Correction of hypoalphalipoproteinemia in LDL receptor-deficient rabbits by lecithin:cholesterol acyltransferase

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Abstract **Familial hypercholesterolemia (FH), a disease caused by a variety of mutations in the low density lipoprotein receptor (LDLr) gene, leads not only to elevated LDL-cholesterol (C) concentrations but to reduced high density lipoprotein (HDL)-C and apolipoprotein (apo) A-I concentrations as well. The reductions in HDL-C and apoA-I are the consequence of the combined metabolic defects of increased apoA-I catabolism and decreased apoA-I synthesis. The present studies were designed to test the hypothesis that overexpression of human lecithin:cholesterol acyltransferase (hLCAT), a pivotal enzyme involved in HDL metabolism, in LDLr defective rabbits would increase HDL-C and apoA-I concentrations. Two groups of hLCAT transgenic rabbits were established: *1*) hLCAT+/LDLr heterozygotes (LDLr+/−) and *2*) hLCAT+/LDLr homozygotes (LDLr−/−). Data for hLCAT+ rabbits were compared to those of nontransgenic (hLCAT−) rabbits of the same LDLr status. In LDLr+/− rabbits, HDL-C and apoA-I concentrations (mg/dl), respectively, were significantly greater in hLCAT+ (62 ± 8, 59 ± 4) relative to hLCAT− rabbits (21 ± 1, 26 ± 2). This was, likewise, the case when hLCAT+/LDLr−/− (27 ± 2, 19 ± 6) and hLCAT−/LDLr−/− (5 ± 1, 6 ± 2) rabbits were compared. Kinetic experiments demonstrated that the fractional catabolic rate (FCR, d^{-1}) of apoA-I was substantially delayed in hLCAT+ (0.376 ± 0.025) versus hLCAT− (0.588) LDLr+/− rabbits, as well as in hLCAT+ (0.666 ± 0.033) versus hLCAT− (1.194 ± 0.138) LDLr−/− rabbits. ApoA-I production rate (PR, $mg \cdot kg \cdot d^{-1}$) was greater in both hLCAT+/LDLr+/− (10 ± 2 vs. 6) and hLCAT+/LDLr−/− (9 ± 1 vs. 4 ± 1) rabbits. Significant correlations ($P < 0.02$) were observed between plasma LCAT activity and HDL-C ($r = 0.857$), apoA-I FCR ($r = -0.774$), and apoA-I PR ($r = 0.771$), while HDL-C correlated with both apoA-I FCR (−0.812) and PR (0.751). In summary, these data indicate that hLCAT overexpression in LDLr defective rabbits increases HDL-C and apoA-I concentrations by both decreasing apoA-I catabolism and increasing apoA-I synthesis, thus correcting the metabolic defects responsible for the hypoalphalipoproteinemia observed in LDLr deficiency.—**Brousseau, M. E., J. Wang, S. J. Demosky, Jr., B. L. Vaisman, G. D. Talley, S. Santamarina-Fojo, H. B. Brewer, Jr., and J. M. Hoeg. **Correction of hypoalphalipoproteinemia in LDL receptor-deficient rabbits by lecithin:cholesterol acyltransferase.** *J. Lipid. Res.* 39: **1558–1567.**

Supplementary key words familial hypercholesterolemia • high density lipoproteins • apolipoprotein A-I • metabolism • lecithin:cholesterol acyltransferase • WHHL rabbit

Familial hypercholesterolemia (FH) is caused by a variety of mutations in the low density lipoprotein receptor (LDLr) gene, leading to elevated concentrations of LDL in heterozygous and, most notably, homozygous patients (1). FH is also associated with significantly reduced concentrations of both high density lipoprotein cholesterol (HDL-C) and its principal protein component, apolipoprotein (apo) A-I (2–4). In vivo kinetic studies have demonstrated that these reductions are the result of the combined metabolic defects of increased apoA-I catabolism and decreased apoA-I synthesis (4, 5).

A rabbit model for FH, the Watanabe heritable hyperlipidemic (WHHL) rabbit, was discovered at Kobe University and reported in 1980 (6, 7). These rabbits have a spontaneously arising mutation in the LDLr gene that encodes a 4-amino acid deletion in the cysteine-rich ligand-binding domain of the protein, severely disrupting receptor function (8). Homozygous WHHL rabbits are markedly hypercholesterolemic from birth and suffer from tendon xanthomas and atherosclerosis, both of which exhibit remark-

Abbreviations: alpha-LCAT activity, exogenous proteoliposome assay; apo, apolipoprotein; CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; FCR, fractional catabolic rate; FH, familial hypercholesterolemia; FPLC, fast protein liquid chromatography; HDL-C, high density lipoprotein cholesterol; LCAT, lecithin:cholesterol acyltransferase; LDLr, low density lipoprotein receptor; NZW, New Zealand White; PR, production rate; RT, residence time; WHHL, Watanabe-heritable hyperlipidemic.

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able pathological resemblance to those observed in human FH (6). Also analogous to the human condition, plasma HDL-C and apoA-I levels are abnormally low in these animals (5, 9, 10).

Plasma HDL-C concentrations are regulated by a complex series of metabolic processes involving synthesis, catabolism, and lipoprotein particle remodeling. Critical to the proper functioning of the HDL metabolic cascade are a number of different enzymes, including hepatic and lipoprotein lipases, cholesteryl ester transfer protein (CETP), and lecithin:cholesterol acyltransferase (LCAT). LCAT is the key enzyme involved in the esterification of intravascular free cholesterol (11). The esterification of free cholesterol by LCAT is essential for HDL particle maturation, converting discoidal particles into spherical HDL only when sufficient cholesteryl esters are generated for the hydrophobic core (12, 13). The obligatory role of LCAT in the regulation of HDL-C levels is clearly evidenced by individuals with LCAT gene defects, who have marked hypoalphalipoproteinemia due to hypercatabolism of both apoA-I and apoA-II (14, 15).

