP mass and activity. In contrast to these results, transgenic mice in which the expression of the CETP minigene was controlled by the metallothionein promoter showed no induction of CETP mRNA or plasma CETP mass or activity in response to a high cholesterol diet. The results suggest that the increase in hepatic CETP mRNA and plasma CETP induced by a high cholesterol diet is entirely mediated by increased transcription of the CETP gene. The increase in gene transcription requires the natural flanking sequences of the human CETP gene, suggesting that these regions contain a novel cholesterol response element (ChRE) that mediates increases in gene transcription in response to alteration in dietary cholesterol.

ALTERNATIVE SPLICING OF CETP mRNA

An alternatively spliced variant of CETP was discovered while cloning the cDNA from adipose tissue (56). In this form of the CETP gene transcript, exon 8 sequences are directly joined to exon 10 sequences, resulting in the precise removal of exon 9-derived sequences. The transcript appears to be otherwise identical to the full-length CETP cDNA. The alternatively spliced CETP mRNA is present in all human tissues containing the CETP mRNA, but its abundance varies from about 20% of total CETP mRNA in liver to 40–60% in spleen. Exon 9 contains 180 nucleotides within the amino acid encoding sequence of the CETP mRNA. The splicing of exon 9 results in the removal of 60 amino acids from the CETP sequence, without alteration of the translational reading frame. Expression of an exon 9-deleted cDNA leads to the synthesis of a smaller protein. This is poorly secreted by Cos cells and CHO cells, and is inactive in cell lysates using standard lipid transfer assays. The smaller protein is absent from plasma, but may be detected in homogenates of human spleen. The function of alternative splicing of the CETP mRNA is unknown. In general, the function of alternative splicing of genes is either to act as a regulatory switch (for example, the splicing-regulated on-off switch which underlies sex determination) or to produce isoproteins that have specialized functions in different tissues and/or at different stages of development (57). The failure of cells to secrete the exon 9-deleted protein suggests that alternative splicing of the CETP mRNA could help to control the amount of active CETP secreted by cells. In the default mode, exon 9-derived sequences would be omitted and the resulting protein would be retained and degraded in the endoplasmic reticulum (ER). In response to certain unknown stimuli (e.g., a cytokine or hormone or sterol), the full-length form of CETP would be synthesized and secreted by the cells.

MECHANISM OF NEUTRAL LIPID TRANSFER BY CETP

The plasma CETP can mediate the transfer or exchange of a variety of hydrophobic lipids among plasma lipoproteins. Morton and Zilversmit (58) showed that CETP can mediate the net transfer of neutral lipids by stimulating the hetero-exchange of cholesteryl esters (CE) and triglycerides (TG). For example, by mediating the exchange of cholesteryl esters in HDL for triglycerides in very low density lipoprotein (VLDL), CETP stimulates the net transfer of CE into VLDL, and of TG into HDL. This hetero-exchange mechanism accounts for the approximately equimolar exchange of VLDL triglyceride for HDL cholesteryl ester in incubated plasma (59).

However, CETP can also promote net transfer of CE without an equimolar back-transfer of triglycerides. For example, purified CETP was found to transfer CE from HDL to a triglyceride/phospholipid emulsion with only one-tenth the molar back-transfer of triglyceride from the emulsion to HDL (11). Recently, Barter and colleagues (60) and Clay, Newnham, and Barter (61) have shown that enrichment of HDL with fatty acids (by direct addition, or as a result of the activity of hepatic lipase) stimulates net transfer of CE from HDL to triglyceride-rich particles without concomitant back-transfer of triglycerides.

Kinetic studies performed with partially purified CETP have yielded results consistent with either a carrier-mediated mechanism of transfer (62) or one involving the formation of a ternary collision complex, consisting of CETP and donor and acceptor lipoproteins (63). Consistent with a carrier-mediated mechanism, Swenson, Brocia, and Tall (64) showed that CETP has binding sites for neutral lipids and phospholipids. Either CE or TG was rapidly transferred to CETP from unilamellar vesicles of egg phosphatidylcholine (PC). The CETP with bound lipids was isolated and incubated with LDL, resulting in the transfer of lipids to LDL. The CETP bound up to 0.9 mol CE or 0.2 mol TG and 11 mol PC/mol as determined by nonequilibrium gel filtration chromatography. Under various conditions, the CETP was isolated either as an apparent monomer with bound lipid or in complexes with vesicles. These results indicated that CETP has binding sites for CE, triglyceride, and PC that readily equilibrate with lipoprotein lipids and suggest that the CETP can act as a carrier of lipid between lipoproteins.

In human plasma most of the CETP is found associated with HDL particles (29, 65). Since diffusion is inversely proportional to the Stokes radius of a particle and since the Stokes radii of CETP or HDL-CETP would only be different by a factor of 2 to 4, the aqueous diffusion rates of free CETP or HDL-bound CETP are quite similar. The diffusion of either free CETP with bound lipid or HDL-associated CETP with bound lipid could both be considered as carrier-mediated mechanisms of lipid transfer. A distinctive mechanism would be one in which CETP mediates fusion between the surface phospholipid monolayers of different lipoprotein particles leading to direct mixing of their contents. At the moment the available experimental evidence does not unequivocally distinguish between these possibilities. However, a CETP mutant with impaired neutral lipid transfer, but normal phospholipid transfer activity, suggests that neutral and phospholipid transfer activities are mediated by different domains of CETP, tending to exclude a general fusion mechanism (66).

Three potential mechanisms of lipid transfer by CETP are illustrated in **Fig. 1.** All mechanisms show lipid binding sites on CETP as intermediates in the transfer process. The first mechanism features CETP acting as a free carrier of lipids between the lipoproteins. The second involves a ternary complex; according to the scheme shown, CETP with bound lipid diffuses with HDL and mediates lipid transfer during the formation of a transient ternary complex. The third scheme is similar to the second but includes a dimer of CETP. CETP does form dimers, but there is presently little evidence to indicate that dimer formation is necessary for the transfer mechanism.

