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BOOK TITLE: Lipid transfer proteins, transporters, and binding proteins

2. Lipid transfer proteins

2.2. Cholesteryl ester transfer protein and lipid metabolism

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Summary

In this chapter, we present the major advances in CETP research since the detection, isolation and characterization of its activity in the plasma of humans and several species. Since CETP is a major modulator of HDL plasma levels, the clinical importance of CETP activity was recognized very early. We describe the participation of CETP in reverse cholesterol transport, conflicting results in animal and human genetic studies, possible new functions of CETP and the results of the main clinical trials on CETP inhibition. Despite major setbacks in clinical trials, the hypothesis that CETP inhibitors are anti-atherogenic in humans is still being tested.

Introduction

Cholesteryl ester transfer protein (CETP) was first isolated from plasma and characterized in 1978^{1,2}. Its activity consists of promoting a net transport of cholesteryl ester (CE) from HDL to VLDL and LDL in exchange for triglycerides (TG). This plasma activity was found in many species, including primates, rabbits, hamsters, reptiles and fishes, but it is not present in mice, rats and dogs^{3,4}. The protein purification⁵ and cDNA cloning in 1987⁶ and human gene cloning in 1990⁷ fomented investigations on CETP. Since then, a growing interest in CETP research has been observed (**Figure 1**).

Chemical purification showed that CETP is a plasma hydrophobic glycoprotein consisting of 476 amino acids with an apparent molecular weight of

66–74 kDa⁵. Protein purification and gene cloning allowed for the production of recombinant protein, antibodies and transgenic mouse models that were very important for further studies on the structure, function, gene expression regulation, human polymorphism screening and relationship with cardiovascular diseases⁸. However, it took approximately 30 years after the isolation of CETP plasma activity to resolve its molecular structure by Qiu and collaborators in 2007⁹. This milestone finding allowed for the elucidation of the CETP mechanism of action and a more refined drug design targeting CETP inhibition. In fact, the current view supports the formation of a ternary complex of CETP with donor and acceptor lipoproteins, allowing the transfer of neutral lipids through a hydrophobic tunnel inside its structure¹⁰.

The human CETP gene is located on chromosome 16 (16q13), spans 25 kb and contains 17 exons. It is conserved in chimpanzees, rhesus monkeys, chickens, zebrafish, and frogs. Orthologs of the human CETP gene have been described in 217 organisms (Gene ID: 1071, <https://www.ncbi.nlm.nih.gov/gene/1071>). There is a major CETP gene alternative splicing product that may occur at high frequency¹¹. The alternatively spliced product, missing exon 9, is in-frame, and the loss of 60 amino acids in the central region of the protein leads to its inactivation¹². Cotransfection of wild-type (full length) and exon 9-deleted constructs significantly reduces wild-type secretion and activity^{12,13}.

Structurally, CETP belongs to the BPI-like protein family, which includes bactericidal/permeability-increasing protein (BPI), LPS (lipopolysaccharide)-binding protein (LBP), phospholipid transfer protein (PLTP) and CETP. While BPI and LBP are known to be involved in innate immunity against bacteria through their ability to sense lipopolysaccharides, PLTP and CETP are characterized by lipid exchange among plasma lipoproteins¹⁴. These proteins are also classified within a large family of extracellular and intracellular membrane contact site proteins that contain the tubular lipid binding (TULIP) domain¹⁵.

Reverse Cholesterol Transport

Much of the scientific interest in CETP arises from the fact that it causes a reduction in HDL cholesterol plasma levels. A well-documented negative

correlation between plasma concentrations of HDL and cardiovascular risk has been established since the late 1970s^{16,17}. Although the protective mechanisms of HDL were not known at that time, the concept that it was the main player in a process called reverse cholesterol transport (RCT) was emerging. In this process, first proposed by Glomset¹⁸, HDL facilitates the uptake of cholesterol from peripheral tissues and its transport to the liver for catabolism and excretion, thus avoiding accumulation of cholesterol in the plasma compartment and hence in the arteries (**Figure 2**). In theory, CETP may play a dual role in RCT. It may add an alternative route for delivering cholesterol to the liver via LDL receptor and LDL-related receptor protein (LRP) pathways (often called indirect RCT). However, if these pathways are malfunctioning, CETP activity results in increased LDL cholesterol levels and a risk of atherosclerosis development (**Figure 2**).

