Increased preβ-HDL levels, cholesterol efflux, and LCAT-mediated esterification in mice expressing the human cholesteryl ester transfer protein (CETP) and human apolipoprotein A-I (apoA-I) transgenes

Omar L. Francone,[1] Lori Royer, and Mehrdad Haghpassand

Department of Cardiovascular and Metabolic Diseases, Central Research Division, Pfizer Inc., Eastern Point Road, Groton, CT 06340

Abstract The effects of cholesteryl ester transfer protein (CETP) on the distribution of apolipoprotein (apo) A-I between high density lipoprotein (HDL) subspecies and its impact on efflux and esterification of cell-derived cholesterol was studied in transgenic mice expressing either the human apoA-I (HuAITg) or both the human apoA-I and CETP (HuAICETPTg) transgenes. The simultaneous expression of the human CETP and apoA-I transgenes induced a 2-fold increase in the proportion of human apoA-I in the preβ-HDL fraction and 1.4- and 2.2-fold increases in the HDL_{3a} and HDL_{3c} fractions, respectively, at the expense of the larger HDL_{2b} population. HuAICETPTg mouse plasma has a greater ability to cause efflux of cholesterol from ^{3}H-labeled fibroblasts than plasma from HuAITg mice. Furthermore, the LCAT-mediated esterification of cell-derived cholesterol is increased 1.7-fold in mice expressing the human apoA-I and CETP transgenes compared to HuAITg mouse plasma. LCAT activity (measured with an exogenous substrate) was increased 1.4-fold and LCAT mRNA levels were increased 1.3-fold as a result of CETP expression. Taken together, these data indicate that, in vivo, the expression of CETP resulted in an increase in the proportion of apoA-I in the preβ-HDL fraction and a stimulation of the efflux and esterification of cell-derived cholesterol.—**Francone, O. L., L. Royer, and M. Haghpassand.** Increased preβ-HDL levels, cholesterol efflux, and LCAT-mediated esterification in mice expressing the human cholesteryl ester transfer protein (CETP) and human apolipoprotein A-I (apoA-I) transgenes. *J. Lipid Res.* 1996. **37:** 1268–1277.

Supplementary key words preβ-HDL • α-HDL • lecithin:cholesterol acyltransferase • transgenic mice • reverse cholesterol transport

Epidemiological studies have suggested an inverse correlation between the plasma concentration of high density lipoproteins (HDL) and the incidence of cardiovascular diseases (1, 2). Although the mechanism by which HDL prevents atherosclerosis remains poorly understood, several lines of evidence suggest that HDL plays a pivotal role in the transport of cholesterol in plasma. HDL participates in a multi-step physiologic process by which cell-derived cholesterol is transported from peripheral tissues back to the liver for excretion by conversion to bile acids (3). This process has been named reverse cholesterol transport (RCT). During the first step in the RCT, free cholesterol is removed out of the cell membrane on to HDL and esterified by lecithin:cholesterol acyltransferase (LCAT). HDL cholesteryl esters can be subsequently transferred to very low density lipoproteins (VLDL) and low density lipoproteins (LDL) by the cholesteryl ester transfer protein (CETP). Finally, lipoprotein cholesterol is taken up by the liver. Thus, factors affecting the structure, composition, or concentration of plasma HDL particles are likely to affect the homeostasis of plasma cholesterol and the RCT process.

Human HDL is heterogeneous, consisting of several distinct populations of particles with differences in size, protein composition, and electrophoretic mobility. On the basis of protein composition, HDL can be separated into two main populations: one containing only apoA-I (LpAI) and one containing apoA-I and apoA-II (LpAI/AII). LpAI can be divided into two metabolically linked subfractions, those with α-electrophoretic migration (α-HDL) and those with preβ mobility (preβ-HDL) (4).

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; HuAITg, transgenic mice expressing human apoA-I; HuAICETPg, transgenic mice expressing human apoA-I and CETP transgenes; MAb, monoclonal antibody; RCT, reverse cholesterol transport; PLTP, phospholipid transfer protein.

[1]To whom correspondence should be addressed.

Kunitake, Mendel, and Hennessy (5) proposed that apoA-I cycles between the preβ and α-HDL species, and that this cycling is controlled by the activity of enzymes and transfer proteins involved in the RCT pathway. Studies in vitro (6, 7) and in transgenic mice (8) demonstrated that the conversion of preβ-HDL to α-HDL is mediated by LCAT.

