Altered lipoprotein metabolism in transgenic mice expressing low levels of a human receptor-binding-defective apolipoprotein E variant

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Abstract Transgenic mouse lines were produced that expressed low levels of a receptor-binding-defective variant of human apolipoprotein (apo) E, apoE(Arg112, Cys142). In transgenic mice, the human apoE was produced only by the kidney, whereas endogenous mouse apoE was produced mainly by the liver. The plasma concentration of the transgenic protein was about half that of endogenous apoE. The expression of transgenic apoE did not affect total plasma cholesterol and triglyceride levels, but the distribution of the human variant differed from that of endogenous apoE in the intermediate size and density range, where the transgenic protein accumulated selectively. Immunoblots of agarose gels of lipoprotein fractions showed that the transgenic protein occurred primarily on large α-migrating particles (HDL_1). This phenomenon was not observed in transgenic mice expressing normal human apoE-3, which distributed like endogenous apoE, suggesting that the defective apoE variant perturbed HDL_1 metabolism. In mice fed a high-fat, high-cholesterol diet, the transgenic apoE associated primarily with the apoB-containing lipoproteins. A significantly higher increase in very low density lipoprotein cholesterol was observed in fat-fed transgenics compared to fat-fed nontransgenic mice, suggesting a metabolic perturbation of apoB-containing lipoproteins. Thus, the receptor-binding-defective variant, apoE(Arg112, Cys142), expressed at low levels by the kidney, alters lipoprotein metabolism in transgenic mice, presumably by interfering with apoE-mediated removal of the lipoproteins from circulation.—**Fazio, S., Y. Horie, W. S. Simonet, K. H. Weisgraber, J. M. Taylor, and S. C. Rall, Jr.** Altered lipoprotein metabolism in transgenic mice expressing low levels of a human receptor-binding-defective apolipoprotein E variant. *J. Lipid Res.* 1994. **35:** 408–416.

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Apolipoprotein (apo) E is a constituent of several classes of lipoproteins, including liver-derived very low density lipoproteins (VLDL) and their remnants, intestine-derived chylomicron remnants, and certain subclasses of high density lipoproteins (HDL) (1). The major role of apoE in plasma of humans and other animal species is to mediate uptake of lipoproteins by specific lipoprotein receptors on cell surfaces (2). ApoE is a ligand for both the low density lipoprotein (LDL) receptor (3) and the LDL receptor-related protein (LRP) (4).

The critical role of apoE in catabolism of lipoproteins has been demonstrated in a variety of ways. Accelerated catabolism of apoB-containing remnants occurs when animals (normolipidemic or hypercholesterolemic) are injected or infused with apoE (5, 6). These types of studies suggest that apoE availability is a rate-limiting step in efficient lipoprotein remnant removal from plasma. Impaired catabolism of remnants occurs in humans with the lipid disorder type III hyperlipoproteinemia (HLP), which can arise either because of the expression of any of about a dozen receptor-binding-defective apoE mutants or because of apoE deficiency (7, 8).

ApoE also appears to be important in HDL metabolism, where it plays a role in the process of reverse cholesterol transport (9). In this process, excess peripheral cell cholesterol becomes associated with HDL, and through the action of lecithin:cholesterol acyltransferase (LCAT), the resulting cholesteryl esters become part of the neutral lipid core of HDL. ApoE appears to facilitate the expansion of this lipid core (10, 11), creating apoE-enriched particles of larger size and lower density that are termed HDL_1 (especially in humans and rodents) or HDL_c (cholesterol-induced HDL in fat-fed animals) (12). ApoE can mediate the catabolism of these cholesteryl ester-enriched HDL species by the liver (13, 14), complet-

Abbreviations: apo, apolipoprotein; CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein(s); HLP, hyperlipoproteinemia; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein(s); LRP, LDL receptor-related protein; VLDL, very low density lipoprotein(s).

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ing the reverse cholesterol transport process. Presumably, impaired catabolism of these HDL_1 particles could occur in the presence of a dysfunctional apoE.

The availability of small animal models affords an opportunity to investigate the above processes in more detail. Mice are an attractive model for the study of HDL metabolism because, unlike humans and some other animal models, they carry a large portion of their cholesterol in HDL rather than in the apoB-containing lipoproteins (15). This is due in large part to the absence of cholesteryl ester transfer protein (CETP) activity in their plasma (16). In CETP-deficient animals, apoE-mediated catabolism of HDL_1 can be a major route for cholesterol metabolism (17, 18).

Because LDL is not a major lipoprotein in mice, the most important ligand for cholesterol metabolism in this species is apoE, rather than apoB (19). The extremely efficient action of apoE in mice has been shown in several ways and is probably at least partly responsible for the resistance to atherosclerosis of most mouse strains (19). Overexpression of either rat apoE (20, 21) or normal human apoE3 (22) in transgenic mice leads to lower levels of VLDL, more efficient clearance of apoB-containing lipoproteins, and appearance of larger, less dense HDL_1 species compared to normal mice. Conversely, recently created null apoE mice (23, 24) and transgenic mice overexpressing dysfunctional apoE (25) have severe hyperlipidemia on normal diets due to accumulation of apoB-containing lipoproteins in the plasma.

We have created transgenic mice that express a receptor-binding-defective human apoE variant, apoE(Arg112, Cys142). This variant apoE has been described in a single family in which all seven subjects in four generations who are heterozygous for this apoE variant have type III HLP (26–28). When expressed in transgenic mice at high levels, this dysfunctional apoE variant yields a phenotype very similar to human type III HLP (25). However, as demonstrated in this report, even expression of only modest levels of this receptor-binding-defective apoE variant in transgenic mice was sufficient to perturb lipoprotein metabolism, presumably by interference with apoE-mediated removal of lipoproteins from circulation.

MATERIALS AND METHODS

Materials

The ICR mice were purchased from Jackson Laboratories, Bar Harbor, ME. Plasmid pBSSK was purchased from Pharmacia, Uppsala, Sweden. Nitrocellulose paper was purchased from Schleicher & Schuell, Keene, NH. The Superose 6 column, purchased from Pharmacia, was used on a Gilson Fast Protein Liquid Chromatography system. The Centricon concentration filters were from Amicon (Lexington, MA). Cholesterol and triglyceride standards were from Abbott (North Chicago, IL) and Boehringer (Indianapolis, IN), respectively; the automated system for lipid analysis (Kinetic Microplate Reader) was from Molecular Devices, Menlo Park, CA. All the reagents for lipoprotein agarose gels were from Ciba Corning, Palo Alto, CA. The ECL chemiluminescence detection kit for Western blots was purchased from Boehringer. Metofane (methoxyflurane) was purchased from Pitman-Moore (Mundelein, IL).

