Expression of Cholesteryl Ester Transfer Protein in Mice Promotes Macrophage Reverse Cholesterol Transport

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Background—Cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from high-density lipoproteins to apolipoprotein (apo) B-containing lipoproteins and in humans plays an important role in lipoprotein metabolism. However, the role that CETP plays in mediation of reverse cholesterol transport (RCT) remains unclear. We used a validated in vivo assay of macrophage RCT to test the effect of CETP expression in mice (which naturally lack CETP) on macrophage RCT, including in mice that lack the low-density lipoprotein receptor or the scavenger receptor class B, type I.

Method and Results—A vector based on adeno-associated virus serotype 8 (AAV8) with a liver-specific thyroglobulin promoter was used to stably express human CETP in livers of mice and was compared with an AAV8-lacZ control vector. The RCT assay was performed 4 weeks after vector injection and involved the intraperitoneal injection of acetylated low-density lipoprotein cholesterol–loaded and 3H-cholesterol–labeled J774 macrophages in mice with plasma sampling at several time points, liver and bile sampling at 48 hours, and continuous fecal collection to measure 3H-sterol as an integrated readout of macrophage RCT. In apobec-1–null mice, CETP expression reduced plasma high-density lipoprotein cholesterol levels but significantly increased fecal 3H-sterol excretion. In low-density lipoprotein receptor/apobec-1 double-null mice, CETP expression reduced high-density lipoprotein cholesterol levels and had no effect on fecal 3H-sterol excretion. Finally, in scavenger receptor class B, type I–null mice, CETP expression reduced high-density lipoprotein cholesterol levels and significantly increased fecal 3H-sterol excretion.

Conclusion—The present results demonstrate that CETP expression promotes macrophage RCT in mice, that this effect is dependent on the low-density lipoprotein receptor, and that CETP expression restores to normal the impaired RCT in mice deficient in scavenger receptor class B, type I. (Circulation. 2007;116:1267-1273.)

Key Words: atherosclerosis ■ cholesterol ■ lipids ■ lipoproteins ■ macrophages

Cholesteryl ester transfer protein (CETP) is a 74-kDa hydrophobic plasma glycoprotein that has an established role in mediation of neutral lipid transport among lipoproteins.1 One net effect of CETP activity is a mass transfer of cholesteryl esters (CEs) from high-density lipoproteins (HDLs) to apolipoprotein (apo) B–containing lipoproteins in exchange for triglycerides. CETP plays a major role in lipoprotein metabolism in humans, as genetic deficiency of CETP is associated with markedly increased HDL cholesterol (HDL-C) and modestly reduced low-density lipoprotein cholesterol (LDL-C) levels.2 It has been suggested that the CETP pathway may also play an important role in reverse cholesterol transport (RCT), the process by which excess peripheral cholesterol is effluxed to HDL-based acceptors and ultimately returned to the liver for excretion in the bile and feces. Indeed, studies in humans have indicated that the majority of HDL CEs that are ultimately excreted in the bile are returned to the liver by apoB-containing lipoproteins and not HDL.3 The issue of whether CETP is important for normal RCT in humans is particularly relevant to the concept of pharmacological inhibition of CETP. Whereas CETP inhibition clearly raises plasma HDL-C levels, the development of torcetrapib, a CETP inhibitor, was recently terminated because of increased death rate in a large clinical outcome trial.4 Thus, it is possible that CETP inhibition could retard RCT and thus have adverse effects on atherosclerosis, despite an increase in HDL-C levels.5

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It is difficult at present to quantify RCT in humans. We previously developed and validated a method to specifically assess macrophage-to-feces RCT in mice.6–9 In the present studies we applied this method to the question of whether CETP influences the rate of RCT. Because mice naturally lack CETP, our approach was to stably introduce the expression of CETP as compared with controls and then perform the
RCT assay. We used a gene transfer vector that is based on adeno-associated virus serotype 8 (AAV8) and the liver-specific thyroglobulin (TBG) promoter to express human CETP in the livers of different mouse models. Our conclusions are that CETP expression promotes macrophage RCT in mice despite a reduction in plasma HDL-C levels, that this requires the presence of the LDL receptor, and that CETP restores to normal the rate of RCT in scavenger receptor class B, type 1 (SR-BI)-knockout (KO) mice.