Several proteins have been reported to promote the dissociation of apoA-I from α-migrating HDL that results in the formation of preβ-HDL. For example, the triacylglycerol activity of hepatic lipase generates $preβ_1$-HDL when human HDL_2 is perfused through rat livers or incubated in vitro with purified rat hepatic lipase (9). The PLTP-mediated enlargement of the HDL species promotes the dissociation of apoA-I (10), forming small particles that resemble preβ-HDL (11). CETP has also been proposed to participate in the formation of preβ-HDL. In vitro, the incubation of plasma or lipoprotein fractions with purified CETP induces the formation of preβ-HDL from either the LpAI or LpAI/AII fractions of α-HDL (12–14). However, in these studies, non-physiological conditions were used, e.g., long incubation times and excess CETP over physiological levels (relative to HDL). If CETP promotes the dissociation of apoA-I and increases the formation of preβ-HDL, the newly formed preβ-HDL could enter the interstitial space, stimulate cholesterol efflux, and thus affect the rate of transport of cell-derived cholesterol by HDL.

To begin exploring the in vivo effects of CETP on the formation of the preβ-HDL species, and its consequences on the first steps of the RCT, transgenic mice expressing either the human apoA-I gene (HuAITg) or both the human apoA-I and CETP transgenes (HuAICETPTg) were used. HuAITg mice contain very small amounts of circulating murine apoA-I (15). These mice have an HDL profile that closely resembles that of human plasma (16, 17), and have approximately 20% of their total apoA-I migrating in the position corresponding to human preβ-HDL (8). Mice expressing the human CETP transgene represent a good model to study the effects of CETP, as mice normally lack CETP activity (18). Moreover, CETP does not bind to murine HDL but strongly interacts with HDL in the HuAITg mice (19). In this report, we have shown that the expression of human CETP in transgenic mice increases the proportion of human apoA-I in the preβ-migrating HDL fraction and increases the cell–cholesterol efflux and LCAT-mediated esterification. Taken together, these data strongly suggest an important role for CETP in the first steps of RCT.

MATERIALS AND METHODS

Transgenic mice

The transgenic mice used in the present study were described previously (19, 20). HuAITg mice (line 179) were obtained from Charles River Laboratories. Mice expressing both human apoA-I and CETP transgenes were obtained by mating HuAITg mice (line 179) with CETP heterozygous transgenic mice. Mice expressing the human apoA-I transgene (HuAITg) or both the human apoA-I and CETP transgenes (HuAICETPTg) were identified by PCR. At 12–16 weeks of age, the animals were placed in metabolic cages with free access to chow diet and water supplemented with 25 mM $ZnSO_4$. Mice were fasted overnight and blood was drawn in tubes containing 2 mM EDTA, 50 μg/ml gentamicin sulfate, and 0.05% sodium azide. Plasma was obtained by centrifugation at 3000 rpm for 15 min at 4°C.

Plasma lipid and apoA-I quantitation

Mouse HDL was separated from apoB-containing lipoproteins by dextran sulfate precipitation as described elsewhere (8, 20). Plasma and lipoprotein total cholesterol levels were determined with cholesterol oxidase (21) in the presence or absence of cholesterol esterase (kit No. 1127568, Boehringer Mannheim, Indianapolis, IN). Plasma human apoA-I concentrations were measured by a sandwich enzyme-linked immunosorbent assay (22).

TABLE 1. Distribution of human apoA-I among preβ and α-HDL species in HuAITg and HuAICETPTg mouse plasma

Transgenic Mouse Line	Human ApoA-I	CETP Mass	CETP Activity	% Total Human ApoA-I: Preβ-HDL	% Total Human ApoA-I: α-HDL
	mg/ml	*μg/ml*	*nmol/ml·h*		
HuAITg (n = 6)	2.46 ± 0.68	-	-	18.70 ± 5.44	81.30 ± 5.42
HuAICETPTg (n = 6)	2.65 ± 0.15	2.32 ± 0.53	58.18 ± 8.62	40.47 ± 5.91[a]	59.53 ± 5.90[a]

Lipoprotein fractions from HuAITg and HuAICETPTg mice were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes as described under Materials and Methods. Human apoA-I was visualized with a goat polyclonal antibody to human apoA-I. Areas containing preβ and α-migrating HDL were visualized and quantitated using a Phosphorimager. Values shown are mean ± SD from 6 mice.

[a] $P < 0.001$.

Determination of plasma CETP concentration and activity

Plasma CETP levels were determined by a monoclonal antibody (MAb) based immunoassay (23). CETP activities in HuAICETPTg mice plasma were measured as the rate of transfer of [^{3}H]cholesteryl ester from LDL to HDL (23). Plasma samples were incubated at 37°C for 18 h, apoB-containing lipoproteins were precipitated with $MnCl_2$ (10 mM final concentration), and labeled cholesteryl esters in the HDL fraction were determined by liquid scintillation spectrometry.

