The effect of cholesteryl ester transfer protein overexpression and inhibition on reverse cholesterol transport

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Aims Cholesteryl ester transfer protein (CETP) has a well-established role in lipoprotein metabolism, but the effect of its overexpression or inhibition on the efficiency of reverse cholesterol transport (RCT) is unclear.

Methods and results Neither overexpression of CETP nor treatment with CETP inhibitor Torcetrapib of RAW 264.7 macrophages or HepG2 hepatocytes affected cholesterol efflux in vitro. Overexpression of CETP or treatment with Torcetrapib, respectively, stimulated or inhibited HDL cholesteryl ester uptake by HepG2 but not by RAW 264.7 cells. When RAW 264.7 cells transfected with CETP or ATP binding cassette transporter A1 (ABCA1) were injected intraperitoneally into mice, cholesteryl egress from macrophages was elevated for ABCA1- but not for CETP-transfected macrophages. Systemic expression of CETP in mice by adenoviral infection stimulated egress of cholesteryl to plasma and liver without affecting HDL levels. Treatment with Torcetrapib did not affect appearance of macrophage cholesteryl in plasma and liver, but inhibited its excretion into feces. Treatment of hamsters with Torcetrapib led to elevation of HDL cholesterol, an increase in the capacity of plasma to support cholesterol efflux, and increased egress of cholesteryl from macrophages to plasma and feces in vivo.

Conclusion Both increased (mice study) and decreased (hamster study) CETP activity could result in enhanced RCT.

KEYWORDS Cholesteryl ester transfer protein; Reverse cholesterol transport; CETP inhibition; Lipoproteins; Cholesterol; Atherosclerosis

1. Introduction

The success in treating dyslipidaemia reducing risk of atherosclerosis and consequently that of cardiovascular diseases has delivered a 30% reduction in the risk of cardiovascular disease for the last three decades. This success was mainly based on limiting cholesterol flow to the vessel wall by statins and/or dietary intervention. Further progress in treating atherosclerosis critically depends on enhancing the pathway responsible for the flow of cholesterol in the opposite direction, reverse cholesterol transport (RCT). A number of approaches have been developed to stimulate RCT with one of more advanced is use of the inhibitors of cholesteryl ester transfer protein (CETP).1

CETP mediates transfer of cholesterol from antiatherogenic HDL to atherogenic TG-rich apoB-containing lipoproteins. Inhibition of CETP consistently and significantly elevates HDL cholesterol levels in animal models and in human studies.2,3 There were early indications that this is associated with reduced risk of atherosclerosis in animals and humans;4 however, disappointing results of the recent clinical trials2,5 emphasized a complexity of connection between HDL levels and its anti-atherogenic effect. CETP is also a part of remodelling cascade required for the effective interaction of HDL with SR-B1 and selective uptake of cholesteryl esters by liver.6 Further, CETP may be involved in intracellular cholesterol metabolism. It was demonstrated that CETP inhibitor increased apoA-I synthesis,7 while overexpression of CETP stimulated cholesterol efflux8 and selective uptake of cholesteryl esters from HDL.9 Complexity of CETP involvement in cholesterol and lipoprotein metabolism requires further investigation of the consequences of CETP inhibition on various levels and its impact on lipid metabolism. Two issues are of primary importance. First is whether inhibition of plasma CETP would also inhibit cellular CETP and if so what would be the effect of this intervention on intracellular lipid metabolism. Second, how changes in HDL composition induced by inhibition of CETP would impact on...
HDL functionality, most importantly on its ability to support cholesterol efflux and RCT in vitro and in vivo.

In this study, we investigate how CETP overexpression and inhibition impact on cholesterol efflux and RCT in vitro and in vivo in two species with very dissimilar HDL metabolism, hamster, and mice. We conclude that contribution of intracellular CETP to lipoprotein metabolism is limited. This study also revealed that the impact of CETP on RCT is complex since both increased (in mice) and decreased (in hamsters) CETP activity resulted in enhanced rate of RCT.

