ROLES OF PLASMA LIPID TRANSFER PROTEINS IN REVERSE CHOLESTEROL TRANSPORT

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1.　ABSTRACT

Plasma lipid transfer proteins include plasma cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). Plasma CETP facilitates the transfer of cholesteryl ester (CE) from high-density lipoprotein (HDL) to apolipoprotein (apo) B-containing lipoproteins, and is a key protein in reverse cholesterol transport which protects vessel walls from atherosclerosis. The importance of plasma CETP in lipoprotein metabolism was highlighted by the discovery of CETP-deficient subjects with a marked hyperalphalipoproteinemia (HALP). The deficiency of CETP causes various abnormalities in the concentration, composition, and functions of both HDL and low-density lipoprotein (LDL). Although the significance of CETP in terms of atherosclerosis has been controversial, the *in vitro* evidence showed that large CE-rich HDL particles in CETP deficiency are defective in cholesterol efflux. Recent epidemiological studies in Japanese-Americans and in Omagari area where HALP subjects with the intron 14 splicing defect of CETP gene are markedly frequent, have demonstrated an increased incidence of coronary atherosclerosis in CETP-deficient patients. Similarly, scavenger receptor BI (SR-BI) knockout mice show a marked increase in HDL-cholesterol but accelerated atherosclerosis in atherosclerosis-susceptible mice. Thus, CETP deficiency is a state of impaired reverse cholesterol transport which may possibly lead to the development of atherosclerosis.

PLTP transfers phospholipids from triglyceride (TG)-rich lipoproteins to HDL during lipolysis. Human plasma PLTP has a 20% sequence homology to human CETP and human PLTP gene has a marked similarity in the exon-intron organization. Both CETP and PLTP belong to the lipid transfer/lipopolysaccharide binding protein (LBP) gene family, which also includes LBP and bactericidal/permeability-increasing protein (BPI). Although these 4 proteins possess different physiological functions, they share marked biochemical similarities. The
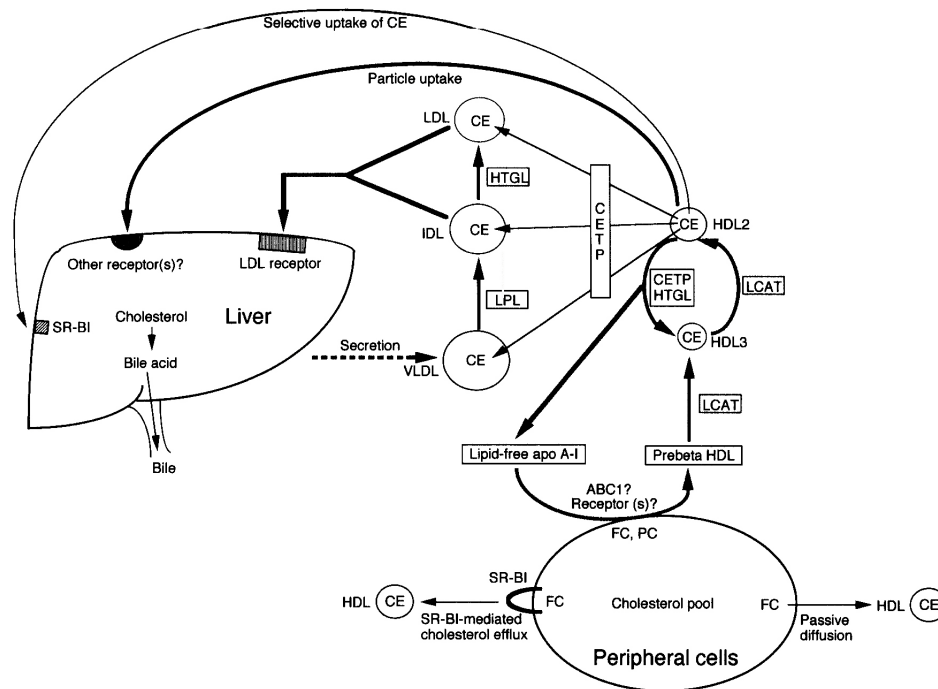
Figure 1: Roles of plasma CETP in the reverse cholesterol transport. CETP is involved in the transfer of cholesteryl ester from HDL particles to apo B-containing lipoproteins. CETP is also involved in the remodeling of HDL particles in conjunction with HL.

current review will also focus on the molecular genetics and function of plasma lipid transfer proteins, including CETP and PLTP.

2. INTRODUCTION

A number of epidemiological studies have shown that serum HDL-cholesterol levels are negatively correlated with the incidence of coronary heart disease (1). Furthermore, patients with a genetic deficiency of HDL are often accompanied by atherosclerotic cardiovascular diseases (2). Thus, HDL plays an essential role in the protection of blood vessels from atherosclerosis. HDL serves as a shuttle, delivering excess cholesterol from peripheral tissues to the liver for excretion into the bile. This pathway was named "reverse cholesterol transport" (3). The interaction between HDL and peripheral cells such as fibroblasts and macrophages is the initial step of reverse cholesterol transport system. The molecular mechanism of this interaction has yet to be clarified, however it is postulated to include aqueous diffusion, scavenger receptor class B type I (SR-BI)-mediated cholesterol flux, and lipid-free apolipoprotein membrane microsolubilization (4-7). The cholesterol picked up by HDL from peripheral tissues is esterified by lecithin:cholesterol acyltransferase (LCAT) to form cholesteryl ester (CE), which is subsequently transferred by plasma CETP to very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL) (8-9). IDL and LDL are catabolized via the LDL receptor in the liver. Furthermore, the CE moiety of HDL is taken up selectively by the liver via SR-BI (10). The HDL becomes enriched with TG after

the CETP-mediated transfer of CE and the TG is hydrolyzed by hepatic lipase (HL). Thus, the size of HDL particle gets smaller to take up more cholesterol.

Plasma HDL particles are continuously modulated by various enzymes and proteins such as LCAT, LPL, HL and plasma lipid transfer proteins. Plasma lipid transfer proteins include (1) plasma CETP which transfers CE from HDL to apo B-containing lipoproteins to remodel HDL particles, and (2) plasma phospholipid transfer protein (PLTP) which transfers phospholipids from TG-rich lipoproteins to HDL during lipolysis. The cloning and sequencing of PLTP cDNA have demonstrated that PLTP belongs to the lipid transfer/lipopolysaccharide binding protein (LBP) family. This family includes CETP, bactericidal permeability increasing protein (BPI), and lipopolysaccharide-binding protein (LBP). These proteins share some common functional and structural properties. The current review will focus on the recent findings on the molecular biology and functional properties of both CETP and PLTP, which are the key proteins in the reverse cholesterol transport system.

3. ROLES OF PLASMA CETP AND PLTP IN LIPOPROTEIN METABOLISM

3.1. Roles of plasma CETP in lipoprotein metabolism and conversion of HDL

The functions of plasma CETP and PLTP in lipoprotein metabolism are illustrated in Figure 1. Plasma CETP facilitates the transfer of CE from HDL to VLDL, IDL and LDL (8-9). CE is bi-directionally transferred and
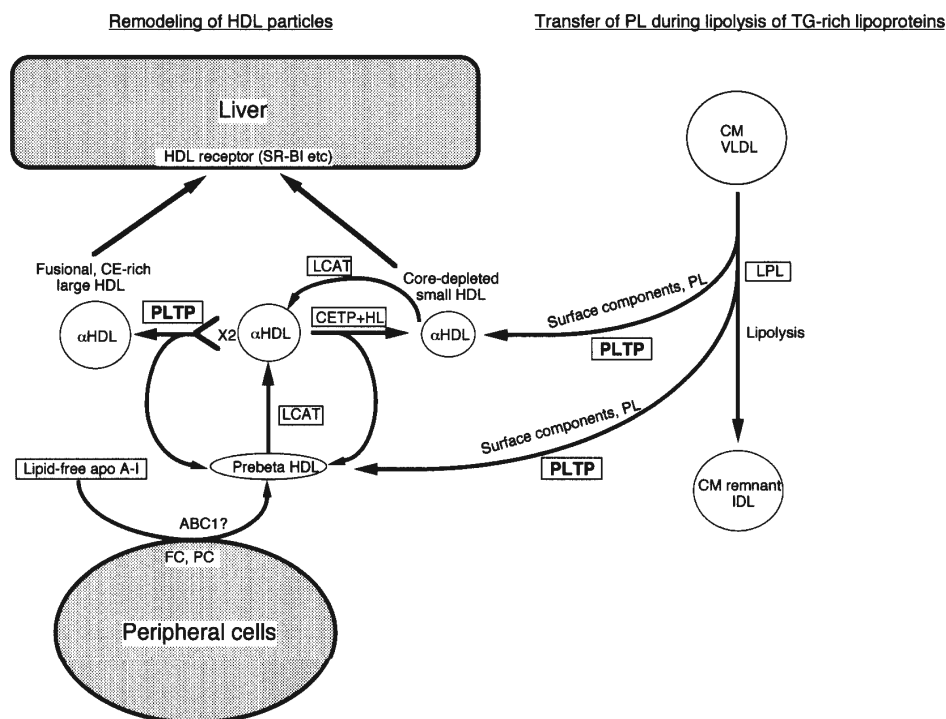
Figure 2: Roles of plasma PLTP in lipoprotein metabolism and HDL conversion. PLTP is involved in the transfer of phospholipids from TG-rich lipoproteins to HDL and in the size conversion of HDL particles.