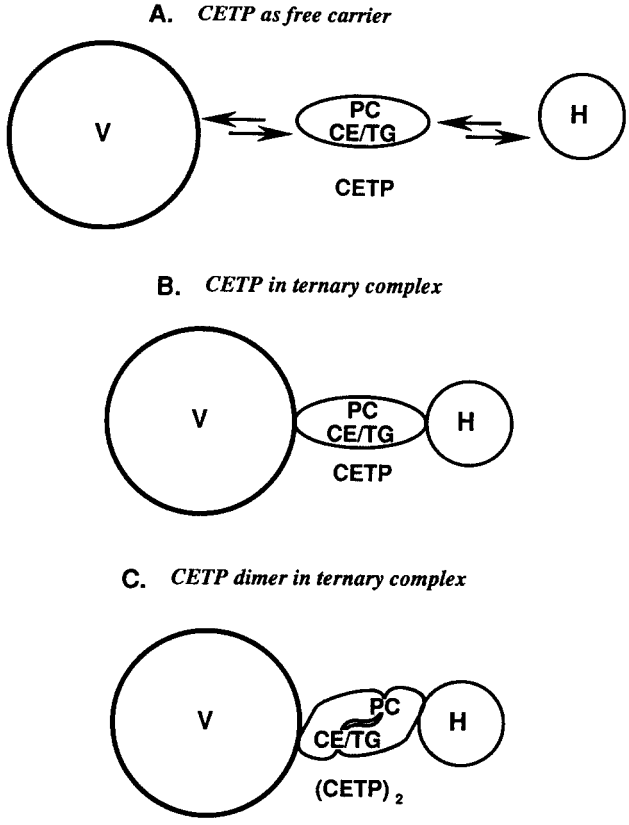

Fig. 1. Three potential models of lipid transfer by CETP. A: CETP is shown acting as a free carrier of lipid between donor lipoprotein (H, HDL) and acceptor lipoprotein (V, VLDL). CETP binds to the donor, takes up lipid (PC and CE or TG), dissociates from the donor, diffuses, collides with the acceptor (V, VLDL), binds to the acceptor, exchanges its bound lipids with the acceptor, dissociates from the acceptor, etc. Some of the factors influencing the efficiency of transfer are the K_d values for binding to donor and acceptor lipoproteins and the availability of lipids in the surface of the lipoproteins. B: The transfer mechanism involves a ternary complex of donor and acceptor lipoproteins and CETP. The CETP may initially bind the donor, take up lipids from the donor, diffuse while still bound to the donor, bind the acceptor, then mediate lipid transfer in the ternary complex. It is likely that many lipid transfer events could occur in the ternary complex, i.e., CETP acts as a hydrophobic bridge between donor and acceptor. This model implies separate and possibly distinct binding sites on CETP for donor and acceptor lipoproteins. C: Transfer also occurs in a ternary complex but it involves a dimer of CETP. The dimer could help to form a lipid binding site on CETP, or it could help to mediate the ternary complex. Presently, there is little experimental evidence to indicate that dimer formation is necessary for lipid transfer. All of the suggested mechanisms involve intermediary lipid binding sites on CETP. It is suggested that CE and TG share a flexible hydrophobic lipid binding site involving the C-terminus of CETP, while PC binds to different sites on CETP.

STRUCTURE-FUNCTION RELATIONSHIPS OF THE CETP MOLECULE

Three approaches have been taken to define regions of the CETP molecule that are important in function: limited digestion with proteases, linker insertion scanning mutagenesis, and production and epitope analysis of CETP monoclonal antibodies.

In an attempt to define an active domain of the protein, fragments of CETP were obtained by limited digestion of the native, plasma-derived protein with various proteases (67). Although proteolysis resulted in extensive fragmentation of CETP and loss of the intact 74 kDa molecule as shown by SDS-PAGE, CE transfer activity was well maintained. Analysis by molecular sieve chromatography showed that the CE transfer-active products of this proteolysis consisted of polypeptide fragments that remained associated, retaining the native molecular weight of CETP. These proteolyzed complexes were resistant to dissociation by dithiothreitol, 8 M urea, or delipidating agents. No evidence was found for an independently active subunit of CETP. Together, the results indicated that CETP possesses a distinct and highly stable tertiary structure that is required for CE transfer catalytic activity. Another approach to identifying sites in CETP necessary for activity used linker insertion scanning mutagenesis (68). The majority of the mutant proteins had relatively normal cholesteryl ester transfer activity, indicating considerable tolerance to local structural perturbation of the polypeptide backbone, similar to the conclusion suggested by the protease studies (67). Selected insertions in three regions were found to impair CE transfer activity. These insertions probably alter the local secondary structure, in regions important for CETP structure or function.

The third and most informative approach to the analysis of structure-function relationships of CETP has been the production and study of CETP monoclonal antibodies (mAbs) (67). The epitope of the neutralizing CETP mAb TP2 was localized to the C terminal 26 amino acids of CETP (30). Two other CETP mAbs, TP6 and TP14, which do not inhibit neutral lipid transfer activity, have different non-C-terminal epitopes. Fab fragments of the neutralizing mAb caused parallel inhibition of neutral lipid transfer activity and of the binding of neutral lipid to CETP, suggesting that the TP2 mAb inhibits lipid transfer by somehow blocking the uptake of lipid by CETP. The simplest interpretation of these results is that the epitope of the neutralizing mAb comprises a region of CETP that is in, or near, or required for entry into the neutral lipid binding site(s) of CETP. A monoclonal antibody has been described that neutralizes TG but not CE transfer activity of CETP (69). However, these properties resemble those of Fab fragments of mAb TP2 (30), and could be due to the larger size of the TG molecule, without requiring the conclusion that CE and TG bind to different sites on CETP. Site-directed mutagenesis shows that the C-terminal region of CETP is directly involved in both neutral lipid transfer activities (66).

A surprising and unexpected finding was that the neutralizing mAb TP2 enhanced the binding of CETP to plasma lipoproteins and to phospholipid vesicles (30). This result was particularly striking for the association of CETP with VLDL and LDL, where there was a marked increase in the binding of CETP to these lipoproteins in the presence of the TP2 Fab fragments, and TP2-CETP-VLDL (or LDL) complexes were readily isolated. These results suggested that the binding of TP2 to CETP induces a conformational change causing enhanced interaction between a non-C-terminal portion of the CETP molecule and VLDL or LDL. The ability of the mAb to induce this conformational change could resemble the effect of a natural process involving the C-terminal region of CETP, such as the binding of neutral lipid to CETP.

Deletional and site-directed mutagenesis of the C-terminal region of CETP (476 amino acids) shows that the epitope of TP2 is located between amino acids 463 and 475, and also involves aspartate 460 (66, 70). Deletion of amino acids 465 to 475 gives rise to well-secreted mutant proteins with low CE transfer activity [0–20% of wild type CETP (66)]. The deletion mutant protein (Δ 470–475) had markedly impaired CE and TG transfer activity but normal or increased phospholipid transfer activity depending on the phospholipid. These findings suggest a specific involvement of amino acids 470–475 in neutral lipid transfer, and indicate that impaired activity of the C-terminal deletion mutants does not arise from a general defect, such as decreased binding to lipoprotein or global malfolding. The high level of secretion of this deletion mutant, as well as a similar pattern of partial digestion with proteases compared to wild-type, also suggests that the deletion mutant is normally folded. The C-terminal polypeptide chain may represent a flexible tail, which can be removed without detriment to overall folding of the protein.

