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The Effect of Natural LCAT Mutations on the Biogenesis of HDL

Panagiotis Fotakis,[†,‡] Jan Albert Kuivenhoven,[§] Eugene Dafnis,[∥] Dimitris Kardassis,[‡] and Vassilis I. Zannis*[,†]

[†]Molecular Genetics, Boston University School of Medicine, 700 Albany Street, W509, Boston, Massachusetts 02118-2394, United States

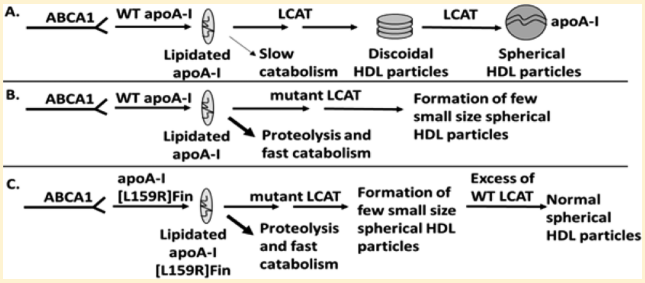
[‡]Department of Biochemistry, University of Crete Medical School and Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology of Hellas, GR-74100 Heraklion, Greece

[§]Department of Pediatrics, Section Molecular Genetics, Groningen, University of Groningen, University Medical Center Groningen, 9700 Groningen, The Netherlands

[∥]Department of Nephrology, University of Crete Medical School, GR-74100 Heraklion, Greece

S *Supporting Information*

ABSTRACT: We have investigated how the natural LCAT-[T147I] and LCAT[P274S] mutations affect the pathway of biogenesis of HDL. Gene transfer of WT LCAT in LCAT$^{-/-}$ mice increased 11.8-fold the plasma cholesterol, whereas the LCAT[T147I] and LCAT[P274S] mutants caused a 5.2- and 2.9-fold increase, respectively. The LCAT[P274S] and the WT LCAT caused a monophasic distribution of cholesterol in the HDL region, whereas the LCAT[T147I] caused a biphasic distribution of cholesterol in the LDL and HDL region. Fractionation of plasma showed that the expression of WT LCAT increased plasma apoE and apoA-IV levels and shifted the distribution of apoA-I to lower densities. The LCAT[T147I] and LCAT[P274S] mutants restored partially apoA-I in the HDL3 fraction and LCAT[T147I] increased apoE in the VLD/IDL/LDL fractions. The in vivo functionality of LCAT was further assessed based on is its ability to correct the aberrant HDL phenotype that was caused by the apoA-I[L159R]$_{FIN}$ mutation. Co-infection of apoA-I$^{-/-}$ mice with this apoA-I mutant and either of the two mutant LCAT forms restored only partially the HDL biogenesis defect that was caused by the apoA-I[L159R]$_{FIN}$ and generated a distinct aberrant HDL phenotype.



INTRODUCTION

LCAT (lecithin cholesterol acyltransferase) is a 414 amino acid long plasma enzyme that is reversibly bound to lipoproteins.[1] LCAT mRNA is found predominantly in the liver and to a much lesser extent in the brain and the testes.[2,3] LCAT plays an important role in maintaining plasma and HDL cholesterol homeostasis and has a pivotal role in the maturation of HDL.[4−6] LCAT bound to HDL is activated by apoA-I and catalyzes the transfer of the 2-acyl group of lecithin or phosphatidylcholine to the free hydroxyl residue of cholesterol to form cholesteryl ester.[7] It also catalyzes the reverse reaction of esterification of lysolecithin to lecithin.[8] The esterification of the HDL cholesterol contributes to the maturation of HDL and converts precursor preβ and discoidal HDL to spherical particles.[7] Although the preferred substrate of LCAT is free cholesterol on HDL, it also esterifies the cholesterol on LDL particles using apoE as an activator.[9] The esterification of free cholesterol on HDL by LCAT is known as α-LCAT activity, whereas the esterification of free cholesterol on LDL by LCAT is known as β-LCAT activity.[10]

Several LCAT mutations have been found in humans[10,11] that result in two clinical syndromes. The first is the familial LCAT deficiency (FLD), characterized by loss of both α- and β-LCAT activity. The clinical manifestation of FLD is corneal opacity, anemia, and sometimes proteinuria and renal disease.[10,12] FLD is characterized by very low LCAT mass or LCAT activity in the plasma, low HDL-C, apoA-I, apoA-II, and apoB levels, and in some cases increased triglycerides. The HDL particles generated in FLD are small, lipid-poor, disc-shaped, and have preβ and α4-HDL electrophoretic mobility.[13,14] The second clinical phenotype is the fish eye disease (FED) that is characterized by partial loss of α-LCAT activity but retention of β-LCAT activity.[10] FED is clinically a milder condition in which only corneal opacity is present and the cholesterol esterification rates are not altered due to the retention of β-LCAT activity of the enzyme. The plasma HDL cholesterol levels in FED are low, and the LCAT mass is partially reduced. Despite the atherogenic lipid/lipoprotein profile of the above two LCAT syndromes, their association with increased atherosclerosis and premature coronary artery disease is not unambiguously established.[15−19]

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The role of LCAT in the pathogenesis of atherosclerosis has been studied in different animal models and has generated inconclusive results. LCAT deficiency has been shown to either protect or enhance atherosclerosis in mice fed different atherogenic diets.[20,21] Furthermore, the human LCAT transgenic mice are not protected from diet-induced atherosclerosis or have even enhanced development of aortic lesions when LCAT is overexpressed despite the increased HDL cholesterol levels.[22,23] In contrast, human LCAT transgenic rabbits are protected from diet induced atherosclerosis.[24]

Several naturally occurring apoA-I mutations are associated with low HDL levels, and 17 of these mutants have reduced capacity to activate LCAT.[4,25] The majority of these mutations are clustered predominantly in or at the vicinity of residues of helix 6 (residues 145−164) of apoA-I and some of them predispose to atherosclerosis.[26−29]

In the present study, we investigated the in vitro and in vivo properties of two naturally occurring mutations, LCAT[P274S] (E. Dafnis and J. A. Kuivenhoven, unpublished data) and LCAT[C337Y]),[12] associated with FLD, and one mutation (LCAT[T147I]) associated with FED.[15] In vivo gene transfer studies in LCAT$^{-/-}$ mice of the LCAT[P274S] and LCAT T147I mutants that express the endogenous WT mouse apoA-I, reproduced the FLD, and FED phenotypes, respectively, observed in humans carrying these mutations.

The ability of the LCAT[P274S] and LCAT T147I mutants to restore the aberrant HDL phenotype caused by apoA-I mutations was tested by gene transfer experiments in apoA-I$^{-/-}$ mice of the human apoA-I[L159R]$_{FIN}$ alone or in the presence of either of the two mutant LCAT forms. These experiments tested the possibility that specific LCAT mutations may restore the interactions of the mutant apoA-I with a specific LCAT mutant and thus correct partially or totally the aberrant HDL phenotype associated with a specific apoA-I mutation. Although this scenario may be rare, we have been able previously to correct the efflux capacity of a mutant form of SR-BI using a mutant apoA-I form.[30] The studies showed that compared to WT LCAT that was shown previously to restore completely the aberrant HDL phenotype caused by specific apoA-I mutations,[5,31−33] coexpression of the apoA-I[L159R]$_{FIN}$ mutant with either LCAT[T147I] or LCAT[P274S] caused only partial restoration of the HDL biogenesis. Moreover, each LCAT mutant produced a distinct aberrant HDL phenotype.

