Lecithin: Cholesterol Acyltransferase Expression Has Minimal Effects on Macrophage Reverse Cholesterol Transport In Vivo

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Background—Lecithin:cholesterol acyltransferase (LCAT) catalyzes the formation of plasma cholesteryl ester, plays a key role in high-density lipoprotein metabolism, and has been believed to be critical in the process of reverse cholesterol transport (RCT).

Methods and Results—The role of LCAT in RCT from macrophages was quantified with a validated assay involving intraperitoneal injection in mice of 3H-cholesterol–labeled J774 macrophages and monitoring the appearance of tracer in plasma, liver, bile, and feces. Human LCAT overexpression in human apolipoprotein A-I transgenic mice substantially increased plasma high-density lipoprotein cholesterol levels but surprisingly did not increase macrophage RCT. Even in the setting of coexpression of scavenger receptor BI or cholesteryl ester transfer protein, both of which promoted the transfer of LCAT-derived high-density lipoprotein cholesterol ester to the liver, LCAT overexpression still had no effect on RCT. Serum from LCAT-overexpressing mice had reduced ability to promote cholesterol efflux from macrophages ex vivo via ABCA1. To determine the effect of LCAT deficiency on macrophage RCT, LCAT−/− and LCAT+/− mice were compared with wild-type mice. Despite extremely low plasma levels of high-density lipoprotein cholesterol, LCAT-deficient mice had only a 50% reduction in RCT. LCAT+/− mice had normal RCT despite a significant reduction in high-density lipoprotein cholesterol. Serum from LCAT-deficient mice had increased ability to promote ABCA1-mediated cholesterol efflux from macrophages ex vivo.

Conclusions—These results demonstrate that LCAT overexpression does not promote an increased rate of macrophage RCT. Although LCAT activity does become rate limiting in the context of complete LCAT deficiency, RCT is reduced by only 50% even in the absence of LCAT. These data suggest that macrophage RCT may not be as dependent on LCAT activity as has previously been believed. (Circulation. 2009;120:160-169.)

Key Words: apolipoproteins ■ atherosclerosis ■ cholesterol ■ lipids ■ metabolism

Lecithin:cholesterol acyltransferase (LCAT) is a key enzyme in the metabolism of high-density lipoprotein (HDL). It is responsible for the synthesis of cholesteryl esters (CEs) in human plasma by catalyzing a reaction in which a fatty acyl residue from the sn-2 position of phosphatidylcholine is transferred to the 3-beta-hydroxy group of cholesterol, resulting in the formation of CE.1 The hydrophobic CE moves to the core of the HDL particle, contributing to the generation of mature HDL particles and their progressive enlargement. The importance of LCAT in normal HDL metabolism is clear from the genetic conditions LCAT deficiency and fish-eye disease,2,3 both of which are associated with markedly reduced levels of plasma HDL cholesterol (HDL-C). Inability to synthesize cholesteryl ester via LCAT results in rapid catabolism of HDL-associated apolipoprotein (Apo) A-I and ApoA-II.

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In addition to its role in HDL metabolism, LCAT has been proposed to have a critical and central role in reverse cholesterol transport (RCT), the process by which excess peripheral cholesterol is effluxed to HDL-based acceptors and returned to the liver for biliary excretion. Glomset4 proposed that LCAT was central to RCT by removing free cholesterol from the surface of HDL, thus helping to maintain a gradient of free cholesterol from cells to HDL. Although this has been a widely accepted paradigm, there are several reasons to question the critical nature of LCAT
in RCT. First, although passive diffusion was previously considered the dominant mechanism of cellular cholesterol efflux, it is now recognized that facilitated transport via ABCA1, ABCG1, and possibly SR-B1 is quantitatively important and is likely to be less sensitive to a free cholesterol gradient. Second, neither LCAT-deficient humans nor mice appear to be at the markedly increased risk of atherosclerosis that might be expected if they had a major defect in RCT. Given this uncertainty, we used a previously validated method for specifically assessing macrophage-to-feces RCT in mice5–9 to address the role of LCAT in macrophage RCT in mice. Our conclusions are that neither LCAT overexpression nor loss of function has the expected effects on macrophage RCT based on the current paradigm and suggest that LCAT may not be a critical regulator of RCT, at least from macrophages.