Quantification of HDL subspecies

To determine the proportion of preβ and α-HDL species in the two groups of transgenics, plasma containing 1.5 mM DTNB (to inactivate LCAT activity) were electrophoresed in 0.75% (wt/vol) agarose gel in 50 mM barbital buffer on Gelbond (FMC, Rockville, ME) and transferred to two Nitro Plus transfer membranes (Micron Separation Inc., Westboro, MA) as previously described (8). To identify the human apoA-I-containing HDL species, nitrocellulose membranes were incubated for 2 h at room temperature with 2% milk in 10 mM phosphate buffer, pH 7.4, and then with a biotinylated goat polyclonal antibody to human apoA-I (2 h at room temperature) in 2% milk in 10 mM phosphate buffer. ApoA-I-containing HDL species were visualized with ^{125}I-labeled Streptavidin (Amersham, Arlington Heights, IL). Unbound ^{125}I was washed 4 times with 1% milk in phosphate buffer and nitrocellulose membranes were exposed to Fuji XLS film at -70°C. No cross-reactivity was observed between this antibody and the murine apoA-I. The relative abundance of the human apoA-I among the α- or preβ-HDL species was calculated by quantitative scanning using a Phosphorimager (Fuji, Stamford, CT). The distribution of human apoA-I among the α-migrating HDL species was determined by two-dimensional gradient gel electrophoresis. The first dimensional gel electrophoresis was run on an agarose gel as described above. The agarose strip was placed on a 3–16% polyacrylamide gradient gel (Integrated Separation Systems, Natick, MA) in 25 mM Tris-glycine buffer (pH 8.3). Electrophoresis was carried out for 4.5 h. Plasma proteins were transferred to Nitro Plus transfer membranes and then immunoreacted with a goat polyclonal antibody to human apoA-I as described above. The proportion of apoA-I among the HDL fractions was determined by quantitative scanning using a Phosphorimager. Nitrocellulose membranes were exposed to Fuji imaging plates composed of radiosensitive phosphor crystals. Imaging plates were scanned with a He-Ne laser beam, recorded, and read as high digital data at 200 μm per pixel and 8 bit pixel depth. Each subfraction was individually quantitated by a customized box which isolated one fraction from another. Various exposure times were used to optimize the separation of the individual subspecies. Background was uniformly subtracted.

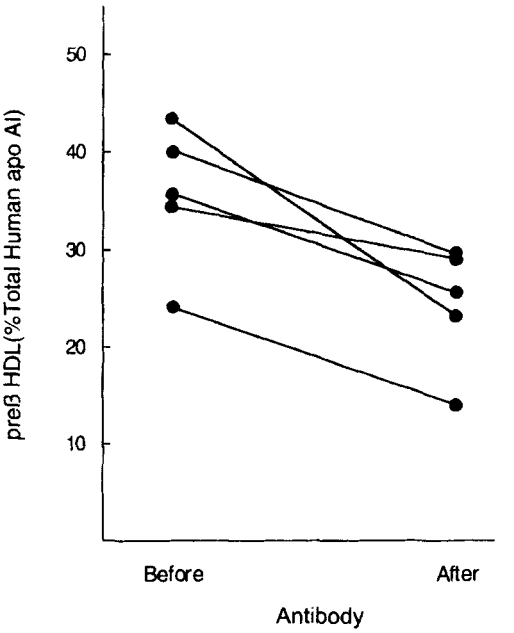

Fig. 1. *Effect of the inhibition of CETP activity on preβ-HDL levels.* HuAICETPTg mice were given intraperitoneal injections of either 3 mg/kg of a MAb to human CETP or a control IgG. CETP activity and preβ-HDL levels were determined initially and 24 h after the administration of the antibody as described in Materials and Methods.

Cell culture and cholesterol efflux

Normal human skin fibroblasts (ATCC CRL 1635) were grown in 12-well plates containing Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, and supplemented with 0.35 mg/ml glutamine and 40 μg/ml gentamicin sulfate. Cells were labeled for 48 h with 0.2 mCi [1,2-^{3}H]cholesterol (DuPont-New England Nuclear, MA) complexed to fetal calf serum. The final specific activity in the cells was 1.5–2.0 × 10^6 cpm/μg cholesterol. Cell cholesterol efflux was determined as previously described (24). Briefly, confluent fibroblasts were washed 4 times with DMEM medium supplemented with gentamicin sulfate, and then incubated at 37°C in an orbital shaker with 200 μl of either HuAITg or HuAICETPTg plasma for various times (1–15 min). Media samples were taken at various intervals for determination of radioactivity. The distribution of cell-derived cholesterol between plasma lipoproteins was assessed by dextran sulfate precipitation.

Determination of LCAT activities

Plasma LCAT activity in HuAITg and HuAICETPTg mice was determined essentially as described elsewhere

