Inhibition of Cholesteryl Ester Transfer Protein by Torcetrapib Modestly Increases Macrophage Cholesterol Efflux to HDL

Laurent Yvan-Charvet, Fumihiko Matsuura, Nan Wang, Mark J. Bamberger, Tu Nguyen, Franz Rinninger, Xian-Cheng Jiang, Charles L. Shear, Alan R. Tall

Objective—This study examines the effects of pharmacological inhibition of cholesteryl ester transfer protein (CETP) on the ability of high-density lipoprotein particles (HDL) to promote net cholesterol efflux from human THP-1 macrophage foam cells.

Methods and Results—Two groups of 8 healthy, moderately hyperlipidemic subjects received the CETP inhibitor torcetrapib at 60 or 120 mg daily for 8 weeks. Torcetrapib increased HDL cholesterol levels in both groups by 50% and 60%, respectively. Compared with baseline, torcetrapib 60 mg daily increased HDL-mediated net cholesterol efflux from foam cells primarily by increasing HDL concentrations, whereas 120 mg daily torcetrapib increased cholesterol efflux both by increasing HDL concentration and by causing increased efflux at matched HDL concentrations. There was an increased content of lecithin:cholesterol acyltransferase (LCAT) and apolipoprotein E (apoE) in HDL-2 only at the 120 mg dose. ABCG1 activity was responsible for 40% to 50% of net cholesterol efflux to both control and T-HDL.

Conclusions—These data indicate that inhibition of CETP by torcetrapib causes a modest increase in the ability of HDL to promote net cholesterol efflux at the 60 mg dose, and a more dramatic increase at the 120 mg dose in association with enhanced particle functionality. (Arterioscler Thromb Vasc Biol. 2007;27:1132-1138.)

Key Words: CETP • torcetrapib • high-density lipoprotein • ABC transporters • macrophages

In recent years, therapeutic intervention targeting HDL have become a major focus of research on the treatment of atherosclerotic cardiovascular disease.1 The HDL-mediated removal of excess free cholesterol (FC) from macrophage foam cells is thought to play a major role in the protection against the development of atherosclerosis.2 However, current therapies for raising HDL are limited. Despite the favorable effects of statins on coronary heart disease, these agents have only modest effects on HDL-C levels.3,4 Fibrates and niacin can raise HDL-C, but the increases are rarely >30% and only some of the fibrate trials have shown prevention of coronary events in patients with low HDL, and niacin is often not well tolerated.5,6

Novel targets to raise HDL-C have emerged from the recent understanding of HDL synthesis, maturation, and catabolism. In humans, cholesteryl ester (CE) generated by the lecithin:cholesterol acyltransferase (LCAT) enzyme in HDL is transferred to apoB-lipoproteins by the cholesteryl ester transfer protein (CETP). CETP promotes the removal of cholesteryl ester (CE) from antiatherogenic HDL to atherogenic apoB-containing particles in exchange for triglycerides (TGs).7 The marked increase in HDL cholesterol associated with human deficiency of CETP has suggested CETP inhibition as a potential strategy to treat atherosclerotic disease.8,9 However, there has been concern that HDL particles accumulating in CETP deficiency might be dysfunctional.10 We recently reported that large CE-rich HDL particles from 4 subjects with complete CETP deficiency (CETP-D) showed increased ability to promote cholesterol efflux from macrophage foam cells.11 Central to this observation was the key role of LCAT and apolipoprotein E (apoE) present at high levels in CETP-D HDL driving net cholesterol efflux. Cholesterol efflux from cells to HDL can occur by passive diffusion or may be facilitated by different transporters.12 ATP-binding cassette (ABC) transporters can promote net movement of cholesterol from cells to acceptors in medium. ABCA1, the defective gene in Tangier disease,13 promotes net cholesterol efflux to lipid-poor apolipoproteins, such as apoA-I and apoE,14,15 initiating the formation of HDL. In contrast, ABCG1 promotes net cholesterol efflux to nascent
or mature HDL particles, and preliminary results using RNA interference suggest an important role for this receptor in CETP-D HDL-mediated cholesterol efflux.