The engineering of CETP transgenic mice in the early 1990s^{19,20,21} was very useful to study its functions, gene expression regulation and susceptibility to diseases. In humans, CETP mRNA is mostly expressed in the liver, spleen and adipose tissue⁶. These data are confirmed in the Human Protein Atlas that analyzed 27 tissue samples from 95 human individuals²² and included lymph nodes and placenta as high CETP-expressing tissues. Hepatic synthesis is the major source of CETP found in the plasma of primates²³, with Kupffer cells being the predominant source in humans²⁴. Human CETP transgenic mice expressing a natural promoter-driven CETP minigene reproduced a similar human pattern of tissue expression²⁰. Thus, it was possible to study gene regulation in this model. For instance, CETP gene expression was shown to be upregulated by dietary cholesterol^{20,25,26}, thyroid hormones²⁷, fish oil and fibrate treatment²⁸, while it is downregulated by corticosteroids²⁹ and hyperinsulinemia³⁰. When CETP is expressed in hyperlipidemic atherosclerosis-prone models, such as the LDL receptor knockout mouse or apo E knockout mouse, an acceleration of diet-induced atherosclerosis is observed^{31,32,33}. On the other hand, other experimental mouse model data support the concept that CETP may have a protective role against atherosclerosis in conditions where LDL receptor function is preserved. For instance, CETP expression decreased atherosclerotic lesions in hypertriglyceridemic mice³⁴, when cholesterol esterification rates were high due to LCAT overexpression^{35,36}, in castrated mice^{37,38}, in diabetic mice^{39,40} and in SR-BI knockout mice^{41,42}.

Human genetic studies

The importance of plasma CETP in lipoprotein metabolism was long ago demonstrated by the discovery of CETP-deficient subjects with marked hyperalphalipoproteinemia in the Japanese population⁴³. Among several mutations of the CETP gene, two are common mutations in that population: an intron 14 splicing defect (Int14 + 1 G --> A) and an exon 15 missense mutation (D442G). Although elevated levels of HDL cholesterol are a marker for protection against atherosclerosis, subjects with CETP deficiency show a variety of abnormalities in the composition and function of HDL that impair RCT⁴⁴. Epidemiological studies in Japanese Americans living in Hawaii and Japanese in the Omagari area of Japan, where the intron 14 splicing defect is markedly frequent, have shown a relatively increased incidence of coronary atherosclerosis in CETP deficiency^{45,46}. On the other hand, the TaqIB polymorphism-B2 allele, with low CETP mass and moderate increases in HDL cholesterol, has been associated with a decreased risk for coronary heart disease in many studies, including the Framingham Offspring Study^{47,46}. However, controversy remains, since subsequent reports of the Framingham Heart study and Honolulu Heart Program presented discrepant results⁴⁸. A more recent meta-analysis confirmed an association between the TaqIB polymorphism and the risk of myocardial infarct. Their study suggests that the B2B2 genotype of the CETP TaqIB polymorphism is a protective factor against the development of myocardium infarct⁴⁹. However, not all CETP-reducing polymorphisms are protective. A large meta-analysis of selected studies with 3 common and 3 uncommon CETP polymorphisms evaluated the associations of CETP genotypes, phenotypes, lipid levels, and coronary risk. The authors concluded that three CETP genotypes that are associated with moderate inhibition of CETP activity (and, therefore, modestly higher HDL-C levels) show weak (but significant) inverse associations with coronary risk, compatible with the expected reductions in risk for equivalent increases in HDL-cholesterol concentration⁵⁰. Another meta-analysis suggests that two (out of seven) CETP polymorphisms (rs708272 [C>T] and rs1800775 [C>A]) may contribute to myocardium infarct susceptibility, especially among Caucasians⁵¹. Thus, it seems that the associations between CETP levels (due to

gene polymorphisms) and cardiovascular diseases may be population-specific, highly dependent on the phenotype (level of CETP activity, LDL and HDL concentration/composition) and highly influenced by environmental factors.

Possible new function of CETP: Anti-inflammatory

Similar to its family members, BPI and LBP, CETP may have an anti-inflammatory function that may be relevant not only for atherogenesis but also for immune responses⁸. Experimentally, inhibition of CETP with torcetrapib did not reduce atherosclerosis beyond the statin effect but induced more proinflammatory lesions in hypertriglyceridemic apoE3Leiden/CETP-expressing mice⁵². Normolipidemic human CETP-expressing mice were protected from mortality induced by bacterial lipopolysaccharide acute infusion⁵³ or polymicrobial sepsis induced by cecal ligation and puncture⁵⁴. Regarding humans, the first large-scale trial with the CETP inhibitor torcetrapib was interrupted because of increased mortality due to several reasons, including infections (22% of noncardiovascular deaths)⁵⁵. In addition, the mortality rate of patients with sepsis correlated with a reduction in plasma CETP concentrations⁵⁶. However, this issue is still enigmatic. A recent report described that a gain-of-function variant (s1800777-A) of CETP promotes a profound reduction in HDL levels and reduced survival in patients with sepsis when compared with noncarriers⁵⁷. Therefore, CETP anti-inflammatory effects may be a direct nonlipid transporting function or indirectly mediated by modulation of HDL size, composition and concentration⁵⁸.

