Increased plasma cholesterol esterification by LCAT reduces diet-induced atherosclerosis in SR-BI knockout mice

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Abstract LCAT, a plasma enzyme that esterifies cholesterol, has been proposed to play an antiatherogenic role, but animal and epidemiologic studies have yielded conflicting results. To gain insight into LCAT and the role of free cholesterol (FC) in atherosclerosis, we examined the effect of LCAT over- and underexpression in diet-induced atherosclerosis in scavenger receptor class B member I-deficient [Scarab(−/−)] mice, which have a secondary defect in cholesterol esterification. Scarab(−/−)×LCAT-null [Lcat(−/−)] mice had a decrease in HDL-cholesterol and a high plasma ratio of FC/total cholesterol (TC) (0.88 ± 0.033) and a marked increase in VLDL-cholesterol (VLDL-C) on a high-fat diet. Scarab(−/−)×LCAT-transgenic (Tg) mice had lower levels of VLDL-C and a normal plasma FC/TC ratio (0.28 ± 0.005). Plasma from Scarab(−/−)×LCAT-Tg mice also showed an increase in cholesterol esterification during in vitro cholesterol efflux, but increased esterification did not appear to affect the overall rate of cholesterol efflux or hepatic uptake of cholesterol. Scarab(−/−)×LCAT-Tg mice also displayed a 51% decrease in aortic sinus atherosclerosis compared with Scarab(−/−) mice (P < 0.05). In summary, we demonstrate that increased cholesterol esterification by LCAT is atheroprotective, most likely through its ability to increase HDL levels and decrease pro-atherogenic apolipoprotein B-containing lipoprotein particles.—Thacker, S. G., X. Rousett, S. Esmail, A. Zarzour, X. Jin, H. L. Collins, M. Sampson, J. Stonik, S. Demosky, D. A. Malide, L. Freeman, B. L. Vaisman, H. S. Kruth, S. J. Adelman, and A. T. Remaley.

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The reverse cholesterol transport (RCT) pathway plays a key role in protecting against atherosclerosis. During RCT, excess cholesterol is effluxed from peripheral cells, such as aortic macrophages, and is transported back to the liver where it can be excreted or converted to a bile salt. LCAT plays a vital role in this process (1), as it is the only known plasma enzyme that esterifies free cholesterol (FC). The esterification of cholesterol promotes net cholesterol efflux by trapping it in the hydrophobic core of lipoproteins, thus preventing its back exchange (2). Cholesterol esterification also promotes cholesterol efflux by increasing the concentration gradient of FC between cells and extracellular lipoprotein acceptors (2, 3). Radiolabeled cholesterol tracer studies in humans indicate that once esterified by LCAT, cholesteryl esters are preferentially returned to the liver (2), in part by scavenger receptor class B member I (SR-BI) (4, 5). As a consequence of its proposed anti-atherogenic effects, LCAT is now being investigated as a possible therapeutic target (6–9).

In addition to promoting RCT, the esterification of cholesterol may have other benefits in preventing the development of atherosclerosis. Excess FC in plasma membranes can adversely affect the function of numerous plasma membrane proteins, and high levels of FC are cytotoxic (10, 11). Inhibition of the intracellular esterification of...
cholesterol by ACAT inhibitors has been shown to accelerate atherosclerosis and increase cardiovascular events in clinical trials (12). Recently, the enrichment of FC in red blood cell (RBC) plasma membranes has also been implicated in the development of atherosclerosis (13–15), and RBC cholesterol content has been proposed as a possible cardiovascular biomarker (15, 16). Patients with a wide variety of primary dyslipidemias, such as abetalipoproteinemia, for example, are known to have elevated levels of FC in RBC membranes due to an altered exchange of excess FC on lipoproteins with circulating RBCs (2, 17). The enrichment of FC in RBC membranes can lead to altered cell shape and function (18, 19). FC on lipoproteins is also known to exchange with the plasma membrane of platelets, and cholesterol enrichment has been demonstrated to promote platelet activation (20–22).

To better understand the role of LCAT, particularly in the context of increased FC, we describe here the development of two novel transgenic (Tg) mice, namely SR-BI-deficient [Scarab(−/−)]xLCAT-null [Leat(−/−)] mice (S L−), which lack LCAT, and Scarab(−/−)xLCAT-Tg mice (S L++), which overexpress human LCAT. RBCs and platelets from Scarab(−/−)xLeat(+/+) (S L−/) mice are known to have elevated levels of FC (19), and HDL from these mice is increased in size and displays an impairment in cholesterol esterification, as only 40–50% of the cholesterol is esterified compared with approximately 75% observed in WT mice (23–28). The decrease in cholesterol esterification in S L−/ mice is presumably because of the decreased efficiency of large HDL particles to activate LCAT (29, 30). Additionally, S L−/ mice have been described as having an increased propensity to diet-induced atherosclerosis despite their increased HDL-cholesterol (HDL-C) levels, possibly because of decreased hepatic delivery of cholesterol (23–28).

