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In the Absence of Endogenous Mouse Apolipoprotein E, Apolipoprotein E*2(Arg-158 → Cys) Transgenic Mice Develop More Severe Hyperlipoproteinemia than Apolipoprotein E*3-Leiden Transgenic Mice*

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Apolipoprotein E*2(Arg-158 → Cys) (APOE*2) transgenic mice were generated and compared to the previously generated apolipoprotein E*3-Leiden (APOE*3-Leiden) transgenic mice to study the variable expression of hyperlipoproteinemia associated with these two APOE variants. In the presence of the endogenous mouse *Apoe* gene, the expression of the APOE*3-Leiden gene resulted in slightly elevated levels of serum cholesterol as compared with control mice (2.7 ± 0.5 *versus* 2.1 ± 0.2 mmol/liter, respectively), whereas the expression of the APOE*2(Arg-158 → Cys) gene did not affect serum cholesterol levels, even after high/fat cholesterol feeding. The extreme cholesterol level usually found in apoE-deficient mice (*Apoe*$^{-/-}$ mice; 23.6 ± 5.0 mmol/liter) could be rescued by introducing the APOE*3-Leiden gene (APOE*3-Leiden·*Apoe*$^{-/-}$; 3.6 ± 1.5 mmol/liter), whereas the expression of the APOE*2(Arg-158 → Cys) gene in *Apoe*$^{-/-}$ mice minimally reduced serum cholesterol levels (APOE*2·*Apoe*$^{-/-}$; 16.6 ± 2.9 mmol/liter). *In vivo* very low density lipoprotein (VLDL) turnover studies revealed that APOE*2·*Apoe*$^{-/-}$ VLDL and APOE*3-Leiden·*Apoe*$^{-/-}$ VLDL display strongly reduced fractional catabolic rates as compared with control mouse VLDL (4.0 and 6.1 *versus* 22.1 pools/h). *In vitro* low density lipoprotein (LDL) receptor binding studies using HepG2 and J774 cells showed that APOE*2·*Apoe*$^{-/-}$ VLDL is completely defective in binding to the LDL receptor, whereas APOE*3-Leiden·*Apoe*$^{-/-}$ VLDL still displayed a considerable binding activity to the LDL receptor. After transfection of APOE*2·*Apoe*$^{-/-}$ and APOE*3-Leiden·*Apoe*$^{-/-}$ mice with adenovirus carrying the gene for the receptor-associated protein (AdCMV-RAP), serum lipid levels strongly increased (15.3 to 42.8 and 1.4 to 15.3 mmol/liter for cholesterol and 5.0 to 35.7 and 0.3 to 20.7 mmol/liter for triglycerides, respectively). This indicates that RAP-sensitive receptors, possibly the LDL receptor-related protein (LRP), mediate the plasma clearance of both APOE*2·*Apoe*$^{-/-}$ and APOE*3-Leiden·*Apoe*$^{-/-}$ VLDL.

We conclude that *in vivo* the APOE*2 variant is completely defective in LDL receptor binding but not in binding to LRP, whereas for the APOE*3-Leiden mutant both LRP and LDL receptor binding activity are only mildly affected. As a consequence of this difference, APOE*2·*Apoe*$^{-/-}$ develop more severe hypercholesterolemia than APOE*3-Leiden·*Apoe*$^{-/-}$ mice.

Apolipoprotein E is one of the major structural components of chylomicron and very low density lipoprotein (VLDL)[1] remnants and serves as a ligand in the receptor-mediated uptake of these particles from the blood by the liver (for review, see Refs. 1–3). Mutant forms of apoE can lead to an impaired clearance and subsequent accumulation of remnant lipoproteins in the circulation. This condition is known as familial dysbetalipoproteinemia (FD) or Type III hyperlipoproteinemia (for review, see Refs. 2 and 4), and can be inherited either as a recessive trait or as a dominant trait. The recessive inheritance pattern occurs in FD patients carrying the APOE*2(Arg-158 → Cys) mutation. Although about 1% of the population is homozygous for this defective APOE*2 allele, only a small percentage (4%) of these homozygous carriers develop hyperlipidemia, indicating that secondary metabolic or genetic factors are required for clinical expression of disease. Several rare mutations show a dominant inheritance pattern, including APOE*3(Arg-142 → Cys), APOE*2(Arg-145 → Cys), APOE*1(Lys-146 → Glu), APOE*2(Lys-146 → Gln), and the APOE*3-Leiden mutation (a 7-amino acid tandem repeat of residues 120–126). Also in the case of the dominantly inherited forms of FD, additional environmental and genetic factors do modulate the severity of the disease (4, 5).

Several groups have studied the biochemical characteristics of the different mutant forms of apoE. *In vitro* studies showed that apoE2(Arg-158 → Cys) was characterized by defective binding to the LDL receptor (2% of normal apoE3 binding activity), while binding to heparan sulfate proteoglycans

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[1] The abbreviations used are: VLDL, very low density lipoproteins; apo, apolipoprotein; LDL, low density lipoproteins; HDL, high density lipoproteins; FD, familial dysbetalipoproteinemia; HFC, high fat/cholesterol diet; SRM-A, standard rat mouse diet; FCR, fractional catabolic rate; LRP, LDL receptor-related protein; RAP, receptor-associated protein; HSPG, heparan sulfate proteoglycans; Ad, adenoviral vector; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; HCR, hepatic control region.

(HSPG) and the LDL receptor-related protein (LRP) were conserved (3, 4, 6). In contrast, mutant forms of apoE associated with the dominant mode of inheritance showed different binding affinities to the LDL receptor (20–100% of normal E3 binding activity), but were defective in binding to HSPG and LRP (6). These *in vitro* studies suggested a correlation between the mode of inheritance of the specific apoE mutation and the binding to HSPG and LRP.

Previously, we reported the generation of apolipoprotein E*3-Leiden (APOE*3-Leiden) transgenic mice. These mice proved to be very useful in studying the (variable) expression of hyperlipoproteinemia associated with this dominant APOE variant *in vivo* (7–9). In the present paper we report the generation of APOE*2(Arg-158 → Cys) transgenic mice. We compared the *in vivo* functions of the recessive APOE*2 mutation with the dominant APOE*3-Leiden mutation, using transgenic mice expressing these apoE variants either on a wild type apoE ($Apoe^{+/+}$) or an apoE-deficient ($Apoe^{-/-}$) background. We found that in the presence of the endogenous *Apoe* gene, the *APOE*2* and *APOE*3-Leiden* genes indeed behave like a recessive and dominant mutation, respectively, like in humans. However, on an apoE-deficient background, APOE*2-expressing mice displayed a much more severe hyperlipidemic phenotype than APOE*3-Leiden-expressing mice. *In vivo* RAP adenovirus transfection experiments and *in vitro* LDL receptor binding studies showed that APOE*2 remnant lipoproteins were cleared via a RAP-sensitive receptor pathway only, most likely the LRP, whereas in APOE*3-Leiden mice remnant lipoproteins were cleared via both the RAP-sensitive pathway and the LDL receptor pathway.

