Lecithin:Cholesterol Acyltransferase Overexpression Generates Hyperalpha-lipoproteinemia and a Nonatherogenic Lipoprotein Pattern in Transgenic Rabbits*

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Cholesterol esterification within plasma lipoprotein particles is catalyzed by lecithin:cholesterol acyltransferase (LCAT). The impact of the overexpression of this enzyme on plasma concentrations of the different plasma lipoproteins in an animal model expressing cholesteryl ester transfer protein was evaluated by generating rabbits expressing human LCAT. A 6.2-kilobase human genomic DNA construct was injected into the pronuclei of rabbit embryos. Of the 1002 embryos that were injected, 3 founder rabbits were characterized that expressed the human LCAT gene. As in mice and humans, the principal sites of mRNA expression in these rabbits is in the liver and brain, indicating that the regulatory elements required for tissue-specific expression among these species are similar. The α-LCAT activity correlated with the number of copies of LCAT that integrated into the rabbit DNA. Compared with controls, the high expressor LCAT-transgenic rabbits total and high density lipoprotein (HDL) cholesterol concentrations were increased 1.5–2.5-fold with a 3.1-fold increase in the plasma cholesterol esterification rate. Analysis of the plasma lipoproteins by fast protein liquid chromatography indicates that these changes reflected an increased concentration of apolipoprotein E-enriched, HDL1-sized particles, whereas atherogenic apolipoprotein B particles disappeared from the plasma. The concentrations of plasma HDL cholesterol were highly correlated with both human LCAT mass (r = 0.93; p = 0.001) and the log LCAT activity (r = 0.94; p < 0.001) in the transgenic rabbits. These results indicate that overexpression of LCAT in the presence of cholesteryl ester transfer protein leads to both hyperalphalipoproteinemia and reduced concentrations of atherogenic lipoproteins.

The esterification of cholesterol with fatty acid in the plasma is mediated by the enzyme lecithin:cholesterol acyl transferase (LCAT)† (1). Sperry first demonstrated that a plasma enzyme could both esterify and de-esterify cholesterol in 1935 (2). However, the physiologic impact of this enzyme in vivo awaited the first descriptions of LCAT deficiency by Norum and Gjone in 1967 (3). By evaluating the plasma LCAT activity in controls and in patients with deficient plasma LCAT activity, Glomset (1) suggested that the LCAT reaction, occurring on the surface of HDL particles, was key to the net transport of cholesterol from peripheral tissues to the liver (4). This 63-kDa protein circulates in the plasma bound to lipoprotein particles and converts cholesterol and phosphatidylcholine to cholesteryl esters and lysophosphatidylcholine (5). A great deal has been learned about the activity, substrate characteristics, and importance of cofactors of this enzyme using in vitro assays (6–9). The physiologic role of this enzyme in vivo has been inferred from patients lacking LCAT activity in their plasma (3). These individuals have severely depressed concentrations of HDL cholesterol and accumulate cholesterol in specific tissues (5). Therefore the esterification of cholesterol in the plasma by LCAT is important in both HDL metabolism and in human cholesterol homeostasis.

The recent cloning of the human LCAT gene (10) has permitted further understanding of the LCAT enzyme itself as well as the ability to explore the physiologic and pathophysiologic consequences of differing degrees of LCAT expression. The characterization of mutations underlying LCAT deficiency have structure-function implications for this enzyme (11, 12). In addition, we have undertaken the investigation of the impact of LCAT overexpression on plasma lipoprotein metabolism. Overexpression of human LCAT in transgenic mice led to elevations in both total and HDL cholesterol concentrations (13, 14). The enhanced esterification of cholesterol in the plasma of transgenic mice led to the accumulation of large HDL1-sized particles with a high correlation between LCAT activity and HDL cholesterol concentrations in the plasma. Therefore reduced plasma LCAT activity leads to hypoalphalipoproteinemia and increased LCAT activity generates hyperalphalipoproteinemia in vivo.