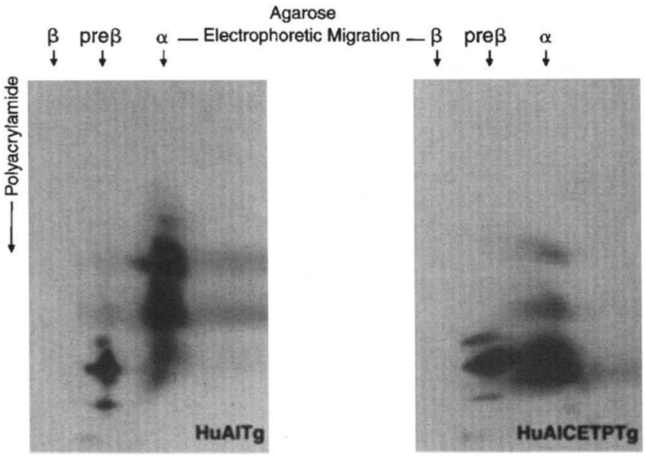

Fig. 2. Distribution of human apoA-I in plasma from HuAITg and HuAICETPTg mice. Fifteen µl of plasma from HuAITg and HuAICETPTg mice was electrophoresed in 0.75% agarose gel and then placed on a 3–16% polyacrylamide gradient gel. Plasma proteins were transferred to nitrocellulose membranes and the human apoA-I was immunoreacted with a goat polyclonal antibody to human apoA-I as described in Material and Methods.

(8). LCAT activity was determined as the rate of [^{3}H]cholesteryl ester synthesis from unilamelar vesicles prepared by French Press (25) and activated with human apoA-I (Sigma Chemical Co., St. Louis, MO) (26) to form discoidal synthetic lipoproteins. Briefly, the enzyme substrate consisted of egg lecithin (800 µg/ml), unesterified cholesterol (100 µg/ml, specific activity 5×10^5 cpm/µg) and human apoA-I (Sigma Chemical Co.). Each assay mixture contained 50 µl of substrate, 10 µl (1:10 diluted) mouse plasma, 50 µl recrystallized human albumin (15% wt/vol) in 60 mM phosphate buffer at pH 7.4, and 190 µl 150 mM NaCl in a total assay volume of 300 µl. The assay mixtures were incubated at 37°C for 20 min and the reaction was stopped with 900 µl chloroform–methanol–H_2O 4:4:1 (v/v/v), and lipids were extracted. [^{3}H]cholesteryl esters were separated from unesterified labeled cholesterol by TLC plates developed in hexane–diethyl ether–acetic acid 83:16:1 (v/v/v). Cholesteryl ester radioactivity was determined by liquid scintillation spectrometry. In agreement with previous studies (8, 27) the esterification of cholesterol by LCAT was linear up to 30 min and independent of the concentration of plasma lipoproteins (endogenous substrate). Therefore, an increase in the esterification rate corresponds to an increased level of LCAT protein.

Preparation and analysis of RNA by Northern blot

Total liver RNA was isolated from HuAITg and HuAICETPTg mice by the single step method using TRISOLV™. After extraction, RNA was precipitated with isopropanol and its integrity was assessed by agarose gel electrophoresis. Eight µg of RNA was separated in a denaturing 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred to nylon membranes (Schleicher & Schuell, Keene, NH), cross-linked and hybridized with an exon 6 murine LCAT cDNA probe using the rapid hybridization system (Amersham Corp., Arlington Heights, IL) according to the manufacturer's conditions. To verify equal loading of RNA, the LCAT probe was stripped and the membranes were rehybridized with a mouse β-actin probe (Clontech Laboratories, Inc., Palo Alto, CA).

Radioactive signals were recorded on a phosphor screen and scanned with a Phosphorimager (Fuji, Stamford, CT) set to detect ^{32}P radioactive emissions over a 5-order linear range of sensitivity. The hybridization signals were digitized and quantified. Background values were determined from equivalent surface areas near each hybridization signal and were subtracted from each value before normalization.

Statistical analysis

Results are expressed as mean ± standard deviation. The statistical significance of the differences between the groups was estimated by the Student's *t*-test. A P value of less than 0.05 was considered significant.

RESULTS

Quantification of preβ-HDL

To determine the proportion of preβ- and α-HDL species, HuAITg and HuAICETPTg mice were prescreened for human apoA-I and CETP plasma concentrations. HuAITg and HuAICETPTg with comparable levels of apoA-I and CETP to those reported for human plasma were selected for this study. Native plasma from HuAITg and HuAICETPTg mice were fractionated by agarose gel electrophoresis and immunoreacted with a goat polyclonal antibody to human apoA-I. In agreement with a previous study (8), HuAITg mice contain 18.70 ± 5.44% of the total human apoA-I (**Table 1**) in the preβ-HDL fraction. In the HuAITg mice, a strong positive correlation (r = 0.68, n = 17, $P < 0.05$) between preβ-HDL levels and apoA-I concentrations were observed. No significant differences in preβ-HDL levels were observed between male and female mice.

