Cholesterol Efflux, Lecithin−Cholesterol Acyltransferase Activity, and Pre-β Particle Formation by Serum from Human Apolipoprotein A-I and Apolipoprotein A-I/Apolipoprotein A-II Transgenic Mice Consistent with the Latter Being Less Effective for Reverse Cholesterol Transport[†]

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ABSTRACT: Studies assessing fatty streak formation in mice have revealed that human apolipoprotein A-I (apoAI) transgenic mice (TgAI) have 15-fold less atherosclerosis susceptibility than combined human apolipoprotein A-I/human apolipoprotein A-II (apoAI:AII) transgenics (TgAI:AII) and 40-fold less than nontransgenic control mice. In order to examine the biochemical mechanisms underlying those *in vivo* observations, we have compared *in vitro* properties of serum from the different groups of animals for participation in cholesterol efflux, LCAT activation, and pre-β particle formation. Analysis of cholesterol efflux from both Fu5AH hepatoma and Ob1771 adipose cells revealed serum from the TgAI to be the most efficient in promoting efflux. The two-dimensional electrophoresis of mouse serum shows that control mice have exclusively apoAI in α particles. TgAI and TgAI:AII mice have 30 and 38% of total apoAI in particles with pre-β electrophoretic mobility, respectively. The distribution of cell-derived cholesterol between these apoAI-containing lipoprotein subspecies after 1 and 60 min of incubation with Fu5AH hepatoma cells was examined. This revealed after a 1 min incubation 66 $\pm$ 8 and 83 $\pm$ 9% of the counts in particles with pre-β mobility for TgAI and TgAI:AII mice, respectively; while after 60 min of incubation, only 6 $\pm$ 2% of counts remained in pre-β particles from the TgAI and 30 $\pm$ 3% for the TgAI:AII. This suggests faster movement of cholesterol from pre-β to α particles in plasma from the TgAI. Consistent with this is the observation that LCAT activity with both exogenous and endogenous substrate increased in the TgAI versus the TgAI:AII mice. The previously observed decrease in fatty streak formation in the TgAI versus the TgAI:AII and control mice is consistent with the *in vitro* studies presented here and suggests that HDL containing human apoAI is a more effective participant in the postulated early steps in reverse cholesterol transport than HDL containing both human apoAI and human apoAII, and/or murine HDL.

It has been demonstrated that C57BL/6 transgenic mice expressing high amounts of the human apolipoprotein A-I (apoAI)[1] are protected for development of fatty streak lesions on a high-fat diet (Rubin et al., 1991) and that this protection is diminished in transgenic mice expressing human apolipoprotein A-II (apoAII) in addition to apoAI (Schultz et al., 1993). The biochemical mechanism underlying the observation of how human apoAI-containing lipoproteins inhibit atherogenesis is still unknown. The most widely held hypothesis is the process of reverse cholesterol transport, whereby apoAI-containing lipoproteins participate in the transport of cholesterol from peripheral cells to the liver for catabolism and removal from the body (Fielding & Fielding, 1995).

The initial step in the process of reverse cholesterol transport is believed to involve interaction of a subgroup of small lipid-poor high-density lipoproteins with cell membranes (Castro & Fielding, 1988). Studies have suggested that particles involved in this process contain exclusively apoAI and have pre-β mobility upon electrophoresis. It has been suggested that the protein composition of the high-density lipoproteins (HDLs) involved in reverse cholesterol transport is important in the efficiency of this process. Although results from several studies, both *in vivo* and *in vitro*, are not entirely consistent, the majority of the studies have suggested that particles containing apoAI may be more efficient for the capture of cell-derived cholesterol than particles containing both apoAI and apoAII (Fielding & Fielding, 1981; Barbaras et al., 1987; Lagrost et al., 1995).

Lecithin−cholesterol acyltransferase (LCAT) is an enzyme involved in the esterification of free cholesterol. It thus plays a crucial role in the early steps of the cholesterol efflux

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[1] Abbreviations: apoAI, apolipoprotein A-I; apoAII, apolipoprotein A-II; LCAT, lecithin−cholesterol acyltransferase; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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process through conversion of the unesterified cholesterol taken up from the peripheral cells by lipoprotein particles and conversion to cholesteryl esters, which are then internalized within the lipoprotein particle core (Jonas, 1991). LCAT is activated by apoAI on the surface of HDL particles and likely is involved in the increase in size of particles from small pre-β particles to α-migrating particles (Miida et al., 1992).

In an attempt to correlate the differences observed *in vivo*, with regard to fatty streak formation in the human apoAI versus the human apoAI:AII transgenic mice, we have examined *in vitro* the early steps of reverse cholesterol transport. Plasma from the apoAI and the apoAI:AII transgenic mice was assessed with regard to cholesterol efflux, formation of pre-β migrating lipoprotein particles, and LCAT activation. These studies, consistent with the *in vivo* studies, suggest that plasma from the apoAI transgenic mice is more effective in the postulated early steps of reverse cholesterol transport.