After the initial esterification of cholesterol by LCAT on HDL particles, the metabolism of cholesteryl ester within HDL is modulated by cholesteryl ester transfer protein (CETP) (15). This plasma protein leads to the net transfer of cholesteryl ester from HDL particles to apoB particles in exchange with triglycerides that also reside in the hydrophobic particle core. CETP is present in man as well as in animal models that develop atherosclerosis, and it is absent in those animal species resistant to atherogenesis (15). Within animal species that

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‡ The abbreviations used are: LCAT, lecithin:cholesterol acyl transferase; HDL, high density lipoproteins; VLDL, very low density lipoproteins; LDL, low density lipoproteins; CETP, cholesteryl ester transfer protein; apoA-I, apolipoprotein A-I; apoA-I, apolipoprotein B; FPLC, fast protein liquid chromatography.
develop arterial lesions, plasma CETP activity has been correlated with the severity of atherogenesis. The metabolism of HDL cholesterol ester in man involves both CETP and LCAT. In order to evaluate the potential protection conferred by the hyperalpalipoproteinemia induced by LCAT overexpression, we have extended our studies to include the rabbit, which both expresses CETP and develops diet-induced atherosclerosis.

Rabbits have long been used in lipoprotein and atherosclerosis research (16). The methods for generating transgenic mice has been extended to rabbits. Using the human LCAT gene that was successful in overexpressing functional enzyme in transgenic mice, we have developed transgenic rabbits expressing this human gene in the presence of substantial plasma CETP activity. The overexpression of human LCAT leads to hyperalpalipoproteinemia, reflecting an increased concentration of HDL \( \alpha \), that directly correlates with the degree of LCAT expression. In addition, in the presence of CETP, LCAT overexpression led to a reduction in the concentration of apolipoprotein B particles. These changes in the plasma lipoproteins may change not only the plasma cholesterol ester metabolism but may alter the susceptibility these animals have for the development of atherosclerosis.

### MATERIALS AND METHODS

Animals—New Zealand White rabbits were purchased from Hazelton Laboratories (Denver, PA). Rabbits were housed in separate cages. The use of the research protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. Generation and Characterization of Transgenic Rabbits—Embryos were isolated from superovulated does aged 7–14 months 16–24 h after mating. Donor does were pre-anesthetized with ketamine (33 mg/kg), xylazine (5 mg/kg), and glycopyrrolate (1.1 μg/kg), intubated, and placed on isoflurane inhalation anesthesia. During the harvesting laparotomy, embryos were obtained from the donor does by retrograde flushing of the oviducts. Those embryos in the one cell stage were micromanipulated with 1 pl of solution containing approximately 1,000 copies of human LCAT genomic DNA (5 × 10\(^{-3}\) pg) as previously outlined. Recipient does were mated with vasectomized male rabbits the day prior to implantation. After the embryos had been harvested from the does and injected with DNA, the recipient does underwent laparotomy, and 10–25 injected embryos were aseptically inserted into the recipient oviducts.

Identification of kits with integration of human LCAT DNA was assessed by Southern blot analysis. After the weaning at 5–7 weeks of age, DNA was isolated from snippets of tails from the potentially transgenic kits. Southern blots were performed using 10 μg of rabbit DNA that had been digested with PsI. Hybridization with a 446-base pair fragment of the LCAT gene was performed as previously outlined (13). The number of LCAT copies per genome was quantitated by comparing the intensity of the hybridization signal by both direct Betagen scintigraphy and by scanning laser densitometry (LK,B, Sweden). The degree of hybridization of transgenic samples was compared with the intensity of the hybridization signal with human LCAT DNA standards.

The sites of expression of the transgene were identified by detecting the gene in the RNA of the tissues. After phenobarbital-induced euthanasia, the RNA species from LCAT transgenic rabbit spleen, liver, kidney, intestine, heart, brain, muscle, lung, and adrenal gland were isolated. After separation by 0.7% formaldehyde-agarose gel electrophoresis and transfer to a nitrocellulose membrane, the sample RNA was probed with the same \(^{32}\)P-labeled 446-base pair LCAT probe and the 412-base pair DraI–RsaI fragment (17) of the human β-actin cDNA (18). The nitrocellulose membrane was autoradiographed using Kodak XAR-2 film.