MATERIALS AND METHODS

Materials. Materials not mentioned in the experimental procedures have been obtained from sources described previously.[30,34]

Origin of the LCAT Mutants. The LCAT[T147I] mutation has been reported in probands and members of the affected families with fish eye disease syndrome.[15] The clinical manifestations were very low HDL-C levels, corneal opacification and in some cases coronary heart disease in the homozygous carriers of the mutation.[15] The LCAT[C337Y] mutation was detected in a family of Moroccan origin with low HDL-C levels, total absence of LCAT activity in the plasma, corneal opacification, proteinuria, and nephropathy at a very early age.[12] The LCAT[P274S] mutation was detected in a subject from Greece suffering from renal disease (E. Dafnis and J. A. Kuivenhoven, unpublished data). The nomenclature of the mutations is based on the guidelines of the Human Genome Variation Society and includes the 24 aa leader sequence.

Recombinant Adenoviruses Construction. The human LCAT cDNA, containing the WT LCAT or the mutant forms ([T147I] or [P274S]), in the pENTR221 vector was generated in Dr. Jean Albert Kuivenhoven's laboratory. The LCAT cDNA was amplified using 5′-3′ primers that contained restriction sites for Bgl-II and EcoRV, respectively, and was subsequently digested with Bgl-II and EcoRV and cloned into the corresponding sites of the pAdTrack-CMV vector. The recombinant viruses were constructed using the Ad-Easy-1 system, where the recombinant adenovirus construct is generated in bacteria BJ-5183 (purchased from Stratagene).[35] The recombinant adenoviruses were packaged in 911 cells, amplified in human embryonic kidney 293 (HEK 293) cells, purified, and titrated as described.[32]

The recombinant adenoviruses expressing the apoA-I-[L159R]$_{FIN}$ mutation has been described previously.[32] The recombinant adenoviruses were packaged and titrated as described.[32]

Animal Studies, RNA Isolation, and apoE mRNA Quantitation. ApoA-I$^{-/-}$ (ApoA1^{tm1Unc}) C57BL/6J mice[36] were purchased from Jackson Laboratories (Bar Harbor, ME). Mice deficient for LCAT were a gift of Dr. Santa-Marina Fojo.[37] The mice were maintained on a 12 h light/dark cycle and standard rodent chow. All procedures performed on the mice were in accordance with National Institutes of Health guidelines and following a protocol (AN-14219.2012.10) approved by IACUC. ApoA-I$^{-/-}$ or LCAT$^{-/-}$ mice, 6−8 weeks of age, were injected via the tail vein with 8×10^8 to 1×10^9 pfu of recombinant adenovirus per anima,l and the animals were sacrificed 4 days postinjection following a 4 h fast. At least four mice were used for each set of experiments.

Secretion of WT and Mutant LCAT and apoA-I Forms. To assess the secretion of WT and mutant LCAT forms, SW1783 human astrocytoma (HTB-13) cells, grown to 80% confluence in Leibovitz's L-15 medium containing 2% heat-inactivated horse serum in 6-well plates, were infected with adenoviruses expressing WT and mutant LCAT forms at a multiplicity of infection of 5, 10 and 20. Twenty-four h postinfection, the cells were washed twice with PBS and incubated in serum-free medium for 2 h. Following an additional wash with PBS, fresh serum-free medium was added and 24 h later was collected along with the cell lysate and analyzed by SDS-PAGE and Western blotting to assess LCAT expression using a human antibody for LCAT (gift from Dr. J. S. Parks). The secretion of WT and mutant forms of apoA-I was assessed similarly and has been described previously.[32,33]

Plasma Lipids and apoA-I Levels, FPLC Fractionation, and Two-Dimensional Gel Electrophoresis. The concentration of total cholesterol, free cholesterol, and phospholipids of plasma drawn 4 days postinfection was determined using the Total Cholesterol E, Free Cholesterol C, and Phospholipids C reagents, respectively (Wako Chemicals USA, Inc., Richmond, VA). Triglycerides were determined using the Infinity triglycerides reagent (ThermoScientific, Waltham, MA), according to the manufacturer's instructions. Plasma apoA-I levels were determined by a turbidometric assay using AutoKit A-I (Wako Chemical USA, Inc., Richmond, VA).[31,32] For FPLC analysis, 20 μL of plasma obtained from mice were loaded onto a Sepharose 6 PC column (Amersham Biosciences, Piscataway, NJ) in a SMART micro FPLC system (Amersham Biosciences, Piscataway, NJ) and eluted with PBS. A total of 25 fractions of 50 μL volume each were collected for further analysis. The concentration of lipids in the FPLC fractions was determined as

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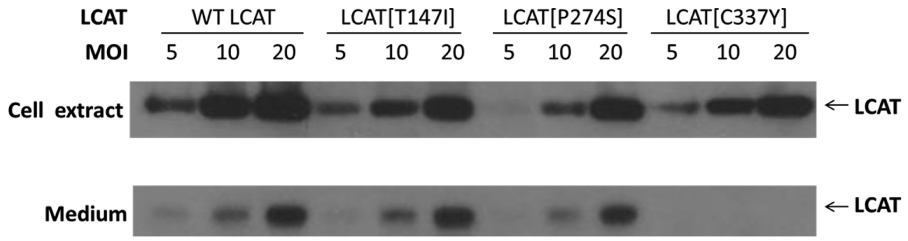

Figure 1. Expression of WT LCAT and the LCAT[T147I] and LCAT[P274S] mutants forms following infection of HTB-13 cells with the corresponding LCAT-expressing adenoviruses. SDS-PAGE and Western blot of 45 μg of total cell extract and 600 μL of culture medium (concentrated to 25 μL) from HTB-13 cells infected with adenoviruses expressing the WT and mutant LCAT forms at a MOI of 5, 10, and 20 as indicated.

described above. The plasma HDL subpopulations were separated by two-dimensional electrophoresis. The proteins were then transferred to a nitrocellulose membrane, and apoA-I was detected by immunoblotting, using a goat polyclonal antihuman apoA-I antibody AB740 (Chemicon International, Billerica, MA)[38] or a rabbit antimouse apoA-I antibody K23500R (Biodesign).

Fractionation of Plasma by Density Gradient Ultracentrifugation and Electron Microscopy (EM) Analysis of the apoA-I Containing Fractions. For this analysis, 240 μL of plasma obtained from adenovirus-infected mice was diluted with saline to a total volume of 0.5 mL and fractionated by density gradient ultracentrifugation. Following ultracentrifugation, 0.5 mL fractions were collected from the top of the tube, and analyzed by SDS-PAGE as described.[38] Fractions 6−7 obtained by the ultracentrifugation, that floated in the HDL region, were analyzed by electron microscopy using a Philips CM-120 electron microscope.

ApoA-I and LCAT mRNA Quantification. Total hepatic RNA was isolated by the Trizol method (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples were adjusted to 0.1 μg/μL, and cDNA was produced using the high capacity reverse transcriptase cDNA kit (Applied Biosystems, Foster City, CA). ApoA-I or LCAT mRNA was quantified using the Applied Biosystems 7300 Real-Time PCR instrument.[39] The Applied Biosystems Gene Array TaqMan primers for apoA-I cDNA (catalogue no. Hs00985000_g1) and LCAT cDNA (catalogue no. Hs00173415_m1) and the primers for 18s rRNA (catalogue no. 4319413E) along with the TaqMan Gene expression PCR Master Mix (catalogue no. 4370048), used for mRNA quantitation, were purchased from Applied Biosystems, Foster City, CA.