EXPERIMENTAL PROCEDURES

DNA Construct—The APOE*2-HCR construct (Fig. 1*A*) was generated from plasmid pJS276 kindly provided by Dr. J. D. Smith (The Rockefeller University, New York, NY), carrying both the *APOE* gene (from the −650 base pair *Bgl*II site to the +1.9-kilobase pair *Hin*dIII site) and a 5.5-kilobase pair *Bam*HI fragment from the region adjacent to APOC-I′, including the hepatic control region (HCR) (10). The APOE*2 gene was introduced into pJS276 by exchanging a 2-kilobase pair *Eco*RI fragment encompassing exon 4 of the APOE*3 gene with the similar fragment from a cosmid carrying APOE*2. The resulting insert (APOE*2-HCR) was excised from the plasmid using the restriction enzymes *Kpn*I and *Hin*dIII.

Generation and Analysis of Transgenic Mice—Transgenic mice expressing human APOE*3-Leiden were generated previously (11). These mice carry the human APOE*3-Leiden including an HCR fragment and are different from APOE*3-Leiden transgenic mice of the earlier described line 2, which co-express human apoCI (7–9). Transgenic and non-transgenic littermates were obtained by breeding with C57BL/6J mice (The Broekman Institute, Someren, The Netherlands). Mice of the F4 generation, were included in the experiments. Transgenic mice were identified by sandwich ELISA for the presence of human apoE in the serum (9). Transgenic mice, expressing human APOE*2 were generated according to Hogan *et al.* (12). Transgenic offspring were identified by polymerase chain reaction analysis and Southern blot analysis on genomic tail-derived DNA (7). Three founders were obtained from which one line with high liver expression of the APOE*2 transgene was bred with C57BL/6J mice. Mice of the F3 generation have been used for the current experiments.

ApoE-deficient ($Apoe^{-/-}$) mice were created as described previously (13–15). LDL receptor-deficient ($Ldlr^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). APOE*3-Leiden and APOE*2 transgenic mice were cross-bred with $Apoe^{-/-}$ mice to obtain APOE*2·$Apoe^{-/-}$ and APOE*3-Leiden·$Apoe^{-/-}$ mice. The resulting breeding offspring was analyzed for the presence of the transgenic human apoE protein by ELISA and the endogenous $Apoe^{-/-}$ genotype through tail tip DNA analysis, as described earlier (13).

For experiments, female mice 8–12 weeks of age were included. Mice were housed under standard conditions in conventional cages and given free access to food and water.

Diets—Mice were fed a regular mouse diet (SRM-A; Hope Farms, Woerden, The Netherlands). In case of dietary treatment, mice were fed for four weeks a semi-synthetic high fat/cholesterol diet (HFC/0.5%). This HFC/0.5% diet (purchased from Hope Farms) is a basic semi-synthetic diet, which was composed essentially according to Nishina *et al.* (16), supplemented with cocoa butter (15%, by weight), cholesterol (1%, by weight), and cholate (0.5%, by weight).

Human ApoE mRNA Measurements—Total RNA was isolated from brain, heart, kidney, liver, muscle, skin, and spleen using the RNAzol procedure (Cinna/Biotecx, Houston, TX). RNA samples (10 μg/lane) were separated by electrophoresis through a denaturing agarose gel (1.2% w/v) containing 7.5% formaldehyde and transferred to a nylon membrane (Hybond N^+, Amersham) according to the manufacturer's recommendations. Blots were subsequently hybridized with a ^{32}P-labeled probe of human APOE cDNA (17) and a rat glyceraldehyde-3-phosphate dehydrogenase cDNA at 55 °C in a solution containing 50% formamide. For liver tissue, the intensity of the hybridization signal was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amounts of APOE mRNA were related to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Lipid, Lipoprotein, and ApoE Measurements—Mice were fasted from 8 a.m. to 1 p.m. and weighed, and approximately 150 μl of blood was obtained from each individual mouse through tail-bleeding. Total serum cholesterol and triglyceride levels (without measuring free glycerol) were measured enzymatically using commercially available kits: 236691 (Boehringer Mannheim, Mannheim, Germany) and 337-B (Sigma).

Lipoprotein fractions were separated by FPLC chromatography using a 25-ml Superose 6B column as described previously (9).

For determination of serum mouse apoE concentrations, some 2 μl of serum was subjected to a 4–20% gradient SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) followed by incubation with polyclonal rabbit antisera against mouse apoE (kindly provided by Dr. K. Weisgraber, Gladstone Foundation Laboratories for Cardiovascular Disease, San Francisco, CA). Donkey anti-rabbit ^{125}I-IgG (Amersham, Litle Chalfont, United Kingdom) was used as a secondary antibody, and detection was performed by scanning the blots with a PhosphorImager. Mouse serum apoE level is expressed relative to the mouse apoE level of pooled serum of normal mice fed the standard chow diet. Human apoE concentrations were measured by sandwich ELISA as described previously (9).

Isolation, Characterization, and Labeling of VLDL—After a 5-h fasting period, blood was collected from 7–15 female mice (60 female mice in case of a wild type mice). Sera were pooled and ultracentrifuged to obtain the VLDL fraction ($d < 1.006$ g/ml). Total and free cholesterol, triglyceride (without glycerol), and phospholipid content of the VLDL were measured enzymatically, using commercially available kits (236691 and 310328 from Boehringer Mannheim, 337-B from Sigma, and 990-54009 from Wako Chemicals GmbH, Neuss, Germany, respectively). VLDL protein was determined using the method of Lowry (18). To determine apolipoprotein composition of the VLDL, some 4.5 μg of VLDL protein was subjected to a 4–20% gradient SDS-PAGE. Proteins were either stained with Coomassie Brilliant Blue R or transferred to nitrocellulose membranes (Schleicher & Schuell) followed by incubation with polyclonal rabbit antisera against mouse apoB, human apoE, and mouse apoE. Donkey anti-rabbit ^{125}I-IgG (Amersham) was used as a secondary antibody, and detection was performed by scanning the blots with a PhosphorImager. The polyclonal rabbit anti-human apoE shows some cross-reaction with mouse apoE, at least in Western blotting experiments. VLDL was radiolabeled with ^{125}I by the iodine monochloride method (19). The fraction of ^{125}I-label present in apoB(100+48) was determined by isopropanol precipitation (20, 21) and ranged from 30 to 65% of total label. The isopropanol method precipitates apoB100 and apoB48 completely, whereas other VLDL apolipoproteins are not precipitated. This was confirmed for the various mouse VLDL samples by apolipoprotein analysis of post-precipitation supernatant, using SDS-PAGE and subsequent Coomassie staining of the proteins as described above.