TG is transferred simultaneously in the opposite direction with an equimolar ratio of CE to TG. The HDL becomes enriched with TG after the transfer of CE and is then hydrolyzed by HL. The HDL particles may be recycled to accelerate cholesterol efflux from peripheral tissues, or catabolized via a putative HDL receptor. The CE of HDL is selectively taken up by the liver via SR-BI (10). CETP also mediates the bi-directional transfers of cholesteryl linoleate hydroperoxide and of cholesteryl linoleate hydroxide between HDL and LDL (11). HDL-associated cholesteryl linoleate hydroperoxide is reduced to cholesteryl linoleate hydroxide. Both forms of oxidized cholesteryl linoleate are rapidly removed via a selective uptake and detoxified by the liver (12). CETP could facilitate the removal and hepatic detoxication of oxidized lipids from the site of high concentration such as atherosclerotic plaques. Furthermore, CETP was demonstrated to play some roles in the removal of cholesterol from lipid-laden macrophages, fibroblasts and smooth muscle cells (13-14).

To clarify the effect of CETP inhibition on plasma lipoproteins, monoclonal or polyclonal antibodies to CETP were intravenously injected in rabbits. The lipoprotein profiles were dramatically changed and the HDL-cholesterol increased markedly after injection (15-16). An intravenous injection into cholesterol-fed rabbits of antisense oligodeoxynucleotides against rabbit CETP coupled with asialoglycoprotein carrier molecules inhibited the liver specific expression of CETP, and the activity and mRNA of CETP were reduced at 24-96 h after injection. Total cholesterol, VLDL-cholesterol and LDL-cholesterol concentrations were decreased, while plasma HDL-cholesterol was increased at 48 h (17).

Plasma CETP also plays some roles in the conversion of HDL particles. The incubation of HDL_3 and VLDL with LCAT and CETP caused a shift of HDL_3 into HDL_{2b}-like subfraction (18). CETP and HL were demonstrated to synergistically transform large HDL particles into very small HDL particles (19). Different effects of CETP and plasma phospholipid transfer protein (PLTP) were shown on the size distribution of HDL (20). CETP promotes the formation of HDL_{3b} particles at the expense of HDL_{2a}, while PLTP accelerates the formation of HDL_{2b} particles at the expense of HDL_{3a}.

3.2. Roles of plasma PLTP in lipoprotein metabolism and conversion of HDL

The role of PLTP in lipoprotein metabolism is illustrated in Figure 2. Plasma PLTP was initially shown to transfer a variety of phospholipids from TG-rich lipoproteins to HDL during lipolysis. Furthermore, PLTP can promote the size redistribution of initial alpha-HDL of intermediate size. Both PLTP and CETP can contribute to the formation of prebeta-HDL, however only PLTP favours the appearance of large alpha-HDL. In contrast, CETP is involved in the appearance of small alpha-HDL in combination with TG-rich lipoproteins and HL. PLTP can dissociate apo A-I from the HDL surface, which constitutes a crucial step in the conversion of HDL particles. Prebeta-HDL particles thus formed have been shown to have an enhanced capacity for cholesterol efflux. PLTP is able to act on both HDL_2 and HDL_3 as well as LpA-I and LpA-I/A-II particles, but with a lower efficiency with apo A-II-containing lipoproteins.

PLTP also transfers cholesterol, diacylglyceride, sphingomyelin, cerebroside, and alpha-tocopherol (21-23). PLTP as well as CETP belongs to the lipid transfer/lipopolysaccharide binding protein (LBP) gene family, which also includes the lipopolysaccharide binding protein (LBP) and bactericidal/permeability-increasing protein (BPI). PLTP, CETP and LBP can transfer phospholipids with different specificity. CETP only exchanges phospholipids, while PLTP can affect a net mass transfer of phospholipids. PLTP can bind and transfer lipopolysaccharide between lipoproteins, suggesting a role of PLTP in antimicrobial defence. However, the physiological role of PLTP on the regulation of the cellular response to lipopolysaccharide remains to be clarified. PLTP is attributed to the majority of phospholipid transfer in plasma, while LBP plays a dominant role in the transfer of phosphatidylinositol. Specific anti-PLTP antibodies blocks plasma phospholipid transfer activity almost completely in a liposome-HDL experimental system, therefore PLTP could play a major role in the transfer of phospholipids from large TG-rich lipoproteins to HDL *in vivo* (24).

In addition to its role in the transfer of phospholipids between lipoproteins, PLTP may be involved in the transfer of phospholipids between lipoproteins and cells. Plasma PLTP activity was positively correlated with its ability to promote cholesterol efflux from Fu5AH hepatoma cells (25). PLTP might be involved in the initial step of reverse cholesterol transport pathway by generating prebeta1-HDL since treatment of plasma with PLTP was accompanied by an increase in cholesterol efflux from fibroblasts (26). PLTP is also involved in the remodeling of HDL. Both CETP and PLTP contribute to the formation of prebeta-HDL, an acceptor of cholesterol from cells, only PLTP can favor the emergence of large alpha-HDL. In the case of PLTP, two alpha-HDLs of intermediate size can fuse, leading to the concomitant formation of prebeta-HDL as well as large CE-rich alpha-HDL. PLTP may exert a beneficial effect on reverse cholesterol transport via the concomitant generation of prebeta-HDL, which is an initial acceptor of cellular cholesterol, and large CE-rich HDL, which can deliver a large amount of cholesterol back to the liver. Furthermore, recent data suggest that PLTP prevents vascular endothelium dysfunction by delivering alpha-tocopherol to endothelial cells (27).

As mentioned earlier, CETP and PLTP are the members of the lipid transfer/lipopolysaccharide binding protein gene family, which also includes LBP and BPI. These 4 proteins possess distinct physiological functions, but they share marked biochemical similarities. All of these 4 proteins can bind lipopolysaccharides (8,28) and phospholipids (29-30) as well as a variety of other lipids. CETP, PLTP and LBP are associated with plasma HDL, however BPI exists on the membranes of secretory granules of neutrophils. The crystal structure of BPI has recently been elucidated. BPI is a long boomerang-shaped molecule that has two domains at the NH2- and COOH-terminus with similar folds shaped like barrels (28). BPI contains a central beta-sheet domain which forms an interface between the barrels. Each barrel has a pocket occupied by a phosphatidylcholine molecule. Since phospholipid binding was coupled with the release of bound lipopolysaccharide, lipopolysaccharide may bind to the same pockets (30).

4.   SYNTHESIS OF CETP AND PLTP

4.1. Synthesis of CETP

Human plasma CETP is a very hydrophobic glycoprotein with an Mr of 70-74 kD, consisting of 476 amino acid residues (8,31). Amino acid analysis of purified CETP demonstrated an unusually high content (45%) of nonpolar residues. CETP is synthesized by the liver, small intestines, spleen, adipose tissues, adrenal gland, kidney, heart, and skeletal muscles. CETP is secreted from cultured monocyte-derived macrophages, B-lymphocytes (32), adipocytes (33-34), hepatocytes and a human hepatoma cell line, HepG2 cells, and CaCo-2 cells (a model of enterocytes). CETP activity varies markedly between species; CETP activity is very low in mice and rats and high in rabbits. The CETP activity in humans is intermediate between mice and rabbits (35). By Northern blot analysis CETP mRNA can be detected in the liver, small intestines, spleen, adrenal gland, heart, skeletal muscle and adipose tissues. In cynomolgous monkeys, high levels of CETP mRNA were detected in the liver and thoracic aorta, whereas low but detectable levels of CETP mRNA were shown in mesenteric fat, adrenal gland, spleen and abdominal aorta (36). Tissues containing LPL such as adipose tissues and skeletal muscles are the major sources of CETP mRNA in hamsters (37).