In this study, we show that the alteration of LCAT expression in Scarab(−/−) mice results in profound changes in the percentage of cholesterol esterified and in the distribution of cholesterol on lipoproteins, RBCs, and platelets. Furthermore, we demonstrate that decreased cholesterol esterification caused by the loss of LCAT in S L−/ mice further accentuates the development of atherosclerosis when compared with S L+/ mice.

**MATERIALS AND METHODS**

**Animal procedures**

LCAT-Tg mice, containing 240 copies of the human LCAT gene, but with only a 2-fold increase in the rate of plasma cholesterol esterification (31), and Leat(−/−) mice (32), both on a C57BL/6N background, were crossed with S L−/ mice (Jackson Laboratory, stock number 3379) which were backcrossed three times onto the C57BL/6N background. The resultant new strains were designated as either S L−/ or S L+/ mice (31, 32). Probucol was added to the chow of S L−/ mice to facilitate breeding (33). C57BL/6N mice (WT) were purchased from Taconic (Albany, NY). Mice were housed under controlled conditions, with a 12:12 h light/dark cycle, and fed either a standard rodent autoclaved chow diet containing 4.0% fat (NIH31 chow diet; Zeigler Brothers Inc., Gardners, PA) or a Western diet (TD.88137 Adjusted Calories Diet; Harlan Teklad, Madison, WI). Only female mice were used in this study and, starting at 2 months of age, were fed ad libitum with a Western diet or chow diet for 7 months. All animal procedures were approved by a National Institutes of Health Institutional Animal Care and Use Committee (protocol H-0050R2).

**Plasma lipid and lipoprotein analysis**

Unless otherwise noted, all chemicals used were obtained from Sigma-Aldrich (St. Louis, MO). Blood samples were collected from the periorbital sinus of eyes, with a heparinized capillary tube, and EDTA-plasma was obtained by centrifugation for 10 min at 3,000 g at 4°C. EDTA-whole blood (500 μl) was used to determine blood cell populations, using a Cell-Dyn 3700 (Abbott, Abbott Park, IL). For lipid extraction, blood cells were washed with normal saline and centrifuged at 4°C, 3,000 g for 10 min. Lipids were extracted by lysing the RBCs with an equal volume of distilled water followed by extraction of lipids by an overnight incubation with 20 vol of chloroform/methanol (2:1). Total cholesterol (TC), FC, and phospholipids were analyzed by enzymatic assays from Wako Chemicals (Richmond, VA). Triglyceride levels were measured by an enzymatic assay from Roche Diagnostics (Indianapolis, IN). Lipoprotein profiles in plasma were obtained by fast-protein LC (FPLC) (Akta FPLC; GE Healthcare) on two Superose-6 columns in series. For each group, 400 μl of pooled plasma from at least four mice was used for FPLC and lipids were measured enzymatically.

To analyze the protein content of FPLC fractions, 50 μl from four fractions corresponding to 2 ml of elution volume were pooled together and 2 μg of lipid removal agent (Sigma) were added to concentrate lipoprotein samples. After washing, lipoproteins were eluted from the lipid removal agent with SDS loading buffer (Life Technologies, Grand Island, NY). For immunoblotting, equal volumes of sample were loaded onto a 4–12% Bis-Tris gel (Life Technologies) and electrophoresed at 200 V in MOPS buffer. Proteins were transferred to polyvinylidene difluoride membrane (Life Technologies), using a Pierce G2 Fast Blotter (Thermo Scientific, Rockford, IL) for 15 min at 25 V and 3.2 A. Following the protein transfer, membranes were blocked with 5% milk and 3% BSA. Membranes were stained sequentially with the following antibodies: mouse anti-mouse apoB-100/48 (5 μg/ml, 1:500) (kindly provided by Dr. Steve Young), rabbit anti-mouse apoA-I serum (1:2,000), and apoE serum (1:1,000) (Meridian Life Science, Inc., Memphis, TN). Primary antibodies were detected with the appropriate secondary antibody conjugated to HRP (Abcam, Cambridge, MA). Staining was visualized on an Omega Lum C (Aplegen, San Francisco, CA) using the WesternBright Quantum detection kit (Advanta Inc., Menlo Park, CA). FPLC fractions were pooled into three peaks corresponding to VLDL, LDL, and HDL and labeled 1, 2, and 3, respectively (Fig. 2). Lipoproteins were concentrated using lipid enrichment of FC in RBC membranes has also been implicated in the development of atherosclerosis (13–15), and RBC cholesterol content has been proposed as a possible cardiovascular biomarker (15, 16). Patients with a wide variety of primary dyslipidemias, such as abetalipoproteinemia, for example, are known to have elevated levels of FC in RBC membranes due to an altered exchange of excess FC on lipoproteins with circulating RBCs (2, 17). The enrichment of FC in RBC membranes can lead to altered cell shape and function (18, 19). FC on lipoproteins is also known to exchange with the plasma membrane of platelets, and cholesterol enrichment has been demonstrated to promote platelet activation (20–22).

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from frozen liver tissue using RNAlater®-ICE. RNA was extracted from thawed tissue, using Trizol, and reverse transcription was carried out using Moloney murine leukemia virus reverse transcriptase oligo(dT) primers, and 1 μg of total RNA. Gene expression analysis was performed with TaqMan® Universal PCR Master Mix and commercially available primers for murine ApoB, Hhang, Ldlt, and Psek9 on an ABI 7900. Values were normalized to murine Actb and then normalized to expression of each gene in the WT mice. Values are expressed as log(2^-ΔΔCT).

