Expression of human lecithin:cholesterol acyltransferase in transgenic mice: effects on cholesterol efflux, esterification, and transport

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Abstract Human lecithin:cholesterol acyltransferase (LCAT) is a key enzyme in the plasma metabolism of cholesterol and is postulated to participate in a physiologic process called reverse cholesterol transport. We have used transgenic mice expressing the human LCAT transgene to study the effect of increased plasma levels of LCAT in each of the proposed steps involved in the reverse cholesterol transport pathway. High density lipoprotein (HDL) from LCAT transgenic mice was 44% more efficient than control mouse HDL in the efflux of cholesterol from human skin fibroblasts. Esterification of cell-derived cholesterol was also markedly increased in mice expressing the human LCAT transgene. The rate of plasma clearance of HDL cholesteryl ester was virtually the same in both types of animals whereas the HDL cholesteryl ester transport rate was significantly increased in mice expressing the human LCAT transgene (152.3 ± 16.9 μg/h vs. 203.1 ± 30.9 μg/h in control and transgenic mice, respectively). Liver cholesteryl ester uptake was significantly increased in mice expressing human LCAT (58.0 ± 1.4 μg/h/g liver vs. 77.9 ± 1.7 μg/h/g liver in control and transgenic mice, respectively). These studies indicate that LCAT modulates the rate by which cholesterol is effluxed from cell membranes onto HDL, esterified, and transported to the liver.—**Francone, O. L., M. Haghpassand, J. A. Bennett, L. Royer, and J. McNeish.** Expression of human lecithin:cholesterol acyltransferase in transgenic mice: effects on cholesterol efflux, esterification, and transport. *J. Lipid Res.* 1997. **38:** 813–822.

Supplementary key words LCAT • cholesterol efflux • HDL turnover • reverse cholesterol transport • transgenic mice

Lecithin:cholesterol acyltransferase (LCAT; phosphatidylcholine:sterol O-acyltransferase; EC 2.3.1.43) is a 416-amino acid glycoprotein that circulates in plasma with a complex of lipids and apoproteins in the high density lipoprotein (HDL) fraction (1). It is proposed that LCAT, through its role in the esterification of cholesterol, maintains a free cholesterol gradient between the cell membrane and plasma, allowing the unloading of cholesterol from peripheral tissues into the plasma compartment. This process is named reverse cholesterol transport (RCT). It involves the release of cholesterol from extrahepatic tissues onto HDL, esterification by LCAT, and its subsequent transfer to very low density (VLDL) and low density lipoprotein (LDL) by cholesteryl ester transfer protein (CETP). Finally, cholesteryl esters (CE) are taken up by the liver via LDL receptor or LDL receptor-related protein (LRP). Alternatively, HDL-CE can be internalized by the liver directly (HDL particle uptake) or through the nonendocytotic uptake of the CE moiety (selective uptake) (2, 3).

Most of the evidence supporting the role of LCAT in the efflux of cholesterol has been drawn from in vitro experiments using cultured cells. In these studies, the removal of cellular cholesterol from either human skin fibroblasts or rat aortic smooth muscle cells was similar regardless of LCAT activity or cholesterol esterification rate (4, 5). Similar findings were reported using rat hepatoma cells (Fu5AH) (6) and mouse peritoneal macrophages (7).

The biochemical and clinical characteristics of patients with either partial or total deficiency in LCAT activity support the role of this enzyme in the plasma metabolism and transport of cholesterol. The absence of LCAT activity is characterized by very low levels of CE and elevated amounts of unesterified cholesterol resulting in multiple abnormalities affecting composition, shape, and size distribution of all lipoprotein classes (8,

Abbreviations: CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); FCR, fractional catabolic rate; HuLCATTg, transgenic mice expressing the human LCAT transgene; LCAT, lecithin:cholesterol acyltransferase; RCT, reverse cholesterol transport; TR, transport rate.

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9). Moreover, cholesterol and phospholipids accumulate in peripheral tissues leading to tissue dysfunction (10). Additional information regarding the role of LCAT in plasma cholesterol metabolism, substrate specificity, and maturation of HDL species has recently been obtained in human LCAT transgenic (HuLCATTg) mice (11–13) and rabbits (14). Mice expressing the human LCAT transgene have a significant increase in HDL-cholesterol and CE resulting in a lipoprotein profile that in humans is associated with a reduced risk of atherosclerosis. Expression of both human LCAT and human apoA-I demonstrates that human LCAT has a marked preference for human-like HDL, and determines *in vivo* the distribution and concentration of the preβ and α-HDL species (13), indicating an essential role of LCAT in the biogenesis and composition of HDL. These studies, however, did not determine whether the increase in plasma levels of LCAT affects the proposed steps involved in the RCT pathway.

In this report, we have used transgenic mice expressing human LCAT to study the effect of increased plasma levels of LCAT in each of the proposed steps involved in the RCT pathway: cell-derived cholesterol efflux, LCAT-mediated cholesterol esterification, HDL-CE turnover, and flux of CE to the liver. Our studies indicate that LCAT modulates not only the rate by which cholesterol is effluxed and esterified but also the mass of CE transported to the liver via HDL.

MATERIALS AND METHODS

Transgenic mice

Transgenic mice expressing the human LCAT gene were produced by pronuclear injection according to established protocols (15, 16). A 7.6 kb DNA fragment containing the entire human LCAT gene, 550 bp of 3′ untranslated region, and the murine albumin promoter and enhancer sequences were injected at a concentration of 3 μg/ml into pronuclei of 1-day-old superovulated female C57BL6 embryos. Previous studies using the same murine albumin enhancer-promoter have shown that sequences linked to this promoter are expressed exclusively in the liver of transgenic animals (13, 17). Four founders were identified by PCR amplification and confirmed by Southern blot using a full-length LCAT cDNA probe as previously described (13). Founders were mated to C57BL6 mice to identify germline transmission of the transgenic lines to the F1 generation. All studies reported here were performed using offspring of these transgenic lines maintained on the C57BL/6 inbred genetic background. The C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, ME) and housed in a pathogen-free facility.

Plasma lipids and lipoprotein analysis

Mouse HDL was separated from apoB-containing lipoproteins by dextran sulfate precipitation as described elsewhere (13, 18). Plasma and lipoprotein total cholesterol levels were determined using kit No. 1127568 from Boehringer Mannheim (Indianapolis, IN). Free cholesterol was determined using a commercially available kit (Wako, Osaka, Japan). Triglyceride concentrations were determined using the Triglyceride/GB kit 450032 from Boehringer Mannheim. Phospholipid concentrations were measured using Phospholipids B reagent (Wako).