Human monocyte-derived macrophages in culture synthesize and secrete CETP activity in a time-dependent manner over 24 h. CETP expression is induced during the differentiation of monocytes into macrophages (38). The secretion of CETP activity into culture medium is facilitated by phorbor myristate acetate, and is also increased when macrophages are loaded with acetylated LDL or free cholesterol. The secretion of CETP is positively correlated with the intracellular accumulation of CE, suggesting that CETP may function to maintain intracellular cholesterol homeostasis during differentiation and in response to an excess of cholesterol accumulation. A human adipocyte-derived liposarcoma cell line, SW872, produces 50-fold more CETP than HepG2 cells with cholesterol loading (39). CETP mRNA abundance in human adipose tissue was shown to be a function of membrane cholesterol content rather than lipid droplet cholesterol. CETP mRNA increased in parallel with cholesterol enrichment via chylomicron remnants (34). CaCo-2 cells, a human intestinal cell line, secrete CETP in a vectorial manner from the basolateral membrane, and its secretion is increased 3-fold by adding fatty acids into the culture medium (40). CETP activity is also detected in seminal fluid and cerebrospinal fluid, which is about 12% of that of plasma (41). CETP activity can be detected in a conditioned medium from human neuroblastoma and neuroglioma cells and from sheep choroid plexus, suggesting that CETP is synthesized and secreted by the brain and may play some roles in the transport and redistribution of lipids within the central nervous system.

4.2. Synthesis of PLTP

Human PLTP contains 476 amino acid residues. Human PLTP has a 93% homology with pig PLTP and an 83% homology with mouse PLTP. Human PLTP is highly expressed in the placenta, adipose tissue, pancreas and lung. Intermediate levels of expression are detected in the liver, heart and kidney (42-43). PLTP expression in the lung suggests some role of PLTP in the synthesis or delivery of pulmonary surfactants. In mice, PLTP mRNA was also observed in brain and testis with intermediate levels (44). The synthesis of PLTP in the liver, adipose tissue, and lung is assumed to contribute to most of the plasma PLTP mass in humans and mice.

5. STRUCTURE AND REGULATION OF HUMAN CETP GENE

5.1. Structure of human CETP gene

The CETP cDNA was cloned from humans, rabbits, cynomolgous monkeys, and hamster (37,45-47). Human CETP cDNA has an 80% homology to that of rabbits. The CETP cDNA sequence of cynomolgous monkeys demonstrated a 95% homology to that of humans (47). Human CETP gene, located in chromosome 16 (16q12-16q21) near the LCAT gene, spans over 25 kb and consists of 16 exons and 15 introns (48). In the promoter region of the human CETP gene, sequences resembling a "TATA" box and an SP1 binding site were identified upstream of the translation initiation codon (48). In the promoter region of human CETP gene, there is a sequence resembling the binding site for the transcription factor CCAAT/enhancer-binding protein (C/EBP), which may be preserving CETP gene promoter activity (49). There is also a 105-bp tissue-restricted promoter which contains a nuclear hormone receptor response element required for transcriptional activity (50). Three specific binding sites of nuclear extracts were identified within the promoter region. Nuclear hormone receptors, including ARP-1 and its homologue Ear-3/COUP-TF, were occupants of the region -93 to -118 in HepG2 and Caco-2 cells. ARP-1 was shown to play a dichotomous role as both a transcriptional repressor and a transcriptional activator, depending on the promoter context.

There are two isoforms of CETP mRNA in human tissues expressing the CETP gene; one is a full-length form which produces an active Mr 74,000 CETP, and the other is a shorter variant in which exon 9-derived sequences had been removed (51). The shorter form of CETP mRNA is produced by an alternative splicing of the CETP gene transcript. By transfection of the exon 9-deleted cDNA to COS cells, a shortened form of CETP is poorly secreted and inactive. By co-transfection of full-length and exon 9-deleted cDNAs to COS cells, the secretion of full-length active CETP was inhibited, suggesting that the exon 9-deleted protein may have a dominant negative effect on the expression of full-length CETP and that alternative splicing of CETP gene may modulate the level of active CETP (52). The Caco-2 cells, an intestinal cell line, show a reduction of active CETP mRNA under basal conditions and about 60% of reverse transcribed CETP cDNA corresponds to exon 9-deleted transcripts, while addition of sodium oleate into the culture medium induces a 2-fold increase in full-length CETP cDNA transcripts without affecting exon 9-deleted transcripts, resulting in an enhancement of CETP activity secreted into the medium (53).

5.2. Regulation of CETP synthesis

Plasma CETP activity is enhanced when rabbits are fed a high-fat, high-cholesterol diet (54), which is mainly due to the increased hepatic production of CETP (55-56). CETP activity is increased in WHHL rabbits compared with wild-type rabbits, suggesting that endogenous hypercholesterolemia may also increase the expression of CETP gene. As will be mentioned later, in the human CETP transgenic mice with its natural promoter, a marked increase in CETP mRNA and protein is observed in response to a high-fat, high-cholesterol diet, whereas the same transgene under the control of the mouse metallothionein promoter exhibits no significant change in CETP mRNA or protein in response to an atherogenic diet (57). Thus, the sequences necessary for the increment of CETP mRNA in response to dietary cholesterol may be located in the natural flanking regions. The sequences in the natural flanking regions which may be responsive to sterols and important for tissue-specific expression of CETP mRNA were examined, using transgenic mice (58). CETP expression was not changed in response to dietary cholesterol when the downstream flanking sequence was deleted. In contrast, by deletion of the 232-bp sequences between –138 to –370 bp upstream of the transcription start site, the response to dietary cholesterol was abolished, suggesting that this region contains element(s) that is responsive to sterols. Moreover, the CETP transgenic mouse line containing an additional 200-bp sequence upstream of the flanking region –138 to –570 bp showed a more remarkable increase in response to dietary cholesterol, indicating that there are sequences in this region affecting the response to dietary cholesterol (58).

The upstream sequence responsible for an authentic tissue distribution of CETP and its induction in response to dietary or endogenous hypercholesterolemia was further examined, using transgenic mice expressing 6 DNA constructs that contain different amounts of natural flanking sequence of the CETP gene (58). The upstream flanking sequence between –138 to –370 bp of CETP gene contained sequence with homologies with promoters of other genes responsive to sterols. Figure 3 shows the upstream flanking sequence of the CETP gene. There was a tandem repeat of a sequence with identity to the sterol regulatory element (SRE) in the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase promoter that is responsible for the sterol-mediated regulation of the HMG-CoA reductase gene (59). The tandem repeat of SRE element was identical to that in the HMG-CoA reductase promoter except for the last nucleotide of the second repeat. By gel shift assays, this element was shown to bind SRE binding protein (SREBP-1) (60) and Red25 (61), which is identical to the transcription factor Yin Yang-1(YY-1). Point mutations were prepared in the SRE-like element, designated MUT1- and MUT2-CETP promoter fragments, which resulted in

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        ------>  <-----                                      ------> ------>
-580  ttctggggtacccagatgcagcgtcaaatgccatggaatactacagtgaggacattatcctttcaagctttcaaatcaga  -50
          /
       Tg C, D  ━━▶
                                                        MLC1F/MLC3F-DR
                   ------->  <-------
-500  gcaagggaaaggtcgatgctagagtttctctagcacccatgaagccctctcccttttttctactgagttttactttacagg  -42
                                                                                    ****
                                                                            Block1a
                              ____DR4____                                     _HNF-1_
       >    ---->         ----->      ----->                 ---->            <---
-420  caacagcaggcttcaagcttggggtcattgtcgggcaacagtatctggcaagaattcaatgtctttttctcatagtcatt  -34
       *     *****                                               /
                                                              Tg E  ━━▶

       __
       -
-340  gtattttgccctctttctatttatggcaactgagagagaaagcttattcctagatatatgtatttaagtaaaaataaat  -26


           _C/EBPα_                         NF-1    _Red25-like (x2)_
                                                    ------>  ------>
-260  gaattcatggaaacatattaagcaattatccagataacataagggatggcaaaaatggtgcagatggtggaggggagaca  -18

                                                          NHR
                              ----->                      ----->
-180  agtagaagttggggtgctcttgttgaatgtctggctctgaactctagaggaggccgcaggggctgggcaggaaggaggtg  -10
                                      /******                ******
                                     / Block2a              Block2b
                                    Tg F  ━━▶
       NHR   PEA-3           PEA-3                 Sol       TATA
             ----->          ----->
-100  aatctctggggccaggaagaccctgctgcccggaagagcctcatgttccgtgggggctgggcggacatacatatacgggc  -2

                      +1
-20   tccaggctgaacggctcgggccacttacacaccactgcctgataaccATGCTGGCTGCCACAGTCCTGACCCTGGCCCTG  +60
                                                     MetLeuAlaAlaThrValLeuThrLeuAlaLeu
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B