EXPERIMENTAL PROCEDURES

Animals

The transgenic mice expressing human apoAI or apoAI:AII used in this study have been previously described (Schultz et al., 1993). The animals were housed in individual cages with ad lib access to food and water. Male mice 8−12 weeks old were given a regular mouse laboratory diet (Purina Chow 5001). For serum samples, blood was taken from overnight-fasted animals from the retro-orbital plexus under chloroform anesthesia. For plasma samples, blood obtained by intracardiac puncture was collected on streptokinase in PBS [pH 7.4 and 150 IU/(mL of blood)]. Samples were kept and centrifuged at 4 °C and serum and plasma used immediately.

Apolipoprotein and Lipid Measurements

Mouse apoAI and apoAII concentrations were measured by immunonephelometry using polyclonal antibodies raised in rabbits against synthetic peptides constructed with mouse apolipoprotein sequences nonhomologous to the corresponding sequences of human apolipoprotein. The antibodies showed no cross-species reactivity. Human apoAI and human apoAII were measured by electroimmunodiffusion with SEBIA plates. Lipids were quantified using kits from Boehringer Mannheim.

Isolation of HDL Fractions

HDL fractions used for cholesterol efflux studies were isolated from serum by gel filtration chromatography using a FPLC Pharmacia system with one Superose 6 and one 12HR column in series. The column fractions were assayed for their content of cholesterol (Boehringer Mannheim), and the HDL fractions were pooled. The protein concentration was determined by the method of Lowry (Lowry et al., 1951). Human apolipoprotein concentrations were measured with Sebia plates.

Analysis by Two-Dimensional Electrophoresis

The apolipoprotein distribution was determined by nondenaturing two-dimensional electrophoresis as previously described (Castro & Fielding, 1988). Briefly, the first dimension was carried out in 0.75% agarose gel (Pharmacia) in 50 mM barbital buffer (pH 8.6) on Gel bond (FMC bioproducts) at 7 °C (200 V) for 2 h. The second dimension was carried out with 2 to 15% gradient polyacrylamide gel electrophoresis at 120 V for 16 h. Two pieces were cut from the agarose gel and laid on the top of the polyacrylamide gel. Each polyacrylamide gel would therefore contain two patterns, and after both electrophoreses, the gel was divided in half, longitudinally. One part was conserved at 4 °C, and the other part was transferred to nitrocellulose sheet. Transfer was carried out in 0.025 M Tris and 0.192 M glycine at pH 8.3 for 2 h at 225 mA in semidry conditions (Electrophoresis-Atta). To situate the apoAI, the nitrocellulose sheets were blotted with rabbit polyclonal antibodies anti-human and anti-murine apoAI in 50 mM Tris and 0.5% NaCl buffer with 5% BSA and 0.2% Nonidet P40 (Sigma) for 60 min at room temperature. Then, the nitrocellulose sheets were blotted with protein A labeled with ^{125}I (Markwell, 1982). To quantify apoAI on the blot, a standard curve was done with known quantities of apoAI applied directly to the same nitrocellulose sheet. Identification of apoAII was done with rabbit polyclonal antibodies anti-human and anti-murine apoAII. The antibodies anti-murine apolipoproteins were the same used for apolipoprotein nephelometric measurements. Radioactivity was detected by autoradiography at −80 °C on XAR 5 Kodak film, and nitrocellulose sheet areas were cut out and counted by use of a Beckman Gamma 5500 B instrument. Identification of lipoproteins on the second part of the polyacrylamide gel was carried out using the anti-apoAI blot as a template. Lipoprotein areas were cut out and the lipids extracted with chloroform/methanol (1:1, v:v), and radioactivity was counted using a liquid β scintillation counter (Wallac 1410). To determine the molecular mass of the different lipoproteins after two-dimensional electrophoresis, protein molecular mass standards were run to equilibrium in the two-dimensional electrophoresis and stained with Coomassie Blue (R 250).

Cholesterol Efflux

Fu5AH Hepatoma Cells. Cellular cholesterol efflux was determined using rat Fu5AH hepatoma cells following the procedure described (de la Llera et al., 1994). Briefly, the cells were grown for 48 h in minimal essential medium (MEM) containing 5% fetal calf serum. Fu5AH cells (25 000 per milliliter) were plated on 2.4 cm multiwell plates using 2 mL/well. Two days after plating, cellular cholesterol was labeled during 60 h of incubation with [^{3}H]cholesterol (1 μCi/well). To allow equilibration of the label, the cells were rinsed and incubated for 24 h in MEM with 0.5% BSA. For determination of cholesterol efflux, the cells were washed once with PBS and incubated at 37 °C for 2 or 4 h with a 5% dilution of mouse serum, or the indicated concentration of isolated HDL fraction. At the end of each incubation, the medium was removed and centrifuged; the monolayer cells were washed three times with PBS and harvested with 0.5 mL of 0.1 M NaOH. Radioactivity was then measured in both medium and cells and the percentage of cholesterol efflux calculated.