This study was undertaken to determine the cholesterol efflux potential of HDL accumulating in subjects treated with the CETP inhibitor, torcetrapib (T-HDL). Torcetrapib effectively raised HDL in human, but a phase 3 clinical trial with torcetrapib was recently halted because of adverse clinical events. A secondary goal of the study was to determine the relative importance of different transporters in the ability of T-HDL to mediate net cholesterol efflux from cells.

Materials and Methods

Study Subjects

A total of 16 subjects with moderate hypercholesterolemia and without CVD were enrolled in this trial. The study consisted of an 8-week period, during which torcetrapib was administered at doses of 60 and 120 mg daily (8 subjects in each group). Subjects were not taking any other lipid modifying therapy. Blood was collected before and after treatment.

Plasma HDL Preparation

After 12-hour fasting, blood samples were collected from all subjects into tubes containing 0.1% EDTA. ApoB-containing particles was precipitated from serum by adding 100 μL of serum to 40 μL of 20% polyethylene glycol (PEG, Sigma P-2139) in 200 mM/L glycine, pH10 solution. This mixture was incubated at room temperature for 15 minutes. After this incubation, the solution was centrifuged at 4000 rpm for 20 minutes. The supernatant, containing the apoE-poor or rich HDL particles, was removed and used for experiments. HDL-2 fraction was isolated from plasma by ultracentrifugation as previously described.

Human THP-1 Macrophages

THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO2. Cells were treated with 100 μmol/L PMA (Phorbol myristate acetate) for 24 hour to facilitate differentiation into macrophages. Then, adherent macrophages were incubated with 50 μg/mL acetyl-LDL and 3 μmol/L LXR agonist (TO901317) for 24 hours before cholesterol efflux studies.

Mouse Peritoneal Macrophages

Peritoneal macrophages from wild-type, ABCG1 knockout, and SR-BI knockout mice were collected 3 days after an i.p. injection of thioglycollate and seeded on 24-well plates in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS. Small interfering RNA (siRNA) experiments were performed as previously described. Briefly, macrophages were transfected with scrambled or ABCA1 siRNA for 32 hours and then treated with 50 μmol/L acetyl-LDL and 3 μmol/L TO901317 in DMEM media containing 0.2% BSA. Cells were treated for 16 hours before cholesterol efflux studies.

Cholesterol Mass Analysis

Cholesterol efflux was performed in DMEM containing 0.2% BSA in presence of HDL as described in figure legends. After incubation with HDL, the lipid fractions were extracted from the collected media with hexane in presence of β-sitosterol (5 μg per sample) added as the internal standard. The mass of total cholesterol dissolved in hexane was subject to gas-liquid chromatography. The HDL-mediated net cholesterol efflux was calculated by subtraction of cholesterol mass of the medium cultured with or without cells.

Sphingomyelin and Phosphatidylcholine Determinations

Analysis of SM and PC content in HDL was measured as previously described. Results are expressed as the ratio between SM and PC.

Western Blot Analysis

Aliquots of 20 μg of HDL-2 were boiled at 95°C for 10 minutes in SDS buffer (6.25.10^-3 mol/L Tris-HCl pH6.8, 2% SDS, 5% 2-mercaptoethanol, 10% sucrose and 0.002% Coomassie blue). Then, HDL proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Primary antibodies for LCAT (400-107A2), apoE (ab1906), or apoA-I (ab17278) were purchased from Novus Biologicals and Abcam. Specific protein signals were revealed using the ECL detection system (Amersham Biosciences).

