Increased plasma HDL cholesterol levels and biliary cholesterol excretion in hamster by LCAT overexpression

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Abstract Lecithin cholesterol acyltransferase (LCAT) is a key enzyme in the metabolism of high density lipoprotein (HDL), which has been found inversely correlated with atherosclerosis. Adenovirus mediated overexpression of human LCAT (hLCAT) in hamsters resulted in increased levels of plasma total cholesterol, HDL cholesterol, phospholipids and enlarged particle size of HDL. It also increased cholesterol and total bile acid concentrations in bile. Hepatic mRNA level of cholesterol 7α-hydroxylase increased 2.7-fold in hamsters. However, such effects were not observed in mice in a parallel experiment. This study suggests that overexpression of hLCAT in hamsters facilitated reverse cholesterol transport. Similar metabolic changes in humans might modify atherogenic risk.

Keywords: Lecithin cholesterol acyltransferase; High density lipoprotein; Bile acid; Adenovirus; Gene; Hamster

1. Introduction

It is well established that reduced high density lipoprotein cholesterol (HDL-C) is an independent risk factor for atherosclerosis, a leading cause of cardiovascular disease and death [1]. HDL intervention study also showed that an increase of 1% in HDL-C is associated with a 3% reduction in the risk of developing clinical atherosclerosis [2].

Lecithin cholesterol acyltransferase (LCAT) is a key enzyme in cholesterol metabolism and has been hypothesized as a potential therapeutic target for raising HDL-C and modulating atherosclerosis. In LCAT transgenic rabbits, HDL levels were significantly increased with reduced atherosclerotic lesions as expected [3,4]. However, studies using human LCAT transgenic (hLCAT-Tg) [5–8] and LCAT-deficient mice [9–11] have yielded controversial results. Either enhanced [5] or unchanged atherosclerosis [7,8] were documented in LCAT-Tg mice. However, overexpression of hLCAT in chlolesteryl ester transfer protein (CETP) transgenic mice, which is naturally absent in this species, reduced diet-induced atherosclerosis compared with LCAT-Tg mice [6]. It has also been shown that LCAT knockout mice fed different diet had either reduced [9,11] or increased atherosclerosis [10] in LDLr−/− and apoE−/− background, even though HDL levels were significantly reduced in both situations.

On the other hand, lack of hepatic lipase (HL) and ApoAII [12,13] in rabbits, both been important players in cholesterol homeostasis, might affect the direct extrapolation of the results obtained in rabbits to humans. It is therefore necessary to find more appropriate models to address this issue further. In this study, hamster was used for the first time to assess the role of hLCAT overexpression on plasma lipids, as well as on liver lipids homeostasis and biliary cholesterol excretion. Adenovirus mediated hLCAT overexpression in mice was performed in a parallel experiment.

2. Materials and methods

2.1. Chemicals and reagents

The adenoviral vectors expressing human LCAT (Ad-hLCAT) and human alkaline phosphatase (Ad-hAP) were generated according to the application manual of AdEasy vector system (Qbiogene, CA, USA). Mouse anti-hLCAT monoclonal antibody was a gift from Kobori (Diagnostics Research Laboratories, Daiichi Pure Chemicals, Japan). Other materials were purchased from following sources: horseradish peroxidase-conjugated secondary antibody from Santa Cruz (CA, USA); chemiluminescence regents, lecithin, human ApoA-I from Sigma–Aldrich (MO, USA); [^{3}H]cholesterol from Amersham Biosciences (Buckinghamshire, UK).

2.2. Animal procedures

Three months old male golden Syrian hamsters or two months old C57BL/6J male mice were housed and allowed free access to tap water and commercial rodent diet. The 'Principles of Laboratory Animal Care' (NIH Publication No. 85-23, revised 1996) were followed. Animals were anesthetized with pentobarbital sodium (45 mg/kg), and Ad-hLCAT or control virus Ad-hAP at 1×10^{10} pfu in 0.5 ml PBS (for hamsters) or 2×10^9 pfu in 0.2 ml PBS (for mice) was injected through jugular vein under direct visualization. Six days after virus infection, animals were anesthetized. Blood was taken from retro-orbital plexus in overnight fasting animals. Hepatic bile was collected for 2 h via the common bile duct cannulation with polyethylene tubing after cystic duct was ligated (for mice, bile sample was drawn directly from the gallbladder using an insulin syringe). Tissue samples were then harvested, snap-frozen in liquid N_2 and stored at −80 °C freezer.

