Expression of Human Lecithin-Cholesterol Acyltransferase in Transgenic Mice

Effect of Human Apolipoprotein AI and Human Apolipoprotein AII on Plasma Lipoprotein Cholesterol Metabolism

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Abstract

Human (Hu) lecithin-cholesterol acyltransferase (LCAT) is a key enzyme in the plasma metabolism of cholesterol. To assess the effects of increased plasma levels of LCAT, four lines of transgenic mice were created expressing a Hu LCAT gene driven by either its natural or the mouse albumin enhancer promoter. Plasma LCAT activity increased from 1.2- to 1.6-fold higher than that found in control mouse plasma. Lipid profiles, upon comparing HuLCAT transgenics to control animals, revealed a 20 to 60% increase in total and cholesteryl esters that were mainly present in HDL. The in vivo substrate specificity of Hu LCAT was assessed by creating animals expressing Hu apo AI + Hu LCAT (HuAI/LCAT), Hu apo AI + Hu apo AII + Hu LCAT (HuAI/AII/LCAT), and Hu apo AII + Hu LCAT (HuAII/LCAT). Plasma cholesterol was increased up to 4.2-fold in HuAI/LCAT transgenic mice and two fold in the HuAI/AII/LCAT transgenic mice, compared with HuAI and HuAI/AII transgenic mice. HDL cholesteryl ester levels were increased more than twofold in both the HuAI/LCAT and HuAI/AII/LCAT mice compared with the HuAI, HuAI/AII, and HuLCAT animals. The HDL particles were predominantly larger in the HuAI/LCAT and the HuAI/AII/LCAT mice compared with those in HuAI, HuAII/LCAT, and HuLCAT animals. The increase in LCAT activity in the HuAI/LCAT and HuAI/AII/LCAT mice was associated with 62 and 27% reductions respectively, in the proportion of Hu apo AI in the preβ-HDL fraction, when compared with HuAI and HuAI/AII transgenic mice. These data demonstrate that moderate increases in LCAT activity are associated with significant changes in lipoprotein cholesterol levels and that Hu LCAT has a significant preference for HDL containing Hu apo AI. (*J. Clin. Invest.* 1995. 96:1440–1448.) Key words: high density lipoproteins • reverse cholesterol transport • cholesterol metabolism • apolipoproteins • transgenic animals

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Introduction

HDL plays a pivotal role in the transport of plasma cholesterol and is postulated to participate in a physiologic process called reverse cholesterol transport (1)—a process by which peripheral cell-derived cholesterol is transported through plasma to the liver for catabolism. Factors affecting this process by way of changes in the structure, composition, or concentration of plasma HDL particles are likely to have a profound effect on the homeostasis of plasma cholesterol.

Lecithin-cholesterol acyltransferase (LCAT)[1] is a 416–amino acid glycoprotein produced mainly by the liver that circulates in the plasma compartment as a functional unit with the cholesteryl ester transfer protein (CETP) and apo AI and apo D primarily in the HDL density range (2, 3). LCAT, through its role in the esterification of cholesterol, promotes the packaging of this polar lipid into the core of the HDL particles, maintaining a concentration gradient for the diffusion of free cholesterol from peripheral tissues to HDL. Therefore, changes in the concentration and/or activity of this enzyme are likely to affect the transport of cholesterol by HDL. apo AI is the major protein component of HDL and the most potent activator of LCAT (4).

The physiologic substrates for LCAT are nascent and mature HDL. Human (Hu) HDL is very heterogeneous both in particle size (5) and protein composition (6). HDL can be separated into two subpopulations on the basis of protein composition, one containing both apo AI and apo AII (LpAI/AII) and one containing apo AI but no apo AII (LpAI). Both are heterogeneous in size (7). The size heterogeneity of Hu HDL distinguishes it from the monodisperse population of HDL particles present in the plasma of mice (8).

LCAT is associated with transformation and remodeling of HDL. Plasma from LCAT-deficient patients (10, 11) contains small, discoidal HDL particles that probably represent nascent HDL (12). These particles undergo marked changes in composition and morphology when LCAT is added, suggesting an essential role of this enzyme in the biogenesis of circulating HDL.

In this report, we established multiple lines of transgenic mice expressing the Hu LCAT gene at variable levels. To assess the impact of expressing Hu LCAT and to examine its substrate specificity, HuLCAT transgenic animals were cross-bred with transgenic mice expressing Hu apo AI, Hu apo AII, and both Hu apo AI and apo AII transgenes. The effect of expression on

1. *Abbreviations used in this paper:* CETP, cholesteryl ester transfer protein; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid; Hu, human; HuAI/LCAT, human apo I + LCAT; HuAI/AII/LCAT, human apo I + apo II + LCAT; HuAII/LCAT, human apo AII + LCAT; LCAT, lecithin-cholesterol acyltransferase.

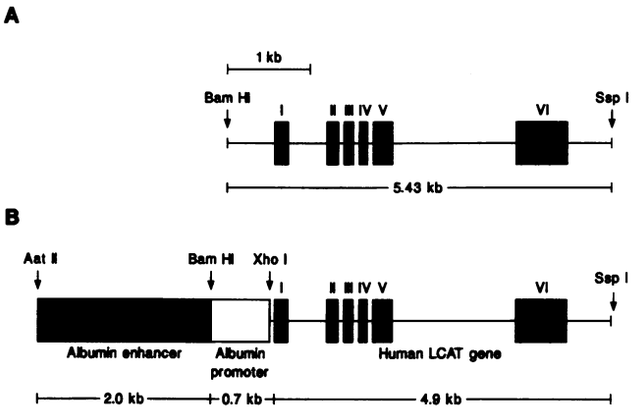

Figure 1. Hu LCAT constructs used to create transgenic mice. *(A)* A Hu LCAT genomic fragment containing 561 bp of 5′ untranslated region followed by 6 exons with the corresponding introns and 550 bp of 3′ untranslated region. This construct contains the natural promoter elements and the polyadenylation sites. *(B)* The construct used to direct Hu LCAT expression in liver. This construct has a 2.74-kb fragment containing the murine albumin enhancer-promoter linked to a promoterless Hu LCAT genomic fragment.

the various combinations of Hu transgenes on HDL size and cholesterol metabolism was studied.