Preparation of constructs for microinjection

The human apoE-3 construct was the previously described HEG1 (29), containing the entire gene for apoE-3, 5 kb of 5' flanking region and 1.5 kb of 3' flanking region. This construct is expressed primarily in the kidney of transgenic mice (29, 30). The apoE(Arg112, Cys142) construct was prepared from HEG1 as follows. The 1.9-kb *Eco*RI fragment containing the entire fourth exon was excised from HEG1 and subcloned into pBSSK. The subclone was digested with *Sty*I and *Nar*I to excise the DNA fragment spanning codons 94–169. As the mutations are contained inside the *Sty*I/*Nar*I fragment (Arg for Cys at codon 112 and Cys for Arg at codon 142), a previously prepared prokaryotic expression vector was used to obtain the same *Sty*I/*Nar*I fragment with the mutations (28). The mutant *Sty*I/*Nar*I fragment was religated into the *Eco*RI fragment, which was then religated into the HEG1 vector. Positive transformants were identified by restriction analysis of plasmid DNA, by polymerase chain reaction of the mutated region followed by digestion with *Hha*I (31), and confirmed by sequencing of the mutated region and ligation boundaries. Upon transfection into COS cells, the constructs produced a protein of the expected size that reacted with human-specific anti-apoE antibodies. We also used a liver-specific construct (obtained from Dr. E. M. Rubin, Berkeley, CA), which contained 5 kb of the human apoA-I promoter to drive the expression of the human apoE gene. Into this construct, we inserted the appropriate mutations for apoE(Arg112, Cys142) (25).

Preparation and analysis of transgenic mice

Transgenic mice were prepared as described (29) using the microinjection method (32). Resultant offspring were weaned at 3–4 weeks of age, and DNA was prepared from tails as described (32). After digestion with *Eco*RI and electrophoresis on a 1% agarose gel, the Southern blot was performed as described (33). The founder animals identified were used to generate transgenic F1 mice. The transgene was transmitted to the offspring with the expected Mendelian pattern of inheritance. All the analyses presented in this study relate to the kidney-expressing mice, although similar analyses (except diet studies) have been performed on the liver-expressing line, which yielded comparable results.

Animal maintenance and diets

Mice of the outbred strain ICR were kept on a light cycle from 7:00 AM to 7:00 PM. Water and Purina mouse chow were given ad libitum. In the diet experiment, mice were kept for 5 weeks on a Purina-based diet containing 15% coconut oil, 1.25% cholesterol, and 0.5% cholic acid (Teklad, Madison, WI). Blood for determination of cholesterol and triglycerides was taken by tail-bleeding. Blood for separation of lipoproteins by Superose 6 chromatography was taken by cardiac puncture with the mouse in deep anesthesia, which was induced by exposure to metofane (methoxyflurane), according to the guidelines of the University of California Animal Care and Use Committee.

Preparation and analysis of total RNA

Total cellular RNA was isolated as described (34). Antisense RNA probe for human apoE mRNA was transcribed using bacteriophage T3 RNA polymerase in the presence of [^{32}P]UTP (35) from a cDNA fragment that had been cloned in Bluescript vector (Stratagene). The human apoE mRNA protected 192 bases of the labeled probe. Protected fragments were analyzed in 6% polyacrylamide gels containing 7 M urea, followed by radiography of the dried gel.

Sequential ultracentrifugation of mouse plasma

Plasma was prepared from blood by centrifugation at 14,000 rpm (microfuge) for 20 min at 4°C. Plasma was ultracentrifuged sequentially using a Beckman tabletop centrifuge at 100,000 rpm for 2.5 h at 4°C with the following density ranges: d<1.006, d = 1.006–1.02, d = 1.02–1.04, d = 1.04–1.06, d = 1.06–1.08, d = 1.08–1.10, d = 1.10–1.21, and d>1.21 g/ml. An aliquot of each fraction was then directly applied to an agarose gel for lipid staining or Western blotting.

Column chromatography and lipid analyses

Aliquots (200 μl) of plasma were chromatographed on a Superose 6 column using a procedure similar to that originally described by Kieft, Bocan, and Krause (36). Fractions were analyzed for cholesterol and triglyceride content (37) and apoE distribution. In some cases, fractions were pooled in four groups representing the major lipoprotein classes (fractions 16–19, VLDL; fractions 20–23, IDL/LDL; fractions 24–28, LDL/HDL$_1$; and fractions 29–35, HDL) (28, 37), and applied to an agarose gel for lipid staining or Western blotting. The large HDL identified by chromatography correspond to the lighter HDL floating in the 1.02–1.08 g/ml density range and are termed HDL$_1$.

Lipoprotein agarose gels and Western blots

The pooled chromatographic fractions were reduced in volume from 2 ml to 200 μl using Centricon filters. Aliquots (5 μl) were loaded onto a precast agarose gel (1% agarose, 1.3% barbital buffer) according to the manufacturer's instructions (Ciba Corning, Lipoprotein System), as described by Chappell (38). After electrophoresis for 35 min, the gel was either dried for lipid staining with Fat Red 7B or blotted onto nitrocellulose and incubated with the specified antibody. The human-specific antisera against apoE showed very little cross-reactivity with mouse apoE. In a slot blot assay using different dilutions of purified mouse or human apoE, this antisera reacted with a 20-fold lower affinity with mouse apoE than with human apoE. For determination of mouse apoE, we used a rat-specific anti-apoE antisera that recognized mouse apoE but did not cross-react with human apoE. In the Western blot assays, a semi-quantitation of apoE levels was achieved by comparing the densitometry readings of the sample bands with those of different concentrations of purified mouse and human apoE. In some cases, agarose gels also were blotted with apoB-specific or apoA-I-specific antisera to differentiate apoB-containing lipoproteins from HDL species.