The simultaneous expression of the apoA-I and CETP transgenes did not change the electrophoretic migration of the preβ- or α-HDL but significantly ($P < 0.001$) increased the levels of preβ-HDL. The plasma concentrations of human apoA-I were similar for both groups of transgenic mice (2.46 ± 0.86 and 2.65 ± 0.15 mg/ml for HuAITg and HuAICETPTg mice, respectively), indicating that increases in the proportion of preβ-HDL

TABLE 2. Proportion of human apoA-I in the α-migrating HDL species

Transgenic Mouse Line	HDL_{2a}	HDL_{3a}	HDL_{3c}
HuAITg (n = 6)	51.4 ± 5.0	33.0 ± 4.5	16.5 ± 2.1
HuAICETPTg (n = 8)	17.5 ± 2.0	47.0 ± 2.6	35.5 ± 2.4

HDL species from HuAITg and HuAICETPTg mice were separated by two-dimensional gradient gel electrophoresis. Human apoA-I on nitrocellulose membranes was visualized with a goat polyclonal antibody to human apoA-I and quantitated using a Phosphorimager. Values shown are mean ± SD from n mice.

result from a redistribution of apoA-I within the HDL fractions.

To determine whether increases in the proportion of preβ-HDL were due to the presence of CETP, HuAICETPTg mice were given intraperitoneal injections of either 3 mg/kg of a MAb to human CETP (2E7) (23) or a control IgG. CETP activity and preβ-HDL levels were determined initially, and 24 h after the administration of the antibody. CETP activity (56.12 ± 3.84 nmol/ml · h) was inhibited by 77.1 ± 12.3% (n = 5) whereas the plasma concentration of apoA-I remained unchanged (3.20 ± 0.48 and 3.18 ± 0.31 mg/ml before and 24 h after injection of the antibody, respectively). As shown in **Fig. 1,** the injection of the MAb 2E7 significantly decreased the proportion of human apoA-I in the preβ-HDL fraction from 35.5 ± 7.4% to 24.9 ± 6% ($P < 0.01$) before and after injection of the antibody, respectively. These results strongly suggest that CETP induces the formation of preβ-HDL. No changes in CETP activity or preβ-HDL levels were observed in mice injected with control IgG.

Two-dimensional gradient gel electrophoresis of native plasma from HuAITg and HuAICETPTg mice

The effects of CETP on HDL size distribution was studied by two-dimensional nondenaturing gradient gel electrophoresis. As shown in Table 1 plasma from HuAICETPTg mice show a marked increase in preβ-HDL as compared to HuAITg mice (**Fig. 2**). Preβ-HDL were distributed into 2–3 populations located within the $preβ_1$ size interval described for human plasma (28). When plasma was run in a two-dimensional gradient gel to equilibrium, no apoA-I with a molecular weight corresponding to a lipid free apoprotein was observed. Although these data do not rule out a poorly lipidated form of apoA-I, they do suggest that all the apoA-I migrating in the preβ position have the same electrophoretic characteristics as human $preβ_1$-HDL. Interestingly, compared to human plasma, HuAITg and HuAICETPTg mouse plasma does not show human apoA-I migrating in the larger size preβ-HDL ($preβ_2$- and $preβ_3$-HDL), therefore suggesting that the formation of a larger preβ-migrating HDL species might be different between human and mouse.

HuAITg mouse α-HDL consists of three distinct populations of particles with sizes corresponding to the human HDL_{2b}, HDL_{3a}, and HDL_{3c} (17, 19). HuAITg mouse HDL have a predominant population of particles within the size range of HDL_{2b} (**Table 2**). The simultaneous expression of the human CETP and apoA-I transgenes induced major changes in the distribution of the human apoA-I within the α-HDL species (Table 2) resulting in 1.4- and 2.2-fold increases in the HDL_{3a} and HDL_{3c} fractions, respectively, at the expense of the larger HDL_{2b} population.

Cell-derived cholesterol efflux

To examine the effects of CETP on the first step of RCT, we determined the efflux of cholesterol from labeled human fibroblasts to plasma from HuAITg and HuAICETPTg mice. When ^{3}H-labeled human fibroblasts were incubated for short periods of time (1–15 min) with plasma from HuAITg or HuAICETPTg mice, there was a rapid and linear accumulation of radioactivity in the medium up to 15 min (**Fig. 3**). The distribution of cell-derived cholesterol among plasma lipoproteins was assessed by selective precipitation of apoB-containing particles by dextran sulfate. Labeled free cholesterol (64 ± 4.8%, n = 4) was recovered in the HDL fraction after a 15-min incubation, indicating that, similar to human plasma, HDL is the major acceptor of cell-derived cholesterol (24). Total efflux over the 15-min period was 1–2% of the total labeled cell cholesterol, consistent with previous data (24).

As reported earlier (19), the expression of the human CETP transgene in HuAITg mice reduced the plasma concentrations of apoA-I and HDL-cholesterol (**Table 3**) further suggesting that CETP not only decreases the HDL-particle size but also decreases the number of circulating HDL particles. Interestingly, plasma from HuAICETPTg mice has an increased capacity to efflux cholesterol from labeled human fibroblasts than plasma from HuAITg mice (Fig. 3 and Table 3). This finding indicates that the efficiency of either one or more of the HDL species to stimulate efflux of cholesterol was increased in the HuAICETPTg mice.