Human VLDL and LDL were isolated from fasted serum of healthy volunteers by density gradient ultracentrifugation as described by Redgrave *et al.* (22).

VLDL Kinetic Studies—After a 5-h fasting period, SRM-A-fed female mice were intravenously injected with 0.2 ml of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 10 μg of ^{125}I-labeled autologous VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t = 5, 10, 30, 60, and 90 min after injection. The serum content of ^{125}I-labeled apoB(100+48) was measured by isopropanol precipitation followed by counting ^{125}I-label in the pellet. A bi-exponential model was used to estimate the area under the ^{125}I-apoB decay curve and subsequent calculation of VLDL-apoB fractional catabolic rate (FCR).

Production rate of hepatic VLDL was determined by intravenous injection of Triton WR 1339 as described (9).

Binding of VLDL to HepG2 and J774 Cells—HepG2 cells were cultured in 24-well plates as described (23). Twenty-four hours prior to each experiment, the cells were washed with Dulbecco's modified Eagle's medium containing 1% (v/v) human serum albumin and further incubated with Dulbecco's modified Eagle's medium containing 5% (v/v) of lipoprotein-deficient serum ($d < 1.21$ g/ml) instead of fetal calf serum.

The receptor-mediated binding of ^{125}I-labeled VLDL to the cells was determined after a 3-h incubation at 4 °C with indicated amounts of ^{125}I-labeled lipoprotein, either in the presence or in the absence of a 200 μg/ml excess of unlabeled lipoprotein or human VLDL, exactly as described earlier (23).

To study whether the respective VLDL samples bind the LDL receptor, competition experiments were performed using J774 cells (24). Therefore, J774 cells were incubated for 3 h at 4 °C with 10 μg/ml ^{125}I-labeled human LDL in the presence of indicated amounts of unlabeled VLDL lipoprotein samples. Thereafter, cells were washed and binding at 4 °C was measured as described (24).

Adenovirus Transfections—The generation of the recombinant adenoviral vectors expressing RAP (AdCMV-RAP) and LacZ (AdCMV-LacZ) under control of the CMV promotor has been described and were kindly provided by Dr. T. Willnow and Dr. J.Herz (25). The recombinant adenovirus was propagated and titrated on the Ad5 E1-transformed human embryonic kidney cell line 911 as described (26). For storage, the virus was supplemented with mouse serum albumin (0.2%) and glycerol (10%), and aliquots were flash-frozen in liquid N_2 and stored at −80 °C. Routine virus titers of the stocks varied from 1 to 5 × 10^{10}/ml.

For *in vivo* adenovirus transfection, on day zero, 1.5 × 10^9 plaque forming units in a total volume of 200 μl (diluted with phosphate-buffered saline) were injected into the tail vein of the SRM-A-fed female mice. Fasted blood samples were drawn from the tail vein of fasted mice at 2, 3, and 4 days after virus injection.

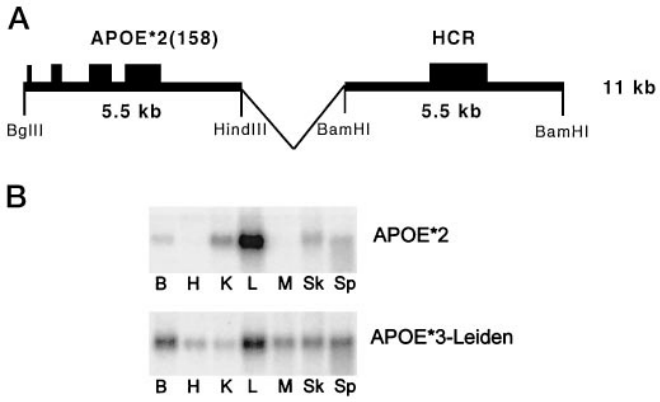

FIG. 1. *A*, schematic representation of the APOE*2-HCR construct used for microinjection. 11-kilobase pair DNA was construct used for microinjection. APOE*2 and HCR sequences (*solid black boxes*) and relevant restriction sites are indicated (see "Experimental Pocedures"). *B*, tissue transgene expression pattern in APOE*2 and APOE*3-Leiden mice. Total RNA was isolated from brain (*B*), heart (*H*), kidney (*K*), liver (*L*), muscle (*M*), skin (*Sk*), and spleen (*Sp*) of SRM-A-fed female APOE*2·*Apoe*$^{-/-}$ (*top panel*) and APOE*3-Leiden·*Apoe*$^{-/-}$ mice (*bottom panel*). Some 10 μg was used for Northern blot analysis, followed by hybridization with a probe of human APOE cDNA and rat glyceraldehyde-3-phosphate dehydrogenase cDNA as a reference (data not shown).

RESULTS

Generation of Transgenic Mice—Three strains of APOE*2 transgenic mice were generated, of which two strains showed high level expression of human apoE mRNA in the liver. One strain was used for further studies. Analysis of a series of different tissues by Northern blotting demonstrated that the expression of the APOE*2 transgene was mainly confined to the liver (Fig. 1*B*, *top panel*). Hepatic human APOE mRNA levels were about 30% higher in the APOE*2 transgenic mice as compared with the previously generated APOE*3-Leiden mice (100.0 ± 19.7 *versus* 67.9 ± 10.5%). In addition to transgene expression in the liver, APOE*3-Leiden mice also express the transgene at high level in brain and at lower levels in the other tissues examined (Fig. 1*B*, *bottom panel*).

Serum Lipid, Lipoprotein, and ApoE Levels in Various ApoE Transgenic Mice—As presented in Table I, APOE*3-Leiden mice show significantly elevated levels of serum cholesterol levels as compared with non-transgenic mice. This increase in serum cholesterol was confined to the VLDL/LDL-sized lipoprotein fractions (data not shown). On the regular chow diet (SRM-A), APOE*2 transgenic mice did not show elevated serum cholesterol levels as compared with non-transgenic mice.

On a high/fat cholesterol diet, APOE*3-Leiden transgenic mice had 2-fold higher serum cholesterol level as compared with non-transgenic mice, which was mainly due to increased levels of VLDL/LDL-sized lipoproteins (data not shown). In contrast, serum cholesterol levels in cholesterol-fed APOE*2 transgenic were similar to non-transgenic mice. Hence, in the presence of the mouse *Apoe* gene, APOE*2 transgenic mice are normolipidemic, even under dietary stress, whereas APOE*3-Leiden transgenic mice develop (diet-induced) hypercholesterolemia.