To isolate HDL from plasma, 500 μl of plasma containing 1.5 mM 5,5′ dithio-bis(2-nitrobenzoic acid] (DTNB) from HuLCATTg and C57BL6 control mice on chow diet was fractionated using two tandem Superose 6 columns (Pharmacia LKB Biotechnology, Piscataway, NJ) as previously described (19). Tubes corresponding to the HDL fraction were pooled and concentrated in Centriprep concentrators (Amicon, Inc., Beverly, MA). Lipids were determined enzymatically as described above whereas apoA-I concentrations were determined by ELISA.

Size fractionation of HDL was determined by computer-assisted scanning densitometry as described (20).

Quantitation of apoA-I

Mouse apoA-I levels were determined by an ELISA. Polyclonal rabbit anti-mouse apoA-I antibody (Biodesign, Kennebunk, ME) was purified on a HiTrap protein A affinity column (Pharmacia Biotech, Piscataway, NJ) and biotinylated. ELISA Maxisorb plates (Nunc, Roskilde, Denmark) were coated overnight at room temperature with 5 μl/ml purified anti-mouse apoA-I in phosphate-buffered saline containing 0.02% azide (PBS-A). Plates were blocked for 1 h at room temperature with PBS-A containing 1% (w/v) bovine serum albumin (type V). Plasma samples were diluted in PBS-A buffer containing 1% bovine serum albumin and 0.004% Tween-20. The standard curve was generated using a calibrated mouse plasma (kindly provided by Dr. B. Ishida). Plates were incubated overnight at 37°C. The next day, plates were washed three times with PBS-A containing 0.1% Tween-20, incubated with biotinylated anti-mouse apoA-I (1:600 in blocking buffer) for 3 h at room temperature, washed three times with PBS-A containing 0.1% Tween-20, and then incubated for 1 h with alkaline phosphatase-conjugated streptavidin (1:500) (Amersham) in blocking buffer containing 2 mM $MgCl_2$. Unbound avidin was removed by washing the plates four times with PBS-A containing 0.1%

Tween-20. Alkaline phosphatase substrate (Sigma, St. Louis, MO) was added at 1 mg/ml in bicarbonate buffer at pH 9.5. The reaction was stopped by adding 50 µl of 0.2 N NaOH. The optical density at 405 nm was determined on a UVmax plate reader (Molecular Devices, Sunnyvale, CA).

Determination of LCAT activity

LCAT activity in control and HuLCATTg mouse plasma was determined as previously described (21). LCAT activity was measured as the rate of synthesis of [^{3}H]CE from unilamellar vesicles prepared with a French pressure cell (22) and activated with human apoA-I (Sigma). LCAT activity was linear up to 30 min of incubation time and independent of the concentration of plasma lipoproteins (endogenous substrate). Therefore, an increase in the esterification rate corresponds to an increased level of LCAT protein.

Cell culture and cholesterol efflux

Normal human skin fibroblasts (ATCC CRL 1635) were grown in 12-well plates in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal calf serum, supplemented with 0.35 mg/ml glutamine and 40 µg/ml gentamicin sulfate. Cells were labeled for 48 h with 0.2 mCi [1,2-^{3}H]cholesterol (DuPont-New England Nuclear, Boston, MA) complexed to fetal calf serum. The final specific activity in the cells was 3.13 ± 0.19 × 10^6 cpm/µg cholesterol (n = 22). Cell cholesterol efflux was determined as previously described (21). Briefly, confluent fibroblasts were washed 4 times with serum-free D-MEM supplemented with gentamicin sulfate and glutamine. Next, cells were incubated at 37°C in an orbital shaker with 200 µl of plasma from either control or HuLCATTg mice for various times (1–60 min). Media samples were taken at each time point for determination of radioactivity. The cellular cholesterol efflux assay, however, does not measure net cholesterol efflux, the parameter most relevant to atherosclerosis.

To determine the esterification of cell-derived cholesterol by LCAT, plasma from HuLCATTg and control mice was incubated with [^{3}H]cholesterol-labeled fibroblasts as described above. Aliquots were taken at various time intervals. Lipids were extracted and the CE was separated from free cholesterol by thin-layer chromatography. [^{3}H]CE radioactivity was determined by liquid scintillation spectrometry. The mass of cholesterol effluxed and esterified was determined from cell cholesterol specific activity.

Preparation of [^{3}H] cholesteryl ether-labeled HDL

HDL was isolated in the density interval 1.09–1.21 g/ml from fasted control and HuLCATTg mouse plasma containing 1.5 mM DTNB, 1 mM EDTA, and a cocktail of protease inhibitors (Boehringer Mannheim) using sequential ultracentrifugation according to standard techniques (23). To remove any remaining apoB-containing lipoproteins, the HDL fraction was further purified by FPLC. Tubes containing the HDL were pooled, concentrated, and extensively dialyzed against a buffer containing 0.15 M NaCl, 0.01 M phosphate, and 0.02% sodium azide (pH 7.2). The purity of the HDL was assessed by polyacrylamide and agarose gel electrophoresis. The HDL preparation was then stored at 4°C in the presence of 1.5 mM DTNB prior to labeling.

Mouse HDL was labeled with [^{3}H]cholesteryl oleoyl ether (Amersham). To enhance the efficacy of labeling, recombinant CETP isolated from cultured CHO cells stably transfected with the human CETP cDNA (24) was used. Purified HDL was adjusted to a protein concentration of 1 mg/ml in 0.15 mM NaCl, 0.01 M phosphate buffer at pH 7.2. Aliquots of 1.5 mg were incubated in screw-top glass tubes which had 100 µCi [^{3}H]cholesteryl oleoyl ether dried onto 50 mg Celite 545-AW (Supelco Inc., Bellefonte, PA) and 25 µg of purified CETP. Incubation was performed for 18 h at 37°C. At the end of the incubation, celite was removed by filtration through 0.22-µm Millipore filters. HDL was extensively dialyzed against a buffer containing 0.15 M NaCl, 0.01 M phosphate, and 1 mM EDTA at pH 7.4 to remove any unbound tracers, and filter sterilized before injection. The distribution of the [^{3}H]cholesteryl ether radioactivity was assessed by agarose gel electrophoresis. More than 95% of the [^{3}H]cholesteryl oleoyl ether migrated with the HDL. Specific activity of the [^{3}H]-labeled HDL was 4–5 × 10^6 cpm/µg of CE. The data shown are from experiments in which HDL from control mice was labeled and injected in both normal and LCAT transgenics; identical results were obtained when HDL from HuLCATTg mice was labeled and used in both transgenic and control mice.