Surprisingly, mutation of single amino acids between amino acids 468 to 475 showed that the amino acids necessary for the binding of TP2 are different from those required for CE transfer (70). The amino acids needed for transfer were hydrophobic residues, whereas those needed for binding of TP2 were more hydrophilic or charged. The results can be understood in the light of the predicted secondary structure of this region of CETP. When amino acids 468 to 475 are arranged on an Edmunson helical wheel, it is apparent that the amino acids mediating binding of TP2 are found on the hydrophilic face of the helix, while those necessary for CE transfer are found on the hydrophobic face (**Fig. 2**). Thus, the mAb probably binds to the hydrophilic face of this helical segment of CETP,

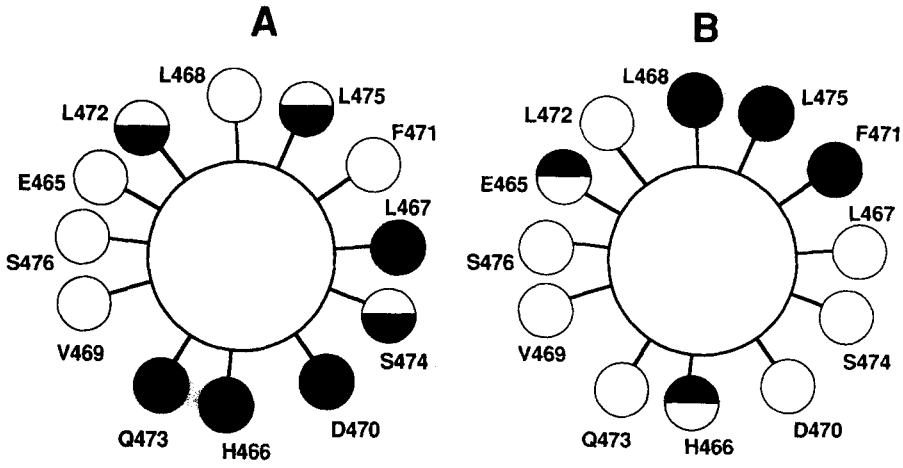

Fig. 2. A helical wheel model of the carboxyl terminus of CETP (from Glu^{465} to Ser^{476}) showing locations of amino acid residues that caused weakening of mAb TP2 binding (A) and decreased CE transfer specific activity (B) when mutated. The side chains of the amino acids are symbolized by the small circles surrounding the helix (large circle in the center) as viewed in cross-section. On the left wheel (A), the fully and half-shaded circles represent the amino acids with TP2 binding < 20% or 21–50% of that of WT CETP, respectively. On the right wheel (B), the fully and semishaded circles represent the mutants with CE transfer specific activity < 41% and 42–80% of that of WT CETP, respectively. For multiple mutants at one particular amino acid residue, only the mutant with lowest TP2 binding (A) or CE transfer activity (B) is represented.

while the hydrophobic face of the helix is directly involved in neutral lipid transfer. The neutralizing antibody may immobilize the flexible helical tail of CETP.

Low activity mutants in the putative C-terminal helix as well as in the contiguous upstream region of CETP all involved the bulky hydrophobic amino acids leucine or phenylalanine (notably L475, F471, L468, F461, and F454) (70). However, most of these mutants showed only partial reductions of activity, showing that it is the general hydrophobic character of the region which is important for function (i.e., an intermediate level of specificity) rather than highly specific side-chain interactions. The hydrophobic face of the C-terminal helix, as well as an adjoining region of hydrophobic amino acids, particularly F454 and F461, may form part of an extensive hydrophobic surface involved in neutral lipid transfer. The precise function of this hydrophobic C-terminal region is unknown. One possibility is that the C-terminal helix embeds in the lipoprotein surface so that the hydrophobic face makes contact with neutral lipid molecules. Movement of the helix could lead to transfer of neutral lipid into a contiguous hydrophobic binding site on CETP. A putative hinge sequence (glycine-phenylalanine-proline) immediately N-terminal of the helix may provide flexibility. The hydrophobic face of the C-terminal helix, as well as the highly hydrophobic sequences upstream of the hinge (especially F454 and F461), may form part of a flexible hydrophobic pocket at the C-terminal of CETP, as suggested in the speculative model of **Fig. 3.**

Au-Young and Fielding (71) have also characterized deletion mutants in the C-terminal region of CETP. The three large deletion mutants (of 26, 48, and 66 amino acids) were all found to be inactive and to have impaired binding to lipoproteins. Since these mutants contained a common sequence phe-leu-leu-leu (amino acids 454 to 457), which is also found in LCAT, BPI, LBP, and several lipid metabolizing enzymes, these authors hypothesized

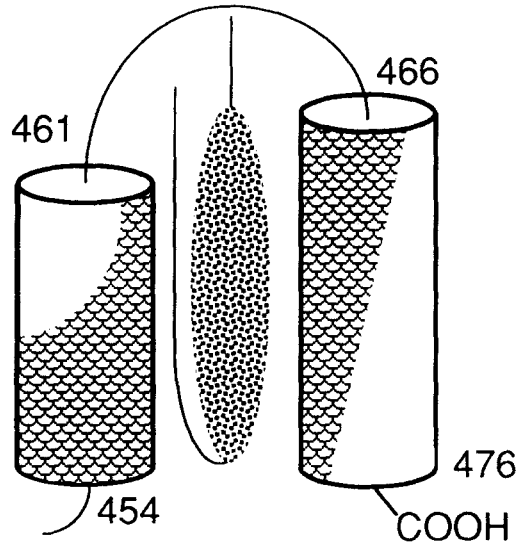

Fig. 3. A speculative mode for how the C-terminal region of CETP might form a neutral lipid binding site. The amphipathic C-terminal helix (amino acids 466 to 476) of CETP is shown with its hydrophobic face in direct contact with a cholesteryl ester molecule. A turn between amino acids 461 to 466 allows the hydrophobic amino acids (454 to 461) to form another face of the hydrophobic surface in contact with cholesteryl ester. The secondary structure of this region could involve an alpha-helix, as shown, or beta-strand. It is highly likely that other parts of the CETP molecule would be involved in forming the hydrophobic surface in contact with neutral lipid. It is suggested that the C-terminal helix forms a mobile segment which makes direct contact with neutral lipid during the lipid transfer process (see text).

that these amino acids might be necessary for normal lipoprotein binding and effective catalysis of neutral lipid transfer. Point mutagenesis of the FLLL sequence does confirm a small reduction in activity for the F:A mutant (70). The importance of the FLLL sequence may be that it is part of a larger hydrophobic surface, as suggested above.

MODULATION OF LIPID TRANSFER ACTIVITY BY ALTERATIONS IN SUBSTRATE LIPOPROTEINS

Lipolysis of VLDL by lipoprotein lipase stimulates the CETP-mediated transfer of CE from HDL to VLDL (16). This phenomenon is related to accumulation of fatty acids in the surface of VLDL, which greatly enhances the binding of CETP to these particles (72). Also, lipolysis increases the binding of CETP to HDL, as a result of enrichment of HDL with phospholipids and fatty acids. HDL isolated from postprandial plasma is phospholipid-enriched and acts as a more efficient substrate for CETP than fasting HDL (9). A similar stimulation of transfer can be produced by the action of hepatic lipase (61) or by the direct addition of fatty acids to the lipoprotein mixture (72, 73). Barter and colleagues (73) have shown that the addition of fatty acids to a mixture of VLDL and HDL enhances unidirectional net transfer of CE from HDL to VLDL, without reciprocal transfer of TG. This promotes the formation of very small HDL particles, probably equivalent to the pre-beta HDL, thought to be involved in the initial step of cholesterol efflux from cells into HDL (74).