Methods

Vector Construction

The AAV8 TGB human (h) LCAT vector contains the hLCAT cDNA insert followed by SV-40 poly A tail and is driven by the liver-specific human TGB promoter. The AAV8 hLCAT was produced with a chimeric packaging construct in which the AAV2 rep gene is fused with the cap gene of serotype 8.10–11 The control vector consisted of the LacZ gene packaged into serotype 8.

Plasma Lipid Analysis

Plasma total cholesterol, HDL-C, and triglycerides were measured on a Cobas FARA; plasma 3H-cholesterol was counted by liquid scintillation counting. Plasma total cholesterol, HDL-C, and triglycerides were measured by fast protein liquid chromatography (FPLC) gel chromatography. Plasma Lipid Analysis Plasma total cholesterol, HDL-C, and triglycerides were measured on a Cobas FARA; plasma 3H-cholesterol was counted by liquid scintillation counting. Pooled plasma (160 μL) from mice was analyzed by fast protein liquid chromatography (FPLC) gel filtration (Amersham Pharmacia Biotech, Piscataway, NJ). The cholesterol concentrations in the FPLC fractions were determined by an enzymatic assay (Wako Pure Chemical Industries Ltd, Osaka, Japan). Free cholesterol and esterified cholesterol in the samples were separated by thin-layer chromatography and quantified by liquid scintillation counting to determine percent CE.12

LCAT Activity Assay

Plasma cholesterol esterification rate (expressed in nmol · h⁻¹ · mL⁻¹ plasma) was determined by use of a modification of the Stocke and Norum13 assay. Briefly, each plasma sample is equilibrated overnight (4°C) in a glass tube with a paper disk containing trace amount (0.3 μCi) of 3H-cholesterol; on the next day, the reaction takes place during a 30-minute incubation at 37°C and it is terminated by adding 1 mL of 100% ethanol. After a 20-minute centrifugation at 3200 rpm at room temperature, the liquid phase is removed and evaporated to dryness under nitrogen gas. Free cholesterol and CE in the samples are then separated by thin-layer chromatography.

Immunoblotting

We used 5 μL of pooled plasma or 2 μL of each fraction plasma per lane for immunoblotting. The plasma sample was resolved on NuPAGE 4% to 12% Bis-Tris SDS-PAGE (Invitrogen, Carlsbad, Calif) and transferred to ECL Hybond membrane (Amersham Pharmacia Biotech, Piscataway, NJ). ApoA-1 was detected with an anti-human ApoA-1 primary antibody (Wako Pure Chemical Industries Ltd, Osaka, Japan) and horseradish peroxidase–conjugated donkey anti-goat IgG. The LCAT was detected with an anti-LCAT primary antibody (NOVUS Biologicals, Littleton, Colo) and horse radish peroxidase–conjugated donkey anti-rabbit IgG.

CE Studies

J774 macrophages were plated in 12-well plates (0.14×10⁶/mL) in RPMI 1640 supplemented with 10% (vol/vol) FBS plus gentamycin. After 1 day, cells were labeled for 24 hours with 3μCi/mL 3H-cholesterol in the presence of 5.0% FBS. The experiments were performed in the presence of 2 μg/mL of the ACAT inhibitor CP113,818 to prevent cellular accumulation of CE. Cells were washed extensively with MEM HEPES with 1% BSA, and then J774 macrophages were equilibrated with or without 0.3 mM/L cyclic AMP (cAMP), which activates mainly ABCA1, in 0.2% BSA-containing medium for 14 hours.14 At the end of the equilibration period, cell monolayers were washed once with MEM HEPES containing 1% BSA and once with MEM HEPES. Subsequently, efflux of cholesterol was induced by incubation for 4 hours with each pooled plasma diluted to 2.5%. Free cholesterol efflux was obtained by measuring the release of radiolabeled cholesterol into the medium as previously described.15