Cholesterol efflux capacity and ex vivo LCAT activity

apoB-depleted serum was prepared by precipitation with polyethylene glycol (20%, v/v, in glycine buffer, pH 7.4). Global cholesterol efflux capacity of serum HDL samples was determined as described in detail elsewhere (35–37). In brief, global cholesterol efflux was measured using [774 mouse macrophage cells in the presence of cAMP. Cells were preincubated with [3H]cholesterol and ACAT inhibitor Sandoz 58-035 (but not preloaded with mass cholesterol) overnight. Cells were then incubated overnight in 0.2% BSA with cAMP. After washing, cells were incubated for 4 h with the serum HDL samples (apoB-depleted serum) added at 2.8% (v/v), [3H]cholesterol released to serum after 4 h was measured by liquid scintillation counting. Cholesterol efflux is expressed as the radiolabel released as a percentage of [3H]cholesterol within cells before addition of serum. All efflux values were corrected by subtracting the small amount of radioactively cholesterol released from cells incubated with serum-free medium. When indicated, J774 cells for some experiments were first loaded with Ac-LDL (180 μg/ml) for 2 h. Following cholesterol loading, these cells were incubated with 2.8% apoB-depleted serum overnight. TC was determined in cell layers and standardized to total protein as described below.

For determination of ex vivo LCAT activity, lipid was extracted from an aliquot of the efflux medium, and thin-layer chromatography was performed to quantitate the proportion of [3H]FC and [3H]cholesterol ester in the sample. Ex vivo LCAT activity is expressed as the proportion of [3H]cholesterol ester formed as a percentage of the total [3H]cholesterol released during the 4 h efflux period.

Cellular cholesterol mass uptake

For mass cholesterol uptake assay, 1.5 × 10^6 RAW cells (ATCC, Manassas, VA) were added to a 6-well tissue culture plate (Corning Inc., Corning, NY). Opti-MEM (Invitrogen) plus 3% mouse serum was added to the cells and incubated overnight. Cells were washed three times in PBS (Invitrogen), then lipids were extracted with 2:1 hexane:isopropanol solution. After removal of the organic solvent, protein from cells was extracted with 0.1 N NaOH/0.1% SDS. Protein concentration from each well was determined by BCA assay (Pierce, Rockford, IL). Lipids were resuspended in 100% ethanol, and levels of cholesterol were measured using the Amplex Red assay (Invitrogen). Hepatic cell uptake was performed in Fu5AH rat hepatoma cells, as previously described (38), with the following modifications. Briefly, labeled media from the efflux assay were pooled and then applied to Fu5AH hepatoma cells. [3H]cholesterol uptake from serum, after incubation for 2 h, was measured by liquid scintillation counting. Cholesterol uptake is expressed as the radiolabel within cells as a percentage of [3H]cholesterol that was added to cells.

Fluorescence-activated cell sorting analysis of platelets and erythrocytes

To detect the level of cholesterol in erythrocytes and platelets, 15 μl of whole blood was washed once with ice-cold PBS. Cells were resuspended in 80 μl of antibody solution and incubated at room temperature for 20 min. After incubation, cells were fixed with 4% paraformaldehyde for 1 h at 4°C. Following fixation, cells were washed one time with PBS and resuspended in 500 μl of a 120 μg/ml filipin solution (Polysciences, Inc., Warrington, PA) supplemented with 2% dextran. Antibodies for erythrocytes and platelets, TER-119 and CD41 (BD Biosciences, San Jose, CA), respectively, were used at a 1/100 dilution. Cells were kept on ice until they could be analyzed by flow cytometry, using an LSRFortessa or FACsAria (BD Biosciences).

To assess the level of reticulated platelets, cells were stained as previously described (39, 40). Briefly, 1 μl of whole blood was stained with antibodies against CD41, as described above. Following staining, the sample was diluted with 1 ml of PBS containing 100 ng/ml of thiazole orange (Sigma). Cells were kept on ice until they could be analyzed by flow cytometry, using an LSRFortessa (BD Biosciences).

Analysis of atherosclerotic lesions

Following euthanasia, the vasculature was washed by perfusion through the heart with PBS. The aorta was then isolated from its origin in the heart to the ileal bifurcation. After removal of adventitial fat, the aorta was placed in 4% paraformaldehyde solution for 18 h. The fixed aorta was stained with a Sudan IV solution for 25 min, destained for 25 min in 80% ethanol, and washed in water. Then the aorta was cut longitudinally, embedded in glycerin, and sealed between a glass slides. Quantification of the area of the aortic plaques was performed with Image-Pro Plus version 4.1 software (Media Cybernetics, Inc., Bethesda, MD).