LCAT Assays. LCAT was purified as described[34] from the culture medium of human HTB13 cells infected with an adenovirus expressing the human LCAT cDNA.[40] For the α-LCAT assay, the reconstituted HDL (rHDL) particles used as the substrate contained cholesterol and [^{14}C] cholesterol, β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine (POPC), and apoA-I. rHDL was prepared by the sodium cholate dialysis method as described previously.[41] rHDL particles without ^{14}C-cholesterol containing mutant forms of apoA-I were prepared with the same procedure in order to measure their size by EM. The size of these particles was determined from the negatives of the EM images. The enzymatic reactions and the derivation of the apparent V_{max} and K_m were carried out as described previously.[5]

For the β-LCAT assay, the IDL/LDL was prepared and labeled with ^{3}H-cholesterol as described previously.[42,43] Briefly, the IDL/LDL lipoproteins were isolated from plasma by ultracentrifugation at densities between 1.007 and 1.063 g/mL and dialyzed against a dialysis buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM EDTA for 24 h at 4 °C with two exchanges every 8 h. The IDL/LDL was heat inactivated at 56 °C for 30 min. For the labeling with ^{3}H-cholesterol, IDL/LDL containing 18 nmol of free cholesterol was labeled with 0.15 μCi $^{[1,2-3H(N)]}$cholesterol (1 mCi/mL of specific activity 50 Ci/mmol; PerkinElmer Life Sciences, Inc.) for 20 h. The IDL/LDL was dialyzed against the aforementioned dialysis buffer for 24 h to remove the ^{3}H-cholesterol that was not incorporated in the lipoproteins. An aliquot of 4.5 μL of concentrated cell culture medium containing the WT LCAT or the mutant LCAT proteins were added to a mixture containing the labeled IDL/LDL, 5 mM β-mercaptoethanol, and 1.5% bovine serum albumin (essentially fatty acid free) to a final volume of 0.4 mL (in the dialysis buffer). The reaction mixture was incubated at 37 °C for 24 h, and the reaction was terminated by the addition of 2 mL of ethanol. The mixture was centrifuged at 2000 rpm for 10 min, and the supernatant containing the cholesterol and the cholesteryl ester was transferred to a new glass tube. The samples were dried under N_2 and resuspended in 75 μL of chloroform. The sample was spotted on ITLC paper, and chromatography was performed using a mixture of 25:15:1 (v/v/v) petroleum ether:ethyl ether:acetic acid as described previously.[5] The chromatograph was treated with iodine (Fisher, catalogue no. UN1759), and the spots corresponding to free cholesterol and cholesteryl esters were quantified by liquid scintillation. The results were expressed as the percentage of free cholesterol that was esterified and provides the rate of LCAT-catalyzed cholesteryl ester formation in the isolated IDL/LDL fractions.

Statistical Analysis. Data are presented as the mean ± standard deviation. Statistically significant differences between two groups of mice were determined using Student's t test with unequal variance.

RESULTS

The goal of this study was to investigate the effects of the naturally occurring LCAT[T147I], LCAT[P274S], and LCAT-[C337Y] mutations on the biogenesis of HDL using adenovirus-mediated gene transfer.

Expression and Secretion of the LCAT Mutants in Cell Cultures. Initial gene transfer experiments in HTB-13 cells showed that in contrast to the LCAT[T147I] and LCAT-[P274S] mutants, the LCAT[C337Y] mutant was not secreted into the culture medium (Figure 1). This finding explains the total absence of plasma LCAT activity and the very low HDL levels in the subjects carrying this mutation,[12] and therefore this mutant was not studied further. On the basis of the cell culture studies of Figure 1, the amounts of LCAT secreted into the

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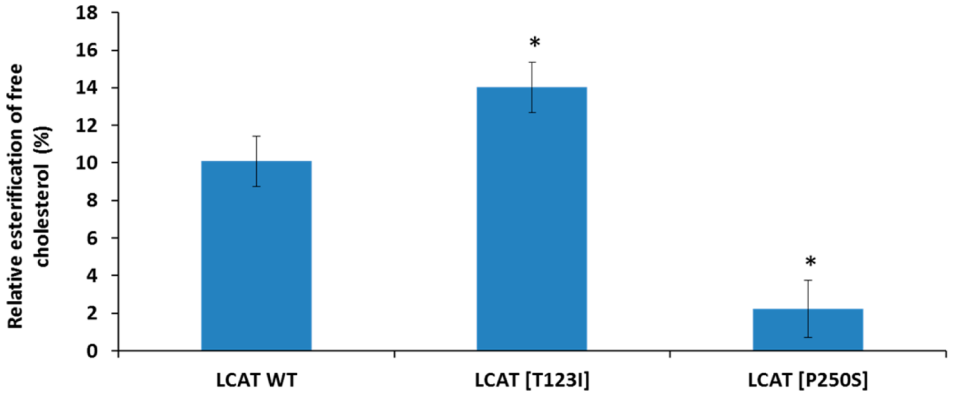

Figure 2. Determination of the β-LCAT activity of the WT LCAT and the two mutant forms of LCAT (LCAT[T147I] and LCAT[P274S]). The β-LCAT activity was assessed by the ability of LCAT to esterify cholesterol on IDL/LDL. The data represent the average from three independent experiments performed in duplicates. Significant difference between the WT and the mutants at $P < 0.05$ are indicated with (*).

Table 1. Plasma Lipids and Hepatic LCAT mRNA Levels of LCAT$^{-/-}$ Mice Expressing Wild-Type (WT) and the Mutant Form of LCAT As Indicated[a]

protein expressed	cholesterol (mg/dL)	CE/TC	phospholipids (mg/dL)	triglycerides (mg/dL)	relative LCAT mRNA (%)
C57BL[b]	70 ± 2			21 ± 3	
C57BL/6[c]	88 ± 10	0.7 ± 0.1	150 ± 15	31 ± 7	
LCAT$^{-/-}$	31 ± 6	0.3 ± 0.1	44 ± 14	30 ± 21	
WT LCAT	368 ± 47^	0.8 ± 0.1^	263 ± 34^	65 ± 20	100 ± 33*
LCAT[T147I]	162 ± 20+^	0.8 ± 0.1^	132 ± 58+^	60 ± 17	100 ± 38
LCAT[P274S]	91 ± 13+^	0.6 ± 0.2+^	151 ± 21+^	65 ± 17	110 ± 26

[a]Values are means ± standard deviation based on analysis of 4−5 mice per experiment. Expression of LCAT mRNA is relative to the expression of WT LCAT and is indicated by a (*). Statistical significant differences in cholesterol, triglyceride and LCAT mRNA levels at $p < 0.05$ between mice expressing the WT LCAT, and the LCAT mutants are indicated by a (+) and between mice expressing GFP and WT LCAT or the mutant form of LCAT are indicated by a (^). [b]Reference 55. [c]Reference 31.

medium of cells expressing the WT and the two LCAT mutants are comparable.