Does CETP have an intracellular function?

As a lipid binding and transport protein, hypothetically, CETP may play an intracellular physiological role.

Zhang and collaborators showed that transient transfection of CETP cDNA into COS-7 cells induced higher cholesterol efflux compared with mock-transfected cells, while lipid uptake was not affected. Conversely, the efflux of free cholesterol from macrophages obtained from CETP-deficient subjects was significantly decreased compared with that from normal subjects⁵⁹. These data

suggest that local intracellular CETP expression in macrophages plays an anti-atherogenic function, facilitating the removal of cholesterol from the cells.

Because the protein product of the major alternative splice variant of CETP (exon 9 deleted) is retained within the endoplasmic reticulum (ER)¹², Lira and collaborators studied whether the expression of CETP variants could induce ER stress¹³. Transient CETP transfections were performed in a human liposarcoma cell line (SW872) and a human embryonic kidney cell line (HEK293S). Surprisingly, not only the alternative spliced variants of CETP but also the full-length protein expression caused an induction of genes linked to the ER stress response¹³. Thus, although CETP is a secreted protein, intracellular CETP plays a complex role in modulating ER stress or the unfolded protein response.

Morton and collaborators have shown that CETP expression modulates cholesterol and triglyceride homeostasis in the SW872 human liposarcoma cell line. They showed that short-term partial inhibition of CETP in these cells using antisense oligonucleotides induces a phenotype characterized by inefficient mobilization of CE stores leading to CE accumulation⁶⁰. In subsequent studies, CETP was chronically repressed with stably transfected oligonucleotides. CETP-deficient cells had decreased CE and TG biosynthesis and inefficient CETP-mediated translocation of CE and TG from the ER to their site of storage^{61,62}. On the other hand, when they stably overexpressed full-length CETP, SW872 cells accumulated 50% less TG due to a decrease in TG synthesis and a higher TG turnover rate, resulting in the formation of smaller and more metabolically active lipid droplets⁶³. In agreement, Zhou and collaborators previously showed that transgenic mice expressing CETP under the control of an adipocyte-specific promoter (aP2) exhibited decreased adipocyte lipid content and size⁶⁴. More recently, Izem et al. showed that exon 9-deleted CETP (the nonsecreted isoform) inhibits full-length CETP synthesis and promotes cellular triglyceride storage⁶⁵.

CETP inhibition as a target to decrease cardiovascular diseases

From most human and experimental studies, it is clear that CETP is important clinically and has been a target for drug development. Dozens of review articles on CETP inhibition have been published in the last 5 years, most of them debating the viability of this strategy because of minor or no benefits of the tested

molecules in reducing cardiovascular events so far. Despite the setbacks, the hypothesis that CETP inhibitors are anti-atherogenic in humans is still being tested. A summary of the results of the main trials with CETP inhibitors is presented in Box 1 and will be discussed below.

The concept that *in vivo* CETP inhibition could be anti-atherogenic was experimentally demonstrated in rabbits. CETP was inhibited with antisense oligodeoxynucleotides against CETP, reducing CETP mass and increasing HDL cholesterol (HDL-C), resulting in a reduced aortic lesion area⁶⁶. In addition, anti-CETP immunotherapy in rabbits was able to reduce CETP activity, increase HDL, decrease LDL, and diminish atherosclerotic lesions⁶⁷.

TORCETRAPIB

The first pharmacological molecule designed to inhibit CETP was torcetrapib. In two trials, ILLUSTRATE and ILLUMINATE, patients received atorvastatin + torcetrapib or atorvastatin alone and were followed for 12 or 24 months^{55,68}. Compared to atorvastatin monotherapy, torcetrapib + atorvastatin was able to increase HDL-C (61-72%) and decrease LDL-C (20-25%). However, an important side effect was the elevation of systolic blood pressure by approximately 5 mm Hg. There was a small or no favorable effect on atheroma volume⁶⁸. Because torcetrapib-treated patients showed an increased number of cardiovascular events and death from both cardiovascular and noncardiovascular causes⁵⁵, the clinical trial was terminated prematurely. The increase in aldosterone levels and blood pressure were considered off-target toxicity rather than a CETP inhibition effect *per se*, increasing expectations for further generation of CETP inhibitors.