Bone marrow macrophages were isolated from hApoA-1 transgenic (tg) mice and plated. Cells were labeled with 3H-cholesterol (2 μCi/mL; Amersham Pharmacia Biotech) and loaded with or without 25 μg/mL acetyl low-density lipoprotein (ac-LDL) for 24 hours. Then, cells were washed and equilibrated overnight in the presence of GW3965 (10 μmol/L) in serum-free medium. For cholesterol efflux, cells were treated with or without 10 μmol/L protocol for 2 hours.16 Subsequently, 2% pooled plasma samples for ac-LDL–loaded macrophage or 1.75% pooled plasma samples for ac-LDL–unloaded macrophage were added to cells as acceptors. After 4 hours, aliquots of the medium were removed, and the 3H-cholesterol released was measured by liquid scintillation counting. The 3H-cholesterol present in the cells was determined by extracting the cell lipids in isopropanol.17

RCT Studies

RCT studies were performed as previously described.5–9 J774 cells, obtained from American Type Culture Collection (Manassas, Va), were grown in suspension in RPMI 1640 supplemented with 10% FBS. Cells were radiolabeled with 5μCi/mL 3H-cholesterol and cholesterol enriched with 100 μg/mL ac-LDL for 48 hours. These foam cells were washed twice, equilibrated in medium with 0.2% BSA for 6 hours, spun down, and resuspended in RPMI medium immediately before use.

Independent experiments were performed in hApoA-1 tg mice (Jackson Laboratories, Bar Harbor, Me) injected with 1×10¹² genome copies (GC) of AAV8 hLCAT, 1×10¹⁰ GC of AAV8 hLCAT, 1×10¹⁰ GC of AAV8 hLCAT+1×10¹⁰ GC of AAV8 human cholesterol ester transfer protein (hCETP), or 1×10¹⁰ GC of AAV8 hLCAT+1×10¹¹ GC of AAV8 murine SR-B1 to determine the effect of LCAT overexpression on RCT and in LCAT /- mice (in-house bred), LCAT /- mice, and wild-type (WT; Jackson Laboratories) mice to determine the effect of LCAT deficiency. All mice were fed a standard chow diet. In the initial experiment, mice were divided into 2 groups. The 1×10¹² GC of AAV8 hLCAT (n=8) or AAV8 LacZ (n=7) was injected intraperitoneally in 12- to 16-week-old hApoA-1 tg male mice, and on day 26, animals were injected intraperitoneally with 3H-cholesterol–labeled J774 cells as described previously.3 They 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. Feces were collected continuously from 0 to 48 hours and stored at 4°C before being counted by liquid scintillation counting. At study termination (48 hours after injection), mice were exsanguinated and perfused with cold PBS, and portions of the liver were removed and flash-frozen for lipid extraction and gene expression analysis.

In sequential experiments, 1×10¹² GC of AAV8 hLCAT (n=8) or LacZ (n=8), 1×10¹⁰ GC of AAV8 hLCAT+1×10¹⁰ GC of AAV8 hCETP (n=8) or 1×10¹⁰ GC of AAV8 LacZ+1×10¹⁰ GC of AAV8 hCETP (n=6), 1×10¹⁰ GC of AAV8 hLCAT+1×10¹¹ GC of AAV8 murine SR-B1 (n=8), or 1×10¹⁰ GC of AAV8 LacZ+1×10¹¹ GC of AAV8 murine SR-B1 (n=7) was injected intravenously in 12- to 16-week-old hApoA-1 tg male. On day 26, animals were injected intraperitoneally with 3H-cholesterol–labeled J774 cells, and RCT study was performed in the manner
described above. Furthermore, RCT study was performed in 12- to 16-week-old WT female mice (Jackson Laboratories; n=8), LCAT+/− female mice (in-house bred; n=8), and LCAT−/− female mice (in-house bred; n=8).

Liver, Bile, and Fecal Analyses
Liver lipids were extracted according to the procedure of Bligh and Dyer. The lipid layer was collected, evaporated, resuspended in toluene, and counted by liquid scintillation counting. Results were expressed as a percentage of injected counts per minute/liver. The bile was diluted up to 200 μL with PBS and counted by liquid scintillation counting. The total feces collected from 0 to 48 hours were weighed and soaked in distilled water (1 mL water/100 mg feces) overnight at 4°C. The next day, an equal volume of ethanol was added, and the samples were homogenized. Finally, 2 mL of the homogenized samples was combined with 2 mL ethanol and counted by liquid scintillation counting.