Methods

Vector Construction

The AAV8 TBG human CETP (hCETP) vector contains the human CETP cDNA insert followed by SV-40 poly A tail and is driven by the liver-specific human thyroglobulin (TBG) promoter. AAV8 hCETP was produced with a chimeric packaging construct in which the AAV2 rep gene was fused with the cap gene of AAV serotype 8. Control vector consisted of the LacZ gene packaged into AAV serotype 8 and driven by the TBG promoter.

Macrophage RCT Studies

RCT studies were performed as previously described. J774 cells were obtained from American Type Culture Collection (Manassas, Va) and were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were radiolabeled with 5 μCi/mL 3H-cholesterol and cholesterol enriched with 100 μg/mL of acetylated LDL for 48 hours. These labeled foam cells were washed 6 hours, spun down, and resuspended in RPMI medium immediately before use.

Experiments were performed in apobec-1–KO mice, LDLR/apobec-1–double-knockout (DKO) mice, and SR-BI–KO mice. Breeders were obtained from Jackson Labs (Bar Harbor, Me) and bred in house for the present studies. All mice were fed a standard chow diet. For each experiment, 12 male mice (n=6 per group) received intraperitoneal injections of AAV8-TBG-hCETP (1×1011 GC) or AAV8-TBG-LacZ (1×1011 GC). On day 26 after vector injection, animals received intraperitoneal injections of 3H-cholesterol-labeled J774 cells as described previously and were caged individually with unlimited access to food and water. Blood was collected at 6, 24, and 48 hours, and plasmas were used for liquid chromatography (FPLC) gel filtration (Amersham Pharmacia Biotech, Uppsala, Sweden). The cholesterol concentrations in the FPLC fractions were determined with an enzymatic assay (Wako Pure Chemical Industries Ltd, Osaka, Japan). The 3H-tracer distribution across the FPLC profile was determined with LSC. Plasma CETP concentrations were measured with CETP ELISA Kit (Wako Pure Chemical Industries Ltd).

Liver, Bile, and Fecal Analyses

Liver lipids were extracted according to the procedure of Bligh and Dyer. Briefly, a 50-mg piece of tissue was homogenized in water, and lipids were extracted with a 2:1 (vol/vol) mixture of chloroform/methanol. The lipid layer was collected, evaporated, resuspended in toluene, and counted in an LSC. Results were expressed as percentage of counts per minute injected (whole liver). The bile was diluted across the FPLC profile was determined with LSC. Plasma CETP concentrations were measured with CETP ELISA Kit (Wako Pure Chemical Industries Ltd).

Statistical Analysis

Values are presented as mean±SD. Results were analyzed with Student t test, 1-way repeated-measures ANOVA, and Tukey’s multiple-comparison test with the use of GraphPad Prism Software (San Diego, Calif). Shapiro-Wilk testing was performed to check the normality of the data. Most of the data were normally distributed despite the small numbers (n=6 per group). In the event that a group failed to pass the normality test, the Mann-Whitney test was performed for these specific analyses and gave the same results as the t test.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Determination of the Optimal Dose of AAV8-TBG-hCETP

In previous (unpublished) studies, we determined that the intraperitoneal route of administration for AAV8-based gene transfer vectors is as effective as the intravenous (tail vein) route and therefore used intraperitoneal injection for the present studies. We then performed a dose-ranging study of AAV8-TBG-hCETP in LDLR/apobec-1–DKO mice to determine the optimal dose for the RCT studies. Doses of 1×1011 CETP, 3×1010 CETP, 1×1010 CETP, and 1×1011 LacZ were given by intraperitoneal injection, and plasma was obtained at 7, 14, 28, and 42 days after injection. Plasma CETP levels (Figure 1A) were dose dependent, reached a plateau 2 weeks after injection, and remained stable through 6 weeks, at which time point determined by ELISA (day 0, 7, 14, 28, 42). B, The change of plasma HDL expression level of each time point determined on a Cobas FarA. The differences between each CETP group and 1×1011 LacZ group were evaluated. *P<0.05 versus 1×1011 LacZ group.
time the experiment was terminated. Plasma HDL-C levels were reduced by CETP expression in a dose-dependent fashion (Figure 1B). We concluded that the optimal vector dose for the RCT studies was 1×10^{11} GC and a good time point for the studies was day 28 after injection.