In another set of experiments designed to determine the distribution of cell-derived cholesterol within the apoAI-containing lipoprotein species, cells were cultured; labeled; and equilibrated in the same way, but using 100 μCi/well [^{3}H]cholesterol. The cells were washed and incubated with freshly isolated mice plasma. After 1 and 60 min of

Table 1: Apolipoproteins and Lipid Concentrations[a]

	apoAI		apoAI						
mice	murine	human	murine	human	cholesterol total	cholesterol free	triglycerides	phospholipids	cholesterol HDL
control	61 ± 8	−	31 ± 8	−	54 ± 8	18 ± 3	53 ± 6	93 ± 9	43 ± 9
TgAI	<12	323 ± 110	21 ± 4	−	137 ± 37	37 ± 7	31 ± 7	166 ± 21	107 ± 32
TgAI:AII	<12	222 ± 33	23 ± 5	56 ± 10	103 ± 23	36 ± 7	55 ± 8	123 ± 12	93 ± 10

[a] All data are expressed as milligrams per deciliter of plasma; mean value ± SD ($n = 8$ mice per group).

incubation at 37 °C on an orbital shaker (Prolabo, France), samples were taken for determination of radioactivity and its distribution among plasma lipoproteins isolated by two-dimensional gel electrophoresis. In some experiments, plasma collected after 1 min from the labeled cells was transferred to a second dish of unlabeled cells and the further transfer of labeled cholesterol followed as a function of time. Samples of plasma medium were taken at 2 min for analysis.

Ob 1771 Adipose Cells. The characterization of the Ob1771 preadipocyte clonal line has been reported previously (Amri et al., 1986). Cells were plated at 2500 cells/cm^2 in multiwell plates (Nunc) and grown in Dulbecco's modified Eagle's medium supplemented with 10% (v:v) fetal calf serum, 200 units/mL penicillin, 50 μg/mL streptomycin, 33 mM biotin, and 17 mM pantothenate. After confluence, cells were maintained in the same medium supplemented with 17 nM insulin and 2 nM triiodothyronine. Isobutylmethylxanthine (100 μM) was added to the medium during the first 2 days after confluence. Under these conditions, differentiation occurred within 10 days. The medium was changed every other day.

To promote cholesterol accumulation in the cells, differentiated Ob1771 cells were first maintained at 37 °C for 48 h in a medium containing 10% lipoprotein-deficient serum and then exposed for 48 h to the same medium supplemented with LDL labeled with [1α,2α(n)-^{3}H]cholesteryl linoleate, according to Craig et al. (1982) [400 cpm/(μg of cholesterol) and 150 μg of cholesterol/mL]. Subsequently, the cells were washed with PBS (pH 7.4) containing 1% bovine serum albumin and then twice with phosphate-buffered saline. In the cholesterol efflux experiment, a 5% dilution of mouse serum was incubated for 2 or 4 h with the cholesterol-loaded cells. After incubation, media were removed and centrifuged to discard cell debris and then counted for radioactivity. The cells were washed twice with PBS/BSA and twice with PBS at 4 °C and dissolved in 1 mL of 0.1 M NaOH. The alkaline digest (0.5 mL) was used for radioactivity counts and the remainder to assay the protein concentration.

LCAT Activity

LCAT activity was determined following the exogenous substrate method as described by Chen and Albers (1982). Transgenic mice serum (10 μL) was incubated for 1 h at 37 °C with a modified proteoliposome substrate containing apoAI, [^{14}C]cholesterol, and POPC. After incubation, lipids were extracted with chloroform/methanol (2:1, v:v) and separated by thin-layer chromatography on silica gel plates using a solvent system composed of petroleum ether/diethyl ether/acetic acid (90:10:5, v:v:v). Cholesteryl ester and free cholesterol radioactivity were measured on a liquid scintillation β counter.

Cholesterol esterification was also estimated after a 10 min incubation with the [^{3}H]cholesterol-labeled Fu5AH cells and the medium extracted and separated by thin-layer chromatography as indicated above for the determination of LCAT activity.

Cholesteryl Ester Formation

To determine the endogenous capacity of cholesteryl esterification, the serum samples were incubated for 1 h at 37 °C in the presence or absence of the Fu5AH hepatoma cells. Free cholesterol and the individual classes of cholesteryl esters were separated by reverse phase high-performance liquid chromatography (HPLC) using a Varian System apparatus. Total lipid extracts of the samples were prepared by extraction with chloroform/methanol after the method of Folch et al. (1957); following evaporation of the extraction solvent, samples were redissolved in 2-propanol and injected into a reverse phase C18 Bondapack column (Waters). HPLC solvent consisted of acetonitrile/2-propanol (80:20). The mass of cholesteryl esters in the samples was calculated from standard curves of mass versus peak area and corrected for loss during extraction and injection.