In vitro lipoprotein association of ^{125}I-labeled apoE(Arg112, Cys142)

A plasmid vector containing the cDNA for apoE(Arg112, Cys142) was expressed in *E. coli* and the apoE was purified as described (28). A 50-μg sample of the purified protein was iodinated using the Bolton-Hunter procedure according to a previously described protocol (39). A 200-ng sample of ^{125}I-labeled apoE (specific activity of about 400 cpm/ng) was incubated for 2 h at 37°C in 200 μl fresh plasma from either nontransgenic mice or apoE(Arg112, Cys142) transgenic mice. Plasma was then separated on a Superose 6 column, concentrated as described above, and electrophoresed on agarose gels. The gels were stained with Fat Red 7B and exposed directly to autoradiographic film.

RESULTS

Four founder mice expressing apoE(Arg112, Cys142) were identified. Lines were developed from the two founders expressing the highest amount of protein in plasma, as determined by Western blot analysis. Southern blot analysis indicated that both lines incorporated about 10 copies of the transgene (data not shown). A ribonuclease protection assay of various mouse tissues demonstrated that the expression of the transgenic protein occurred exclusively in the kidney, while the endogenous mouse apoE was produced primarily in the liver (data not shown), consistent with previous observations of transgenic mice produced with this construct (29, 30). Despite the ectopic site of synthesis and secretion, transgenic human apoE associated with lipoproteins and was present in plasma at a concentration about half that of endogenous mouse apoE (data not shown).

Total plasma lipids were not significantly affected by the expression of the transgenic protein in plasma. Total plasma cholesterol and triglycerides were 106 ± 18 mg/dl and 83 ± 50 mg/dl, respectively, in nontransgenic mice and 100 ± 25 mg/dl and 67 ± 38 mg/dl, respectively, in transgenic mice. However, the expression of the human apoE variant altered the distribution of lipoprotein cholesterol (**Fig. 1**). Compared to nontransgenic littermates, transgenic mice reproducibly had somewhat higher (about 8 mg/dl) cholesterol values in the intermediate region of the chromatographic profile (fractions 24–28) that bordered on statistical significance (Student's *t* test, $P = 0.05$, n = 4). The distribution of human apoE among the chromatographic fractions as analyzed by Western blotting (**Fig. 2**) also suggested differences in the intermediate-size lipoprotein fraction. In both transgenic lines, the apoE(Arg112, Cys142) variant accumulated in the intermediate region, which contains both LDL and HDL_1. By contrast, in transgenic lines expressing normal human apoE3, the distribution (Fig. 2) was similar to that of endogenous mouse apoE (not shown), with most of the apoE3 in VLDL and the major HDL peak. It has been reported previously that human apoE3 and mouse apoE distribute nearly identically among plasma lipoproteins of transgenic mice (40). The distribution of apoE(Arg112, Cys142) among plasma lipoproteins of mice expressing low levels of the transgenic protein from the liver was indistinguishable from that observed in kidney-expressers (data not shown), indicating that the site of synthesis of apoE does not influence this phenomenon.

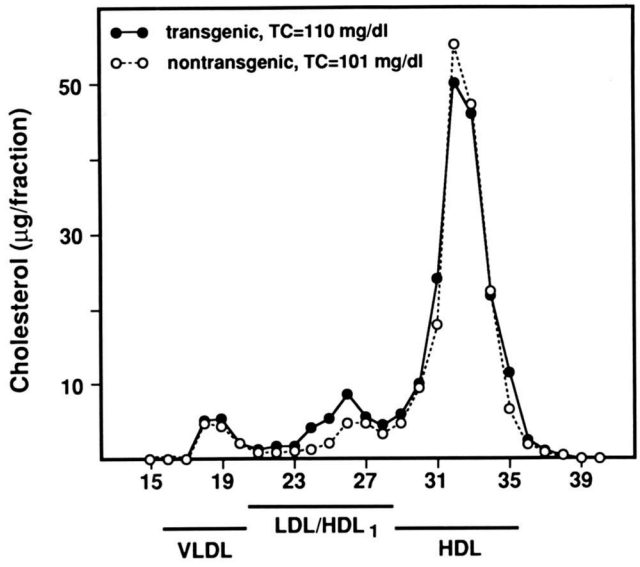

Fig. 1. Superose 6 chromatography of transgenic apoE(Arg112, Cys142) and nontransgenic mouse plasma. Plasma was isolated as described in Materials and Methods. A 200-μl aliquot was injected into a Superose 6 column, and 55 fractions of 0.5 ml each were collected. Cholesterol mass was determined on a portion of each fraction using an automated enzymatic method. The major lipoprotein classes are indicated. HDL_1 refers to apoE-enriched HDL of lower density and larger size than the major HDL; O--O, nontransgenic plasma; ●--●, transgenic plasma; TC, total plasma cholesterol.

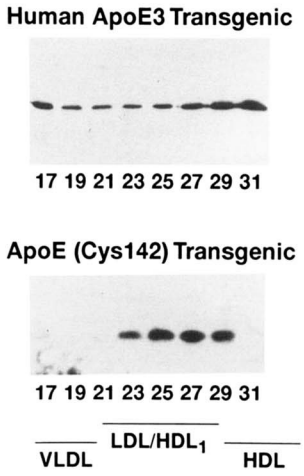

Fig. 2. Transgenic apoE distribution among mouse lipoproteins. Chromatography fractions were collected as described in the legend to Fig. 1. A 100-μl aliquot from each odd-numbered fraction from 17 to 35 was precipitated with Cab-O-Sil, separated on a 12% SDS-polyacrylamide gel, blotted onto nitrocellulose, and probed with a human-specific anti-apoE antiserum. Upper panel: normal human apoE-3 transgenic. Lower panel: apoE(Cys142) transgenic. The corresponding lipoprotein classes are indicated at the bottom of the figure.

Because the intermediate size (and density) region of the Superose 6 profile (Fig. 1) contains both apoB-containing LDL and α-migrating HDL_1 (40), pooled fractions were concentrated, electrophoresed on agarose gels, and immunoblotted to determine which lipoprotein(s) carried the apoE. As shown in **Fig. 3,** panel A, endogenous mouse apoE distributed to all lipoprotein fractions, including VLDL and its remnants and the main HDL fraction, in agreement with previous results (40). In contrast, in the same animal, the human apoE variant (Fig. 3, panel B) was associated almost exclusively with HDL_1. Less of the human apoE variant was detected in the major HDL peak and none in the VLDL fraction, confirming the results in Fig. 2. A similar predominance of human apoE variant in HDL_1 fractions was obtained when the plasma lipoproteins were separated by sequential density gradient ultracentrifugation (data not shown).