The evaluation of atherosclerosis in aortic valve leaflets was performed as previously described (41). Briefly, the heart and attached section of ascending aorta were dissected en bloc and embedded in OCT (Sakura Finetek, Torrance, CA). The aortic sinus was sectioned serially (10 μm intervals) using a cryostat (model CM3050 S; Leica Microsystems, GmbH, Wetzlar, Germany). Sections were fixed in a 4% paraformaldehyde solution for 15 min and stained for neutral lipids with Oil Red O (Poly Scientific R&D Corp., Bay Shore, NY). The images were acquired using a Leica DM 4000 B microscope and Leica DFC 500 camera (Leica Microsystems, GmbH). Five sections per animal were evaluated to calculate the area of lesions in the aortic sinus.

Confocal and polarization microscopy

To visualize FC in tissues, fixed frozen tissue was stained with filipin (100 ug/ml) at room temperature. After staining, slides were mounted in a water-based mounting medium (Polysciences, Inc.). Tissue sections were examined using a HC-PL-IRAP0 40X/1.1 NA water immersion objective (WD = 0.6 mm) on a Leica TCS SP5-AB5 5-channel confocal system (Leica Microsystems) equipped with multiline argon, diode 405 nm, 561 nm, HeNe 594 nm, and HeNe 633 nm visible lasers. Filipin fluorescence was detected using 405 nm excitation. Quantification of the intima area of the aorta that stained with filipin was performed in ImageJ (42). Cholesterol crystals were imaged using polarization microscopy (43) and appear white in photomicrographs.

Statistical analysis

Results represent the mean ± SEM and all statistics were calculated using Prism 6 (GraphPad Software, Inc., La Jolla, CA). Groups were compared using a one- or two-way ANOVA as indicated. Post hoc analysis using Bonferroni’s test was used to compare multiple groups. Statistics reported on each graph were calculated by comparing S’L’ mice to each of the other strains or, when indicated, each strain was compared on a standard chow versus Western diet basis. All statistical comparisons are shown in supplementary Table 1.
RESULTS

LCAT alters plasma lipid and lipoprotein profiles in SR-BI-deficient mice

To test the hypothesis that increased expression of LCAT could overcome the cholesterol esterification defect in HDL and the FC enrichment in blood cells observed in Scarab (−/−) mice, the following lines of mice were purchased or created: 1) C57BL/6 (WT), 2) S’L’, 3) S’L”, and 4) S’L+++.

On a normal chow diet, all three strains of mice on the Scarab (−/−) background had higher TC than the WT mice, with the S’L+++ mice having the highest level of TC (Fig. 1A). Following 7 months on a high-fat diet, all mouse strains displayed an increase in plasma levels of TC, FC, cholesteryl esters, and phospholipids (Fig. 1A–D). Only S’L’ and S’L− mice, however, showed increased levels of triglycerides after the Western diet (Fig. 1E). The majority of the increase in TC in the S’L+++ mice appears to be due to an increase in cholesteryl esters, presumably from increased esterification by LCAT. In contrast, S’L’ and S’L− mice had higher levels of FC than cholesteryl esters on both the normal and the Western diet. The FC/TC ratio in plasma from WT mice was close to 0.25 on both the chow and the Western diet, whereas in S’L++ mice, the ratio was between 0.5 and 0.6 (Fig. 1F), which is in agreement with previous reports (40). The S’L− mice had an even higher FC/TC ratio than the S’L+ mice on both diets, between 0.88 and 0.62.

Interestingly, LCAT overexpression in the S’L+++ mice was able to restore the altered FC/TC ratio due to Scarab (−/−) deficiency when on a normal chow diet and to limit the increase in the FC/TC to 0.36 following a Western diet (Fig. 1F). As expected, the S’L− mice had very low levels of cholesteryl esters on the chow diet due to the absence of LCAT, but there was a significant increase in plasma cholesteryl esters after the Western diet, most likely from intracellular esterification by ACAT (44–46).

As expected, cholesterol in WT mice was found mainly on HDL, but following the Western diet, a peak of cholesterol corresponding to LDL was also observed (Fig. 2A). As previously described (47), the S’L− mice had a marked increase in cholesterol in larger sized HDL fractions, and increased levels of cholesterol in peaks corresponding to VLDL and LDL after a Western diet (Fig. 2B). As expected, loss of LCAT resulted in an almost total loss of HDL and an accumulation of VLDL- and LDL-sized particles in S’L+++ mice (Fig. 2C). S’L+++ mice showed a similar lipoprotein distribution as the S’L− mice on the normal chow diet, but appeared to have a slightly larger HDL peak. Compared with the S’L− mice (Fig. 2B), the increased LCAT activity in the S’L+++ mice (Fig. 2D) appeared to cause a marked decrease in VLDL and LDL, and to significantly raise HDL.

By Western blot analysis, the large HDL in S’L− mice on the Western diet was enriched in apoE (Fig. 2B), as has been previously described (47). In the case of the S’L− mice (Fig. 2C), most of the apoE was found in larger size fractions corresponding to LDL and VLDL. The apolipoprotein distribution for the S’L+++ mice (Fig. 2D) was similar to the S’L− mice (Fig. 2B). The main difference was that apoE was found in larger size fractions that also contained apoB, suggesting that the front shoulder of the large HDL peak observed in these mice may also contain some apoB-containing lipoproteins. This was confirmed by agarose gel electrophoresis, which showed a small amount of LDL in S’L+++ mice. The S’L− mice, which had no HDL, had even more LDL/VLDL than the S’L+++ mice (supplementary Fig. 2).