### TABLE I

| Founder line | Integrated copy number | LCAT mass | LCAT activity | HDL-C |
|--------------|------------------------|-----------|---------------|-------|
| T-1          | 1436                   | 54.0      | 3217          | 106   |
| T-2          | 284                    | 4.2       | 723           | 64    |
| T-3          | 38                     | 1.9       | 219           | 18    |
| Control      | 68                     | 202±37    | 25±2          |       |

Plasma Lipid and Lipoprotein Analyses—From 5–7 ml of blood was drawn on rabbits after a 12-h fast. EDTA plasma was analyzed for total cholesterol and triglyceride concentrations (Sigma) and free cholesterol and phospholipids (Wako Chemicals U. S. A., Inc., Richmond, VA) using enzymic methods on a Hitachi 911 Autoanalyzer (Boehringer-Mannheim, Indianapolis, IN). The esterified cholesterol was calculated by subtracting the free from the total cholesterol. The HDL cholesterol concentration was determined on plasma that had been diluted with phosphate-buffered saline (1:1, v/v) and then precipitated with dextran sulfate (19). The HDL cholesterol was calculated by subtracting the cholesterol concentration in the supernatant after precipitation from the total plasma cholesterol concentration.

Rabbit apoA-I concentrations in plasma were determined using a mouse monoclonal anti-rabbit antibody in an enzyme-linked immunosorbent assay. The cholesterol esterification rate was determined in duplicate 250-μl plasma samples by evaluating the incorporation of \(^{14}\)C cholesterol into cholesteryl ester in the presence of control and transgenic rabbit plasma as originally outlined by Stokke and Norum (20). LCAT mass was determined by radiomunnoassay (21), and α-LCAT activity was determined using 10 μl of plasma in a proteoliposome assay (22).

Plasma CETP activity was determined by assessing the transfer of \(^{14}\)C-labeled HDL \( \alpha \) cholesteryl ester to the \( < 1.060 \) g/ml lipoproteins as described by Albers and co-workers (23). This was performed after incubation for 18 h at 37°C with or without the addition of 5 μl of plasma as a source of CETP. The HDL \( \alpha \) and the \( < 1.060 \) g/ml lipoproteins were separated by heparin-MnCl\(_2\) precipitation, and the radioactivity in the supernatant (HDL \( \alpha \)) was then determined. The increase in CETP activity was linearly increased during 18 h of incubation.

### Statistical Analyses

- **Remodeling of Lipoproteins in LCAT Transgenic Rabbits**
- **Identification of kits with integration of human LCAT DNA** was assessed by Southern blot analysis. After the weaning at 5–7 weeks of age, DNA was isolated from snippets of tails from the potentially transgenic kits. Southern blots were performed using 10 μg of rabbit DNA that had been digested with PsI. Hybridization with a 446-base pair fragment of the LCAT gene was performed as previously outlined (13). The number of LCAT copies per genome was quantitated by comparing the intensity of the hybridization signal by both direct Betagen scintigraphy and by scanning laser densitometry (LK,B, Sweden). The degree of hybridization of transgenic samples was compared with the intensity of the hybridization signal with human LCAT DNA standards.

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RESULTS

Embryos were harvested from does that had undergone superovulation. Of the 1704 embryos that were harvested, 59% were in the cell stage, lacked a mucinous coat, and were injectable. Of these, 5% led to live births and 0.5% integrated the human genomic LCAT DNA. Of the five transgenic founder animals, three expressed the human LCAT gene. The three transgenic lines T-1, T-2, and T-3 all integrated human LCAT DNA that lead to the 690-base pair insert (data not shown).

Using known concentrations of construct DNA, the number of copies of LCAT DNA that integrated could be calculated with the copy number for these three lines and ranged from 38 to 1436 copies per genome.