Methods

Plasmid construction. Clone pUCLCATBamNsi was a kind gift from Dr. John McLean (Genentech, South San Francisco, CA). The Hu LCAT gene is divided into six exons and spans 4,200 bp in the genome (13). The LCAT genomic fragment used in this study (Fig. 1 *A*) contained 561 bp of 5′ untranslated region, including its promoter elements, followed by the six exons and their corresponding introns. The 3′ untranslated sequence extended 550 bp after the stop codon.

A second construct was created by inserting a 2,737-bp ApaI/XhoI mouse albumin enhancer-promoter segment (obtained from plasmid PALBSVPA, kindly provided by E. Schmidt, Massachusetts General Hospital, Boston MA) before the start of transcription (Fig. 1 *B*). The natural promoter region on the LCAT gene was removed by SphI digestion followed by ApaI partial enzyme digestion. Cohesive ends were blunted with T_4 DNA polymerase (New England Biolabs, Beverly, MA) and ligated together with T_4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, IN). A XhoI/KpnI 1,407-bp promoterless segment was cloned, replacing the original fragment. This construct was named pGEMAlbLCAT. Plasmids pUCLCATBamNsi and pGEMAlbLCAT were transfected in DH5α *Escherichia coli* cells and grown until stationary phase in terrific broth glucose medium supplemented with ampicillin.

Plasmid DNA was purified by alkaline hydrolysis (14) followed by two rounds of ultracentrifugation. Genomic DNA fragments were obtained by BamHI/NsiI or AatII/SspI digestion of clones pUCLCATBamNsi and pGEMAlbLCAT, respectively (Fig. 1), separated from vector sequences by agarose gel electrophoresis, extracted from gels using the Geneclean kit (Bio 101, La Jolla, CA), and dialyzed in injection buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

Creation of transgenic mice. Methods utilized in the creation of transgenic mice have been previously described (15). Fertilized embryos used for the microinjection were derived from matings of inbred FVB mice (Charles River Laboratories, Wilmington, MA). The Hu apo AI and Hu apo AII transgenic mice used in these studies have previously been described (16, 17). These transgenic lines were created and maintained in the C57BL/6 background. In all the studies involving combinations of Hu apo AI, Hu apo AII, and LCAT transgenes, the genetic background of each of the transgenic and control mice was (FVB × C57BL/6) F1 hybrids.

Preparation and analysis of DNA and RNA by Southern and Northern blot. Tail tip DNA from 3-wk-old mice was screened for integration of Hu LCAT gene sequences by PCR. Primers were directed to synthesize the exon 6 of the Hu LCAT gene. Amplification reaction using nontransgenic mouse DNA as a template yielded no amplification products. In some experiments, integration of the full-length genomic construct was detected by Southern blot hybridization. 10 μg genomic DNA was digested overnight with 5 U PstI/μg DNA, electrophoresed in a 1% agarose gel, and transferred to a nylon membrane (Sigma Chemical Co., S. Louis, MO). DNA was cross-linked to the membranes (Stratagene, La Jolla, CA) and hybridized with a ^{32}P-radiolabeled full-length Hu LCAT cDNA as a probe.

RNA was isolated from transgenic and control mouse tissues (ovaries, brain, muscle, small intestine, kidney, heart, liver, spleen, and lung). Tissue samples were homogenized in RNAzol®B (Cinna/Biotecx, Friendswood, TX), extracted, and precipitated with isopropanol. RNA integrity was assessed by agarose gel electrophoresis. To determine tissue expression, 20 μg total RNA/tissue was separated according to size by electrophoresis through a denaturing agarose gel containing formaldehyde. RNA was subsequently transferred to nylon membranes and hybridized with either a full-length ^{32}P-labeled Hu LCAT cDNA or an exon 6 murine LCAT DNA probe using the rapid hybridization system (Amersham Corp. Arlington Heights, IL) according to the manufacturer's conditions. No cross-reactivity was detected between the Hu LCAT cDNA probe and the mouse LCAT mRNA.

Determination of LCAT activity. After the control and transgenic mice were fasted overnight, blood was collected into tubes containing anticoagulant (2 mM EDTA, 50 μg/ml gentamycin sulfate, 0.05% sodium azide). LCAT activity was measured as the rate of synthesis of ^{3}H cholesteryl esters from unilamellar vesicles prepared with French pressure cell (18) and activated with Hu apo AI (Sigma Chemical Co) to form discoidal synthetic lipoproteins (19). 1,2 [^{3}H]cholesterol (New England Nuclear, Boston, MA) was purified by TLC on silica gel plates developed in cyclohexane/ethylacetate (60:40, vol/vol). Liposomes contained egg lecithin (800 μg/ml, Sigma Chemical Co.), unesterified cholesterol (100 μg/ml, specific activity 5×10^5 cpm/μg) and Hu apo AI (100 μg/ml). Each assay mixture contained 50 μl of the dispersed lipid, an equal volume of recrystallized Hu albumin (15%, wt/vol) in buffer containing 60 mM phosphate at pH 7.4, 198 μl 150 mM NaCl, and 2 μl mouse plasma in a total assay volume of 300 μl. LCAT activities were determined over 20 min at 37°C. The reaction was stopped by adding 900 μl chloroform/methanol/H_2O (4:4:1, vol/vol/vol), and lipid extracted. After separation of the phases, the content of [^{3}H]cholesteryl ester radioactivity in the organic phase was determined by TLC on silica gel plates developed in hexane/diethyl ether/acetic acid (83:16:1, vol/vol/vol). Cholesteryl ester radioactivity (Rf 0.9–1.0 in this system) was determined by liquid scintillation spectrometry. LCAT activity was linear up to 30 min of incubation at 37°C and independent of the concentration of plasma lipoproteins in all transgenic lines used in this study. Therefore, an increase in the esterification rate corresponds to an increased level of LCAT protein.