**CETP Expression Promoted Macrophage RCT in Wild-Type and Apobec-1–KO Mice**

In preliminary studies in wild-type mice, expression of CETP was associated with a reduction of plasma HDL-C levels but with increased macrophage-to-feces RCT (data not shown). We turned to apobec-1–KO mice because the lack of apoB RNA editing in the liver and resultant apoB-100 in plasma more closely resembles human apoB-lipoprotein metabolism, which is particularly important given our expression of human CETP in the present studies. Apobec-1–KO mice received intraperitoneal injections of 1×10^{11} GC AAV8-hCETP or AAV8-LacZ, and blood was taken 4 weeks after injection for lipid analysis. The mean plasma CETP mass concentration was 20.7±4 μg/mL. CETP expression resulted in the expected 71% reduction in HDL-C level (61±4.6 versus 17±2.7 mg/dL). At 4 weeks, the labeled J774 cells were injected for the RCT study. The ^3H-cholesterol counts in plasma, expressed as a percentage of total label injected, at each time point were not different between the CETP and control groups (Figure 2A). In FPLC analysis, the ^3H-cholesterol in plasma tracked closely with the cholesterol mass in plasma lipoprotein distribution, with reduced counts in the HDL fraction and increased counts in the apoB-lipoprotein fractions in the CETP-expressing mice (Figure 2, B and C). There was no difference between the 2 groups with regard to ^3H-sterol in the liver or the bile at the 48-hour time period (Figure 3, A and B). However, the CETP-expressing mice excreted a significantly greater (46%) tracer in the feces over 48 hours than the control mice (Figure 3C). Thus, in apobec-1–KO mice, expression of CETP resulted in reduced HDL-C levels but increased flux of ^3H-cholesterol from macrophages to feces, consistent with promotion of RCT.

**CETP Expression Failed to Promote Macrophage RCT in Apobec-1–KO Mice That Lack the LDL Receptor**

We hypothesized that CETP expression promoted RCT in apobec-1–KO mice by facilitating the transfer of HDL CEs to apoB-containing lipoproteins, which were then cleared in the liver via the LDL receptor. Therefore, we tested the hypoth-

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**Figure 2.** Macrophage RCT in apobec-1–KO mice injected with hCETP, AAV8, or LacZ as a control (n=6/group). Mice received intraperitoneal injections of 1×10^{11} GC hCETP, AAV8, or LacZ and then 26 days later received intraperitoneal injections of ^3H-labeled cholesterol J774 foam cells (5.3×10^{6} cells that contained 4.8×10^{6} cpm per 0.5 mL medium). A, ^3H-labeled cholesterol in liver of each group. B, ^3H-labeled cholesterol lipoprotein profile of pooled plasma samples drawn 48 hours after injection of J774 cells. C, Cholesterol mass profile of pooled plasma samples drawn 48 hours after injection of J774 cells.