Statistical Analysis
Values are presented as mean±SD. Results were analyzed with the use of GraphPad Prism Software. Results involving LCAT overexpressing mice compared with controls, including plasma lipid and tracer levels, fecal excretion of tracer, and assays of cholesterol efflux, were analyzed by Student’s t test. Results involving LCAT homozygous and heterozygous knockout mice compared with controls, including the same parameters, were analyzed by 1-way ANOVA with Tukey multiple-comparison test.

Results
LCAT Overexpression Substantially Increased HDL-C Levels but Not Macrophage RCT
In Vivo
In a pilot study, adeno-associated viral vectors, with the liver-specific promoter TBG, encoding human LCAT (AAV8-TBG-hLCAT) or LacZ (AAV8-TBG-lacZ) were injected intraperitoneally into hApoA-I tg mice at a dose of 1×1012 GC. Blood was drawn at baseline and 2 and 4 weeks after injection. Plasma LCAT protein and activity increased by 2 weeks after injection and were stable at 4 weeks (Figure 1A). Plasma HDL-C levels also were significantly increased at 2 weeks and stable at 4 weeks (Figure 1B).

To determine the effect of LCAT overexpression on RCT, 1×1012 GC of AAV8-TBG-hLCAT was injected intraperitoneally into hApoA-I tg mice and compared with the same dose of control AAV8-TBG-LacZ. Four weeks after injection, plasma LCAT activity was 6.7-fold higher in the LCAT-expressing group compared with the control group (603.5 versus 90.3 nmol · mL−1 · h−1), resulting in a 5.8-fold increase in HDL-C levels (1614±86 versus 276±17 mg/dL). Cholesterol-loaded and 3H-cholesterol-labeled J774 macrophages were injected at 4 weeks to assess macrophage RCT. LCAT-overexpressing mice had higher plasma 3H-cholesterol counts at all time points compared with controls (P<0.05; Figure 2A). FPLC analysis at 24 hours confirmed that the lipoprotein distribution of 3H-cholesterol in plasma was similar to that of cholesterol mass and demonstrated markedly increased cholesterol mass and 3H-cholesterol counts, particularly in a very large HDL fraction (Figure 2B). Despite the increase in plasma HDL-C mass and tracer, there was a significant 20% decrease (5.48±0.9% versus 4.40±1.0% of injection per total liver) in 3H-sterol in liver (P<0.05), a significant 66% decrease (0.34±0.005% versus 0.011±0.003% of injection/μL bile) in 3H-sterol in bile (P<0.01), and a significant 35% decrease in fecal 3H-sterol over the 4-hour period of the experiment (P<0.01; Figure 2C).

We performed an experiment with more modest overexpression of human LCAT using injection of only 1×1010 GC (2 orders of magnitude less) of AAV8-TBG-hLCAT into hApoA-I tg mice. Four weeks after injection, the plasma LCAT activity in LCAT-expressing mice was 2.1-fold higher than in control mice (348.7 versus 163.3 nmol · mL−1 · h−1), resulting in a more modest 2.9-fold increase in HDL-C (419±165 versus 145±7 mg/dL). On performance of the RCT study, LCAT-overexpressing mice had higher plasma 3H-cholesterol counts at all time points compared with controls (P<0.05; Figure 3A). FPLC analysis confirmed substantially increased cholesterol mass and 3H-cholesterol counts in the HDL fraction in the LCAT group (Figure 3B). There was no significant change in 3H-sterol in bile and liver at 48 hours (data not shown), but there was a significant 24% decrease in feces over the 48-hour period (P<0.01; Figure 3C). These results indicate that although both substantial and modest LCAT overexpression increased plasma HDL-C levels, LCAT overexpression did not increase RCT from macrophages to feces in vivo.