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                  Small intestine
                  Brain
                  Muscle           Adrenal
                  (Lung)           Kidney
                  (Gonads)         Liver
       Liver      (Adipose)        Spleen
       Spleen                                    +1
       |----//----|-------|---------|--------|-------|
      -3.4      -0.57   -0.37     -0.138 Kbp
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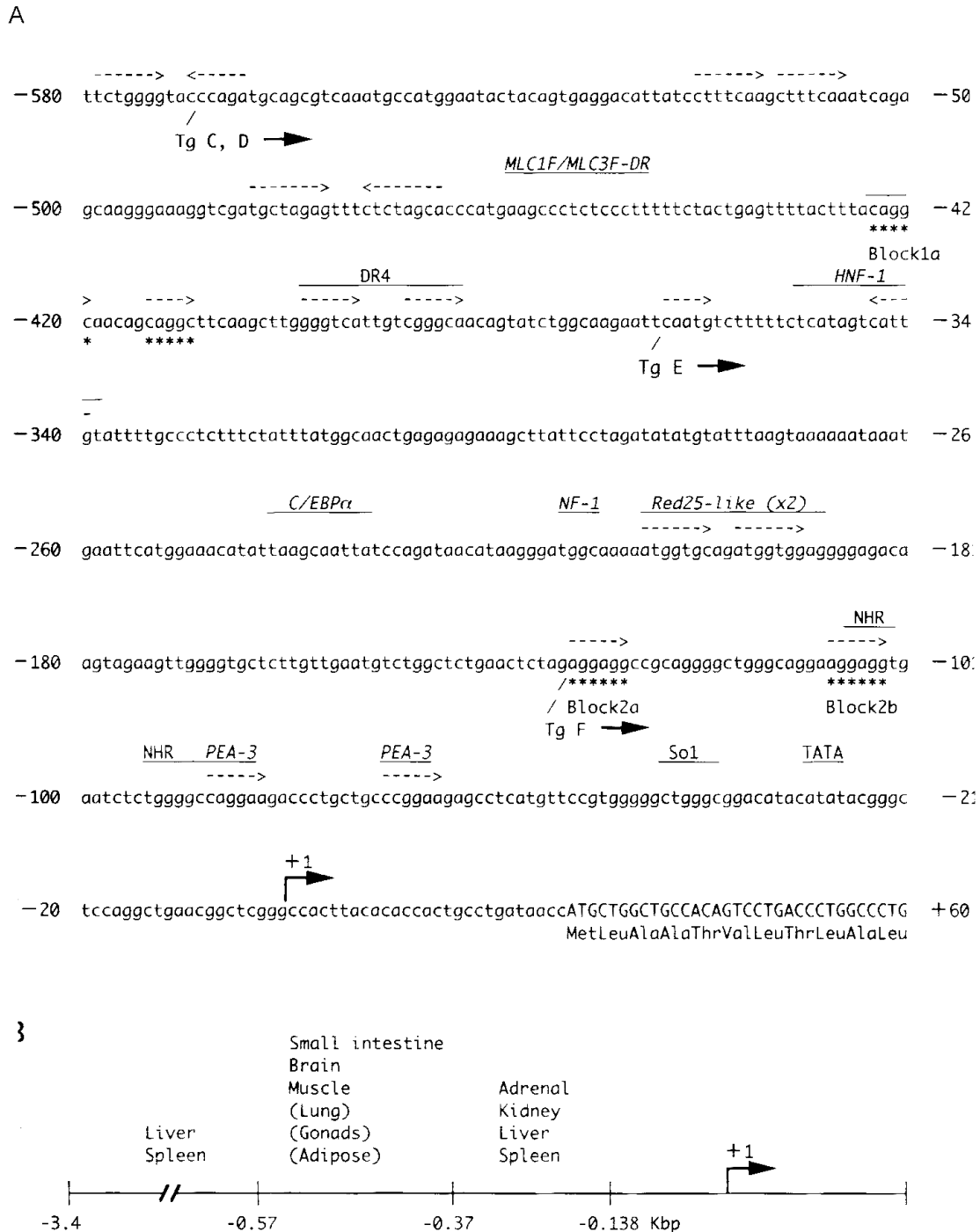
Figure 3. (A) Complete nucleotide sequence between +60 and –580 of the human CETP promoter region. Consensus elements for known transcription factors are demonstrated. There is a tandem repeat of the sterol regulatory element of the HMG-CoA reductase gene (Red 25), the ubiquitous nuclear factor-1 (NF-1), C/EBPalpha, hepatocyte nuclear factor-1 (HNF-1), a nuclear hormone receptor (NHR), PEA-3, Sp1, and the muscle-specific element MLC1F. Arrows show direct and inverted repeats. Asterisks indicate sequences found using MACAW program in the proximal promoter of rat, mouse, hamster, and human cholesterol 7 alpha-hydroxylase, human PLTP, and human, rat, and mouse apo E. Tg C, D, E, and F show the 5' ends of transgenes that were used to study the in vivo expression of CETP in transgenic mice. (B) Distribution of tissue-specific elements in the 3.4 kbp of the human CETP promoter. Tissue names in parenthesis indicate the presence of regulatory elements with minor effects (Reproduced and modified from Reference 58).

decreased binding of SREBP-1 in MUT1 or SREBP-1 and YY-1 in MUT2 (62). CETP transgenic mice expresssing these promoter point mutations were prepared, and both MUT1 and MUT2 transgenic mice expressed CETP activity and mass. Both MUT1-CETP and MUT2-CETP showed an induction of plasma CETP activity upon loading high-fat and high-cholesterol diets. Using a stable transfectant of adipocyte cell line (3T3-L1) with MUT1 and MUT2 CETP promoter-reporter genes, a significant induction of reporter activity was observed in response to sterols. CETP transgenic mice with natural flanking region (NFR) and those with MUT1-CETP promoter were crossed with SREBP-1 transgenic mice to elucidate transactivation by SREBP-1. Induction of SREBP transgenes in the liver with a low carbohydrate diet resulted in a 3-fold increase in plasma CETP activity in NFR-CETP/SREBP transgenic mice, whereas CETP activity was not significantly changed in MUT1-CETP/SREBP transgenic mice. Therefore, the NFR-CETP transgene is transactivated *in vivo* by SREBP-1 through the interaction with the CETP promoter SREs. However, this interaction is not necessary for positive sterol induction of CETP gene transcription. Taken together, CETP gene may be independently regulated by SREBP-1 and a distinct positive sterol response factor (62). A fragment between nucleotides –361 and –138 was involved in a positive response to cholesterol loading (63). Both SREBP-1a and YY-1 were shown to *trans*-activate the luciferase activity of constructs harboring the cholesterol response element. SREBP-2 was also shown to *trans*-activate the luciferase construct, although much less effectively than SREBP-1.

Plasma CETP levels and hepatic CETP mRNA are also markedly increased in response to endogenous hypercholesterolemia as observed in the LDL receptor and apo E knockout mice. These increases were due to an enhanced transcription of CETP gene in the liver and peripheral tissues. Since plasma cholesterol level positively correlated with plasma CETP in CETP transgenic mice and humans (64-65), it is speculated that CETP gene expression is driven by a mechanism which senses high levels of plasma cholesterol independent of apo E and LDL receptors (66). Orphan nuclear receptors such as LXRalpha (NR1H3), LXRbeta (NR1H2), and SF-1 (NR5A1) have recently been shown to be activated by sterols in cell culture (67-70). LXRs were also activated by hydroxy sterols at physiological concentrations (67-68). A retinoic acid receptor element (RARE) was identified in the –165/–134 region of the CETP gene (71). A direct repeat of a nuclear receptor binding sequence separated by 4 nucleotides (DR4 element, –384 to –399) was identified in the CETP promoter region (72). To evaluate the induction of CETP gene, mice carrying normal or mutated promoter sequences were prepared. DR4 element was responsible for the induction of CETP gene after cholesterol loading. Both LXRalpha/RXRalpha and LXRalpha/RXRbeta transactivated the CETP promoter via its DR4 element in a sterol-responsive manner, suggesting that the positive response of CETP gene to sterols is mediated by a nuclear receptor binding site which is activated by LXRs. There is a binding site for LXR in the promoter of the *Cyp7a* gene that encodes cholesterol 7 alpha-hydroxylase, the first rate-limiting enzyme in the pathway converting cholesterol to bile. LXRalpha was shown to transactivate the *Cyp7* alpha promoter (69). Taken together, LXRalpha coordinates the regulation of HDL-CE catabolism and bile acid synthesis in the liver.

5.3. Determinants of plasma CETP levels

Which is the predominant source of CETP that regulates the plasma level of CETP? A positive correlation was observed between plasma CETP and hepatic CETP mRNA abundance, and the liver was shown to be one of the predominant sources of plasma CETP in primates (73). Although hepatocytes were assumed to be a primary site of CETP synthesis, it was suggested that non-parenchymal liver cells (sinusoidal cells) are a predominant source of plasma CETP in primates (74). CETP mRNA was detected only in non-parenchymal cells, while apo A-I and B were produced only by parenchymal cells, and apo E by both types of cells. *In situ* hybridization showed that hepatic sinusoidal cells in primates were responsible for the high CETP mRNA levels.