2. Methods

2.1 Cells

RAW 264.7 and J774 mouse macrophage cells and THP-1 human monocyte-macrophages were maintained in RPMI 1640 medium; HepG2 human hepatoma cells and HeLa cells were maintained in DMEM; all media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L sodium pyruvate, and 2 mmol/L glutamine. RAW 264.7 macrophages, and HepG2 cells were transiently transfected with hCETP-pCDNA3.1+ as previously described.10

2.2 Generation of cholesteryl ester transfer protein recombinant adenoviruses and viral production in mammalian cells

The pAdTrack-CMV vector recombined with CETP has a CMV promoter and a polyadenylation site. It also included a GFP gene with a separate CMV promoter. Generation of CETP recombinant adenovirus and production of adenovirus in mammalian cells were performed as previously reported.11 To propagate virus, HEK 293 cells were infected with 600 μL of virus suspension. Virus was collected 2-3 days post infection and purified by centrifugation of cesium chloride gradient.

2.3 Cholesterol efflux and selective uptake of cholesteryl esters

To label cellular cholesterol, cells were incubated in serum-containing medium with [3H]cholesteryl oleate (specific radioactivity 1.81 TBq/mmol, final radioactivity 74 KBq/mL) for 48 h in a CO2 incubator. After labelling, cells were washed and further incubated for 18 h in serum-free medium containing LX-10 901317 (1 μmol/L). Cells were then washed and incubated for 2 h at 37°C in serum-free medium containing either lipid-free apoA-I (30 μg/mL) or 3% plasma. Cholesterol efflux was expressed as a percentage of labelled cholesterol transferred from cells to the medium.

HDL was labelled with [3H]cholesteryl oleate according to Sattler et al.12 The uptake of HDL-derived cholesteryl esters was determined as described by Vassiliou et al.13

2.4 In vivo model of cholesterol efflux

2.4.1 Mice

Cholesterol efflux in vivo was measured as described by Rader14,15 with modifications. Briefly, transfected RAW 264.7 cells were radio-labelled and loaded with cholesterol by incubation for 48 h with 1.1 GBq/mL [3H]cholesterol and acetylated LDL (100 μg/mL) for 48 h. Cells were washed, incubated for 24 h in serum-free medium and resuspended at a concentration of 107 cells/mL. Cells were injected intraperitoneally into male C57BL/6 mice (2×106 cells containing 5×105 cpm per mice, each group consisted of 6 animals). After 24 h, mice were euthanized and blood, liver, and feces collected. Aliquots of plasma were counted and cholesterol from liver and feces was extracted by Folch method. Plasma lipid analysis was performed by FPLC. To assess distribution of labelled cholesterol between HDL and non-HDL subfractions of plasma, apoB-containing lipoproteins were precipitated with PEG8000 according to Chiba et al.16 and the radioactivity in the supernatant and the pellet was counted.

For systemic expression of CETP C57BL/6 mice were intravenously injected with 3 mg/kg/day. Experiments were conducted 10 days after infection or introduction of Torcetrapib.

2.4.2 Hamsters

J774A.1 cells were radiolabelled and loaded with cholesterol by incubation for 48 h with 4 μCi/mL [3H] cholesterol and acetylated LDL (final concentration 50 μg/mL) for 48 h. Cells were washed, incubated for 24 h in serum-free medium, harvested, and resuspended at a concentration of 107 cells/mL.

For a period of 5 days preceding treatment, Golden Syrian hamsters (Lak:LVG(SYR)) received a defined portion of 10 g chow diet every morning. The daily serving of 10 g was mixed with 10 mL of water to produce a paste. This daily portion was subsequently mixed with torcetrapib powder at the dose of 3 or 30 mg/kg/day for 12 days. Hamsters consistently ate 90-95% of their daily serving. On day 10 of the treatment period, radiolabelled cells were injected intraperitoneally into hamsters (2×106 cells containing 5.6×108 cpm per hamster). After 72 h hamster were euthanized and blood, liver, and feces collected as described for mice.

Both mice and hamster studies were approved by the appropriate Animal Ethics Committees. The study conforms with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996).

