In LDL receptor-deficient mice, catabolism of remnant lipoproteins requires a high level of apoE but is inhibited by excess apoE

Ko Willems van Dijk,[1,2,*] Bart J. M. van Vlijmen,[1,†,§] H. Belinda van't Hof,[§] Andre van der Zee,[*] Silvia Santamarina-Fojo,[**] Theo J.C. van Berkel,[†] Louis M. Havekes,[§,††] and Marten H. Hofker[*]

Department of Human Genetics,* Leiden University Medical Center; Department of Biopharmaceutics,† Leiden/Amsterdam Center for Drug Research; TNO Prevention and Health,§ Gaubius Laboratorium, Leiden, The Netherlands; Molecular Disease Branch,** National Institutes of Health, Bethesda, MD; and Departments of Cardiology and Internal Medicine,†† Academic Hospital Leiden, Leiden, The Netherlands

Abstract **To investigate the quantitative requirement for apolipoprotein (apo) E in the clearance of lipoproteins via the non-low density lipoprotein (LDL) receptor mediated pathway, human APOE was overexpressed at various levels in the livers of mice deficient for both the endogenous *Apoe* and *Ldlr* genes (*Apoe*−/− · *Ldlr*−/−) using adenovirus-mediated gene transfer. We found that a low level of APOE expression, that was capable of reducing the hyperlipidemia in *Apoe*−/− mice, did not result in a reduction of the hyperlipidemia in *Apoe*−/− · *Ldlr*−/− mice. Surpisingly, a very high level of APOE expression also did not result in a reduction of hypercholesterolemia in *Apoe*−/− · *Ldlr*−/− mice, despite very high levels of circulating apoE (>160 mg/dl). Only a moderately high level of APOE expression resulted in a reduction of serum cholesterol level (from 35.2 ± 6.7 to 14.6 ± 2.3 mmol/l) and the disappearance of VLDL from the serum. Moreover, the very high level of APOE expression resulted in a severe hypertriglyceridemia in *Apoe*−/− · *Ldlr*−/− mice and not *Apoe*−/− mice (25.7 ± 8.9 and 2.2 ± 1.8 mmol/l, respectively). This hypertriglyceridemia was associated with an APOE-induced increase in the VLDL triglyceride production rate and an inhibition of VLDL-triglyceride lipolysis. We conclude from these data that, for efficient clearance, the non-LDL receptor-mediated pathway requires a higher level of APOE expression as compared to the LDL receptor, but is more sensitive to an APOE-induced increase in VLDL production and inhibition of VLDL-triglyceride lipolysis.**—van Dijk, K. W., B. J. M. Van Vlijmen, H. B. van't Hof, A. van der Zee, S. Santamarina-Fojo, T. J. C. van Berkel, L. M. Havekes, and M. H. Hofker. **In LDL receptor-deficient mice, catabolism of remnant lipoproteins requires a high level of apoE but is inhibited by excess APOE.** ***J. Lipid Res.*** **1999.** 40: **336–344.**

Supplementary key words adenovirus-mediated gene transfer • LDL receptor-related protein • hypertriglyceridemia • VLDL-triglyceride lipolysis • hepatic VLDL triglyceride production

Apolipoprotein (apo) E plays a key role in the metabolism of chylomicron and very low density lipoprotein (VLDL) remnants by functioning as a ligand for receptor-mediated uptake of these lipoproteins by the liver (for review, see ref. 1). The important role of apoE was recognized from studies on patients with type III hyperlipidemia, which is characterized by serum accumulation of chylomicron and VLDL remnants and is associated with mutations in the APOE gene (for reviews, see refs. 2, 3). Additional evidence was provided by the analysis of *Apoe*-deficient mice, which demonstrate massive accumulation of lipoprotein remnants (4–6). One important receptor involved in remnant lipoprotein uptake is the LDL receptor, an apoB/E specific receptor which can be responsible for up to 75% of total remnant uptake (7–10). In the absence of the LDL receptor, the LDL receptor-related protein (LRP) is thought to provide the backup mechanism (11–13). This was demonstrated by the accumulation of chylomicron and VLDL remnants in the serum of LDL receptor-deficient mice injected with an adenovirus carrying the receptor-associated protein (RAP), a potent inhibitor of LRP ligand interaction (14, 15). More recently, similar observations have been made after liver specific inactivation of the LRP gene (16). Additional liver recognition sites for remnant lipoproteins have been postulated, including proteoglycans (17), the remnant receptor (18), and the lipolysis-stimulated receptor (19), although their significance remains to be elucidated.

At present it is unclear which factor(s) determine the specificity of remnants for the different receptor systems

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LDLR, LDL receptor; LRP, LDL receptor-related protein; RAP, receptor-associated protein; HSPG, heparan sulphate proteoglycans; Ad, adenoviral vector; CMV, cytomegalovirus.

[1] These authors contributed equally to this study.

[2] To whom correspondence should be addressed.

in vivo. Data from rat liver perfusion studies suggest that the size of the remnant particle is an important determinant in receptor recognition (20). This has been confirmed in vivo using apoE-enriched artificial 'neo-chylomicrons' (21). In addition to size, the apoE content of a remnant particle influences receptor specificity. From ligand-blotting experiments it was concluded that binding of β-VLDL to the LRP requires apoE enrichment, despite the already abundant presence of apoE on these particles (22). To account for these observations, in vivo uptake of remnants via the LRP has been postulated to occur after enrichment with apoE that is bound to cell surface heparan sulfate proteoglycans (HSPG) in the space of Disse (the so-called secretion re-capture process (23–25)).