Determination of the α- and β-LCAT Activity of WT LCAT and the LCAT Mutants. LCAT secreted by HTB-13 cells expressing the WT LCAT or the LCAT[T147I] and LCAT[P274S] mutants was used to measure their α- and β-LCAT activity. The α- and the β-activity of these mutants were determined as described in the experimental procedures using reconstituted HDL (rHDL) and plasma human IDL/LDL respectively as substrate. This analysis showed that the in vitro α-LCAT activity of the two mutants was greatly diminished as compared to that of WT LCAT (1.5% and 3.5% for LCAT[T147I] and LCAT[P274S], respectively) (Supporting Information Figure 1). In contrast, the β-LCAT activity of the LCAT[T147I] mutant was 139% as compared to the WT LCAT, whereas the β-LCAT activity of the LCAT[P274S] mutant was 22% of the WT LCAT (Figure 2). These findings are consistent with the classification of the LCAT[T147I][15] and LCAT[P274S] (E. Dafnis and J.A. Kuivenhoven, unpublished data) mutants as FED and FLD mutations, respectively.

Gene Transfer Experiments in Mice. The LCAT[T147I] and LCAT[P274S] mutants were used in gene transfer experiments in LCAT$^{-/-}$ mice to assess their ability to promote formation of HDL using the endogenous WT mouse apoA-I and to reproduce the FED and FLD phenotypes respectively observed in humans carrying these mutations. In another set of gene transfer experiments, we evaluated the ability of the LCAT[T147I] and the LCAT[P274S] mutants to restore the HDL and apoA-I levels in apoA-I$^{-/-}$ mice, following coexpression of these mutants with apoA-I[L159R]$_{FIN}$. This apoA-I mutant was shown previously to have defects in the pathway of the biogenesis of HDL that can be corrected by overexpression of the human LCAT.[32,33] Following gene transfer, we measured hepatic mRNA levels, plasma lipids, lipoproteins, and the formation of HDL particles.

Plasma Lipids, apoA-I mRNA Levels, and FPLC Profiles Following LCAT Gene Transfer. To examine the effect of the LCAT mutations of the biogenesis of HDL, LCAT$^{-/-}$ mice were injected with 8×10^8 pfu of adenoviruses expressing GFP as control, the WT LCAT, or either of the LCAT[T147I] and the LCAT[P274S] mutants. Total plasma cholesterol, free cholesterol, triglycerides, phospholipids, and hepatic apoA-I mRNA levels were determined 4 days postinfection. The LCAT$^{-/-}$ mice had low total cholesterol, triglycerides, and phospholipids and CE/TC ratio (Table 1). Gene transfer of WT human LCAT in LCAT$^{-/-}$ mice increased significantly total plasma cholesterol and phospholipid levels as well the CE/TC ratio (Table 1). Expression of the LCAT[T147I] increased the total plasma cholesterol and phospholipids to levels corresponding to 44% and 50% of WT LCAT and normalized the CE/TC to a ratio of 0.8 (Table 1). Expression of the LCAT[P274S] increased the total plasma cholesterol and phospholipids to levels corresponding to 25% and 57% of WT LCAT and partially corrected the CE/TC to a ratio of 0.6. The triglyceride levels in mice expressing the WT LCAT, and the two mutant forms of LCAT were increased 2-fold compared to LCAT$^{-/-}$ mice, but remained low (Table 1).

Fractionation of the plasma by FPLC and analysis of the distribution of the cholesterol in the resulting fractions showed that in mice expressing the WT LCAT there was a large peak of

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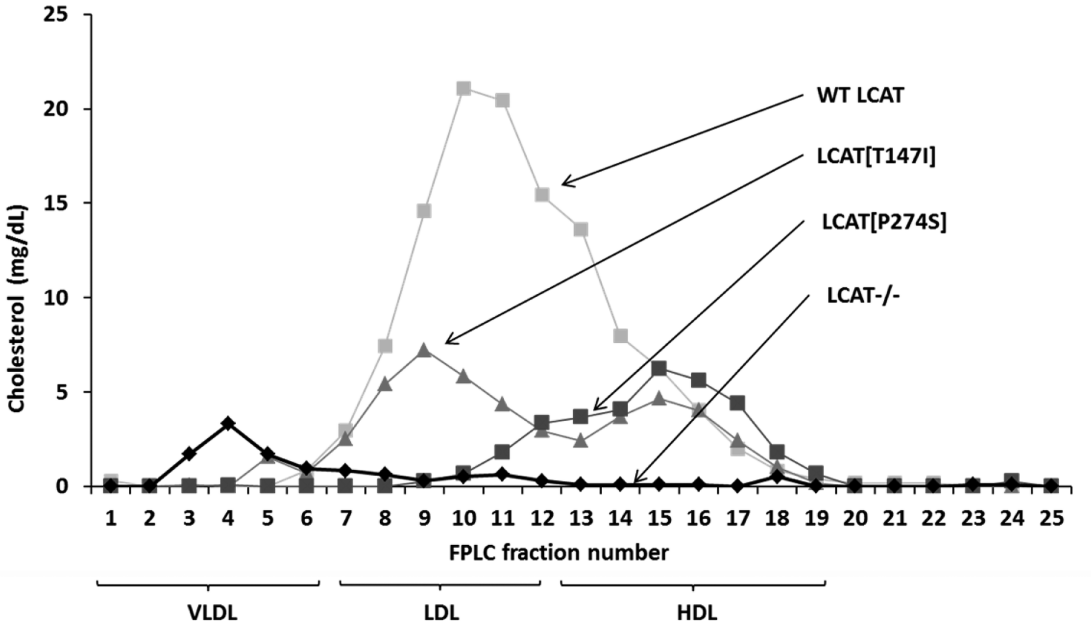

Figure 3. Plasma FPLC cholesterol profile of LCAT$^{-/-}$ mice expressing WT LCAT or the LCAT[T147I] or the LCAT[P274S] mutants as indicated.

cholesterol covering the LDL and to a lesser extend the HDL region. The cholesterol peak in mice expressing the LCAT-[P274S] mutant was significantly decreased as compared to that of mice expressing the WT LCAT, and the cholesterol was distributed predominantly in the HDL fractions. In mice expressing the LCAT[T147I] mutant, there were two discrete cholesterol peaks covering the HDL and the LDL regions (Figure 3).

Fractionation of Plasma by Density Gradient Ultracentrifugation and Analysis of the Resulting Fractions by SDS-PAGE and Electron Microscopy of the HDL Fractions: Two-Dimensional Gel Electrophoresis of the Plasma. Fractionation of plasma by density gradient ultracentrifugation and subsequent analysis of the resulting fractions by SDS-PAGE served two purposes: it gave important information on the distribution of apoA-I and other apolipoproteins in the different lipoprotein fractions and provided the HDL fractions that were used for EM analysis. This analysis showed that in LCAT$^{-/-}$ mice, we could not detect apoA-I or apoA-IV in any of the fractions and there were only traces of apoE in the HDL2 and LDL fractions (Figure 4A). In LCAT$^{-/-}$ mice expressing the WT LCAT, the mouse apoA-I was predominantly distributed in the HDL2/HDL3 fractions and to a lesser extent in the LDL fraction (Figure 4B). Mouse apoE and apoA-IV were present in all lipoprotein fractions but predominantly in the LDL and HDL2 fraction. ApoB-48 was present in the in the VLDL/IDL/LDL fractions and to a lesser extent in the HDL2 fractions (Figure 4B). The presence of these apolipoproteins in the LDL region is consistent with the formation of apoE- and apoA-IV-containing HDL particles.[44,45] The presence of apoE is also expected to contribute to the esterification of the cholesterol of LDL and explains the formation of a predominant LDL cholesterol peak with a shoulder in the HDL region (Figure 3). In LCAT$^{-/-}$ mice expressing the LCAT[T147I] mutant, apoA-I was predominantly distributed in the HDL3 fractions and to a lesser extend in the LDL fraction (Figure 4C). A small amount of apoE and apoB-48 was found in the VLDL/IDL region and traces of apoA-IV in the HDL3 region (Figure 4C). This is compatible with the formation of an LDL as well as HDL cholesterol peak (Figure 3). In LCAT$^{-/-}$ mice expressing the LCAT[P274S] mutant, apoA-I was predominantly distributed in the HDL3 fractions and to a lesser extend in the HDL2 fractions (Figure 4D). This is compatible with the formation of only an HDL cholesterol peak (Figure 3). On the basis of the data of Figure 4A–D, the levels of the murine apoA-I in LCAT deficient mice or mice expressing WT or mutant LCAT forms were different. This reflects the limited capacity of the mutant LCAT forms to produce mature HDL. Particles that do not achieve maturation are removed rapidly from plasma.[46,47]