The accumulation of the human apoE variant on HDL_1 could be due either to preferential physical association or to a retarded clearance of HDL_1 enriched in the receptor-binding-defective apoE. To differentiate between these two possibilities, we incubated ^{125}I-labeled purified apoE(Arg112, Cys142) produced by recombinant DNA techniques (26) with plasma of an apoE(Arg112, Cys142) transgenic mouse. Apolipoprotein E has a great affinity for plasma lipoproteins and has been shown to distribute in vitro completely to lipoproteins in 2 h (41). The exogenously added apoE(Arg112, Cys142) distributed mostly to

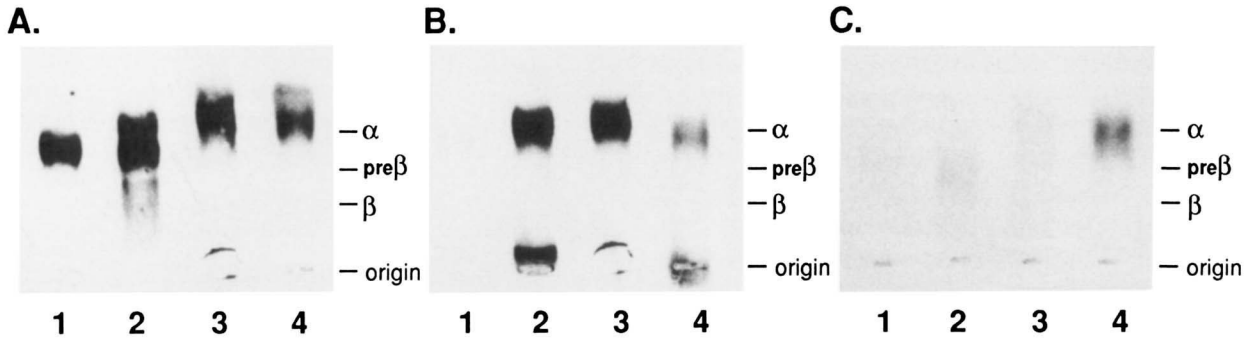

Fig. 3. In vivo association of endogenous mouse apoE (panel A) and transgenic apoE (panel B) and in vitro association of ^{125}I-labeled apoE(Arg112, Cys142) (panel C) with transgenic mouse lipoproteins. For lipoprotein localization of endogenous mouse apoE and transgenic apoE in an apoE(Arg112, Cys142) mouse, lipoproteins were separated on a Superose 6 column as in Fig. 1. Fractions encompassing the major lipoprotein peaks (as determined by the distribution of cholesterol and triglycerides) were pooled and concentrated to 200 μl. A 5-μl aliquot of each fraction was then loaded on a precast agarose gel and electrophoresed for 35 min. The lipoproteins were then blotted onto nitrocellulose and probed with either a rat-specific anti-apoE antiserum, which recognizes mouse apoE but not human apoE (panel A), or a human-specific anti-serum (panel B). The immunoreactive material visible near the origin in panels A and B is probably due to degradation or dissociation of apoE-containing material from the lipoprotein particle. For in vitro association of ^{125}I-labeled apoE(Arg112, Cys142) with lipoproteins of an apoE(Arg112, Cys142) transgenic mouse, apoE(Arg112, Cys142) produced by recombinant DNA techniques was iodinated as described in Methods, and 200 ng was added to 200 μl of fresh plasma from an apoE(Arg112, Cys142) transgenic mouse. After a 2 h incubation at 37°C, plasma was chromatographed on a Superose 6 column, and fractions were pooled, concentrated, and electrophoresed on agarose gels as described above. The agarose gel was stained with Fat Red 7B and exposed to autoradiographic film to detect the iodinated protein; 1, fractions 16–19 (VLDL); 2, fractions 20–23; 3, fractions 24–28; 4, fractions 29–35 (HDL). The origin and the migration distances of α-, pre-β-, and β-lipoproteins are indicated.

the major HDL peak (Fig. 3, panel C), in contrast to the endogenously produced apoE(Arg112, Cys142), which accumulated primarily on HDL_1 (Fig. 3, panel B) in the same plasma. Therefore, the association of the endogenously synthesized apoE variant with the HDL_1 of transgenic mice is a metabolic phenomenon.

The phenomenon of HDL_1 accumulation could also be demonstrated in transgenic hybrids produced by crossing F1 ICR transgenic mice with C57BL/6 mice. Compared to nontransgenic littermates, offspring from this cross carrying the human apoE(Arg112, Cys142) variant showed a readily detectable increase in HDL_1 (**Fig. 4**). In this case, the accumulation of the HDL_1 was visible by lipid staining because C57BL/6 mice have a lower basal level of HDL_1 than do ICR mice (H. de Silva, J. Más-Oliva, R. Pitas, J. Taylor, R. Mahley, unpublished results).

As the human apoE variant did not associate to a significant extent with mouse apoB-containing lipoproteins, it was unclear whether the low level of expression of this apoE variant could perturb the metabolism of apoB-containing lipoproteins. Therefore, a circumstance was sought in which the transgenic protein could be made to associate with apoB-containing lipoproteins. As the apoB-containing lipoproteins will increase in mice upon fat-feeding, nontransgenic and apoE(Arg112, Cys142) transgenic mice were fed a high-fat, high-cholesterol diet (Purina mouse chow with 15% coconut oil, 1.25% cholesterol, and 0.5% cholic acid) for 5 weeks. As shown in **Fig. 5,** this diet period was sufficient to cause a drastic change in lipoprotein cholesterol distribution, resulting in a reduction in HDL and a major increase in VLDL, which now contained the majority of total plasma cholesterol. Similar qualitative changes after diet were observed in the nontransgenic littermates, in which most of the cholesterol was also in VLDL (data not shown). The high-fat diet also affected apoE distribution among lipoproteins. As shown in **Fig. 6,** the human apoE variant associated primarily with apoB-containing lipoproteins, but was virtually absent in the major HDL fraction and much