Results

Patients and HDL Responses

A total of 16 subjects were studied and were divided into 2 groups of 8 subjects receiving either 60 mg (group 1) or 120 mg of torcetrapib (group 2) per day for 8 weeks. At baseline, the 2 groups did not show significant differences with respect to demographic and lipid characteristics (supplemental Table 1). Inhibition of CETP with torcetrapib increased plasma concentrations of HDL by 50% in group 1 (0.53±0.04 versus 0.80±0.09 mg/mL cholesterol; P<0.01) and 60% in group 2 (0.57±0.06 versus 0.90±0.06 mg/mL cholesterol; P<0.005). At the 120 mg dose one subject showed no change in HDL-C; this subject, however, was included in the analysis. We determined key features of HDL-2 composition because CETP deficiency (CETP-D) or inhibition primarily leads to an increase in HDL-2 rather than HDL-3. Immunoblot analysis of LCAT, apoE, and apoA-I in duplicate pooled HDL-2 samples from patients before and after 60 mg torcetrapib treatment did not reveal any difference (supplemental Figure IA). By contrast, in group 2, T-HDL-2 exhibited an increase in LCAT (1.7-fold) and apoE proteins (1.4-fold) compared with control HDL (supplemental Figure IA). The level of apoA-I was not appreciably changed as expected because samples were matched for total protein content and apoA-I is the major protein of HDL. Because decreased sphingomyelin/ phosphatidylcholine (SM/PC) ratio was previously found in CETP-D HDL, we also determined this ratio in the individual HDL fractions. Supplemental Figure IB shows that 60 mg torcetrapib treatment did not modify the SM/PC ratio of HDL. However, a significant decrease was observed in T-HDL from subjects treated with 120 mg torcetrapib as compared with control HDL (0.29±0.02 versus 0.25±0.02, respectively; P<0.05; supplemental Figure IB). Although the changes in LCAT content and SM/PC ratio were smaller than observed in 4 CETP-D subjects, they confirm similar changes in normal subjects treated with 120 mg torcetrapib. Interestingly, these changes were seen in group 2 but not in group 1, suggesting they only occur at higher levels of CETP inhibition.

Effects of Torcetrapib on HDL-Mediated Cholesterol Efflux

To compare the functionality of HDL particles before (control HDL, C-HDL) and after torcetrapib treatment (T-HDL), we measured HDL-mediated cholesterol efflux for each
subject in human THP-1 macrophages previously treated with AcLDL (50 μg/mL) and TO901317 (LXR agonist, 2 μmol/L) for 24 hours. To avoid ultracentrifugation-induced artifacts in HDL properties, this study used PEG supernatants, in which HDL was separated from apoB-lipoproteins by PEG precipitation. In the initial studies, C- and T-HDL were added to media for 8 hours at the same cholesterol concentration (12 μg/mL). At this concentration, T-HDL from group 1 subjects caused similar levels of cholesterol efflux compared with C-HDL (Figure 1A). No single subject showed decreased cholesterol efflux potency of their HDL after torcetrapib treatment compared with their baseline HDL (data not shown). HDL from group 2 subjects exhibited a significant 2.5-fold increase in CE accumulation in media and an insignificant 1.4-fold increase in total cholesterol efflux (Figure 1B). Notably, in group 2 subjects, the increased CE accumulation in media was associated with increased LCAT, apoE and decreased SM/PC ratio in HDL-2 (Figure 1B; supplemental Figure I), consistent with increased cholesterol esterification activity in the HDL.

Dose-Response Curve for Cholesterol Efflux Mediated by HDL From Torcetrapib-Treated Subjects

Because torcetrapib treatment results in increased HDL concentration as well as changes in HDL composition, we next compared cholesterol efflux using 3 different concentrations of pooled HDL (ie, 12, 36, and 72 μg/mL cholesterol) to determine whether increasing concentrations of T-HDL would improve cholesterol efflux in similar fashion to C-HDL. For the 60 mg torcetrapib samples, the dose–response curves were not significantly different, comparing HDL from subjects before and after treatment. However, at the 120 mg dose, there were significantly higher levels of cholesterol efflux at the 2 higher HDL concentrations, primarily reflecting increased CE formation in media (Figure 2A and 2B). Thus, the study suggests that matched for cholesterol concentration in media, T-HDL shows either normal or enhanced cholesterol efflux properties. HDL from both groups showed significantly higher levels of efflux at higher (72 μg/mL cholesterol) compared with lower HDL concentration (36 μg/mL cholesterol). To confirm the increased cholesterol efflux potential in subjects receiving the 60-mg torcetrapib dose (the dose that was used in Phase III studies), pooled HDL from subjects before and after treatment with 60 mg torcetrapib was added to cells using the same volume of PEG supernatant. T-HDL promoted a modest increase in net cholesterol efflux and CE accumulation in media as compared with C-HDL at each of the 3 volumes used (approximately 1.5-fold; Figure 3). Taken together, these data suggest that 60 mg torcetrapib treatment modestly improved cholesterol efflux by increasing HDL levels, whereas 120 mg torcetrapib more dramatically increased efflux both by increasing HDL levels and also by improving the ability of HDL particles themselves to promote net cholesterol efflux.