2.5 Analytical methods

CETP activity was determined using fluorometric assay (ROAR) according to the manufacturer instructions. Plasma lipoproteins of individual plasma were separated and identified by size-exclusion Superose-6 gel Chromatography [FPLC, AKTA system (Pharmacia)]. Total cholesterol in the fractions was quantified using a fluorometric assay. Lipoprotein distribution was calculated assuming a Gaussian distribution for each peak, using a non-linear, least-squares curve fitting procedure to calculate the area under the curve. Total and HDL cholesterol levels and triglyceride levels were also analysed by fluorometric assay.

2.6 Statistical analysis

All experiments were reproduced two to four times and representative experiments are shown. Unless otherwise indicated, experimental groups consisted of quadruplicates; means±SEM are presented. The Student’s t-test was used to determine statistical significance of the differences.

3. Results

3.1 Heterologous expression of cholesteryl ester transfer protein

Transfection of RAW 264.7 mouse macrophages and HepG2 human hepatoma cells with CETP plasmid resulted in expression of CETP as determined by the appearance of the 70 kDa protein reacting with anti-CETP antibodies in both RAW 264.7 (Figure 1A inset) and Hep G2 cells (Figure 1B inset). The apparent molecular weight was slightly lower than that of plasma CETP (74 kDa) probably due to different post-translational modifications of the protein. Transfection with the plasmid carrying reporter
gene instead of CETP revealed that over 75% of cells were transfected. The activity of CETP also increased in both RAW 264.7 cells (Figure 1A) and HepG2 cells (Figure 1B). It is of interest that untransfected RAW 264.7 cells did have some CETP activity despite being derived from mice, a species that does not express CETP. It is not clear however if this activity belongs to CETP or is a result of activity of another protein, such as phospholipid transfer protein. A small proportion of CETP has been secreted to the medium during 48 h incubation (Figure 1), but no detectable amount of CETP was found in the medium after 2 h incubation (a duration of the efflux incubation).

### 3.2 Cholesterol efflux and cholesteryl ester uptake in vitro

To assess the effect of intracellular overexpression or inhibition of CETP on cholesterol efflux, RAW 264.7 macrophages or HepG2 hepatoma cells were either transiently transfected with CETP or treated for 24 h with CETP inhibitor Torcetrapib (200 nmol/L). Neither overexpression of CETP nor inhibition with Torcetrapib changed the rate of cholesterol efflux from RAW 264.7 macrophages to human apoA-I or 3% human plasma over 2 h as assessed described in ‘Methods.’ Open bars, mock transfected cells; filled bars, CETP transfected cells. Means ± SEM of quadruplicate determinations are shown; *P < 0.01 vs. samples without CETP overexpression; #P < 0.05 vs. samples without Torcetrapib.

by incubation for 18 h with 50 μg/mL of acetylated LDL; transfection of cholesterol-loaded cells with CETP had no effect on cholesterol efflux to apoA-I or plasma (not shown). Essentially, the same was observed when HepG2 cells were either transfected with CETP or treated with Torcetrapib (Figure 1D). In some experiments, both RAW 264.7 and HepG2 cells were transfected with CETP and treated with Torcetrapib at the same time, Torcetrapib was kept in the incubation mixture during the efflux incubation, however again there was no change in cholesterol efflux to apoA-I due to this treatment (not shown). It was recently demonstrated that CETP can mediate selective uptake of HDL-cholesteryl esters by HepG2 cells (A) or RAW 264.7 macrophages (B). HepG2 hepatoma cells (A) or RAW 264.7 macrophages (B) were transiently transfected with CETP or mock transfected as described in ‘Methods.’ Both transfected and mock-transfected cells were incubated for 18 h in the presence of indicated concentrations of Torcetrapib. Selective uptake of HDL-cholesteryl esters was assessed as described in ‘Methods.’ Open bars, mock transfected cells; filled bars, CETP transfected cells. Means ± SEM of quadruplicate determinations are shown; *P < 0.01 vs. samples without CETP overexpression; #P < 0.05 vs. samples without Torcetrapib.