Cell-derived cholesterol esterification, LCAT activities, and liver LCAT mRNA levels in HuAITg and HuAICETPTg mice

The utilization of cell-derived cholesterol by the LCAT reaction was examined by measuring the percent of [^{3}H]free cholesterol esterified when native plasma was incubated with [^{3}H]cholesterol-labeled fibroblasts. As previously reported for human plasma (29), labeled cholesteryl esters could only be detected in plasma after

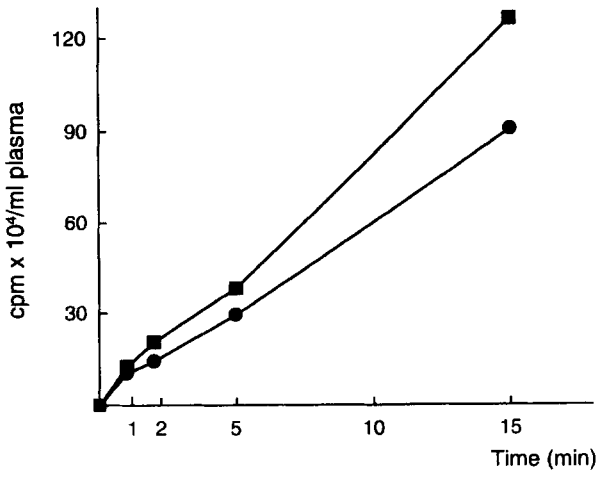

Fig. 3. Rate of appearance of [^{3}H]cholesterol from labeled human fibroblasts. Normal human skin fibroblasts were grown in 12-well plates containing DMEM + 10% fetal calf serum, supplemented with L-glutamine and gentamicin sulfate. Cells were labeled for 48 h with 0.2 mCi [1,2-^{3}H]cholesterol complexed to fetal calf serum. Cell cholesterol efflux was determined as described under Material and Methods. Two hundred μl of either HuAITg or HuAICETPTg mouse serum was incubated at 37°C for various times (1–15 min). Samples were taken at intervals for determination of medium radioactivity. HuAITg, closed circles; HuAICETPTg, closed squares.

5 min of incubation (data not shown). Further incubation led to the linear accumulation of labeled cholesteryl ester in plasma. As shown in **Table 4,** 7.24 ± 1.15% of the [^{3}H]cholesterol found in the plasma of HuAITg mice was esterified at the end of a 1-h incubation. The percent esterification of labeled cholesterol released from fibroblasts to HuAITg mice plasma is substantially higher than the value previously reported for human serum (22). Compared to HuAITg mice, plasma from HuAICETPTg mice had a 1.7-fold increase in the esterification of cell-derived cholesterol ($P < 0.001$). We then determined whether the increase in the esterification of cholesterol was due to a change in the plasma concentration of the enzyme or solely as a result of a better lipoprotein substrate for the LCAT reaction. Although the plasma LCAT mass was not measured due to the absence of antibodies to murine LCAT, plasma LCAT activity was determined using an excess of discoidal synthetic lipoproteins. Under these conditions, LCAT activity is proportional to the levels of LCAT protein. By this measure, LCAT increased by 1.4-fold in HuAICETPTg compared to HuAITg mice (Table 4). This result suggests that the increase in the esterification of cell-derived cholesterol observed in HuAICETPTg mice plasma can be explained by an increase in the plasma level of LCAT. Further support for this hypothesis comes from the cellular levels of LCAT mRNA in the two groups of transgenic mice. When comparing LCAT mRNA levels in HuAITg versus HuAICETPTg mice, a substantial and significant increase is found in the HuAICETPTg mice, indicating that the expression of the mouse LCAT gene is stimulated when the human CETP gene is expressed.

DISCUSSION

In this report, we have used transgenic mice expressing the human apoA-I and CETP transgenes to show that, in vivo, human CETP promotes the formation of small HDL with a preβ electrophoretic mobility, and increases cell-derived cholesterol efflux and esterification. Taken together, these results point to an important role of CETP in the initial steps of the RCT pathway and suggest that CETP could significantly influence the homeostasis of plasma cholesterol.

Preβ-HDL are small, phospholipid-rich, apoA-I only-containing HDL (28, 30) particles that are present in different species (6, 31, 32). In humans, the average concentration of preβ-HDL is about 5–10% of the plasma apoA-I (33). Higher concentrations are found in lymph (34), aortic intima (35), and plasma from hypertriglyceridemic subjects (36). Although several studies (37–39) have proposed a key role for this HDL fraction in the initial steps of cell-derived cholesterol transport, the origin and molecular mechanisms responsible for their formation remain poorly understood. It has been proposed that preβ-HDL are formed, at least in part, by the dissociation of apoA-I as a consequence of the shedding of redundant surface constituents from HDL. Released apoA-I can then be complexed with phospholipids released from several sources such as VLDL and chylomicrons that are undergoing lipolysis (40), cell membranes (41), or PLTP-mediated transfer from a range of lipoproteins (42).