The large variation number of copies of LCAT that integrated into the three founder LCAT transgenic rabbit varied reflected marked differences in the degree of LCAT expression in the plasma in these animals (Table I). The integrated copy number was correlated with the plasma LCAT mass (r = 0.86). In addition, the human LCAT mass correlated well with both the α-LCAT activity (r = 0.99) and the HDL cholesterol concentration (r = 0.76) in these three founder animals. Although the high expressing transgenic line integrated an astonishing number of copies, this high copy number is most likely not required for high levels of expression, because the high copy number most likely represents serial tandem repeats that do not function to the same extent.

The sites of expression of human LCAT in these rabbits was evaluated by Northern blot analysis (Fig. 1). Liver, heart, brain, and muscle all have detectable quantities of LCAT mRNA. Compared with the β-actin mRNA, which served as a control for message expression for the tissues analyzed, the liver was the principal site of expression. The brain also had a considerable degree of expression. This tissue distribution is similar to that of LCAT transgenic mice (13). These rabbits appear to be neurologically intact and have no aberrant behavior.

The marked changes in the plasma LCAT activity were paralleled by changes in the plasma lipid and lipoprotein concentrations in these animals (Table II). The greater the degree of LCAT expression, the higher the concentrations of the plasma total (r = 0.92), free (r = 0.86), and esterified cholesterol (r = 0.94) in littermate controls at the time of weaning. In addition, the ratio of cholesteryl ester to free cholesterol was higher with greater LCAT mass (r = 0.98) and LCAT activity (r = 0.95). Apolipoprotein A-I and plasma phospholipid concentrations also increased in parallel with the rises in the plasma cholesterol levels.

The differences in the concentrations of these plasma lipid constituents reflect changes in the plasma lipoproteins induced by LCAT overexpression in these rabbits. Plasma lipoproteins were characterized by Superose-6 column chromatography (Fig. 2). Nontransgenic NZW rabbits, shown in the solid lines in Fig. 2, have the plasma total, free, and esterified cholesterol distributed in particles corresponding to VLDL, IDL + LDL, and HDL (Fig. 2a). The lowest level of LCAT expression present in T-3 did not result in a significant change in the FPLC profile compared with nontransgenic controls (data not shown). In contrast, the highest expressing transgenic founder T-1 has virtually no detectable cholesterol in VLDL and IDL + LDL. There is also a virtual absence of cholesterol in particles the size typical of HDL. The marked elevation in total, free, and esterified cholesterol in this rabbit is in large HDL$_1$ particles. The transgenic rabbit with an intermediate degree of LCAT expression, T-2, also has a shift of cholesterol to a larger HDL particle species but is intermediate to that of the high expressor.

The consistent changes in the distribution of cholesterol parallels the particle distribution of phospholipids and triglycerides (Fig. 2b). The high expressor transgenic has no detectable amount of either of these analytes outside of an HDL$_1$ particle distribution, whereas the intermediate expressor has a shift in the fraction containing phospholipids and triglycerides to a larger HDL particle. The distribution of the human LCAT, determined by immunoblot, is identical to the phospholipid distribution in the transgenic rabbits. The LCAT in the highest expressor is principally associated with the HDL$_1$ particles. However, some of the LCAT is not particle-associated. The intermediate level expressor has almost all of the human LCAT present in the large, phospholipid-rich HDL. Therefore human LCAT overexpression leads to a redistribution of lipids into large HDL-sized particles.

These changes in the particle distribution of the plasma lipid is associated with marked changes in the apolipoproteins present in these particles. Immunoblot analysis of the FPLC column fractions was performed for the principal rabbit apolipoproteins, apoA-I and apoB (Fig. 3). The major isoproteins of...
apoB, apoB-48, and apoB-100 were detected in the fractions containing VLDL and IDL + LDL in the fasted NZW control (Fig. 3, top panel). In contrast, apoA-I was detected to some extent in almost all of the column fractions, with most of the apoA-I detected in HDL. The apolipoprotein distribution in the FPLC fraction from T-1 (Fig. 3, bottom panel) differs from nontransgenic control for both apoB and apoA-I. No apoB can be detected from the immunoblot from T-1. These results extend to the progeny of this rabbit. The plasma apoB concentration is markedly reduced in rabbits expressing more than 6-fold the LCAT activity shown in control rabbits. In addition, the apoA-I particle distribution has shifted to the larger HDL1 particles.