It was recently demonstrated that CETP can mediate selective uptake of HDL-cholesteryl esters by liver.9 We tested the effect of CETP overexpression on HDL-CE uptake by HepG2 hepatocytes and RAW 264.7 macrophages. Consistent with findings of Gauthier et al.,8 overexpression of CETP in HepG2 cells enhanced HDL-CE uptake, while CETP inhibitors reduced it (Figure 2A). At low Torcetrapib concentration, the effect of CETP overexpression on HDL uptake was still detectable, while higher concentration of Torcetrapib had completely eliminated the effect of CETP overexpression. When tested in RAW 264.7 cells neither...
CETP overexpression nor inhibition affected HDL-CE uptake (Figure 2B). The lack of the effect of inhibition may be due to the lack of CETP in RAW 264.7 cells (Figure 1).

3.3 Cholesterol efflux ex vivo

Cholesterol efflux depends both on the ability of cells to release cholesterol and the ability of plasma to support cholesterol efflux. To test the latter experiments were conducted using human and animal plasmas. First, human plasma was incubated in the presence or absence of Torcetrapib (200 nmol/L) for up to 24 h at 37°C. Presence of Torcetrapib did not affect the ability of plasma to support cholesterol efflux from RAW 264.7 macrophages (Figure 3A). Next, hamsters, species that express CETP, were given Torcetrapib (3 mg/kg) or placebo for 7 days. Treatment of hamsters with Torcetrapib led to a predictable elevation of their total plasma cholesterol and plasma HDL-C levels, the latter was due to elevated levels of large HDL, their levels almost quadrupled (Table 1). The ability of 3% hamster plasma to support cholesterol efflux from macrophages has almost doubled when plasma from animals treated with Torcetrapib was compared with that from animals treated with placebo (Figure 3B, left panel). Further, there was a clear correlation between plasma HDL-C levels and the ability of plasma to support cholesterol efflux ($r = 0.8$, $P < 0.02$) (Figure 3B, right panel). This finding indicates that the enhanced ability of plasma from animals treated with Torcetrapib to support cholesterol efflux was entirely due to elevated HDL concentrations.

To establish a specific pathway responsible for the increase of cholesterol efflux to plasma, we assessed cholesterol efflux from HeLa cells transiently transfected with ABCA1, ABCG1, or mock-transfected; the expression of the transporters was verified by western blot (Supplementary material online, Figure S1). Cholesterol efflux from cells transfected with ABCA1 to lipid-free apoA-I and 3% hamster plasma was three-fold and 25% higher compared to mock-transfected cells. Cholesterol efflux from ABCG1 transfected cells to isolated HDL was 30% higher, but the efflux to 3% hamster plasma was identical to that from mock-transfected cells. ABCA1-dependent cholesterol efflux was defined as a difference between cholesterol effluxes from ABCA1-transfected cells vs. mock-transfected cells. There was no difference in ABCA1-dependent cholesterol efflux to plasma from hamsters treated with Torcetrapib and plasma from control animals (Figure 3C). There was also no difference between plasma from control hamsters and hamsters treated with Torcetrapib in efflux from HeLa cells transfected with ABCG1 (not shown). Thus, the

Figure 3 The effect of CETP inhibition on cholesterol efflux ex vivo. (A) Human plasma was incubated for the indicated periods of time in the presence or absence of 200 nmol/L of Torcetrapib and then used as an acceptor at concentration of 3% in cholesterol efflux assay as described in ‘Methods.’ (B) Hamsters were fed with Torcetrapib (3 mg/kg) for 7 days. Plasma was taken and used as an acceptor at concentration of 3% in cholesterol efflux assay as described in ‘Methods.’ On the right panel, a correlation between HDL-C and cholesterol efflux is shown; *$P < 0.01$. (C) Cholesterol efflux to 3% plasma from HeLa cells transfected with ABCA1 minus cholesterol efflux from mock-transfected HeLa cells. (D) Mice were infected with CETP adenovirus as described in ‘Methods’; CETP in plasma was detected by Western Blot and CETP activity was assessed as described in ‘Methods’ and compared with human and hamster plasma. (E) Mice were infected with CETP adenovirus as described in ‘Methods’ and fed with Torcetrapib (3 mg/kg) or control chow. Plasma was taken on day 10 and used as an acceptor at concentration of 3% in cholesterol efflux assay as described in ‘Methods.’ Means ± SEM of quadruplicate determinations are shown.
increased cholesterol efflux to plasma from animals treated with Torcetrapib was due to higher levels of HDL in the plasma of these animals; and was not ABCA1 or ABCG1-specific.