Electron microscopy analysis of fractions 6 and 7 obtained by density gradient ultracentrifugation, which correspond to HDL, showed that in LCAT$^{-/-}$ mice expressing GFP, the HDL fractions contained very few small size spherical particles (6.1 ± 1.3 nm diameter) (Figure 4E). The HDL fractions obtained from LCAT$^{-/-}$ mice expressing the WT LCAT contained larger spherical HDL particles (9.1 ± 2 nm diameter) (Figure 4F). In contrast, the HDL fractions in LCAT$^{-/-}$ mice expressing the LCAT[T147I] or the LCAT[P274S] mutant contained numerous small size particles of approximately 7 nm diameter (Figures 4G,H). Two dimensional gel electrophoresis of plasma showed that LCAT$^{-/-}$ mice generated predominantly two preβ HDL subpopulations and one small size α4-HDL subpopulation (Figure 4I). LCAT$^{-/-}$ mice expressing the WT LCAT formed α2-, α3- and larger size α1-HDL subpopulations (Figure 4J). LCAT$^{-/-}$ mice expressing the LCAT[T147I] mutant formed α2- and α3-HDL subpopulations in addition to the preβ and α4-HDL subpopulations seen in LCAT$^{-/-}$ mice (Figure 4K). LCAT$^{-/-}$ mice expressing the LCAT[P274S] mutant also formed α2-, α3-HDL subpopulation as well as α4- and preβ HDL subpopulations (Figures 4L). In vivo gene transfer studies of the LCAT[P274S] and LCAT[T147I] mutants in LCAT$^{-/-}$ mice that express the endogenous WT mouse apoA-I reproduced the FLD and FED phenotypes respectively observed in humans carrying the LCAT[P274S] and LCAT[T147I] mutations.

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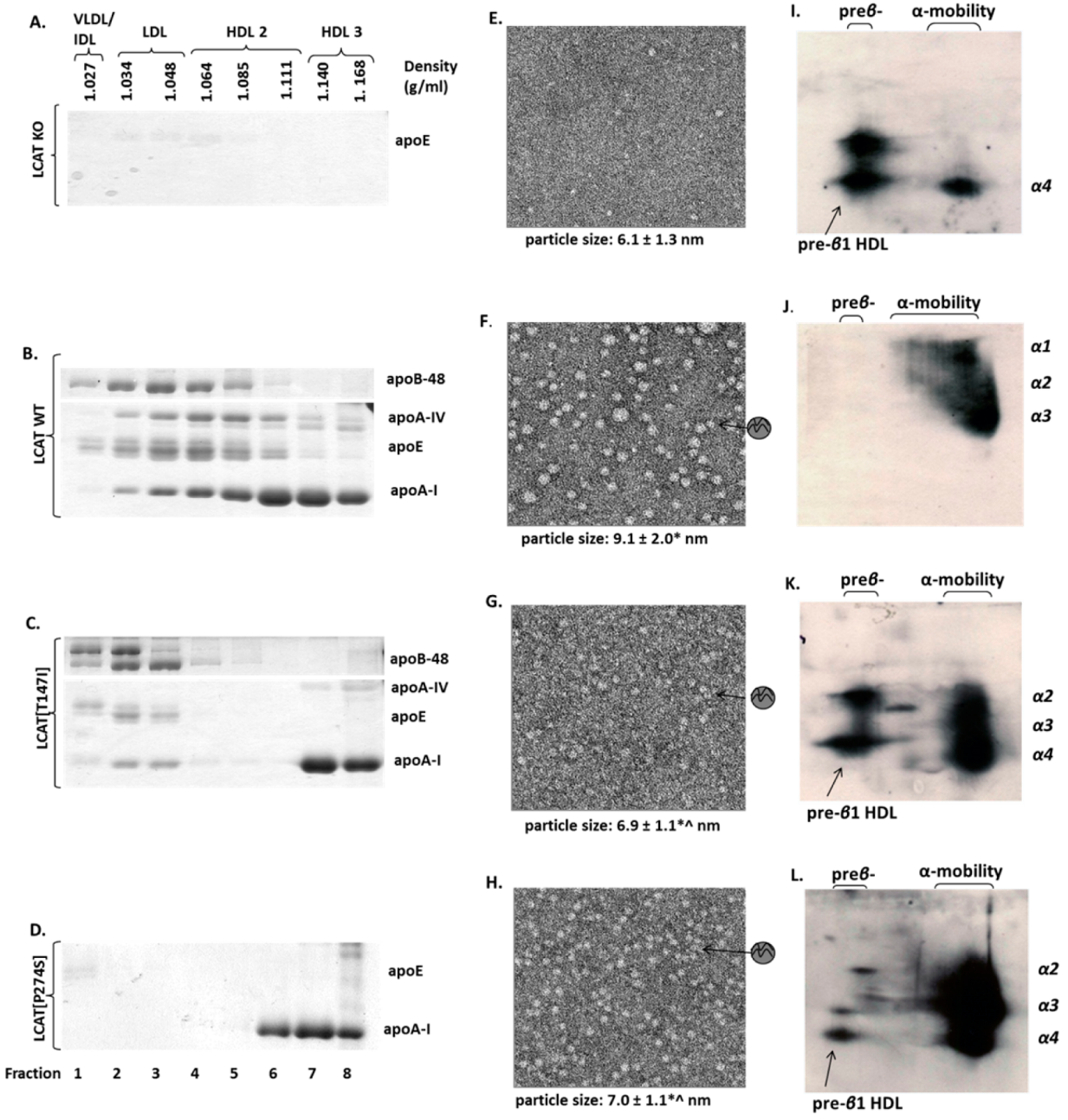

Figure 4. (A–L) Analysis of plasma of LCAT$^{-/-}$ mice infected with adenoviruses expressing GFP, the WT LCAT, or the LCAT[T147I] and LCAT[P274S] mutants by density gradient ultracentrifugation and SDS-PAGE (A–D), EM analysis of HDL fractions 6–7 (E–H), and two-dimensional gel electrophoresis of plasma of (I–L). Statistically significant differences at $p < 0.05$ in the size of the HDL particles relative to that of mice expressing GFP is indicated by (*) and relative to the size of HDL of mice expressing WT LCAT by (^).