For studying the *in vivo* functional properties of the mutant apoE forms in absence of a functional mouse *Apoe* gene, APOE*2 and APOE*3-Leiden transgenic mice were cross-bred with *Apoe*$^{-/-}$ mice (13–15) (designated as APOE*2·*Apoe*$^{-/-}$ and APOE*3-Leiden·*Apoe*$^{-/-}$ mice, respectively).

On a regular SRM-A diet, the expression of the APOE*3-Leiden transgene resulted in an almost complete rescue of the extremely hypercholesterolemic phenotype usually found in

TABLE I

*The effect of the human APOE*3-Leiden and the APOE*2 transgene on serum lipids, lipoproteins, and apolipoprotein E levels in mice with or without the wild type mouse Apoe alleles*

Female mice of 2–3 months of age were fed a SRM-A diet or a HFC/0.5% diet. After 4 weeks of feeding, mice were fasted and blood samples were drawn from the tail vein. Total serum cholesterol (TC), triglycerides (TTG), and ApoE (mouse and human) values are the mean ± S.D. of 4–7 in case of mice with the *Apoe*$^{+/+}$ background or 15–18 mice in case of the *Apoe*$^{-/-}$ background. RU, relative units; ND, not determined.

Diet	APOE transgene	Endogenous *Apoe* genotype						
		Apoe$^{+/+}$				*Apoe*$^{-/-}$		
		TC	TTG	Mouse apoE[a]	Human apoE	TC	TTG	Human apoE
		mmol/liter	*mmol/liter*	*RU*	*mg/dl*	*mmol/liter*	*mmol/liter*	*mg/dl*
SRM-A		2.1 ± 0.2	0.5 ± 0.2	1.0 ± 0.2		23.6 ± 5.0	0.5 ± 0.3	
SRM-A	APOE*3-Leiden	2.7 ± 0.5[b]	0.8 ± 0.4	1.0 ± 0.3	2.7 ± 0.4	3.6 ± 1.5[b]	0.3 ± 0.2	0.5 ± 0.1
SRM-A	APOE*2	2.1 ± 0.2[c]	0.6 ± 0.2	1.0 ± 0.3	0.9 ± 0.2[c]	16.5 ± 2.9[b,c]	2.4 ± 0.8[b,c]	9.2 ± 0.8[c]
HFC/0.5%		6.5 ± 1.1	0.1 ± 0.0	1.6 ± 0.6		ND	ND	ND
HFC/0.5%	APOE*3-Leiden	13.7 ± 2.5[b]	0.2 ± 0.1[b]	2.4 ± 0.7	4.5 ± 0.4	ND	ND	ND
HFC/0.5%	APOE*2	6.8 ± 1.5[c]	0.1 ± 0.1[b]	1.6 ± 1.0	1.1 ± 0.2[c]	ND	ND	ND

[a] Mouse apoE levels are expressed relative to mouse apoE levels present in pool serum of normal female mice fed the regular SRM-A diet.
[b] $p < 0.05$, significantly different from non-transgenic mice fed the same diet, using nonparametric Mann-Whitney tests.
[c] $p < 0.05$, indicating significant difference between APOE*3-Leiden and APOE*2 transgenic on the same diet and the same Apoe genotype, using non-parametric Mann-Whitney tests.

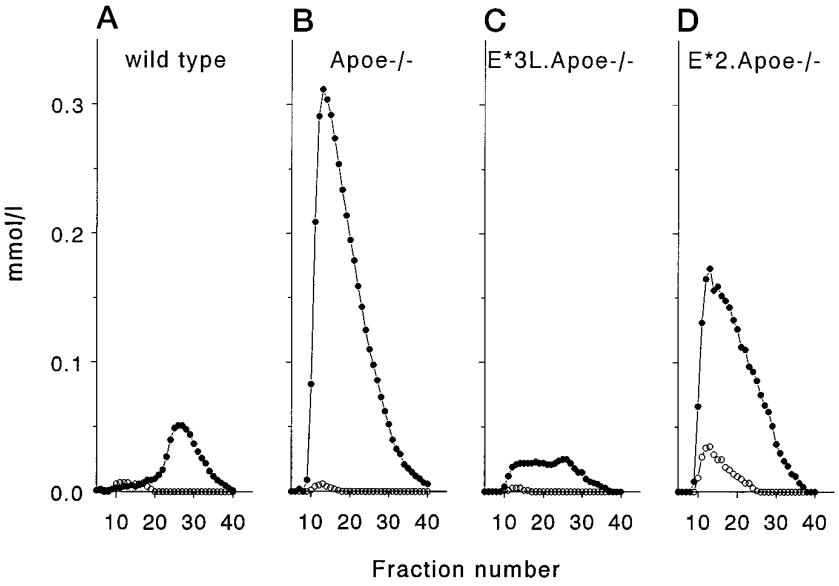

FIG. 2. **Distribution of serum cholesterol and triglycerides among lipoprotein fractions.** Lipoprotein fractions were separated by FPLC permeation chromatography using a 25-ml Superose 6B column, and fractions were analyzed for cholesterol (●) and triglycerides (○). Lipoprotein profiles are shown for SRM-A-fed female wild type (*panel A*), *Apoe*$^{-/-}$ (*panel B*), APOE*3-Leiden·*Apoe*$^{-/-}$ (*panel C*), and APOE*2·*Apoe*$^{-/-}$ SRM-A-fed mice (*panel D*). Each run is performed with a fasted pool serum of at least 12 mice of the same group. Fraction numbers 10–23 and 24–40 correspond to VLDL/LDL and HDL, respectively.

TABLE II
The lipid composition of the d < 1.006 lipoproteins

SRM-A-fed female mice (>10) were fasted and bled via orbital puncture. $d < 1.006$ lipoproteins were isolated from pool serum by density gradient ultracentrifugation. $d < 1.006$ fraction was analyzed for total, free, and esterified cholesterol, triglycerides, and phospholipids. TC, total cholesterol; CE, cholesterol ester; FC, free cholesterol; TTG, total triglycerides; PL, phospholipids.