In the next series of experiments, mice were infected with either human CETP adenovirus or empty adenovirus. Introduction of human CETP into mice without other genetic manipulations (such as concomitant introduction of human apoA-I or k/O of LDL receptor) was shown to only minimally affect HDL-C concentration in this species.\(^{17}\) This makes the model suitable for investigating the effects of CETP and CETP inhibition that are independent from changes in HDL-C concentrations. Mice infected with CETP adenovirus had time-dependent increase of both CETP mass and activity in their plasmas (Figure 3D) as well as in the liver (Supplementary material online, Figure S2). Maximum CETP concentration in mouse plasma was reached after 10 days, while maximum of CETP activity was reached after 7 days after infection, 10 days post-infection mice were used in further experiments. Neither expression of CETP nor treatment with Torcetrapib caused statistically significant changes in plasma total or HDL cholesterol levels or triglyceride concentrations (Table 2), which is consistent with what was found by Hayek et al.\(^{17}\) No significant change in cholesterol efflux from RAW 264.7 macrophages to 3% plasma was observed in between plasma from control and CETP infected mice (Figure 3E). Torcetrapib did not affect the ability of plasma to support cholesterol efflux independently of whether animals were infected with CETP adenovirus or empty adenovirus (Figure 3E). Thus, introduction of CETP or its inhibitor without concomitant effect on HDL-C levels does not affect functionality of HDL toward cholesterol efflux.

### 3.4 Cholesterol efflux in vivo

To assess the effect of systemic CETP overexpression as well as overexpression of CETP in macrophages, in vivo model of cholesterol efflux previously described by Rader et al.\(^{14}\) was utilized.

First, the effect of overexpression of CETP in macrophages on cholesterol efflux in vivo was examined. RAW 274.1 cells were transiently transfected with CETP before being loaded and labelled with cholesterol and injected into intraperitoneal cavity of wild-type, uninfected mice. The amount of labelled cholesterol in plasma, liver, and feces (cholesterol and bile acid combined) was similar for CETP transfected and mock transfected cells (Figure 4). At the same time, transient transfection of macrophages with ABCA1 led to a substantial elevation of labelled cholesterol in plasma, liver, and feces (Figure 4). This finding is consistent with the in vitro data where cholesterol efflux was not affected by overexpression of CETP in macrophages. At the same time, overexpression of CETP in macrophages is apparently insufficient to introduce effective quantities of CETP into plasma.

Next, mice were infected with recombinant adenovirus encoding for human CETP as described above. Expression of human CETP resulted in elevation of the amount of labelled cholesterol in plasma, liver, and fecal bile acids (but not in fecal cholesterol) indicating that CETP enhanced RCT in this model (Figure 5). Notably, there was no change in HDL-C level in these mice (Table 2) indicating that these effects were not related to HDL levels. The ratio of HDL to non-HDL \(^{[3H]}\)cholesterol was sharply decreased in CETP-expressing mice indicating that CETP is active in these mice (Figure 5A). Feeding mice with Torcetrapib completely eliminated the effect of CETP expression on cholesterol distribution and efficiency of RCT (Figure 5). While Torcetrapib itself did not affect the amount of labelled cholesterol in plasma and liver, it reduced the amount of labelled cholesterol in feces (Figure 5C) as well as the ratio of HDL/non-HDL cholesterol in plasma (Figure 5A). The former effect of Torcetrapib may be connected with the inhibition of HDL-derived cholesteryl ester uptake shown in vitro. However, uninfected mice do not express CETP and this effect of Torcetrapib may be not related to its CETP inhibiting activity.