LCAT-mediated lipoprotein cholesterol esterification. Blood was drawn from control and transgenic mice after an overnight fast in tubes containing 2 mM EDTA, 50 μg/ml gentamycin sulfate, and 0.05% sodium azide. Plasma was obtained by centrifugation at 3,000 rpm for 15 min at 4°C. LCAT activity was immediately inhibited by adding 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to a final concentration of 1.5 mM (20). Cholesterol esterification in total plasma was determined by the method described by Stokke and Norum (21). Briefly, the incubation mixture containing 36 μl DTNB-treated plasma and 9 μl albumin-stabilized emulsion of 1,2 [^{3}H]cholesterol ($7–10 \times 10^3$ cpm/μl) was incubated at 37°C for 4 h to allow tracer equilibration with endogenous lipoprotein cholesterol. The enzyme was reactivated by adding β-mercaptoethanol to a final concentration of 10 mM. Synthesis

of lipoprotein-derived cholesteryl ester was monitored for 30 min at 37°C. The rate of cholesterol esterification is linear up to 60 min. ^{3}H-labeled cholesteryl esters were determined by TLC as described earlier. In some experiments, the distribution of [^{3}H]cholesteryl esters within the major lipoprotein classes was determined by agarose gel electrophoresis as previously described (2). The migration of apo B- and apo AI-containing lipoproteins was visualized with Sudan black. The lipoprotein areas were cut out, the agarose was digested with β-Agarase I (New England Biolabs) and the lipids extracted with chloroform/methanol (1:1, vol/vol). [^{3}H]cholesteryl esters were determined by TLC.

Determination of plasma lipid and lipoprotein analysis. Mouse HDL was separated from apo B-containing lipoproteins by dextran sulfate precipitation as described elsewhere (22, 23). No significant amount of apo AI was precipitated in the apo B fraction as determined by solid phase immunoassay.

To determine lipid composition and Hu apo AI concentrations in HDL, 200 μl pooled plasma from nontransgenic FVB control, Hu LCAT, HuAI/LCAT transgenic mice was applied to two Superose 6 columns in a series by fast protein liquid chromatography (FPLC), (Pharmacia Fine Chemicals Piscataway, NJ). Tubes containing the HDL lipoprotein fractions were pooled and concentrated in Macrosep centrifugal concentrators (Filtron Technology Corp. Northborough, MA). Hu and mouse apo AI in each sample were measured by standardized ELISA (24) using purified capture antibodies and biotinylated detection antibodies (International Immunology Corp., Murrieta, CA) in a noncompetitive sandwich-style immunoassay.

Lipid determinations. Plasma and lipoprotein total cholesterol levels were determined enzymatically with an autoanalyzer (Roche Cobas Mira). Total and free cholesterol levels were measured with cholesterol oxidase (25) in the presence and absence of cholesterol esterase, respectively. The plasma cholesteryl ester level is calculated as the difference between total and free cholesterol. Phospholipid concentrations were determined by the method described by Barlett (26). Triglyceride concentrations were assayed using a diagnostic kit (44119; Roche Biomedical Laboratories, Burlington, NC). Values were corrected for the glycerol content.

Size fractionation of HDL. Total plasma lipoproteins ($\delta \leq 1.21$ g/ml) were isolated by ultracentrifugation of 50 μl fasted mouse plasma. Plasma was spun in a 42.2 rotor for 24 h at 40,000 rpm at 10°C. Total lipoproteins were collected and sieved in a 4-30% nondenaturing gradient gel electrophoresis (GGE) until equilibrium. HDL fractions were visualized by Coomassie blue staining of proteins, and the particle size distribution was determined by computer-assisted scanning densitometry as described (27). The particle size intervals for the five major HDL subpopulations by GGE are HDL_{2b}, 12.9-9.7 nm; HDL_{2a}, 9.7-8.8 nm; HDL_{3a}, 8.8-8.2 nm; HDL_{3b}, 8.2-7.8 nm; and HDL_{3c}, 7.8-7.2 nm.

Quantification of HDL subspecies. HDL species with pre-β-mobility are lost during ultracentrifugation from the $\delta \leq 1.21$ g/ml lipoprotein fraction (28). Therefore, to determine the proportion of preβ- and α-migrating HDL species in the different groups of transgenic mice, 15 μl native plasma was electrophoresed in 0.75% (wt/vol) agarose gel as previously described (2) and transferred to two nitrocellulose membranes (to avoid loss of apo AI migrating through the first membrane). To identify the Hu apo AI containing HDL species, nitrocellulose membranes were incubated for 2 h at room temperature with 2% milk in 10 mM phosphate buffer at pH 7.4, and then with a protein G-purified ^{125}I-labeled rabbit anti-Hu apo AI antibody in 1% milk (1.5×10^6 cpm/ml), 10 mM phosphate buffer for 90 min. Unbound antibody was washed four times with 1% milk in phosphate buffer, and nitrocellulose membranes were exposed overnight to Kodak XAR-2 film (Eastman Kodak Rochestar, NY) at -70°C. No cross-reactivity was observed between this antibody and the murine apo AI HDL species in the nitrocellulose membrane, and the areas were cut out and counted in a gamma-spectrophotometer.

Statistical analysis. The results are expressed as means±SE. The statistical significance of the differences between the groups was estimated by the Student's t test. A t value greater than $t_{0.05}$ ($P > 0.05$) was considered significant.

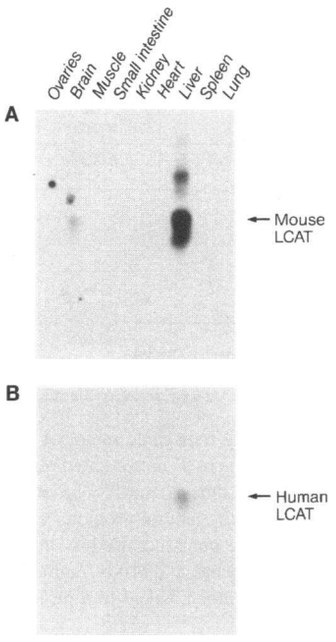

Figure 2. Northern blot analysis of *(A)* nontransgenic control and *(B)* HuLCAT transgenic mice. Total RNA was isolated from nontransgenic control mice and HuLCAT mice containing the natural promoter sequence as described in Methods. 20 μg of total RNA were electrophoresed in agarose gels, transferred to a nylon membrane, and then hybridized with *(A)* a ^{32}P-labeled exon 6 murine LCAT or *(B)* a full-length Hu LCAT cDNA as probe.