In vivo HDL-CE turnover studies

Before injection, blood was drawn from HuLCATTg and control mice after an overnight fast for measurement of HDL-CE and apoAI concentrations. Mice were injected in the tail vein with 4.9 × 10^5 dpm of ^{3}H-labeled HDL. The injected HDL mass was less than 0.5% of the mouse HDL-CE pool. Mice were fasted throughout the 24-h study period but had free access to water. Blood (50 µl) was withdrawn from the retroorbital plexus at 2 min, 1 h, 3 h, 6 h, 9 h, 12 h, 21 h, and 24 h after tracer injection. Aliquots of plasma were assayed for ^{3}H radioactivity. The fractional catabolic rate (FCR) was calculated from plasma disappearance curves analyzed by a two-pool model described by Matthews (25), and kinetic parameters were estimated using the nonlinear least-squares curve fitting program kinetic (G. A. McPer-

TABLE 1. HDL-cholesterol and LCAT activity in the plasma of control and HuLCATTg mice

Mice	n	HDL-Cholesterol	LCAT Activity
		mg/dl	*nmol CE/ml/h*
Controls	7	45.5 ± 6.4	71.7 ± 6.9
HuLCATTg10	29	62.2 ± 2.9[a]	197.7 ± 32.1[a]
HuLCATTg11	4	70.1 ± 4.6[b]	529.1 ± 46.2[b]
HuLCATTg12	6	86.0 ± 9.4[a]	744.2 ± 134.5[b]
HuLCATTg13	4	65.4 ± 9.3[a]	771.8 ± 147.0[b]

HDL-cholesterol levels were determined enzymatically after precipitation of apoB-containing lipoproteins by dextran sulfate. LCAT activity was measured as the rate of synthesis of [^{3}H]CE from unilamellar vesicles activated with apoA-I. Values shown are mean ± SD of n mice. CE, cholesteryl esters.

Statistically significant differences from control mice: [a]$P < 0.01$; [b]$P < 0.001$.

son, Elsevier-Biosoft, Cambridge, UK). Plasma transport rates (TR) were calculated using the equation TR = FCR × M, where M is the HDL-CE plasma pool size (26).

Twenty-four hours after the injection of the tracer, mice were anesthetized with sodium nembutal. The liver was perfused through the portal vein and drained from the right atrium with Dulbecco's phosphate-buffered saline (Gibco, BRL). The liver was excised and weighed immediately. The radioactivity present in the liver was determined by liquid scintillation spectrometry after lipid extraction. The fraction of the plasma pool of the labeled HDL cleared by the liver per h/g of tissue (liver FCR) was calculated as follows: liver FCR = plasma FCR × [fraction of total degradation in liver/weight of liver]. The absolute amount of CE mass taken up by the liver was obtained by multiplying liver FCR by the plasma HDL-CE pool size (27).

RESULTS

Plasma lipids and lipoprotein analysis

Plasma LCAT activities and HDL-cholesterol levels were determined in the human LCAT transgenic lines. Plasma LCAT activities in transgenic mice increased by 2.6- to 10.8-fold over the non-transgenic controls (**Table 1**). Compared to controls, plasma from HuLCATTg mice had a 1.4- to 1.9-fold increase in HDL-cholesterol levels. The transgenic line, HuLCATTg11, was further propagated and used for the subsequent studies. Plasma lipids and HDL-cholesterol concentrations from control and HuLCATTg11 mice fasted overnight are in agreement with previous reports (11, 13). Compared to nontransgenic controls, mice expressing the LCAT transgene had a statistically significant increase in the plasma concentrations of cholesterol and CE (25% and 36%, respectively). In HuLCATTg mice the total cholesterol level (mg/dl ± SD) was 107.4 ± 13.4, compared with 90.8 ± 10.5 in the nontransgenic animals ($P < 0.005$). Cholesteryl ester levels were 80.4 ± 10.4 and 64.0 ± 7.1 ($P < 0.001$) in HuLCATTg and controls, respectively. Plasma triglycerides were significantly decreased in transgenic mice (23.1 ± 8.9 and 10.7 ± 2.3 mg/dl ($P < 0.001$) for control and HuLCATTg mice, respectively) consistent with the proposed up-regulation of hepatic lipase in mice expressing the human LCAT transgene (12). The increase in plasma cholesterol and CE was found exclusively in the HDL fraction. Plasma concentrations of apoA-I were not affected in transgenic mice (207.2 ± 36.1 and 244.8 ± 38.5 mg/dl for control and HuLCATTg mice, respectively) confirming previous reports (13) that the overexpression of LCAT up to 10-fold, has no effect on apoA-I levels.

Cholesterol efflux

To examine the effect of increased plasma levels of LCAT on the efflux of cholesterol, we determined the potential of HuLCATTg and control mouse plasma to mediate cholesterol efflux from [^{3}H]cholesterol-labeled human fibroblasts. In agreement with a previ-

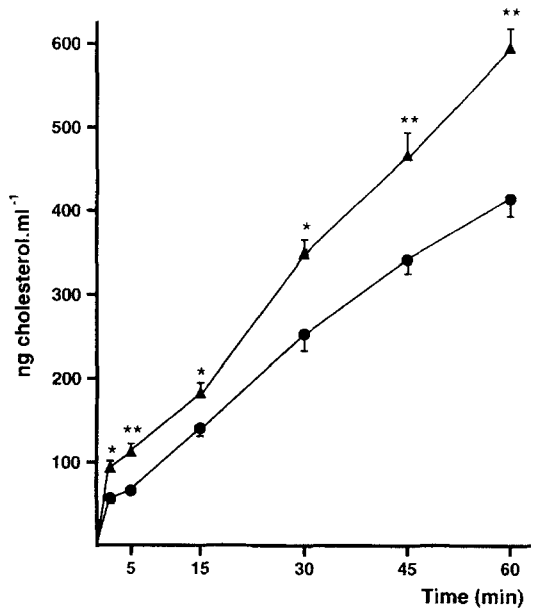

Fig. 1. Rate of appearance of [^{3}H]cholesterol from labeled human skin fibroblasts. Normal human skin fibroblasts were grown in 12-well plates containing DMEM + 10% fetal calf serum, supplemented with L-glutamine and gentamicin sulfate. Cells were labeled with 0.2 mCi [1,2-^{3}H]cholesterol as described under Materials and Methods. Cell cholesterol efflux was determined by incubating 200 µl of plasma from either controls (closed circles) or HuLCATTg mice (closed triangles) at 37°C for various times (1–60 min). Media samples were taken at various intervals for determination of radioactivity. Data are presented as mean ± SD (n = 5) for each time.