Role of Different Transporters in Mediating Net Cholesterol Efflux to C- and T-HDL

Our previous studies using RNA interference and overexpression suggested an important role of ABCG1 in mediating cholesterol efflux to CETP-D HDL.11 This was confirmed for both control and T-HDL in the present study using ABCG1 knock-out (ABCG1−/−) mouse peritoneal macrophages previously treated with AcLDL and TO901317 for 16 hours. These cells displayed an approximately 40% and 50% decrease in cholesterol efflux using C-HDL and T-HDL, respectively, as compared with WT macrophages (Figure 4A). We next investigated the role of ABCA1 and SR-BI in the ability of T-HDL to promote net cholesterol efflux from macrophage foam cells. Knock-down of ABCA1 by siRNA in WT macrophages resulted in a slight increase in T-HDL–mediated cholesterol efflux (1.2-fold), possibly reflecting a compensatory induction of ABCG1 (Figure 4A). In contrast, knockdown of ABCA1 in ABCG1-deficient cells led to a small but significant further decrement in cholesterol efflux to both control and T-HDL compared with ABCG1−/− cells. Somewhat surprisingly, knockout of SR-BI had no effect on net cholesterol efflux to either C- or T-HDL (Figure 4B).

Effects of SR-BI and ABCG1 Overexpression on Cholesterol Efflux Mediated by T-HDL

It has been reported that SR-BI expression decreases with macrophage cholesterol loading,22 possibly accounting for the lack of effect of SR-BI deficiency on cholesterol efflux in studies using cultured macrophages (Figure 4B). To determine net cholesterol efflux in cells expressing high levels of SR-BI, we transiently transfected HEK293 cells with the murine SR-BI cDNA and cholesterol efflux was determined.
after addition of C- and T-HDL for 6 hours. ABCG1 cDNA transfection was used as control. The ABCG1-mediated net cholesterol efflux to T-HDL was significantly increased compared with that of C-HDL (1.3-fold) confirming the role of this transporter in increased efflux by T-HDL (Figure 5). SR-BI-transfected cells did not exhibit significant net cholesterol efflux to either C-HDL or T-HDL (Figure 5). Thus, ABCG1 but not SR-BI appears to play a key role in control and T-HDL promoted net cholesterol efflux.

Discussion
This study was undertaken to assess the functionality of HDL accumulating in subjects treated with the CETP inhibitor torcetrapib, at 2 different doses. Matched for HDL cholesterol concentration, there was no difference in the ability of pre- and post-treatment HDL to promote net cholesterol efflux from human THP-1 macrophage foam cells at the 60-mg dose. However, because of the increase in HDL concentration resulting from the treatment with 60 mg torcetrapib, there was a modest increased level of cholesterol efflux when HDL samples were matched by volume. In contrast, the higher dose (120 mg) led to higher levels of cholesterol efflux matched for particle cholesterol content, as well as the likely effects of higher HDL concentration resulting from the treatment. Increased functional capacity per particle was driven by an increase in media CE formation, reflecting an increased content of apoE and LCAT and an increased HDL PC/SM ratio at the 120 mg dose.