Ability of LCAT Mutants to Correct the Defective HDL Phenotype Observed in the apoA-I[L159R]Fin Mutant That Is Associated with Low HDL Levels. In previous studies, we have shown that LCAT can correct the aberrant HDL phenotypes caused by the apoA-I[L159R]$_{FIN}$ mutant.[32] We investigated to what extent the two LCAT mutants can correct the aberrant HDL phenotype of apoA-I$^{-/-}$ mice expressing the apoA-I[L159R]$_{FIN}$ mutant. For this set of experiments, one group of apoA-I$^{-/-}$ mice was injected with adenoviruses expressing GFP at a dose of 1.5×10^9 pfu as described in Materials and Methods. Two other groups were injected with 1×10^9 pfu of adenoviruses expressing either WT apoA-I or the apoA-I[L159R]$_{FIN}$ along with 5×10^8 pfu of the control adenovirus expressing GFP. Three other groups of mice were injected with a combination of 1×10^9 pfu of adenoviruses expressing the apoA-I[L159R]$_{FIN}$ mutant along with 5×10^8 pfu of adenoviruses expressing either the WT LCAT or the LCAT[T123I] or the LCAT[P274S] mutants (Table 2). Plasma obtained 4 days post infection was analyzed for total cholesterol, CE/TC ratio, triglycerides, apoA-I protein levels, and hepatic human LCAT and apoA-I mRNA levels. In all cases, the apoA-I and LCAT mRNA levels were comparable (Table 2). ApoA-I$^{-/-}$ mice expressing GFP had very low total cholesterol levels (Table 2). Expression of WT apoA-I increased 9-fold the total cholesterol levels. Expression of apoA-I[L159R]$_{FIN}$ did not increase cholesterol levels as compared to mice expressing GFP (Table 2). In mice expressing apoA-I[L159R]$_{FIN}$ mutant, the apoA-I protein levels

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Table 2. Plasma Lipids, apoA-I Levels, and Hepatic mRNA Levels of Human apoA-I and LCAT in apoA-I$^{-/-}$ Mice Expressing GFP, the WT apoA-I, and apoA-I[L159R]$_{FIN}$ Alone or in Combination with the WT Human LCAT or the Mutant LCAT[T147I] and LCAT[P274S] Forms As Indicated[a]

protein expressed	cholesterol (mg/dL)	CE/TC	triglycerides (mg/dL)	apoA-I protein (mg/dL)	relative apoA-I mRNA (%)	relative LCAT mRNA (%)
GFP	27.5 ± 6.5	0.68 ± 0.04	37.3 ± 11.1			
apoA-I WT	251.7 ± 70.6	0.85 ± 0.12	41.6 ± 12.1	191 ± 88	100 ± 32*	
apoA-I[L159R]FIN	26.4 ± 3^{+}	0.75 ± 0.1	26.5 ± 13	25 ± 4^{+}	100 ± 50	
apoA-I[L159R]FIN + LCAT	380.5 ± 76^{+}	0.79 ± 0.04	44.5 ± 5	381 ± 93^{+}	120 ± 37	115 ± 21*
apoA-I[L159R]FIN + LCAT[T147I]	46.3 ± 6+$\hat{}$	0.85 ± 0.04	28.6 ± 7	107 ± 21+$\hat{}$	116 ± 52	98 ± 29
apoA-I[L159R]FIN + LCAT[P274S]	45.1 ± 21+$\hat{}$	0.70 ± 0.09	38.7 ± 16	136 ± 19$\hat{}$	130 ± 17	100 ± 40

[a]Values are means ± standard deviation based on analysis of 4−5 mice per experiment. The symbol (*) indicates that apoA-I and LCAT mRNA levels are relative to WT apoA-I and WT LCAT mRNA levels, respectively. Statistical significant differences in cholesterol, triglyceride, mRNA, and protein levels at $p < 0.05$ between mice expressing the WT apoA-I and the apoA-I mutants are indicated by a (+) and between mice expressing the mutant form of apoA-I along with WT LCAT and the mice expressing the mutant forms of apoA-I along with the mutant forms of LCAT are indicated by a (^). No significant differences in mRNA levels of apoA-I or LCAT mutants were observed as compared to their respective WT forms.

were very low as compared to mice expressing the WT apoA-I (Table 2). The coexpression of the apoA-I[L159R]$_{FIN}$ with WT LCAT increased significantly the total cholesterol and plasma apoA-I levels as compared to mice expressing the apoA-I mutant alone (Table 2). In contrast, the coexpression of apoA-I[L159R]$_{FIN}$ mutants with either of the LCAT[T147I] or LCAT[P274S] mutant forms showed a moderate increase in total cholesterol and apoA-I levels as compared to mice expressing the apoA-I mutant alone (Table 2). In all cases, the changes in plasma triglyceride levels and the CE/TC ratio were not substantial (Table 2).

FPLC analysis showed that in apoA-I$^{-/-}$ mice expressing the WT apoA-I, a large cholesterol peak in the HDL region was formed (Figure 5A). In mice expressing apoA-I[L159R]$_{FIN}$ mutant, the cholesterol peak was barely detectable but it increased dramatically when the apoA-I[L159R]$_{FIN}$ was coexpressed with WT LCAT and was shifted to lower densities as compared to the HDL cholesterol peak of mice expressing the WT apoA-I (Figure 5A). In mice coexpressing the apoA-I[L159R]$_{FIN}$ and LCAT[T147I], there was a modest increase in the cholesterol peak and its distribution was biphasic, covering the HDL and LDL regions (Figure 5A). In mice coexpressing the apoA-I[L159R]$_{FIN}$ mutant and LCAT[P274S], there was a modest increase in the cholesterol peak that was distributed predominantly in the HDL and to a lesser extent in the LDL region (Figure 5A). The distribution of cholesterol caused by LCAT[T147I] and LCAT[P274S] in this set of experiments is similar but not identical with the distribution of cholesterol observed in LCAT$^{-/-}$ mice expressing LCAT[T147I] and LCAT[P274S] (compare Figure 5A with Figure 3).

The potential correction of the aberrant phenotypes caused by the apoA-I[L159R]$_{FIN}$ mutants by the LCAT mutants was further assessed by density gradient ultracentrifugation of the plasma, electron microscopy of the HDL fractions, and two-dimensional gel electrophoresis of the plasma.

These analyses showed that in mice coexpressing the apoA-I[L159R]$_{FIN}$ mutant and either LCAT[T147I] or LCAT-[P274S], the apoA-I was predominantly found in the HDL3 region (Figure 5B,C). Traces of apoE were also detected in the LDL region (Figure 5B). EM analysis of the HDL fractions 6 and 7 obtained by density gradient ultracentrifugation showed the formation of small size spherical particles of average diameter 7.5 nm (Figure 5D,E). Two dimensional electrophoresis showed that coexpression of the apoA-I[L159R]$_{FIN}$ and either LCAT[T147I] or LCAT[P274S] mutants generated preβ subpopulation along with α3-, α4-HDL subpopulations (Figure 5F,G).

In comparison, following coexpression of the apoA-I[L159R]$_{FIN}$ mutant and WT LCAT, plasma apoA-I was increased and was distributed predominantly in the HDL2/HDL3 region and to a lesser extent in other lipoprotein fractions. The HDL fractions 6 and 7 contained spherical particles of diameter 9.9 and 10.9 nm, respectively, and the HDL contained few pre-β and normal α-HDL subpopulations (Table 2 and compare panels of Supporting Information Figure 2C,F,J, with the corresponding panels B−G of Figure 5).