To further investigate the quantitative requirement of apoE in the clearance of lipoproteins via the non-LDL receptor-mediated pathway in vivo, human APOE was overexpressed at various levels in the livers of mice deficient for both the endogenous *Apoe* and *Ldlr* genes (*Apoe*−/− · *Ldlr*−/−) using adenovirus-mediated gene transfer. We have found that in the absence of the LDL receptor, low APOE expression was not sufficient for remnant removal. High APOE expression was needed for removal of remnants via the non-LDL receptor-mediated route, but this effect was counteracted by the APOE-induced increase in liver VLDL-triglyceride production and inhibition of LPL-mediated VLDL-triglyceride lipolysis. Thus, the APOE expression level is an important factor determining serum lipid levels when LDL receptor-mediated clearance is disturbed.

METHODS

Generation and analysis of transgenic mice

ApoE-deficient (*Apoe*−/−) mice were created as described previously (6). LDL receptor-deficient (*Ldlr*−/−) mice (26) were purchased from the Jackson Laboratory (Bar Harbor, ME). *Apoe*−/− mice were cross-bred with *Ldlr*−/− mice to obtain mice that lack both the apoE and LDL receptor gene (*Apoe*−/− · *Ldlr*−/− mice). The resulting breeding offspring was analyzed for the endogenous *Apoe*−/− and *Ldlr*−/− genotype through tail-tip DNA analysis, as described earlier (6, 26). C57Black/6J/Icco mice were purchased from the Broekman Institute, Someren, The Netherlands.

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 (i.e., a standard chow diet) and water.

Adenovirus transfections

The generation of the recombinant adenoviral vectors expressing APOE (Ad-APOE) under control of the CMV promotor has been described (27). The recombinant adenoviral vector expressing LacZ (Ad-LacZ) under control of the CMV promotor was kindly provided by Dr. T. Willnow and Dr. J. Herz (15). The recombinant adenovirus was propagated and titrated on the Ad5 E1-transformed human embryonic kidney cell line 911 as described (28). 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, 0.5×10^9 − 2.0×10^9 plaque-forming units in a total volume of 200 μl (diluted with PBS) were injected into the tail vein of female mice. Fasted blood samples were drawn from the tail vein of fasted mice at 5, 8, 11, and 14 days after virus injection.

Lipid, lipoprotein, and apoE measurements

Adenovirus-injected mice were fasted from 9 am to 1 pm and approximately 100 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 Chemical Co., St. Louis, MO).

For determination of the serum lipoprotein distribution, some 100 μl of pooled serum was subjected to density gradient ultracentrifugation according to Redgrave, Roberts, and West (29). After ultracentrifugation, the volume was fractionated in fractions of 0.5 ml and density was measured using a DMA 602M densitometer (Paar, Germany). Fractions with density d < 1.006, 1.006–1.019, 1.019–1.063, and 1.063–1.120 g/ml correspond to VLDL, IDL, LDL, and HDL, respectively. After dialysis against PBS, containing 10 μm EDTA (pH 7.4), lipoprotein fractions were analyzed enzymatically for cholesterol and triglyceride content, using kits #236691 and #701904 (Boehringer Mannheim, Germany), respectively.

Human apoE concentrations were measured by sandwich ELISA as described previously (30).

Characterization of VLDL

Total and free cholesterol, triglyceride (without glycerol), and phospholipid content of the VLDL (d < 1.006 g/ml) fraction were measured enzymatically, using commercially available kits (#236691 and #310328: Boehringer Mannheim, Mannheim, Germany; #337-B:Sigma Chemical Co., St. Louis, MO; and 990-54009: Wako Chemicals GmbH, Neuss, Germany, respectively). VLDL protein was determined using the method of Lowry et al. (31). Human apoE levels were measured by sandwich ELISA as described previously (30). Particle size of the d < 1.006 g/ml lipoproteins was determined by photon correlation spectroscopy using a Malvern 4700C system (Malvern Instruments, UK).

Immunohistochemical procedures for light microscopy

Fasted adenovirus-injected animals were anesthetized with 7.5 ml/kg body weight of a mixture of Hypnorm (Janssen-Cilag, Animal Health, Sandilon, UK), Ketamin (Nimatek®, Eurovet, Bladel, The Netherlands), Thalamonal (Janssen-Cilag bv, Tilburg, The Netherlands). Livers were perfused through the portal vein with ice-cold saline (flow rate 1 ml/min for 3 min), followed by 5 min perfusion with ice-cold 2% paraformaldehyde in PBS (pH 7.4). Paraformaldehyde-fixed, paraffin-embedded 3-μm sections were subjected to immunohistochemistry. For apoE immunostaining, two primary antibodies were used: rabbit anti-mouse apoE polyclonal antibody (kind gift from Dr. P. Groot, Smith-Kline Beecham Pharmaceuticals, Harlow, UK) and a rabbit polyclonal antibody anti-human apoE. Both antisera were diluted to 1:1000 with PBS, containing 0.1% Tween 20 and 2% normal goat serum. The sections were incubated with the respective antibodies overnight at 4°C. After washing with PBS/0.1% Tween 20, the sections were incubated for 2 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG (Nordic Immunology, Tilburg, The Netherlands) diluted 1:500 with PBS, containing 2% Tween 20 and 2% normal goat serum. Finally, peroxidase detection was performed using diaminobenzidine as a substrate.