Lipases and transfer proteins participate in the remodelling of HDL and could contribute to the dissociation of apoA-I from HDL. For example, the triacylglycerol lipase activity of hepatic lipase decreases the lipid core of triacylglycerol-rich HDL and promotes the dissociation of apoA-I and possibly phospholipids, inducing the formation of preβ-HDL (9). In vitro, the incubation of HDL in the presence of LDL or VLDL supplemented with CETP indicates that a portion of the apoA-I dissociates from α-migrating HDL (12–14) and migrates in a preβ position that is identical to that of human preβ-HDL. In the present study, we have tested whether a similar mechanism occurs in vivo. We have used transgenic mice with plasma levels of apoA-I and CETP similar to those reported for human plasma (43). Although our measurements reflect a steady state rather than a dynamic conversion determined by kinetics, it seems reasonable to assume, as in the experiments performed in vitro, that the increase in preβ-HDL levels

TABLE 3. Cell-cholesterol efflux, HDL-cholesterol and human apoA-I concentrations in plasma from HuAITg and HuAICETPTg mice

Transgenic Mouse Line	Cholesterol Efflux	HDL-Cholesterol	Human ApoA-I
	ng cholesterol/min	*mg/dl*	*mg/ml*
HuAITg	8.80 ± 0.79	82.60 ± 12.15	2.92 ± 0.35
	(10)	(10)	(10)
	10.81 ± 0.46[a]	44.84 ± 4.99[a]	1.91 ± 0.29[a]
HuAICETPTg	(9)	(9)	(10)

Two hundred μl of plasma from either HuAITg or HuAICETPTg mice was incubated with [^{3}H]cholesterol-labeled fibroblasts from 1–15 min as described in Materials and Methods. HDL-cholesterol was determined after precipitation of apoB-containing lipoproteins with dextran sulfate. Plasma apoA-I was determined by ELISA. Values shown are mean ± SD from n mice.

[a]Statistically significant differences (*t*-test).

is a consequence of the presence of CETP. Further support for this hypothesis comes from the experiments in which the inhibition of the CETP activity by a MAb results in decreased levels of preβ-HDL. The plasma concentrations of preβ-HDL in HuAITg and HuAICETPTg mice are substantially higher than those reported for human plasma. This interesting observation could eventually explain the increased protection to diet-induced atherosclerosis observed in transgenic mice expressing the human apoA-I transgene (15).

Despite the lower levels of HDL-cholesterol and apoA-I, plasma from HuAICETPTg mice had a greater ability to stimulate the efflux of cholesterol from fibroblasts than plasma from HuAITg mice. This finding was somewhat surprising in view of the well-documented positive correlation among HDL-cholesterol, apoA-I levels and cholesterol efflux (22). The decrease in the plasma concentration of human apoA-I and HDL-cholesterol observed in HuAICETPTg mice is consistent with a decrease in the number of HDL particles. Thus, the CETP-induced remodelling of HDL must increase the efficiency of either one or all of the HDL populations to accept cell-cholesterol. Because HDL is very heterogeneous in protein and lipid composition, it is difficult to assess whether the increase in efflux observed in HuAICETPTg mice is due to changes in size, lipid, or apolipoprotein composition of the HDL subclasses. However, the results of this study and the evidence presented by others (28), showing that about 50% of the cholesterol released from cells in short-term (1–5 min) efflux studies is associated with preβ-HDL, strongly suggest that increases in the preβ-HDL fraction contribute to increases in the cell-cholesterol efflux observed in HuAICETPTg mice plasma. In fact, despite the lower plasma concentration of apoA-I, HuAICETPTg mice have a higher concentration of preβ-HDL than HuAITg mice (0.77 mg/ml vs. 0.55 mg/ml, respectively).

It is likely that there is not a single HDL subclass that is totally responsible for the efflux of cholesterol, but rather that a number of particles with different lipid compositions and sizes contribute to the removal of cell-cholesterol. In agreement with a previous study (19), our findings show that the simultaneous expression of human CETP and apoA-I transgenes not only increased the proportion of apoA-I in the preβ-HDL fraction but also produced a marked redistribution of α-migrating HDL resulting in a significant increase in the small HDL_3 fractions at the expense of the larger HDL_{2b} population. The increase in HDL_3 may also contribute to the stimulation of efflux observed in HuAICETPTg mice. In agreement with this hypothesis, several studies have indicated that cholesterol efflux is inversely proportional to HDL size (44) and positively correlated to the levels of LpAI and HDL_3 (45).