Results

Production of Hu LCAT transgenic mice and RNA expression. Two DNA fragments containing either the natural promoter element (Fig. 1 *A*) or the murine albumin enhancer-promoter linked to the LCAT gene (Fig. 1 *B*) were microinjected into fertilized eggs from FVB mice. Four transgenic founder animals containing construct A and three containing construct B were derived. HuLCAT14 and HuLCAT31 transgenic mice (derived from construct A) and Hu(a)LCAT22 and Hu(a)LCAT57 transgenic mice (derived from construct B) were propagated and chosen for further analysis.

The tissue-specific expression of LCAT was analyzed by Northern blot hybridization with species-specific probes. Total RNA isolated from several tissues of control (Fig. 2 *A*) and transgenic mice containing the natural promoter elements (Fig. 2 *B*) was hybridized to the LCAT probes. The murine LCAT mRNA was abundant in the liver, while only small amounts were detected in the brain. Transgenic mice expressed the Hu transgene exclusively in the liver. No signal was detected in the brain. Previous transgenic studies that used exactly the same murine enhancer-promoter used in these studies have shown that sequences linked to this promoter express exclusively in the liver of transgenic animals (29).

LCAT activity. Plasma LCAT activities of transgenic mice containing either the natural or the albumin enhancer-promoter increased by 1.2- to 1.6-fold over the nontransgenic control (Table I). The greatest LCAT activity was observed in the HuAI/LCAT and HuAI/AII/LCAT mice, whereas the lowest levels were obtained in HuAI, HuAI/AII, and HuAI, HuAI/AII, and HuAII/LCAT transgenic mice. The simultaneous presence of Hu apo AI and Hu LCAT was associated with the highest LCAT activities, whereas Hu apo AII and Hu LCAT

Table I. Lipid Profile of Plasma and Lipoprotein Fractions in Nontransgenic Controls, HuLCAT, HuAI, HuAI/LCAT, HuAI/AII, HuAI/AII/LCAT, HuAII, and HuAII/LCAT Transgenic Mice

Mice	LCAT activity (nmol/ml/h)	Plasma		(VLDL + LDL)		HDL	
		TC	CE	TC	CE	TC	CE
Nontransgenic controls	23.0±1.1	166.1±6.0	118.4±4.4	81.4±3.7	53.5±3.0	84.7±4.5	64.9±3.0
HuLCAT14	25.1±2.1	219.7±12.1	149.8±6.2	101.8±6.8	58.7±4.3	117.9±8.1	91.2±4.3
HuLCAT31	30.1±3.5	219.0±14.2	157.3±7.1	94.6±7.0	61.8±2.9	124.4±8.3	95.5±3.2
Hu(a)LCAT22	30.4±2.6	199.6±6.8	169.9±10.2	77.9±8.2	57.3±4.9	121.7±7.5	109.6±8.8
Hu(a)LCAT57	37.0±2.8	240.8±12.0	191.5±5.8	88.2±4.9	66.1±6.0	152.6±7.2	125.4±6.8
HuAI	25.6±1.3	154.8±6.2	112.5±5.2	55.7±3.8	33.3±2.7	99.1±2.7	77.4±2.1
HuAI/LCAT31	36.7±2.7	393.0±10.0	256.8±9.4	146.0±6.0	64.3±5.0	247.0±4.7	192.4±5.2
HuAI/(a)LCAT22	72.6±3.8	656.0±14.8	459.7±18.3	367.6±19.2	214.3±10.3	288.4±15.0	245.4±16.4
HuAI/AII	24.2±2.0	120.0±9.8	90.6±8.1	45.4±3.5	27.6±1.5	74.7±10.3	63.0±7.0
HuAI/AII/LCAT31	58.6±1.2	248.5±7.3	201.6±12.9	61.7±4.8	43.2±2.1	186.8±9.0	153.3±3.9
HuAII	21.7±1.2	66.5±5.0	57.2±5.8	20.3±4.7	14.4±3.3	46.2±9.7	42.8±9.0
HuAII/LCAT31	22.8±3.1	74.0±4.8	63.4±3.0	35.0±3.1	27.1±2.8	39.0±3.6	36.3±2.7

Values shown are mean±SE (n = 4–6 mice per group). All lipid values are expressed in milligrams per deciliter of plasma. TC, total cholesterol; CE, cholesteryl esters.

had levels similar to the HuLCAT mice, indicating the failure of Hu apo AII to preferentially associate with Hu LCAT or to sustain LCAT reaction.

The distribution of the LCAT activity among plasma lipoproteins was assessed by measuring the enzyme activity in plasma before and after precipitation of apo B–containing lipoproteins with dextran sulfate and $MgCl_2$. In agreement with previous data reported in humans (2, 30), essentially all the LCAT activity (94.8±7.6%; n = 5) was associated with the HDL lipoprotein fraction in nontransgenic control and HuLCAT transgenic mice. The high homology in the primary amino acid sequence between species (95%) (31) did not allow us to obtain a specific antibody that will recognize and distinguish between mice and Hu LCAT. Thus, HuLCAT mass, and the effect of Hu LCAT on murine LCAT in the HuLCAT transgenic mice, was not determined.