The changes in the apoA-I and apoB are paralleled by changes in plasma apoE (Fig. 4). Using sequential immunoblots for apoA-I and apoE in FPLC fractions containing LDL, HDL1, and HDL particles, a high expressing LCAT transgenic from the T-1 line has detectable apoE in both LDL and HDL1. An LCAT transgenic rabbit with low levels of expression has faintly detectable amounts of apoE, but it is evident only the HDL1 particles. In contrast, no detectable apoE is present in any of these fractions in nontransgenic control rabbits. These changes indicate that human LCAT overexpression leads to marked changes in apoB, apoE, and apoA-I particle metabolism.

LCAT-T1 is a male and the LCAT gene was expanded into his F1 generation. Because all of the male progeny of this animal expressed human LCAT at a high level, the principal site of integration is into the Y chromosome. The plasma lipid and lipoprotein concentrations of the male offspring of LCAT-T1 are summarized in Table III and compared with age-matched nontransgenic males on a standard rabbit chow diet. As with the original founder, the total, free, and esterified cholesterol concentrations are significantly increased, whereas the ratio of cholesteryl ester to free cholesterol is unchanged. Triglyceride concentrations were unchanged, and the phospholipid and HDL cholesterol concentrations were significantly increased, just as in the founder rabbit. The cholesterol esterification rate and the cholesteryl ester transfer protein activity were also determined in this F1 generation. These animals had a 3-fold increase in the cholesteryl esterification rate. As expected, these rabbits had a substantial degree of CETP activity. Overexpression of LCAT did not alter the CETP activity.

The correlations of the plasma LCAT activity and human LCAT mass were evaluated on the F1 generation of LCAT-Tg1. Human LCAT mass and the log LCAT activity were both highly and significantly correlated with total (r = 0.92, p < 0.001; r = 0.94, p < 0.001), free (r = 0.66, p < 0.05; r = 0.73, p < 0.03), and esterified cholesterol (r = 0.91, p < 0.001; r = 0.91, p < 0.001) concentrations. The HDL cholesterol (r = 0.92, p < 0.001) and apoA-I concentrations (r = 0.62, p < 0.04) were also significantly correlated with the log LCAT activity. The ratio of cholesteryl ester to free cholesterol, phospholipids, and CETP were not correlated with the level of LCAT expression. Of interest, the fasting plasma triglyceride concentrations were inversely correlated with the level of log LCAT activity (r = −0.43; p =...
DISCUSSION

The generation of transgenic animals overexpressing LCAT was undertaken in order to address several remaining questions regarding this enzyme's role in lipoprotein metabolism. Is this enzyme rate-limiting in the metabolic pathways of HDL, LDL, and plasma cholesterol? Does overexpression of LCAT lead to a recognizable lipoprotein phenotype? What other gene products interact with LCAT in modulating lipoprotein metabolism? Finally, can alteration of plasma LCAT activity affect reverse cholesterol transport and atherogenesis? The overexpression of the LCAT gene in different animal models for lipoprotein metabolism can address these issues (27). We first overexpressed human LCAT in the mouse (13). The degree of LCAT overexpression in the mouse correlated with both the size and concentration of the HDL particles in these animals (13, 14). There are several reasons for extending these studies in LCAT overexpression in mice to the rabbit: 1) Rabbit VLDL are similar in their chemical composition, apolipoprotein content, and electrophoretic mobility with agarose gel electrophoresis to human VLDL (28). 2) ApoB is evident in rabbit IDL and LDL closely resembling that seen in man (28). 3) Like man, the rabbit expresses CETP, which not only permits the transfer of HDL-derived cholesteryl ester to apoB-containing lipoprotein particles, it also is likely to play a role in the diet-induced atherosclerosis that rabbits develop (15). 4) The atherosclerosis in the rabbit not only resembles that of human arterial disease (29) but also the macrovascular lesions that develop in the rabbit can be detected and quantitated in vivo (30).