**Figure 3.** Macrophage RCT in apobec-1–KO mice injected with hCETP, AAV8, or LacZ as a control (n=6/group). Mice received intraperitoneal injections of 1×10^{11} GC hCETP, AAV8, or LacZ and then 26 days later received intraperitoneal injections of ^3H-labeled cholesterol J774 foam cells (5.3×10^{6} cells that contained 4.8×10^{6} cpm per 0.5 mL medium). A, ^3H-labeled cholesterol in liver of each group. B, ^3H-labeled cholesterol in bile of each group. C, ^3H-labeled cholesterol in feces of each group. †P<0.05.
The results indicate that CETP expression would be ineffective in promoting RCT in the absence of the LDL receptor. LDLR/Apoec-1–DKO mice received intraperitoneal injections of $1 \times 10^{11}$ GC hCETP, AAV8, or LacZ and then 26 days later received intraperitoneal injections of $^3$H-labeled cholesterol J774 foam cells (5.3 × 10^6 cells that contained 4.8 × 10^6 cpm in 0.5 mL medium). A, The change of $^3$H-labeled cholesterol in plasma. B, $^3$H-labeled cholesterol lipoprotein profile of pooled plasma samples drawn 48 hours after injection of J774 cells. C, Cholesterol mass profile of pooled plasma samples drawn 48 hours after injection of J774 cells. *P<0.05 versus LacZ group.

We previously demonstrated that SR-BI–KO mice have markedly impaired macrophage RCT,7 which potentially accounts for the increased risk of atherosclerosis in these animals.16 We tested the hypothesis that CETP expression would overcome the absence of hepatic SR-BI and restore macrophage RCT to normal in SR-BI–KO mice. SR-BI–KO mice received intraperitoneal injections of $1 \times 10^{11}$ GC AAV8-hCETP or AAV8-LacZ. At 4 weeks after injection, CETP expression resulted in a significant 46% reduction in total cholesterol (268 ± 18 versus 144 ± 27 mg/dL) and a significant 48% reduction in HDL-C levels (189 ± 15 versus 98 ± 13 mg/dL) in control mice versus CETP-expressing mice, respectively. At 4 weeks, the labeled macrophages were injected. Plasma $^3$H-cholesterol at each time point was substantially lower in CETP-expressing mice (Figure 6A). FPLC analysis confirmed that the $^3$H-cholesterol in plasma tracked closely with the cholesterol mass, which demonstrated reduced cholesterol mass and $^3$H-cholesterol in HDL as well as increased mass and tracer in apoB-containing lipoproteins in the CETP-expressing mice (Figure 4, B and C), consistent with CETP-mediated transfer of CEs from HDL to apoB-containing lipoproteins. Interestingly, whereas mice that expressed CETP were found to have a significant 49% increase in $^3$H-sterol in the liver at 48 hours (Figure 5A), there was not an increase in $^3$H-sterol in bile or in feces over the 48-hour period (Figure 5, B and C). These results indicate that, in the absence of the LDL receptor, CETP expression did not promote RCT from macrophage to feces but did result in increased uptake of macrophage-derived cholesterol to liver, potentially via a pathway that is not linked to effective biliary excretion.

**CETP Expression in SR-BI–KO Mice Restored Markedly Impaired Macrophage RCT to Normal**

We previously demonstrated that SR-BI–KO mice have markedly impaired macrophage RCT,7 which potentially accounts for the increased risk of atherosclerosis in these animals.16 We tested the hypothesis that CETP expression would overcome the absence of hepatic SR-BI and restore macrophage RCT to normal in SR-BI–KO mice. SR-BI–KO mice received intraperitoneal injections of $1 \times 10^{11}$ GC AAV8-hCETP or AAV8-LacZ. At 4 weeks after injection, CETP expression resulted in a significant 46% reduction in total cholesterol (268 ± 18 versus 144 ± 27 mg/dL) and a significant 48% reduction in HDL-C levels (189 ± 15 versus 98 ± 13 mg/dL) in control mice versus CETP-expressing mice, respectively. At 4 weeks, the labeled macrophages were injected. Plasma $^3$H-cholesterol at each time point was substantially lower in CETP-expressing mice (Figure 6A). FPLC analysis confirmed that the $^3$H-cholesterol in plasma tracked closely with the cholesterol mass in plasma lipoproteins and that CETP expression substantially decreased the $^3$H-cholesterol counts in HDL (Figure 6, B and C). Whereas there was...
no effect of CETP expression on 3H-sterol in the liver at 48 hours (Figure 7A), CETP expression resulted in a highly significant 52% increase in fecal sterol excretion of 3H-sterol over 48 hours (Figure 7B). This indicates that CETP expression restored macrophage RCT to normal in SR-B1–KO mice.