Assay of lipolysis with lipoprotein lipase in solution

Lipolysis assays were performed at 37°C in 0.1 m Tris(hydroxymethyl)-aminiomethane (Tris) buffer, pH 8.5, for 5 min with bovine lipoprotein lipase (LPL; 0.2 U, Sigma, St. Louis, MO) in the presence of 2% (w/v) albumin (essentially FFA-free). The reaction was stopped by the addition of 50 mm KH_2PO_4, 0.1% Triton-X100, pH 6.9, and placed on ice. To obtain a time zero control, the reaction was prevented by adding Triton prior to the addition of LPL and samples were placed on ice. Free fatty acids (FFA) were measured enzymatically with a NeFa-C kit (Wako Chemicals GmbH, Neuss, Germany). The rate of FFA release by 0.2 U LPL was linear for 5 min as used in this assay. The assay was performed at VLDL-triglyceride concentration of 0.2 mmol/l with duplication of FFA determination.

In vivo hepatic VLDL-triglyceride production

After a 4-h fasting period, mice were injected intravenously with Triton WR1339 (500 mg/kg body weight) (32) using 15% (wt/vol) Triton solution in 0.9% NaCl. At 1, 10, and 20 min after injection, blood samples were drawn and analyzed for triglycerides as described above. Production of hepatic triglyceride was calculated from the slope of the curve and expressed as mmol/h per kg body weight.

Statistical analysis

Differences in lipid parameters of comparable groups after different treatment regimens or of the same group of mice at different time points were evaluated using the GraphPad Prism software (San Diego, CA). As the measured parameters showed non-Gaussian distributions, the non-parametric Mann-Whitney rank sum test was used. *P* values less than 0.05 were regarded as significant.

RESULTS

Serum lipid, apoE, and lipoprotein levels after adenovirus-mediated gene transfer of APOE in *Apoe*−/− and *Apoe*−/−·*Ldlr*−/− mice

To determine the role of apoE in the clearance of lipoproteins by the liver in the absence of the LDL receptor, APOE was over-expressed at different levels in *Apoe*−/−·*Ldlr*−/− mice using adenovirus-mediated gene transfer. *Apoe*−/− mice were included for comparison. To obtain different APOE expression levels, 5×10^8 PFU (low dose) and 2×10^9 PFU (high dose) of adenovirus carrying the human APOE3 cDNA driven by the CMV promotor (Ad-APOE) were injected into the tail vein of *Apoe*−/− and *Apoe*−/−·*Ldlr*−/− mice. As a control, similar groups of mice were injected with a high dose of an adenovirus carrying the gene encoding β-galactosidase driven by the CMV promotor (Ad-LacZ). The mice were followed for up to 14 days.

As can be seen in **Fig. 1A**, both the low and high dose of Ad-APOE resulted in complete rescue of hypercholesterolemia of *Apoe*−/− mice on day 5 after injection, whereas a high dose of Ad-LacZ had no effect. From day 5 onwards, concurrent with the decrease of serum apoE level, serum cholesterol level gradually increased (Figs. 1A and E). A mild hypertriglyceridemia was evident on day 5 in the *Apoe*−/− mice injected with the highest dose of Ad-APOE (Fig. 1C).

In contrast, neither the low dose nor the high dose Ad-APOE had any effect on serum cholesterol level in

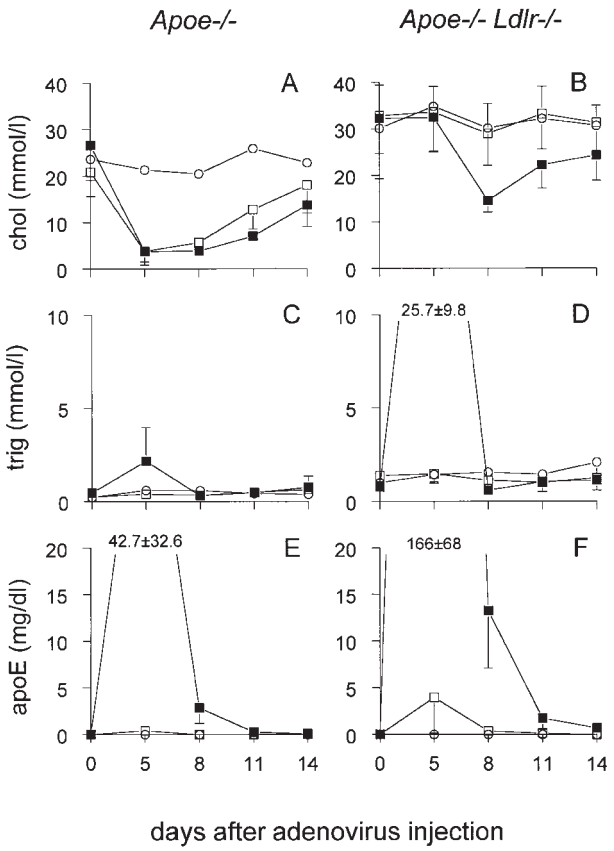

Fig. 1. Serum cholesterol, triglycerides, and apoE levels after adenovirus-mediated gene transfer of APOE in *Apoe*−/− and *Apoe*−/−·*Ldlr*−/− mice. *Apoe*−/− (left panels) and *Apoe*−/−*Ldlr*−/− mice (right panels) were injected intravenously with 5×10^8 PFU (open squares, n = 5 per group) and 2×10^9 PFU (black squares, n = 6 per group) of Ad-APOE. As a control, similar mice were injected with 2×10^9 PFU of Ad-LacZ (open circles, n = 2–3 per group). Serum cholesterol (panels A and D), triglycerides (panels B and E) and apoE levels (panels C and F) were followed for up to 14 days. Values are represented as the mean ± SD.