RESULTS

Apolipoproteins and Lipid Measurements. We examined the apolipoproteins and lipids in the plasma of the apoAI transgenic, apoAI:AII transgenic, and control mice (Table 1). As previously noted (Schultz et al., 1993), murine apoAI concentrations were dramatically decreased in animals expressing the human apoAI transgene. Murine apoAII levels were decreased in both groups of transgenic mice, with a less significant decrease than the one observed for murine apoAI. Both the apoAI and the apoAI:AII transgenic mice had a greater than 2-fold increase in total HDL cholesterol, which was concomitant with the increase in total apoAI. Since it has previously been shown that the first acceptor of cellular cholesterol is apoAI-containing lipoprotein particles of pre-β mobility (Castro & Fielding, 1988), we characterized plasma from the various transgenic and nontransgenic control animals for the presence of such particles by bidimensional electrophoresis, followed with exposure to species-specific apoAI antisera. The nontransgenic control mice showed exclusively particles with α mobility (Figure 1A), while in contrast, the apoAI transgenic and the apoAI:AII transgenic mice both showed pre-β and α particles. Both groups of transgenic mice showed a nearly identical pattern with a pre-β profile consisting of five particles and some free human apoAI, with apparent molecular masses for these particles estimated to be 43, 70, 92, 116, and 256 kDa. The corresponding pre-β apoAI percentages were 30 ± 6 and 38 ± 8% in the apoAI transgenic and the apoAI:AII transgenic mice, respectively. Analysis of the pre-β migrating lipoproteins in the apoAI and apoAI:AII transgenic mice, with species-specific apoAI antisera, revealed that the 70 and 43 kDa particles reacted exclusively with human apoAI

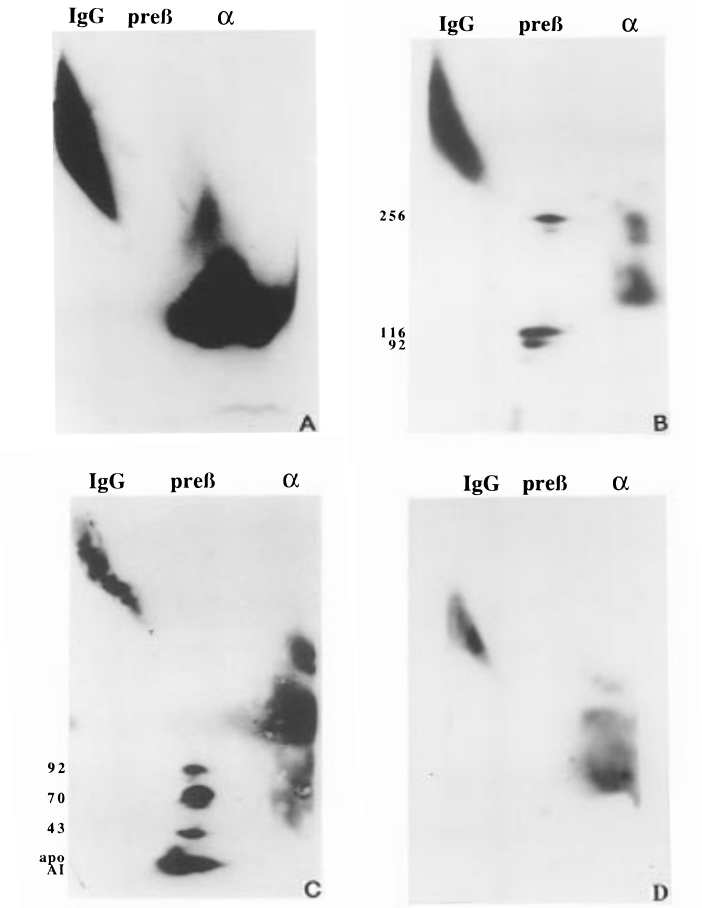

FIGURE 1: Two-dimensional electrophoresis. Twenty microliters of mice serum was used to separate lipoprotein particles by bidimensional electrophoresis as described in Experimental Procedures. Fractions were visualized by immunoblotting with anti-murine and/or anti-human apoAI and/or apoAII antibodies. (1A) Control mice, immunoblot anti-murine apoAI. (1B) ApoAI transgenic mice, immunoblot anti-murine apoAI. (1C) ApoAI transgenic mice, immunoblot anti-human apoAI. Similar results were obtained for the apoAI:AII transgenic mice. (1D) ApoAI:AII transgenic mice, immunoblot anti-human apoAII. Similar patterns were observed for all groups of animals when blotting with anti-murine apoAII antibodies.

antisera, while the 256 and 116 kDa particles reacted with murine-specific apoAI antisera. The 92 kDa particle reacted with both human and murine apoAI antisera (panels B and C of Figure 1). These studies demonstrate for the first time significant amounts of pre-β migrating lipoproteins in animals expressing the human apoAI transgene, but lacking in the nontransgenic control animals. Blotting with both murine and human anti-apoAII antibodies showed that all apoAII is concentrated in particles with α electrophoretic mobility for all groups of animals (Figure 1D), results that correspond to what is observed in human plasma (Castro & Fielding, 1988).