DALCETRAPIB

The second molecule to go on to clinical trial was dalcetrapib (dal-OUTCOMES). Although the increase in systolic blood pressure was modest, dalcetrapib was less efficient at increasing HDL-C and reducing LDL-C, so the risk of major cardiovascular outcomes was not significantly altered. Dalcetrapib is considered a relatively weak inhibitor of CETP, meaning that a more potent CETP inhibitor could still be effective regarding clinical benefits in cardiovascular diseases (CVD)⁶⁹.

EVACETRAPIB

A potent CETP inhibitor, evacetrapib, was then evaluated in the ACELLERATE trial⁷⁰. Indeed, evacetrapib caused marked favorable changes in the lipoprotein profile, increasing HDL-C by approximately 130% and reducing LDL-C by 37% compared to placebo. However, there were no significant benefits for CVD risks and events, and the trial was stopped due to futility at 28 months of treatment. Some could raise the possibility that longer treatment could show CVD improvements.

ANACETRAPIB

The most successful CETP inhibitor to date is anacetrapib. It in fact reduced the incidence of major coronary events⁷¹. Anacetrapib was added to intensive statin treatment in the REVEAL trial, a much larger and longer trial than the previous ones. Patients were followed up for 4 years, and at midpoint, HDL-C was increased by 104% in the anacetrapib group. The incidence of the primary outcome was reduced in the anacetrapib group (10.8% vs. 11.8% in the placebo group). Although there was no significant difference between groups during the first year of follow-up, the incidence of major coronary events after one year was significantly lower in the anacetrapib group (rate ratio, 0.88; 95% CI, 0.81 to 0.95; $P = 0.001$). Anacetrapib is a highly lipophilic drug that accumulates in adipose tissue, explaining its prolonged elimination profile⁷². No serious adverse events were identified, and there was only a slightly higher blood pressure (0.7 mm Hg) in the group of patients receiving anacetrapib. Another unexpected good finding of anacetrapib was its association with a lower incidence of new-onset diabetes cases. A recent meta-analysis of CETP inhibitor trials showed that CETP inhibitor therapy was associated with a significant 12% reduction in the incidence of diabetes and concluded that the improvement in glucose metabolism is at least in part related to the increase in HDL-C concentration⁷³.

Although CETP inhibitors are expected to increase HDL-C levels, their impact on reducing LDL-C has gained special attention. In the anacetrapib trial, LDL-C levels were reduced by 40%, as indicated by the 'direct method' or by 17% when measured by beta-quantification. This discrepancy discloses the importance of understanding what different methods for LDL cholesterol quantification truly quantify. It is important to discover differential inhibitor effects across the whole

spectrum of atherogenic apoB-containing lipoproteins⁷⁴. CETP inhibition may also reduce the concentrations of triglyceride-rich remnant lipoproteins rather than affect size-specific LDL particles⁷⁵. Regarding the mechanism of action, anacetrapib reduces LDL-C levels by increasing its catabolism, while the LDL-apoB-100 production rate is unchanged⁷⁶.

TA-8995 (OBICETRAPIB)

Another promising CETP inhibitor compound is TA-8995 (Obicetrapib), which is well tolerated and shows beneficial effects on lipid and apolipoprotein profiles in healthy subjects and patients with mild dyslipidemia^{77,78}. TA-8995 was tested in a randomized, double blind, placebo-controlled phase 2 trial (TULIP)⁷⁸. The safety and efficacy of TA-8995 were tested as a monotherapy and combined with statins. This CETP inhibitor reduces LDL cholesterol and apoB levels by 45% and 34%, respectively, conferring an additional decrease in LDL-C in combination with statins. HDL-C and apoA-1 increased up to 179% and 63%, respectively. TA-8995 also reduces lipoprotein(a) ranging from 27% to 37%⁷⁸. TA-8995 is now registered in a new clinical trial (*ClinicalTrials.gov identifier (NCT number): NCT02241772*) to evaluate its effects on subjects with elevated Lp(a). A subsequent study advanced the possible mechanisms of TA-8995. Plasma samples from TULIP trial patients treated with TA-8995 were shown to dose-dependently increase total and ABCA1-specific cholesterol efflux capacity from the J774 macrophage cell line. These findings suggest that TA-8995 not only increases HDL-C and pre-Beta1-HDL particle levels but also promotes the functional properties of these particles. Whether these changes in HDL particle composition and functionality have a beneficial effect on cardiovascular outcome requires formal testing⁷⁹. Thus, a cardiovascular disease outcome trial with TA-8995 is still needed to translate these effects into a reduction in cardiovascular disease events.

