Overexpression of lecithin:cholesterol acyltransferase in transgenic rabbits prevents diet-induced atherosclerosis
(high density lipoprotein cholesterol/low density lipoprotein cholesterol/atherogenic diet/cholesterol esters)


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ABSTRACT Lecithin:cholesterol acyltransferase (LCAT) is a key plasma enzyme in cholesterol and high density lipoprotein (HDL) metabolism. Transgenic rabbits overexpressing human LCAT had 15-fold greater plasma LCAT activity than nontransgenic control rabbits. This degree of overexpression was associated with a 6.7-fold increase in the plasma HDL cholesterol concentration in LCAT transgenic rabbits. On a 0.3% cholesterol diet, the HDL cholesterol concentrations increased from 24 ± 1 to 39 ± 3 mg/dl in nontransgenic control rabbits (n = 10; P < 0.05) and increased from 161 ± 5 to 200 ± 21 mg/dl (P < 0.001) in the LCAT transgenic rabbits (n = 9). Although the baseline non-HDL concentrations of control (4 ± 3 mg/dl) and transgenic rabbits (8 ± 4 mg/dl) were similar, the cholesterol-rich diet raised the non-HDL cholesterol concentrations, reflecting the atherogenic very low density, intermediate density, and low density lipoprotein particles observed by gel filtration chromatography. The non-HDL cholesterol rose to 509 ± 57 mg/dl in controls compared with only 196 ± 14 mg/dl in the LCAT transgenic rabbits (P < 0.005). The differences in the plasma lipoprotein response to a cholesterol-rich diet observed in the transgenic rabbits paralleled the susceptibility to developing aortic atherosclerosis. Compared with nontransgenic controls, LCAT transgenic rabbits were protected from diet-induced atherosclerosis with significant reductions determined by both quantitative planimetry (~86%; P < 0.003) and quantitative immunohistochemistry (~93%; P < 0.009). Our results establish the importance of LCAT in the metabolism of both HDL and apolipoprotein B-containing lipoprotein particles with cholesterol feeding and the response to diet-induced atherosclerosis. In addition, these findings identify LCAT as a new target for therapy to prevent atherosclerosis.

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The development of human atherosclerosis is inversely related to the concentration of the high density lipoproteins (HDL) (1). High and low concentrations of plasma HDL are associated with decreased and increased risk of developing premature atherosclerotic cardiovascular disease, respectively (2). It has been proposed that a 1% increase in the concentration of HDL would lead to a 3% reduction in risk for developing coronary heart disease in man (1). An important enzyme in modulating plasma HDL concentrations is lecithin:cholesterol acyltransferase (LCAT). LCAT catalyzes the transfer of fatty acid from the sn-2 position of lecithin to the free hydroxyl group of cholesterol and was first proposed nearly 30 years ago that cholesterol esterification would be a key step in transfering cholesterol from the tissues of the body to the liver (3). This process, termed reverse cholesterol transport (4), was proposed to facilitate the removal of cholesterol from the body. Thus reverse cholesterol transport is one of several proposed mechanisms by which HDL provides protection from cardiovascular disease (5–10).

Inborn errors of metabolism can point to the in vivo effects of either overexpression or loss of enzymatic function. Recent studies have established mutations in the gene encoding LCAT that lead to the total or partial loss of LCAT activity in the plasma (11–14). This leads to reduced concentrations of HDL cholesterol and accumulation of cholesterol in the kidney and cornea (14). To assess the impact that overexpression of LCAT has on the plasma lipoproteins, we and others have generated transgenic mice (15, 16) and rabbits (17) expressing high levels of human LCAT. In both species, human LCAT raises the plasma HDL cholesterol concentrations. In addition, the concentration of the non-HDL lipoprotein particles was unexpectedly reduced in transgenic rabbits expressing high levels of human LCAT (17). Because the cholesterol-fed rabbit is the classic model for the study of diet-induced atherosclerosis (18), we have tested the hypothesis that overexpression of LCAT in cholesterol-fed rabbits would not only increase the HDL cholesterol concentrations in these animals but also prevent the development of atherosclerosis. In addition to raising plasma HDL cholesterol concentrations, the concentrations of the atherogenic very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and low density lipoprotein (LDL) particles were substantially lower in the cholesterol-fed LCAT transgenic rabbits than the concentrations in the controls. These results indicate that LCAT may protect against diet-induced atherosclerosis by modulating both HDL and non-HDL particle metabolism.