We have previously described the generation of transgenic New Zealand White (NZW) rabbits which express human LCAT to varying degrees (16). The overexpression of human LCAT in this model resulted in significant elevations of plasma HDL-C and apoA-I concentrations, as well as in alterations of HDL composition and size. The latter changes, in turn, ultimately led to gene dose-dependent reductions in HDL particle catabolism, such that high expressors of hLCAT had the largest HDL particles with the longest plasma half-life (17). With these concepts in mind, the present studies were designed to test the hypothesis that overexpression of hLCAT in LDLr defective rabbits would lead to increased concentrations of HDL-C and apoA-I relative to nontransgenic controls of the same LDLr status. Our data support this hypothesis by demonstrating that hLCAT overexpression increases HDL-C and apoA-I concentrations in both heterozygous and homozygous LDLr defective rabbits. Moreover, the elevated levels of HDL-C and apoA-I are attributable to both delayed apoA-I catabolism and increased apoA-I synthesis; thus, correcting the dual metabolic defects responsible for the hypoalphalipoproteinemia observed in LDLr deficiency.

METHODS

Animals

The human LCAT gene was introduced into LDLr defective rabbits by selective breeding. Rabbits were determined to be transgenic for human LCAT by Southern blot analysis. Four experimental groups were defined by the presence or absence of the hLCAT gene, as well as by LDL receptor status. Each group was comprised of three age-matched males, with the exception of the hLCAT−/LDLr+/− group which consisted of two females and one male. Because of the gender differences known to exist in HDL metabolism (18), only the kinetic data for the male rabbit of this group are presented in Table 2. However, the data provided in Table 1 represent the mean of all three rabbits in this group.

The mutation in WHHL rabbits involves a 12 base pair in-frame deletion that removes four amino acids from the fourth ligand binding repeat of the LDL receptor. Heterozygosity or homozygosity for the LDLr defect in the present study was established by PCR, using genomic DNA that was isolated from whole blood. A 306 bp portion of exon 4 of the rabbit LDLr gene, including the 12 bp mutant region from 369 to 380 which contains a *Bgl* I restriction site (Fig. 1A), was amplified with the following primers: 5′-primer (5′-GCC CAA GAC GTG CTC CCA GGA C-3′) and 3′-primer (5′-GCC GCT GCC ACA GTG GAA CTC GTG C-3′). PCR products were then applied to a 20% TBE-polyacrylamide gel (Novex, San Diego, CA) and electrophoresed for 2 h at 120 V. Gel-purified PCR products were digested with *Bgl* I at 37°C overnight and applied to a 20% TBE-polyacrylamide gel. Gels were stained with ethidium bromide and visualized by UV light. After digestion with *Bgl* I, LDLr+/+ rabbits (wild type) generated two PCR products, 212 and 94 bp in size, whereas LDLr heterozygotes were defined by the presence of three PCR product bands of 294, 212, and 94 bp. LDLr homozygotes generated only a single band 294 bp in length, regardless of *Bgl* I digestion.

Plasma lipids, apolipoprotein A-I, and LCAT activity

Samples were collected from the central ear artery of each rabbit at each designated time point and were added to tubes containing tripotassium EDTA. Plasma was isolated by centrifugation at 2500 rpm for 30 min at 4°C. Total cholesterol (TC) and triglycerides (TG) (Sigma, St. Louis, MO) and free cholesterol (FC) and phospholipids (PL) (Wako Chemicals USA, Inc., Richmond, VA) in both plasma and FPLC fractions were measured with a Hitachi 911 Autoanalyzer (Hitachi USA, Indianapolis, IN) using enzymic reagents. Plasma HDL cholesterol was determined after dextran sulfate-Mg^{2+} precipitation of very low density and low density lipoproteins (19). Cholesteryl ester (CE) values were calculated by subtracting free cholesterol from total cholesterol concentrations. Plasma apoA-I concentrations were determined by a competitive ELISA assay, utilizing a monoclonal antiserum directed against purified rabbit apoA-I. α-LCAT activity was determined in duplicate using an exogenous proteoliposome assay, as previously described (20).

Gel filtration chromatography

Two-hundred microliters of rabbit plasma was applied to a fast protein liquid chromatography (FPLC) system consisting of two Superose 6 columns connected in series (Pharmacia Biotech, Inc., Piscataway, NJ). Lipoproteins were eluted at 0.3 ml/min with phosphate-buffered saline, containing 1 mm EDTA and 0.02% (wt/wt) sodium azide (21). After the initial 10 ml was eluted, the next 30 ml was collected in 0.5 ml fractions. The distribution of ^{131}I-labeled apoA-I among the lipoproteins was assessed by quantitating the radioactivity in each FPLC fraction on a Packard Cobra gamma counter (Packard Instrument Co., Downers Grove, IL). Total, free, and esterified cholesterol concentrations, as well as phospholipids and triglycerides, were determined for each fraction.

Two-dimensional lipoprotein electrophoresis

Lipoproteins were analyzed by two-dimensional electrophoresis according to the method of Asztalos et al. (22). Twenty microliters of fresh whole rabbit plasma was applied to a 0.7% agarose gel (first dimension), followed by separation on a 2–36% nondenaturing polyacrylamide gradient gel (second dimension). After electrophoresis, lipoproteins were transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), probed with a monoclonal antibody directed against rabbit apoA-I, and visualized using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA).

In vivo metabolic studies

Rabbit apoA-I was purified (23) and then radiolabeled with ^{131}I (Dupont/NEN, Boston, MA), using a modification of the iodine monochloride method previously described (24, 25). ^{131}I-labeled apoA-I was reassociated with autologous rabbit plasma for 30 min at 39°C, the normal body temperature of a rabbit. Unbound iodine was removed by extensive dialysis against 0.1% (vol/vol) PBS, 0.1% (wt/vol) EDTA. Each preparation was filter-sterilized (0.22 μm Millex-GV filters, Millipore, Bedford, MA) prior to injection.

While there is evidence in the literature that suggests that exchange-labeled HDL is cleared more rapidly from the plasma than is whole-labeled HDL (26, 27), the present study used methodology similar to that described by Schaefer et al. (28) where exchange-labeled HDL, which were generated in vivo by incubating radiolabeled apoA-I with autologous plasma, were not cleared at a faster rate than were whole-labeled HDL. These data are corroborated by the results of Vega et al. (29) which provide further evidence that isolated radioiodinated apoA-I has kinetic behavior similar to apoA-I radiolabeled as part of intact HDL. Additionally, Ikewaki et al. (30) have shown that endogenous labeling of apoA-I with a stable isotope generated kinetic parameters which were highly comparable with those obtained by exchange-labeling.