Apoe−/−·*Ldlr*−/− mice on day 5 after injection (Fig. 1B), despite appropriate APOE expression (serum apoE levels of 4.0 ± 3.8 and 166 ± 67.7 mg/dl for low and high dose, respectively; Fig. 1F). However, on day 8, when adenovirus-mediated APOE expression was reduced, in *Apoe*−/−·*Ldlr*−/− mice injected with the high dose of Ad-APOE (serum apoE level of 10.5 ± 8.2 mg/dl; Fig. 1F), serum cholesterol was significantly ($P < 0.05$) lowered (from 32.2 ± 7.5 to 14.6 ± 2.5 mmol/l; Fig. 1B), approaching the serum cholesterol level of *Apoe*+/+ *Ldlr*−/− mice (8–10 mmol/l). At the peak of APOE expression on day 5, the high dose of Ad-APOE resulted in severe hypertriglyceridemia (serum triglyceride of 25.7 ± 9.8 mmol/l, Fig. 1D). The low dose of Ad-APOE did not result in a reduction of serum cholesterol at any time point evaluated, nor did it affect serum triglyceride levels. Thus, reduction of hypercholesterolemia in *Apoe*−/−·*Ldlr*−/− mice was only evident within a certain window of APOE expression and a high serum apoE level was associated with hypertriglyceridemia.

The lipoprotein fractions of *Apoe*−/− mice and *Apoe*−/−·*Ldlr*−/− mice 5 and 8 days after adenovirus

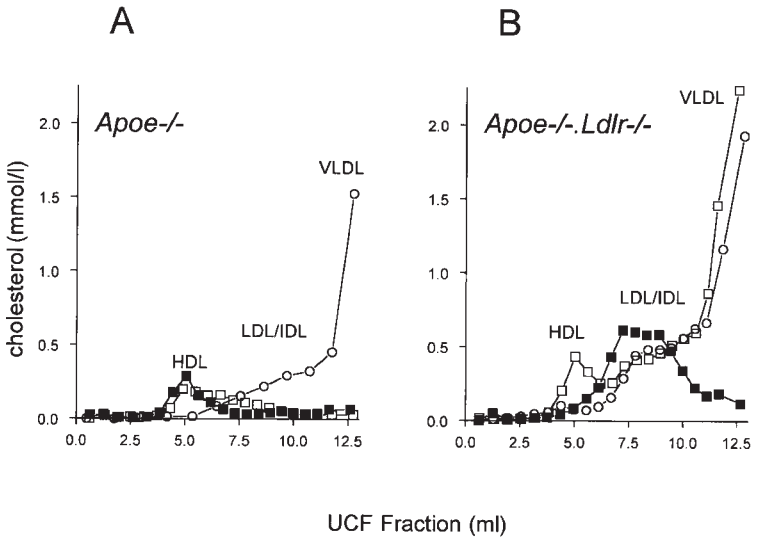

Fig. 2. Distribution of serum cholesterol among lipoprotein fractions after adenovirus-mediated APOE gene transfer. The lipoprotein fractions of *Apoe*−/− (panel A) mice and *Apoe*−/−·*Ldlr*−/− (panel B) mice after 5 (open squares) and 8 days (black squares) after adenovirus injection (2×10^9 PFU) were analyzed by ultracentrifugation density gradient fractionation. Ad-LacZ (2×10^9 PFU)-injected mice (open circles) are shown as control. Each run was performed with a pool of serum from 2–5 fasted mice of the same group. Fractions with density d < 1.006, 1.006–1.063, and 1.063–1.120 g/ml correspond to VLDL, IDL/LDL, and HDL, respectively. The triglycerides were predominantly associated with the VLDL fractions and are not shown.

injection were analyzed by ultracentrifugation density gradient fractionation (**Fig. 2**). In *Apoe*−/− mice injected with Ad-LacZ, a prominent peak is present in the VLDL-sized region on day 5, whereas in *Apoe*−/− mice injected with the high dose of Ad-APOE, lipoproteins from the VLDL-sized range had completely disappeared both on day 5 and day 8 (Fig. 2A). These latter two ultracentrifugation profiles were identical to those of non-treated wild-type mice (data not shown). In *Apoe*−/−·*Ldlr*−/− mice injected with the high dose of Ad-APOE, a large peak was present in the VLDL-sized region on day 5, similar to the Ad-LacZ-injected mice (Fig. 2B), the only difference being the appearance of an HDL-sized peak in the Ad-APOE-injected group. On day 8, IDL/LDL peaks were still present, whereas the VLDL peak had completely disappeared from the *Apoe*−/−·*Ldlr*−/− mice injected with the high dose of Ad-APOE, indicating rescue of the *Apoe*−/−·*Ldlr*−/− phenotype towards that of a *Ldlr*−/− mouse (26).

Characterization of the d < 1.006 lipoproteins after adenovirus-mediated APOE gene transfer

To further investigate the basis for the difference in serum VLDL levels after Ad-APOE administration, the apoE and lipid content of d < 1.006 lipoproteins (VLDL) were determined on day 5 and day 8 after injection of the high dose Ad-APOE (**Table 1**). The main difference in the composition of the VLDL between the different mice and time points was in the apoE and triglyceride content. Circulating VLDL in *Apoe*−/− mice that had received a high dose of Ad-APOE contained 6-fold more apoE on day 5 as compared to day 8 (7.4 and 1.3 μg/mg protein, respectively). In addition, the VLDL from *Apoe*−/− mice given the high dose of Ad-APOE was enriched in triglycerides on day 5 as compared to day 8 (6.5 and 0.7 μmol/mg protein, respectively).