MATERIALS AND METHODS

Control and Transgenic Animals. The full-length human LCAT gene was used to generate transgenic animals (15, 17). This genomic construct contained all of the introns and 851 bp of the 5′ and 1134 bp of the 3′ untranslated regions of the human LCAT gene. The generation of transgenic rabbits was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. New Zealand White rabbits were purchased from Hazeltone Research Products (Denver, PA). Rabbits were housed in separate cages. A

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; CETP, cholesteryl ester transfer protein.

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total of 9 LCAT transgenic and 10 control males 5–6 months of age were studied.

Plasma LCAT, Lipid, and Lipoprotein Analyses. Blood was drawn for analysis in tubes containing EDTA from rabbits after a 12-h fast. Plasma α-LCAT activity was determined using 10 μL of plasma in a proteoliposome assay (19). CETP activity was determined as described by Iverius and Brunzell (21). The total cholesterol and triglyceride concentrations (Sigma) as well as unesterified cholesterol and phospholipids (Wako Chemicals, Richmond, VA) were determined on plasma samples using enzymic methods on a Hitachi 911 Autoanalyzer (Boehringer Mannheim). The esterified cholesterol was calculated by subtracting unesterified from the total cholesterol. The non-HDL cholesterol concentration was determined by quantitating the cholesterol concentration in the supernatant of plasma that had been diluted with PBS (1:1 vol/vol) and then precipitated with dextran sulfate (22). The HDL cholesterol was calculated by subtracting the cholesterol concentration in the supernatant after precipitation from the total plasma cholesterol concentration.

Gel filtration chromatography was performed on plasma samples as described (17). Briefly, 200 μL of plasma was applied to a fast protein liquid chromatography system consisting of two Superose 6 columns in series (Pharmacia) and eluted with 1 mM EDTA and 0.02% (wt/wt) sodium azide in PBS (23). The first 10 mL of eluate was discarded, and the remaining 30 mL containing the plasma lipoprotein fractions, was collected in 0.5-ml aliquots. The total cholesterol, unesterified cholesterol, phospholipid, and triglyceride concentrations of each of these 0.5-mL fractions were determined as outlined above.

Diet-Atherosclerosis Study. Rabbits were fed a daily ration of 120 g of 0.3% cholesterol diet (Ziegler Brothers, Gardener, PA; product number 4109000). After 17 weeks, the rabbits were killed using intravenous sodium pentobarbital. The aortas were harvested and stained with Sudan IV, and the percentage of the surface area stained was determined by planimetry of the digitized image (24). One-millimeter slices of aorta just inferior to the left subclavian artery ostia were harvested from each aorta, stained, and analyzed. The extent of intimal cellular proliferation was quantitated using a ratio of the intima to media (25).

Hepatic Lipid and Cholesterol Quantitation. After harvesting of the aorta, approximately 200 mg of liver (wet weight) was weighed, minced, and extracted in 20 mL of chloroform/methanol (2:1, vol/vol) using the method by Folch and coworkers (26). After drying of the organic phase under nitrogen, lipid content was determined gravimetrically, and the lipids were resolubilized in 2 mL of isopropanol. Protein content was quantitated on the remaining tissue after overnight solubilization in 5 mL of NaOH (27) using the enhanced protocol of the bicinchoninic acid method (Pierce). Total cholesterol content was assayed using the Cholesterol CII Enzymatic Colorimetric Method (Wako Chemicals). Unesterified cholesterol content was ascertained using the Free Cholesterol Enzymatic Colorimetric Method (Wako Chemicals). Esterified cholesterol was calculated by subtraction.