The stimulation of CE transfer by lipolysis is an in vitro phenomenon that requires the presence of limiting concentrations of albumin to permit the build-up of fatty acids in the lipoproteins (72). It remains uncertain whether such conditions exist in vivo. However, nonperturbing nuclear magnetic resonance (NMR) methods suggest that fatty acids begin to redistribute from albumin to lipoproteins at fatty acid/albumin molar ratios greater than 1.0, rather than 3.0 as previously thought (75). High ratios of fatty acid/albumin occur in uncontrolled diabetes, nephrosis, and sepsis or after prolonged fasting or exercise (75). In vivo there may be a local increase in concentration of fatty acids in lipoproteins at sites of lipolysis by lipoprotein or hepatic lipase. The major tissues containing lipoprotein lipase (i.e., adipose tissue, heart, and skeletal muscle) are also relatively abundant sources of CETP mRNA in several species. In hamsters CETP mRNA abundance in adipose tissue shows a stronger inverse correlation with HDL levels than plasma CETP concentration, suggesting that CETP that is locally active in adipose tissue may play a more significant role in HDL catabolism than circulating CETP (23). In vivo evidence supporting a role of lipolysis in neutral lipid transfer was obtained in a recent study of the transfer of retinyl palmitate from chylomicrons to other lipoproteins in normal subjects or those with LPL deficiency (76). In subjects with complete or partial LPL deficiency, triglyceride and retinyl palmitate clearance curves were coincident after ingestion of a fatty meal, whereas they were clearly dissociated in normal subjects, suggesting transfer of retinyl palmitate from chylomicrons to the non-chylomicron fraction. The ensemble of information suggests a physiological role for the stimulation of CETP-mediated lipid transfer by products of lipolysis. Accelerated cholesteryl ester transfer between lipoproteins has been shown in insulin-dependent diabetics, perhaps reflecting accumulation of fatty acids in lipoproteins (77, 78).

The enrichment of lipoproteins with unesterified cholesterol may also result in enhanced net CE transfer from HDL to VLDL (79). This process is thought to result from decreased back-transfer of triglyceride from the VLDL to HDL, possibly related to a decrease in the surface concentration of CE in the VLDL surface due to increased free cholesterol. These findings may have relevance to pathophysiological states such as human dysbetalipoproteinemia, where there is build-up of free cholesterol in lipoproteins and accelerated transfer of CE from HDL to triglyceride-rich lipoproteins in some studies (80). In general, the concentration of neutral lipids in the phospholipid surface of substrate particles appears to be an important determinant of lipid transfer activity (81). CETP-mediated lipid transfer between emulsions and LDL is enhanced by the presence of apolipoproteins, such as apoA-I (82). There do not appear to be specific protein co-factors influencing CETP activity. However, it has recently been shown that apoA-II inhibits the production of smaller HDL by CETP (83). Also, Kinoshita et al. (84) have recently shown, by apoE supplementation of the plasma of an apoE-deficient subject, that apoE enhances lipid transfer from HDL to VLDL by enhancing the affinity of apoE for VLDL.

PROTEIN INHIBITORS OF CETP

One or more plasma proteins of M_r 29,000–35,000 inhibit cholesteryl ester transfer mediated by CETP (85). The activity of the protein inhibitor can be overcome by increasing the concentration of lipoprotein substrates, suggesting that the inhibitor acts on lipoproteins and not directly on CETP (86). Consistent with this suggestion, the inhibitor decreases the binding of CETP to HDL (65). The significance of inhibition of lipid transfer by proteins that compete for the lipid–water interface is uncertain, as these properties are also displayed by apoA-I (87). However, baboons with higher levels of HDL may have an HDL-associated protein inhibitor which might be responsible for the lipoprotein phenotype (88). Also, sub-

jects with abetalipoproteinemia have decreased CE transfer activity in the d > 1.21 g/ml fraction, apparently due to the activity of an inhibitor (89). The purification of cholesteryl ester transfer activity from species with low levels of plasma activity has led to the suggestion that inhibitors may mask the expression of CETP in these species (87). However, expression of human CETP in transgenic mice resulted in a plasma protein with specific activity similar to that in human plasma, tending to refute this suggestion (90). Thus, the identity and physiological significance of CETP inhibitors remain uncertain.

ROLE OF CETP IN SIZE CHANGES OF HDL SUBCLASSES

In addition to its role in mediating neutral lipid transfer between lipoproteins, Lagrost et al. (91) have suggested that CETP is an essential component of the HDL "conversion" process, i.e., the conversion of HDL_3 into both larger- and smaller-sized particles upon incubation. The HDL conversion process was induced by the addition of partially purified CETP and blocked by the addition of CETP mAbs. ApoA-IV may interact with CETP to enhance the conversion process (92). Compared to incubations with CETP alone, incubations of CETP and apoA-IV increased the size range of redistribution of HDL_3 particles, particularly by promoting the formation of very small-sized particles (91). This study suggested that CETP can mediate an HDL size conversion even in the absence of lipid transfers between HDL and other lipoprotein fractions. However, a recent report is in conflict with these findings and indicates that the conversion process is mediated by the phospholipid transfer protein and not by CETP (93). The conversion of HDL to smaller sized particles by partially purified CETP is accelerated by the presence of fatty acids (73), a process dependent on both the length and unsaturation of the fatty acid (94). Analogous studies by Kunitake et al. (95) have shown that CETP promotes the movement of apoA-I from alpha-migrating HDL to prebeta-migrating HDL (equivalent to HDL "conversion"), whereas LCAT induces the formation of alpha HDL from prebeta HDL.

SPECIES VARIATION IN CETP

In different species plasma cholesteryl ester transfer activity ranges from none detectable to several times human levels (96). In a broad species comparison, the level of plasma cholesteryl ester transfer activity was found to be correlated with VLDL CE concentration, but not with HDL CE concentration. However, only species deficient in plasma cholesteryl ester transfer activity accumulate appreciable levels of large CE and apoE-enriched HDL (HDL_c) in response to a high cholesterol diet (9). With the exception of the pig, there is a general positive correlation between the susceptibility to atherosclerosis of different species and their plasma CE transfer activity.

Rats and mice with little detectable plasma neutral lipid transfer activity nonetheless have a gene homologous to that of humans and hamsters; rats also may have CETP-like mRNA in adipose tissue (23). The function of the CETP-like gene in species with minimal plasma neutral lipid transfer activity is an enigma. Given the existence of synthetic CETP mutants with phospholipid, but not neutral lipid transfer activity (see above), it is possible that the CETP homolog in rats and mice might be a phospholipid transfer protein. Alternatively, the neutral lipid transfer activity might be restricted to certain tissues, such as adipose tissue.