Twenty-five microcuries of radiolabeled, autologous plasma preparation was infused into the marginal ear vein of each rabbit. Blood samples were collected at 5 min and at 1, 3, 6, 9, 24, 48, 72, and 96 h after injection. Plasma was isolated by centrifugation at 2500 rpm, 4°C for 30 min. Five-hundred microliters of each sample was analyzed for radioactivity on a Packard Cobra gamma counter. Residence time (RT) was determined from the area under the plasma radioactivity decay curve, using a multiexponential computer curve-fitting program (SAAM 31) (31). Fractional catabolic rate (FCR) was calculated as the reciprocal of the RT. ApoA-I pool size was derived from the formula: [plasma volume (dl) × plasma apoA-I concentration (mg/dl)]/body weight (kg). Plasma volume was estimated as 3.28% of body weight (32). Production rate (PR) was calculated as the product of FCR and pool size.

Statistical analysis

Data within each group were assessed for significance with Student's nonpaired, two-tailed *t* test, and correlation coefficients were determined by the method of Pearson. In all cases, statistical significance was set at $P < 0.05$. Data presented in the text, tables, and figures represent mean ± SEM.

RESULTS

Determination of LDL receptor status

Heterozygosity or homozygosity for the 12 base pair deletion in exon 4 of the rabbit LDLr gene was established by PCR, as described in Methods. A 306 bp portion of exon 4, including the 12 bp mutant region from 369 to 380 which contains a *Bgl* I restriction site, was amplified using primers 1 and 2 (**Fig. 1A**). PCR products were then gel-purified prior to digestion with *Bgl* I. Figure 1B shows the gel-purified PCR products of LDLr+/+, LDLr+/−, and LDLr−/− rabbits, with the exception of lane 2 which shows the undigested PCR product of an LDLr+/+ animal prior to gel purification. Subsequent to gel purification and *Bgl* I digestion, LDLr+/+ rabbits generated two product bands of 212 and 94 bp (lane 3). In the case of LDLr+/− rabbits, two bands, 306 and 294 bp, were generated before digestion (lane 4), whereas three product bands were observed in LDLr+/− animals after incubation with *Bgl* I (lane 5) due to the presence of one normal and one mutant allele. In contrast, LDLr−/− rabbits generated only one band, 294 bp in size, both before (lane 6) and after (lane 7) incubation with *Bgl* I.

Plasma lipids and lipoprotein composition

Table 1 summarizes the mean plasma lipid data of hLCAT transgenic LDLr heterozygotes and homozygotes, as compared with those of their respective controls. Neither plasma total, free, or esterified cholesterol concentrations were significantly different when these two groups were compared. However, the lipoprotein distribution of cholesterol was dramatically dissimilar between the transgenic and control LDLr heterozygotes. The mean plasma HDL-C concentration of the hLCAT+/LDLr+/− group was three times greater than that of the hLCAT−/LDLr+/− group. Conversely, nontransgenic LDLr heterozygotes had a greater than 4-fold elevation in nonHDL-C when compared with heterozygotes overexpressing hLCAT. Although plasma phospholipids were elevated (139%) in nontransgenic LDLr heterozygotes, this difference was not statistically significant. Interestingly, plasma triglycerides were increased 3-fold in hLCAT−/LDLr+/− rabbits relative to the hLCAT+/LDLr+/− group.

As was the case for the LDLr+/− groups, no significant differences were noted in either plasma total, free, or esterified cholesterol concentrations when the two LDLr−/− groups were compared. The most substantial difference between hLCAT+ and hLCAT− LDLr homozygotes was in HDL-C concentration, with a greater than 5-fold elevation observed in hLCAT+/LDLr−/− rabbits. However, in contrast to LDLr heterozygotes, LDLr homozygotes overexpressing hLCAT did not have lower nonHDL-C concentrations when compared with those of their nontransgenic controls. When plasma phospholipids and triglycerides were compared between the two LDLr−/− groups, only the 56% reduction in triglycerides observed in the hLCAT+ rabbits was statistically significant.

In **Fig. 2**, panels A–D, FPLC profiles from representative hLCAT+ and hLCAT− LDLr heterozygotes and homozygotes are given. In agreement with the plasma lipid determinations, the FPLC profile of hLCAT+/LDLr+/− rabbits (A) was characterized by the presence of a large CE- and PL-enriched HDL, analogous to HDL_1, the levels of which were markedly elevated relative to the hLCAT−/LDLr+/− group (B). In contrast, IDL+LDL cholesterol concentrations were significantly lower in LDLr heterozygotes overexpressing hLCAT relative to those of their nontransgenic controls. Thus, HDL was the predominant lipoprotein class in the plasma of hLCAT+/LDLr+/− rabbits, whereas the presence of CE-rich apoB-containing lipoproteins characterized the plasma of nontransgenic LDLr heterozygotes. As shown in panels C and D, the latter was, likewise, the case for LDLr homozygotes, independent of the degree of LCAT expression, such that apoB-containing lipoproteins were far more