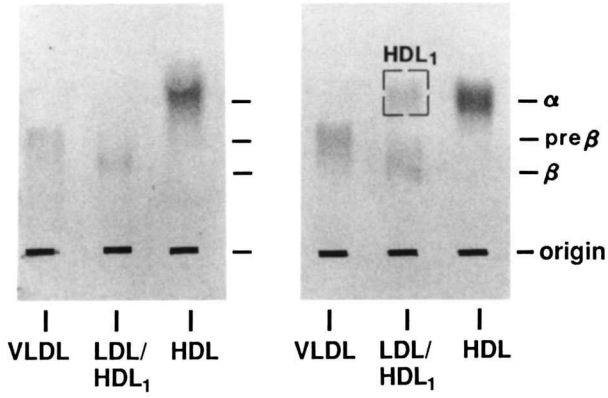

Fig. 4. Lipoprotein of ICR × C57BL/6 hybrid mice. Plasma of first generation hybrids was chromatographed on a Superose 6 column, and the fractions corresponding to the major lipoproteins (VLDL, LDL/HDL_1, HDL) were pooled and separated on an agarose gel. Lipoproteins were stained with Fat Red 7B. Left panel: nontransgenic ICR × C57BL/6. Right panel: transgenic ICR × C57BL/6 expressing apoE(Arg112, Cys142). The prominent HDL_1 band from the transgenic hybrid is boxed. The origin and the migration distances of α-, pre-β-, and β-lipoproteins are indicated.

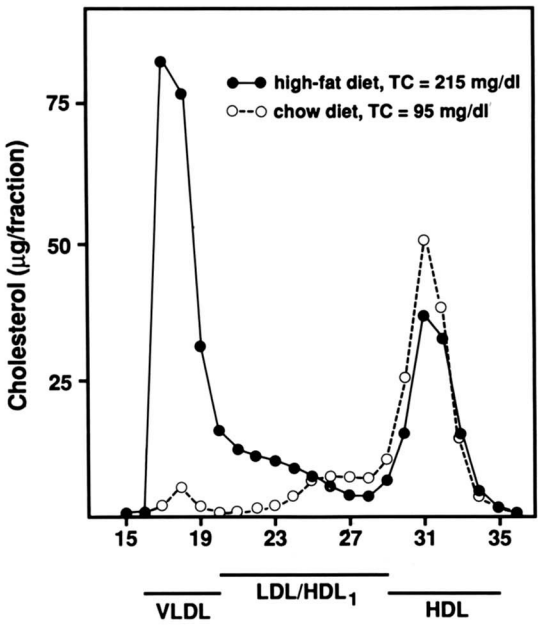

Fig. 5. Superose 6 chromatography of plasma from apoE(Arg112, Cys142) transgenic mice on either normal chow or high-fat, high-cholesterol diet. Transgenic mice were maintained on one of the two diets for 5 weeks. Chromatography and analysis were performed as described in the legend to Fig. 1. Lipoprotein classes are indicated as in Fig. 1: O--O, chow diet; ●--●, high-fat diet; TC, total plasma cholesterol.

reduced in HDL_1 (compare this distribution to that in Fig. 3, panel B). Almost all the endogenous mouse apoE was associated with the apoB-containing lipoproteins in both transgenic and nontransgenic animals (data not shown). However, a significant quantitative difference was observed between the transgenic and nontransgenic mice on the high-fat diet. Total cholesterol levels, which were similar in both groups of mice on the chow diet, increased significantly more in fat-fed apoE(Arg112, Cys142) transgenic mice than in the nontransgenic littermates (**Table 1**).

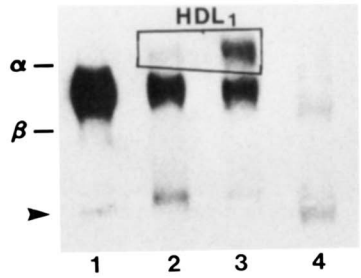

Fig. 6. Distribution of apoE(Arg112, Cys142) among mouse lipoproteins after a 5-week high-fat, high-cholesterol diet. Plasma (200 µl) was chromatographed on Superose 6, and chromatography fractions were collected as described in the legend to Fig. 1. Fractions corresponding to the major lipoprotein classes were pooled as described in the legend to Fig. 3. Each pool was concentrated using a Centricon filter, electrophoresed on an agarose gel, and immunoblotted with human-specific anti-apoE antiserum. The positions of HDL_1 are boxed. The origin is indicated by the arrow; the migration distances of α- and β-lipoproteins are also indicated.

TABLE 1. Plasma lipids of nontransgenic and apoE(Arg112, Cys142) transgenic mice[a]

Mice	Cholesterol		Triglycerides	
	Chow Diet	High-fat Diet	Chow Diet	High-fat Diet
	mg/dl		*mg/dl*	
Nontransgenic	106 ± 18	192 ± 64	83 ± 50	22 ± 19
(n)	(7)	(7)	(7)	(7)
Transgenic	100 ± 25	290 ± 81[b]	67 ± 38	33 ± 13
(n)	(9)	(9)	(9)	(9)

[a]Data are presented as mean ± standard deviation.
[b]P = 0.01 compared to nontransgenic mice on high-fat diet, pooled t-test.

In contrast, triglyceride levels in transgenic and nontransgenic animals were similar before diet and fell similarly after diet. The fall in triglyceride levels upon fat feeding is a typical response in mice as well as other animal species (42, 43).

To ascertain which lipoproteins accounted for the increased plasma cholesterol in the fat-fed transgenic animals, three mice with total cholesterol levels nearest that of the average were selected from the transgenic and nontransgenic fat-fed groups (Table 1), and their plasma was subjected to Superose 6 chromatography. **Fig. 7** shows the mean cholesterol profile of the three mice from each group. The results indicate that the plasma cholesterol difference between transgenic and nontransgenic mice is a result of higher VLDL levels in the fat-fed transgenic mice. Agarose electrophoresis of this fraction from each

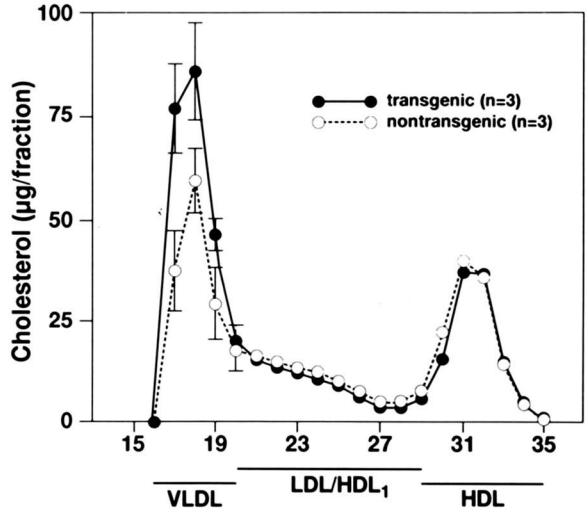

Fig. 7. Superose 6 chromatography of plasma from nontransgenic mice and apoE(Arg112, Cys142) transgenic mice after a 5-week high-fat, high-cholesterol diet. A 200-µl aliquot of plasma was chromatographed on a Superose 6 column, and the fractions containing the lipoproteins were analyzed for their cholesterol content. Three mice were analyzed in each group and the mean profile for each group was plotted (vertical bars in the VLDL peak represent standard deviation). Lipoprotein classes are indicated as in Fig. 1: O--O, nontransgenic (n = 3); ●--●, transgenic (n = 3).

group of mice failed to show a discernible difference between them; both had a broad VLDL band extending from the β-migrating to the pre-β-migrating position (data not shown).