RESULTS
Plasma LCAT activity was 101 ± 11 nmol/ml/h in the nontransgenic control rabbits. In contrast, the LCAT activity in the LCAT transgenic rabbits was 1593 ± 101 nmol/ml/h and was significantly higher than controls (P < 0.0001). Compared with controls, LCAT transgenic rabbits had a marked increase in both total (617%; P < 0.001) and HDL cholesterol concentrations (671%; P < 0.001; Table 1). On the cholesterol diet, control rabbits had 19-fold and 127-fold increases in total and non-HDL cholesterol concentrations, respectively, as well as a 74% increase in the plasma triglyceride concentrations (Table 1). In contrast, the plasma total and non-HDL cholesterol concentrations in the LCAT transgenic rabbits increased only 2-fold and 11-fold, respectively, on the cholesterol diet. The LCAT activity in the transgenic rabbits on the cholesterol diet remained more than 3-fold that of controls and was associated with an increase of HDL cholesterol concentration to more than 5-fold that of control rabbits. Analysis of the plasma lipoproteins by gel filtration chromatography demonstrated that cholesterol-fed control rabbits had cholesterol principally in VLDL, IDL, and LDL particles (Fig. 1). In contrast, a substantial fraction of the plasma cholesterol in the LCAT transgenic rabbits was present in large HDL particles (Fig. 1.4). The majority of the cholesterol in both controls and LCAT transgenic rabbits was esterified (Fig. 1B). However, compared with nontransgenic control rabbits, the HDL in the LCAT transgenic rabbits were enriched in triglycerides and especially phospholipids (Fig. 1 C and D). These differences in the plasma lipoproteins were reflected in the total/HDL cholesterol ratio. The total cholesterol/HDL cholesterol ratio, a sensitive indicator of clinically detectable human atherosclerosis (28), increased in the control group by more than 12-fold. In contrast, in LCAT transgenic rabbits, the total/HDL ratio rose less than 2-fold (Table 1) and remained below the ratio of 5, which provides an average risk for atherosclerosis in man (29).

Both the CETP and post-heparin hepatic lipase activities were determined in these rabbits. On a regular chow diet, the CETP activity (expressed as percent per 5 μL per 18 h) in the LCAT transgenic rabbits (30 ± 2) was twice that of controls (15 ± 1; P < 0.001). In addition, there was a significant difference in hepatic lipase activity. The activity of post-heparin hepatic lipase in controls (56 ± 4 nmol/ml/h) was significantly higher than in the LCAT transgenic (34 ± 1 nmol/ml/h; P < 0.02). Cholesterol feeding increased the activities of both of these plasma proteins in both control and transgenic rabbits. The CETP activity in control rabbits increased to 35 ± 3, and the LCAT transgenic rabbit CETP activity increased to 58 ± 4. These values from cholesterol-fed animals were significantly higher than those from chow-fed animals in both strains of rabbit (P < 0.001), and the LCAT transgenic CETP activity remained 66% higher than controls (P < 0.005). The hepatic lipase activities were also significantly

<p>| Table 1. Plasma lipoproteins before and after cholesterol feeding in control and LCAT transgenic rabbits |
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<table>
<thead>
<tr>
<th></th>
<th>Total cholesterol, mg/dl</th>
<th>Total triglycerides, mg/dl</th>
<th>HDL cholesterol, mg/dl</th>
<th>non-HDL cholesterol, mg/dl</th>
<th>Total cholesterol/HDL cholesterol, mg/dl</th>
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<tr>
<td>Control (n = 10)</td>
<td></td>
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<tr>
<td>Baseline</td>
<td>29 ± 3</td>
<td>39 ± 4</td>
<td>24 ± 1</td>
<td>4 ± 3</td>
<td>1.17 ± 0.12</td>
</tr>
<tr>
<td>Cholesterol-fed</td>
<td>548 ± 57*</td>
<td>107 ± 15*</td>
<td>39 ± 3*</td>
<td>509 ± 57*</td>
<td>14.98 ± 2.13*</td>
</tr>
<tr>
<td>LCAT-transgenic (n = 9)</td>
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<tr>
<td>Baseline</td>
<td>179 ± 7†</td>
<td>43 ± 4</td>
<td>161 ± 5†</td>
<td>18 ± 4</td>
<td>1.11 ± 0.02</td>
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<tr>
<td>Cholesterol-fed</td>
<td>396 ± 33††</td>
<td>81 ± 8*††</td>
<td>200 ± 21††</td>
<td>196 ± 14††</td>
<td>2.03 ± 0.07††</td>
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*Differs from baseline; P < 0.05.
††Differs from control values; P < 0.05.
Table 2. Hepatic lipid content in control and LCAT transgenic rabbits after cholesterol feeding

