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

Hiroyuki Tanigawa, MD, PhD; Jeffrey T. Billheimer, PhD; Jun-ichiro Tohyama, MD; YuZhen Zhang, MD, PhD; George Rothblat, PhD; Daniel J. Rader, MD

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

Clinical Perspective p 1273

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.6–8 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

Received March 21, 2007; accepted June 29, 2007.

From the Institute for Translational Medicine and Therapeutics and the Cardiovascular Institute (H.T., J.T.B., J.-i.T., Y.Z.Z., D.J.R.), University of Pennsylvania School of Medicine, Philadelphia, and the Children’s Hospital of Philadelphia (G.R.), Pa.

Correspondence to Daniel J. Rader, MD, University of Pennsylvania Medical Center, 654 Biomedical Research Building II/III, 421 Curie Blvd, Philadelphia, PA 19104. E-mail rader@mail.med.upenn.edu

© 2007 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.107.704254

1267
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\textsuperscript{10,11} 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.\textsuperscript{8,10,11} The 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.\textsuperscript{6–9} 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 twice, equilibrated in medium with 0.2% bovine serum albumin for 6 hours, spun down, and resuspended in RPMI medium immediately before use.

Experiments were performed in apobec-1–KO mice,\textsuperscript{12} LDLR/apobec-1–double-knockout (DKO) mice,\textsuperscript{13} and SR-BI–KO mice.\textsuperscript{14} 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 each group) received intraperitoneal injections of AAV8-TBG-hCETP (1×10\textsuperscript{11} GC) or AAV8-TBG-LacZ (1×10\textsuperscript{11} 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 scintillation counting (LSC). Feces were collected from 0 to 48 hours were weighed and soaked in Millipore (Billerica, Mass) water (1 mL water per 100 mg feces) overnight at 4°C. An equal volume of ethanol was added the next day, and the samples were homogenized; 2 mL of the homogenized samples was combined with 2 mL ethanol and counted in an LSC.

**Liver, Bile, and Fecal Analyses**
Liver lipids were extracted according to the procedure of Bligh and Dyer.\textsuperscript{15} 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 up to 200 μL with PBS and counted in an LSC. The total feces collected from 0 to 48 hours were weighed and soaked in Millipore (Billerica, Mass) water (1 mL water per 100 mg feces) overnight at 4°C. An equal volume of ethanol was added the next day, and the samples were homogenized; 2 mL of the homogenized samples was combined with 2 mL ethanol and counted in an LSC.

**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 W 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 1×10\textsuperscript{10} CETP, 3×10\textsuperscript{10} CETP, 1×10\textsuperscript{10} CETP, and 1×10\textsuperscript{11} 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 point...
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 hypo-

**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-

---

**Figure 2.** Macrophage RCT in apobec-1–KO mice injected with hCETP, AAV8, or LacZ as a control (n=6/each group). Mice received intraperitoneal injections of $1 \times 10^{11} \text{GC hCETP, AAV8, or LacZ}$ and then 26 days later received intraperitoneal injections of $^{3}$H-labeled cholesterol J774 foam cells ($5.3 \times 10^6$ cells that contained $4.8 \times 10^6$ counts per minute [cpm] in 0.5 mL medium). A, The change of $^{3}$H-labeled cholesterol in plasma. B, $^{3}$H-labeled cholesterol in bile of each group. C, $^{3}$H-labeled cholesterol in feces of each group. †