DISCUSSION

Background. Lecithin cholesterol acyltransferase (LCAT) is a key enzyme that esterifies the free cholesterol of the HDL particles and thus regulates the plasma levels of HDL.[10,18,48]

On the basis of the proposed 3D model of LCAT, it was proposed that the mutations associated with FLD, including the LCAT[P274S] mutant, are in the vicinity of the catalytic residues of the enzyme and the mutations associated with FED including the LCAT[T147I] mutant are on the hydrophilic surface of the amphipathic α helices of the enzyme that may interact with apoA-I and HDL.[49,50]

In the present study, we have investigated the impact of two FLD (LCAT[P274S] and LCAT[C337Y]) and one FED (LCAT[T147I]) mutation on the in vitro and in vivo activities of LCAT and their ability to be secreted by the cells. The in vivo activities were assessed by the characteristics of the HDL formed in LCAT$^{-/-}$ mice that express the endogenous mouse apoA-I as well as their ability to correct the aberrant HDL phenotypes generated following expression of the apoA-I[L159R]$_{FIN}$ mutants in apoA-I$^{-/-}$ mice.[5,32] The two LCAT mutants studied here were previously characterized in human carriers by measuring the LCAT mass and/or activity and the lipids/lipoproteins and HDL levels [ref 15 and (E. Dafnis and J. A. Kuivenhoven, unpublished data)]. This rudimentary analysis is highly augmented by the comprehensive experiments of Figures 3−5 that include FPLC profiles, density gradient ultracentrifugation, EM, and two-dimensional gel electrophoresis. The mouse models studied here mimic the homozygous human subjects.

The C337Y Mutations in LCAT Prevent the Secretion of the Mutant Enzyme. Gene transfer of WT and mutant LCAT forms in HTB-13 cells showed that the WT LCAT and the two mutants, LCAT[T147I] and LCAT[P274S], are

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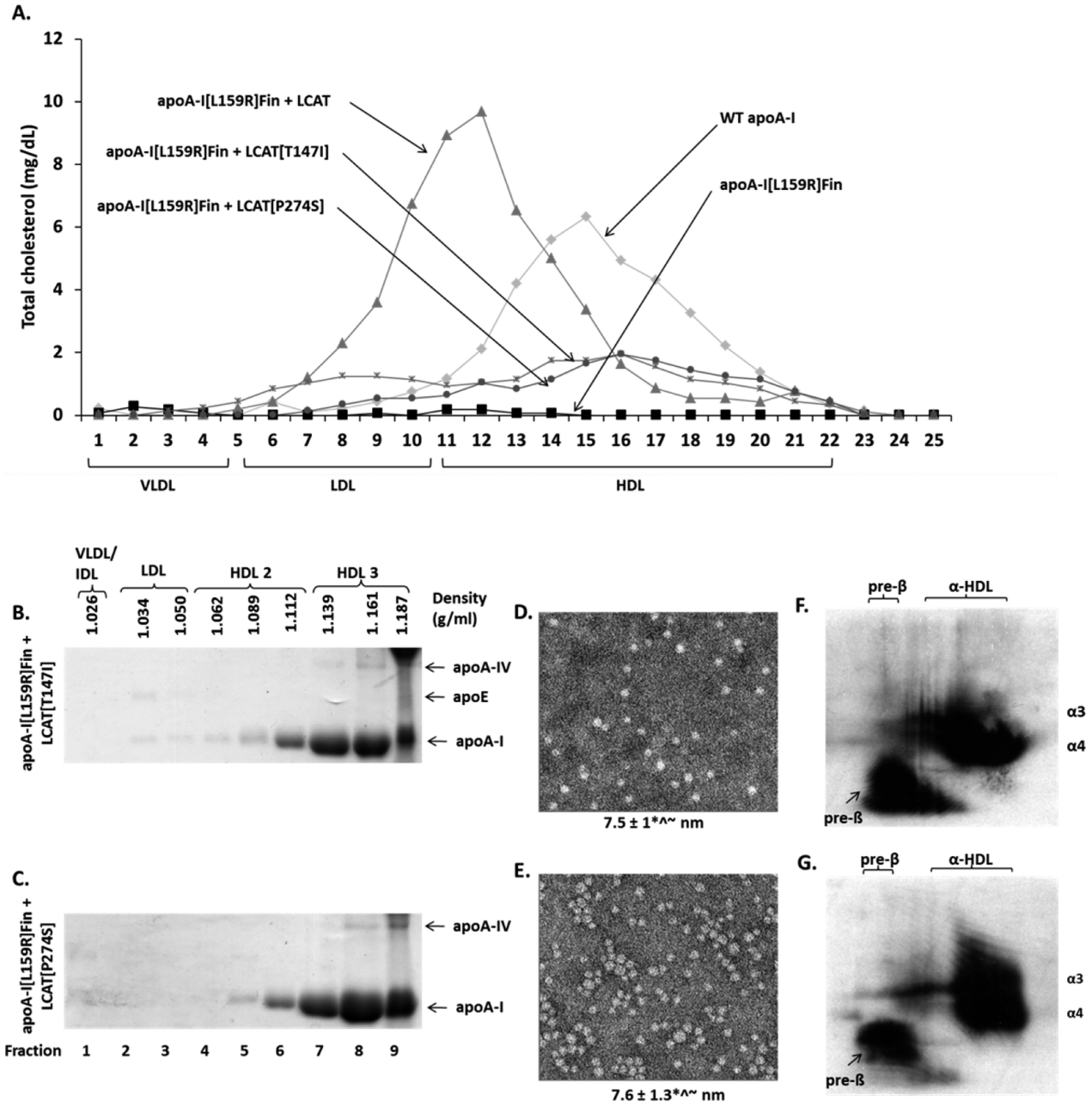

Figure 5. (A–G) Plasma FPLC cholesterol profile of apoA$^{-/-}$ mice expressing WT apoA-I and apoA-I[L159R]$_{FIN}$ alone or in combination with either WT LCAT or the LCAT[T147I] and the LCAT[P274S] mutants as indicated (A). Analysis of the plasma of apoA-I$^{-/-}$ mice infected with adenoviruses expressing the apoA-I[L159R]$_{FIN}$ in combination with either the LCAT[T147I] or the LCAT[P274S] mutants by density gradient ultracentrifugation and SDS-PAGE (B,C), EM of HDL fractions 6–7 (D,E) and two-dimensional gel electrophoresis of plasma (F,G). Statistically significant differences at $p < 0.05$ in the size of the HDL particles of mice expressing WT apoA-I as compared to particles generated in mice expressing the apoA-I[L159R]$_{FIN}$ alone or in the presence of either WT LCAT or LCAT[T147I] and LCAT[P274S] is indicated by (*) (compare with Supporting Information Figure 2). Statistically significant differences at $p < 0.05$ in the size of the HDL particles of mice expressing the apoA-I[L159R]$_{FIN}$ alone as compared to particles generated in mice expressing the apoA-I[L159R]$_{FIN}$ in the presence of either WT LCAT or LCAT[T147I] and LCAT[P274S] is indicated by (^) (compare with Supporting Information Figure 2). Statistically significant differences at $p < 0.05$ in the size of the HDL particles of mice expressing the apoA-I[L159R]$_{FIN}$ in the presence of WT LCAT as compared to particles generated in mice expressing the apoA-I[L159R]$_{FIN}$ in the presence of LCAT[T147I] and LCAT[P274S] is indicated by (~) (compare with Supporting Information Figure 2).

secreted efficiently by the cells, whereas the LCAT[C337Y] is not secreted. The lack of secretion of the LCAT[C337Y] mutant is consistent with the total absence of plasma LCAT activity in humans carrying this mutation.[12] It is reasonable to assume that loss of a critical cysteine in the 337 residue that participates in the formation of a disulfide bridge with residue 380 prevents correct folding of the enzyme and results in its intracellular degradation.