Plasma lipids and lipoprotein analysis. The increase in plasma LCAT activities observed in HuLCAT transgenic mice (Table I) is associated with a 20–60% increase in plasma cholesterol and cholesteryl ester levels compared with nontransgenic controls. The increase in the cholesteryl esters was found almost exclusively in the HDL fraction. The ratio of cholesteryl esters to total cholesterol in HDL was very similar between the control mice and the different HuLCAT transgenic lines (ranging between 0.77 and 0.88 in the different lines). These data indicate that changes in the level of cholesteryl esters observed in HuLCAT transgenic mice are accompanied by similar increments in HDL-free cholesterol levels. To determine whether the increase in HDL-cholesterol is accompanied by a parallel increase in apo AI concentration, and thus an increase in the HDL number, murine apo AI levels were determined in pooled plasma from nontransgenic controls and HuLCAT transgenic mice containing either the natural promoter (HuLCAT14 and HuLCAT31) or the albumin enhance-promoter sequences (Hu(a)LCAT22 and Hu(a)LCAT57). The apo AI concentration was 1.95 mg/dl in nontransgenic controls, 2.10 mg/ml and 2.13 mg/ml in HuLCAT14 + Hu LCAT31 and Hu(a)LCAT22 + Hu(a)LCAT57, respectively, suggesting that increasing amounts of plasma Hu LCAT do not associate with significant increases in plasma apo AI concentration.

Effect of Hu apo AI and Hu apo AII in plasma and lipoprotein cholesterol levels in HuLCAT transgenic mice. Hu HDL is very heterogeneous and differs from murine HDL in both particle size distribution and protein composition. Thus, the in vivo substrate specificity and the impact of Hu apo AI and Hu apo AII (the two major proteins of Hu HDL) on Hu LCAT were assessed in transgenic mice expressing HuAI/LCAT, HuAI/AII/LCAT, and HuAII/LCAT.

The cholesterol levels in plasma of HuAI/LCAT31 and HuAI/(a)LCAT22 mice increased by 1.8- and 3.3-fold, respectively, over those found in HuLCAT31 and Hu(a)LCAT22 (Table I). These changes were associated with increases in both cholesteryl esters and plasma free cholesterol, whereas the ratio of cholesteryl ester to total cholesterol was unaffected. Non-HDL cholesterol also increased in HuAI/LCAT transgenic mice. HuAI/LCAT31 transgenic mice had a 1.5-fold increase in VLDL + LDL-cholesterol compared with the HuLCAT31 transgenic mice. HuAI/(a)LCAT22 transgenic mice, despite doubling the LCAT activity, did not substantially increase the HDL-cholesterol compared with HuAI/LCAT31, but had a 4.7-fold increase in VLDL + LDL-cholesterol compared with the Hu(a)LCAT22 transgenic line. To determine whether the increase in VLDL + LDL-cholesterol is the consequence of an increased transfer of newly synthesized cholesteryl ester to apo B–containing lipoproteins, plasma containing tracer amounts of ^{3}H-free cholesterol from HuAI and HuAI/LCAT transgenic mice was incubated for 30 min at 37°C. The distribution of ^{3}H-labeled cholesteryl ester within plasma lipoproteins was determined by agarose gel electrophoresis. Only 6.1±2.0% (n = 4) and 9.8±1.5% (n = 4) of the labeled cholesteryl esters was detected in the apo B–containing lipoproteins in HuAI and HuAI/LCAT transgenic mice, respectively, indicating that a small proportion of cholesterol esterified by LCAT is present in VLDL + LDL in both groups of transgenic mice.

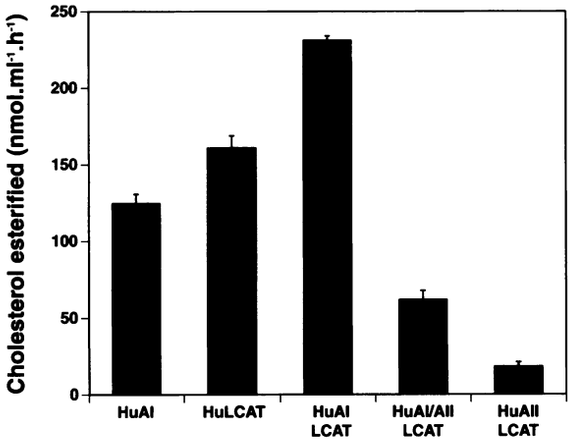

Figure 3. LCAT-mediated esterification of lipoprotein-derived cholesterol. Blood was obtained from control and transgenic mice after an overnight fast. Plasma was obtained by centrifugation, and the LCAT activity was immediately inhibited with DTNB. Lipoprotein cholesterol was labeled with an albumin-stabilized emulsion of 1,2-[^{3}H] cholesterol as described in Methods. LCAT activity was reactivated by adding β-mercaptoethanol, and the synthesis of ^{3}H-lipoprotein-derived cholesteryl ester was monitored for 30 min at 37°C. Lipids were extracted, and the content of ^{3}H-labeled cholesteryl ester in the organic phase was determined by TLC as described in Methods.

Compared with HuLCAT31 mice, the HuAI/AII/LCAT31 transgenic line had a more moderate increase in plasma and HDL-free and esterified cholesterol levels than that observed in the HuAI/LCAT31 line. HuAII/LCAT31 transgenic mice showed no increase in the cholesterol content of plasma and lipoprotein cholesterol compared with HuLCAT transgenic mice. The functionality of the plasma lipoproteins as substrate for LCAT was examined in the various combinations of transgenes (Fig. 3). The simultaneous presence of Hu apo AI and Hu LCAT was associated with the highest LCAT-mediated esterification of lipoprotein-derived cholesterol, whereas the presence of Hu apo AII does not increase but rather decreases the synthesis of cholesteryl esters when compared with HuAI or HuLCAT transgenic mice. Taken together these results suggest that the presence of Hu apo AI is a major factor determining the in vivo substrate specificity of Hu LCAT, whereas Hu apo AII does not have a positive impact on cholesteryl ester formation by Hu LCAT.

HDL particle characterization. HDL was isolated from nontransgenic FVB controls, HuLCAT, HuAI, and HuAI/LCAT mice to determine lipid and Hu apo AI concentrations. Whole plasma was used instead of total lipoproteins ($\delta \leq 1.21$ g/ml) to avoid losses of apo AI because of ultracentrifugation (28, 32). HDL isolated by FPLC copurifies with plasma proteins, and therefore its protein concentration cannot be determined. Hu apo AI levels were thus used to monitor changes in particle size. The HDLs from HuLCAT and HuAI/LCAT transgenic mice (Table II) show an increase in free and esterified cholesterol compared with controls. However, the identical ratio of free cholesterol to cholesteryl ester in both groups of animals and the similar apo AI concentration observed in HuAI and HuAI/LCAT transgenic mice suggest Hu LCAT overexpression led to an increase in the HDL size rather than in the number of particles.