EFFECTS OF CETP EXPRESSION ON PLASMA LIPOPROTEINS IN TRANSGENIC MICE

In order to evaluate the effects of CETP expression on lipoprotein metabolism, CETP transgenic mice were prepared. A human CETP minigene was placed under the control of a mouse metallothionein-I promoter, resulting in the generation of a line of mice in which basal levels of CETP were similar to those in humans [approx. 2 μg/ml (90)]. With Zn induction of the transgene, plasma CETP concentrations were doubled, thus achieving levels of CETP similar to those in certain human hyperlipidemias or in patients receiving probucol (54, 97). The Zn induction also resulted in a lowering (by 20–30%) of HDL cholesterol, consistent with the concept that increased plasma CETP concentration, such as occurs in certain human hyperlipidemias, can cause lowering of plasma HDL cholesterol (90).

Although the reduction of HDL was significant, it was less than expected, given the large effects on HDL of human genetic CETP deficiency. To evaluate the possibility that mouse HDL is a poor substrate for CETP, CETP transgenic mice were crossed with human apoA-I transgenic mice. The offspring bearing both human apoA-I and human CETP displayed much more pronounced reductions of HDL cholesterol than animals expressing CETP alone, despite similar levels of plasma CETP activity (98). There was also a much more pronounced decrease in HDL size in animals expressing both human genes. Western blotting analysis of nondenaturing gels of plasma revealed that only about 20% of CETP was HDL-associated in the CETP transgenic mice, whereas 100% was HDL-associated in the human apoA-I/CETP transgenic animals. Thus, CETP has a much more profound effect on HDL cholesterol in transgenic animals expressing human apoA-I. This may be due to an enhanced interaction of CETP with human apoA-I compared to mouse apoA-I or with the HDL particles produced by human apoA-I. It is known that expression of human apoA-I

in mice leads to the formation of distinct HDL_2 and HDL_3 subspecies resembling that in humans (99).

Marotti et al. (100) have also reported the plasma lipoprotein profile of CETP transgenic mice. In general, effects on HDL were similar to those reported by Agellon et al. (90) and Hayek et al. (98). However, an additional effect of CETP expression to markedly increase plasma cholesterol, VLDL plus LDL cholesterol, and apoB levels was found (100). The reason for these different results may be related to the much higher level of expression of CETP, the expression of monkey CETP, or the different genetic background of the animals (100). Groener, van Gent, and van Tol (101) found decreases in HDL and also increases in VLDL and LDL cholesterol and apoB when partially purified CETP was injected to give high levels of CETP activity (to 1.5- to 4.0-fold human levels) in rats. The increase in apoB implies an effect of high level CETP expression on apoB-particle numbers. CETP levels are strongly correlated with LDL cholesterol and apoB levels in monkeys fed atherogenic diets (44). Monkeys have CETP levels about 10 × higher than humans. These results suggest that high level CETP expression may result in increases in VLDL and LDL cholesterol and in apoB.

HUMAN GENETIC CETP DEFICIENCY

Koizumi et al. (102) first described a patient with very high levels of plasma HDL and very low plasma cholesteryl ester transfer activity. Using two different CETP mAbs, Brown et al. (3) showed that the plasma of this subject contained no detectable CETP. Analysis of the proband's CETP gene revealed that he was homozygous for a point (G–A) mutation at the first position of the fourteenth intron. The first nucleotide of a normal intron is never an A. Mutations of the strictly conserved G at this position prevent normal splicing and consequently there is no formation of normal mRNA or protein. In some cases splice site mutations lead to activation of nearby cryptic splice sites or use of an alternative splice site (exon skipping), resulting in an abnormal transcript and protein. No CETP is detected in plasma, using antibodies recognizing CETP sequences derived from transcript on either side of intron 14. Thus, even if an alternative splice site is used or an exon is skipped in the mutant gene, no stable CETP polypeptide appears in plasma.

To assess the frequency and phenotype of CETP deficiency due to this gene splicing defect, we screened 11 additional families with high HDL levels and found the same CETP gene mutation in four families from three different regions of Japan (103). Analysis of restriction fragment length polymorphism (RFLP) of the mutant CETP allele showed that all probands were homozygous for the identical haplotype. Family members homozygous for CETP deficiency (n = 10) had moderate hypercholesterolemia and markedly increased levels of HDL cholesterol (100–250 mg/dl) and apoA-I (147–282 mg/dl). They also tended to have decreased levels of LDL cholesterol (35–150 mg/dl) and apoB (31–73 mg/dl). Members heterozygous for the deficiency (n = 20), whose CETP levels were in the lower part of the normal range, had moderately increased levels of HDL cholesterol and apoA-I and a more marked increased ratio of HDL_2/HDL_3 as compared to unaffected family members. Correlational analysis revealed a strong inverse relationship between the ratio HDL_2/total HDL and plasma CETP concentration, a positive correlation between CETP and LDL cholesterol levels (also found with homozygotes excluded), and a positive correlation between CETP and apoB levels. Even though several homozygotes were moderately hypertriglyceridemic and plasma triglycerides spanned a wide range, there was no inverse correlation between plasma triglycerides and HDL cholesterol levels amongst homozygotes. By contrast, the usual inverse relationship was observed in heterozygotes and unaffected family members.

More recently, VLDL, IDL, and LDL were analyzed in further detail in families with CETP deficiency (104). These studies showed that CE levels in intermediate density lipoprotein (IDL) were lower in homozygotes than in controls. Furthermore, the CE/TG molar ratio in both IDL and VLDL showed a graded reduction comparing homozygotes –heterozygotes–unaffected family members (0.19, 0.38, 0.57 for VLDL; 0.31, 1.1, 1.83 for IDL). There was a strong positive correlation between plasma CETP and CE/TG ratios in VLDL and IDL.

A detailed analysis of particles found within the LDL density range in CETP deficiency has been reported by several groups. In homozygous CETP deficiency the HDL are markedly enlarged and include particles extending into the LDL size range (105). Thus, in homozygous CETP deficiency the LDL density range contains both an apoE-enriched enlarged HDL (resembling HDL_c) as well as the apoB-containing lipoproteins (105, 106). These apoE-enriched HDL particles were isolated and shown to react efficiently with the LDL receptor of fibroblasts (107). Isolation of the authentic "LDL" by apoB-affinity chromatography showed that it was enriched with triglyceride (105). Also, the apoB-containing particles had an increased ratio of cholesteryl oleate/cholesteryl linoleate, compared to the subject's own HDL or to LDL of normal subjects; in the CETP-deficient subjects this ratio was similar to that of their VLDL and IDL. The results suggest reduced exchange of cholesteryl linoleate-rich, LCAT-derived CE for cholesteryl oleate-rich, ACAT-derived CE in CETP deficiency. The detailed sizing pattern of LDL, as determined by native polyacrylamide gel electrophoresis, has been reported by two groups; a similar pattern was observed in all subjects. The LDL in homozygous CETP deficiency is much more polydisperse than in normal subjects and consists of four or five distinct subpopu-

lations, which include distinct particles both larger and smaller than the LDL subclasses of normal subjects (105, 106).

In summary, CETP deficiency appears to be a fairly frequent cause of increased HDL in the population of Japan, possibly because of a founder effect (103). A recent survey suggests that the mutant CETP allele (with the intron 14 splice donor defect) may be present in about 1% of the Japanese population, and might be commonly observed in subjects with HDL cholesterol > 60 mg/dl (108). In Japan the high frequency of this mutation could have a significant impact on the epidemiology of HDL. To date the gene splicing defect underlying CETP deficiency in the Japanese has not been found in United States subjects (56).