2.3. RT-PCR and real-time RT-PCR

Total RNA was isolated from homogenized hamster tissues using Trizol Reagent (Invitrogen, CA, USA). First-strand cDNA was

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Abbreviations: CYP7, cholesterol 7α-hydroxylase; HDL, high density lipoprotein; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LCAT, lecithin cholesterol acyltransferase; RCT, reverse cholesterol transport; TBA, total bile acid

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generated in 20 μl reverse transcription mixture containing 1 μg total RNA using RT kit (Invitrogen, CA, USA).

Quantitative real-time RT-PCR was performed using following primer sets: hLCAT (5′-GGATGTTTCCCTCTCGCATG-3′; 5′-GGGGATGCAGGGGGACCCTG-3′); hamster β-actin (5′-TCTGGCACCACACCTTCTAC-3′; 5′-AATGCCAGTGGTACGACC-3′); hamster 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR): (5′-CATGCAGCAAACATCGTCAC-3′; 5′-AGGATTGTCTTTGCA- CGCTC-3′); hamster cholesterol 7α-hydroxylase (CYP7): (5′-GGGATACTTCAAAGCCAGAC-3′; 5′-AGACGTATCAGTTCCGAGAC-3′).

One microliter of the RT product and 1× SYBR green (Molecular Probes, Eugene, USA) were included in the 25 μl reaction mixture (10 mM Tris–HCl, pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$, 200 μM of dNTP mix, 0.2 μM of each primer and 1 unit of *Taq* DNA polymerase) for real-time RT-PCR. Thirty-five cycles of amplification were performed using an opticon continuous fluorescence detection system (MJ Research, MA, USA). Each cycle consisted of a 45-s denaturation at 94 °C, a 45-s annealing at 56 °C, and a 60-s extension at 72 °C. The mRNA levels were normalized to β-actin by using the comparative cycle threshold (C_T) method [14].

2.4. Western blot

Hamster plasma samples were subjected to electrophoresis on 12% gels and transferred to nitrocellulose membranes (Sigma). After blocking within 5% bovine serum albumin for 1 h, the membrane was probed with 1 μg/ml mouse anti-human LCAT monoclonal antibody, and followed by horseradish peroxidase-conjugated secondary antibody (goat anti-mouse, 1:2000). The reaction was detected by chemiluminescence and exposed to Kodak X-Omat film (Kodak, Rochester, USA).

2.5. Plasma LCAT activity and plasma lipids analysis

LCAT activity was determined by proteoliposome substrate as described by Chen and Albers [15]. The substrate contained apoA-I, [^{3}H]cholesterol, and lecithin at a molar ratio of 0.8:12.5:250. The reaction mixture containing 15 μl of plasma sample or 50 μl of culture medium sample was incubated at 37 °C for 30 min or 2 h, respectively. Then the reaction was terminated by adding 1 ml of absolute ethanol. Unesterified cholesterol and cholesteryl esters were separated using thin-layer chromatography in petroleum ether/diethyl ether/acetic acid (70:12:1, v/v) and the radioactivity was determined by scintillation counting.

Plasma total cholesterol (TC) and triglyceride (TG) (Sigma kits), as well as phospholipids (PL) (Wako kit, Osaka, Japan) were determined by using enzymatic methods. Plasma HDL-C was measured after precipitation of apoB-containing lipoproteins with an equal volume of a 20% polyethylene glycol solution, as described by Liu et al. [16].

2.6. Non-denaturing gradient gel electrophoresis

Plasma HDL particle sizes were analyzed by non-denaturing gradient PAGE (polyacrylamide gel electrophoresis) as described by Rainwater et al. [17]. A Hoefer SG gradient maker (Amersham Biosciences, Buckinghamshire, UK) was employed to generate a 3–32% linear gradient polyacrylamide gel. Plasma samples were prestained with 1% Sudan black B in ethylene glycol (3:1, v/v) for 6 h, then mixed with 40% sucrose (4:1, v/v), and 8 μl was applied to the gel. Electrophoresis was carried out in a slab gel apparatus (Bio-Rad, USA) at 125 V constant voltage at room temperature for 10 h in a buffer containing 90 mM Tris, 80 mM boric acid, and 3 mM EDTA, pH 8.35.