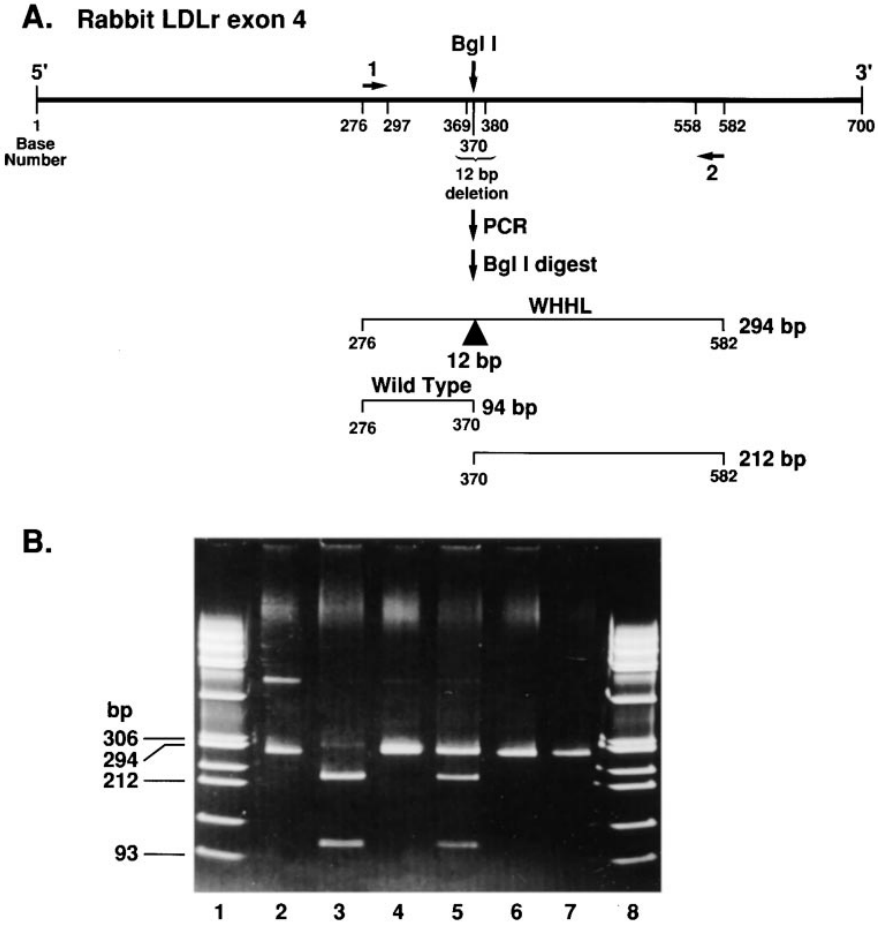

Fig. 1. Determination of LDL receptor status by PCR. A 306 bp portion of exon 4, including the 12 bp mutant region from 369 to 380 which contains a *Bgl* I restriction site, was amplified with primers 1 and 2, and PCR products were gel-purified prior to overnight digestion with *Bgl* I at 37°C. Molecular weight markers are shown in lanes 1 and 8. Lane 2 shows the undigested PCR product of an LDLr+/+ rabbit prior to gel purification. Subsequent to gel-purification and *Bgl* I digestion, LDLr+/+ rabbits generated two products of 212 and 94 bp in size (lane 3), whereas LDLr+/− rabbits generated two (306 and 294 bp) prior to digestion (lane 4) and three (294, 212, and 94 bp) after *Bgl* I digestion (lane 5). LDLr−/− animals, however, generated only one product (294 bp) both before (lane 6) and after (lane 7) *Bgl* I digestion due to the presence of the 12 bp deletion in both LDLr alleles.

prevalent in the plasma of both hLCAT+ and hLCAT- LDLr homozygotes than were HDL. However, an enrichment of CE and PL was also noted in the HDL of hLCAT+/LDLr−/− rabbits.

Isotopic distribution of apoA-I

The distribution of radiolabeled rabbit apoA-I in the 1-h post-injection plasma samples of LDLr heterozygous and homozygous control and hLCAT overexpressing rabbits was as-

TABLE 1. Plasma LCAT activity and lipids of control and hLCAT transgenic heterozygous and homozygous LDLr-deficient rabbits

Group	LCAT Activity	TC	FC	CE	HDL-C	Non HDL-C	PL	TG
	nmol/ml per h				*mg/dl*			
LDLr+/−								
hLCAT+	297 ± 57[d]	76 ± 14	13 ± 3	59 ± 14	62 ± 8[c]	14 ± 6[c]	68 ± 7	17 ± 1[b]
hLCAT−	55 ± 11	84 ± 11	26 ± 5	58 ± 6	21 ± 1	63 ± 13	95 ± 7	50 ± 6
LDLr−/−								
hLCAT+	219 ± 50[c]	602 ± 31	138 ± 11	464 ± 22	27 ± 2[a]	575 ± 31	305 ± 16	69 ± 16[c]
hLCAT−	62 ± 4	554 ± 20	163 ± 7	391 ± 13	5 ± 1	549 ± 20	350 ± 14	157 ± 18

Values are mean ± SEM (n = 3 per group). Plasma lipid values represent the mean of at least three different determinations for each animal. Data for hLCAT transgenic rabbits were compared to those of nontransgenic control rabbits of the same LDLr status.

[a] $P < 0.0004$; [b] $P < 0.007$; [c] $P < 0.03$; [d] $P < 0.05$.