The phenotype of human CETP deficiency shows that CETP plays a role in the metabolism of all lipoprotein classes. The most salient finding is the markedly increased HDL, indicating that CETP plays a major role in the catabolism of HDL cholesteryl esters (3). The secondary elevation of apoA-I shows that apoA-I levels may be regulated by lipid transfer processes, and suggests that apoA-I may be catabolized more slowly from larger HDL particles (3). Turnover studies in a homozygote with CETP deficiency have shown an FCR of 0.13 pools/day for HDL_2 apoA-I, compared to a normal value of 0.26 pools/day (109). The relatively normal levels of apoA-II in CETP deficiency suggest that CETP does not play a role in the catabolism of apoA-II. The accumulation of apoE-enriched HDL_c-like particles within the LDL density range demonstrates that these particles form in appreciable amounts in the absence of CETP. CETP deficiency is also required to maintain normal levels of cholesteryl esters in all classes of apoB-containing lipoproteins. The reduced levels of plasma apoB in some homozygotes suggests that the formation or catabolism of apoB-containing lipoproteins is abnormal in CETP deficiency. The distinctive subspeciation of LDL in homozygous CETP deficiency indicates that the metabolic input into LDL includes four or five distinct kinds of particles which are normally remodeled by CETP to particles with more similar overall CE content and size. In the absence of CETP, LCAT may act on some but not all of the LDL species (e.g., LpB-apoC-I but not LpB), generating large size differences.

Since subjects with heterozygous CETP deficiency for the most part have CETP levels within the lower normal range, a comparison of heterozygotes and control subjects may give some insight into the effects on lipoproteins of CETP variation within the normal range in subjects who do not have genetic CETP deficiency. These comparisons predict weak effects of CETP on total HDL cholesterol and apoA-I levels, but stronger effects on HDL_2/HDL_3 ratio. The heterozygote data also suggest that normal or abnormal variation of CETP is likely to influence the CE/triglyceride ratio of VLDL and IDL. There may also be weaker effects of CETP variation on plasma apoB concentration and the content of CE in LDL.

DISTRIBUTION OF CETP IN HUMAN PLASMA

Analysis of human plasma by gel filtration or apoA-I immunoaffinity chromatography indicates that the major part (> 80%) of CETP is associated with HDL particles (110). Although CETP binds with similar affinity to VLDL, LDL, and HDL, the molar concentration of plasma HDL is much higher than that of plasma VLDL or LDL, explaining the predominant association with HDL in plasma (65). CETP dissociates from lipoproteins during ultracentrifugation.

Francone, Gurakar, and Fielding (111) and Marcel et al. (112) reported the association of CETP with pre-beta migrating HDL. The former report found CETP signals in particles with a size range as wide as that of apoA-I, and found that nearly a third of plasma CETP comigrated with a fraction also containing apoA-I, apoD, and LCAT, identified as LpA-I pre-beta 3 and of a size larger than the major apoA-I lipoproteins. By contrast, the latter report concluded that the major fraction of CETP resided in HDL_3-sized particles. However, since CETP activity per se can lead to formation of pre-beta migrating HDL particles (95), the possibility that CETP becomes associated with pre-beta particles during electrophoresis of plasma needs to be considered. Although the concept of a particle containing both LCAT and CETP activities may seem functionally appealing, the evidence is inconclusive. In fact, Cheung et al. (110) found that the bulk of plasma LCAT and CETP activities was present on different particles, as determined by gel filtration chromatography. Furthermore, inhibition of plasma CETP by neutralizing mAb led to no change in the rate of the plasma LCAT reaction, indicating the lack of a tight coupling of CETP and LCAT activities in normolipidemic plasma (59).

VARIATION OF PLASMA CETP CONCENTRATION IN HUMAN POPULATIONS

By solid phase competitive displacement RIA, plasma CETP concentration was found to be 1.50 ± 0.26 μg/ml in normolipidemic males and 1.92 ± 0.52 μg/ml in normolipidemic females (112). Females have been confirmed to have CETP values about 25% higher than males in subsequent studies of other populations. Correlational analysis of plasma CETP values with lipoprotein concentrations in normolipidemic subjects revealed weak positive correlations between plasma CETP concentration and HDL TG (r = .31), apoA-I (0.38) and apoE (0.43) (112). Studies of plasma CETP concentration in hyperlipi-

demic subjects revealed a small increase in subjects with hypercholesterolemia (+26%) or combined hyperlipidemia (+25%), with no change in subjects with moderate hypertriglyceridemia (97). Plasma CETP concentration was more markedly increased in subjects with dysbetalipoproteinemia (+68%) and severe chylomicronemia (+85%). Similar results were obtained for hyperlipidemic patients attending lipid clinics in Montreal and Leeds. Correlational analysis in both populations showed a strong positive correlation between plasma CETP and total plasma cholesterol (r = 0.52), tending to confirm the earlier observations based on activity measurements (113), and no correlation with HDL-cholesterol or apoB levels. In the Montreal group, which contained more patients with hypertriglyceridemia than the Leeds group, there was a strong positive correlation between plasma CETP concentration and VLDL cholesterol (r = 0.63); in the Leeds group this was not found, but a correlation between plasma CETP and LDL CE was observed (r = 0.37). Also, there was a significant correlation between plasma CETP and apoE concentrations. Dietary treatment of subjects with dysbetalipoproteinemia resulted in a marked decrease in plasma CETP associated with a major rise in HDL-cholesterol levels. Comparison of CETP mass measurements determined directly by radioimmunoassay (RIA) and inferred by isotopic transfer assay (in diluted plasma in the presence of excess substrates) showed strong correlations in normolipidemics (r = 0.86) and hypertriglyceridemics (r = 0.94) and moderate correlations in hypercholesterolemics (r = 0.72), validating the use of transfer assays to approximate CETP mass in most plasma samples, with the possible exception of hypercholesterolemic subjects. Inazu et al. (108) found increases in cholesteryl ester transfer activity (CETA) in lipoprotein-free plasma in heterozygotes and homozygotes with familial hypercholesterolemia. There were positive correlations between CETA and the CE/TG ratio in VLDL and IDL, and inverse correlations with the ratio of HDL cholesterol/apoA-I.

Plasma CETP concentration has been found to be increased (+58%) in patients with nephrotic syndrome (NS) (52). Subjects with nephrotic syndrome have increased levels of VLDL and LDL cholesterol, increased plasma apoB, and reduced HDL cholesterol. They were also found to have an increased cholesteryl ester concentration within VLDL, with a CE/TG ratio about twice that in normolipidemic or hypertriglyceridemic controls. The increase in plasma CETP was proportional to the severity of the NS. Treatment with corticosteroids resulted in normalization of plasma CETP and in the CE/TG ratio of VLDL, without normalization of the other lipid parameters, or of NS itself. Correlational analysis showed a strong positive correlation between plasma CETP concentration and the CE/TG ratio of VLDL + IDL (r = 0.59), and between plasma CETP concentration and plasma apoB levels (r = 0.6). There was no correlation between CETP and HDL cholesterol; however, there was a weak inverse correlation between HDL-2 levels and CETP concentration, and an inverse correlation between plasma CETP concentration and HDL cholesterol in nephrotic subjects with hypertriglyceridemia. Overall, these data in nephrotic patients suggest that increases in CETP are causally related to enrichment of VLDL + IDL with CE, possibly related to increases in apoB, and may have a minor influence on HDL-2 levels and on HDL cholesterol in hypertriglyceridemic subjects.