Cholesterol Efflux. In order to examine the properties of serum from the different groups of animals to promote cholesterol efflux *in vitro*, we measured the efflux of labeled cholesterol from the Fu5AH hepatoma cells after 2 and 4 h of incubation (Figure 2). An approximately 30% greater efflux of cellular cholesterol to diluted serum from the apoAI transgenics compared to the apoAI:AII transgenics and control mice was observed after 4 h of incubation. When values for control mice are taken as 100% to allow comparison of results obtained in different cell experiments,

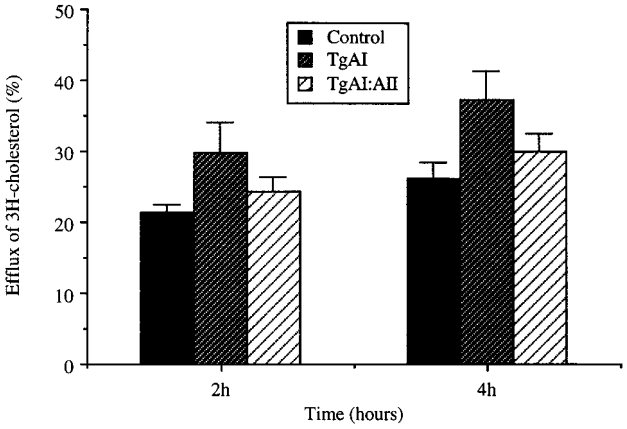

FIGURE 2: Cellular cholesterol efflux from Fu5AH cells. Two days after plating, cellular cholesterol was labeled during 60 h of incubation with [^{3}H]cholesterol (l μCi/well). To allow equilibration of the label, the cells were incubated for 24 h in MEM with 0.5% BSA. For determination of cholesterol efflux, the cells were washed and incubated at 37 °C for 2 and 4 h with a 5% dilution of murine serum. The medium was then removed and centrifuged, and the monolayer cells were washed and harvested with 0.5 mL of 0.1 M NaOH. The radioactivity was then measured in both media and cells and the percentage of cholesterol efflux calculated. Figures represent mean values ± SD of triplicate determinations and are representative of five separate experiments.

statistical differences become more significant between both groups of transgenic animals ($p < 0.005$): transgenic apoAI mice (127.5 ± 17.9, $n = 22$) versus transgenic apoAI:AII mice (105.4 ± 9.8, $n = 15$) after 4 h of incubation.

Reported roles of apoAI and apoAII in the initial removal of cholesterol from cells are still controversial (Johnson et al., 1991a). Indeed, cholesterol efflux was shown to be either decreased/unchanged or increased in the presence of apoAI-containing lipoproteins as compared with that with HDL particles containing both apoAI and apoAII (Lagrost et al., 1995; Mahlberg & Rothblat, 1992; Mahlberg et al., 1991; Johnson et al., 1991b; Oikawa et al., 1993; Barkia et al., 1991; von Hodenberg et al., 1991).

Discrepancies among reported cholesterol efflux measurements could be related to the cell types used, possibly reflecting differences in cholesterol pools. To minimize this issue, we decided then to test efflux to mouse plasma with another cell type, the adipocyte Ob1771.

Incubation of the serum from the various strains of mice with the Ob1771 adipocytes showed a pattern similar to that obtained with the hepatoma cells: apoAI transgenic (131 ± 9%) versus apoAI:AII transgenic (106 ± 1%) ($p < 0.05$, $n = 6$), when control mice are taken as 100%. Both cell types demonstrated plasma from the apoAI transgenics to be most effective in promoting cholesterol efflux.

In order to determine if the higher cholesterol efflux observed in both cell lines was related to the higher concentration of cholesterol HDL described in these animals or to the quality of the HDL fractions, we isolated the HDL fractions from the three groups of animals. This material was then incubated with the Fu5AH hepatoma cells on the basis of 50 μg of protein per milliliter. At this concentration, absolute values of cholesterol efflux are lower than those measured with total serum, but the differences between groups of animals are more impressive; assuming the cholesterol efflux induced by the HDL from the control animals as 100%, the HDL from apoAI transgenic mice that contain mainly LpAI particles resulted in 260% efflux, while

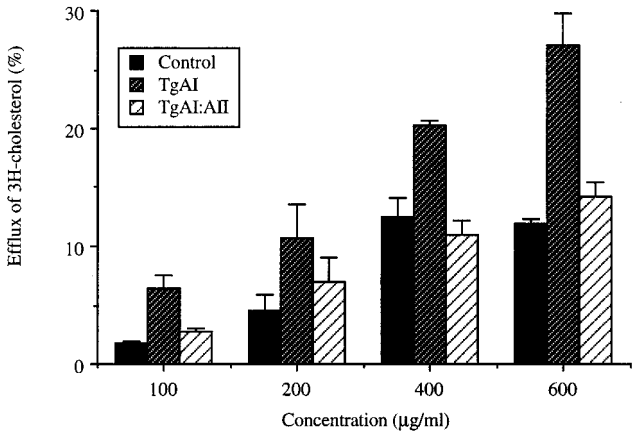

FIGURE 3: Concentration-dependent effect of mice HDL on cholesterol efflux from Fu5AH hepatoma cells. [^{3}H]Cholesterol-labeled cells were incubated for 4 h at 37 °C in the presence of various concentrations of mice HDL. Values are mean ± SD of triplicate determinations.