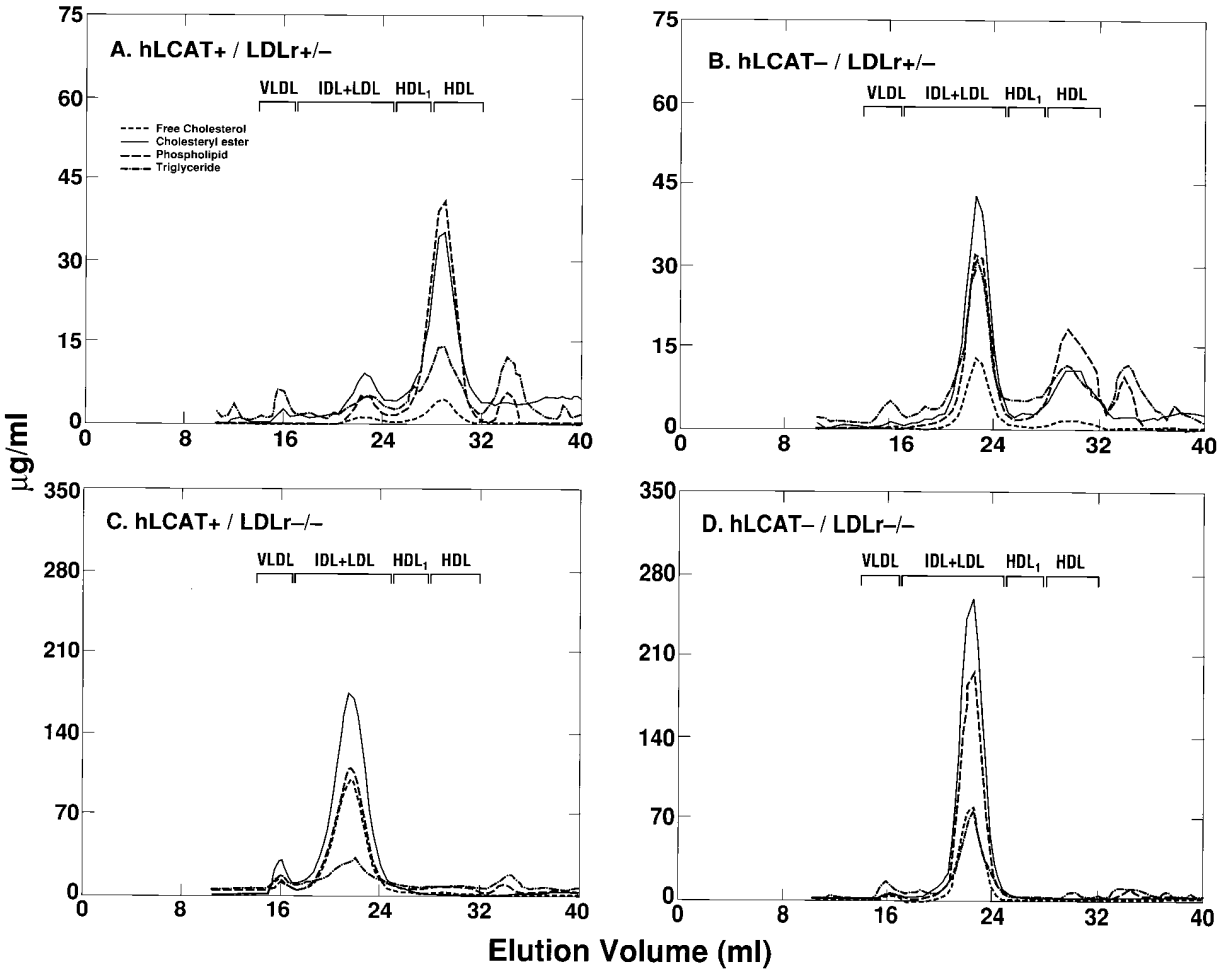

Fig. 2. Compositional analysis of gel filtration chromatography fractions. Panels A–D illustrate the concentrations of free cholesterol, cholesteryl ester, phospholipid, and triglyceride in the FPLC fractions of representative hLCAT+/LDLr+/− (A), hLCAT−/LDLr+/− (B), hLCAT+/ LDLr−/− (C), and hLCAT−/LDLr−/− rabbits (D). HDL was the predominant lipoprotein class in the plasma of hLCAT+/ LDLr+/− rabbits, whereas the presence of cholesteryl ester-rich apoB-containing lipoproteins characterized the plasma of hLCAT−/ LDLr+/−, hLCAT+/ LDLr−/−, and hLCAT−/LDLr−/− animals.

sessed by gel filtration chromatography, as depicted in **Fig. 3**, panels A and B, respectively. In both the LDLr+/− and LDLr−/− groups, the animals overexpressing hLCAT had a shift in apparent HDL particle size, such that their HDL were larger than those of their respective nontransgenic controls. These data also revealed an incremental shift in HDL size which correlated with both the level of LCAT expression and LDLr status, with hLCAT+/LDLr+/− rabbits having the largest HDL followed by hLCAT+/LDLr−/− > hLCAT−/ LDLr+/− > hLCAT−/ LDLr−/−. As expected, radiolabeled apoA-I was only associated with HDL in vivo.

Two-dimensional electrophoresis

As shown in **Fig. 4**, panels A and B, the HDL size shifts observed in LDLr+/− and LDLr−/− rabbits overexpressing hLCAT were confirmed by two-dimensional lipoprotein electrophoresis using fresh plasma samples, subsequent to immunolocalization with a monoclonal antibody directed against rabbit apoA-I. The relatively larger HDL of the LDLr heterozygotes overexpressing hLCAT can be observed on the right-hand side of panel A. The most intriguing observation of this analysis was the presence of a large pre-β_2 HDL particle in the plasma of hLCAT+/ LDLr+/− rabbits, a particle that was not present in the plasma of their nontransgenic controls. No differences were noted in the pre-β_1 subpopulations of HDL in LDLr heterozygotes. In contrast, when assessing the results in panel B, it is apparent that, in addition to having a larger α-migrating HDL, hLCAT+/LDLr−/− rabbits had an increase in pre-β_1-migrating HDL, as compared with their nontransgenic controls. Not only were these levels elevated in hLCAT+/ LDLr−/− animals, but increased heterogeneity in this subpopulation was observed as well. Interestingly, both groups of LDLr−/− rabbits had increased concentrations of pre-β_1-migrating HDL relative to LDLr+/− rabbits of the same LCAT status.

Metabolic parameters of HDL apoA-I

The kinetic parameters of HDL apoA-I are shown in **Table 2**. Within the LDLr+/− group, the mean plasma apoA-I pool size was dramatically increased (283%) in the rabbits overexpressing hLCAT, as compared with the one

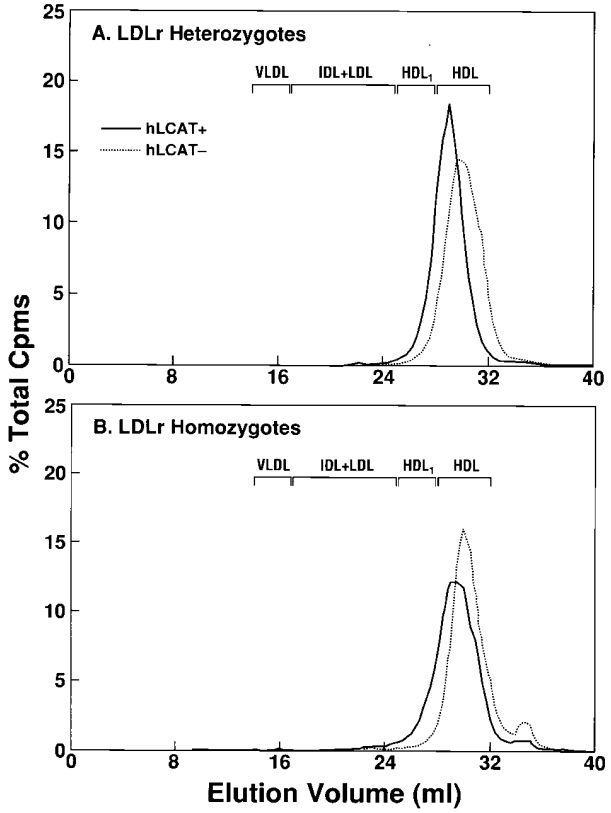

Fig. 3. Distribution of radiolabeled apoA-I in the 1-h post-injection plasma samples of representative hLCAT+/LDLr+/−, hLCAT−/LDLr+/−, hLCAT+/ LDLr−/−, and hLCAT−/LDLr−/− rabbits, as assessed by gel filtration chromatography. In both the LDLr+/− (A) and LDLr−/− (B) groups, the hLCAT transgenic animals had an increase in apparent HDL particle size relative to that of their respective control group.