Moreover, CETP is also synthesized by adipose tissues (34,37). CETP mRNA is detected in preadipocytes and is present throughout differentiation (75). In contrast, LPL and adipsin are detected by day 2 or 3 of adipocyte differentiation. The transcription factors such as peroxisome proliferator-activated receptor (PPAR) gamma, ADD1/SREBP1 and C/EBPalpha are expressed by day 2. CETP expression is induced at an early stage of adipocyte lineage and may be activated by transcription factor(s), which are not members of the PPAR, ADD1/SREBP1 or C/EBP families (75). In an attempt to explain the low plasma HDL-cholesterol levels in obese subjects, Arai et al showed that both CETP activity/mass and postheparin hepatic lipase activity were increased in obese subjects, while postheparin LPL activity was significantly decreased (76). Plasma HDL-cholesterol level was correlated positively and independently with plasma CETP activity, but not with LPL or HL activity. CETP activity in obese subjects was positively correlated with body weight, body mass index (BMI), body fat mass and subcutaneous fat area determined by CT scan, while it was negatively correlated with the ratio of visceral fat area / subcutaneous fat area. Therefore, low plasma HDL-cholesterol level in obese subjects is attributed mainly to the increased CETP activity produced by adipose tissues, especially subcutaneous fat tissues, which may be a predominant source of plasma CETP in obese subjects (76). In contrast, Dusserre et al. reported that CETP mRNA levels were more abundant in visceral fat tissues than in abdominal subcutaneous fat (77).

6. STRUCTURE AND REGULATION OF HUMAN PLTP GENE

Human plasma PLTP has a 20% sequence homology to human CETP (78). It is noteworthy that human PLTP gene has a marked similarity in the exon-intron organization (79). Two distinct basic amino acid residues, Lys233 and Arg259, were implicated in the ionic interaction of CETP with negatively charged groups (80). The two specific basic amino acid residues of CETP are remarkably conserved

among all the members of the lipid transfer/LBP family, and Arg218 and Arg245 of PLTP correspond to Lys233 and Arg259 of CETP, respectively. Thus, Arg218 and Arg245 of PLTP might mediate the electrostatic interaction of PLTP with lipoprotein negative charges (81). The N-terminal regions of PLTP, CETP, LBP and BPI are markedly homologous, while the C-terminal regions are less conserved. It is suggested that C-terminal region may possess the specific properties of PLTP and CETP, namely the binding and transfer of surface lipids in the case of PLTP and the binding and transfer of apolar lipids in the case of CETP, respectively.

PLTP activity and mRNA are increased in response to a high-cholesterol diet in mice (44). Mice fed a high-fat, high-cholesterol diet showed a significant increase in plasma phospholipid transfer activity in association with PLTP mRNA in the lung. PLTP promoter shows no strong homology to known SREs. The promoters of human and mouse PLTP genes show 5 consensus sequences for the transcription factor Sp1 that is necessary for PLTP transcription (82-83). Injection of lipopolysaccharide into mice was shown to downregulate PLTP expression (44).

7. STRUCTURE-FUNCTION RELATIONSHIP OF CETP

The carboxyl-terminal sequences of CETP (26 amino acids) are important for neutral lipid transfer activity, which are the epitope of a neutralizing monoclonal antibody (TP2) (84). The binding sites of CE and TG may be different since some monoclonal antibodies completely inhibit TG transfer, but not CE transfer (85). The changes of amino acids 48-53, amino acid 165, and amino acids 373-379 by linker insertion scanning mutagenesis markedly impaired CE transfer activity (86). By expressing full-length and mutant human CETP cDNAs with overlapping deletions in the C-terminal region in baculovirus-transfected Sf9 insect cells, the characteristics of mutant proteins were analyzed (87). Both CE and TG transfer were impaired in the mutant proteins and the C-terminal sequence -Phe-Leu-Leu-Leu- (residues 454-457) was essential for normal binding of CETP to each lipoprotein and the effective transfer of CE and TG possibly together with the other sequences in the C-terminal region. Several other proteins that are reported to function in binding non-polar lipids, including LCAT, cholesterol 7 alpha-hydroxylase, cholesterol esterase, and hormone-sensitive lipase possess similar conserved sequences. The *in vitro* point mutagenesis and expression in COS cells demonstrated that the binding of TP2 to CETP was abolished by mutations of charged amino acids existing periodically within the sequence between His^{466} and Leu^{475} and at Asp^{460}, although CE transfer activity was well preserved in these mutants (88). In contrast, mutations of hydrophobic amino acids in this region (especially Phe^{454}, Phe^{461}, Leu^{468}, Phe^{471}, and Leu^{475}) caused a marked reduction in CE transfer, but normal binding of TP2. Therefore, these paradoxical effects of mutations on TP2 binding and CE transfer activity suggest that the hydrophobic face of a C-terminal helix of CETP is

involved in CE transfer and that TP2 inhibits activity by local sterical hindrance. The hydrophobic character of this region due to the abundance of hydrophobic amino acids Leu and Phe may be crucial for CE transfer.

The Mr of CETP deduced from the cDNA is 53,108, however purified plasma CETP appears as a broad band containing two different molecular forms (89). This microheterogeneity can be explained by the variable N-linked glycosylation. There are 4 potential N-linked glycosylation sites in the CETP cDNA sequence at amino acid positions 88, 240, 341 and 396 (90). Site-directed mutagenesis analysis demonstrated that the mutant proteins 88N:Q and 396N:Q were poorly secreted, while the 341N:Q protein was well secreted with a higher CE transfer activity compared to the wild type (90). Thus, plasma CETP consists of a mixture of two forms reflecting a variable glycosylation at Asp 341. Rabbit CETP was expressed and secreted by *Pichia pastoris* (91), and the recombinant CETP was synthesized as an inactive polypeptide that is processed and secreted as a functional protein. N-linked glycosylation was essential for activity of recombinant rabbit CETP.

As mentioned earlier, although CETP and BPI have a relatively low sequence similarity (23% identity at the nucleotide and amino acid level), both proteins are homologous in the whole structures. When the sequences of CETP and BPI are aligned, the C terminus of the 476 amino acid-long CETP extends by 12 extra residues, which are predicted to form an amphiphilic helix (88) and the epitope of a monoclonal antibody that inhibits the neutral lipid (but not phospholipid) transfer activity of CETP. Based upon the binding characteristics of anti-CETP monoclonal antibodies, a model of CETP structure was presented. Four epitopes composed of CETP residues 215-219, 219-223, 223-227, and 444-450 were predicted to be present on the external surface of the central beta-sheet and a fifth epitope (residues 225-258) on an extended linker that connects the two domains of the molecule (92). A possible lipid transfer mechanism for CETP was hypothesized; the initial step involves the disordering of lipids in the lipoprotein surface, followed by the flipping and entry of a lipid molecule into the hydrophobic lipid-binding pocket (93). A monoclonal antibody against rabbit CETP was developed, which selectively inhibited TG transfer without affecting CE transfer (94). The epitope of this monoclonal antibody was the segment 465-473 (EHLLVDFLQ) of human CETP or 485-493 (KHLLVDFLQ) of rabbit CETP (core epitope), although neither peptide by itself bound to the antibody (95). Circular dichroism analysis showed that the limited interaction of monoclonal antibodies with a common lipid interaction site causes selective inhibition of TG transfer that has possibly lower priority than CE for the CETP reaction.

8. TRANSGENIC ANIMAL MODELS OF CETP AND PLTP

8.1. CETP transgenic animal models

CETP transgenic mouse lines with mouse metallothionein promoter were developed (57). Plasma

CETP concentration was increased approximately 1.4 - 1.6 fold and the CETP activity was proportionally elevated. The transgene overexpression after zinc induction was detected in the liver and small intestines. CETP transgene expression caused a reduction in serum cholesterol due to a 20-30% decrease in HDL-cholesterol and HDL particle size, although no significant changes were observed in total cholesterol in VLDL and LDL fraction. Transgenic mice expressing cynomolgus monkey CETP were also developed (96). The alterations in plasma lipoprotein profile were partly similar to those of CETP transgenic mice with metallothionein promoter (57), but more striking increases in plasma cholesterol, (VLDL+LDL)-cholesterol, and apo B were demonstrated probably due to much higher CETP levels (96). There was a strong negative correlation between plasma CETP activity and total plasma cholesterol, plasma apo A-I levels and apo A-I/apo B ratio. The size of apo A-I-containing lipoproteins became smaller because of the disappearance of HDL1 and HDL2 particles. Several lines of CETP transgenic mice with natural flanking sequences of human CETP gene were developed, using a minigene linked to the natural flanking sequences of the human CETP gene (64). A transgene containing 3.2 kb of upstream and 2.0 kb of downstream flanking sequence was utilized. A 4 to 10-fold increase in liver CETP mRNA was noted in response to a high fat, high cholesterol diet. The increase in liver CETP mRNA was associated with a 5-fold increment of the transcription rate of CETP transgene and a 2.5-fold increase in plasma CETP activity/mass. In contrast, CETP transgenic mice with the CETP minigene linked to metallothionein promoter rather than to its own flanking sequences, demonstrated no significant changes in liver CETP mRNA in response to cholesterol loading. Taken together, the natural flanking sequences of CETP gene may contain elements which mediate authentic tissue-specific expression.