DISCUSSION

Transgenic mice have been produced that express the human apoE variant, apoE(Arg112, Cys142). The DNA construct used did not contain the downstream regulatory element necessary for liver expression (29, 44), and the transgenic apoE was produced exclusively in the kidney. Despite the ectopic site of expression, we had reasons to expect that, upon secretion into the plasma, this human apoE variant would associate with lipoproteins and influence metabolic processes. It has been shown that normal human apoE3 produced from the same HEG1 construct distributes among plasma lipoproteins similarly to the liver-derived mouse apoE (40). Moreover, recent data showed that apoE secretion is not coupled to changes in VLDL output in the human hepatoma line HepG2 (37), suggesting that the presence of apoE on the VLDL is not essential at the time of secretion.

The transgenic mice expressed the human apoE variant in plasma at a level of about 2.5 mg/dl, roughly half of the endogenous mouse apoE level, which in most strains is in the range of 5–10 mg/dl (15). The plasma levels of human apoE expressed from this construct are comparable to those previously reported (29, 30). In mice on chow diet, although no significant differences in total plasma cholesterol and triglycerides were observed between transgenic and nontransgenic mice, HDL_1 were more apparent in transgenic mice than in nontransgenic littermates (Figs. 1 and 4). These HDL_1 contained most of the human apoE variant (Figs. 2 and 3). The in vitro incubation experiment presented in Fig. 3, panel C, suggests that the accumulation of apoE(Arg112, Cys142) on HDL_1 has a metabolic origin and does not result simply from a physical association preference of this apoE variant for HDL_1 particles. In support of this argument, we found that another receptor-binding-defective apoE variant, apoE-Leiden, which has a completely different structural mutation (45), also accumulated on HDL_1 in transgenic mice without having a physical preference for those particles (46). The most likely explanation for the observed results is that because of diminished interaction with mouse lipoprotein receptors, the apoE variant retarded the clearance of those HDL_1 particles containing a high ratio of the human variant apoE to the endogenous apoE. This proposed explanation is consistent with the postulated role for apoE in reverse cholesterol transport, specifically, that HDL_1 are cleared from the circulation through apoE-mediated catabolism. Koo, Innerarity, and Mahley (11) demonstrated the importance of apoE in the formation of large cholesterol-rich HDL (HDL_1/HDL_c). They showed that the apoE-deficient macrophage line J774 was able to transform HDL_3 into HDL_1, after cholesterol loading and in the presence of lecithin:cholesterol acyltransferase, only when exogenous apoE was added to the medium. These apoE-rich HDL_1 then acquired the ability to bind to the LDL receptor. Funke et al. (14) showed that apoE-rich HDL_1 are actively taken up by hepatocytes of both dogs and rats. The dysfunctional apoE produced in low levels by the apoE(Arg112, Cys142) mice reported here associates with the HDL and probably is involved in the expansion of the lipoprotein core and its transformation into HDL_1. The accumulation of HDL_1 might be a result of retarded clearance due to defective binding to lipoprotein receptors, or might be due to overproduction driven by the increased content in total apoE. Because of the low levels of expression of the defective human apoE variant and the presence of endogenous mouse apoE, the perturbations are not large.

The ectopic site of expression of the human apoE apparently does not influence the observed effects on HDL_1 metabolism. Transgenic mice have been prepared that express the same human apoE variant, apoE(Arg112, Cys142), but by the liver rather than the kidney. High expresser mice develop a spontaneous hyperlipidemia (25). In the present study, we analyzed a line expressing 2.5 mg/dl of the apoE variant, comparable to that reported here for the kidney-expressing lines, that also accumulated the transgenic protein on HDL_1. The results of the liver-expressing and kidney-expressing lines were essentially indistinguishable (data not shown).

The absence of CETP activity in mouse plasma could account, at least in part, for the specific effect of apoE(Arg112, Cys142) on HDL_1 metabolism and the lack of an effect on VLDL metabolism when the apoE variant is expressed at low levels in transgenic mice on a normal chow diet. However, VLDL metabolism was altered significantly in the absence of CETP when apoE(Arg112, Cys142) was expressed in transgenic mice at high enough levels (25). Furthermore, even in the presence of large amounts of β-VLDL in those mice, the effect of the apoE variant on HDL_1 accumulation persisted (S. Fazio, unpublished observation). It is quite possible that in the presence of CETP activity, HDL_1 accumulation would be eliminated if the HDL_1 are good substrates for CETP. Likewise, the presence of CETP may lead to a perturbation in VLDL metabolism in chow-fed mice expressing low levels of apoE(Arg112, Cys142). We are testing these and other hypotheses by crossing the apoE(Arg112, Cys142) transgenic lines with CETP transgenic mice (47).

When the transgenic mice reported here were fed a high-fat, high-cholesterol diet for 5 weeks, the human apoE variant associated mostly with the apoB-containing lipoproteins (Fig. 6) and led to an increase in total plasma and VLDL cholesterol that was significantly greater than

that in fat-fed nontransgenic mice (Table 1 and Fig. 7). In contrast, no difference was noted in response to a high-fat, high-cholesterol diet between nontransgenics and normal human apoE transgenics that expressed human apoE in the plasma at levels up to twice that of endogenous apoE (E. M. Rubin, personal communication). Therefore, our results indicate that the presence of the receptor-binding-defective apoE variant causes accumulation of apoB-containing lipoproteins over and above that induced by the high-fat diet. This finding suggests an additional, specific metabolic perturbation of apoB-containing lipoproteins, again consistent with impaired catabolism caused by the presence of the receptor-binding-defective apoE. Although the increased cholesterol levels after diet were limited to the VLDL (Fig. 7), the fat feeding studies demonstrated that metabolism of apoB-containing lipoproteins can be perturbed in mice expressing low levels of this human apoE variant.