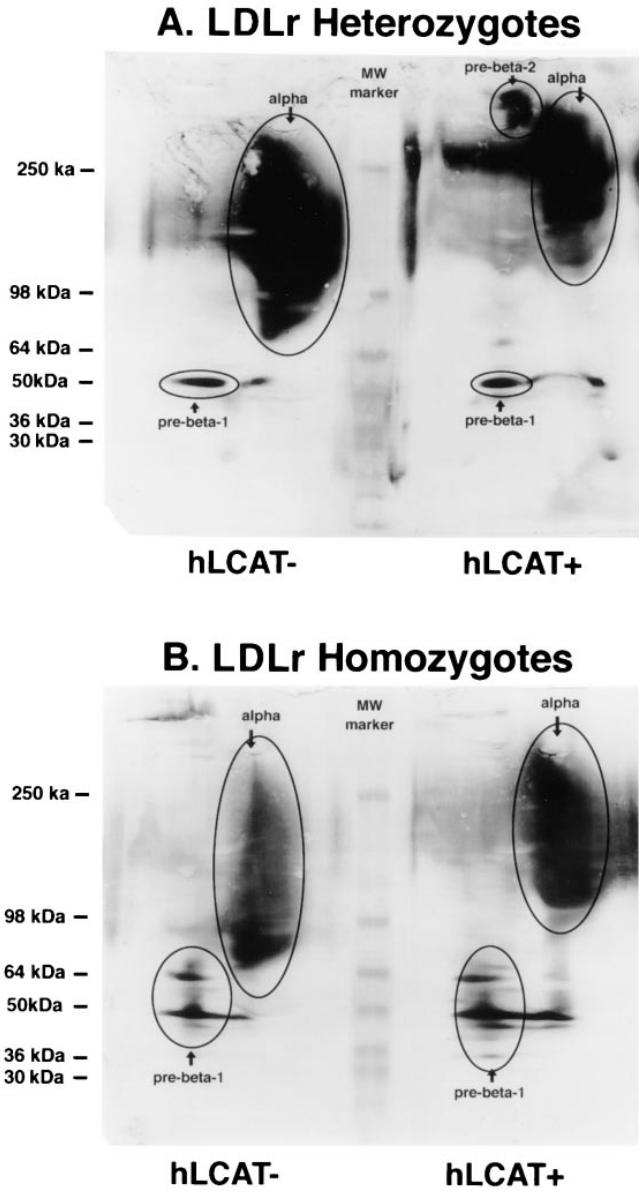

Fig. 4. Two-dimensional lipoprotein electrophoresis. Twenty microliters of whole rabbit plasma as applied to a 0.7% agarose gel (first dimension), followed by separation on a 2–36% nondenaturing polyacrylamide gradient gel (second dimension), as described in Methods. In addition to having larger α-migrating HDL, the plasma of hLCAT+/LDLr+/− rabbits (A) was distinguished by the presence of a large pre-β_2 particle that was not present in the plasma of their nontransgenic controls. In contrast, hLCAT+/LDLr−/− rabbits (B) had an increase in pre-β_1-migrating HDL relative to their controls, with no pre-β_2 observed in either hLCAT+ or hLCAT- LDLr homozygotes.

age-matched littermate male which was studied. This increase in apoA-I pool size was due to the combined effects of both increased apoA-I synthesis and delayed apoA-I catabolism. The apoA-I PR of LCAT+/LDLr+/− rabbits was almost twice that of their nontransgenic control, while, additionally, their mean apoA-I FCR was reduced by 36%. The latter corresponded with apoA-I residence times of 65 and 41 hours, respectively, for LCAT+/LDLr+/− and LCAT−/LDLr+/− animals.

Analogous results were obtained when the LDLr−/− groups were compared. In this case, the mean apoA-I pool size was four times greater in the animals overexpressing hLCAT relative to that of their nontransgenic littermates. This significant elevation in circulating apoA-I levels was due to both the approximately 2.5-fold increase in apoA-I PR, as well as to the 44% reduction in apoA-I clearance. The mean apoA-I residence time for the LCAT+/LDLr−/− group was 36 h, whereas that of the LCAT−/LDLr−/− group was 21 h. As illustrated in **Fig. 5**, the LCAT+/LDLr−/− group cleared apoA-I at a rate similar to that of the LCAT−/LDLr+/− rabbit, indicating that the presence of LCAT significantly improved the hypercatabolism of apoA-I associated with complete LDLr deficiency. Taken together, the preceding indicates that LCAT improves the hypoalphalipoproteinemia observed in LDLr deficiency by both delaying apoA-I catabolism and increasing apoA-I synthesis.

Relationships between LCAT activity, apoA-I metabolic parameters, and plasma lipids

The results of linear regression analyses are shown in **Table 3**. A strong positive correlation was observed between α-LCAT activity and plasma HDL-C concentration, whereas a significant negative correlation was noted be-

TABLE 2. Metabolic parameters of HDL apoA-I in control and hLCAT transgenic heterozygous and homozygous LDLr-deficient rabbits

Group	ApoA-I Pool Size	ApoA-I PR	ApoA-I FCR
	mg/kg	*mg·kg·d*$^{-1}$	*pools/d*
LDLr+/−			
hLCAT+	85 ± 19	10.2 ± 1.7	0.376 ± 0.025
hLCAT−	30	6.0	0.588
LDLr−/−			
hLCAT+	33 ± 1[a]	8.5 ± 0.7[b]	0.666 ± 0.033[c]
hLCAT−	8 ± 1	3.5 ± 0.4	1.194 ± 0.138

Values are mean ± SEM (n = 3 per group), with the exception of the hLCAT−/LDLr+/− group where only 1 age-matched littermate male was studied. Data for hLCAT transgenic rabbits were compared to those of nontransgenic control rabbits of the same LDLr status.

[a]$P < 0.0001$; [b]$P < 0.004$; [c]$P < 0.02$.

TABLE 3. Correlation Coefficient Analysis

Parameter	LCAT Activity	ApoA-I FCR	ApoA-I PR
LCAT activity		−0.774[d]	0.771[d]
HDL-C	0.857[a]	−0.812[b]	0.751[d]
nonHDL-C	−0.697[e]	0.709[e]	−0.392
FC	−0.786[d]	0.814[c]	−0.577
CE	−0.609	0.623	−0.249
PL	−0.798[c]	0.805[c]	−0.596
TG	−0.856[b]	0.827[c]	−0.810[d]

Data from the three animals of each group were pooled, for an n of 12 for most correlations, with the exception of those for apoA-I FCR and apoA-I PR where the data for the 2 females were not included in any of the analyses.