Human APOE transgene	Endogenous *Apoe* genotype									
	Apoe$^{+/+}$					*Apoe*$^{-/-}$				
	TC	CE	FC	TTG	PL	TC	CE	FC	TTG	PL
	μmol/mg protein					*μmol/mg protein*				
	2.0	0.7	1.3	9.1	1.3	20.0	14.8	5.2	0.3	3.0
APOE*3-Leiden	4.9	2.6	2.3	9.2	2.3	22.3	17.0	5.2	2.2	3.9
APOE*2	2.3	1.1	1.2	7.7	1.2	20.6	14.5	6.1	5.3	4.7

Apoe$^{-/-}$ mice (Table I). While in *Apoe*$^{-/-}$ mice the cholesterol was confined to the VLDL/LDL fractions, APOE*3-Leiden·*Apoe*$^{-/-}$ mice showed an equal distribution of cholesterol in both VLDL/LDL and HDL-sized lipoprotein fractions (Fig. 2). However, APOE*2·*Apoe*$^{-/-}$ mice were severely hypercholesterolemic and, in addition, showed a relatively mild hypertriglyceridemia. The increased levels of serum cholesterol and triglycerides were confined to the VLDL/LDL-sized lipoprotein fractions (Fig. 2). Strikingly, serum human apoE levels in APOE*2·*Apoe*$^{-/-}$ mice were much higher than in APOE*2·*Apoe*$^{+/+}$ mice (9.2 *versus* 0.9 mg/dl). Opposite, APOE*3-Leiden·*Apoe*$^{-/-}$ mice had lower human apoE levels than APOE*3-Leiden·*Apoe*$^{+/+}$ mice (0.5 *versus* 2.7 mg/dl).

Composition of d < 1.006 Lipoproteins Isolated from the ApoE Transgenic Mice—From the various SRM-A-fed female apoE transgenic mice the $d < 1.006$ g/ml (VLDL) were isolated by density gradient ultracentrifugation, and lipid and apolipoprotein compositions were determined. As shown in Table II, VLDL isolated from hyperlipidemic APOE*3-Leiden·*Apoe*$^{+/+}$ mice were 2-fold higher in free and esterified cholesterol and phospholipids as compared with VLDL isolated from *Apoe*$^{+/+}$ (wild type) and APOE*2·*Apoe*$^{+/+}$ mice. Strikingly, in the absence of endogenous mouse apoE, all VLDL samples were strongly enriched in cholesterol (free plus esterified) and phospholipids and contained less triglycerides. Although reduced, triglyceride content of APOE*2·*Apoe*$^{-/-}$ VLDL was still high when compared with *Apoe*$^{-/-}$ and APOE*3-Leiden·*Apoe*$^{-/-}$ VLDL and is in line with the observed high serum triglyceride levels observed in these mice (Table I).

As shown in Fig. 3, VLDL isolated from the different *Apoe*$^{+/+}$ (transgenic) mice all contained both apoB100 and apoB48. After cross-breeding to endogenous *Apoe* gene deficiency, VLDL contained no (*Apoe*$^{-/-}$), hardly (APOE*3-Leiden·*Apoe*$^{-/-}$) or little (APOE*2·*Apoe*$^{-/-}$) apoB100 as compared with mice with the respective *Apoe*$^{+/+}$ background. APOE*2·*Apoe*$^{-/-}$ VLDL was relatively rich in human apoE when compared with APOE*3-Leiden·*Apoe*$^{-/-}$ VLDL.

VLDL-ApoB Kinetics in Various ApoE Transgenic Mice—To study the underlying mechanism of the different hyperlipoproteinemias in the SRM-A-fed female *Apoe*$^{-/-}$, APOE*3-Leiden·*Apoe*$^{-/-}$, and APOE*2·*Apoe*$^{-/-}$ mice, *in vivo* VLDL-apoB kinetic studies were performed. Mice were injected with 10 μg of autologous ^{125}I-labeled VLDL, and the ^{125}I-apoB disappearance from the circulation was determined. VLDL-apoB clearance rate was clearly reduced in all apoE transgenic mice (Fig. 4, Table III) in the order: wild type ≫ APOE*3-Leiden·*Apoe*$^{-/-}$ > and APOE*2·*Apoe*$^{-/-}$ > *Apoe*$^{-/-}$ mice. To investigate whether an increase in VLDL production contributes to the observed accumulation of VLDL-sized lipoproteins, we determined hepatic VLDL-triglyceride production rate directly from serum triglyceride increase after injection of Triton WR 1339. *Apoe*$^{-/-}$ and APOE*3-Leiden·*Apoe*$^{-/-}$ mice had a significant 2-fold reduction in hepatic VLDL triglyceride production rate as compared with wild type mice, whereas APOE*2·*Apoe*$^{-/-}$ mice had a hepatic VLDL production rate comparable with wild type mice (Table III).

The above described results indicate that the accumulation of VLDL-sized lipoproteins in APOE*2·*Apoe*$^{-/-}$ mice was due to a strong decreasing effect on VLDL clearance. In contrast, the mild accumulation of VLDL-sized lipoproteins observed in APOE*3-Leiden·*Apoe*$^{-/-}$ mice seems to be due to a reduced VLDL clearance that is partly compensated by a reduction in

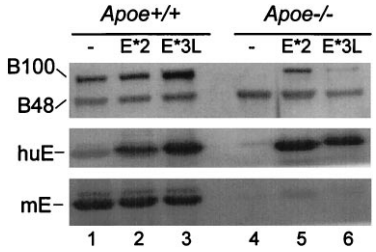

FIG. 3. **Western blot analysis of the d < 1.006 lipoproteins of the various apoE transgenic mice.** *d* < 1.006 lipoproteins were isolated from fasted pool serum of SRM-A-fed female wild type (*Apoe*$^{+/+}$, *lane 1*), APOE*2·*Apoe*$^{+/+}$ (*lane 2*), APOE*3-Leiden·*Apoe*$^{+/+}$ (*lane 3*), *Apoe*$^{-/-}$ (*lane 4*), APOE*2·*Apoe*$^{-/-}$ (*lane 5*), and APOE*3-Leiden·*Apoe*$^{-/-}$ mice (*lane 6*). 4.5 μg of VLDL-protein was subjected to SDS-gel electrophoresis (4–20% gradient gels) and transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antisera against mouse apoB (*B100* and *B48*), human apoE (*huE*), and mouse apoE (*mE*). Note: the polyclonal rabbit anti-human apoE shows some cross-reaction with mouse apoE.