We hypothesized that cholesterol efflux via the ABCA1 pathway may have been reduced in LCAT-overexpressing mice because of reduced availability of lipid-poor ApoA-I in the setting of increased cholesterol esterification and formation of mature HDL particles. To test this hypothesis, plasma from mice injected with 1×1012 GC of AAV8-hLCAT was used for ex vivo cholesterol efflux studies compared with plasma from control AAV8-lacZ–injected mice. Plasma from LCAT-overexpressing mice had significantly reduced capacity to promote ABCA1-specific ef-
flux by 64% from J774 macrophages ($P<0.01$) (Figure 4A) and by 19% from cholesterol-loaded ($P<0.01$) or non–cholesterol-loaded ($P<0.05$) bone marrow macrophages (Figure 4B and 4C). However, there was reciprocal increased capacity to promote non–ABCA1-mediated efflux (Figure 4A through 4C), likely resulting from the combination of ABCG1-mediated efflux and passive diffusion. Nevertheless, the reduced ABCA1-mediated cho-
Lesterol efflux may have contributed to the overall reduced macrophage RCT seen in the context of LCAT overexpression.

**LCAT Overexpression Did Not Promote Macrophage RCT Even in the Setting of SR-BI Overexpression or CETP Expression**

We previously demonstrated that hepatic SR-BI is an important determinant of macrophage RCT, with increased RCT observed on overexpression of SR-BI. We therefore hypothesized that in the setting of LCAT overexpression, hepatic SR-BI is rate limiting in the RCT pathway. To test this hypothesis, we overexpressed LCAT in the setting of SR-BI overexpression in hApoA-I tg mice. On the background of SR-BI overexpression, LCAT overexpression resulted in a 168% increase in HDL-C level (96 ± 6 versus 36 ± 13 mg/dL), but HDL-C levels were much lower than with LCAT overexpression alone. In the RCT study, on the background of SR-BI overexpression, LCAT overexpression resulted in higher plasma 3H-cholesterol counts at all time points compared with controls (P < 0.05; Figure 5A). FPLC analysis demonstrated increased cholesterol mass and 3H-cholesterol counts in the HDL fraction in the LCAT-expressing group (Figure 5B) but without evidence of the very large HDL particle seen with LCAT overexpression alone (Figure 3B). There were no significant differences in 3H-sterol in bile and liver at 48 hours (data not shown) or in feces collected over the 48-hour period (Figure 5C). Thus, even in the setting of SR-BI coexpression, LCAT overexpression failed to promote RCT. However, overexpression of SR-BI did prevent the decrease in RCT observed with LCAT coexpression. Ex vivo plasma efflux experiments demonstrated that plasma from mice overexpressing LCAT and SR-BI had significantly reduced ABCA1-dependent (P < 0.05) and total cholesterol efflux (P < 0.01) compared with mice overexpressing only SR-BI (Figure 5D).

We previously showed that CETP expression promotes macrophage RCT in mice and that CETP expression restores normal the impaired RCT in SR-BI–deficient mice. We hypothesized that CETP expression might allow greater flux of cholesteryl esters from HDL to liver in the setting of LCAT overexpression and thus permit LCAT overexpression to promote RCT. To test this hypothesis, we expressed human

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**Figure 4. Efflux study using pooled plasma taken from hApoA-1 tg mice injected with 1 x 10^12 GC of hLCAT AAV8 or LacZ as a control using J774 macrophage and bone marrow macrophage. A, Total (with cAMP), ABCA1-independent (without cAMP), and ABCA1-dependent efflux in J774 macrophage. B, Total, ABCA1-independent (with probucol), and ABCA1-dependent efflux in bone marrow macrophages with ac-LDL. C, Total, ABCA1-independent (with probucol), and ABCA1-dependent efflux in bone marrow macrophage without ac-LDL. Data shown are mean ± SD. *P < 0.05, **P < 0.01.**
CETP in the setting of human LCAT overexpression in hApoA-I tg mice. On the background of CETP expression, LCAT overexpression resulted in a 107% increase in HDL-C level (48/11006/6 versus 23/11006/5 mg/dL), but HDL-C levels were much lower than with LCAT expression alone. In the RCT study, on the background of CETP expression, LCAT expression had no effect on plasma 3H-cholesterol counts compared with controls (Figure 6A). FPLC analysis demonstrated greatly increased cholesterol mass and 3H-cholesterol counts in both the LDL fraction and the small HDL fraction in the LCAT-overexpressing group (Figure 6B). There were no significant differences in 3H-sterol in bile and liver at 48 hours (data not shown) or in feces collected over the 48-hour period (Figure 6C). Thus, CETP expression did not have the effect of permitting LCAT overexpression to promote RCT but did prevent the decrease in RCT observed with LCAT overexpression alone. Indeed, ex vivo plasma efflux experiments again demonstrated that plasma from mice expressing LCAT and CETP had significantly reduced ABCA1-dependent cholesterol efflux compared with mice expressing only CETP (P<0.05; Figure 6D).