The human CETP transgenic mice were mated with human apo A-I transgenic mice (97). Overexpression of both human CETP and apo A-I genes resulted in a more prominent reduction in HDL-cholesterol level and HDL particle size than mice expressing only CETP, although plasma level of CETP activity was similar. The CETP in CETP/apo A-I transgenic mice was associated with HDL particles, however only one-fifth of CETP was bound to HDL in the CETP transgenic mice, suggesting that the HDL containing human apo A-I may be a better substrate for CETP (97). The CETP transgenic mice were also mated with hypertriglyceridemic human apo C-III transgenic mice (98). These CETP/apo C-III transgenic mice showed markedly decreased levels of serum HDL-cholesterol and apo A-I and the HDL size. These changes were due to an increased fractional catabolic rate of HDL-CE and apo A-I. A mouse model with some of the features of familial combined hyperlipidemia was developed by crossing mice carrying the human apo C-III transgene with LDL-receptor knockout mice (99). The human CETP transgene was introduced onto the LDL-receptor (-/-)/apo C-III transgenic mice, which resulted in redistribution of cholesterol from HDL and IDL-LDL to VLDL and of TG from VLDL to IDL-LDL and HDL. In the transgenic mice expressing both human apo B and human CETP, the percentages of total cholesterol within the HDL, LDL and VLDL fractions were 30%, 65% and 5%, respectively, under a normal chow diet (100). These lipoprotein profiles were similar to those of normolipidemic human subjects. The liver LDL receptor was downregulated at the transcriptional level in the apo B100/CETP double transgenic mice compared with the nontransgenic mice (101).

Regarding the atherogenicity of CETP overexpression, the rate of progression of arterial lesions was accelerated in the transgenic mice expressing simian CETP (102). To create transgenic mice, they used C57BL/6 mouse, an inbred strain in which the development of atherosclerosis was well characterized. The plasma (VLDL+LDL)-cholesterol and apo B levels increased to a greater extent in the CETP transgenic mice than in the non-transgenic mice when fed atherogenic diet. The plasma HDL-cholesterol level in the CETP transgenic mice was significantly lower than that of non-transgenic mice when they were consuming a normal chow. The overexpression of CETP caused an increase in (VLDL+LDL)/HDL ratio, which positively correlated with the mean lesion area in aorta. However, the atherosclerotic lesion area was not significantly different between the LDL-receptor (-/-)/apo C-III transgenic mice and the LDL-receptor (-/-)/apo C-III/CETP transgenic mice despite the marked decrease in HDL-cholesterol (99). In contrast, CETP overexpression was shown to inhibit the development of early atherosclerotic lesions in hypertriglyceridemic apo C-III transgenic mice (103). In the human LCAT transgenic mice, plasma HDL-cholesterol levels are elevated, but enhanced atherosclerosis is observed (104). The LCAT transgenic mice were crossbred with CETP transgenic mice (105). The CETP expression could normalize both the plasma clearance of CE from HDL and the hepatic uptake of CE from HDL in LCAT transgenic mice. On the atherogenic diet, the mean aortic lesion area was decreased by 41% in LCAT/CETP double transgenic mice compared to the LCAT transgenic mice. Thus, the overexpression of CETP was assumed to reduce atherosclerosis in LCAT transgenic mice by correcting the dysfunctional properties of LCAT-transgenic mouse HDL and promoting the hepatic uptake of HDL-CE.

8.2. PLTP transgenic animal models

The transgenic mice expressing moderate levels (~30% increase) of human PLTP do not exhibit marked changes in lipoprotein metabolism, whereas the PLTP transgenic mice expressing human apo A-I showed a significant increase in the plasma levels of alpha-HDL and prebeta-HDL (106). The little changes in lipoprotein distribution in the PLTP transgenic mice may be explained by the substantial levels of PLTP that are already present in control animals, but these data suggest that prebeta-HDL particles are generated by PLTP reaction. In contrast, adenovirus-mediated overexpression of human PLTP cDNA in the liver resulted in a 10- to 40-fold increase in plasma PLTP activity in mice (107). These mice were characterized by the increased prebeta-HDL levels, an increased fractional catabolic rate of HDL, and enhanced hepatic uptake of HDL-CE compared with the wild-type mice, suggesting the role of PLTP in stimulating reverse cholesterol transport *in vivo*.

The PLTP knockout mice on a chow diet showed a marked decrease in HDL phospholipid, cholesterol and apo A-I, but no significant change in non-HDL lipid or apo B levels, compared with the wild-type littermates (108). On a high-fat diet, HDL levels were similarly decreased, but there was also an increase in VLDL and LDL phospholipids, free cholesterol and cholesteryl ester without changes in apo B levels, demonstrating the important role of PLTP-mediated transfer of surface components of TG-rich lipoproteins in the maintenance of HDL levels. Interestingly, lamellar lipoproteins were observed in the free cholesterol- and phospholipid-rich IDL/LDL fraction. Furthermore, the HDL of the PLTP knockout mice was enriched with protein and poor in phosphatidylcholine, and turnover studies showed a 4-fold increase in the catabolism of HDL protein and CE compared with that of wild-type mice (109). Thus, the impairment of phospholipid transfer from TG-rich lipoproteins into HDL may lead to hypoalphalipoproteinemia characterized by hypercatabolism of HDL protein.

9. HUMAN PLASMA CETP DEFICIENCY

9.1. Abnormal properties and functions of plasma lipoproteins in CETP-deficiency

Hyperalphalipoproteinemia (HALP) is caused by a variety of genetic and environmental factors, and is also associated with certain diseases. Families of HALP with hypobetalipoproteinemia accompanied by longevity due to a low incidence of coronary heart disease were previously described (110). Matsuzawa et al reported two patients with a marked HALP and premature corneal opacity, which is a sign of lipid depositions in tissues, and one of these patients complained of angina pectoris (111-112). Thus, it was suggested that HALP may be a condition of "impaired reverse cholesterol transport" and could be accompanied by atherosclerosis. The most important cause of primary HALP is a genetic CETP deficiency. Patients with CETP-deficiency show markedly elevated serum HDL-cholesterol levels, while their serum total cholesterol is moderately increased (113-115). Serum apo A-I, C-III and E levels are also very high, whereas apo B is normal or slightly decreased. The increment of HDL-cholesterol is due to the increase in HDL_2-cholesterol, while HDL_3-cholesterol is not increased. The HDL of the patients is more enriched with CE and poorer in TG than control HDL. In contrast, the CE content of the VLDL and LDL fraction is reduced. LDL-cholesterol level is normal or slightly elevated. However, it is noteworthy that the ultracentrifugally separated LDL fraction (d=1.019-1.063) contains apo E-rich HDL with a slow alpha-mobility in agarose gel electrophoresis in addition to the apo B-containing LDL (116). These HDLc-like particles have a high affinity to the LDL receptor of fibroblasts. The HDL particles of CETP-deficient patients resemble partly those of SR-BI knockout mice in their characteristics (117). SR-BI is an HDL receptor involved in the selective uptake of CE from HDL and is abundantly expressed in the liver and steroidogenic tissues (118). SR-BI knockout or SR-BI attenuated mice show a marked increase in serum cholesterol, especially HDL-cholesterol, while serum apo A-I level is not altered. The size of apo A-I-containing lipoproteins was much enlarged (117,119). These changes of lipoproteins in SR-BI

knockout mice are in part similar to those in CETP-deficient patients, suggesting that both CETP and SR-BI may regulate the concentration and size of HDL particles. However, the contribution of hepatic SR-BI expression to the regulation of HDL-cholesterol levels in humans remains to be clarified.