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As in the mouse, overexpression of LCAT in the rabbit leads to elevated concentrations of HDL cholesterol (Tables I and III). This hyperalphalipoproteinemia reflected a dose-response relationship with the degree of human LCAT mass and total LCAT activity in the plasma. These particles are larger than typical HDL particles (Fig. 3a) and are associated with phospholipid, LCAT, and apoA-I (Fig. 3b and Table II). Because rabbits do not express apoA-II (31), these apoA-I only particles may be particularly effective in removing free cholesterol from cells (32). The increased concentration of apoA-I and the increased size of these particles would be expected to lead to a reduced removal of apoA-I-containing particles from the circulation (33, 34).

In contrast to the mouse, the rabbit has very high levels of CETP activity (35). The substantial CETP activity (17 ± 1% [5 μl/18 h]) in the plasma of transgenic rabbits leads to transfer of nascent cholesteryl ester to apoB-containing lipoprotein particles. This may then affect the metabolism of the apoB particle pathway. Immunodot analysis indicates that the apoB-48 and apoB-100 present in VLDL, IDL, and LDL in nontransgenic rabbits is not evident in the transgenic rabbits (Fig. 4). This reduced apoB particle concentration could be due to enhanced clearance of these particles from the circulation or a reduced production of apoB-containing lipoprotein particles. The removal of apoB from the circulation could be modulated by the presence of CETP. In addition to remodeling the apoB particles, the uptake of these particles could be affected by the delivery of cholesterol to the hepatocyte mediated by CETP. Alternatively, LCAT overexpression within the secretory pathway of the hepatocyte could modify production of apoB. The assembly and secretion of nascent apoB requires cofactors for particle assembly, and alteration of this process could enhance the intracellular degradation of nascent apoB. These results indicate that LCAT overexpression not only leads to hyperalphalipoproteinemia, but it also affects the concentration apoB particles in plasma.

The endogenous LCAT activity in rabbits using the proteoliposome assay is about one third that of human plasma (36), yet it was increased from 5- to 15-fold in the LCAT transgenic compared with nontransgenic control rabbits. In addition, the cholesteryl esterification rate in the transgenic rabbits, a measure of endogenous LCAT activity in plasma, was increased more than 3-fold. Yet the increase was not as striking as the LCAT activity determined by the α-proteoliposome assay. These observations suggest that the in vitro α-proteoliposome assay might not reflect the changes in the esterification rate in vivo in the transgenic rabbits.

These findings may have direct physiologic and pathologic relevance to man. Albers and co-workers demonstrated that in adults aged 20–59 years in the Pacific Northwest Bell Telephone Company study the plasma activity of LCAT ranged 3-fold, from 2.87–8.56 μg/ml (37). Subgroup analysis in this study indicated that women and nonsmokers, groups known to have lower risk for developing cardiovascular disease, had significantly higher LCAT mass concentrations than men or smokers, respectively. The transgenic rabbits in this study expressed human LCAT comparable (LCAT-T; 4.2 μg/ml) with that of normal human concentrations to 10-fold the median value for man (LCAT-Tg; 54.0 μg/ml). As with the data in the Pacific Northwest Bell Telephone Company study, the degree of human LCAT gene expression in rabbits was highly correlated with the plasma cholesterol concentrations ($r^2 = 0.85$; $p < 0.001$).

The combined results of this study establish that the overexpression of human LCAT in the rabbit leads to markedly increased concentrations of large HDL particles containing apoE. In addition, the substantial plasma CETP activity in the rabbit markedly reduces the concentrations of the apoB-containing VLDL and IDL + LDL particles. The dose dependence that we observed indicates that LCAT can be rate-limiting in affecting these particle concentrations in vivo.

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