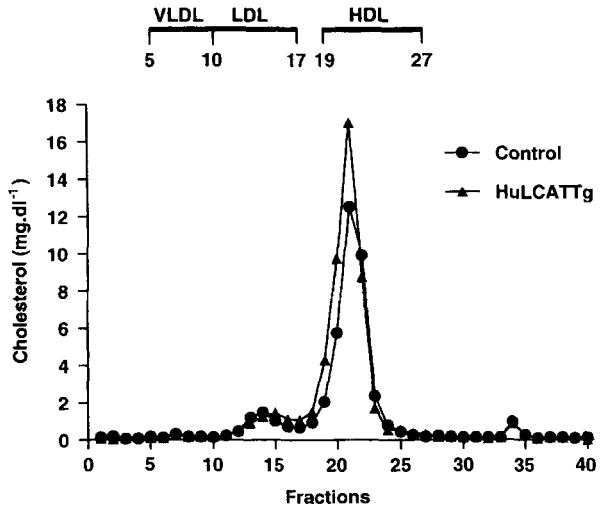

Fig. 2. Lipoprotein profiles of control and HuLCATTg mice. Pooled mouse plasma (500 µl) containing 1.5 mM DTNB from HuLCATTg and C57BL6 control mice was fractionated using Superose 6 columns as described under Materials and Methods. The cholesterol content was determined and plotted as a function of column fraction. The positions at which known lipoproteins eluted from the column are indicated. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

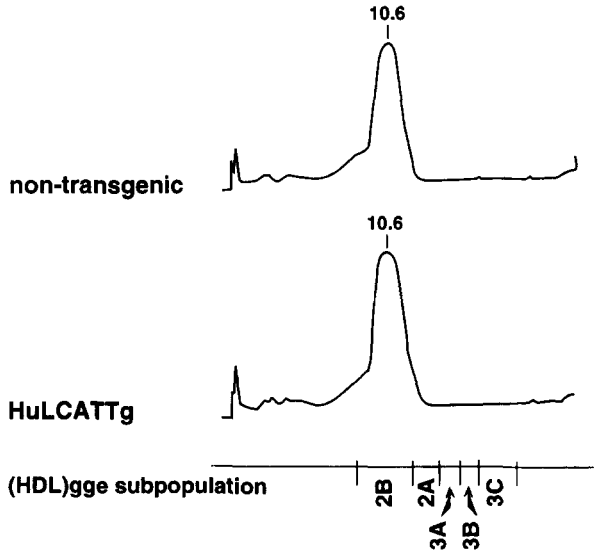

Fig. 3. Densitometric scan profiles of lipoproteins isolated from control and HuLCATTg mouse plasma. Total plasma lipoproteins were isolated by ultracentrifugation, collected, and sieved in a 4–30% nondenaturing gradient gel electrophoresis. HDL particle size distribution was assessed as described (20).

ous study (21), the rate of cholesterol efflux by control C57BL6 mouse plasma was 6.91 ± 0.34 ng of cholesterol/min per ml of plasma (**Fig. 1**). Plasma from HuLCATTg mice had an increased efflux rate of 44% over controls (9.95 ± 0.35 vs. 6.91 ± 0.34, $P < 0.005$). To determine whether the increased efflux is solely the consequence of the HDL's capacity to accept cholesterol, we determined the distribution of [^{3}H]cholesterol within plasma lipoproteins by agarose gel electrophoresis. Plasma from control and HuLCATTg mice was incubated with labeled fibroblasts for 2 min, removed, and immediately separated by agarose gel electrophoresis. More than 90% of the radioactivity found in the plasma co-migrated with the HDL fraction in both groups of mice. There was no increase in the proportion of labeled cholesterol in the apoB-containing lipoproteins (results not shown). To further characterize the HDL fraction, plasma from fasted C57BL6 control and HuLCATTg mice was fractionated by FPLC. As previously reported (13), HDL from human LCAT transgenic mice eluted from the column in the same fractions as the HDL from control mice indicating that its apparent size remains unchanged (**Fig. 2**). Identical results were obtained when the HDL particle size distribution was determined by gradient gel electrophoresis (**Fig. 3**). The increased esterification of cholesterol by moderate LCAT overexpression, led to an accumulation of CE in HDL (2.3-fold more than HDL from controls, relative to apoA-I, **Table 2**). Compared to controls, triglyceride content of HDL from HuLCATTg mice was nearly reduced by half. As one molecule of triglyceride is about 1.5 times larger than one CE molecule (27), it is likely that the decrease in the triglyceride content of HuLCATTg mouse HDL leads to an accumu-

TABLE 2. HDL lipid composition in HuLCATTg and C57BL6 control mice

Mice	TG	PL	FC	CE	PL/ApoA-I	CE/ApoA-I
Controls	2.0 (1.6%)	79.6 (65.7%)	11.1 (9.2%)	28.4 (23.5%)	0.65	0.23
HuLCATTg	0.9 (0.7%)	75.5 (62.3%)	10.4 (8.6%)	34.5 (28.4%)	1.14	0.52

Pooled plasma samples from controls and HuLCATTg mice were used to isolate HDL by FPLC as described under Experimental Procedures. Fractions containing the HDL were pooled and concentrated. Lipids and apoA-I concentrations were determined as described under Materials and Methods and expressed in mg/dl. TG: triglycerides; PL: phospholipids; FC: free cholesterol; CE: cholesteryl ester; %, percentage composition relative to the sum of TG, PL, FC, and CE values.

lation of CE in the core of the HDL without affecting their size. Interestingly, relative to apoA-I, HuLCATTg HDL contained 1.8-fold more phospholipids than HDL from controls.