2.7. Liver lipids analysis

Approximately 100 mg of liver (wet weight) was weighed and homogenized in 1 ml PBS. Lipids were extracted as described by Folch et al. [18] and dissolved in 100 μl 3% Triton X-100. The determination of TC, TG and PL was carried out using enzymatic methods as described above.

2.8. Bile analyses

Bile flow was determined gravimetrically assuming a density of 1 g/ml. The total bile acid (TBA) concentration was measured using 3α-hydroxysteroid dehydrogenase method [19]. Bile lipids were extracted as described by Folch et al. [18] and dissolved in 3% Triton X-100. Ch and PL were determined by similar kits.

2.9. Statistical analysis

All data are presented as means ± S.E.M. Statistical comparison between two groups was performed using Student's *t* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of hLCAT in hamsters and mice

Through pilot experiment, the viral dose of 1×10^{10} pfu/100 g body weight and the day 6 after viral injection were chosen for current study. The hLCAT mRNA was detected in the liver of Ad-hLCAT infected hamsters by RT-PCR (Fig. 1(a)). Majority of hLCAT mRNA (78.5%) was expressed in the liver (Fig. 1(b)). A substantial level of hLCAT mRNA were also expressed in lung (11.3%), spleen (4.4%) and heart (3.5%). There was barely detectable level of the hLCAT mRNA expression in kidney (1.8%) and testis (0.5%). By Western blot, a molecular weight of 63-kDa protein, which is consistent with the size of hLCAT, was detected in the plasma of Ad-hLCAT infected hamsters (Fig. 2(a)). In contrast, this band was absent in the control hamsters.

Plasma LCAT activity increased 10-fold in the hamsters infected with Ad-hLCAT, as compared with the Ad-hAP control (Fig. 2(b)). Mice infected with 2×10^9 pfu Ad-hLCAT showed 23-fold increase of plasma LCAT activity, at a level similar to that of hamster due to lower LCAT activity in control mice (Fig. 2(b)).

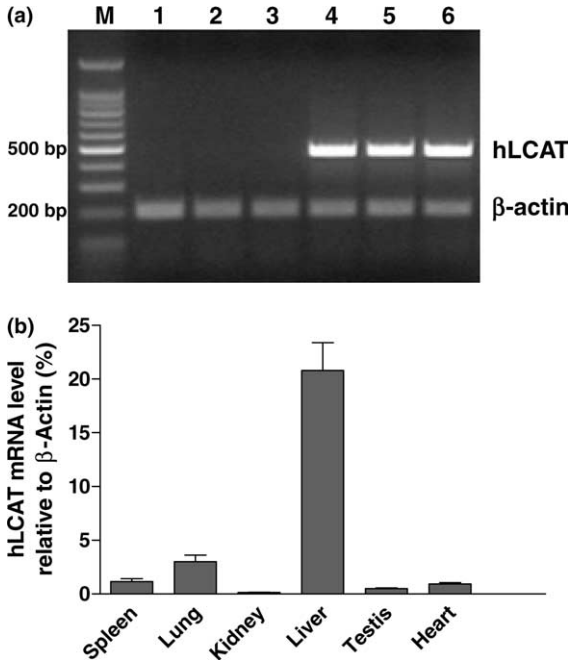

Fig. 1. The expression of hLCAT transgene in hamsters. (a) hLCAT mRNA expression in hamster liver verified by RT-PCR. The size of amplified fragments is 546 bp (for human LCAT) and 232 bp (for hamster β-actin). Lanes 1–3: Ad-hAP treated hamsters; lanes 4–6: Ad-hLCAT treated hamsters; lane M were 100 bp DNA ladder. (b) The tissue expression pattern of human LCAT transgene determined by real-time RT-PCR ($n = 4$). The mRNA level of human LCAT was presented as percent level relative to β-actin.