The apoB-containing lipoproteins in the S’L− mice were mainly in LDL and VLDL fractions (Fig. 2E). Similarly, apoB-containing lipoproteins were found in VLDL and LDL after a Western diet (Fig. 2F). As expected, loss of LCAT resulted in an almost total loss of HDL and an accumulation of VLDL- and LDL-sized particles in S’L+++ mice (Fig. 2G). S’L+++ mice showed a similar lipoprotein distribution as the S’L− mice on the normal chow diet, but appeared to have a slightly larger HDL peak. Compared with the S’L− mice (Fig. 2F), the increased LCAT activity in the S’L+++ mice (Fig. 2G) appeared to cause a marked decrease in VLDL and LDL, and to significantly raise HDL.

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The same FPLC lipoprotein

\[\text{Effect of LCAT on total plasma lipids and cholesterol esterification.} \]

\[\begin{align*}
\text{A: Total cholesterol} \\
\text{B: Free Cholesterol} \\
\text{C: Cholesteryl Esters} \\
\text{D: Phospholipids} \\
\text{E: Triglycerides} \\
\text{F: FC/TC Ratio}
\end{align*}\]

\text{Fig. 1. Effect of LCAT on total plasma lipids and cholesterol esterification. A–F: Display levels of TC, FC, cholesterol ester, phospholipids, triglycerides, and FC/TC ratio, respectively, on normal chow or Western diet. P values were determined by a two-way ANOVA. The # denotes comparisons between normal and Western diet for each strain and * denotes a comparison between S’L− mice and each strain on the same diet. *,#P = 0.05; **,##P = 0.01; ###,###P = 0.001; ####,####P = 0.0001; n = 6–8 mice per group.\]
and macrophages (Fig. 3). Despite the relatively large differences in lipoprotein profiles of the four lines of mice, there were only minor differences in radiolabeled cholesterol efflux from J774 cells on the chow or Western diet, using apoB-depleted serum (Fig. 3A). Cholesterol efflux was also monitored from cholesterol-loaded J744 cells after treatment with apoB-depleted serum on a Western diet by monitoring FC and cholesteryl ester mass from cells (Fig. 3B). Compared with WT mice, cell lines treated with serum from all three lines of mice lacking Scarab (H11002/H11002) had higher FC levels, but the levels were only statistically higher for the S/H11002L + mice.

Effect of LCAT on cholesterol efflux and uptake

As LCAT has been proposed to affect multiple steps in the RCT pathway, we investigated the ability of serum from the four lines of mice to promote cholesterol efflux from cells, to esterify cholesterol, and to donate cholesterol to liver cells and macrophages (Fig. 3). Despite the relatively large differences in lipoprotein profiles of the four lines of mice, there were only minor differences in radiolabeled cholesterol efflux from J774 cells on the chow or Western diet, using apoB-depleted serum (Fig. 3A). Cholesterol efflux was also monitored from cholesterol-loaded J744 cells after treatment with apoB-depleted serum on a Western diet by monitoring FC and cholesteryl ester mass from cells (Fig. 3B). Compared with WT mice, cell lines treated with serum from all three lines of mice lacking Scarab (−/−) had higher FC levels, but the levels were only statistically higher for the S’L’ mice.

In addition to monitoring cholesterol efflux, we also measured the esterification of cholesterol in the conditioned media after cholesterol efflux (Fig. 3C). As
Conditioned medium containing radiolabeled cholesterol from the cell cholesterol efflux experiment in Fig. 3A was also used to assess cholesterol uptake into Fu5AH cells. *P values were obtained by two-way ANOVA. The # denotes comparisons between normal and Western diet for each strain and * denotes a comparison between S ’ L’ mice and each strain.

expected, there was no significant cholesterol esterification during the cholesterol efflux experiment in the S ’ L’ mice that lack LCAT (Fig. 3C). The S ’ L’ mice also had an approximate 75% reduction in esterification compared with WT mice (Fig. 3G), which is consistent with the observed decrease in their relative plasma cholesterol ester content. This is most likely due to the fact that large HDL, which accumulates in these mice, is known to be a poor substrate for LCAT (48). Interestingly, apoB-depleted serum from WT and S ’ L’ mice showed less esterification after being placed on the Western diet compared with chow. This is consistent with the observed increase in plasma FC in these mice on the Western diet (Fig. 1), indicating that the esterification of cholesterol by LCAT was less efficient on a high-fat diet. Similar results have been previously shown in Apoe(−/−) and Ldlr(−/−) mice (49). Increased expression of LCAT in S ’ L’ mice resulted in a greater fraction of the effluxed cholesterol being esterified compared with the S ’ L’ mice on the Western diet (Fig. 3C). Again, the decreased esterification observed on the Western diet is likely due to the increased size of the HDL (48) (Fig. 2), but increased LCAT in the S ’ L’ mice was able to largely overcome this defect. In fact, cholesterol esterification for the S ’ L’ mice almost approached the level observed in WT mice when on a Western diet.