Furthermore, the LDL particles of CETP-deficient patients are small and very heterogeneous (designated as polydisperse LDL) and HDL particles are markedly large when examined by native polyacrylamide gradient gel electrophoresis or analytical ultracentrifugation (115,120). In analyses using equilibrium density gradient ultracentrifugation, the LDL of CETP-deficient patients is composed of heterogeneous lipoprotein particles distributed in a wide density range without any prominent peak (120). To the contrary, the LDL of controls comprises of a homogeneous group of lipoprotein particles distributed in a narrower density range with a single sharp peak (120). The LDL in each subfraction obtained from the patients is poor in CE and enriched with TG. In native polyacrylamide gradient gel electrophoresis, each subfraction of control LDL contains only one species of homogeneous LDL particles that decreases in size with an increase in the density. In contrast, each subfraction of patients' LDL contains two species of LDL particles; smaller LDL particles are present in addition to the LDL particles that are identical to the control LDL particles observed in the corresponding subfractions. The IDL of the patients also comprises of two species of lipoproteins. Thus, two metabolic pathways were hypothesized in the process of mature LDL formation (120). VLDL is secreted from the liver as two species of lipoprotein particles different in size. Each species of VLDL is successively metabolized to LDL through IDL by a separate pathway. Various modulations in plasma might take place to produce cholesterol-rich LDL particles with a high affinity for LDL receptors. The hydrolysis of TG by lipoprotein lipase and HL is important for this process. Moreover, CETP may play a crucial role in converting small TG-rich LDL particles to large CE-rich homogeneous LDL particles by transferring CE from HDL.

Since the lipoprotein particles from CETP-deficient patients show marked changes in their composition and size, they also exhibit functional abnormalities. The HDL_2 particles from control subjects can protect macrophages from cholesterol accumulation and enhance cholesterol efflux from cells when incubated with acetylated LDL. In contrast, the CE-rich large HDL_2 particles from CETP-deficient patients have a reduced capacity for inhibiting acetyl LDL-induced accumulation of CE in macrophages when HDL is added to the culture medium together with acetylated LDL (121). The HDL_2 from the patients has a less capacity than normal HDL_2 for cholesterol efflux from macrophages loaded with acetylated LDL, suggesting that large CE-rich HDL_2 particles from CETP-deficient patients do not possess anti-atherogenic functions. Human plasma contains two types of HDL particles; HDL particles with only apo A-I (LpA-I) and those with both apo A-I and A-II (LpA-I/A-II). The LpA-I is reduced in CETP-deficient patients, resulting in a significant decrease in the efflux and LCAT-mediated esterification of cholesterol compared to normal controls (122). In a stable isotope
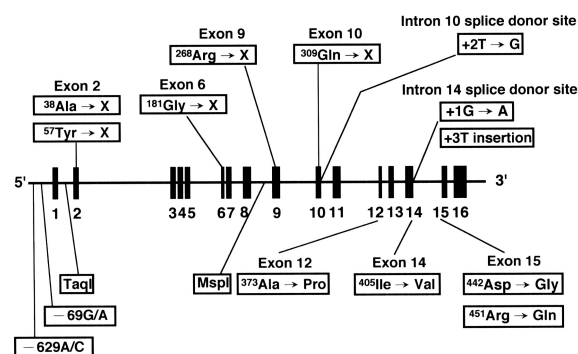
Figure 4: Mutations and polymorphisms in the human CETP gene reported so far. The intron 14 splicing defect and 442 D:G mutation are the most predominant in the Japanese subjects with HALP.

study, the fractional catabolic rates (FCRs) of apo A-I and A-II were significantly lower in the CETP-deficient patients than in the controls, while the rates of apo A-I and apo A-II production were normal (123). Thus, homozygous CETP deficiency causes a markedly delayed catabolism of apo A-I and A-II. Furthermore, the apo B-containing TG-rich LDL particles from CETP-deficient patients have a reduced affinity to the LDL receptor of fibroblasts compared with the control LDL particles (124). These LDL particles may be susceptible to the *in vivo* oxidation. Taken together, CETP deficiency causes both compositional changes and functional abnormalities of both HDL and LDL.

These changes in the lipoproteins from CETP-deficient patients can be modified by CETP *in vitro*. The addition of CETP into the patients' plasma caused a disappearance of large HDL particles together with a formation of very high density lipoprotein (VHDL)-like particles and the addition of HL further accelerated this process (125). The VHDL-like particles isolated by gel permeation chromatography were enriched with phospholipids and protein and the protein moiety was almost exclusively apo A-I. The VHDL-like particles which migrated at the prebeta-position and partly at the alpha-position in agarose gel electrophoresis showed a more increased capacity than normal HDL_3 for cholesterol efflux from acetyl LDL-loaded macrophages. Prebeta-HDL levels, cholesterol efflux and LCAT-mediated esterification *in vivo* were increased in mice overexpressing human CETP and human apo A-I transgenes (126). The HDL particles of human CETP transgenic mice removed cholesterol from macrophages more efficiently than those of non-transgenic mice. The co-expression of both human apo A-I and CETP further improved the cholesterol efflux efficiency of HDL particles in the human apo A-I / CETP transgenic mice when compared with the human apo A-I transgenic mice (127). Therefore, CETP may play essential roles in remodeling of large HDL particles into small ones that possess a potent anti-atherogenic function.

9.2. Gene defects in CETP deficiency

Most of the CETP-deficient patients have been reported so far from Japan. A G-to-A mutation in the 5'-

splice donor site of intron 14 was first identified in a Japanese patient with CETP deficiency (128). This defect was also identified in other Japanese CETP-deficient subjects (129-130) and is thus common in the Japanese HALP subjects and in the general population (130-131). A missense mutation (442D:G) in the exon 15 was later identified (132). Although the patients were heterozygous for this defect, they showed a 3-fold increase in plasma HDL-cholesterol and markedly decreased plasma CETP activity and mass, suggesting that the 442D:G mutation may have dominant effects on CETP and HDL level *in vivo*. Expression *in vitro* of wild-type and mutant proteins in COS cells confirmed this possibility. The 442 D:G mutation is also common in the Japanese HALP subjects and general population (133-134). A nonsense CETP mutation in exon 10 which causes premature stop codon and another nonsense mutation in exon 6 (G181X) were also identified (135-136). The latter mutation is also common, although its frequency in the Japanese HALP subjects is lower than that of intron 14 splicing defect and 442 D:G mutation. Furthermore, a defect in the intron 10 splice donor site of CETP gene was found, which causes exon 10 skipping, resulting in abnormal downstream splice site selection (137). Most of CETP-deficient subjects have been found in the Japanese population, however several mutations have recently been identified in the HALP subjects from the German, Caucasian and Asian populations (138-141). In the CETP-deficient patient with a heterozygous nonsense mutation (^{57}Tyr->stop), the presence of a null allele in addition to the allele with the nonsense mutation was suggested. The patient was associated with a marked postprandial lipemia (141). The site of gene mutations and polymorphisms in the CETP gene reported so far is summarized in Figure 4.

9.3. Atherogenicity of CETP deficiency

A positive correlation was reported between the extent of coronary atherosclerosis and plasma LDL-cholesterol or CETP concentrations in cynomolgus monkeys fed a high-fat, high-cholesterol diet (142). Aortic atherosclerosis was more marked in some CETP transgenic mouse lines after cholesterol feeding compared to non-transgenic mice (96,102). Antisense oligodeoxynucleotides against CETP coupled to asialoglycoprotein carrier molecules targeted to the liver were intravenously injected, which inhibited plasma CETP activity and increased plasma HDL-cholesterol in cholesterol-fed rabbits (143). Furthermore, the injection of antisense but not sense oligodeoxynucleotides attenuated aortic atherosclerosis in the cholesterol-fed rabbits (144). A CETP inhibitor has been shown to attenuate aortic atherosclerosis in cholesterol-fed rabbits (145). Thus, the pro-atherogenic function of plasma CETP was previously hypothesized in animal models (146). However, these data under unphysiological conditions such as cholesterol feeding in animal models do not preclude the possibility that CETP may play an anti-atherogenic role *in vivo* in humans.

A high prevalence of stroke was observed in some CETP-deficient families and corneal arcus was noted in CETP-deficient patients (147). Homozygous and heterozygous CETP-deficient subjects were sometimes accompanied by atherosclerotic cardiovascular diseases such as coronary heart disease (147-148), cerebrovascular

disease and arteriosclerosis obliterans (147). The HALP subjects whose CETP and HL activities were both reduced were accompanied by atherosclerotic cardiovascular diseases (147). The increased HDL_2 particles in CETP-deficient subjects were deficient in cholesterol efflux (121), moreover the addition of CETP and HL transformed the large HDL particles of CETP deficiency into small VHDL-like particles with a potent antiatherogenic function (125). The HDL particles isolated from mice expressing CETP showed a 2- to 4-fold increase in SR-BI-mediated HDL-CE uptake compared to those from mice lacking CETP (149). The addition of CETP to HDL in cell culture did not cause an increased selective uptake of HDL-CE by cells. However, when human HDL was enriched with TG by incubation with TG-rich lipoproteins in the presence of CETP followed by treatment with HL, HDL-CE uptake was significantly enhanced. Thus, the remodeling of human HDL by CETP, involving CE-TG exchange, followed by the action of HL, leads to the enhanced uptake of HDL-CE by SR-BI.