HDL particle size distribution. HDL, isolated by ultracentrifugation ($\delta \leq 1.21$ g/ml) from the plasma of nontransgenic FVB mice, consisted of a monodisperse population of particles with a peak diameter of 10.6 nm as determined by GGE. The HuLCAT transgenic mice had a similar size distribution of HDL particles (data not shown). As previously reported (16), HDL isolated from HuAI transgenic mice consisted of a bimodal distribution of particles with peak sizes of 8.9 and 11 nm, corresponding to Hu HDL_{2a} and HDL_{2b} (Fig. 4 *A*). A third but smaller population at 7.8 nm was also observed. Major changes were observed in particle sizes of HDL isolated from the plasma of HuAI/LCAT and HuAI/AII/LCAT transgenic mice. HDL isolated from HuAI/LCAT31 transgenic mice (low expressor) showed predominant peaks (Fig. 4 *B*) of large particles (12.6, 11.2, and 10.2 nm) located within the HDL_{2b} interval. HDL particle distribution from HuAI/(a)LCAT22 transgenic mice (high expressor) (Fig. 4 *B*) consisted of multiple peaks ranging in size from 14.6 to 7.4 nm. Interestingly, the less prominent but distinct peaks of smaller particles (8.2 and 7.4 nm) in the HDL_{3a} and HDL_{3c} intervals only occurred in high LCAT expressors, suggesting a dose effect.

In agreement with the observations of Schultz et al. (17), the HDL isolated from HuAI/AII transgenic mice (Fig. 4 *C*) consisted of two major overlapping populations of particles within the HDL_{3a}-to-HDL_{3b} size intervals. A minor population of particles that appear as a shoulder on the predominant peak with a 10.5-nm diameter was also observed. Expression of Hu LCAT together with Hu apo AI and apo AII results in a marked redistribution of the particle sizes (Fig. 4 *D*). The majority of the HDL particles were distributed into a larger population of particles with the peak particle size in the HDL_{2b} interval, which corresponds to a minor population observed in HuAI/AII animals. HDL from transgenic mice expressing Hu apo AII (Fig. 4 *E*) shows a bimodal distribution, with a predominant peak (9.3 nm) in the HDL_{2a} size interval and a smaller peak at 7.7 nm (HDL_{3c}). When Hu LCAT was overexpressed in the HuAII transgenic mice, there was a slight increase in particle size of the major peak (9.7 nm), but no change in size of the smaller HDL_{3c} peak (7.7 nm). Thus, the expression of Hu LCAT did not significantly alter the size distribution of HDL isolated from HuAII transgenic mice, although the peak particle size of the major HDL subpopulation (HDL_{2a}) was greater in the HuAII/LCAT mice.

Quantification of preβ-migrating HDL levels. Native plasma from human, HuAI, and HuAI/AII transgenic and nontransgenic control mice was fractionated by agarose gel electrophoresis and immunoreacted with a ^{125}I-labeled rabbit antibody to Hu apo AI (Fig. 5). Murine apo AI showed no cross-reaction with an antibody against Hu apo AI. HDL isolated from HuAI and HuAI/AII transgenic mice contained 18.4±3.7% and 7.8±1.2%, respectively, of the total Hu apo AI radioactivity migrating in the position corresponding to the Hu preβ-migrating HDL species (Table III). The balance of the apo AI immunoreactivity was found in the α-migrating HDL. Expression of the Hu LCAT transgene alone did not affect the migration of either preβ- or the α-migrating HDL species. In HuAI/LCAT and HuAI/AII/LCAT transgenic mice, the preβ-migrating HDL fraction decreased to 6.0±1.6% and 5.7%, representing 67 and 27% reductions in the proportion of apo AI in the preβ-migrating HDL fraction, respectively ($P < 0.05$). The total apo AI radioactivity was not significantly different between HuAI/

Table II. HDL Composition in HuLCAT, HuAI, and HuAI/LCAT Transgenic and Nontransgenic Control Mice

	TG (mg/dl)	PL (mg/dl)	FC (mg/dl)	CE (mg/dl)	FC/CE	ApoAI (mg/dl)	CE/ApoAI
Nontransgenic controls	1.9 (13%)	8.1 (56%)	0.8 (6%)	3.6 (25%)	0.2	—	—
HuLCAT	0.7 (5%)	7.4 (56%)	0.9 (7%)	4.2 (32%)	0.2	—	—
HuAI	1.0 (5%)	14.6 (66%)	1.9 (9%)	4.7 (20%)	0.4	0.2	24.0
HuAI/LCAT	1.3 (5%)	13.7 (49%)	3.7 (13%)	9.4 (33%)	0.4	0.2	47.0

200 μl of pooled plasma from nontransgenic control, HuLCAT, HuAI, and HuAI/LCAT mice was used to isolate HDL by FPLC as described in Methods. Tubes containing the HDL lipoprotein fractions were pooled and concentrated. Lipid and Hu apo AI measurements were determined as described in Methods. TG, triglycerides; PL, phospholipids; FC, free cholesterol; CE, cholesteryl ester; %, percentage composition relative to TG, PL, FC, and CE sum.

LCAT and HuAI, HuAI/AII/LCAT, and HuAI/AII transgenic mice.

Furthermore, the plasma apo AI concentration was not different between HuAI/LCAT and HuAI transgenic mice (3.14 and 3.04 mg/ml, respectively), indicating that the lower proportion of preβ-migrating HDL represents a decrease in the plasma concentration of preβ-HDL particles because of a redistribution of apo AI within the HDL fractions.

Discussion

The creation of HuLCAT transgenic mice, as described in this study, provides a model to study the regulation of this gene and its effect on HDL composition and structure. In humans (13), mice (31), and monkeys (33), LCAT gene expression has been primarily detected in the liver and, to a lesser extent, in the testis and the brain. The LCAT genomic construct used in this study expressed exclusively in the liver and suggests that the sequence-specific elements required for liver expression are present in the 561 bp of the 5^1 untranslated region.