Esterification of cell-derived and plasma-derived lipoprotein cholesterol

The plasma esterification of cell-derived cholesterol was quantitated by measuring the amount of [^{3}H]CE formed when plasma from HuLCATTg and control mice was incubated with [^{3}H]cholesterol-labeled human fibroblasts. On the average, 42.3 ± 4.48 ng/ml of plasma CE was synthesized at the end of a 1 h incubation. As shown in **Fig. 4,** compared to control mouse plasma, the esterification of cell-derived cholesterol was markedly increased in mice expressing the human LCAT transgene. To assess the relative contributions of cell- and lipoprotein-derived cholesterol to the LCAT reaction, plasma samples from HuLCATTg and control mice were incubated at 37°C in the presence or absence of labeled fibroblasts. The percent of total free cholesterol mass esterified in plasma, and the percent of labeled cell-derived cholesterol esterified were determined. As shown in **Table 3,** the comparison of these rates and their ratio indicates that mice expressing human LCAT have a 2-fold increase in the contribution of cell-derived cholesterol to the LCAT reaction, suggesting a preferential utilization of cellular cholesterol by LCAT in HuLCATTg mice.

TABLE 3. Esterification of cell-derived and lipoprotein-derived cholesterol by LCAT in control and HuLCATTg mice

Mice	Lipoprotein-Derived Cholesterol Esterified/Hour (a)	Cell-Derived Cholesterol Esterified/Hour (b)	b/a
	%	%	
Control	12.8 ± 1.5	4.0 ± 0.6	0.3
HuLCATTg	8.3 ± 1.1[a]	6.1 ± 1.0[a]	0.7

Esterification of lipoprotein-derived cholesterol by LCAT was determined as the rate of decrease of plasma free cholesterol mass as a function of time; percent esterification is expressed relative to the initial amount of free cholesterol in plasma. The percent of cell-derived cholesterol esterified by LCAT as a function of time is expressed as the ratio of ^{3}H-labeled esterified and free cholesterol. Cell cholesterol efflux and esterification were determined as described under Materials and Methods. Values shown are mean ± SD on 3–6 mice.
[a] $P < 0.05$.

HDL-CE turnover studies

To evaluate in vivo the effect of LCAT on the clearance of HDL cholesterol, HDL was labeled with [^{3}H]cholesteryl oleoyl ether and injected into HuLCATTg and control mice. Several studies conducted in rats and transgenic mice have demonstrated that HDL labeled with cholesteryl ether has identical metabolic and biological activity as native HDL (28–30).

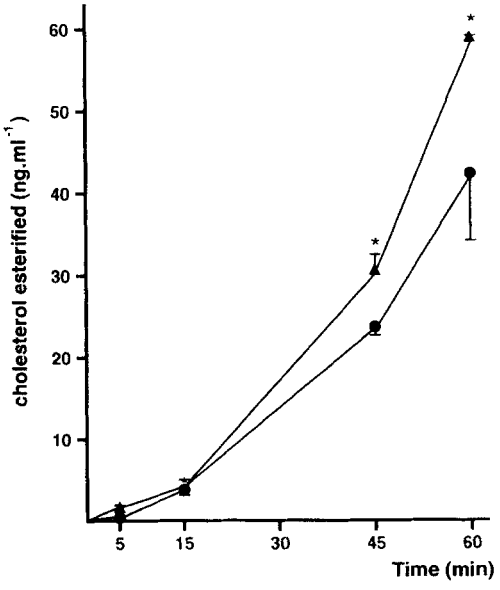

Fig. 4. Rate of appearance of [^{3}H]cholesteryl ester radioactivity from labeled human fibroblasts incubated with unlabeled control or HuLCATTg mouse plasma. LCAT-mediated cholesterol esterification was determined as described under Materials and Methods. Aliquots were taken at various time intervals. Lipids were extracted and the [^{3}H]cholesteryl esters were separated by thin-layer chromatography. Controls, closed circles; HuLCATTg, closed triangles. Data are presented as mean ± SD (n = 4–6) for each time point.

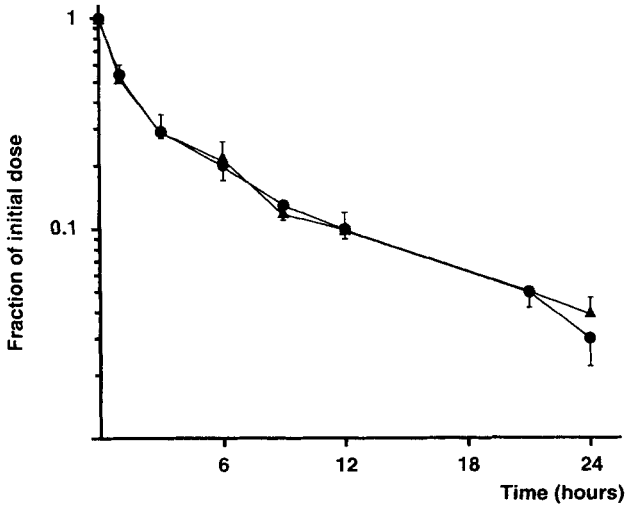

Fig. 5. Radiolabel HDL decay curve in control and transgenic mouse plasma. Control (n = 10) and HuLCATTg (n = 8) mice were injected intravenously with [^{3}H]cholesteryl ether-labeled HDL. Blood (50 µl) was withdrawn from the retroorbital plexus for determination of radioactivity. Control, closed circles; HuLCATTg, closed triangles. Values are mean ± SD.

TABLE 4. In vivo HDL-CE metabolism in HuLCATTg and control mice fed a chow diet

Mice	n	ApoA-I	HDL-CE Pool Size	HDL-CE FCR	HDL-CE TR[#]
		mg/dl	*μg*	*pool/h*	*μg/h*
Controls	10	212.8 ± 23.9	629.5 ± 29.2	0.24 ± 0.02	152.4 ± 16.9
HuLCATTg	8	171.7 ± 38.4	810.9 ± 36.4[a]	0.25 ± 0.02	203.1 ± 30.9[a]

Mouse HDL was labeled with [^{3}H]cholesteryl oleoyl ether as described under Materials and Methods and injected in the tail vein of fasted HuLCATTg and control mice. Fifty μl of blood was withdrawn from the retroorbital plexus at various time intervals and radioassayed for ^{3}H. HDL-CE TR[#] is calculated using the equation TR = FCR × M, where M is the HDL-CE plasma pool size. Statistically significant differences were calculated by Student *t*-test.

[a] $P < 0.005$.