Plasma CETP activity was also shown to enhance plasma CE formation (150). The incubation of CETP transgenic mouse plasma showed a 20% to 40% increase in plasma cholesterol esterification rate compared to control mice. Mean plasma cholesterol esterification rates were lower in CETP-deficient patients than in normal subjects. Since LCAT mass was normal in CETP-deficient patients, genetic changes in CETP levels induce secondary alterations in the plasma LCAT reaction, due possibly to remodeling of HDL by CETP and other proteins *in vivo*. Thus, a moderately impaired plasma LCAT reaction may contribute to an abnormality of reverse cholesterol transport.

Hirano et al identified a unique area in the northern part of Japan named Omagari, where the prevalence of the intron 14 splicing defect was extremely higher than in the other part of Japan (151). In Omagari area, both the prevalence of HALP subjects (serum HDL-cholesterol more than 100 mg/dl) and the frequency of CETP deficiency were significantly lower in subjects over 80 years of age than in those under 80 years. The prevalence of ischemic changes in electrocardiogram was higher in the HALP subjects with serum HDL-cholesterol more than 90 mg/dl than in those whose HDL-cholesterol was 50-70 mg/dl. Furthermore, CETP-deficient HALP subjects showed higher atherosclerotic scores by an ultrasound examination of carotid arteries compared with control CETP-positive subjects. Transesophageal echocardiography also demonstrated an enhanced atherosclerosis in descending aorta (152). Therefore, it could be speculated that CETP-deficient HALP patients are not accompanied by longevity, but by atherosclerotic cardiovascular diseases. Zhong et al reported partly similar findings in Japanese-American men living in Hawaii. The incidence of coronary artery disease was higher in subjects with CETP mutations than in those without mutations, however the difference was significant only in subjects whose HDL-cholesterol level was between 41 and 60 mg/dl and men with increased HDL levels (> 60 mg/dl) in this population had a low risk of coronary artery disease irrespective of the CETP genotype (153). In contrast, a study in the Kochi area showed no significant difference in the prevalence of coronary heart disease between HALP subjects with and without CETP mutations, but HALP subjects (HDL-cholesterol ≥ 80 mg/dl as well as HDL-cholesterol from 60 to 79 mg/dl) appeared to be protected against coronary heart disease irrespective of the presence or absence of CETP deficiency (154). The reason for the discrepancy between the data of Omagari and Kochi remains to be clarified.

As mentioned earlier, CETP-deficient patients have lipoprotein profiles similar to those observed in SR-BI knockout mice, which show a marked increase in serum HDL-cholesterol and HDL size. Significant atherosclerotic lesions in the aortic root and coronary arteries were developed in the SR-BI/apo E double knockout mice (155). Similarly, increased LDL cholesterol and atherosclerosis were demonstrated in LDL receptor knockout mice with attenuated expression of SR-BI, which also showed a slight increase in HDL-cholesterol (156). Thus, reduced expression of hepatic SR-BI may be proatherogenic despite high HDL-cholesterol levels, since reverse cholesterol transport is impaired in this condition. Since CETP-deficient patients show similar lipoprotein patterns, CETP deficiency is also a condition of impaired reverse cholesterol transport which may be accompanied by increased atherosclerosis.

9.4. Pathophysiological significance of polymorphisms in the CETP gene
Several restriction fragment length polymorphisms (RFLPs) have been reported in the human CETP gene, including Taq I (Taq IA and IB) in intron 1, Stu I, MspI (in intron 8) and EcoNI (157-159). The Taq IB polymorphism is a silent base change affecting the 277th nucleotide in the first intron (160) and has been extensively studied. Subjects with the B2 allele (absence of the Taq I site at this polymorphic site) were more likely to have high HDL-cholesterol levels and low levels of CETP activity and mass (161-167). This association was implicated to be population specific (168-169), and influenced by environmental factors such as alcohol consumption and tobacco smoking (170-171). The association between Taq IB polymorphism and plasma HDL-cholesterol concentrations can be influenced by the presence of abdominal obesity and insulin resistance syndrome (172). The association of Taq IB polymorphism with plasma HDL-cholesterol concentration was independent of plasma CETP concentration in diabetic patients (173).

A G-to-A substitution in exon 15, which changes arginine 451 to glutamine, was also identified (174). Men heterozygous for the R451Q mutation showed 27% higher CETP activity than controls with normal genotype. Women heterozygous for the mutation had 16% lower total cholesterol compared to matched controls, but no such difference was detected in men. Isoleucine 405 to valine polymorphism (I405V) in exon 14 was also identified, however this is a neutral mutation since the specific activity of CETP with a valine or an isoleucine at residue 405 is similar (175). The frequency distribution of plasma CETP

concentration among men homozygous for the 405V allele was bimodal, however that for the 405I homozygote was not. Therefore, a subpopulation of these men may carry a functional mutation(s) in linkage disequilibrium with this polymorphism that may influence the expression levels of CETP (175). Three RFLPs at the CETP locus were shown to independently influence plasma CETP levels among subjects matched for factors that affect HDL levels (163). In this study, Taq IB, MspI, and I405V RFLPs accounted for approximately 11%, 17%, and 4%, respectively, of the variance in CETP mass in a healthy Dutch population. The haplotype of Taq I B1-Msp I M1-405I allele was overly represented in the lowest HDL decile, while the Taq I B2-Msp I M2-405V haplotype was predominant in the highest HDL decile (163). Underlying mutation(s) in partial linkage disequilibrium with these loci may be responsible for these effects on plasma CETP levels.

A new functional polymorphism, CETP/–629A/C, has recently been identified in the control subjects from the ECTIM study (176). The –629A allele was associated with lower CETP mass and higher HDL-cholesterol levels than the –629C allele. Nuclear factors Sp1 and/or Sp3 suppressed CETP promoter activity, while the binding of nuclear factors to the –629C allele had no effect on promoter expression. Furthermore, a G to A substitution at the –69 nucleotide in the promoter region (–69G–>A), corresponding to the second nucleotide of PEA3/ETS binding site (CGGAA) located upstream of the putative TATA box, has been identified in HALP subjects with low CETP levels (177). Luciferase reporter gene assay demonstrated that the mutated promoter appeared to lose transcriptional activity in HepG2 cells.

Concerning the relationship of RFLPs in the CETP gene to atherosclerosis, a significant dose-dependent association of Taq I B1B2 genotype marker with atherosclerosis progression was observed in the placebo group, while this link was abolished by pravastatin-treatment (164). Pravastatin attenuated the progression of coronary atherosclerosis in B1B1 carriers (with higher CETP levels), but not in B2B2 carriers (with lower CETP levels). This RFLP appeared to predict whether men with coronary artery disease may benefit from pravastatin treatment to delay the progression of coronary atherosclerosis. The protective effects of B2 allele on the development of coronary heart disease in association with increased HDL-cholesterol and decreased CETP activity were observed in men of the Framingham population, while no significant protective effects were observed in women (178). TaqI RFLPs were examined in type II diabetic patients; HDL-cholesterol was higher and the incidence of coronary heart disease was lower in men with the B2B2 genotype than in those with the B1B1 or B1B2 (179). However, women showed higher HDL-cholesterol levels than men and an equally high incidence of coronary heart disease in B2 homozygotes as in other genotypes, suggesting that the Taq IB polymorphism appears to be sex-dependent and affect HDL-cholesterol level and atherosclerosis only in type II-diabetic men. In the Honolulu Heart Program cohort, the I405V polymorphism was examined to clarify the relationship between the

polymorphism, CETP and HDL levels, and coronary heart disease (165). The V/V genotype was associated with lower CETP concentrations than the I/V or I/I genotype. Plasma HDL-cholesterol levels were higher in men with the V/V genotype than in men with the I/V or I/I genotype, but the increase in HDL-cholesterol was only significant in hypertriglyceridemic men with the V/V genotype. The prevalence of coronary heart disease was not significantly different among the 3 genotypes, however it was significantly higher among V/V than I/V or I/I subjects. Thus, the V/V genotype may be associated with higher HDL-cholesterol levels and increased coronary heart disease in hypertriglyceridemic men. Similar findings have recently been reported in women not treated with hormone replacement therapy, who were heterozygous or homozygous for Val405 (167). These subjects were associated with lower CETP activity and higher HDL-cholesterol level and had a 1.4-fold to 2.1-fold increase in the risk of ischemic heart disease, whereas no significant association was found in men. Furthermore, the Q451 allele was associated with significantly higher plasma CETP activity and reduced intima media thickness in men, and the I405V genotype was associated with lower plasma CETP activity and significantly affected intima media thickness in men with the highest alcohol consumption (180). Taken together, the contribution of RFLPs in the CETP gene to the development of atherosclerosis is still complex and should be evaluated prospectively. Further studies may be necessary in the future.

10. ACKNOWLEDGMENTS

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