On day 5, circulating VLDL in *Apoe*−/−·*Ldlr*−/− mice that had received a high dose of Ad-APOE also contained much more apoE as compared to VLDL circulating on day 8 (114.1 and 5.5 μg/mg protein, respectively). On day 5 the apoE level of *Apoe*−/−·*Ldlr*−/− mice was dramatically higher than that of *Apoe*−/− mice. Apparently, this difference can be attributed to the absence of the LDL receptor. As was the case for the *Apoe*−/− mice, the VLDL from *Apoe*−/−·*Ldlr*−/− mice given the high dose of Ad-APOE was enriched in triglycerides on day 5 as compared to day 8 (respectively, 6.8 and 1.2 μmol/mg protein). The accumulation of the apoE and triglyceride-

TABLE 1. Composition of d < 1.006 g/ml lipoproteins after adenovirus-mediated gene transfer

Mouse Line	Virus	Days Post-inj.	ApoE	TG	TC	CE	FC	PL
			μg/mg			μmol/mg		
Apoe−/−	LacZ	5	0.0	0.9	11.5	8.5	3.0	2.3
	APOE	5	7.4	6.5	1.7	0.7	1.1	1.4
	APOE	8	1.3	0.7	1.0	0.5	0.5	0.4
Apoe−/−·*Ldlr*−/−	LacZ	5	0.0	0.3	7.4	3.8	3.6	2.8
	APOE	5	114.1	6.8	5.2	1.9	3.3	3.7
	APOE	8	5.5	1.2	3.5	1.6	2.0	1.8

Mice (n = 2–5), injected with 2×10^9 PFU adenovirus were fasted and bled via orbital puncture. Lipoproteins (d < 1.006 g/ml) were isolated from pool serum by density gradient ultracentrifugation. The d < 1.006 g/ml fraction was analyzed for human apoE concentration, triglycerides (TG), total cholesterol (TC), cholesteryl ester (CE), free cholesterol (FC), and phospholipids (PL). Values are expressed per mg VLDL protein.

rich VLDL on day 5 in the *Apoe*−/− · *Ldlr*−/− mice clearly indicated a disturbance of the VLDL metabolism that apparently could not be alleviated via the non-LDL receptor-mediated pathway.

Immunohistochemical localization of apoE in the liver after adenovirus-mediated APOE gene transfer

To confirm that adenovirus-derived apoE was available for the secretion–recapture process (22–24) both on day 5 and day 8 after injection of the high dose Ad-APOE in *Apoe*−/− · *Ldlr*−/− mice, immunohistochemical localization studies of apoE in perfusion-fixed livers were performed. As can be seen in **Fig. 3**, apoE was clearly present in the regions lining the sinusoids in *Apoe*−/− · *Ldlr*−/− mice both on day 5 (Fig. 3C) and on day 8 (Fig. 3D) after injection of the high dose Ad-APOE. On day 8, apoE staining was clearly much less intense than on day 5, although at this time point efficient VLDL clearance did occur. The staining pattern of human apoE largely resembled that of mouse apoE in *Ldlr*−/− mice (Fig. 3A). *Apoe*−/− · *Ldlr*−/− mice not injected with Ad-APOE were negative for human apoE (Fig. 3B).

Analysis of the disturbance in triglyceride metabolism after adenovirus-mediated gene transfer of APOE

Five days after injection of a high dose of Ad-APOE, serum triglyceride levels in both *Apoe*−/− mice and *Apoe*−/− · *Ldlr*−/− mice were significantly increased (Fig. 1C, 1D). Specifically, in the *Apoe*−/− · *Ldlr*−/− mice, there was a correlation in individual mice between serum apoE level and serum triglyceride level. This observation was extended by injecting three additional groups of 6–7 mice each with, respectively, 0.75×10^9, 1.0×10^9, and 1.25×10^9 PFU of Ad-APOE. The correlations between serum apoE level and serum triglyceride level and between serum apoE level and cholesterol level are shown in **Fig. 4** (on day 5 after Ad-APOE injection). The strong positive correlation between serum apoE and serum triglyceride ($r = 0.96$, $P < 0.0001$; Fig. 4A) and not serum cholesterol level ($r = 0.37$, $P = 0.05$, Fig. 4B) is indicative of an apoE-related effect on the triglyceride metabolism. This could be the result of an increased VLDL production rate correlated with increased APOE expression or the result of an inhibition of triglyceride lipolysis mediated by the effect of excess apoE on LPL.