The LCAT[P274S] has Diminished α- and β-LCAT Activity, Whereas the LCAT[T147I] has Diminished α- and Slightly Increased β-LCAT Activity. LCAT secreted by HTB-13 cells, grown in a large scale in roller bottles, was used

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to measure the α-LCAT activity using reconstituted HDL (rHDL) containing apoA-I, phospholipids, and cholesterol and the β-LCAT activity using IDL/LDL isolated from human plasma respectively as substrates. The finding that the LCAT[T147I] mutant had diminished α-LCAT activity but maintained the β-LCAT activity is consistent with its classification in the category of FED mutations.[15] Similarly, the fact that the LCAT[P274S] mutant had diminished α- and β-LCAT activity classifies it in the category of FLD mutations.

Effect of LCAT Mutations on the Biogenesis of HDL. The effect of LCAT mutants on the biogenesis of HDL and the features of HDL formed was tested by adenovirus-mediated gene transfer of the LCAT mutants in LCAT$^{-/-}$ mice that express their endogenous apoA-I gene. It is generally recognized that experiments in mice and other animal models is an approximation. Nevertheless, it has been the predominant methodology used to study the in vivo properties of a protein. Regarding this specific system, we reported previously that the HDL phenotypes generated in mice and humans by the apoA-I[L141R]$_{Pisa}$ are very similar.[29,32,51]

The HDL phenotype of the LCAT deficient mice[37] was characterized by lack of HDL cholesterol peak, the loss of apoA-I from all lipoprotein fractions, presence of very small HDL particles, and formation of predominantly preβ and few α-4 HDL particles. This aberrant HDL phenotype was completely corrected by gene transfer of WT LCAT but only partially by gene transfer of the LCAT[T147I] or the LCAT[P274S] mutant. The HDL phenotype observed in LCAT$^{-/-}$ mice expressing the T147I mutant is consistent with that observed in human patients homozygous for this mutation.[14,15]

The formation of small size HDL indicates that these two LCAT mutants have some cholesterol esterification potential, but the particles formed cannot proceed efficiently to generate mature HDL particles. Furthermore, the low apoA-I and HDL cholesterol levels produced by expression of the LCAT mutants in combination with the small HDL particles formed is consistent with accelerated catabolism of the lipoprotein particles generated by these two LCAT mutants.[46] Overall, these analyses provided distinct HDL phenotypes for the two LCAT mutants, indicating that they have different effects on HDL biogenesis.

Interactions of WT LCAT with Mutant apoA-I Forms. Previous studies have shown that certain apoA-I mutants, when expressed in apoA-I$^{-/-}$ mice, have low HDL cholesterol levels and generate aberrant HDL phenotypes as determined by a variety of analyses described in Figures 4−5.[31−33] Thus, expression of apoA-I[L159R]$_{FIN}$ mutant in apoA-I$^{-/-}$ mice decreased total plasma cholesterol, apoA-I, and HDL cholesterol and generated few small size HDL particles. This defect in the biogenesis of HDL was corrected by coexpression of the apoA-I[L159R]$_{FIN}$ mutant with WT LCAT.[32]

Interactions of Mutant LCAT with a Mutant apoA-I. In the present study, we tested the hypothesis that when two mutant proteins (such as apoA-I and LCAT) interact with each other, mutations in one protein may restore the function of the other. Although this scenario is rare, previous studies by us have shown that mutations in apoA-I restored the capacity of the mutant SR-BI to promote cholesterol efflux.[30]

On the basis of the computer derived 3D structure of LCAT, the enzyme contains seven conserved β-strands connected by loops to four α-helices.[52] One of the helices of LCAT (helix α 4−5), located between residues 153−171, is close to the active site of the enzyme.[50,52] It has been suggested that electrostatic interactions between LCAT helices[53] and helix 6 (residues 143−164) of apoA-I may draw the two proteins together on the surface of the lipoprotein particle and drive the activation of LCAT.[54] The L159R mutation located within helix 6 of apoA-I apparently disrupts the apoA-I/LCAT interactions and inhibits the biogenesis of HDL.[32]

We tested the hypothesis that the T147I mutation that is found in the hydrophilic phase of helix α 3−4 of LCAT or the P274S mutation that is found in the sixth β-strand of LCAT[52] may offset the effects of the L159R mutation of apoA-I and thus facilitate the biogenesis of HDL. More precise predictions of the molecular interactions of mutant forms of apoA-I and LCAT will require the determination of a high resolution 3D structure of LCAT.

Mechanistic Insights. Our studies showed that although WT LCAT corrected completely the aberrant HDL phenotype of apoA-I[L159R]$_{FIN}$ mutant, the LCAT[T147I] and LCAT[P274S] mutants corrected only partially the aberrant HDL phenotype of this mutant. The patterns generated with apoA-I[L159R]$_{FIN}$ and the two mutant LCAT forms in apoA-I$^{-/-}$ mice are different and distinct. The LCAT[T147I] caused biphasic distribution of cholesterol in the HDL and LDL region and the LCAT[P274S] caused predominantly monophasic distribution of cholesterol in the HDL region. In both mutants, apoA-I was distributed in the HDL region. Although the LCAT mutants did not normalize the aberrant HDL phenotype generated by the apoA-I[L159R]$_{FIN}$, partial or complete correction may be possible for other sets of apoA-I and LCAT mutants.

Because mice do not express CETP, it will be informative in future experiments to study how mutant apoA-I and mutant LCAT forms interact in the background of mice that express human CETP, a condition that will reflects better the human physiology.

Overall, our studies demonstrate that the LCAT[T147I] and LCAT[P274S] mutations affect in unique ways the biogenesis of HDL and generate distinct aberrant HDL phenotypes. Our findings are consistent with the hypothesis that the immature particles generated by the LCAT mutants are unstable and are removed rapidly from plasma.[46] The accelerated catabolism of the aberrant HDL particles by the kidney is consistent with the renal disease observed in the LCAT deficient patients.[10,12]

■ ASSOCIATED CONTENT

Supporting Information

Representative set of initial velocity graphs that provided the apparent K_m and V_{max} in the α-LCAT assays of WT LCAT, LCAT[T147I], and LCAT[P274S]. Analysis of the plasma of apoA-I$^{-/-}$ mice infected with adenoviruses expressing the WT apoA-I and the apoA-I[L159R]$_{FIN}$ alone or in combination with WT LCAT by density gradient ultracentrifugation and SDS-PAGE, electron microscopy, and two-dimensional gel electrophoresis. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00180.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 617-638-5085. Fax: 617-638-5141. E-mail: vzannis@bu.edu.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; apoA-IV, apolipoprotein A-IV; apo-B48, apolipoprotein B-48; $LCAT^{-/-}$, LCAT-deficient; $apoA\text{-}I^{-/-}$, apoA-I-deficient; BSA, bovine serum albumin; EM, electron microscopy; HEK293, human embryonic kidney 293; HTB-13, SW 1783 human astrocytoma; POPC, β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine; WT, wild-type; FPLC, fast-protein liquid chromatography; pfu, plaque forming units; HDL, High Density lipoprotein; TC, total cholesterol; CE, cholesteryl ester

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