**Homozygous LCAT-Null Mice Had 50% Reduced and Heterozygous LCAT-Null Mice Had Normal Macrophage RCT**

If cholesterol esterification by LCAT is a critical component of the RCT pathway, then LCAT deficiency would be expected to result in severely reduced RCT. To test this hypothesis, WT mice, LCAT+/− mice, and LCAT−/− mice were used in an RCT study. HDL-C levels were 52±9 versus 38±3 versus 3.4±1.5 mg/dL (WT versus LCAT+/− versus LCAT−/−) and correlated with LCAT activity, 192.2 versus 148.6 versus 0.8 nmol · mL⁻¹ · h⁻¹ (WT mice versus LCAT+/− mice versus LCAT−/− mice). In the in vivo RCT study, LCAT−/− mice had significantly decreased plasma 3H-cholesterol counts compared with WT controls (P<0.05), and LCAT+/− mice were intermediate (Figure 7A). The percent of 3H-cholesterol counts in cholesteryl ester in plasma was markedly reduced in LCAT−/− mice (23%) compared with 73% in LCAT+/− mice and 76% in WT mice. FPLC analysis confirmed that the 3H-cholesterol in plasma tracked closely with the cholesterol mass, demonstrating markedly decreased cholesterol mass and 3H-cholesterol counts in the HDL fractions in the LCAT−/− mice and intermediate levels in the LCAT+/− mice compared with controls (Figure 7B). There were no significant differences among the 3 groups in 3H-sterol in bile at 48 hours (data not shown). Fecal excretion of 3H-sterol over 48 hours was significantly reduced by 45% in LCAT−/− mice (P<0.05) but was normal in LCAT+/− mice (Figure 7C). An ex vivo cholesterol efflux study using J774 cells demonstrated that plasma from LCAT−/− mice promoted total cholesterol efflux to the same extent as WT
mice plasma and promoted significantly greater ABCA1-specific efflux. Plasma from LCAT/H11001/H11002 mice promoted more total efflux ($P<0.05$) and more ABCA1-specific efflux ($P<0.05$) compared with WT mice plasma (Figure 7D).

**Discussion**

In these studies, we used overexpression of human LCAT in hApoA-I tg mice as a model to assess effects of LCAT overexpression on macrophage RCT. We found that although both marked and moderate LCAT overexpression substantially raised levels of HDL-C as expected, they unexpectedly resulted in reduced macrophage RCT, accompanied by a reduced ability of the plasma from LCAT-overexpressing mice to promote ABCA1-mediated efflux from macrophages. Even coexpression of SR-BI or CETP, intended to facilitate the return of LCAT-derived HDL-CE to the liver, failed to result in promotion of RCT by LCAT overexpression. In addition, although LCAT-deficient mice did have reduced macrophage RCT, consistent with a role for LCAT in RCT, it was reduced by only 50% despite markedly reduced plasma HDL-C levels. LCAT heterozygotes had normal macrophage RCT despite significantly lower plasma HDL-C levels and plasma LCAT activity. Thus, our results challenge the paradigm that LCAT plays a central role in modulating the rate of macrophage RCT and suggest that therapeutic intervention to increase LCAT activity may not necessarily have an RCT-promoting and thus atheroprotective effect.