Effect of LCAT on hepatic gene expression and VLDL production

To better understand the mechanism behind the observed differences in the level of pro-atherogenic apoB-containing lipoproteins in the different mice (Fig. 2), we examined hepatic gene expression and VLDL production rate on the Western diet (Fig. 5). Interestingly, when compared with WT mice, liver cells from S ’ L’ mice displayed a significant decrease in Hmgcr, Ldlr, and Peso9, and a
and lipoproteins were monitored over 3 h. All three of the *Scarab* (−/−) lines of mice displayed a similar lower rate of hepatic triglyceride-rich lipoprotein secretion compared with the WT mice (Fig. 5B). This suggests that the accumulation of VLDL and LDL observed in the S' L− mice (Fig. 2) is most likely due to decreased plasma clearance. Lastly, when the levels of FC were examined by FLPC in VLDL-sized fractions, we observed that S' L− mice had a greater than 8-fold increase in FC compared with S' L+ mice (Fig. 5C). In contrast, almost all of the cholesterol on VLDL was esterified in WT and S' L++ mice (Fig. 5C).

LCAT increases cell counts and alters the FC content of RBCs and platelets

Because it has previously been reported that *Scarab* (−/−) mice have abnormalities in their cholesterol content and number of RBCs and platelets (19, 32, 40), we examined the ability of LCAT to modulate these parameters. There were no significant differences observed in RBC counts in the four strains of mice on the chow diet. On the Western diet, however, both S' L+ and S' L− mice showed a trend toward lower RBC counts compared with WT and S' L++ mice, although the difference was not significant (Fig. 6A). When the FC content of RBCs was examined, these same mice showed a dramatic increase in FC on the Western diet (Fig. 6B). The increased expression of LCAT in the S' L+ mice appeared to significantly blunt the increase in FC on RBCs (Fig. 6B).

**Fig. 4.** Effect of LCAT on the atherogenic potential of serum. RAW cell uptake of cholesterol mass from whole serum. *P* values were obtained by two-way ANOVA. The # denotes comparisons between normal and Western diet for each strain and * denotes a comparison between S' L− and each strain. *,# *P* = 0.05; **,## *P* = 0.01; ****,#### *P* = 0.0001; n = 3 mice per group.

**Fig. 5.** Effect of LCAT on hepatic gene expression and VLDL secretion. A: Expression of key hepatic genes from livers of the indicated mice. All values normalized to WT animals (n = 4). B: Plasma triglyceride levels of mice after inhibition of LPL with Triton (n = 4). C: Concentration of FC on VLDL. *P* values were obtained by two-way ANOVA. The # denotes comparisons between normal and Western diet for each strain and * denotes a comparison between S' L+ and each strain. *,# *P* = 0.05; **,## *P* = 0.01; ****,#### *P* = 0.0001.

**Fig. 6.** Effect of LCAT on hepatic gene expression and VLDL secretion. A: Expression of key hepatic genes from livers of the indicated mice. All values normalized to WT animals (n = 4). B: Plasma triglyceride levels of mice after inhibition of LPL with Triton (n = 4). C: Concentration of FC on VLDL. *P* values were obtained by two-way ANOVA. The # denotes comparisons between normal and Western diet for each strain and * denotes a comparison between S' L+ and each strain. *,# *P* = 0.05; **,## *P* = 0.01; ****,#### *P* = 0.0001; n = 3 mice per group.
LCAT protects *Scarab* (-/-) mice from developing atherosclerosis

As mice lacking SR-BI have been reported to have increased atherosclerosis in their aortic root (27) and also in their aorta by en face analysis when crossed with *ApoE* (-/-) or *Ldlr* (-/-) mice (23, 27, 52), we determined whether LCAT could modulate diet-induced atherosclerosis. After seven months on a Western diet, only a limited degree of atherosclerosis, as measured by en face analysis, was observed in any of the mouse strains except for the S/H11002 L/H11002 mice, which had more than a 20-fold increase in atherosclerosis compared with S/H11002 L+ mice (Fig. 7A, D). Examination of the aortic root revealed that S/H11002 L+ mice had a trend toward increased atherosclerosis compared with WT mice, similar to what was previously reported (27), but in our study this did not reach statistical significance (*P* < 0.05) when compared with WT mice.

On the chow diet, S/H11002 L+ mice had an approximate 50% decrease in platelet counts (Fig. 6C), as previously described (40), and either increasing or decreasing LCAT levels normalized platelet counts for the S’/H11002 L++ and S’/H11002 L− mice, respectively. On the Western diet, all strains of mice displayed a significant decrease in platelet counts, but increased expression of LCAT in the S’/L+ mice was able to blunt the decrease in platelets. FC levels in platelets, as assessed by filipin staining, showed minimal differences between strains on the chow diet (Fig. 6D), but on the Western diet, S’/L+ mice displayed an enrichment of FC in platelets, as has been previously described (40). The S’/L− mice did not show any enrichment of cholesterol, but these mice and the S’/L+ mice showed a 3.4- and 2.7-fold increased rate of platelet production, respectively, over WT mice, as assessed by staining for nucleic acids with thiazole orange (Fig. 6E), suggesting that differences in the rate of platelet clearance may alter the accumulation of cholesterol-rich platelets in the blood compartment.