The disappearance of radioactivity from plasma was followed for 24 h. The plasma clearance rate for labeled HDL (**Fig. 5**) was virtually identical in control and HuLCATTg mice. The FCR was also the same between controls and HuLCATTg mice. In close agreement with previous studies (31), the FCR values in the present study were 0.24 ± 0.02 and 0.25 ± 0.02 pools/h for controls and HuLCATTg mice, respectively. The HDL-CE TR, calculated as the product of plasma FCR and HDL-CE plasma pool size, was 30% higher in mice expressing the human LCAT transgene compared to control mice ($P < 0.005$), indicating that the increase in HDL-CE observed in HuLCATTg mice is due to an increase in the production of HDL-CE without a major disturbance in their catabolism. Plasma concentrations of apoA-I were not affected by the expression of the human LCAT transgene (**Table 4**), suggesting that the clearance of apoA-I is not impaired in HuLCATTg mice.

Liver uptake of HDL-CE

The uptake of HDL-CE by the liver of HuLCATTg and control mice was determined by using cholesteryl oleoyl ether-labeled HDL. As mice lack cholesteryl ester transfer activity (32), exchange of labeled cholesteryl ether with other lipoprotein fractions does not occur. Thus, the radioactivity present in the liver reflects the uptake of [^{3}H]cholesteryl ethers from HDL itself, not from other lipoprotein fractions labeled secondarily. In this study, liver HDL-CE uptake was determined 24 h after the injection of labeled HDL, when >95% of the injected tracer was cleared from the plasma. After 24 h, the liver contained 38.5 ± 4.6% (n = 10) of the injected radioactivity as expressed per gram of tissue. The fraction of the plasma pool of the traced HDL taken up by the liver (liver FCR) shows no difference between controls and HuLCATTg mice (94.0 ± 4.0 and 89.0 ± 5.0 pool cleared 10^3/h/g, $P = 0.49$). The absolute amount (flux) of HDL-CE going to the liver, calculated by multiplying liver FCR by the plasma HDL-CE pool size (**Table 5**), is markedly increased (35%, $P < 0.0001$) in mice expressing the human LCAT transgene. Taken together, these results indicate that the clearance rate of HDL by the liver is not altered in HuLCATTg mice. However, as a result of increased plasma LCAT levels, the lipid composition and, in particular, the CE content of HuLCATTg mouse HDL is increased, leading to increased HDL-CE pool and the flux of CE to the liver. Expressed as mg of CE per day, normalized to a constant body weight of 1 kg, nontransgenic control mice HDL transports 69.6 mg of CE/kg per day to their livers. HDL in mice expressing the human LCAT transgene transported 93.5 mg of CE/kg per day (an additional 23.9 mg of CE/kg per day).

DISCUSSION

In this report, we have used mice expressing the human LCAT transgene to demonstrate that LCAT determines, at least in part, the rate by which cholesterol is effluxed from cell membranes onto HDL, esterified and transported to the liver.

Cellular cholesterol efflux is the first step of choles-

TABLE 5. Liver HDL-CE uptake in HuLCATTg and control mice

Mice	n	Liver FCR[a]	Mass Flux to Liver
			μg CE/h/g liver
Controls	10	94.0 ± 4.0	58.0 ± 1.4
HuLCATTg	8	89.0 ± 5.0	77.9 ± 1.7[b]

Labeled HDL were injected into control and transgenic mice as described under Materials and Methods. Plasma decays were followed for 24 h after which livers were perfused with PBS, removed, and assayed for radioactivity after lipid extraction. Statistically significant differences were calculated by Student *t*-test.

[a] Liver FCR is expressed as fraction of plasma pool cleared per hour per gram of liver $\times 10^3$.

[b] $P < 0.0001$.

terol transport from peripheral tissues to the liver by the lipoprotein system. Esterification of cholesterol by LCAT is thought to play a major role in this process by maintaining a cholesterol concentration gradient between the plasma membrane and HDL. In this study we used HuLCATTg mice to address whether in vivo elevated levels of LCAT affect the capacity of plasma to remove cholesterol from human fibroblasts. HuLCATTg mouse HDL is 44% more efficient than control mouse HDL in the efflux of cholesterol over the time points taken (2–60 min). The mechanism by which HuLCATTg HDL increases cholesterol efflux is unclear. However, it is likely that the increased capacity of HuLCATTg mouse HDL to promote cholesterol efflux is independent of the esterification of cell-derived cholesterol by LCAT. Further support for this hypothesis comes from the observation that cholesterol efflux is increased as early as 2 min in LCAT transgenic plasma even though no newly synthesized CE can be detected at this time (1, 21). Most probably the increased capacity of HuLCATTg mouse HDL to promote cholesterol efflux is a consequence of LCAT-mediated changes in its lipid composition. Elevated levels of LCAT increase the HDL-CE, leaving size and number of HDL particles unaffected. Interestingly, relative to apoA-I, HuLCATTg mouse HDL has 1.8-fold more phospholipid than control mouse HDL. Studies conducted by Burns and Rothblat (33) have shown that cholesterol efflux from L5178Y cells is influenced by both the type and concentration of phospholipids. More recently, studies in which recombinant HDL particles were incubated with either Ob1771 cells (34) or human skin fibroblasts (35) suggested that the phospholipid to apoA-I ratio may determine the rate by which cholesterol is effluxed from cell membranes to acceptor particles. Our findings are consistent with this hypothesis and strongly suggest that expression of the human LCAT gene causes changes in the lipid composition of HDL leading to an increase in cholesterol efflux.

The metabolism of HDL-CE was studied by using cholesteryl ether-labeled HDL. The rate of plasma and liver clearance of HDL-CE was virtually the same in HuLCATTg and control mice, whereas the plasma HDL-CE TR and flux of cholesterol to the liver were significantly higher in mice expressing the human LCAT transgene. Taken together, these results indicate that the increase in the plasma levels of HDL-CE in HuLCATTg mice is due to an increase in the production rate with no effect on HDL catabolism. It is the increased esterification of cholesterol by LCAT that leads to an accumulation of CE in the core of the HDL, inducing a greater mass flux [Mass flux = liver FCR × HDL-CE plasma pool size] of CE to the liver.