Mann et al. (114) measured plasma net CE transfer and optimum CETP activity (a measure of CETP mass) in primary hypertriglyceridemia (n = 11) and in normolipidemic subjects (n = 15). The hypertriglyceridemic group showed threefold greater net CE transfer leading to enhanced accumulation of CE in VLDL. In normolipidemia, net CE transfer correlated with VLDL TG. In contrast, net CE transfer in hypertriglyceridemia but not in normolipidemia correlated with CETP activity (mass). That net CE transfer depends on VLDL concentration was confirmed by an increase of net CE transfer in normolipidemic plasma supplemented with purified VLDL. Addition of purified CETP to normolipidemic plasma did not stimulate net CE transfer. In contrast, net CE transfer was enhanced by addition of CETP to both plasma supplemented with VLDL and hypertriglyceridemic plasma. The authors concluded that in normal subjects VLDL concentration determines the rate of net CE transfer. CETP becomes rate limiting as VLDL concentration is increased, i.e., in hypertriglyceridemia. One limitation of this kind of analysis is that in vivo transfer rates may be different from those obtained in vitro. As noted above, results in human genetic CETP deficiency strongly suggest an effect of CETP concentration on VLDL and IDL CE content within the normal range of CETP concentrations (104).

FUNCTION OF CETP IN REVERSE CHOLESTEROL TRANSPORT

The plasma CETP participates in a process of reverse cholesterol transport, i.e., the centripetal movement of cholesterol from peripheral tissues to the liver via the plasma compartment. There is direct evidence that plasma CETP regulates the rate of return of cholesteryl ester from plasma to the liver (24, 101, 115). However, it remains uncertain whether CETP can influence the rate of efflux of cholesterol from tissues in vivo (e.g., by influencing the rate of the LCAT reaction). This process of reverse cholesterol transport and the role of CETP in the overall process is illustrated in **Fig. 4.** Reverse cholesterol transport is considered in three settings: *1)* in the absence of CETP (as in species without CETP activity in plasma or in human genetic CETP deficiency); *2)* in

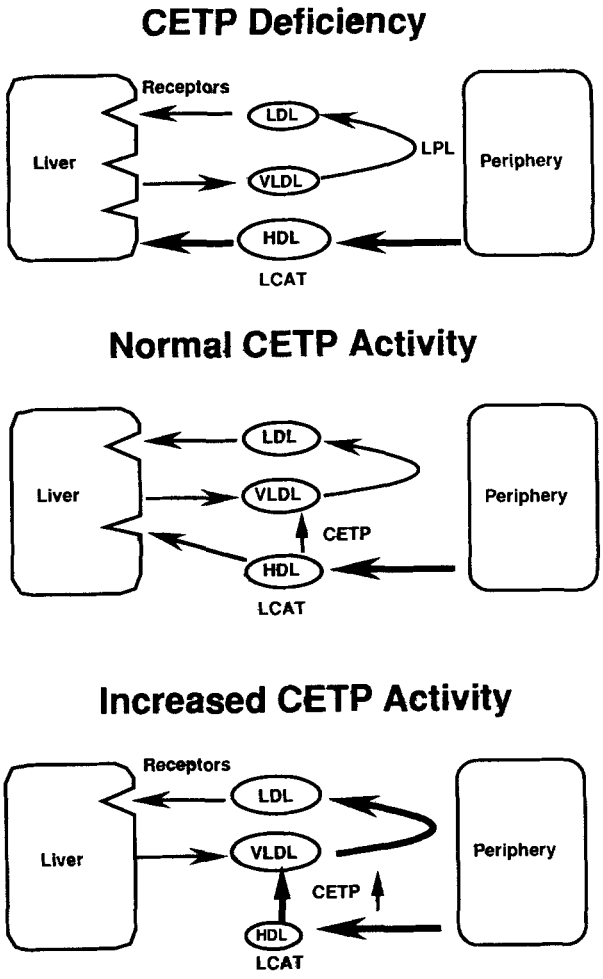

Fig. 4. Models of reverse cholesterol transport (RCT) in three different metabolic settings. A: (Human genetic CETP deficiency or species lacking CETP), RCT occurs primarily through HDL, and involves the clearance of apoE-HDL by LDL receptors in the liver. B: RCT involves both CETP transfer of CE to apoB-containing lipoproteins, as well as some direct clearance of HDL. This may occur in fasting normolipidemic individuals. C: CETP activity is increased (due to an increased concentration of triglyceride-rich lipoproteins and/or an increased concentration of CETP). RCT occurs primarily via the VLDL–LDL pathway. This state may be characterized by low concentrations of HDL and increased CE concentration in VLDL and IDL. See text for further details.

the presence of normal activity of CETP in an animal consuming a low cholesterol diet (normal CETP activity); and *3)* in the presence of increased CETP activity in an animal consuming a high cholesterol diet (increased CETP activity).

Case 1. Reverse cholesterol transport in CETP deficiency. In the initial step dietary or endogenous cholesterol is deposited or synthesized in peripheral tissues. Excessive cholesterol in peripheral tissues is removed by HDL acting in conjunction with LCAT, which provides the driving force for net cholesterol removal. In the absence of CETP and in the presence of continued LCAT activity, HDL particles gradually increase in size and acquire apoE. When sufficient numbers of apoE molecules are obtained, this particle becomes a high affinity ligand for the LDL receptor and is removed by LDL receptors in the liver. In human CETP deficiency this process is relatively inefficient, so that HDL builds up to very high levels. Other processes for removal of HDL lipids may occur, but in humans they appear to be relatively inefficient. The amount of cholesterol transported in VLDL and chylomicron remnants is reduced. The relatively inefficient process of reverse cholesterol transport may be associated with up-regulation of LDL receptors in the liver leading to decreased levels of LpB particles.

Case 2. Reverse cholesterol transport in the presence of normal CETP levels, low cholesterol diet, and normal LDL receptor function. CETP activity results in the transfer of newly synthesized CE from HDL particles into chylomicron and VLDL remnants. Under physiological circumstances much of this transfer may occur during lipolysis, particularly in adipose tissue, heart, and skeletal muscle, due to the stimulation of CETP activity by lipolysis and the local synthesis of CETP in these tissues. The major routes of return of cholesterol to the liver are via chylomicron remnants (involving the chylomicron remnant receptor) and via VLDL remnants and LDL (involving the LDL receptor). Some cholesterol is also returned to the liver in HDL. Relative to CETP deficiency (Case 1), VLDL and LDL carry more cholesterol and HDL carries less cholesterol. However, as long as remnant and LDL removal processes stay normal, VLDL remnants and LDL do not accumulate excessively.