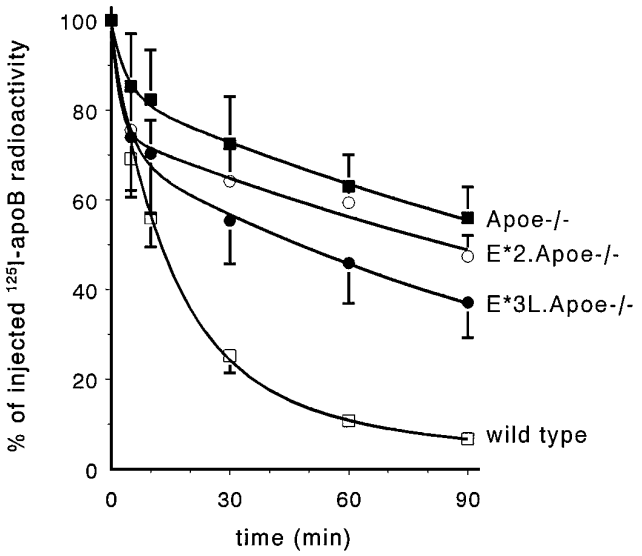

FIG. 4. **VLDL-apoB removal in wild type, *Apoe*$^{-/-}$, APOE*3-Leiden·*Apoe*$^{-/-}$, and APOE*2·*Apoe*$^{-/-}$ mice.** After a 5-h fasting period, SRM-A-fed female wild type (□), *Apoe*$^{-/-}$ (■), APOE*3-Leiden·*Apoe*$^{-/-}$ (●), and APOE*2·*Apoe*$^{-/-}$ (○) mice were injected with autologous ^{125}I-labeled VLDL. 25 μl of blood was drawn at each time point, and ^{125}I-apoB(100+48) radioactivity of the serum sample was measured. Values are the mean ± S.D. of seven mice. Curves were calculated from the mean data using a bi-exponential curve fit model.

VLDL production rate.

Binding of VLDL Isolated from the Various ApoE Transgenic Mice to HepG2 and J774 Cells—To study whether the observed decrease in VLDL FCR in the apoE transgenic mice was due to a reduced binding efficiency of VLDL to hepatic cells, we determined the receptor-mediated binding of the VLDL lipoproteins to HepG2 cells. As shown in Fig. 5, the specific binding of the VLDL was reduced in all transgenic mice as compared with VLDL isolated from wild type mice. Binding efficiency was in the order: wild type > APOE*3-Leiden·*Apoe*$^{-/-}$ > APOE*2·*Apoe*$^{-/-}$ = *Apoe*$^{-/-}$, and corresponds with the order observed for VLDL FCR (Fig. 4, Table III) and level of hypercholesterolemia (Table I).

The interaction of remnant lipoproteins with hepatic cells include the LDL receptor and the LRP. Fig. 6 shows that unlabeled VLDL isolated from wild type mice was most efficient in competing with ^{125}I-labeled human LDL for the binding to J774 cells, whereas VLDL isolated from APOE*3-Leiden·*Apoe*$^{-/-}$ mice was a less efficient in this respect. VLDL isolated from *Apoe*$^{-/-}$ and APOE*2·*Apoe*$^{-/-}$ mice did not compete with ^{125}I-labeled LDL for binding to the J774 cells at all. Thus, apoE-deficient VLDL and VLDL containing apoE2 as the sole apoE protein cannot bind to the LDL receptor.

TABLE III

*VLDL-apoB fractional catabolic rates (FCR) and in vivo hepatic VLDL triglyceride production rate (PR) in APOE*3-Leiden and APOE*2 transgenic mice without endogenous mouse Apoe alleles*

After a 5-h fasting period SRM-A-fed female mice were injected with 10 μg of autologous labeled ^{125}I-labeled VLDL protein. ^{125}I-ApoB (100 + 48) disappearance from circulation was determined, and FCR was calculated (see "Experimental Procedures"). For determining hepatic VLDL production rate fasted SRM-A fed female mice were injected with Triton WR1339. Fasted serum triglycerides were determined just before injection (0 min) and at 30 and 60 min after Triton injection. Production of hepatic triglyceride production rate was calculated from the slope of the curve and is expressed as mmol/h/kg mouse. Values are the mean ± S.D. of 6–7 mice/group.

Mouse	VLDL-apoB FCR	VLDL-triglyceride PR
	pool/h	*mmol/h/kg mouse*
Wild type	22.1 ± 3.4[a]	0.136 ± 0.044[a]
Apoe$^{-/-}$	3.2 ± 0.7[b]	0.076 ± 0.023[b]
APOE*3-Leiden · *Apoe*$^{-/-}$	6.1 ± 1.9[a,b]	0.077 ± 0.017[b]
APOE*2 · *Apoe*$^{-/-}$	4.0 ± 0.5[a,b]	0.128 ± 0.027[a]

[a] $p < 0.05$, significantly different from *Apoe*$^{-/-}$ mice, using nonparametric Mann-Whitney tests.

[b] $p < 0.05$, significantly different from mice with the wild type mouse *Apoe* allele, using nonparametric Mann-Whitney tests.

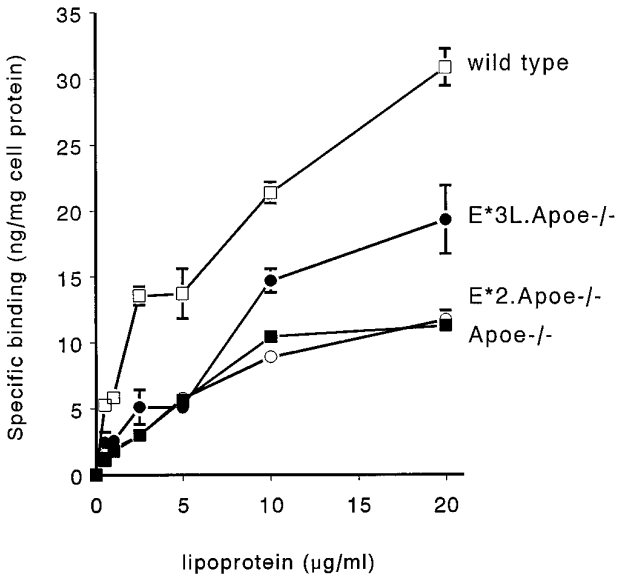

FIG. 5. **Binding of VLDL isolated from wild type, *Apoe*$^{-/-}$, APOE*3-Leiden·*Apoe*$^{-/-}$, and APOE*2·*Apoe*$^{-/-}$ mice.** The binding of VLDL isolated from fasted serum of SRM-A-fed female wild type (□), *Apoe*$^{-/-}$ (■), APOE*3-Leiden·*Apoe*$^{-/-}$ (●), and APOE*2·*Apoe*$^{-/-}$ (○) mice to HepG2 cells was measured upon incubation of the cells with indicated amounts of labeled lipoprotein at 4 °C for a period of 3 h. Binding was determined as described under "Experimental Procedures." Values represent the mean ± S.D. of three measurements.