[a]$P < 0.001$; [b]$P < 0.005$; [c]$P < 0.01$; [d]$P < 0.02$; [e]$P < 0.04$.

tween α-LCAT activity and nonHDL-C concentration. The latter was, likewise, the case for the relationships between LCAT activity and all of the other plasma lipid constituents, with the strongest inverse association observed between LCAT activity and plasma triglyceride concentration. Both plasma LCAT activity and HDL-C were negatively associated with apoA-I clearance rate. Conversely, apoA-I FCR was positively correlated with nonHDL-C, as well as with plasma free cholesterol, phospholipid, and triglyceride concentrations. Significant positive correlations were noted between apoA-I PR and both LCAT activity and HDL-C. Interestingly, triglycerides were the sole plasma lipid parameter associated with apoA-I PR.

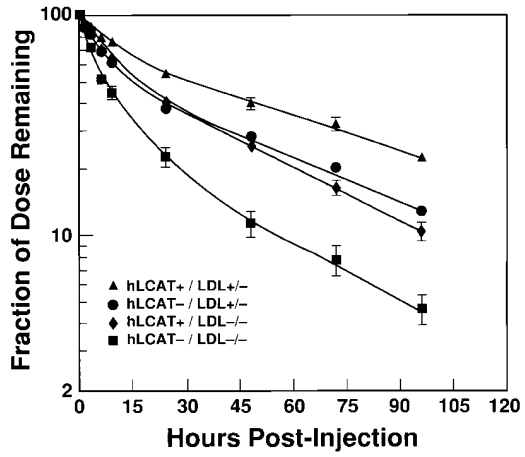

Fig. 5. In vivo metabolism of apoA-I. Purified rabbit apoA-I was radiolabeled and reassociated with autologous plasma, as described in Methods. The radioactivity decay curves for hLCAT+/LDLr+/− (▲), hLCAT−/LDLr+/− (●), hLCAT+/LDLr−/− (◆), and hLCAT−/LDLr−/− (■) rabbits are illustrated using a two-log scale as the ordinate. The plasma clearance of apoA-I was delayed in both groups of hLCAT+ rabbits relative to their respective controls. Moreover, the hLCAT+/LDLr−/− group cleared apoA-I at a rate similar to that of the hLCAT−/LDLr+/− rabbit, indicating that the presence of hLCAT significantly improved the hypercatabolism of apoA-I associated with complete LDLr deficiency.

DISCUSSION

Disorders of LDL and HDL metabolism underlie the pathogenesis of atherosclerotic vascular disease (33–36). Plasma concentrations of LDL and HDL cholesterol are inversely related in several dyslipoproteinemias. Because elevated LDL and reduced HDL cholesterol concentrations are each independent risk factors for coronary heart disease (CHD) (37–39), the potential exists for synergistic effects on disease pathophysiology. An example of this synergism is observed in patients with the autosomal codominant disease familial hypercholesterolemia. FH heterozygotes and homozygotes not only have LDL cholesterol concentrations that are elevated as much as 2- and 6-fold (1), respectively, but both also have significant reductions in HDL cholesterol and apoA-I concentrations. Within both the heterozygous and homozygous FH populations, plasma HDL cholesterol concentrations have been shown to be reciprocally related to the incidence and severity of CHD (2), with homozygotes having a much greater risk relative to heterozygotes. Hence, the low HDL cholesterol levels observed in FH patients may further increase their risk for CHD. This inverse association between LDL and HDL is also observed in the hyperlipidemic LDL receptor-defective rabbit, a well-defined model of human FH (6–8). Similar to their human counterparts, these animals have levels of HDL cholesterol and apoA-I which are abnormally low (5, 9, 10), providing an excellent model with which to investigate the metabolic consequences of various therapeutic interventions.

The strong inverse association that has consistently been demonstrated between plasma apoA-I concentrations and the risk of CHD has generated much interest in elucidating the precise mechanisms that modulate such concentrations in vivo. In general, plasma apoA-I concentrations are subject to regulation at the levels of *a*) synthesis, *b*) catabolism, and *c*) lipoprotein particle remodeling. Critical to these processes are a number of enzymes, including CETP and LCAT. LCAT is the key enzyme involved in the esterification of intravascular free cholesterol and is essential to proper HDL particle maturation. We have previously demonstrated that overexpression of human LCAT in transgenic NZW rabbits resulted in significant eleva-

tions of plasma HDL cholesterol and apoA-I concentrations, the extent of which correlated directly with LCAT gene expression. Thus, with the preceding concepts in mind, the present studies were designed to test the hypothesis that overexpression of human LCAT in WHHL rabbits would correct the hypoalphalipoproteinemia associated with LDL receptor deficiency.

The metabolic basis of the hypoalphalipoproteinemia in FH has been examined in both humans and rabbits. Using stable isotope methodology, Schaefer et al. (4) demonstrated that the reduced concentrations of HDL and apoA-I in a homozygous FH patient were due to the dual metabolic defects of hypercatabolism and decreased synthesis. Similar results were reported by Saku et al. (5) in the WHHL rabbit. The study reported here further establishes that the hypoalphalipoproteinemia observed in FH is the consequence of a combined metabolic defect. The mechanism by which a lack of functional LDL receptors may result in aberrant HDL clearance is uncertain, but one possible explanation involves the enzyme CETP. CETP activity has been shown to be elevated in LDLr-defective rabbits relative to normocholesterolemic controls (40). This, in concert with the expanded pool size of apoB-containing lipoproteins in FH, may result in a greater net transfer of cholesteryl ester from HDL which, in turn, may lead to accelerated HDL catabolism. This concept is supported by the demonstration that patients with CETP deficiency have delayed, rather than accelerated, catabolism of apoA-I (25).