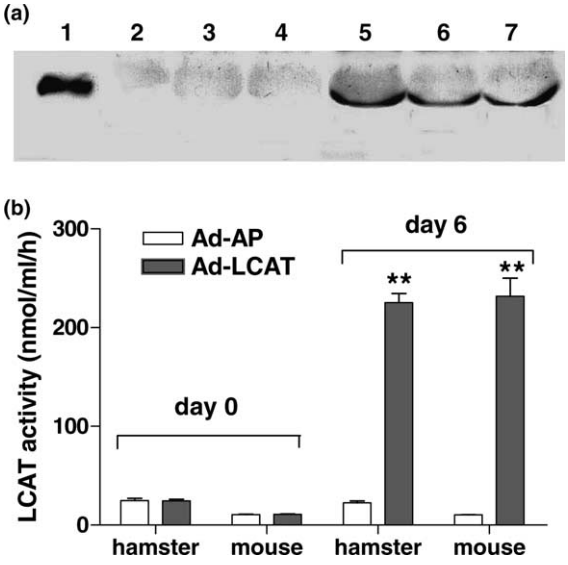

Fig. 2. Mass and activity of hLCAT in the plasma. (a) Western blot analysis of human LCAT protein in hamster plasma. Lane 1: serum free culture medium of Ad-hLCAT infected Cos7 cells, which was used as a positive control; lanes 2–4: Ad-hAP treated hamsters; lanes 5–7: Ad-hLCAT treated hamsters. (b) Plasma LCAT activity in hamsters ($n = 5$ for each group) and mice ($n = 5$ for each group). $^{**}P < 0.01$.

3.2. Effects of hLCAT overexpression on plasma lipids

A 10-fold increase of LCAT activity in hamsters resulted in approximately 3.1-fold and 2.3-fold increase in plasma TC and HDL-C levels, respectively, as compared with the control (Table 1). Overexpression of hLCAT infection also resulted in significant increase of plasma PL levels in hamsters ($P < 0.01$). In Ad-hLCAT infected mice, plasma TC increased 1.7-fold ($P < 0.01$). HDL-C and PL were unchanged between the two groups.

3.3. Alterations of HDL particle sizes in hamster plasma

The expression of hLCAT in hamster resulted in a major alteration of plasma HDL size distribution (Fig. 3). An incremental shift in HDL particle size in hamsters with hLCAT overexpression was revealed by non-denaturing 3–32% gradient PAGE, compared to the control. The pattern of HDL size distribution in control hamsters remained very similar to that of human, which has been well characterized.

3.4. Alterations of lipids concentration in hamster liver

Since liver is the major organ responsible for the clearance of excessive cholesterol in the body, we measured the lipids level in the hamster liver (Fig. 4). There is no significant difference in

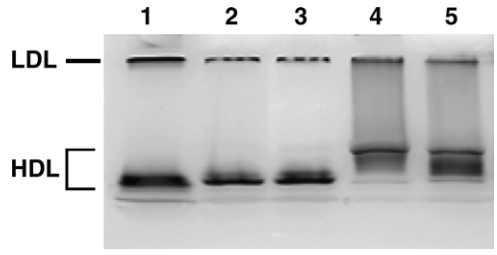

Fig. 3. The analysis of HDL particle size in hamster plasma by non-denaturing 3–32% gradient PAGE. Lane 1: human plasma used for a comparison. Lanes 2 and 3: plasma samples from control hamsters. Lanes 4 and 5: plasma samples from Ad-LCAT infected hamsters.

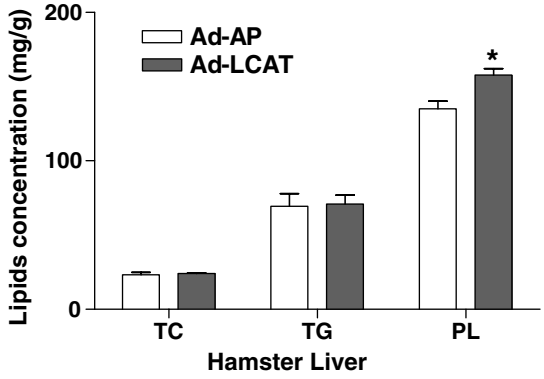

Fig. 4. Liver lipids analysis. TC, TG and PL were analyzed in hamsters ($n = 5$ for each group) 6 days after infection with 1×10^{10} pfu Ad-LCAT or Ad-AP. $^{*}P = 0.01$.