---

**Figure 3.** Macrophage RCT in apobec-1–KO mice injected with hCETP, AAV8, or LacZ as a control (n=6/each group). Mice received intraperitoneal injections of $1 \times 10^{11} \text{GC hCETP, AAV8, or LacZ}$ and then 26 days later received intraperitoneal injections of $^{3}$H-labeled cholesterol J774 foam cells ($5.3 \times 10^6$ cells that contained $4.8 \times 10^6$ cpm in 0.5 mL medium). A, $^{3}$H-labeled cholesterol in liver of each group. B, $^{3}$H-labeled cholesterol in bile of each group. C, $^{3}$H-labeled cholesterol in feces of each group. †
that CETP expression substantially decreased the 3H-cholesterol counts in HDL (Figure 6, B and C). Whereas there was not an increase in 3H-sterol in bile or in feces over the 48-hour period (Figure 5A), FPLC analysis confirmed that the 3H-cholesterol in plasma tracked closely with the cholesterol mass in plasma lipoproteins and that CETP expression resulted in 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, mice were injected with labeled macrophages. In contrast to the apobec-1–KO mice, CETP-expressing DKO mice had higher plasma 3H-cholesterol counts at all time points than controls (Figure 4A). FPLC analysis confirmed that the 3H-cholesterol in plasma tracked closely with the cholesterol mass, which demonstrated reduced cholesterol mass and 3H-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 3H-sterol in the liver at 48 hours (Figure 5A), there was not an increase in 3H-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, which potentially accounts for the increased risk of atherosclerosis in these animals. We tested the hypothesis that CETP expression would overcome the absence of hepatic SR-B1 and restore macrophage RCT to normal in SR-BI–KO mice. SR-BI–KO mice received intraperitoneal injections of 1 × 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 3H-cholesterol at each time point was substantially lower in CETP-expressing mice (Figure 6A). FPLC analysis confirmed that the 3H-cholesterol in plasma tracked closely with the cholesterol mass in plasma lipoproteins and that CETP expression substantially decreased the 3H-cholesterol counts in HDL (Figure 6, B and C). Whereas there was
no effect of CETP expression on \(^3\)H-sterol in the liver at 48 hours (Figure 7A), CETP expression resulted in a highly significant 52% increase in fecal sterol excretion of \(^3\)H-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.\(^4\),\(^5\),\(^7\) 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 CEs and restores normal RCT. Thus, the present studies are consistent with the concept that CETP may be part of a physiological system that facilitates effective RCT, at least when LDL receptor–mediated uptake is functioning efficiently.

We used apobec-1–KO mice because their apoB-lipoprotein metabolism is closer to that of humans; the lack of apoB RNA editing results in the synthesis and secretion of only apoB-100, in contrast with wild-type mice that have a large amount of hepatically derived apoB-48 in plasma. The effects of expressing CETP in these mice were remarkably similar to our previously reported studies of overexpression of SR-B1 in mice:\(^7\) 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 \(^3\)H-cholesterol tracer in plasma from HDL to apoB-containing lipoproteins, as expected. However, due to impaired uptake of apoB-containing lipoproteins, plasma \(^3\)H-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,\(^16\) and we previously showed that they have markedly impaired macrophage RCT.\(^7\) 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/group). Mice received intraperitoneal injections of \(1\times10^{11}\) GC hCETP, AAV8, or LacZ and then 26 days later received intraperitoneal injections of \(^3\)H-labeled cholesterol J774 foam cells (6.7\(\times\)10⁶ cells that contained 5.5\(\times\)10⁶ 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.

**Figure 7.** Macrophage RCT in SR-B1–KO mice injected with hCETP, AAV8, or LacZ as a control (n=6/group). Mice received intraperitoneal injections of \(1\times10^{11}\) GC hCETP, AAV8, or LacZ and then 26 days later received intraperitoneal injections of \(^3\)H-labeled cholesterol J774 foam cells (6.7\(\times\)10⁶ cells that contained 5.5\(\times\)10⁶ cpm in 0.5 mL medium). A, \(^3\)H-labeled cholesterol in liver of each group. B, \(^3\)H-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. 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. 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. 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.

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. 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.

Acknowledgments

We are indebted to Dawn Marchadier, Aisha Wilson, Edwige Edouard, David Chang, Michelle Joshi, Anna DiFlorio, and Linda Morrell for their excellent technical assistance; to Dr Mahlet Tadesse for assistance with biostatistics; and to Drs Colin MacPhee and Max Walker for helpful discussions.

Sources of Funding

This work was supported by P01-HL22633 and P01-HL59407 from the National Heart, Lung, and Blood Institute, and an Alternative Drug Discovery Initiative award to the University of Pennsylvania from GlaxoSmithKline.

Disclosures

Dr Rader has been a consultant to and received research funding from Bristol Myers Squibb, Merck, and Pfizer, and has been a consultant to Roche. The other authors report no potential conflicts of interest.

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

Hiroyuki Tanigawa, Jeffrey T. Billheimer, Jun-ichiro Tohyama, YuZhen Zhang, George Rothblat and Daniel J. Rader

_Circulation_. 2007;116:1267-1273; originally published online August 20, 2007;
doi: 10.1161/CIRCULATIONAHA.107.704254

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/116/11/1267

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/