<table>
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<th>Per mg of tissue (wt weight)</th>
<th>Per mg of cell protein</th>
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<tbody>
<tr>
<td></td>
<td>Total lipid, mg/mg</td>
<td>Cholesteryl ester, µg/mg</td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>0.12 ± 0.03</td>
<td>28.5 ± 7.0</td>
</tr>
<tr>
<td>Transgenic (n = 9)</td>
<td>0.09 ± 0.03</td>
<td>22.4 ± 11.8</td>
</tr>
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higher in both control (95 ± 7) and LCAT transgenic animals (61 ± 5) after cholesterol feeding (P < 0.005). However, the hepatic lipase remained significantly higher in the controls than in the transgenic rabbits (P < 0.001). Therefore, LCAT overexpression was associated with substantial changes in plasma activity of both of these proteins that affect HDL metabolism.

To determine if cholesterol feeding was associated with changes in hepatic cholesterol content, the livers of control and LCAT transgenic rabbits were analyzed. LCAT overexpression did not alter the hepatic lipid content compared with control rabbits (Table 2). Whether expressed per mg of tissue or per mg of hepatic protein content, the total lipid, cholesterol, and cholesteryl ester contents in rabbit liver were not different between control and LCAT transgenic rabbits (Table 2). In addition, the histology of the livers of control and LCAT transgenic rabbits were similar and disclosed only mild hepatocytic lipid accumulation. Finally, serum transaminase concentrations were not affected with cholesterol feeding in either control or LCAT transgenic rabbits (data not shown). Therefore, the changes observed in the plasma lipoproteins with LCAT overexpression were not secondary to hepatotoxicity and did not lead to alterations in hepatic cholesterol content.

To evaluate the potential protective role of LCAT overexpression in the development of diet-induced atherosclerosis, control and LCAT transgenic rabbits were placed on a 0.3% cholesterol diet. After 17 weeks on the 0.3% cholesterol diet, the aortas from control and LCAT transgenic rabbits were harvested, and two methods were used to quantitate the severity of diet-induced atherosclerosis. Sudan IV staining of the lipid droplets was used to quantify the percent of the surface area developing lesions (24). The probability map for aortic lesion development in the transgenic rabbits showed only scattered foci of sudan IV-staining material, whereas control aortas had substantial staining in the majority of the animals (Fig. 2). The aortas of the control group had 35 ± 7% of the surface covered by plaque. In marked contrast, only 5 ± 1% of the aortic surface was covered by plaque in the LCAT transgenic rabbits (P < 0.009; see Figs. 2 and 4).

The substantial differences in the atherosclerosis in aortas between control and LCAT transgenic rabbits were also evident microscopically (Fig. 3). The intima of the control rabbits demonstrated foam cell formation, cellular proliferation, and an increase in the ratio of the intima/media to 0.4 ± 0.11 (Fig. 3 Left). There was virtually no foam cell formation or cellular proliferation in the transgenic rabbits expressing human LCAT (see Figs. 3 and 4 Right). The 0.03 ± 0.01 intima/media ratio was significantly lower than the control (P < 0.009; Fig. 4). Therefore, LCAT overexpression led to an 85–90% reduction in diet-induced atherosclerosis in rabbits.