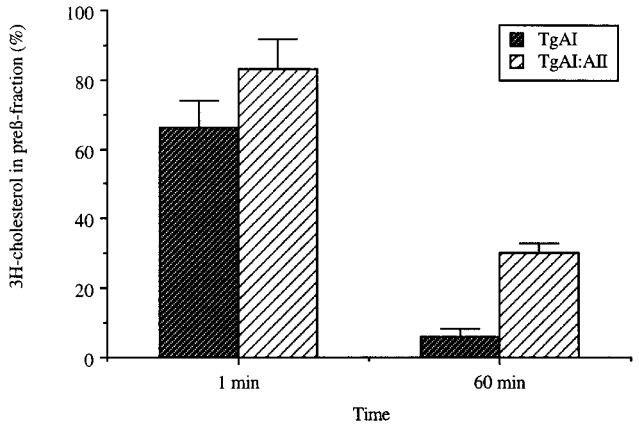

FIGURE 4: Distribution of cell-derived [^{3}H]cholesterol following the incubation of mice plasma with [^{3}H]cholesterol-labeled Fu5AH cells for 1 and 60 min at 37 °C. The vertical axis represents the proportion of total radioactivity recovered under these conditions in the apoAI-containing fractions of pre-β electrophoretic mobility.

that of the apoAI:AII transgenic, containing nearly exclusively LpAI/AII particles, showed a 106% efflux after a 4 h incubation.

To evaluate the levels of HDL that could saturate cholesterol efflux, we tested HDL concentrations up to 600 μg of protein per milliliter (Figure 3). Even at this high level, saturation was not reached, but the slope of the curve concentration was evidently different among the three groups of animals. At all HDL concentrations, the HDL from apoAI transgenic mice produces a higher cholesterol efflux.

The concentration of human apoAI in the HDL fraction was 0.24 mg/mL in the apoAI transgenic mice. HDL from the apoAI:AII transgenic mice contain 0.20 mg/mL apoAI and 0.053 mg/mL apoAII, values that allow the use of total protein concentration to indicate particle concentration.

Distribution of Cell-Derived Cholesterol. To examine efflux to the particles believed to participate in the early steps of efflux, plasma from the different animals was incubated with Fu5AH cells labeled with [^{3}H]cholesterol to a high specific activity, and incorporation of radioactivity in pre-β and α particles following 1 and 60 min of exposure to whole plasma was assessed.

As for the distribution of [^{3}H]cholesterol (Figure 4), we could observe that, after 1 min of incubation, 66 ± 8 and 83 ± 9% of the counts were in the pre-β migrating particles. In contrast, after 60 min of incubation, we observed that only 6 ± 2% of the [^{3}H]cholesterol was in the pre-β migrating particles for apoAI transgenic mice, but 30 ± 3% for the apoAI:AII transgenic mice. For apoAI transgenics, there was a great capacity to transfer [^{3}H]cholesterol from pre-β migrating particles to α particles.

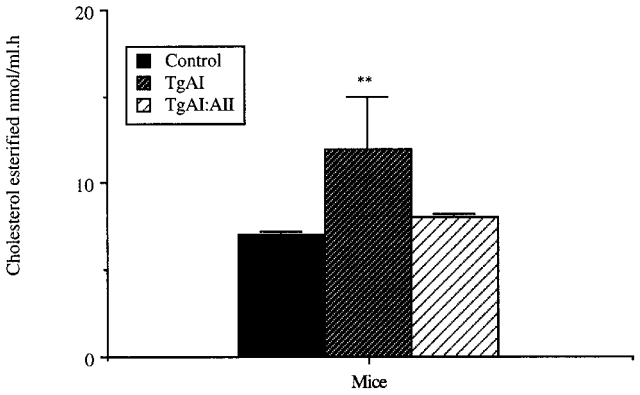

FIGURE 5: LCAT activity determined following an exogenous substrate method. Ten microliters of transgenic mice serum was incubated for 1 h at 37 °C with a proteoliposome substrate containing apoAI, [^{14}C]cholesterol, and POPC at a molar ratio of 1:10:100. $^{**}p < 0.05$ when compared to control by the Mann−Whitney U Test.

To obtain further information of this possibility, plasma that had been labeled for 1 min at 37 °C was transferred to an equivalent dish of unlabeled cells for 2 min under the same conditions, such that the early label on these lipoproteins would be replaced by unlabeled cholesterol. After the 2 min chase, 38 and 85% of the label were in particles with pre-β mobility for the apoAI and the apoAI:AII transgenic mice, respectively. We also estimated the fractional cholesterol efflux after 1 and 5 min of incubation with the labeled cells. The 1 min values were 0.85 and 0.45%; the 5 min values were 11 and 7% of the cellular cholesterol for apoAI and apoAI:AII transgenic mice, respectively.