In this light, a review of the literature on the relationship between LCAT overexpression and atherosclerosis in animal models is of interest. In LCAT tg rabbits fed a cholesterol diet, HDL levels were significantly increased$^{19}$ and atherosclerotic lesions were significantly reduced.$^{20}$ In contrast, using human LCAT tg mice fed an atherogenic diet, Berard et al$^{21}$ reported that overexpression of human LCAT (50- to 116-fold) was associated with markedly increased atherosclerosis. In other studies in human LCAT tg mice fed an atherogenic diet by Mehlum et al, overexpression of LCAT (17-fold) was associated with no difference$^{22}$ or increased$^{23}$ atherosclerosis, depending on the duration of the study. Furbee et al$^{24}$ reported that 10-fold overexpression of human LCAT in tg mice fed an atherogenic diet did not result in increased aortic cholesterol deposition. Mertens et al$^{25}$ used a recombinant adenovirus ($5 \times 10^{10}$ plaque-forming units) encoding human LCAT in LDLR$^{-/-}$ ob/ob double-mutant mice fed a chow diet and reported that there was no change in HDL level despite an increase in plasma LCAT activity and a 42% reduction in aortic root atherosclerosis. The inconsistency in the results may be related in part to varied levels of LCAT overexpression and possibly the specific murine models used.

In the present studies, we used an AAV vector to stably overexpress LCAT at 2 different doses, which increased plasma HDL-C levels in a dose-dependent fashion. Despite increases in HDL-C, we found no evidence of promotion of macrophage RCT by LCAT overexpression, largely consistent with the mouse atherosclerosis results. We initially hypothesized that LCAT overexpression, by generating large cholesterol-enriched HDL particles, may have resulted in a functional block in the efficient transfer of HDL-CE into liver. Indeed, when a human CETP

![Figure 6](image-url)
transgene was introduced into the LCAT tg mice with increased atherosclerosis mentioned above, CETP expression reduced atherosclerosis. Importantly, atherosclerosis in the double-tg mice was still greater than in control mice, indicating that even coexpression of LCAT and CETP was not atheroprotective. We previously showed that overexpression of either SR-BI or CETP, despite reducing HDL-C levels, promotes macrophage RCT. Thus, we tested whether uptake of HDL-CE into liver in LCAT-overexpressing mice was rate limiting by coexpressing either SR-BI or CETP together with LCAT. Although each reduced the plasma HDL-C level as expected, neither converted LCAT overexpression to an intervention that promoted macrophage RCT. We conclude that the inability of LCAT overexpression to promote macrophage RCT is not explained simply by the fact that hepatic uptake of LCAT-derived HDL-CE is rate limiting in this setting.

Notably, LCAT-deficient humans do not develop obviously premature atherosclerotic vascular disease. Reduced LCAT activity has been reported to be associated with increased intima-media thickness of the carotid arteries, but this report is not definitive, and more investigation of this issue is needed. Studies of the effects of LCAT deficiency on atherosclerosis in mice are discordant. Lambert et al showed that LCAT knockout mice in the C57Bl/6 or LDLr/−/− background fed a cholic acid–containing atherogenic diet had smaller atherosclerotic lesions compared with their counterparts with active LCAT. Consistent with that observation, Ng et al showed that aortic atherosclerotic lesions in male LCAT/−/− mice on an apoE/−/− background fed a chow diet had a 52% reduction in atherosclerosis; even LCAT+/− mice had a 24% reduction. In contrast, Furbee et al reported that LCAT deficiency in LDLr/−/− and apoE/−/− mice fed an atherogenic diet resulted in increased aortic cholesterol deposition. In the present study, we found that heterozygous deficiency of LCAT (LCAT+/− mice) was associated with normal macrophage RCT despite significantly reduced plasma HDL-C levels and plasma LCAT activity. Complete deficiency of LCAT, accompanied by marked 93% reduction in HDL-C levels, resulted in a 50% reduction in RCT, indicating that LCAT becomes rate limiting in the context of complete LCAT deficiency. However, substantial RCT from macrophage to feces still occurred in the absence of LCAT activity, highlighting the fact that LCAT is not required for RCT and indicating that substantial RCT can occur via unesterified cholesterol.