Unfortunately, phase III clinical trials of torcetrapib treatment were recently stopped because of an excess number of deaths, heart failure, angina, and revascularization procedures in high risk subjects receiving Torcetrapib 60 mg in combination with Atorvastatin. The reasons for this failure are presently unknown, but there has been concern that large CE-rich HDL particles accumulating in CETP-D might not be antiatherogenic.23 Ishigami et al reported that macrophage mass cholesterol efflux was impaired using apoE-free HDL2 from CETP-D patients.10 Thus, a crucial question is whether CETP inhibition by torcetrapib treatment influenced the functionality of HDL (T-HDL). The present study suggest that functionality of HDL is either unchanged (60 mg dose) or enhanced (120 mg dose), confirming the results of our earlier study done in a small number of subjects with complete CETP deficiency compared with healthy controls.11 It is conceivable that at the 60 mg Torcetrapib dose, improvements in HDL function and incremental LDL lowering24 are too modest to overcome adverse cardiovascular events related to hypertension and associated untoward vascular effects of
torcetrapib. The hypertension induced by torcetrapib appears not to be attributable to CETP inhibition, because subjects with genetic deficiency of CETP do not show hypertension, and other CETP inhibitors have been developed that does not cause hypertension. However, it is also possible that the adverse outcome was partly mechanism-related because studies of genetic CETP deficiency have not provided clear evidence of a beneficial effect on CHD. Nevertheless, the findings in this study raise the possibility that higher levels of inhibition could have more dramatic effects on HDL function.

To gain greater insight into the nature of the changes induced by the different doses of CETP inhibition, the protein composition of control and T-HDL-2 was analyzed. Interestingly, high LCAT and apoE protein levels were observed in T-HDL-2 from subjects treated with the higher dose of torcetrapib, correlating with the finding of the increased HDL particle efficiency that was more prominent in this group. Earlier studies revealed the importance of apoE in conjunction with the action of LCAT, presumably facilitating the expansion of the CE core of HDL and permitting ongoing cholesterol efflux from macrophage foam cells. Consistent with our observation was the increased plasma apoE observed after high-dose torcetrapib treatment in a previous study performed by Clark et al., as well as the increased apoE and LCAT protein content in the HDL-2 fraction from CETP-D subjects. The SM/PC ratio of HDL could have a major impact on these observed changes because it has been previously reported that increased SM/PC ratio decreased the binding of apoE to lipid emulsions as well as the binding and activity of LCAT in reconstituted HDL particles, whereas decreased SM/PC ratio of control HDL-2 increased cholesterol efflux from macrophage foam cells. The CETP-mediated exchange of choline-containing phospholipids (PC, SM) between HDL and apoB-lipoproteins could play a role in determining the SM/PC ratio of HDL and thus the content and activity of apoE and LCAT in HDL.

Previous studies have shown that 2 weeks treatment with 60 mg torcetrapib results in 35% inhibition of CETP activity, whereas at 120 mg the inhibition was 53%. Together these findings could suggest that inhibition of CETP by more than 50% might be required to result in key compositional changes such as apoE enrichment and enhanced particle functionality.

The potential mechanisms by which T-HDL promotes net cholesterol efflux from macrophage foam cells could involve passive diffusion and also ABC transporters or SR-BI. ABCG1 is abundantly expressed in macrophages, especially after cholesterol loading and activation of the transcription factor liver X receptor (LXR), and this transporter promotes net cholesterol efflux from cells to HDL. Our findings revealed a major role of this transporter in the ability of both C-HDL and T-HDL to promote net cholesterol efflux as previously reported for CETP-D HDL. SR-BI facilitates the bidirectional flux of cholesterol between cells and HDL, and it is likely that this receptor efficiently promotes selective uptake of FC and CE from CETP-D HDL in liver. However, our results do not support a role of SR-BI in the ability to T-HDL to promote net cholesterol efflux from macrophage foam cells. ABCA1, which is regulated via similar mechanisms as ABCG1, mediates cholesterol efflux to lipid-poor apoA-I, generating HDL particles that can then interact with ABCG1. A deficiency of ABCA1 led to a slight but significant increase in net cholesterol efflux when macrophages where exposed to T-HDL, perhaps reflecting compensatory induction of ABCG1. This interpretation is strengthened by the fact that suppression of ABCA1 and ABCG1 together abolished this effect. Interestingly, the approximately 60% decrease in net cholesterol efflux induced by knockdown of both ABCA1 and ABCG1 indicates they are major transporters mediating the efflux of cholesterol from macrophage foam cells to control and T-HDL. The small decrement in efflux attributable to knock-down of ABCA1 in ABCG1" cells does not necessarily signify that ABCA1 mediates cholesterol efflux directly to HDL particles. More likely this reflects compensatory induction of ABCA1 and apoE secretion and associated cholesterol efflux in ABCG1-deficient cells and the reversal of these changes by ABCA1 knockdown. The mechanism responsible for the residual 40% of efflux could represent “passive” cholesterol efflux.