The effect of Torcetrapib was further tested in the hamster model, a species that naturally expresses CETP. The model is similar to the mouse model, except that J774 cells were used. RCT in mice vs. hamsters has not been directly compared, but it is known that the rate of whole body cholesterol synthesis decreases as body weight increases.\(^{18}\) Therefore, we also extended the experiments in hamsters to 72 h to account for an eventuality of hamster RCT being slower. In hamster, treatment with Torcetrapib leads to increase in HDL-C levels (Table 1). In contrast to mice, Torcetrapib treatment resulted in dose-dependent elevation of the amount of labelled cholesterol in plasma as well as the amount of labelled cholesterol and bile acids in the feces (Figure 6). The increase in the amount

#### Table 1 The effect of CETP inhibition by Torcetrapib (3 mg/kg) on plasma lipids in hamsters

<table>
<thead>
<tr>
<th>Lipid parameter</th>
<th>Control</th>
<th>Torcetrapib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>222 ± 35</td>
<td>266 ± 35</td>
</tr>
<tr>
<td>VLDL cholesterol (mg/dL)</td>
<td>9.2 ± 4.1</td>
<td>12.7 ± 4.0</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dL)</td>
<td>48.3 ± 15.5</td>
<td>47.5 ± 9.1</td>
</tr>
<tr>
<td>Total HDL (mg/dL)</td>
<td>165 ± 22</td>
<td>206.6 ± 32*</td>
</tr>
<tr>
<td>Small HDL (mg/dL)</td>
<td>144 ± 19</td>
<td>125 ± 11</td>
</tr>
<tr>
<td>Large HDL (mg/dL)</td>
<td>21 ± 12</td>
<td>81 ± 28*</td>
</tr>
</tbody>
</table>

Means ± SD (four hamsters) are shown.

\(^*P < 0.01\).

#### Table 2 The effect of CETP overexpression and inhibition on the levels of plasma lipids in mice

<table>
<thead>
<tr>
<th>Lipid parameter</th>
<th>Control (n = 15)</th>
<th>adCETP (n = 11)</th>
<th>Torcetrapib (n = 12)</th>
<th>adCETP+Torcetrapib (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>88 ± 17</td>
<td>100 ± 30</td>
<td>89 ± 13</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>81 ± 12</td>
<td>77 ± 15</td>
<td>80 ± 8</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>66 ± 3</td>
<td>56 ± 1</td>
<td>56 ± 6</td>
<td>70 ± 5</td>
</tr>
</tbody>
</table>

Means ± SD are shown.
CETP and lipid metabolism

of labelled cholesterol in plasma was entirely due to time-dependent increase of \([^{3}H]\)-cholesterol in HDL, but not in non-HDL fraction (Figure 6B and C); consequently the HDL to non-HDL ratio has almost doubled.