Figure 6. Effect of LCAT on RBC and platelet counts and FC content. A: RBC counts of mice on chow or Western diet (7 months). B: RBC FC content, as determined by filipin staining of mice on chow or Western diet (7 months). C: Platelet counts of mice on chow or Western diet (7 months). D: Platelet FC content, as determined by filipin staining of mice on chow or Western diet (7 months). E: Percentage of reticulated platelets as determined by thiazole orange staining on the Western diet (*n* = 6 or *n* = 10–12 on chow or Western diet, respectively). *P* values were obtained by two-way ANOVA. The # denotes comparisons between normal and Western diet for each strain and * denotes a comparison between S’/L+ and each strain. *,#*P* = 0.05; **,#*P* = 0.01; ###,#*P* = 0.001; ####,#*P* = 0.0001.
In contrast, relatively small amounts of atherosclerosis were detected in the aortic root of S^{-}L^{++} mice and were similar to those observed in WT mice.

Because recent reports have postulated that cholesterol crystals derived from FC may contribute to the initiation of atherosclerosis (27, 53, 54), we examined the aortic sinus of these mice for the presence of cholesterol crystals by polarized light (Fig. 9B–E). We found abundant FC crystals in the lesions of both S^{-}L^{+} and S^{-}L^{-} mice, whereas cholesterol crystals were undetectable in WT and S^{-}L^{++}

Fig. 7. Effect of SR-B1 and LCAT expression on diet-induced atherosclerosis. Whole aortas from mice following 7 months on a Western diet were stained with Sudan IV to visualize lipid deposits. A: S^{-}L^{-} mice displayed increased atherosclerosis following 7 months on a Western diet, while S^{-}L^{+} and S^{-}L^{++} mice did not display any increase in atherosclerosis. B–E: Representative images of whole aorta from WT, S^{-}L^{+}, S^{-}L^{-}, and S^{-}L^{++}, respectively. P values were determined by one-way ANOVA. **P ≤ 0.01; n = 9–12 mice per group.

Fig. 8. Effect of LCAT expression on diet-induced atherosclerosis. Atherosclerosis was assessed in the aortic root by Oil Red O staining. A: Loss LCAT exacerbates atherosclerosis while overexpression of LCAT prevents atherosclerosis in Scarab(-/-) mice. B–E: Representative images of aortic sinus from WT, S^{-}L^{+}, S^{-}L^{-}, and S^{-}L^{++} mice, respectively. Arrows indicate atherosclerotic plaque. Scale bar indicates 200 um. *P<0.05 (n = 8 per group).
mice (Fig. 9B-E). Interestingly, in the intimal regions of S-L- mice, where cholesterol crystals were observed, abundant staining was also observed for FC, as determined by filipin staining (Fig. 9A).

**DISCUSSION**

Results from this study reveal that modulating the expression of LCAT in mice lacking the SR-BI receptor can have a profound effect on numerous parameters and pathways related to lipoprotein metabolism and atherosclerosis. SR-BI deficiency is known to result in an increase of large HDL particles enriched in FC (23, 55, 56), but we found that increased LCAT was largely able to correct this phenotype (Fig. 1). Overexpression of LCAT in Scarab(+/−) mice resulted in a significant decrease in the fraction of unesterified cholesterol, whereas the loss of LCAT in the S-L- mice resulted in an almost total loss of HDL and a marked increase in unesterified cholesterol in plasma.

![Image of figure 9](image)

**Fig. 9.** Effect of LCAT on FC in the aortic sinus. A: Amount of FC in the intima of the aortic sinus was assessed by filipin staining. B–E: Representative images of cholesterol crystals detected by polarized light and the amount of FC detected by filipin stain in the aortic sinus. Scale bar indicates 100 um. ****P < 0.0001 (n = 4 per group).
The loss of HDL in S’L’ mice was also associated with a corresponding marked increase in pro-atherogenic LDL- and VLDL-sized particles. Interestingly, although the S’L’ mice did not show an increase in TC compared with the other two strains of mice lacking the SR-BI receptor, they exhibited the most atherosclerosis. In contrast, the S’L++ mice, which had the highest level of TC, were protected from diet-induced atherosclerosis, most likely because, unlike the S’L’ mice, they had a decrease in cholesterol-containing pro-atherogenic lipoproteins, such as VLDL- and LDL-sized particles, and the majority of their cholesterol was on HDL (Fig. 2). Consistent with our data, it has been shown in Ldlr(−/−) mice that the level of VLDL-cholesterol (VLDL-C) is the best predictor of atherosclerosis (57, 58).

Although many epidemiologic studies have linked elevated levels of HDL-C to a decreased atherosclerotic risk (59, 60), there is evidence in some settings that elevated levels of HDL can in fact be detrimental (61–64). To date, the mixed results seen in the trials using CETP inhibitors suggest that simply elevating the levels of HDL can in fact be detrimental (61–64). To date, pro-atherogenic lipoproteins, such as VLDL- and LDL-sized particles, and the majority of their cholesterol was on HDL. (Fig. 2). Consistent with our data, it has been shown in Ldlr(−/−) mice that the level of VLDL-cholesterol (VLDL-C) is the best predictor of atherosclerosis (57, 58).