TC and TG levels between Ad-hLCAT and Ad-hAP treated hamsters. However, the PL levels in Ad-hLCAT treated hamsters was slightly higher than that of control (157.7 ± 4.4 vs 135.1 ± 5.2 mg/g wet weight, $P = 0.01$).

3.5. mRNA level of two related genes in the liver

Hepatic mRNA level of CYP7 in the Ad-hLCAT treated hamsters increased about 2.7-fold compared to the control ($5.4 \pm 1.1\%$ vs $2.0 \pm 0.4\%$, $P < 0.05$; Fig. 5). However, the hepatic HMGR mRNA level in the Ad-hLCAT treated hamsters decreased about 3.3-fold as compared with the control hamsters ($1.5 \pm 0.3\%$ vs $5.0 \pm 0.4\%$, $P < 0.01$).

3.6. Increased bile cholesterol excretion in hamster

There was no significant difference in bile flow between the two groups of hamsters. However, 10-fold overexpression of hLCAT in hamsters led to a significant increase in bile

Table 1
Plasma lipids (mg/dl) in hamsters and mice infected with Ad-LCAT or Ad-AP

	TC	TG	PL	HDL-C
Hamster				
Ad-hAP ($n = 5$)	111.6 ± 5.1	73.0 ± 8.8	199.3 ± 4.0	53.9 ± 3.8
Ad-hLCAT ($n = 5$)	347.2 ± 46.1[a]	47.7 ± 2.2	289.7 ± 18.8[b]	124.0 ± 9.2[b]
Mouse				
Ad-hAP ($n = 5$)	95.0 ± 1.4	69.6 ± 5.9	275.9 ± 5.6	60.4 ± 1.5
Ad-hLCAT ($n = 5$)	166.2 ± 13.8[b]	51.2 ± 7.7	286.0 ± 11.8	71.3 ± 5.7

[a] $P < 0.05$, as compared with the Ad-hAP-infected control group.
[b] $P < 0.01$, as compared with the Ad-hAP-infected control group.

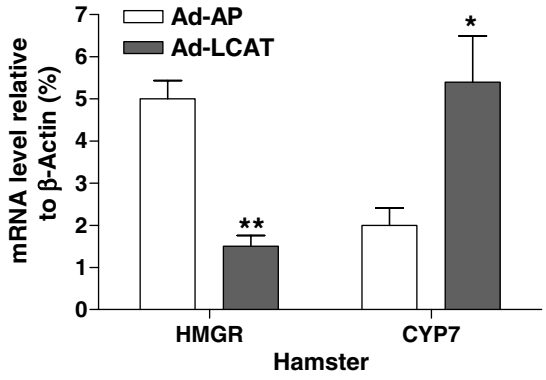

Fig. 5. Hepatic mRNA levels of HMGR and CYP7 in hamsters ($n = 5$ for each group) determined by quantitative real-time RT-PCR. The mRNA level was presented as percent level relative to β-actin. $^{*}P < 0.05$. $^{**}P < 0.01$.

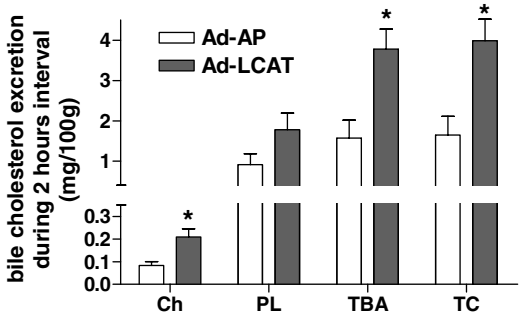

Fig. 6. Bile cholesterol excretion during 2 h interval in hamsters ($n = 5$ for each group). The value of total bile cholesterol excretion was calculated as described in Section 2. $^{*}P < 0.05$.