The atherosclerosis assessed by two different end points used in this study were highly correlated for both the control rabbits (Fig. 5A; r = 0.87; P < 0.001) as well as for the entire study group (r = 0.92; P < 0.001). The severity of atherosclerosis was inversely correlated with the plasma LCAT activity (r = −0.55; P < 0.019). This was true not only with LCAT overexpression but also within the control group. The higher the LCAT activity in the nontransgenic control rabbits, the lower the extent of atherosclerosis (r = −0.64; P < 0.006; Fig. 5B). With LCAT overexpression, both the non-HDL cholesterol (r = 0.82; P < 0.0001) and the total cholesterol/HDL cholesterol ratio (r = 0.89; P < 0.001) correlated with the severity of atherosclerosis. However, the intima/media ratio and the total/HDL cholesterol concentration were also highly correlated within the nontransgenic control group (Fig. 5C

Fig. 1. Gel filtration chromatography of plasma from control (solid lines) and transgenic (dashed lines) rabbits ingesting a 0.3% cholesteryl-enriched diet. The concentrations of cholesterol (A), cholesteryl ester (B), triglycerides (C), and phospholipids (D) were determined on 0.5-ml fractions eluted from the two sequential Sepharose 6 columns.
and D). These dose–response relationships in both LCAT overexpressors and the nontransgenic control group further strengthen the association between diet-induced atherogenesis and the level of LCAT expression.

**DISCUSSION**

This study was undertaken to evaluate the role of LCAT overexpression in modulating the plasma lipoproteins and atherosclerosis. Transgenic rabbits overexpressing human LCAT were generated, and the development of atherosclerosis in rabbits was evaluated after the animals were fed a high cholesterol diet. Rabbit lipoprotein metabolism is unique for several reasons. These animals have elevated concentrations of plasma CETP (30), they do not synthesize apolipoprotein A-II (31–33), and they have reduced plasma hepatic lipase activity (34) compared with murine species. The feeding of cholesterol to rabbits has long been known to lead to atherosclerosis (18). High cholesterol diets lead to the accumulation of lipid in apolipoprotein B-containing VLDL, IDL, and LDL particles in control rabbits (Fig. 1). The increase in plasma concentrations of non-HDL lipoprotein particles in control rabbits was considerably greater than in the transgenic animals overexpressing LCAT after cholesterol feeding (Fig. 1 and Table 1). Non-HDL cholesterol concentrations increased in LCAT transgenic rabbits to only 39% of that observed in nontransgenic controls. In contrast, the plasma HDL concentrations were 5-fold higher in the LCAT-expressing strain than in control rabbits. These differences in the plasma lipoproteins were associated with resistance to diet-induced atherosclerosis in the LCAT transgenic rabbits. Compared with nontransgenic controls, the LCAT-expressing strain experienced an 86% reduction in the surface of the aorta covered by plaque (Figs. 2 and 4) and 93% reduction in the intima/media ratio (Figs. 3 and 4). LCAT overexpression led to marked changes in both the plasma lipoproteins and in cholesterol-induced atherogenesis.

LCAT overexpression had a variety of effects on the impact of a cholesterol-rich diet on the plasma lipoproteins, leading to an antiatherogenic lipoprotein profile. The elevated concentrations of HDL observed in chow-fed LCAT transgenic rabbits (17) were even more pronounced with cholesterol feeding (Table 1 and Fig. 1). The HDL cholesterol concentrations with cholesterol feeding in LCAT transgenic rabbits (200 ± 39 mg/dl) was 5.1-fold that in nontransgenic controls (39 ± 3 mg/dl). The large HDL particles generated in the LCAT transgenic rabbit contained not only cholesteryl ester (Fig. 1B) generated from the LCAT reaction, but these lipoproteins also had a high triglyceride content (Fig. 1C). This triglyceride enrichment reflected, at least in part, the 66%
higher CETP activity and the 31% reduction in hepatic lipase activity in the LCAT transgenic rabbits compared with controls. CETP exchanges cholesteryl ester from the core of HDL particles with triglyceride from the core of apolipoprotein B-containing particles (35, 36). The large HDL particle size, triglyceride content, and particularly the high phospholipid concentration in HDL (Fig. 1D) reflect the lower activity of hepatic lipase in LCAT transgenic rabbits.

![Graph 1](image1.png)

**FIG. 4.** The extent of intimal cellular proliferation as shown in Fig. 2 was quantitated using a ratio of the intima to media (25). The quantitative assessment of both the intima/media ratio (P < 0.003) and the percent of the surface area (P < 0.009) was significantly lower in the transgenic LCAT rabbits than in controls.