Despite the increase in HDL-cholesterol levels observed in HuLCAT transgenic mice, the HDL remained a monodisperse population of particles without a significant change in size or apo AI concentration. This suggests that murine HDL is able to accommodate some increases in free and esterified cholesterol without dramatic size changes. The dramatic changes in HDL size observed in mice with both Hu apo AI and Hu LCAT suggests that failure to observe significant HDL size changes in the HuLCAT mice results from either structural constraints of murine HDL to accept a significantly higher amount of cholesteryl ester or a poor ability of Hu LCAT to associate with and/or be activated by murine HDL. The latter is probably consistent with the higher plasma LCAT levels observed in HuAI/LCAT and HuAI/AII/LCAT transgenic animals compared with the HuLCAT transgenic animals. Alternatively, the different levels of plasma LCAT activity observed in the various combinations of transgenes could reflect the differences in LCAT removal. This agrees with the proposed divergent metabolic pathways of LpAI and LpAI,AII HDL subclasses (34).

The most potent physiologic activator of LCAT is apo AI (4). Several hypotheses have been formulated concerning the mechanism(s) by which this protein cofactor activates LCAT. The specific binding between lipoprotein lipase and its activator apo CII (35) and the tridimensional structure of pancreatic lipase-colipase complex (36) has suggested that the role of these protein cofactors is to assist the association of the enzyme with the substrate interface. LCAT does not require apoproteins to bind to lipid interfaces (37); however, on a pure lipid surface the serine residue in the LCAT active site (38) may not be accessible to the lipid substrates, but in the presence of apo AI, the access of substrates to the active site may be facilitated by either a direct interaction of the enzyme with apo AI or by an activation of the lipid substrates through the protein cofactor. The amino acid sequence of Hu and murine apo AI differs by 32% (39) and has profound effects on HDL particle sizes, which may account for the differences seen in the HuLCAT transgenic mice with and without Hu apo AI. Hu CETP has been shown not to bind to murine HDL particles but to interact strongly with HDL particles containing Hu apo AI in the plasma of transgenic mice (23). This suggests a species-specific HDL structural requirement may be necessary for activation or binding of both Hu CETP and Hu LCAT.

The increase in the HDL-cholesterol levels observed in HuAI/LCAT transgenic mice did not associate with an increase in the concentration of apo AI but rather with an increase in the HDL free and esterified cholesterol and with changes in the HDL size distribution. This observation suggests that the size transformation of HDL particles may occur more readily with Hu apo AI containing HDL by a pathway involving LCAT in a dose-dependent manner. The results of the present study and the evidence presented by others showing two different conformations adopted by Hu apo AI in reconstituted discoidal apo AI–containing particles (40, 41) suggest that the cholesteryl ester formed by LCAT can effect conformational changes in Hu apo AI resulting in a change in HDL particle size. A larger HDL will accommodate more core lipids because of the increase in particle volume and free cholesterol as a consequence of the increase in the surface area. The accumulation of cholesterol observed in the plasma of HuAI/LCAT and HuAI/AII/LCAT transgenic mice raises the possibility that the observed increments are solely a result of higher amounts of total apo AI present in HuAI/LCAT and HuAI/AII/LCAT transgenic mice compared with the HuLCAT mice and nontransgenic controls. This possibility seems unlikely because the concentration of Hu apo AI is only 40–50% higher in HuAI/LCAT transgenic mice compared with HuLCAT mice, and, unlike the "murine-like" HDL isolated from HuLCAT transgenic mice, the increase in HDL cholesterol in HuAI/LCAT and HuAI/AII/LCAT mice was associated with profound changes in HDL size. These results thus suggest that the differences observed between Hu

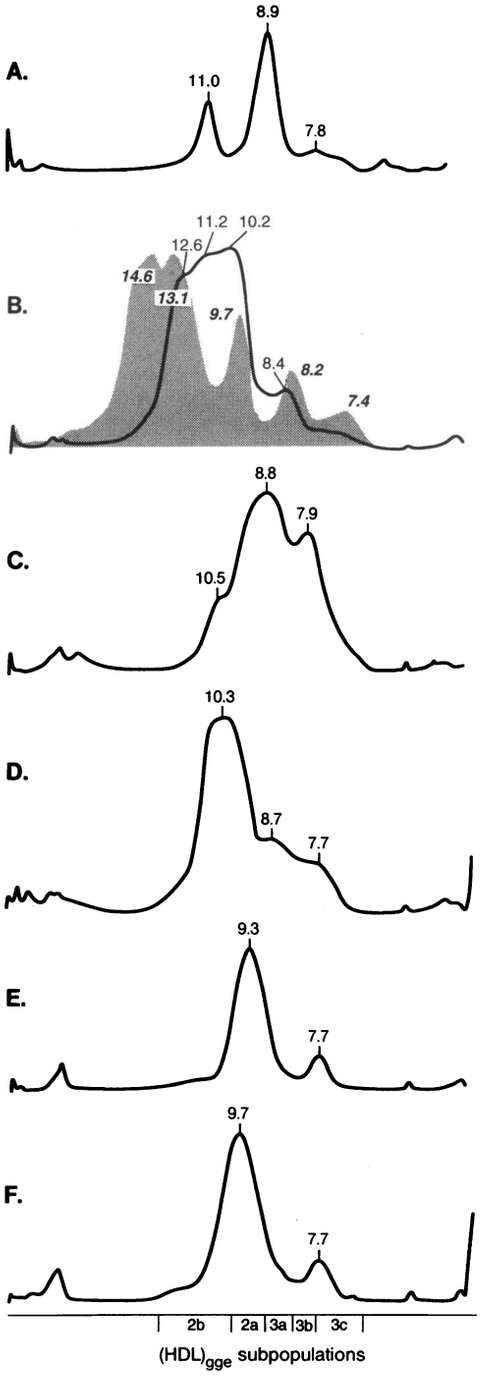

Figure 4. Densitometric scan profiles of lipoprotein fractions isolated from transgenic mice. Total plasma lipoproteins were isolated by ultracentrifugation, collected, and sieved in a 4–30% nondenaturing gradient gel electrophoresis until equilibrium. HDL particle size distribution was assessed by gradient gel electrophoresis as described in Methods. *(A)* HuAI; *(B)* HuAI/LCAT, low expressor *(solid line)*; HuAI/(a)LCAT, high expressor *(shaded area)*; *(C)* HuAI/AII; *(D)* HuAI/AII/LCAT; *(E)* HuAII; and *(F)* HuAII/LCAT. Peak particle sizes are shown in nanometers; peak particle sizes for the shaded area in *B* are shown in italics. Scale at bottom of *F* shows Hu HDL subpopulation size.