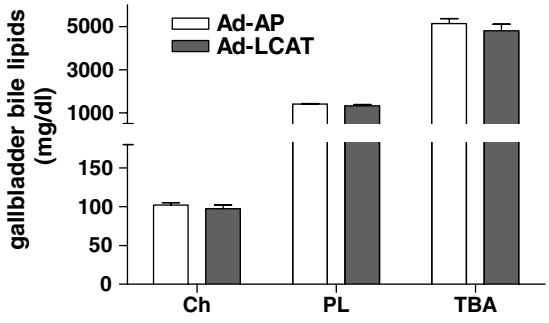

Fig. 7. Gallbladder bile lipids analysis in mice ($n = 5$ for each group). There was no significant change in the concentration of gallbladder bile cholesterol, PL, and TBA.

cholesterol and TBA as compared with the control (Table 2). PL was not changed in Ad-hLCAT treated hamsters. Bile cholesterol excretion, which was calculated by multiplying total bile cholesterol concentration (Ch + TBA) with the bile flow during 2 h interval, increased significantly as compared with the control (3.99 ± 0.53 vs 1.65 ± 0.47 mg/100 body weight, $P < 0.05$; Fig. 6). In Ad-hLCAT treated mice, the bile cholesterol, TBA and PL concentrations remained unchanged as compared with its control (Fig. 7).

4. Discussion

In the present study, we provided evidence that hamster, which has both activities of CETP [20] and HL [21] in plasma, is a better responder to hLCAT than mouse. Ten fold overexpression of hLCAT in hamsters significantly increased plasma HDL-C and bile cholesterol excretion. However, such effects were not observed in mice in a parallel experiment.

Adenovirus-mediated gene transfer has been used to overexpress cholesterol metabolism related genes including apoAI [22], LDLR [23], SR-BI [24] and CYP7 [25] in animals. Except gutted adenovirus [26], transgene expression mediated by commonly used adenoviral vectors in animals is stable for only 7–10 days, due to the development of immune response against the recombinant adenovirus and transgenes [22–25]. Although it is hard to directly evaluate the formation of atherosclerosis by this method, information generated from above studies proves to be valuable for establishing proof of principles and for future research on atherosclerosis. In present study, we therefore employed this method to explore the role of LCAT in relation to reverse cholesterol transport (RCT), which may further affect atherosclerosis ultimately. Consistent with other reports [22–25], hLCAT mRNA was preferentially expressed in the hamster livers. As noted, transient hepatic overexpression of hLCAT raised HDL-C levels, enlarged HDL particle size and enhanced biliary cholesterol excretion. These results implied enhanced RCT, a process that involves the removal of cholesterol from peripheral cells to the liver for catabolism. We therefore postulate that risk of atherosclerosis might be reduced if similar changes last for long time.

Theoretically, the increase of plasma HDL-C by hLCAT overexpression would result in enhanced clearance of HDL cholesteryl ester by the liver. However, such relationship has not been explored yet by studies of LCAT overexpression [3–8,27]. In the present study, we observed expression pattern of two related genes in the liver that implied enhanced clearance of HDL cholesteryl ester by the liver. HMGR and CYP7 are the key enzymes involved in the endogenous cholesterol synthesis and bile acid formation in the liver, respectively. The decrease of HMGR mRNA level in hamster liver reflected decrease of de novo cholesterol synthesis, which may result from increased influx of cholesterol into the liver. On the other hand, the significant increase of CYP7 mRNA level suggests

Table 2
Bile flow and bile lipid analysis in hamster

	Bile flow (μl/min/100 g)	Ch (mg/dl)	TBA (mg/dl)	PL (mg/dl)
Ad-hAP ($n = 5$)	2.1 ± 0.4	36.2 ± 1.4	622.9 ± 67.3	393.1 ± 71.1
Ad-hLCAT ($n = 5$)	3.2 ± 0.4	50.3 ± 3.8^{a}	960.8 ± 51.3^{b}	466.3 ± 44.4

[a] $P < 0.05$, as compared with the Ad-hAP-infected control group.
[b] $P < 0.02$, as compared with the Ad-hAP-infected control group.

an increase in the conversion of cholesterol into bile acid by the liver. The later was confirmed by the results of bile analysis. Due to the inhibition of endogenous cholesterol synthesis and increase of cholesterol clearance, hepatic cholesterol homeostasis remained unchanged though plasma TC and HDL-C increased significantly. These results suggest that the whole RCT was enhanced in hamsters with hLCAT overexpression.

In summary, this study demonstrated that transient hepatic overexpression of human LCAT in hamster significantly raised the HDL cholesterol levels and increased bile cholesterol excretion, facilitating RCT, therefore supporting the antiatherogenic role of LCAT. With respect to HDL metabolism, the hamster is certainly a better model for the study of hLCAT overexpression than mouse.

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