Treatment of Various ApoE Transgenic Mice with Adenovirus Containing RAP cDNA—To investigate whether remnant lipoproteins in the respective apoE transgenic mice were cleared via the LRP, RAP was overexpressed via injection of recombinant adenovirus containing RAP cDNA (AdCMV-RAP) in the various mice. It has previously been shown that injection of AdCMV-RAP efficiently blocks ligand clearance via the LRP (25). Injections of adenovirus containing LacZ (AdCMV-LacZ) were used as control. Four days after AdCMV-RAP injections, LDL receptor-deficient (*Ldlr*$^{-/-}$) mice showed a strong hypercholesterolemic and mild hypertriglyceridemic response, indicating a strong RAP-mediated inhibition of the RAP-sensitive

receptors, likely the LRP (Table IV). Both APOE*3-Leiden·*Apoe*$^{-/-}$ and APOE*2·*Apoe*$^{-/-}$ and not *Apoe*$^{-/-}$ mice displayed a strong hypercholesterolemic response to the Ad-CMV-RAP injection, whereas all three lines showed a strong hypertriglyceridemic effect upon AdCMV-RAP transfection. In addition, the transgenic mice displayed a strong increase in serum human apoE levels upon AdCMV-RAP transfection. These results indicate that RAP-sensitive receptors recognize both apoE2 and apoE3-Leiden proteins.

DISCUSSION

Previously, we used transgenic mice to study the dominant APOE*3-Leiden mutation (7–9). APOE*3-Leiden transgenic mice exhibited a hyperlipoproteinemic phenotype and proved to be very useful in studying the role of subtle environmental and genetic factors in the expression of hyperlipidemia (8, 9) and the development of atherosclerosis (8, 27). In the present study, APOE*2(Arg-158 → Cys) mice were generated and compared with the previously generated APOE*3-Leiden transgenic mice, both when expressed on a wild type apoE or on an apoE-deficient background. A detailed *in vivo* characterization of both the APOE*2(Arg-158 → Cys) and APOE*3-Leiden mutant in mice may help to better understand the differential expression pattern of FD associated with both APOE variants.

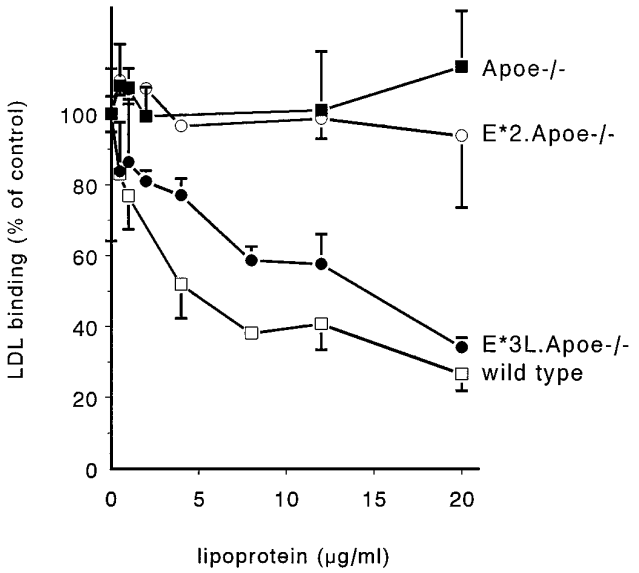

FIG. 6. **Competition for binding of ^{125}I-labeled human LDL to J774 cells by VLDL isolated from wild type, *Apoe*$^{-/-}$, APOE*3-Leiden·*Apoe*$^{-/-}$, and APOE*2·*Apoe*$^{-/-}$ mice.** Competition studies were performed by incubating J774 cells with 10 μg/ml ^{125}I-labeled LDL for 3 h at 4 °C in the presence of the indicated amounts of VLDL isolated from fasted serum of SRM-A-fed female wild type (□), *Apoe*$^{-/-}$ (■), APOE*3-Leiden·*Apoe*$^{-/-}$ (●), and APOE*2·*Apoe*$^{-/-}$ (○) mice. The binding is expressed as percentage of the value in the absence of competitor, and is determined as described under "Experimental Procedures." Values represent the mean ± S.D. of four measurements.

In the present study we showed that APOE*3-Leiden expression in mice can lead to a hyperlipidemic phenotype, already in the presence of normal functioning mouse *Apoe* genes, whereas APOE*2-expressing mice exhibit a hyperlipidemic phenotype only in the complete absence of the normal mouse *Apoe* gene. This implies that in mice the APOE*3-Leiden and APOE*2 mutation behave as a dominant and recessive mutation, respectively, as they do in humans.

The present *in vivo* and *in vitro* data show that apoE2 is unable to bind to the LDL receptor, whereas apoE3-Leiden still exhibits a considerable binding activity to this receptor. These observations are in line with earlier results from *in vitro* studies (2–4, 6). Ji *et al.* (6) concluded that apoE2 binds to the LRP *in vitro* comparable with the wild type isoform apoE3, whereas apoE3-Leiden appeared to be rather defective in this respect (20% of apoE3 binding). Our present data using adenovirus-RAP transfections indicated that *in vivo* both apoE3-Leiden and apoE2 do bind to the LRP. Thus, in spite of a considerable LRP binding, the loss of LDL receptor binding activity of the APOE*2 variant leads to a severely impaired remnant lipoprotein clearance and, consequently, a massive hyperlipidemia. For the APOE*3-Leiden variant, both LRP and LDL receptor binding activity are largely conserved, leading to only a mild hyperlipidemia.

The APOE*2·*Apoe*$^{-/-}$ mice can be compared with E2E2 homozygous subjects as far as their apoE genotype is concerned. However, at least two remarkable differences in hyperlipidemic phenotype became apparent; (i) in humans only a small proportions (4%) of E2E2 homozygotes become hyperlipidemic (2), whereas in the present study all APOE*2·*Apoe*$^{-/-}$ mice exhibited hyperlipidemia, and (ii) in APOE*2·*Apoe*$^{-/-}$ mice the level of plasma cholesterol and triglyceride is extremely high as compared with their hyperlipidemic human counterparts. ApoE-deficient mice also exhibit extreme hypercholesterolemia (13–15) as compared with apoE-deficient humans (28–30). For apoE-deficient mice this extreme hyperlipidemia is assumed to be due to the hepatic editing of apoB100 that occurs in mice and not in humans (31–34). Indeed, an important role of apoB100 as alternative ligand mediating hepatic remnant clearance is clearly illustrated by the complete absence of apoB100 in VLDL accumulating in the plasma of apoE-deficient mice (Fig. 3; Refs. 13–15). Such a reduced availability of apoB100 could also explain the extreme phenotype found in APOE*2·*Apoe*$^{-/-}$ mice relative to E2E2 humans. However, VLDL isolated from APOE*2·*Apoe*$^{-/-}$ transgenic mice did contain relatively high levels of apoB100 (Fig. 3), indicating that in APOE*2·*Apoe*$^{-/-}$ mice apoB100 is not effective as alternative ligand for remnant clearance (Fig. 4, Table III) via binding to LDL receptor (Fig. 6). Since in these mice, the VLDL remnants contain relatively high amounts of apoE, we hypothesize that a high apoE2 content per remnant particle somehow hampers the action of

TABLE IV

Serum lipids and human apoE levels in LDL receptor-deficient and various apoE transgenic mice before and after adenovirus-mediated overexpression of receptor-associated protein (RAP)

SRM-A-fed female Ldlr$^{-/-}$ and apoE transgenic mice were intravenously injected with AdCMV-RAP (± 1.5 × 10^9 plaque-forming units) or AdCMV-LacZ (± 1.5 × 10^9 plaque-forming units). Four days after injection mice were bled and fasted serum lipids and apoE were determined. Values are the mean ± SD of indicated number of mice. TC, total serum cholesterol; TG, total serum triglycerides; apoE, human apolipoprotein E; *n*, number of mice.