Surprisingly, modulation of LCAT expression did not seem to have a major impact on HDL function, as measured by an in vitro radiolabeled cholesterol efflux assay from macrophages, using apoB-depleted serum as the acceptor. It has been previously shown, however, that in vitro cholesterol efflux assays are relatively unaffected by the presence or absence of LCAT, because of the relatively low unphysiologic ratio of donor membranes to acceptor particles used in these studies (3). Using RBCs as a cholesterol donor, only at relatively high concentrations (hematocrit >15%), much higher than the ratio of donor membranes to acceptor particles typically used in in vitro cholesterol efflux assays, was LCAT shown to promote cholesterol efflux (3). We did observe, however, that a much greater fraction of the radiolabeled cholesterol effluxed from the macrophage cell line during the in vitro efflux study was esterified in the conditioned media containing serum from S’L’ mice (Fig. 3B). Based on human tracer studies (4), approximately 80% of esterified cholesterol is returned to the liver as compared with only 4% of FC (2), which suggests that the esterification of cholesterol by LCAT should ultimately lead to increased hepatic excretion of cholesterol.

When we monitored bidirectional flux of cholesterol by measuring cholesterol mass in J744 macrophage cells (Fig. 3B), we found that incubation of apoB-depleted serum from all three strains of mice lacking SR-BI resulted in higher levels of FC than what we found with WT mice. This suggests that the large HDL that accumulates in the absence of SR-BI may, in fact, promote the net delivery of cholesterol to cells, possibly by the SR-BI receptor present on the J744 cells (72). Similarly, we observed (Fig. 3D) an increase in cholesterol uptake by Fu5AH cells, which also abundantly express SR-BI (50), when incubated with apoB-depleted serum from the three lines of mice lacking SR-BI. This again could be due to cellular uptake of cholesterol from HDL.

RBCs from LCAT-overexpressing mice on a Western diet had decreased levels of FC (Fig. 6). By lowering the amount of FC on lipoproteins, LCAT could be reducing the amount of FC that can passively exchange with RBCs (2). In general, increased levels of FC in cells have been shown to be pro-inflammatory and cytotoxic (11, 73), and result in increased atherosclerosis (74). RBCs themselves have also been shown to remove excess cholesterol from peripheral cells by a countercurrent-like exchange mechanism...
and deliver it to the liver for excretion (13). The enrichment of FC on RBCs in the S L− mice could thus potentially interfere with this process and could also lead to increased atherosclerosis. RBCs themselves have also been proposed as a possible source of cholesterol in plaque (15, 16).

Decreased LCAT activity in the S L− and S L+ mice also appeared to be correlated with decreased platelet counts, and in the case of the S L+ mice, cholesterol enrichment of platelets (Fig. 6). A higher rate of platelet production was also observed for both the S L− and S L+ mice despite their lower platelet counts, suggesting that these mice must have a higher rate of platelet turnover. Cholesterol enrichment of platelets is known to lead to increased thrombosis from platelet aggregation also contributes to the development of atherosclerotic plaque (77).

A significant amount of atherosclerosis was only detected in the mice with decreased LCAT activity and an increase in the FC/TC ratio, namely the S L+ and S L− mice (Figs. 7, 8). The difference we observed in the degree of atherosclerosis in the en face compared with the aortic root in the S L+ mice (Figs. 7, 8) is most likely due to differences in disease propensity in different anatomic locations, with plaque usually first developing in the aortic root before other locations. Cholesterol crystals, which are now thought to play a role in the early initiation of the atherosclerosis process (53), were also detected in the plaques of both S L+ and S L− mice (Fig. 9). The increase in the FC/TC ratio on lipoproteins could promote cholesterol crystal formation when they enter the plaque, and could also account for the increase in FC staining by filipin in the S L− mice (Fig. 9).

A limitation of our study is that the mice used in this study, unlike humans, did not contain CETP. Previous studies, however, have shown that CETP expression in mice can lower serum cholesterol in LCAT-Tg mice (78), and that CETP expression can protect Scarab(−/−) mice from the development of atherosclerosis (79). Interestingly, mice that overexpress LCAT are at increased risk of developing atherosclerosis on both the Ldlr(−/−) and Apoel(−/−) background (80, 81), but expression of CETP in these models has been shown to ameliorate the development of atherosclerosis (78). In contrast, LCAT overexpression in rabbits, which express CETP, protected against diet-induced atherosclerosis (82). Based on these results, it is possible that a greater atheroprotective effect from LCAT could be observed in Scarab(−/−) mice that express CETP, but this will have to be tested in future studies. Another potential limitation in our study is the mixed genetic background of the S L+ mice, which could be another source of variation in our study.

In summary, increased LCAT expression in Scarab(−/−) mice was found to lead to an anti-atherogenic lipoprotein phenotype and reduced diet-induced atherosclerosis. Further work is needed, however, to fully understand how LCAT affects the overall functionality of HDL in Scarab(−/−) mice and its role in RCT, as well as in other potential anti-atherogenic pathways.

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