The most important finding of this study concerned the fact that in both heterozygous and homozygous LDLr-defective animals the combined metabolic defects of increased apoA-I catabolism and decreased apoA-I synthesis were corrected by the overexpression of human LCAT. Within the LDLr+/− group, the overexpression of hLCAT resulted in an almost 3-fold elevation in apoA-I pool size. This elevation was due to both the almost 2-fold increase in apoA-I production rate observed in LDLr heterozygotes overexpressing hLCAT, as well as to the 36% decrease in apoA-I FCR. This was similarly the case when the LDLr−/− groups were compared. Thus, hLCAT−/LDLr−/− rabbits had the fastest clearance rate of apoA-I, followed by hLCAT+/ LDLr−/− > hLCAT−/LDLr+/− > and hLCAT+/ LDLr+/− animals. The significant contribution of LCAT to the reduction in apoA-I catabolism in these animals was further made apparent by the fact that LDLr homozygotes overexpressing hLCAT cleared apoA-I at a rate similar to that of the nontransgenic LDLr heterozygote animal. Similarly, the mean apoA-I FCR of the hLCAT+/LDLr+/− group was significantly delayed relative to that which we previously reported in hLCAT−/LDLr+/+ (NZW) rabbits (17).

It has consistently been demonstrated that HDL particle size can influence the metabolism of apoA-I (17, 18, 26, 41, 42). In fact, one study has reported that as much as 70% of the variability in apoA-I FCR was due to variability in estimates of HDL size or density (42). Accordingly, the differences in apoA-I FCR observed in our study were, in part, explained by LCAT-induced compositional changes in HDL which, in turn, resulted in alterations in HDL particle size. Fast protein liquid chromatography analysis of plasma revealed that the HDL of both LDLr heterozyotes and homozygotes overexpressing hLCAT were enriched in cholesteryl ester and phosholipid, relative to those of their respective nontransgenic controls, resulting in increased HDL particle size. These changes in HDL particle size were inversely associated with the rate of HDL catabolism, with the large HDL of hLCAT+/LDLr+/− rabbits having the longest life span and the small HDL of hLCAT−/LDLr−/− rabbits the shortest. This finding is consistent with our previous study in NZW rabbits which showed that HDL particle size correlated directly with the level of hLCAT expression, but reciprocally with the rate of apoA-I catabolism (17).

Our data further indicate that hLCAT overexpression increased HDL cholesterol and apoA-I levels in LDL receptor defective rabbits not only by delaying apoA-I catabolism but by increasing apoA-I synthesis as well. Linear regression analysis indicated that plasma HDL cholesterol levels were significantly associated with both apoA-I FCR and apoA-I PR. This was not the case in our investigation with NZW rabbits expressing normal LDL receptors, where fractional catabolic rate was the predominant mechanism by which LCAT differentially modulated plasma HDL cholesterol and apoA-I concentrations (17). The mechanism responsible for the reduction in apoA-I synthesis in FH is not known, nor is the mechanism by which overexpression of hLCAT in LDLr defective animals increases apoA-I synthesis readily apparent. Saku et al. (43) have reported that hepatic apoA-I mRNA levels in WHHL rabbits were significantly lower than in normal rabbits; however, as most of the circulating apoA-I in the rabbit is intestinally derived, the significance of this finding is unclear. Interestingly, transient reconstitution of LDLr function after infusion of a recombinant adenovirus encoding the human LDL receptor gene in LDLr defective rabbits has been shown to increase plasma apoA-I concentrations, suggesting that a gene–gene interaction may exist between apoA-I and the LDL receptor (44). Consistent with this hypothesis is the work of Mitchell, Fidge, and Griffiths (45), which demonstrated that the treatment of rats with drugs that up-regulated the LDL receptor resulted in a marked increase in apoA-I mRNA levels, and that of Monge and colleagues (46), which showed that LDL uptake by HepG2 cells led to increased levels of apoA-I mRNA. As plasma LCAT activity was inversely associated with nonHDL-C concentrations in this study, the possibility that LCAT overexpression increased apoA-I mRNA abundance in heterozygous animals via up-regulation of the LDL receptor pathway cannot be excluded.

Unexpectedly, we observed that hLCAT overexpression significantly reduced plasma triglyceride concentrations in LDLr+/− and LDLr−/− rabbits. Preliminary studies in these animals have shown that this is, in part, due to accelerated VLDL apoB-100 catabolism. Thus, hLCAT overexpression may up-regulate both VLDL and LDL receptor-mediated pathways. Such alterations in catabolism may be the result of LCAT's ability to influence the phospholipid

and triglyceride content of lipoprotein particles, as well as of cell membranes. Moreover, LCAT-induced changes in VLDL composition could also potentially generate more effective substrates for lipolysis, leading to reduced plasma triglyceride concentrations such as those noted in our study. Alternatively, it is feasible that overexpression of hLCAT, the highest levels of which were in the liver, may have directly influenced VLDL production by increasing intracellular degradation of apoB-100.

Another intriguing finding of our study was the difference observed in pre-β-migrating HDL subpopulations in the LDLr heterozygous and homozygous rabbits overexpressing hLCAT. Pre-β-HDL are thought to function as carriers of cholesterol during the early steps of reverse cholesterol transport, perhaps functioning as the initial acceptors of cellular cholesterol (47–49). This may have significant implications with respect to atherosclerosis susceptibility in this animal model. Although we have previously shown that overexpression of hLCAT in rabbits is anti-atherogenic (50), while in mice it is proatherogenic, further experiments have revealed potential explanations for this apparent discrepancy. Briefly, the enhanced atherosclerosis observed in hLCAT transgenic mice relative to littermate controls was due to the presence of dysfunctional HDL which were less capable of transporting cholesterol to the liver, thus adversely affecting reverse cholesterol transport. Moreover, hLCAT transgenic rabbits, unlike hLCAT transgenic mice, have reduced concentrations of proatherogenic apolipoprotein B-containing lipoproteins, as compared with control animals. Both of the former may be attributable to a key metabolic difference that exists between mice and rabbits, namely the absence of CETP in the mouse. In support of this concept, we recently reported that LCAT × CETP transgenic mice have less atherosclerosis than do hLCAT transgenic mice when fed an atherogenic diet (51). Therefore, we believe that LCAT remains a potential gene therapy candidate for patients with hypoalphalipoproteinemia, as well as for those with FH.

In summary, these data indicate that hLCAT overexpression in LDLr defective rabbits increases HDL cholesterol and apoA-I concentrations by both delaying apoA-I catabolism and increasing apoA-I synthesis; thus, correcting the dual metabolic defects responsible for the hypoalphalipoproteinemia observed in LDLr deficiency. These results identify LCAT as a potential gene therapy candidate for patients with FH.

The authors are grateful to Darlene Allen, Tina Diers, and Santana Flores for their technical assistance.

Manuscript received 21 October 1997, and in revised form 12 January 1998, and in re-revised form 18 March 1998.

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