4. Discussion

CETP inhibitors are a new class of drugs aimed at increasing plasma concentrations of HDL to protect against development of atherosclerosis and reducing the risk of coronary heart disease. A number of CETP inhibitors were examined and all of those tested in animals and in clinical trials consistently showed their ability to significantly elevate plasma HDL and to decrease LDL levels, a change consistent with a reduction of the atherogeneity of lipoprotein profile. In rabbits, CETP inhibitors attenuated development of atherosclerosis. Recent surprising results of the clinical trials of Torcetrapib brought up a question of why the compound that was very effective in animals produced a negative outcome in humans. The complexity of the issue is confounded by the fact that CETP may be playing a role in a number of pathways and by possible ‘off-target’ effects of Torcetrapib. One possibility was that intracellular CETP plays a bigger role than anticipated role in cholesterol metabolism in macrophages and hepatocytes. Our findings argue against this possibility: overexpression or inhibition of intracellular CETP as well as inhibition of plasma CETP did not affect cholesterol efflux in macrophages and hepatocytes, a finding consistent with the report of Westerterp et al. Another possibility was that inhibition of CETP may affect HDL functionality. Again, our findings argue against this possibility: both in mice and in hamsters, the level of cholesterol efflux was fully explained by the variations of HDL-C levels implying that ‘specific activity’ of HDL is unchanged. Recent findings of Yvan-Charvet et al. demonstrated that functionality of HDL is even elevated toward ABCG1-dependent cholesterol efflux pathway also arguing that functionality of HDL is not impaired by inhibition of CETP. It must be recognized however that anti-atherogenic activity of HDL is not limited to its role in cholesterol efflux, other functions of HDL were not tested in this study and could be affected. A third possibility is that inhibition of CETP may have a positive effect through elevating HDL level and a negative effect through blocking an important path of cholesterol disposal via LDL receptors. The end result would be a balance between these two outcomes and could be greatly influenced by the exact metabolic circumstances. We tested the effects of CETP overexpression and inhibition in two animal models, mice and hamsters. Lipoprotein metabolism in both animals is different to that in humans allowing investigation of the effect of metabolic environment. Lipoprotein metabolism in mice is very dissimilar to that of humans: mice do not express CETP, HDL is the main lipoprotein fraction in murine plasma, murine HDL is monodisperse while it is polydisperse in humans, LDL-C levels in murine plasma are low. Overexpression of human CETP, which predominantly reacts with large LpAI particles, has a limited effect on HDL levels unless a genetic modification is introduced to alter distribution of HDL sub-species. In our experiments, overexpression of CETP in mice did not have a statistically significant effect on HDL-C level; however, CETP was active as witnessed by its activity in the plasma, changes in HDL/non-HDL ratio and by the fact that the effects were negated by Torcetrapib. We hypothesize that when HDL level was not reduced, but an alternative pathway of cholesterol disposal was introduced, the balance was shifted toward enhancing of RCT (Figure 7A), a finding consistent with that of Rader and co-workers. In these experiments, Torcetrapib reduced fecal cholesterol excretion, a finding consistent with our observations in vitro. This effect was observed independently of the presence of CETP indicating that it might be an ‘off-target’ effect of Torcetrapib unrelated to its CETP-inhibiting ability. Lipoprotein metabolism in hamsters is more similar to that of humans: hamsters do express CETP, although at

Figure 4 The effect of overexpression of CETP in macrophages on cholesterol efflux in vivo. RAW 264.7 macrophages were transfected or not with CETP or ABCA1, loaded with cholesterol, labelled with \([^{3}H]\)-cholesterol and injected intraperitoneally into mice. The appearance of label in plasma (A), liver (B), and feces (C) was assessed after 24 h as described in ‘Methods.’ Mean ± SEM (n = 6) are shown. *P < 0.01.

Figure 5 The effect of systemic overexpression or inhibition of CETP on cholesterol efflux in vivo in mice. RAW 264.7 macrophages were loaded with cholesterol, labelled with \([^{3}H]\)-cholesterol, and injected intraperitoneally into mice that were either infected with CETP adenovirus, fed with Torcetrapib (3 mg/kg), or both (6 animals per group). The appearance of the label in plasma (A), liver (B), cholesterol fraction of feces (C), and bile acid fraction of feces (D) was assessed after 24 h as described in ‘Methods.’ Table in (A) shows the ratio of cholesterol content in HDL to non-HDL fractions of plasma. Mean ± SEM (n = 6) are shown; *P < 0.01.
Lower levels than humans, and have polydisperse HDL. Inhibition of CETP in hamsters elevated HDL-C levels but did not affect non-HDL cholesterol suggesting a limited impact on the alternative pathway of cholesterol disposal. These changes would shift the balance toward enhancing RCT (Figure 7B). Our findings may be relevant for the situation when CETP inhibition was accompanied with aggressive lowering of LDL-C levels by statins, as was the case in recent trials.2,23 Statins increase the number of LDL receptors in the liver thus enhancing disposal of LDL-C, including cholesterol transferred to LDL from HDL by CETP, presumably making it a predominant pathway of cholesterol disposal. Inhibition of CETP blocks this transfer and diminishes availability of the acceptor, apoB-containing lipoproteins, thus reducing the amount of cholesterol disposed via this pathway (Figure 7C). Torcetrapib is likely to enhance the direct uptake pathway; the outcome is a balance between two opposing influences and the net effect is uncertain. Thus, the efficiency of CETP inhibitors may greatly depend on metabolic environment. For example, CETP inhibitors may be more effective in patients with isolated low HDL/high TG, where the primary target is elevation of HDL rather than lowering of LDL.

Supplementary material
Supplementary material is available at Cardiovascular Research online.
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