Overall, the results of the present study suggest that mouse lipoprotein metabolism can be altered by the expression of only modest levels of a receptor-binding-defective human apoE variant and that the mechanism of action is similar to that involving defective apoE in humans, i.e., impaired apoE-mediated catabolism of lipoproteins due to defective interaction with lipoprotein receptors. These results support the concept of a crucial role for apoE in lipoprotein metabolism in mice (19). In particular, these findings highlight the importance of apoE in the metabolism of HDL and HDL_1, whose levels can be affected even by low level expression of a receptor-binding-defective apoE. This contrasts with the severe disruptions in the metabolism of apoB-containing lipoproteins occurring in the cases of either apoE absence (23, 24) or high level expression of dysfunctional apoE (25). Our results suggest that apoE also may be operable in a reverse cholesterol transport pathway where apoE is involved in the expansion of the HDL core and the eventual removal of cholesterol by apoE-mediated interaction with lipoprotein receptors.

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REFERENCES

1. Mahley, R. W., T. L. Innerarity, S. C. Rall, Jr., and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1294.
2. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
3. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
4. Beisiegel, U., W. Weber, G. Ihrke, J. Herz, and K. K. Stanley. 1989. The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature.* **341:** 162–164.
5. Yamada, N., H. Shimano, H. Mokuno, S. Ishibashi, T. Gotohda, M. Kawakami, Y. Watanabe, Y. Akanuma, T. Murase, and F. Takaku. 1989. Increased clearance of plasma cholesterol after injection of apolipoprotein E into Watanabe heritable hyperlipidemic rabbits. *Proc. Natl. Acad. Sci. USA.* **86:** 665–669.
6. Mahley, R. W., K. H. Weisgraber, M. M. Hussain, B. Greenman, M. Fisher, T. Vogel, and M. Gorecki. 1989. Intravenous infusion of apolipoprotein E accelerates clearance of plasma lipoproteins in rabbits. *J. Clin. Invest.* **83:** 2125–2130.
7. Mahley, R. W., and S. C. Rall, Jr. 1989. Type III hyperlipoproteinemia (dysbetalipoproteinemia): the role of apolipoprotein E in normal and abnormal lipoprotein metabolism. *In* The Metabolic Basis of Inherited Disease. 6th edition. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, Inc., New York. 1195–1213.
8. Rall, S. C., Jr., and R. W. Mahley. 1992. The role of apolipoprotein E genetic variants in lipoprotein disorders. *J. Intern. Med.* **231:** 653–659.
9. Mahley, R. W., K. H. Weisgraber, T. P. Bersot, and T. L. Innerarity. 1978. Effects of cholesterol feeding on human and animal high density lipoproteins. *In* High Density Lipoproteins and Atherosclerosis. A. M. Gotto, Jr., N. E. Miller, and M. F. Oliver, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. 149–176.
10. Gordon, V., T. L. Innerarity, and R. W. Mahley. 1983. Formation of cholesterol- and apoprotein E-enriched high density lipoproteins in vitro. *J. Biol. Chem.* **258:** 6202–6212.
11. Koo, C., T. L. Innerarity, and R. W. Mahley. 1985. Obligatory role of cholesterol and apolipoprotein E in the formation of large cholesterol-enriched and receptor-active high density lipoproteins. *J. Biol. Chem.* **260:** 11934–11943.
12. Mahley, R. W. 1978. Alterations in plasma lipoproteins induced by cholesterol feeding in animals including man. *In* Disturbances in Lipid and Lipoprotein Metabolism. J. M. Dietschy, A. M. Gotto, Jr., and J. A. Ontko, editors. American Physiological Society, Bethesda. 181–197.
13. Morrisett, J. D., H-S. Kim, J. R. Patsch, S. K. Datta, and J. J. Trentin. 1982. Genetic susceptibility and resistance to diet-induced atherosclerosis and hyperlipoproteinemia. *Arteriosclerosis.* **2:** 312–324.
14. Funke, H., J. Boyles, K. H. Weisgraber, E. H. Ludwig, D. Y. Hui, and R. W. Mahley. 1984. Uptake of apolipoprotein E-containing high density lipoproteins by hepatic parenchymal cells. *Arteriosclerosis.* **4:** 452–461.
15. Lusis, A. J., B. A. Taylor, D. Quon, S. Zollman, and R. C. LeBoeuf. 1987. Genetic factors controlling structure and expression of apolipoproteins B and E in mice. *J. Biol. Chem.* **262:** 7594–7604.
16. Jiao, S., T. G. Cole, R. T. Kitchens, B. Pfleger, and G. Schonfeld. 1990. Genetic heterogeneity of lipoproteins in inbred strains of mice: analysis by gel-permeation chromatography. *Metabolism.* **39:** 155–160.
17. Tall, A. R. 1986. Plasma lipid transfer proteins. *J. Lipid Res.* **27:** 361–367.
18. Ha, Y. C., L. B. F. Chang, and P. J. Barter. 1985. Effects of injecting exogenous lipid transfer protein into rats.