It is postulated that HDL-CE can be transported to the liver by three different mechanisms: HDL particle uptake (and thus HDL components) (36, 37), non-endocytotic uptake of the CE moiety without uptake of the particle (selective CE uptake) (2), or by liver LDL receptors after the CETP-mediated transfer of CE to apoB-containing lipoprotein particles (38). As mice do not have detectable plasma CETP activity, the third pathway cannot be addressed in this study. The exact mechanism and contribution of each of the first two pathways for the removal of plasma HDL-CE by the liver remains unclear. However, studies conducted in vivo and in vitro suggest that these processes occur in several species including mice and account for approximately 65% of the total HDL-CE and 40% of apoA-I cleared from the plasma compartment (28, 29). The data from the present study indicate that the flux of CE to the liver is markedly increased in mice expressing human LCAT. This study, however, does not distinguish whether the increase in hepatic cholesterol flux is due to the uptake of HDL particles or selective uptake of CE as both mechanisms predict a greater flux of CE when the CE content of HDL is increased. Although the extent to which this represents cell-derived net RCT is not known because the in vivo contribution of lipoprotein-derived (VLDL and LDL) free cholesterol to the LCAT reaction has not been determined, several lines of evidence support the hypothesis that increased levels of LCAT lead to a greater rate of cell-derived cholesterol transport to the liver. Mice expressing human LCAT have an increase in the relative amount of cell-derived cholesterol supplied to the LCAT reaction. In addition, as fasted control and HuLCATTg mice have very low plasma levels of VLDL and LDL, it is likely that the majority of the lipoprotein cholesterol supplied to the LCAT reaction in vivo is provided by HDL and not LDL as suggested for human plasma (39, 40). Also, in this study, cell-derived cholesterol efflux and esterification were significantly increased in mice with increased plasma levels of LCAT. Finally, several studies have provided direct in vivo evidence that peripheral esterified cholesterol derived from the LCAT reaction is incorporated into HDL and transported to the liver (41–43).

In summary, the results from this study are consistent with the proposed role of LCAT in RCT. However, the exact role of the RCT pathway in the development of atherosclerosis is not yet fully understood. Mice expressing the human LCAT transgene at low levels provide a useful means for addressing this issue.

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REFERENCES

1. Francone, O. L., A. Gurakar, and C. J. Fielding. 1989. Distribution and functions of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in plasma lipoproteins. *J. Biol. Chem.* **264:** 7066–7072.
2. Pittman, R. C., T. P. Knecht, M. S. Rosenbaum, and C. A. Taylor, Jr. 1987. A nonendocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J. Biol. Chem.* **262:** 2443–2450.
3. Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science.* **271:** 518–520.
4. Stein, O., R. Goren, and Y. Stein. 1978. Removal of cholesterol from fibroblasts and smooth muscle cells in culture in the presence and absence of cholesterol esterification in the medium. *Biochim. Biophys. Acta.* **529:** 309–318.
5. Fielding, C. J., and P. E. Fielding. 1981. Evidence for a lipoprotein carrier in human plasma catalyzing sterol efflux from cultured fibroblasts and its relationship to lecithin:cholesterol acyltransferase. *Proc. Natl. Acad. Sci. USA.* **78:** 3911–3914.
6. Ray, E., F. Bellini, G. Stoudt, S. Hemperly, and G. Rothblat. 1980. Influence of lecithin:cholesterol acyltransferase on cholesterol metabolism in hepatoma cells and hepatocytes. *Biochim. Biophys. Acta.* **617:** 318–334.
7. Czarnecka, H., and S. Yokoyama. 1995. Lecithin:cholesterol acyltransferase reaction on cellular lipid released by free apolipoprotein-mediated efflux. *Biochemistry.* **34:** 4385–4392.
8. Norum, K. R., J. A. Glomset, A. V. Nichols, and T. Forte. 1971. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: physical and chemical studies of low and high density lipoproteins. *J. Clin. Invest.* **50:** 1131–1140.
9. Forte, T., K. R. Norum, J. A. Glomset, and A. V. Nichols. 1971. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: structure of low and high density lipoproteins as revealed by electron microscopy. *J. Clin. Invest.* **50:** 1141–1148.
10. Glomset, J. A., G. Assmann, E. Gjone, and K. R. Norum. 1995. *In* The Metabolic and Molecular Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Inc., New York. 1933–1952.
11. Vaisman, B. L., H-G. Klein, M. Rouis, A. M. Bérard, M. R. Kindt, G. D. Talley, S. M. Meyn, R. F. Hoyt, S. M. Marcovina, J. J. Albers, J. M. Hoeg, H. B. Brewer, Jr., and S. Santamarina-Fojo. 1995. Overexpression of human lecithin:cholesterol acyltransferase leads to hyperalphalipoproteinemia in transgenic mice. *J. Biol. Chem.* **270:** 12269–12275.
12. Mehlum, A., B. Staels, N. Duverger, A. Tailleux, G. Castro, C. Fievet, G. Luc, J-C. Fruchart, G. Olivecrona, G. Skretting, J. Auverx, and H. Pritz. 1995. Tissue-specific expression of the human gene for lecithin:cholesterol acyltransferase in transgenic mice alters blood lipids, lipoproteins and lipases towards a less atherogenic profile. *Eur. J. Biochem.* **230:** 567–575.
13. Francone, O. L., E. L. Gong, D. S. Ng, C. J. Fielding, and E. M. Rubin. 1995. Expression of human lecithin-cholesterol acyltransferase in transgenic mice. *J. Clin. Invest.* **96:** 1440–1448.
14. Hoeg, J. M., B. L. Vaisman, S. J. Demosky, Jr., S. M. Meyn, G. D. Talley, R. F. Hoyt, Jr., S. Feldman, A. M. Bérard, N. Sakai, D. Wood, M. E. Brousseau, S. Marcovina, H. B. Brewer, Jr., and S. Santamarina-Fojo. 1996. Lecithin:cholesterol acyltransferase overexpression generates hyperalphalipoproteinemia and a nonatherogenic lipoprotein pattern in transgenic rabbits. *J. Biol. Chem.* **271:** 4396–4402.
15. Hogan, B., F. Costantini, and E. Lacy. 1986. Manipulating the Mouse Embryo: a Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY. 89–173.
16. Gibbs, E. M., J. L. Stock, S. C. McCoid, H. A. Stukenbrok, J. E. Pessin, R. W. Stevenson, A. J. Milici, and J. D. McNeish. 1995. Glycemic improvement in diabetic db/db mice by overexpression of the human insulin regulatable glucose transporter (GLUT4). *J. Clin. Invest.* **95:** 1512–1518.
17. Pinkert, C. A., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter. 1987. An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient liver-specific expression in transgenic mice. *Genes Dev.* **1:** 268–276.
18. Walsh, A., Y. Ito, and J. L. Breslow. 1989. High levels of human apolipoprotein A-I in transgenic mice result in increased plasma levels of small high density lipoproteins (HDL) particles comparable to human HDL_3. *J. Biol. Chem.* **264:** 6488–6494.
19. Cole, T. G., R. Kitchens, A. Daugherty, and G. Schonfeld. 1988. An improved method for separation of triglyceride-rich lipoproteins by FPLC. *Pharmacia FPLC Biocommunique* **4:** 4.
20. Nichols, A. V., P. J. Blanche, and E. L. Gong. 1983. Gradient gel electrophoresis of human plasma high density lipoproteins. *In* Handbook of Electrophoresis. Vol. 3. L. Lewis, editor. CRC Press, Boca Raton, FL. 29–47.
21. Francone, O. L., L. Royer, and M. Haghpassand. 1996. Increased preβ-HDL levels, cholesterol efflux, and LCAT-mediated esterification in mice expressing the human cholesteryl ester transfer protein (CETP) and human apolipoprotein A-I (apoA-I) transgenes. *J. Lipid Res.* **37:** 1268–1277.
22. Hamilton, R. L., J. Goercke, L. S. S. Guo, M. C. Williams, and R. J. Havel. 1980. Unilamellar liposomes made with the French pressure cell: a simple preparative and semiquantitative technique. *J. Lipid Res.* **21:** 981–992.
23. Havel, R. J., H. E. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
24. Stevenson, S. C., S. Wang, L. P. Deng, and A. R. Tall. 1993. Human plasma cholesteryl ester transfer protein consists of a mixture of two forms reflecting variable glycosylation at asparagine 341. *Biochemistry.* **32:** 5121–5126.
25. Matthews, C. M. E. 1957. The theory of tracer experiments with ^{131}I-labeled plasma proteins. *Phys. Med. Biol.* **2:** 36–53.
26. Berman, M. 1982. Kinetic analysis and modeling: theory and applications to lipoproteins. *In* Lipoprotein Kinetics