**Discussion**

The role of CETP in RCT is a critically important question, accentuated by questions about the effect of CETP inhibitors on atherosclerosis in humans. In the present study we used a second-generation AAV vector to express human CETP in different mouse models and then used a validated assay of macrophage RCT to assess the effect of CETP expression compared with controls. The present studies demonstrate several novel observations: First, CETP expression promotes macrophage RCT in wild-type and apobec-1–KO mice despite a reduction in plasma HDL-C levels. Second, this effect of CETP requires the LDL receptor. Third, expression of CETP in SR-B1–KO mice circumvents the inability of the liver to take up HDL CE and restores normal RCT. Thus, CETP expression does not promote RCT as assessed by 48-hour fecal excretion of tracer. Interestingly, liver tracer levels were similar to our previously reported studies of overexpression of SR-B1 in mice: reduction in plasma HDL-C levels but a significant increase in macrophage RCT. Thus, this provides a second example of an intervention in mice that promotes RCT despite reducing steady-state plasma HDL-C levels and further emphasizes the importance of assessing the flux of RCT and not simply using HDL-C as a surrogate for RCT.

These results suggested that CETP promoted RCT by transferring HDL CEs to apoB-containing lipoproteins, followed by their efficient clearance in the liver. We hypothesized that deletion of the LDL receptor might alter this dynamic and carried out similar studies in LDLR/apobec-1–DKO mice. CETP expression effectively resulted in redistribution of CE mass and 3H-cholesterol tracer in plasma from HDL to apoB-containing lipoproteins, as expected. However, due to impaired uptake of apoB-containing lipoproteins, plasma 3H-cholesterol counts were higher than in controls at all time points. Indeed, in the absence of the LDLR, expression of CETP did not promote RCT as assessed by 48-hour fecal excretion of tracer. Interestingly, liver tracer levels were greater in the CETP expressing mice, suggesting that the LDLR-independent mechanisms by which the tracer-containing apoB-containing lipoproteins were taken up by the liver were not effectively linked to pathways of biliary secretion.

SR-B1–KO mice develop markedly accelerated atherosclerosis, and we previously showed that they have markedly impaired macrophage RCT. Here we hypothesized that CETP expression might bypass the inability of the liver to

**Figure 6.** Macrophage RCT in SR-B1–KO mice injected with hCETP, AAV8, or LacZ as a control (n=6/each group). Mice received intraperitoneal injections of 1×10¹¹ GC hCETP, AAV8, or LacZ and then 26 days later received intraperitoneal injections of 3H-labeled cholesterol J774 foam cells (6.7×10⁶ cells that contained 5.5×10⁶ cpm in 0.5 mL medium). A, The change of 3H-labeled cholesterol J774 foam cells. B, 3H-labeled cholesterol lipoprotein profile of pooled plasma samples drawn 48 hours after injection of J774 cells. C, Cholesterol mass profile of pooled plasma samples drawn 48 hours after injection of J774 cells. *P<0.05 versus LacZ group.