We compared the various pre-β particles from serum from the apoAI transgenic mice and their ability to take up cholesterol from the labeled cells. The 70 kDa particle was observed to be the most efficient for both types of transgenic mice. After 1 min of incubation, 25% of the [^{3}H]cholesterol was found in this fraction. This 70 kDa particle from the plasma of the transgenic mouse had a capacity for the uptake of cholesterol similar to that observed previously for the pre-β particles described in human plasma (Castro & Fielding, 1988).

LCAT Activity. In human plasma, cell-derived cholesterol is channeled from pre-β_1 to a second particle with pre-β mobility (pre-β_2), from where it will be esterified by the action of LCAT and transferred to particles with α mobility (Francone et al., 1989). For this reason, LCAT constitutes the driving force for the concentration gradient from cell membranes to the plasma compartment (Fielding, 1984).

A possible explanation for the increased cellular cholesterol efflux and the transfer of cell-derived cholesterol from pre-β to α migrating particles observed in the apoAI versus the apoAI:AII transgenic mice is the increased LCAT activity and cholesterol esterification in the plasma from the apoAI animals. LCAT activity in serum from the various groups of animals using an exogenous substrate was examined (Figure 5). These results show that there was approximately

Table 2: Cholesteryl Ester Formation after 1 h of Incubation with (+) or without (−) Fu5AH Hepatoma Cells[a]

	controls		TgAI		TgAI:AII	
	−	+	−	+	−	+
cholesteryl arachidonate	114 ± 13	154 ± 33	128 ± 61	355 ± 175	116 ± 91	125 ± 0
cholesteryl linoleate	122 ± 29	120 ± 50	136 ± 75	313 ± 124	92 ± 56	121 ± 89
cholesteryl oleate	122 ± 30	128 ± 33	120 ± 74	196 ± 125	115 ± 94	121 ± 94
free cholesterol	102 ± 7	82 ± 22	101 ± 11	90 ± 24	130 ± 51	95 ± 7

[a] Values are the percentage of initial values. To determine the endogenous capacity of cholesteryl esterification, the serum samples were incubated for 1 h at 37 °C in the presence or absence of the Fu5AH hepatoma cells. Free cholesterol and the individual classes of cholesteryl esters were separated by reverse phase high-performance liquid chromatography (HPLC) using a Varian System with a C18 Bondapack reverse column (Waters). The HPLC solvent consisted of acetonitrile/2-isopropanol (80:20). The mass of cholesteryl esters in the samples was calculated from standard curves of mass versus peak area.

a 60% increase in cholesterol esterification in serum from the apoAI transgenic mice versus the other two groups of animals.

In order to investigate the endogenous capacity of cholesteryl ester formation in plasma from the various groups of animals, we incubated plasma samples for 1 h at 37 °C with and without Fu5AH hepatoma cells and quantitated by HPLC the individual classes of cholesteryl ester formed (Table 2). Using measures of cholesteryl esters on plasma not incubated as a relative baseline, esterification following exposure to cells demonstrated that the apoAI transgenics exhibited nearly a 3-fold increase in arachidonate and linoleate cholesteryl ester formation. In addition, when cholesteryl ester formation was estimated after only 10 min of incubation with [^{3}H]cholesterol-labeled cells, we observed that the sera from apoAI transgenic mice produce 10% more cholesteryl ester than that of the apoAI:AII transgenic mice. These results are consistent with the human apoAI-containing lipoprotein being a better activator of mouse LCAT, compared to mouse apoAI or HDL containing both human apoAI and apoAII.

DISCUSSION

Although numerous studies have demonstrated a strong association between elevated levels of HDL and a decreased risk for atherosclerosis, deciphering the mechanism responsible for association has proven to be illusive. The recent availability of genetically engineered mice and the assessment of their susceptibility to atherogenesis provided new substrates so we could begin connecting the *in vivo* atherogenic process to hypotheses regarding mechanisms that may explain HDL's antiatherogenic effects. The most widely held mechanism explaining the potential protective role of HDL is the hypothesis of reverse cholesterol transport, first put forth by Glomset (1968). This model involves HDL participating in the transport of serum cholesterol present in the vessel wall to delivery and removal by the liver. In the present study, we have attempted to correlate the early steps in this process, the uptake of cellular cholesterol by HDL through *in vitro* analysis of plasma from mice with defined differences in HDL apolipoprotein composition and atherosclerosis susceptibility.

Initial studies by Castro and Fielding (1988) have indicated that the initial acceptors of cellular-derived cholesterol are apoAI-containing lipoprotein particles with pre-β electrophoretic mobility. Using a bidimensional electrophoresis system, we characterized the apoAI-containing particles in plasma from the various groups of mice and the generation of both pre-β and α migrating lipoproteins. Comparison among the various groups of animals revealed that both lines of transgenic mice had significant amounts of pre-β migrating lipoprotein particles while the control animals had exclusively α migrating particles. In humans, a 70 kDa pre-β migrating lipoprotein particle has been observed as an efficient acceptor of cellular cholesterol, and in both groups of transgenic mice (apoAI and apoAI:AII), a similar size pre-β migrating particle is abundantly observed.