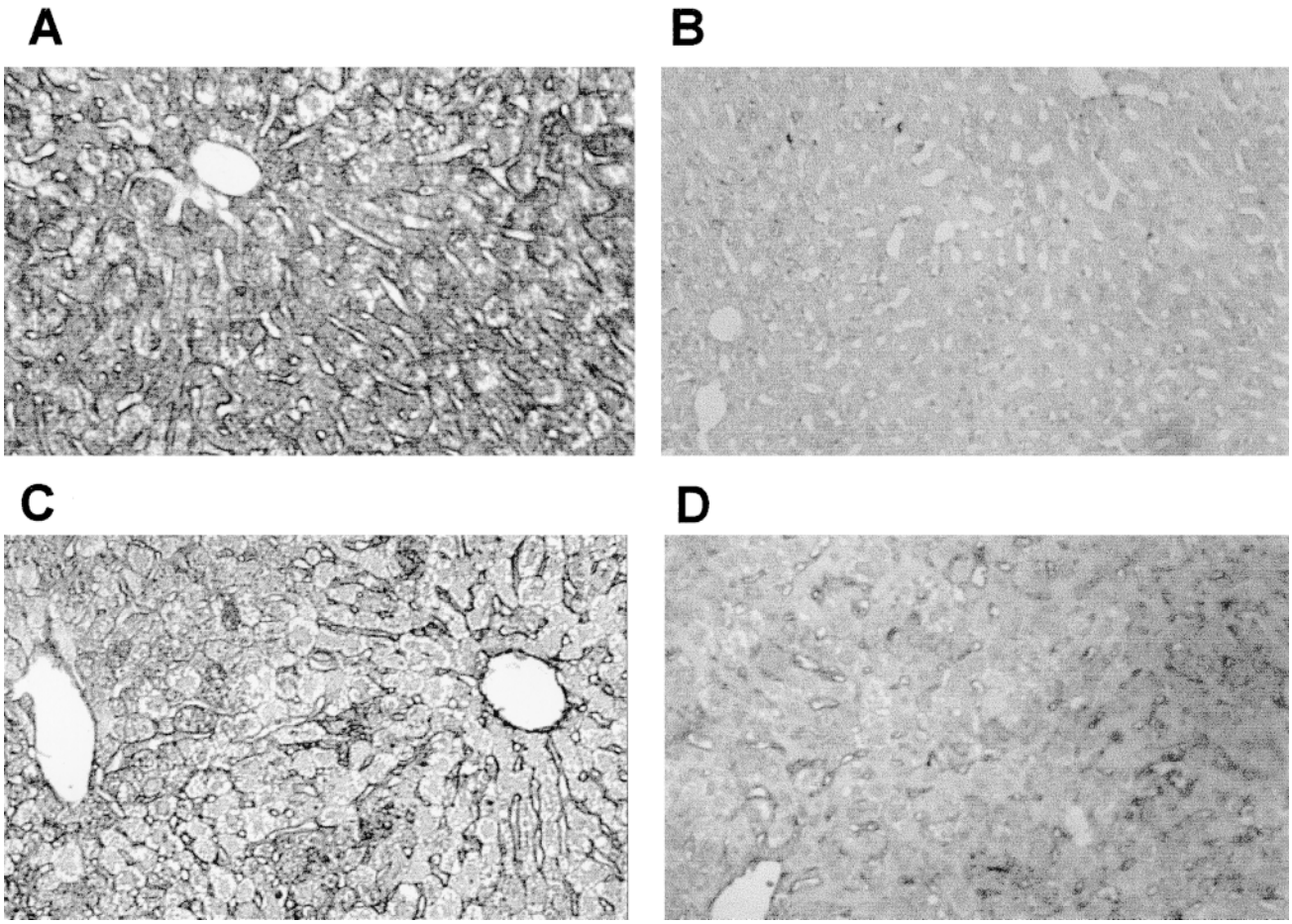

Fig. 3. Immunohistochemical localization of apoE in the liver after adenovirus-mediated APOE gene transfer. Livers of *Apoe*−/− · *Ldlr*−/− mice 5 days (panel C) and 8 days (panel D) after Ad-APOE injection (2×10^9 PFU) were stained for the presence of human apoE (see Methods section). Livers of *Apoe*−/− · *Ldlr*−/− mice 5 days after injection with Ad-LacZ (2×10^9 PFU) (panel B) are shown as control. As a reference, untreated *Ldlr*−/− mice stained for mouse apoE are shown (panel A).

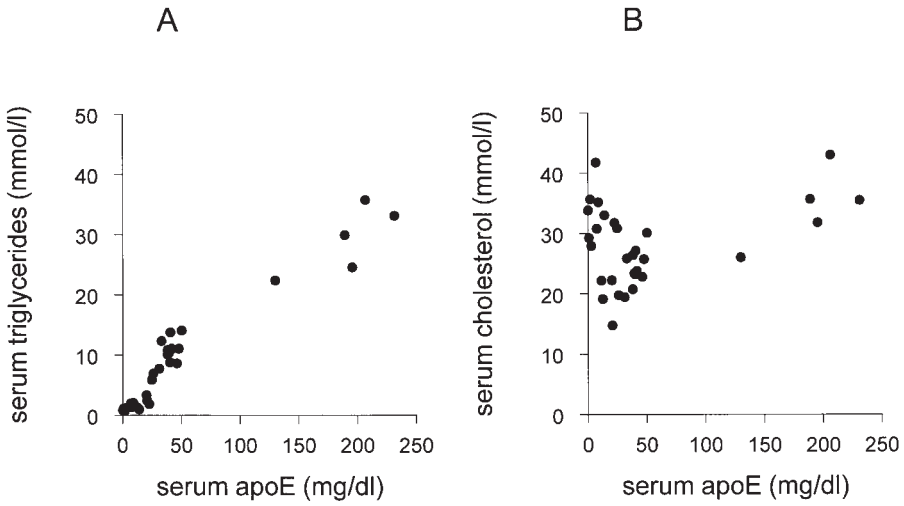

Fig. 4. Serum triglyceride and cholesterol levels plotted against serum apoE level after adenovirus-mediated APOE gene transfer. Serum triglyceride (panel A) and cholesterol (panel B) levels of *Apoe*−/−·*Ldlr*−/− mice injected with 0.5×10^9 (n = 5), 0.75×10^9 (n = 7), 1.0×10^9 (n = 7),1.25×10^9 (n = 6), and 2×10^9 (n = 6) PFU of Ad-APOE 5, plotted against the apoE level, 5 days after injection.