CONCLUDING REMARKS

Major advances in understanding CETP biology were obtained after protein purification, gene cloning and molecular structure resolution. Studies in animal models have elucidated CETP functions, gene expression regulation

and relationships with diseases. Apart from its HDL reducing systemic action, intriguing reports raise the possibility that CETP may have relevant local and novel nonlipid transfer functions. Animal and human studies suggest that CETP is important clinically and worthy as a target for drug development. The pros and cons of inhibiting CETP were discussed. Despite major setbacks in clinical trials, the hypothesis that CETP inhibitors are anti-atherogenic in humans is still being tested.

BOX 1: Main CETP inhibitor trials

Torcetrapib (Pfizer)

Atorvastatin + Torcetrapib (vs. atorvastatin only)

Trial ILLUSTRATE

24-month follow-up

HDL-C increased by 61%

LDL-C decreased by 20%

Increase in systolic blood pressure of 4.6 mm Hg

From small to no favorable effect on atheroma volume

(Nissen et al., 2007)⁶⁸

Trial ILLUMINATE

12-month follow-up

HDL-C increased by 72.1%

LDL-C decreased by 24.9%

Increase in systolic blood pressure (5.4 mm Hg)

Increase in apo-CIII and aldosterone levels

Increase in mortality by CVD, cancer and infections/sepsis

(Barter et al., 2007)⁵⁵

Dalcetrapib (Hoffman-La Roche)

Trial: dal-OUTCOMES

Dalcetrapib (vs. placebo)

31-month follow-up

Increased HDL (25%), blood pressure (0.6 mm Hg) and C-reactive protein (hsPCR)

No changes in LDL and aldosterone

No benefits on CVD events/mortality.

Possible benefits in genetically defined population (ADCY9 gene, adenylate cyclase, AA allele)

(Schwartz et al., 2012)⁶⁹

Evacetrapib (Eli Lilly)

Trial: ACCELERATE

Evacetrapib (vs. placebo)

28-month follow-up

HDL-C increased by 132%

LDL-C decreased by 37%

Increased cholesterol efflux

Increased blood pressure (1.2 mm Hg) and hsPCR

No CVD benefits in 28 months

(Lincoff et al., 2017)⁷⁰

Anacetrapib (Merk)

Trial: **REVEAL**

Anacetrapib (vs. placebo) in patients receiving intensive atorvastatin therapy
4.1-year follow-up

HDL-C increased by 104% (midpoint)

LDL-C decreased by 41% (midpoint)

Slightly higher blood pressure (0.7 mm Hg)

No significant differences in the risk of death, cancer, or other serious
adverse events

Associated with a lower incidence of new-onset diabetes (11%)

Lower incidence of major coronary events (10.8 vs 11.8% CVD events)
(HPS3/TIMI55–REVEAL Collaborative Group, Bowman L, et al., 2017)⁷¹

TA-8995 (obicetrapib)- Funded by Dezima and undertaken by Xention

Trial: **TULIP**

Nine treatments with TA-8995 alone and combined with statins

Analyses at 12 weeks of treatment

LDL-C reduced by 45.3% (apoB reduced by 33.7%)

HDL-C increased by 179% (apoA-1 increased by 63.4%).

Combined with statins: decrease in LDL-C from 39.8% to 50.2%
(Hovingh et al., 2015)

Increased pre β -HDL and cell cholesterol efflux
(van Capelleveen et al., 2016)⁷⁹

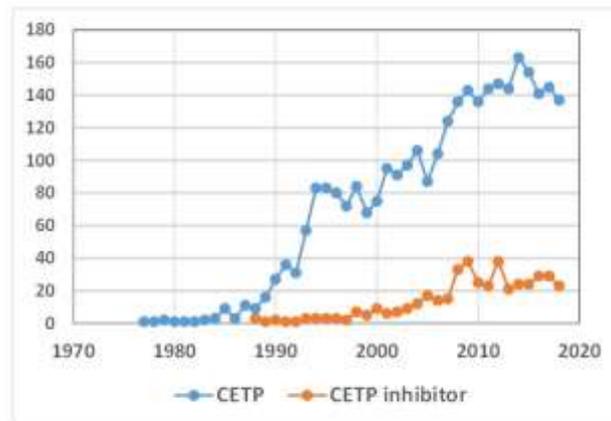


Figure 1 – Number of scientific publications through the years with the key words cholesteryl-ester-transfer-protein or CETP (blue line) and cholesteryl-ester-transfer-protein inhibitor or CETP inhibitor (orange line). PubMed searches were performed on January 13, 2020, limiting the results by the presence of the key words in the field: Title and Abstract.

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