We considered the hypothesis that LCAT expression influences the generation of “pre-β HDL” or lipid-poor ApoA-I that serves as the acceptor for ABCA1-mediated cholesterol efflux. ABCA1 is a critical transporter that facilitates efflux of cellular cholesterol to lipid-poor ApoA-I; macrophage-specific deficiency of ABCA1 resulted in significantly increased atherosclerosis in ApoE/−/− mice fed a chow diet and in LDLr/−/− mice fed a high-cholesterol Western-type diet. Our experiments using plasma from LCAT-overexpressing mice revealed that although there was only a modest change in total efflux to serum, the mechanism of efflux changed greatly. The ability to promote cholesterol efflux from macrophages via

Figure 7. Efflux and macrophage RCT in LCAT−/− knockout mice (n = 8), LCAT+/− knockout mice (n = 8), or WT mice (n = 8). A, The change in 3H-labeled cholesterol in plasma. B, 3H-labeled cholesterol lipoprotein profile and cholesterol mass profile of pooled plasma samples drawn 48 hours after injection of J774 cells. C, 3H-labeled cholesterol in feces of each group. D, Total (with cAMP), ABCA1-independent (without cAMP), and ABCA1-dependent efflux in J774 macrophage. Data shown are mean ± SD. *P < 0.05.
the ABCA1 pathway was significantly reduced by LCAT overexpression, whereas ABCA1-independent efflux was increased. As expected, increased HDL-C levels in LCAT overexpression increased efflux via “non-ABCA1” pathways (such as ABCG1 and possibly SR-BI). Conversely, plasma from LCAT-deficient mice had a significantly greater capacity to promote ABCA1-mediated efflux.

Conclusions

Stable LCAT overexpression failed to increase macrophage RCT despite substantially increasing HDL-C levels. Even in the setting of SR-BI or CETP coexpression, both of which increased the delivery LCAT-derived HDL-CE to the liver, LCAT overexpression failed to increase macrophage RCT. In the heterozygous state, moderate LCAT deficiency did not impair RCT despite lower HDL-C levels. Complete LCAT deficiency reduced RCT by 50% but showed that substantial macrophage RCT can still occur in the absence of LCAT activity. These studies challenge the long-held belief that LCAT is critical for RCT and important in modulating its rate. Although additional experiments are needed, these results bring into question whether pharmacological LCAT activation would necessarily promote RCT and therefore be atheroprotective.

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CLINICAL PERSPECTIVE

Lecithin:cholesterol acyltransferase (LCAT) is a key enzyme in the metabolism of high-density lipoprotein (HDL). It converts “free” cholesterol (form effluxed from macrophages) to cholesteryl ester, permitting the formation and progressive enlargement of mature HDL particles. It has long been believed that LCAT is critical in facilitating the process of reverse cholesterol transport, leading to the concept that upregulation of LCAT activity is a therapeutic target for atherosclerosis, but this has never been experimentally proven. Using a validated assay of macrophage-specific reverse cholesterol transport in mice, we explored the effects of both LCAT overexpression (which raises HDL cholesterol [HDL-C] levels) and LCAT deficiency (which reduces HDL-C levels). Surprisingly, LCAT overexpression, despite markedly raising HDL-C, did not promote macrophage reverse cholesterol transport. Even coexpression of scavenger receptor BI or cholesteryl ester transfer protein, both of which transfer cholesterol ester out of HDL and facilitate its return to the liver, failed to convert LCAT overexpression into an intervention that promotes reverse cholesterol transport. Furthermore, complete LCAT deficiency, despite reducing HDL-C to <5% of normal levels, reduced reverse cholesterol transport by only ~50%, and moderate LCAT deficiency, despite moderately reducing HDL-C levels, had no effect on reverse cholesterol transport. These studies challenge the long-held belief that LCAT is critical for macrophage reverse cholesterol transport and bring into question whether LCAT activation would necessarily be an atheroprotective intervention even though it raises HDL-C levels. The results are consistent with a growing trend focused on HDL quality and function rather than simply plasma HDL-C levels as a target for the development of new therapies.
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