Biochim. Biophys. Acta. **833:** 203–210.
19. Brown, M. S., and J. L. Goldstein. 1992. Koch's postulates for cholesterol. *Cell.* **71:** 187–188.
20. Shimano, H., N. Yamada, M. Katsuki, M. Shimada, T. Gotoda, K. Harada, T. Murase, C. Fukazawa, F. Takaku, and Y. Yazaki. 1992. Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **89:** 1750–1754.
21. Shimano, H., N. Yamada, M. Katsuki, K. Yamamoto, T. Gotoda, K. Harada, M. Shimada, and Y. Yazaki. 1992. Plasma lipoprotein metabolism in transgenic mice over-expressing apolipoprotein E. Accelerated clearance of lipoproteins containing apolipoprotein B. *J. Clin. Invest.* **90:** 2084–2091.
22. de Silva, H. V., S. J. Lauer, J. Wang, Y. Newhouse, L. Shinto, R. W. Mahley, K. H. Weisgraber, and J. M. Taylor. 1992. Opposing effects of human apolipoproteins E and C-III in lipoprotein remnant clearance in transgenic mice. *Circulation.* **86:** I-471 (Abstr.).
23. Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* **258:** 468–471.
24. Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setälä, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* **71:** 343–353.
25. Fazio, S., Y-L. Lee, Z-S. Ji, and S. C. Rall, Jr. 1993. Type III hyperlipoproteinemic phenotype in transgenic mice expressing dysfunctional apolipoprotein E. *J. Clin. Invest.* **92:** 1497–1503.
26. Havel, R. J., L. Kotite, J. P. Kane, P. Tun, and T. Bersot. 1983. Atypical familial dysbetalipoproteinemia associated with apolipoprotein phenotype E3/3. *J. Clin. Invest.* **72:** 379–387.
27. Rall, S. C., Jr., Y. M. Newhouse, H. R. G. Clarke, K. H. Weisgraber, B. J. McCarthy, R. W. Mahley, and T. P. Bersot. 1989. Type III hyperlipoproteinemia associated with apolipoprotein E phenotype E3/3: structure and genetics of an apolipoprotein E3 variant. *J. Clin. Invest.* **83:** 1095–1101.
28. Horie, Y., S. Fazio, J. R. Westerlund, K. H. Weisgraber, and S. C., Rall Jr. 1992. The functional characteristics of a human apolipoprotein E variant (cysteine at residue 142) may explain its association with dominant expression of type III hyperlipoproteinemia. *J. Biol. Chem.* **267:** 1962–1968.
29. Simonet, W. S., N. Bucay, S. J. Lauer, D. O. Wirak, M. E. Stevens, K. H. Weisgraber, R. E. Pitas, and J. M. Taylor. 1990. In the absence of a downstream element, the apolipoprotein E gene is expressed at high levels in kidneys of transgenic mice. *J. Biol. Chem.* **265:** 10809–10812.
30. Smith, J. D., A. S. Plump, T. Hayek, A. Walsh, and J. L. Breslow. 1990. Accumulation of human apolipoprotein E in the plasma of transgenic mice. *J. Biol. Chem.* **265:** 14709–14712.
31. Hixson, J. E., and D. T. Vernier. 1990. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. *J. Lipid Res.* **31:** 545–548.
32. Brinster, R. L., H. Y. Chen, M. E. Trumbauer, M. K. Yagle, and R. D. Palmiter. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci. USA.* **82:** 4438–4442.
33. Paik, Y-K., D. J. Chang, C. A. Reardon, D. W. Walker, E. Taxman, and J. M. Taylor. 1988. Identification and characterization of transcriptional regulatory regions associated with expression of the human apolipoprotein E gene. *J. Biol. Chem.* **263:** 13340–13349.
34. MacDonald, R. J., G. H. Swift, A. E. Przybyla, and J. M. Chirgwin. 1987. Isolation of RNA using guanidinium salts. *Methods Enzymol.* **152:** 219–227.
35. Gilman, M. 1990. Ribonuclease protection assay. *In* Current Protocols in Molecular Biology. Vol. 1. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, editors. John Wiley and Sons, New York. 4.7.1–4.7.8.
36. Kieft, K. A., T. M. A. Bocan, and B. R. Krause. 1991. Rapid on-line determination of cholesterol distribution among plasma lipoproteins after high-performance gel filtration chromatography. *J. Lipid Res.* **32:** 859–866.
37. Fazio, S., Z. Yao, B. J. McCarthy, and S. C. Rall, Jr. 1992. Synthesis and secretion of apolipoprotein E occur independently of synthesis and secretion of apolipoprotein B-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **267:** 6941–6945.
38. Chappell, D. A. 1988. Pre-β-very low density lipoproteins as precursors of β-very low density lipoproteins. A model for the pathogenesis of familial dysbetalipoproteinemia (type III hyperlipoproteinemia). *J. Clin. Invest.* **82:** 628–639.
39. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1986. Lipoprotein-receptor interactions. *Methods Enzymol.* **129:** 542–566.
40. Taylor, J. M., W. S. Simonet, N. Bucay, S. J. Lauer, and H. V. de Silva. 1991. Expression of the human apolipoprotein E/apolipoprotein C-I gene locus in transgenic mice. *Curr. Opin. Lipidol.* **2:** 73–80.
41. Weisgraber, K. H. 1990. Apolipoprotein E distribution among human plasma lipoproteins: role of the cysteine–arginine interchange at residue 112. *J. Lipid Res.* **31:** 1503–1511.
42. Mahley, R. W., and K. H. Weisgraber. 1983. Canine plasma lipoproteins: characterization of lipoproteins from control and cholesterol-fed dogs. *In* CRC Handbook of Electrophoresis. Lipoproteins of Nonhuman Species. Vol. IV. L. A. Lewis, and H. K. Naito, editors. CRC Press, Inc., Boca Raton, Florida. 151–173.
43. Weisgraber, K. H., and R. W. Mahley. 1983. Characterization of rat plasma lipoproteins. *In* CRC Handbook of Electrophoresis. Lipoprotein Studies of Nonhuman Species. Vol. IV. L. A. Lewis, and H. K. Naito, editors. CRC Press, Inc., Boca Raton, Florida. 103–132.
44. Simonet, W. S., N. Bucay, R. E. Pitas, S. J. Lauer, and J. M. Taylor. 1991. Multiple tissue-specific elements control the apolipoprotein E/C-I gene locus in transgenic mice. *J. Biol. Chem.* **266:** 8651–8654.
45. Wardell, M. R., K. H. Weisgraber, L. M. Havekes, and S. C. Rall, Jr. 1989. Apolipoprotein E3-Leiden contains a seven-amino acid insertion that is a tandem repeat of residues 121 to 127. *J. Biol. Chem.* **264:** 21205–21210.
46. Fazio, S., Y. Horie, W. S. Simonet, K. H. Weisgraber, J. M. Taylor, and S. C. Rall, Jr. 1991. Expression of human apolipoprotein E variants in transgenic mice. *Circulation.* **84:** II-18 (Abstr.).
47. Marotti, K. R., C. K. Castle, R. W. Murray, E. F. Rehberg, H. G. Polites, and G. W. Melchior. 1992. The role of cholesteryl ester transfer protein in primate apolipoprotein A-I metabolism. Insights from studies with transgenic mice. *Arterioscler. Thromb.* **12:** 736–744.