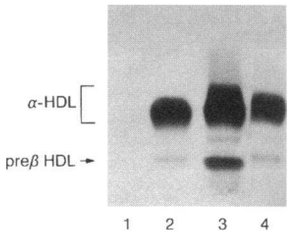

Figure 5. Agarose gel electrophoresis of native plasma. 15 μl plasma was used to separate preβ- and α-migrating HDL species by agarose gel electrophoresis as described in Methods. Hu apo AI was visualized using an ^{125}I-labeled rabbit polyclonal antibody to Hu apo AI. (Lane *1*) Nontransgenic control mouse plasma; (lane *2*) Hu plasma; (lane *3*) HuAI transgenic mouse plasma; and (lane *4*) HuAI/AII transgenic mouse plasma.

LCAT and HuAI/LCAT transgenic mice are due to a specific interaction between Hu LCAT and Hu apo AI or Hu apo AI containing HDL particles. Although in vitro experiments have shown similar activations of Hu LCAT by apo AI of different species (42, 43), the preference of Hu LCAT for HDL containing Hu (rather than mouse) apo AI has not been shown previously.

The effect of apo AII on HDL metabolism and LCAT activation remain unclear. Our results in vivo suggest that particles containing Hu apo AII alone or chimeras containing murine apo AI and Hu apo AII are a poorer substrate for the Hu LCAT than HDL containing Hu apo AI. HDL particles containing both Hu apo AI and apo AII do increase their cholesterol content followed by a transformation to larger particles, whereas HDL containing Hu apo AII, but lacking Hu apo AI, do not undergo a similar size transformation. This supports the importance of Hu apo AI in determining the degree of cholesteryl ester accumulation and subsequent enlargement of HDL by the activity of Hu LCAT. The lower levels of cholesteryl esters formed in HuAI/AII/LCAT compared with HuAI/LCAT mice are possibly the consequence of the proposed effect of apo AII in displacing the Hu apo AI and LCAT from the surface of HDL particles (44) and indirectly modulating LCAT activity.

Table III. Distribution of HuApoAI Among preβ- and α-migrating HDL

	% Total Hu Apo AI	
Transgenic mice line	preβ-HDL	α-HDL
HuAI	18.4±3.7	81.6±3.7
	(9)	(9)
HuAI/LCAT	6.0±1.6	94.0±1.6
	(7)	(7)
HuAI/AII	7.8±1.2	92.2±1.2
	(4)	(4)
HuAI/AII/LCAT	5.7	94.3

Lipoprotein fractions from HuAI, HuAI/AII, HuAI/LCAT, and HuAI/AII/LCAT transgenic mice were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes as described in Methods. Hu Apo AI was visualized with a ^{125}I-labeled rabbit antibody to Hu Apo AI. Autoradiographs have been used as a template to identify the pre-β- and α-migrating HDL species in the nitrocellulose membranes. Areas containing pre-β- and α-migrating HDL were cut out and counted in a gamma-spectrophotometer. Values are mean±SE from the number of mice indicated in parentheses.

The increase in LCAT activity in HuAI/LCAT transgenic mice is associated with an increase in the cholesterol content of VLDL + LDL lipoproteins. This result was unexpected because of the absence of CETP activity in mouse plasma (8). We ruled out the possibility that this increment was the consequence of a transfer of cholesterol from HDL to the apo B-containing particles in HuAI/LCAT transgenic mice. Furthermore, no LCAT activity was associated with the VLDL + LDL lipoprotein fraction. However, it is possible that a fraction of LCAT associates with the VLDL + LDL fraction in HuAI/LCAT transgenic mice and synthesizes cholesteryl esters as proposed in Fish-eye disease (45). Alternatively, the increase in VLDL + LDL cholesterol could be explained by an increase in liver synthesis of cholesterol and subsequent secretion as part of the VLDL.

Of the variety of functions attributed to HDL, perhaps the most clinically relevant are those associated with reverse cholesterol transport. Preβ-migrating HDL are the initial acceptor of cell-derived cholesterol (pre-β_1) (46), but also part of the later esterification and transfer of cell-derived cholesterol (pre-β_2 and pre-β_3) to α-migrating HDL (2). As shown in this study, the expression of Hu apo AI in mice resulted in two major populations of HDL particles with identical electrophoretic mobility, as preβ- and α-HDL migrating species present in Hu plasma. The decrease in the proportion of preβ-migrating HDL observed in HuAI/LCAT and HuAI/AII/LCAT transgenic mice confirms the suggested relationship between preβ-HDL levels and LCAT (47–49). Incubation of plasma at 37°C in the presence or absence of LCAT inhibitors strongly suggests the conversion of preβ-migrating HDL into α-migrating HDL without a net loss of apo AI. Although our measurements probably reflect a steady state rather than a dynamic conversion determined by kinetics, it seems reasonable to assume the decrease in preβ-migrating HDL levels is the consequence of the transformation of preβ-HDL apo AI to α-HDL by the action of LCAT.

The lack of CETP activity in mouse plasma may account not only for the accumulation of large HDL subpopulations but also for the decrease in the level of preβ-migrating HDL observed in HuAI/LCAT and HuAI/AII/LCAT transgenic mice. In this study, we have shown that increasing LCAT activity is associated with a transformation of preβ-HDL and an increase in HDL-cholesterol levels. Further studies, including kinetic and atherosclerosis assays, need to be performed on the various lines of transgenic mice described in the present report to establish a direct link between cell-derived cholesterol transport, LCAT, and its effect on the prevention of atherosclerosis.

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