Recently, Atger et al. (22) determined the fractional and relative efflux potential of diluted serum from mice expressing human apoA-I and/or CETP. Although the efficiency of the HDL particles as cholesterol acceptors (expressed as relative efflux potential) was greater in sera from HuAICETPTg mice than in HuAITg mice, the average cholesterol efflux was significantly lower in HuAICETPTg mice than in HuAITg mouse serum. Our study indicates that even though there was a significant decrease in HDL-cholesterol in HuAICETPTg mice plasma, plasma from HuAICETPTg mice had a greater ability to efflux cholesterol than HuAITg mouse plasma. Differences in experimental conditions such as the incubation times used to examine the cholesterol efflux may explain the apparent differences. The studies by Atger et al. (22) were performed by incubating diluted serum with labeled Fu5AH rat hepatoma cells for periods of time ranging from 30 min to a few hours. Under these conditions, the accumulation of cholesterol in the serum is more likely to represent the movement of cell-cholesterol to a larger HDL species that has a greater capacity for cholesterol (46). In contrast, in the present study, undiluted plasma was incubated with labeled human fibroblasts for short periods of time (1–5 min).

TABLE 4. Esterification of cell-derived cholesterol, LCAT activities and LCAT mRNA levels on HuAITg and HuAICETPTg mice

Transgenic Mouse Line	Cell-Derived Cholesterol Esterification	LCAT Activity	Relative LCAT mRNA Levels
	% labeled FC/h	nmol CE/ml.h	
HuAITg	7.24 ± 1.15	99.30 ± 5.70	1.00 ± 0.13
	(10)	(9)	(6)
HuAICETPTg	12.22 ± 1.47[a]	143.46 ± 6.45[a]	1.34 ± 0.16[a]
	(9)	(9)	(6)

[^{3}H]cholesterol-labeled fibroblasts were incubated for 15 min with plasma at 37°C. An aliquot of medium was extracted with chloroform and methanol, and free and cholesteryl esters were separated by TLC. The percent of cell-derived cholesterol esterified as a function of time is expressed as the ratio of free and cholesteryl ester radioactivity. LCAT activity was determined as the rate of synthesis of ^{3}H-labeled cholesterol and unlabeled egg lecithin and apoA-I as described in Materials and Methods. Eight μg of total liver RNA was electrophoresed, transferred to a nylon membrane, and hybridized to mouse LCAT or mouse β-actin cDNA probes. The β-actin mRNA level in each sample was used as an internal control to normalize the LCAT mRNA values. Values are expressed relative to HuAITg mice and represent the mean ± SD.

[a]Statistically significant differences from HuAITg mice.

Under these conditions, efflux is likely to represent the movement of cell-cholesterol to a high efficiency, but perhaps low capacity, HDL species such as preβ-HDL.

An important observation of our study is that the esterification of cell-derived cholesterol was increased in HuAICETPTg mice compared to HuAITg mice. Several lines of evidence suggest that LCAT and CETP activities are closely coordinated. For example, in humans, the increase in plasma triglycerides, phospholipids, and free cholesterol observed in postprandial lipemia was associated with a parallel increase in the cholesterol esterification and transfer rates to VLDL and LDL (47). In transgenic mice, the expression of human apoA-I and CETP transgenes or human CETP transgene alone (19, 48) decreased the free cholesterol to cholesteryl ester ratio compared to HuAITg and background mice. Furthermore, the fractional esterification of the cholesterol released from Fu5AH cells was increased in mice expressing the CETP transgene (22). Our present data also support the hypothesis of a coordinate regulation of LCAT and CETP. As shown in this report, it is conceivable that the increase in preβ- and small HDL particles induced by CETP may stimulate the LCAT reaction by either increasing the available substrate (29) or enhancing cholesteryl ester removal. The increase in cholesteryl ester synthesis observed in HuAICETPTg mice plasma could also be explained by an increase in the circulating levels of plasma LCAT. In support of this hypothesis, we have shown that plasma LCAT activity and liver LCAT mRNA levels were increased in HuAICETPTg mice suggesting a transcriptional regulation of LCAT. Recent studies have indicated that the transcription of CETP, LDL receptor, and HMG-CoA reductase genes is regulated by the cholesterol status of the cells (49, 50). More recently, Jackson et al. (51) have suggested that the transcriptional regulation of the farnesyl diphosphate synthase and HMG-CoA reductase, two key enzymes in the biosynthesis of cholesterol, requires a specific interaction between the NF-Y transcription factor with the sterol regulatory element-binding protein (SREBP), or related transcription factors. No similar mechanism has yet been proposed for LCAT or other proteins involved in the RCT pathway.

Consistent with our findings, recent investigations in patients with CETP deficiency (52) have shown that the absence of circulating CETP decreases the level of LpAI and causes a significant decrease in the efflux and LCAT-mediated esterification of cholesterol compared to normal controls. Our results and the evidence presented by others suggest that CETP could contribute to RCT not only by delivering cholesteryl esters to the liver via VLDL + LDL but also by generating small HDL species that stimulate the efflux of cholesterol. More recently, Hayek et al. (53) have indicated that CETP expression inhibits the development of early atherosclerotic lesions in hypertriglyceridemic mice. Although these studies suggest an important role of CETP in the development of atherosclerosis, further lesion studies in a different genetic background should be performed to assess the exact contribution of CETP in the RCT pathway and the development of atherosclerosis.

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