Production of VLDL-triglycerides was measured by determining the triglyceride secretion rate after Triton WR1339 injection on day 5 in wild-type C57Black/6J mice injected with a high dose of Ad-APOE or a high dose of Ad-LacZ (**Fig. 5**). C57Black/6J mice injected with a high dose of Ad-APOE displayed a 4-fold higher VLDL-triglyceride production rate as compared to similar mice injected with a high dose of Ad-LacZ (0.200 ± 0.082 versus 0.053 ± 0.005 mmol/kg per h, $P < 0.01$). On day 8, VLDL-triglyceride production rate has normalized in the C57Black/6J mice injected with the high dose of Ad-APOE as compared with the mice injected with the high dose of Ad-LacZ (0.056 ± 0.004 and 0.063 ± 0.011 mmol/kg per h, respectively).

To determine VLDL triglyceride lipolysis rate, VLDL (d < 1.006 lipoproteins) were isolated from serum of *Apoe*−/−·*Ldlr*−/− mice 5 and 8 days after injection of a high dose of Ad-APOE. The VLDL were characterized and the triglyceride lipolysis rate was determined in vitro in a solution assay using purified bovine LPL (**Table 2**). The apoE-rich VLDL on day 5 after adenovirus injection were poor substrates for LPL in this assay as compared with the VLDL isolated on day 8 (8.9 ± 4.9 and 24.2 ± 6.6 μmol FFA/U·min). Thus, both increased VLDL production and inhibited VLDL triglyceride lipolysis contribute to the effect of high APOE expression on serum triglyceride levels.

DISCUSSION

In the present study, we have demonstrated that clearance of remnant lipoproteins via the non-LDL receptor-mediated pathway required more apoE as compared to remnant clearance in the presence of the LDL receptor. This was illustrated by the fact that a low dose of Ad-APOE had no effect on serum lipid levels in *Apoe*−/−·*Ldlr*−/− mice while an identical dose completely rescued the hypercholesterolemia of *Apoe*−/− mice. A relatively high level of APOE expression in *Ldlr*−/−·*Apoe*−/− mice, such as on day 8 after a high dose of Ad-APOE, did result in a reduction of the serum cholesterol level to approximately the level of *Apoe* +/+·*Ldlr*−/− mice.

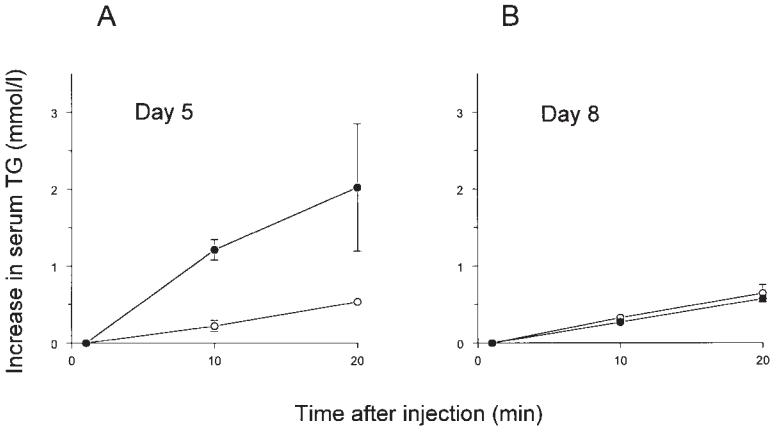

Fig. 5. Production of VLDL-triglycerides after adenovirus-mediated APOE gene transfer. C57Black/6J mice (n = 5–6 per group) injected with a high dose (2×10^9 PFU) of Ad-APOE (black circles) or a high dose of Ad-LacZ (open circles). At 5 (panel A) and 8 days (panel B) after adenovirus injection, mice were fasted for 5 h and injected intravenously with Triton WR1339 (500 mg/kg body weight) (31). At 1, 10, and 20 min after injection, blood samples were drawn and analyzed for triglycerides (TG). The increase in serum triglyceride was normalized to the 1-min point.

TABLE 2. In vitro lipolysis of d < 1.006 g/ml lipoproteins isolated from *Apoe* −/− · *Ldlr* −/− mice 5 and 8 days after Ad-APOE injection

Genotype	Days Post-inj.	ApoE	TG	TC	Size	Lipolysis
		μg/mg	*μmol/mg*	*μmol/mg*	*nm*	*μmol FFA/U·min*
Apoe −/− · *Ldlr* −/−	5	75.4 ± 78.7	3.6 ± 1.5	5.4 ± 2.1	69.3 ± 24.0	8.9 ± 4.9
Apoe −/− · *Ldlr* −/−	8	5.7 ± 2.6[a]	1.7 ± 0.6[a]	5.9 ± 4.5	38.8 ± 2.1[a]	24.2 ± 6.6[b]

Apoe −/− · *Ldlr* −/− mice (n = 4–6), injected with 2×10^9 PFU adenovirus were fasted and bled via orbital puncture. The d < 1.006 g/ml lipoproteins were isolated from serum of individual mice by density gradient ultracentrifugation. The d < 1.006 g/ml fraction was analyzed for human apoE concentration, total cholesterol (TC), and triglycerides (TG) and size. Values are expressed per mg VLDL protein. The rate of lipolysis by LPL in vitro was determined as described in Methods and expressed as free fatty acids (FFA) released per unit LPL per min (U·min).

[a] $P < 0.05$, significantly different from day 5 value, using nonparametric Mann-Whitney tests.

[b] $P < 0.01$, significantly different from day 5 value, using nonparametric Mann-Whitney tests.