and Modeling. M. Berman, S. M. Grundy, and B. Howard, editors. Academic Press, Inc., New York, NY. 3–36.
27. Shen, B. W., A. M. Scanu, and F. J. Kézdy. 1977. Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. USA.* **74:** 837–841.
28. Khoo, J. C., R. C. Pittman, and E. M. Rubin. 1995. Selective uptake of HDL cholesteryl esters is active in transgenic mice expressing human apolipoprotein A-I. *J. Lipid Res.* **36:** 593–600.
29. Glass, C., R. C. Pittman, D. B. Weinstein, and D. Steinberg. 1983. Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesterol ester to liver, adrenal, and gonad. *Proc. Natl. Acad. Sci. USA.* **80:** 5435–5439.
30. Gwynne, J. T., and D. D. Mahaffee. 1989. Rat adrenal uptake and metabolism of high density lipoprotein cholesteryl ester. *J. Biol. Chem.* **264:** 8141–8150.
31. Hayek, T., T. Chajek-Shaul, A. Walsh, L. B. Agellon, P. Moulin, A. R. Tall, and J. L. Breslow. 1992. An interaction between the human cholesteryl ester transfer protein (CETP) and apolipoprotein A-I genes in transgenic mice results in a profound CETP-mediated depression of high density lipoprotein cholesterol levels. *J. Clin. Invest.* **90:** 505–510.
32. Jiao, J., T. G. Cole, T. T. Kitchens, B. Pfleger, and G. Schonfeld. 1990. Genetic heterogeneity of lipoproteins in inbred strains of mice. *Metabolism.* **39:** 155–160.
33. Burns, C. H., and G. H. Rothblat. 1969. Cholesterol excretion by tissue culture cells: effect of serum lipids. *Biochim. Biophys. Acta.* **176:** 616–625.
34. Jonas, A., K. Bottum, N. Theret, P. Duchateau, and G. Castro. 1994. Transfer of cholesterol from Ob1771 cells or LDL to reconstituted, defined high density lipoproteins. *J. Lipid Res.* **35:** 860–870.
35. Agnani, G., and Y. L. Marcel. 1993. Cholesterol efflux from fibroblasts to discoidal lipoproteins with apolipoprotein A-I (LpA-I) increases with particle size but cholesterol transfer from LpA-I to lipoproteins decreases with size. *Biochemistry.* **32:** 2643–2649.
36. DeLamatre, J. G., T. G. Sarphie, R. C. Archibold, and C. A. Hornick. 1990. Metabolism of apoE-free high density lipoproteins in rat hepatoma cells: evidence for a retroendocytic pathway. *J. Lipid Res.* **31:** 191–202.
37. Garcia, A., R. Barbaras, X. Collet, A. Bogyo, H. Chap, and B. Perret. 1996. High-density lipoprotein3 receptor-dependent endocytosis pathway in a human hepatoma cell line (HepG2). *Biochemistry.* **35:** 13064–13071.
38. Schneider, W. J. 1991. Removal of lipoproteins from plasma. *In* Biochemistry of Lipids, Lipoproteins and Membranes. E. E. Vance and J. Vance, editors. Elsevier Press, Amsterdam. 461–487.
39. Fielding, C. J., and P. E. Fielding. 1981. Regulation of human plasma lecithin:cholesterol acyltransferase activity by lipoprotein acceptor cholesteryl ester content. *J. Biol. Chem.* **256:** 2102–2104.
40. Park, M-S. C., B. J. Kudchodkar, J. Frohlich, H. Pritchard, and A. G. Lacko. 1987. Study of the components of reverse cholesterol transport in lecithin:cholesterol acyltransferase deficiency. *Arch. Biochem. Biophys.* **258:** 545–554.
41. Miller, N. E., A. La Ville, and D. Crook. 1985. Direct evidence that reverse cholesterol transport is mediated by high-density lipoprotein in rabbit. *Nature.* **314:** 109–111.
42. Davis, R. A., P. Helgerud, S. Dueland, and C. A. Drevon. 1982. Evidence that reverse cholesterol transport occurs in vivo and requires lecithin:cholesterol acyltransferase. *Biochim. Biophys. Acta.* **689:** 410–414.
43. Mindham, M. A., and P. A. Mayes. 1995. Application of organ perfusion to the study of reverse cholesterol transport. *In* Atherosclerosis III: Recent Advances in Atherosclerosis Research. F. Numano, and R. W. Wissler, editors. *Ann. NY Acad. Sci.* New York, NY. 240–263.