Case 3. Reverse cholesterol transport in the presence of increased CETP activity and a high saturated fat, high cholesterol diet. Under these circumstances, CETP activity becomes increased due to increased CETP gene expression in peripheral and central tissues. Effective CETP activity may also be increased owing to increased concentrations of acceptor lipoproteins (i.e., remnant particles, VLDL and IDL) accumulating in plasma, due to a variety of different metabolic defects, such as overproduction of apoB or impaired removal of remnants. The increased activity of CETP results in an increased rate of transfer of CE from HDL to VLDL and IDL, causing a decrease in concentration of CE in HDL and increased concentration in the acceptors. Increased reverse cholesterol transport occurs through the chylomicron or VLDL remnant pathway. However, LDL receptors are down-regulated due to the excessive deposition of dietary cholesterol in the liver. The down-regulation of LDL receptors may also be augmented by greater rates of return of cholesterol to the liver, as a result of CETP activity. Consequently, cholesteryl ester-enriched LDL particles and VLDL remnants, normally cleared by receptors, accumulate in plasma.

RELATIONSHIP OF CETP TO ATHEROSCLEROSIS

The following evidence suggests an anti-atherogenic effect of CETP deficiency states, and a pro-atherogenic effect of increased CETP activity.

1) The lipoprotein changes of homozygous CETP deficiency (increased HDL, decreased cholesteryl ester in VLDL, IDL, LDL, and decreased apoB) are usually associated with resistance to atherosclerosis. Although genetic CETP deficiency is common in Japan, no documented homozygote has yet been reported with accelerated or obvious atherosclerosis. Anecdotally, some families with CETP deficiency appear to experience longevity (103).

2) The relationship of CETP to atherosclerosis was studied in a group of 28 cynomolgus monkeys fed high fat, high cholesterol diets for 5 years (42). In these animals plasma CETP concentration showed a strong inverse correlation with HDL cholesterol concentration, and a positive correlation with LDL cholesterol concentration and with LDL molecular weight. The extent of coronary artery atherosclerosis was positively correlated with LDL cholesterol concentration ($r = 0.8$) and with plasma CETP concentration ($r = 0.58$). In multiple regression analysis only LDL cholesterol concentration appeared as an independent variable significantly correlated with coronary artery atherosclerosis. Thus, the positive correlation of CETP with atherosclerosis appeared to be due to its relationship with LDL cholesterol concentration. In cynomolgus monkeys the plasma CETP concentration appears to be a major determinant of the atherogenicity of the plasma lipoproteins, probably because CETP transfers CE from non-atherogenic or anti-atherogenic HDL to atherogenic LDL (or its precursors), and also because CETP may influence LDL particle number. Although these relationships could also reflect the activity of a confounding variable, no beneficial effect of CETP on atheroma formation was discerned in this study.

3) In many human dyslipidemias associated with accelerated atherosclerosis, there is an increase in plasma CETP concentration and/or an increase in the rate of net transfer of CE from HDL to apoB-containing lipoproteins in incubated plasma. These include dysbetalipoproteinemia (80), familial hypercholesterolemia (108), nephrotic syndrome (52), hypercholesterolemia (97, 116–118) insulin-dependent diabetes mellitus (77, 78), and peripheral vascular disease (119). Alcoholism, typically associated with increased HDL and decreased atherosclerosis, is associated with diminished CETP concentration (120, 121). Exercise conditioning, associated with increases in HDL and decreases in LDL cholesterol, is associated with decreased CETP concentration (50). An exception to this general trend was the report of blocked CE transfer in incubated plasma in hypercholesterolemia and dysbetalipoproteinemia (122).

4) With the exception of the pig, species with low levels of plasma CETP are resistant to dietary atherosclerosis (9). The introduction of CETP into rats by injection (78) or into mice by transgenesis (90, 100) results in lipoprotein changes normally associated with the development of atherosclerosis.

On the other hand, the following observations suggest that CETP might have an antiatherogenic effect in some settings.

1) CETP and LCAT are key molecules in the process of reverse cholesterol transport. The role of CETP in this process is highlighted by the widespread distribution of CETP mRNA and its induction in central and peripheral tissues by increased dietary cholesterol (13, 23). This suggests that CETP is a highly regulated component of reverse cholesterol transport with local effects in tissues as well as in plasma. Local synthesis of CETP within macrophages or smooth muscle cells of the arterial wall could help to deplete cholesterol from arterial wall foam cells, providing a directly anti-atherogenic action (35). There is some evidence that CETP promotes cellular cholesterol efflux or efflux from vascular interstitium (39, 123, 124). CETP activity may be rate-limiting for LCAT activity on nascent HDL particles (125), such as may be present at sites of lipolysis, or in interstitial fluid. Also, it might promote the formation of small pre-beta HDL which appear to be preferred acceptors of cholesterol from tissues (74). These observations are tempered by the findings that CETP is not rate-limiting for the LCAT reaction in human plasma, and patients with genetic CETP deficiency have normal rates of plasma LCAT (59, 104). Furthermore, they show no signs of accumulation of cholesterol in tissues (as in LCAT deficiency) or in their lipoproteins, i.e., the cholesterol/phospholipid ratios of plasma lipoproteins are normal (104). Finally, even though CETP, LCAT, and HDL mediate reverse cholesterol transport, genetic deficiency states of each of these components show no clear increase in atherosclerosis.

2) Probucol therapy is associated with increased CETP concentration, reduced HDL (54), and reversal of atherosclerosis in animal models. However, probucol also lowers LDL cholesterol and has protective anti-oxidant effects. Thus, it is uncertain that the effects of probucol on CETP are related to its anti-atherogenic properties.

3) A decrease in mean plasma cholesteryl ester transfer activity was found in elderly subjects with angina pectoris, compared to a control group without angina pectoris (126). These potentially important findings need to be confirmed in a larger group, using an immuno-assay for CETP.

In summary, the data suggest that the relationship of CETP and reverse cholesterol transport to atherosclerosis is complex. There may be anti-atherogenic effects due to local synthesis of CETP in cells of the arterial wall, perhaps promoting cholesterol efflux. However, the

lipoprotein changes resulting from CETP activity are typically pro-atherogenic. When remnant lipoproteins accumulate in plasma (as in dysbetalipoproteinemia), CETP may promote decreases in HDL and CE enrichment of remnants. The inhibition of CETP activity or synthesis by drugs would be an interesting experimental approach to the treatment of dyslipidemia and atherosclerosis. Although this strategy would increase HDL and decrease VLDL, IDL, and LDL CE, consequences for atherosclerosis are presently unpredictable. In the future it is likely that further information on the relationship of CETP to atherosclerosis will be obtained from studies of CETP transgenic mice, from the effects of drugs that are CETP inhibitors, and from population-based surveys of CETP in relation to coronary atherosclerosis.

Manuscript received 29 March 1993.

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