Mouse	AdCMV-LacZ				AdCMV-RAP			
	n	TC	TG	ApoE	*n*	TC	TG	ApoE
		mmol/liter	*mmol/liter*	*mg/dl*		*mmol/liter*	*mmol/liter*	*mg/dl*
Ldlr$^{-/-}$	2	9.1 ± 0.0	1.5 ± 0.2		5	23.4 ± 10.8	3.1 ± 1.0	
Apoe$^{-/-}$	2	25.6 ± 2.4	0.8 ± 0.2		5	27.5 ± 4.2	21.5 ± 5.1	
APOE*3-Leiden · *Apoe*$^{-/-}$	2	1.4 ± 0.1	0.3 ± 0.0	1.7 ± 0.1	3	15.3 ± 2.5	20.7 ± 4.5	19.1 ± 6.0
APOE*2 · *Apoe*$^{-/-}$	2	15.3 ± 0.1	5.0 ± 0.2	21.8 ± 4.2	5	42.8 ± 6.0	35.7 ± 7.2	53.3 ± 3.8

apoB100 as alternative ligand in remnant clearance. Such a mechanism might also be an important aggravating factor in the clinical expression of FD in E2E2 subjects.

Several lines of evidence emerged arguing for an important inhibitory role of apoE in the process of triglyceride lipolysis *in vitro* (35–36) and *in vivo* (37). The current observation that APOE*2·*Apoe*$^{-/-}$ transgenic mice display hypertriglyceridemia in addition to high plasma VLDL cholesterol and apoE levels (Table I), suggests that high levels of apoE2 per VLDL particle prevents efficient VLDL-triglyceride lipolysis *in vivo* as well. This is in line with the earlier findings in humans that the metabolic conversion of VLDL into LDL is hampered in E2E2 subjects (38, 39).

Serum human apoE levels in APOE transgenic mice strongly relate to the level of hyperlipidemia in these mice (Table I), since it is a major constituent of the accumulating remnants (Fig. 3). Similar results were observed for APOE*3-Leiden/CI transgenic mice (9) and FD subjects. Remarkably, for APOE*3-Leiden·*Apoe*$^{-/-}$ mice, serum apoE levels are very low despite a considerable accumulation of remnant lipoproteins. At present we still do not have an explanation for this striking observation.

Ji *et al.* (6) studied the interaction of several mutant forms of apoE with HSPG. HSPG may facilitate the interaction of remnant lipoproteins with the LRP for internalization by hepatic cells (40). It was found that dominant apoE mutants, including APOE*3-Leiden, were defective in binding to HSPG. However, the current observation that APOE*3-Leiden mice exhibit only a mild hyperlipidemia, irrespective of the presence or absence of endogenous mouse apoE, suggests that the impaired interaction of APOE*3-Leiden with HSPG is not of major importance *in vivo*, as far as the development of hyperlipidemia is concerned. In *Apoe*$^{-/-}$ mice, the plasma clearance of remnants via binding to HSPG cannot occur because of complete absence of apoE. Thus, the fact that APOE*2·*Apoe*$^{-/-}$ mice exhibit extreme hypercholesterolemia comparable with *Apoe*$^{-/-}$ mice, whereas apoE2 itself does bind to HSPG (6), also argues against an important role of HSPG in plasma clearance of VLDL remnants.

We found that APOE*3-Leiden·*Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$ mice had a decreased production rate of hepatic VLDL-triglyceride (Table III). Whether the APOE*3-Leiden mutant or the absence of hepatic apoE synthesis affect the production of VLDL in the liver will be a subject for further investigation. Remarkably, APOE*2·*Apoe*$^{-/-}$, APOE*3-Leiden·*Apoe*$^{-/-}$, and *Apoe*$^{-/-}$ mice displayed a dramatic increase in serum triglyceride levels after adenovirus-mediated RAP overexpression (Table IV). In *Apoe*$^{-/-}$ mice neither the LDL receptor nor the LRP are involved in the clearance of the VLDL remnants accumulated in the plasma. The observation that RAP transfection leads to an extreme hypertriglyceridemia in these *Apoe*$^{-/-}$ mice suggests that RAP, somehow, inhibits VLDL-triglyceride lipolysis independent of both the LDL receptor and LRP pathway. RAP overexpression in LDL receptor-deficient mice also leads to mice in which both the LDL receptor and LRP activity are eliminated. The observation that these mice do not display hypertriglyceridemia upon RAP treatment suggests that endogenous mouse apoE is able to circumvent the suggested RAP-mediated inhibition of lipolysis. Such an escape then could not be obtained by either apoE3-Leiden or apoE2 (Table IV). An inhibitory effect of RAP on VLDL-triglyceride lipolysis has not been reported before and is currently under further investigation.

Since APOE*3-Leiden and APOE*2 appeared to behave as a dominant and recessive mutation, respectively, in transgenic mice as well, we conclude that the differential expression of FD associated with these apoE mutants can also be studied in mice. From the present results it is strongly suggested that a difference between the two apoE mutants in binding efficiency to HSPG does not represent a major cause for the different expression patterns of FD associated with these apoE mutants in humans. Experimental evidence is accumulating for an important role of apoE in *in vivo* lipolysis of VLDL triglyceride. It is striking that in mice the E2E2 genotype displays a complete penetrance of hyperlipoproteinemia, which is in strong contrast to humans, where E2E2 homozygosity exhibits a low penetrance for hyperlipidemia (4%). Further analyses of the differences between humans and mice in this respect may help in finding the factors involved in VLDL remnant clearance, a process that is commonly assumed to be of major importance in the western societies regarding the risk of development of early atherosclerosis. More specifically, these analyses might help finding major aggravating factors involved in the clinical expression of FD.

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In the Absence of Endogenous Mouse Apolipoprotein E, Apolipoprotein E*2(Arg-158 → Cys) Transgenic Mice Develop More Severe Hyperlipoproteinemia than Apolipoprotein E*3-Leiden Transgenic Mice

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