Quantitative *in vitro* studies examining cholesterol efflux to serum from the various groups of animals gave results that directly correlate with the *in vivo* resistance to atherosclerosis of the various groups of mice. In the two cell lines tested, Fu5AH hepatoma and Ob1771 adipocytes, serum from the apoAI transgenics promoted a greater degree of efflux than that from the apoAI:AII which was more active in promoting efflux than that from the nontransgenic controls. Analysis of efflux with the HDL lipoprotein fraction isolated from the various groups of animals also gave results consistent with analysis of total serum saturation. One of the most difficult aspects of comparing *in vitro* efflux with the clinical situation is the difficulty in defining patient populations. The genetically defined groups of mice used in this study provide us with a somewhat defined "clinical" setting in which to compare our *in vitro* studies. The more efficient efflux of cellular cholesterol to serum or HDL fractions from the apoAI transgenics versus the apoAI:AII transgenics is consistent with the *in vivo* findings of a decrease in fatty streak formation in apoAI versus apoAI:AII animals (Schultz et al., 1993) despite similar levels of total HDL cholesterol. The consistent correlation of the *in vivo* studies and *in vitro* studies adds support to the hypothesis of efflux of cellular cholesterol to HDL as a mechanism for HDL's apparent antiatherogenic properties.

Previous *in vitro* studies have demonstrated the movement of cellular (Castro & Fielding, 1988; Francone et al., 1989) cholesterol from pre-β to α lipoprotein particles. A distinct difference was observed with regard to the distribution of cellular-derived cholesterol between pre-β and α particles in the apoAI versus the apoAI:AII transgenic mice with time. A significantly greater amount of labeled cholesterol was associated with the α migrating lipoprotein particles following a 60 min exposure to cholesterol-labeled cells in plasma from the apoAI versus the apoAI:AII transgenic mice. The movement of unesterified cholesterol from pre-β lipoproteins to α lipoproteins is believed to be the step that follows the initial efflux of cellular-derived cholesterol. The accelerated rate at which this appears to occur in the plasma of the apoAI transgenics again is consistent with the clinical findings of decreased diet-induced atherogenesis in these animals.

The process of esterification of cellular-derived cholesterol is, in large part, believed to be the result of LCAT activity. The increased LCAT activity observed in the apoAI transgenics versus the apoAI:AII transgenics is predicted from the observed [^{3}H]cholesterol associated with α particles observed with time. Two mechanisms to explain the increased LCAT activity in plasma from these animals are (1) increased activation of LCAT by lipoprotein particles containing primarily human apoAI versus human apoAI:AII and (2) increased stability of LCAT in the apoAI transgenic mice. Recently, Golder et al. (1995) have examined the ability of proteoliposomes containing human apoAI versus murine apoAI to serve as an activator of murine LCAT. These studies revealed the human apoAI to be a more potent activator of murine LCAT. Our studies demonstrate increased LCAT activity from the apoAI transgenics compared to the apoAI:AII transgenics on proteoliposomes containing human apoAI. In this assay, the LCAT activity is correlated to the mass of the enzyme. These results thus indicate that there is more mass of LCAT in the plasma of the apoAI transgenic mice and that mice LCAT is better activated by human apoAI. In addition, we were able to demonstrate increased esterification of cellular-derived cholesterol following incubation of plasma from the apoAI versus the apoAI:AII transgenic animals. These results demonstrating (1) that human apoAI is a more effective activator of murine LCAT, (2) increased LCAT activity in plasma from the apoAI transgenic mice, and (3) increased esterification of cellular-derived cholesterol may explain the increased transfer of radiolabeled cholesterol to α migrating lipoprotein particles following the 2 min chase or even more the 60 min exposure to labeled cells.

Although other mechanisms may be invoked to explain the decreased atherogenesis observed in the apoAI transgenics versus the apoAI:AII transgenics and nontransgenic control animals, the *in vitro* studies demonstrated here are consistent with the early steps in the Glomset hypothesis of reverse cholesterol transport. Schultz et al. (1993) using immunoaffinity chromatography demonstrated that in the apoAI:AII transgenic mice 88% of human apoAI was associated with particles also containing human apoAII; meanwhile, the apoAI transgenic had mainly human apoAI particles. Although both the apoAI and the apoAI:AII transgenics contain similar amounts pre-β particles, these *in vitro* studies have demonstrated that HDL containing primarily apoAI is more effective in (1) promoting cholesterol efflux, (2) cell-derived cholesterol esterification, and (3) transfer from pre-β to α lipoproteins than HDL containing both apoAI and apoAII. Our results suggest that, at least in mice, not only is HDL containing exclusively apoAI, a more effective cholesterol acceptor, but also cellular-derived cholesterol is more effectively esterified and transferred to migrating HDL when the protein composition of this lipoprotein is primarily human apoAI versus human apoAI plus human apoAII. By comparison of *in vitro* mechanistic studies with *in vivo* analysis in the transgenic mice, previously unavailable approaches for studying the biological properties of HDL are now possible.

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