The elevated plasma levels of HDL and reduced apolipoprotein B-containing lipoproteins in the LCAT transgenic rabbits were associated with a markedly decreased lipid accumulation and cellular proliferation in the aortic intima (Figs. 2–4). Both methods for quantitation of atherogenesis employed in these studies, the extent of the aortic surface covered by plaque and the intima/media ratio, were significantly reduced in the LCAT transgenic rabbits. The decreased aortic cellular lipid and cholesterol accumulation may be due to both the reduction in the proatherogenic apolipoprotein B lipoproteins as well as the increased HDL lipoproteins. Several lines of evidence support the role that HDL plays in modulating the development of atherosclerosis. Direct infusion of HDL intravenously has been shown to attenuate atherogenesis in lagomorphs (37). Raising HDL concentrations in apolipoprotein A-I transgenic mice resulted in decreased diet-induced atherosclerosis (38).

A number of mechanisms by which HDL could mediate protection from atherosclerosis have been proposed. HDL have been proposed to both induce cholesterol efflux (41, 42) and serve as a mitogen for cellular proliferation (43). HDL can also serve as a buffer to protect apolipoprotein B particles from potentially proatherogenic oxidation (44). HDL is het-

![Graph 2](image2.png)

**FIG. 5.** Significant (P < 0.05) bivariate Pearson correlations of post-diet variables with aortic atherosclerosis in nontransgenic, control rabbits. The intima/media (I/M) ratio correlated well with the planimetry assessment of atherosclerosis (A). The I/M ratio was inversely correlated with LCAT activity (B) and positively correlated with the non-HDL cholesterol (C) and total cholesterol/HDL cholesterol (TC/HDL) (D).
erogeneous and contains several different lipoprotein particles. In man, the HDL-containing apoA-I (LpA-I) has been proposed to be more effective in reverse cholesterol transport than particles containing both apoA-I and apoA-II (45–47). ApoA-I is a potent cofactor enhancing LCAT activity, and the modulation of LpA-I size is sensitive to the presence of apoB-containing particles and LCAT activity (48). Because rabbits express no apoA-II (31–33), these transgenic rabbits have only LpA-I particles. Alternatively, the reduced concentration of atherogenic apolipoprotein B particles with LCAT overexpression may reduce the endothelial damage and cellular proliferation induced by LDL and LDL (49), respectively.

The effects of LCAT overexpression on atherosclerosis was significantly correlated with the changes in the plasma lipoproteins. However, these study end points, which were so striking in the transgenic rabbits, were also apparent within the nontransgenic control rabbits. The LCAT activity in the control group correlated with the total cholesterol/HDL cholesterol ratio (Fig. 5D) and the severity of atherosclerosis (Fig. 5A and C). Therefore, the changes observed in the LCAT transgenics may reflect a more subtle impact (i.e., LCAT may have on the plasma lipoproteins and susceptibility to atherosclerosis at more physiologic levels of expression).

The findings in this study may be of relevance to atherogenesis in man. Although a deficiency in the plasma LCAT activity was not originally associated with atherosclerosis (50), some mutations in the LCAT gene may be more deleterious to apoprotein B metabolism and lead to enhanced cardiovascular disease risk (51). However, the therapeutic impact of gene overexpression may not be predicted by the clinical observations in inborn errors in metabolism. Modulation of lipoprotein metabolism relevant to the prevention of atherogenesis by LCAT may occur in the context of other regulatory steps within the circulation. This may be the case in the general population. Adults aged 20–59 years in the Pacific Northwest Bell Telephone Company study were found to have plasma LCAT masses ranging from 2.87 to 8.56 μg/ml (52). 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 at 10-fold the median value for man. Thus, enhanced LCAT activity may decrease atherogenesis by representing a “no fault” (53) approach to therapy in man.

In conclusion, HDLs are now recognized as key plasma lipoproteins in modulating the development of atherosclerosis. The present studies demonstrate that overexpression of LCAT results in an antiatherogenic lipoprotein profile with reduced apolipoprotein B-containing lipoproteins in addition to elevated plasma levels of HDL. These changes were associated with markedly reduced atherosclerosis. These combined results indicate that modulation of plasma LCAT activity represents a new target for developing pharmacologic and gene therapy strategies to prevent atherosclerosis.

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