**Figure 7.** Macrophage RCT in SR-B1–KO mice injected with hCETP, AAV8, or LacZ as a control (n=6/each group). Mice received intraperitoneal injections of 1×10¹¹ GC hCETP, AAV8, or LacZ and then 26 days later received intraperitoneal injections of 3H-labeled cholesterol J774 foam cells (6.7×10⁶ cells that contained 5.5×10⁶ cpm in 0.5 mL medium). A, 3H-labeled cholesterol in liver of each group. B, 3H-labeled cholesterol in feces of each group. †P<0.05.
take up HDL CEs via SR-BI and thus restore normal RCT. The present results suggest that this is in fact the case, with CETP expression increasing macrophage RCT by >50% in SR-BI–KO mice despite marked reductions in plasma HDL-C levels. These data are consistent with the recent observation that CETP expression attenuated the development of atherosclerosis in SR-BI–KO mice.18 These data could also have direct relevance to human physiology. Schwartz and colleagues have demonstrated that in humans the vast majority of HDL CEs that are ultimately secreted into bile are transported to the liver by apoB-containing lipoproteins.3 This observation suggested that in humans the hepatic SR-BI pathway may not be especially active and that the CETP pathway is the major pathway by which HDL CEs are returned to the liver. In contrast, in mice that lack CETP, the hepatic SR-BI pathway has evolved to be the critical pathway for returning HDL cholesterol to the liver. The present results have implications to explain the conflicting studies of the effects of CETP expression on atherosclerosis in mice. These results would predict that, in mice that have robust pathways for uptake of apoB-containing lipoproteins, the expression of CETP, although it reduces HDL-C levels, might be expected to be antiatherogenic, consistent with published studies.18–21 Conversely, in mice that have markedly defective clearance of apoB-containing lipoproteins, expression of CETP might be expected to promote atherogenesis, also consistent with published studies.22–25

What implications might the present studies have for the role of CETP (and its inhibition) on RCT and atherosclerosis in humans? One hypothesis is that in normolipidemic healthy humans, hepatic SR-BI is expressed at low levels and the CETP pathway is a critical pathway for the hepatic clearance of HDL-derived CEs.3 However, in the setting of impaired clearance of apoB-containing lipoproteins (including not only familial hypercholesterolemia but also other genetic and environmental factors that reduce hepatic apoB-lipoprotein uptake), the CETP pathway may instead be more proatherogenic by transferring HDL CEs to apoB-containing lipoproteins, which are then inefficiently cleared. It would follow from this hypothesis that CETP inhibition in persons with highly effective apoB-lipoprotein clearance (such as patients on high-dose statins) might not be protective (or even proatherogenic), whereas in those with defective clearance it might be antiatherogenic.

In summary, the present studies, which used AAV-mediated expression of hCETP in different mouse models of lipoprotein metabolism, suggest that CETP promotes RCT in the setting of effective clearance of apoB-containing lipoproteins but does not promote RCT in the setting of impaired clearance. Furthermore, CETP expression normalizes RCT in SR-BI–KO mice. The present studies may have implications for CETP inhibition in humans, but direct studies of RCT in humans in the setting of CETP inhibition are needed.

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Disclosures

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References

CLINICAL PERSPECTIVE

Cholesteryl ester transfer protein (CETP) is responsible for the transfer of cholesteryl esters from high-density lipoproteins to apolipoprotein (apo) B-containing lipoproteins in exchange for triglycerides. Inhibition of CETP raises plasma levels of high-density lipoprotein cholesterol but has uncertain effects on atherosclerosis. It has been suggested that CETP may promote the process of reverse cholesterol transport (RCT) (and thus CETP inhibition may inhibit it), but the effect of CETP on RCT is unknown. Mice naturally lack CETP, and in the present studies a gene transfer vector was used to stably express human CETP in different mouse models of lipoprotein metabolism. A validated method to measure RCT from the macrophage through the plasma to the liver and feces was used to assess the effect of CETP expression on RCT. The results indicate that CETP expression, although it reduced high-density lipoprotein cholesterol concentrations, promoted RCT in the setting of effective clearance of apoB-containing lipoproteins; however, it was ineffective in the setting of impaired clearance as a result of deficiency in the low-density lipoprotein receptor. Furthermore, CETP expression normalized RCT in mice deficient in the liver high-density lipoprotein scavenger receptor class B, type I, which is known to have highly defective RCT. The present studies suggest that the CETP pathway may promote RCT and thus be antiatherogenic in the setting of effective clearance of apoB-containing lipoproteins but be proatherogenic in the setting of impaired clearance of apoB-containing lipoproteins. Thus the effectiveness of CETP inhibition as a therapeutic strategy for atherosclerosis may depend in part on the underlying metabolic milieu with regard to the efficiency of apoB-lipoprotein clearance.
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