**Figure 3.** Dose–response curve for cholesterol efflux induced by similar volume of control and T-HDL. THP-1 macrophages were incubated for 24 hours with 50 μg/mL acLDL and 3 μmol/L TO901317. Then, increased volumes of pooled C-HDL (0.53 mg/mL cholesterol) and T-HDL (0.80 mg/mL cholesterol) from 60 mg torcetrapib group were added to 500 μL of media for 8 hours before cholesterol mass analysis. Values are means±SEM of an experiment performed in triplicate. *P<0.05, significant difference vs control HDL.
It should also be pointed out that ABCG1 does not directly bind HDL and that it may act to increase availability of plasma membrane cholesterol to a variety of acceptors, similar to passive diffusion.16

Recent studies have reported mixed results for the effects of macrophage ABCG1 deficiency on atherosclerosis.18,40,41 With the complete absence of ABCG1 there is a secondary induction of ABCA1, a posttranscriptional increase in apoE secretion,18 and enhanced susceptibility to oxidized LDL-induced apoptosis,40 resulting in a modest reduction in atherosclerosis in some but not all studies.18,40,41 However, the relevance of these findings to increasing macrophage cholesterol efflux to HDL via ABCG1 and other pathways is uncertain, and in view of the epidemiology of HDL and the likely central role of HDL-mediated cholesterol efflux from macrophages in explaining the protective effect of HDL, it seems reasonable to assume that increased cholesterol efflux to HDL as observed in this study would most likely be antiatherogenic in vivo.

**Figure 5.** Cholesterol efflux from HEK293 cells transfected with SR-BI or ABCG1 cDNAs. Cells were transiently transfected with plasmid constructs expressing SR-BI, ABCG1, or empty vector, and cholesterol efflux was initiated by addition of 100 μg/mL HDL cholesterol from 120 mg torcetrapib-treated group for 6 hours. Data are means±SEM of an experiment performed in triplicate. *P<0.05, significant difference vs empty vector. §P<0.05, significant difference vs control HDL.

To summarize, our data indicate that the CETP inhibitor torcetrapib increases the ability of HDL to promote cholesterol efflux from macrophage foam cells. The increased LCAT and apoE content in HDL−2 from patients treated with the higher dose of torcetrapib along with the decreased SM/PC ratio of HDL might lead to increased ability of HDL particles to promote cholesterol efflux. Although the limitations of in vitro cholesterol efflux studies,25 decrease their predictive power, they suggest that at the 60 mg dose of torcetrapib, there may be only modest improvements in HDL function that could be outweighed by other off-target effects of Torcetrapib such as hypertension. Higher levels of CETP inhibition are likely to result in greater improvements in cholesterol efflux potential, higher levels of HDL, and significantly more incremental LDL lowering. Only a clinical trial, performed in conjunction with atherosclerosis imaging studies, can determine if this would be beneficial for patients.

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**Disclosures**
Alan R. Tall reports being a consultant to Pfizer, Merck, Boehringer-Ingelheim, and Takeda Pharmaceuticals.

**References**
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Supplemental Table 1. Baseline Characteristics

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Data are presented as mean ± SD. BMI indicates body mass index. There were no significant differences between the groups.
Supplemental Figure 1

(A) Western blot analysis showing the expression of ApoE, LCAT, and ApoAI before and after treatment with Torcetrapib at doses of 60 mg and 120 mg. The images are presented for 0 Weeks and 8 Weeks.

(B) Bar graph showing the SM/PC ratio before treatment and after treatment with Torcetrapib at doses of 60 mg and 120 mg. The bars indicate the mean with standard deviation. The asterisk (*) denotes a significant difference. The y-axis represents the SM/PC ratio ranging from 0 to 0.35.