We (33, 34) and others (14, 15, 35, 36) have shown that, in mice, the non-LDL receptor-mediated pathway for lipoprotein clearance is RAP sensitive, and most likely involves the LRP. This has recently been confirmed by liver specific inactivation of the LRP in *Ldlr* −/− mice (16). In vitro studies have demonstrated that the LRP requires abundant apoE on the particle for binding (22). Our present observation that in vivo the non-LDL receptor-mediated pathway required a high level of apoE for efficient clearance is in line with the LRP being the most likely candidate for mediating clearance in the absence of the LDL receptor.

To facilitate liver uptake of remnants via the LRP in vivo, it has been postulated that lipoproteins are enriched with apoE in the space of Disse, the so-called secretion–recapture process (23–25). Our immunohistochemistry studies indicated that in *Apoe* −/− · *Ldlr* −/− mice on day 5 after injection of a high dose of Ad-APOE, apoE was abundantly present in the space of Disse and on the lipoprotein particles. In addition, clearance studies with α_2-macroglobulin indicated that the LRP is active in *Apoe* −/− · *Ldlr* −/− mice (data not shown) and thus all the presumed conditions for uptake of remnant lipoproteins via a secretion–recapture process were fulfilled. However, in contrast to *Apoe* −/− mice, the *Apoe* −/− · *Ldlr* −/− mice did not show a reduction in serum cholesterol level on day 5 after injection of a high dose of Ad-APOE. Moreover, the *Apoe* −/− · *Ldlr* −/− mice showed a significantly more pronounced hypertriglyceridemia on day 5 than the *Apoe* −/− mice. These results indicate that at high expression levels of APOE, VLDL is poorly cleared via the non-LDL receptor-mediated pathway, due to apoE-induced changes in VLDL composition.

The high dose of Ad-APOE induced hypertriglyceridemia in both *Apoe* −/− mice and *Apoe* −/− · *Ldlr* −/− mice, albeit to a different extent. The latter mice showed a strong positive correlation between serum apoE and triglyceride levels (Fig. 4A), indicative of an apoE-mediated effect on triglyceride production and/or lipolysis. Production of VLDL triglyceride was shown to be dramatically increased on day 5 after administration of the high dose of Ad-APOE to C57/Bl6 mice (Fig. 5) and also *Apoe* −/− mice (data not shown), whereas it was normalized on day 8, both compared to a similar dose of Ad-LacZ (Fig. 5). These data indicate that APOE expression, especially at high levels, results in an increase in VLDL-triglyceride production. This is in line with our recent in vitro and in vivo observations in apoE-deficient hepatocytes and *Apoe* −/− mice, showing that lack of APOE expression resulted in a reduced VLDL triglyceride production rate (37).

The rate of VLDL triglyceride lipolysis was inversely correlated with the apoE content of the particle (Table 2). Several variant forms of human APOE, including APOE2 (146) and APOE3Leiden, have been described that have a direct negative effect on the rate of LPL-mediated triglyceride lipolysis (38–40). In line with previously reported data (41–43), our in vivo data show that also the most common APOE variant, APOE3, when present at high levels on the VLDL particle, can also have a detrimental effect on VLDL-triglyceride lipolysis by LPL. The mechanism underlying the apoE-mediated inhibition of lipolysis has not been investigated. One likely explanation is that excess apoE on the remnant particles results in reduced accessibility of LPL to the core triglycerides of the lipoprotein particle. Alternatively, high levels of APOE could result in the displacement of apoC2, the essential cofactor for LPL-mediated triglyceride lipolysis (44, 45), from the lipoprotein particle (40).

The increase in triglyceride content of the particle was associated with almost doubling of the VLDL particle size (from 38.8 ± 2.1 to 69.3 ± 24.0 nm, Table 2) on day 5 after the high dose Ad-APOE in *Ldlr* −/− · *Apoe* −/− mice. Whether the size of the particles during the hypertriglyceridemia limited the uptake via non-LDL receptor clearance routes remains to be established. This could be either at the level of direct affinity for the binding site or at the level of physically approaching the binding site, i.e., passage through the hepatic fenestrae. However, the latter has been estimated to be restricting only at sizes beyond 100 nm, and the VLDL particles at day 5 are below this size.

APOE is considered a potential candidate gene for the treatment of recessive type III hyperlipidemia, such as associated with the common APOE2(Arg158–Cys) variant. Interestingly, the level of APOE2(Arg158–Cys) expression in transgenic mice is a determining factor resulting in ei-

ther hypolipidemia or hyperlipidemia (46), and hypolipidemia is converted to hyperlipidemia by the absence of the LDL receptor (47). The in vivo demonstration that different levels of APOE3 expression can also have either a hypo- or hyperlipidemic effect has direct implications for the application of apoE as a therapeutic agent. For gene therapeutic approaches aimed at obtaining liver expression of APOE, care should be taken to optimize APOE expression levels, at which clearance of VLDL is enhanced but VLDL production and lipolysis are not affected. Our data indicate that, especially when LDL receptor mediated clearance is sub-optimal, APOE expression level requires optimization. In addition, it can be speculated that variation of APOE gene expression level in the population can have a large impact on individual serum lipid levels, not only through a well-known beneficial effect on lipoprotein clearance, but also through a disadvantageous effect on VLDL production and VLDL triglyceride lipolysis.

We gratefully acknowledge the technical assistance of J. van der Boom, and the input of Dr. M. Gijbels with the interpretation of the histological data. This research was supported by the Netherlands Heart Foundation (projects M93.001 and 96.178). Financial contribution of the specific RTD Programme of the European commission (BIOMED-2, #BMH4-CT96-0898) is gratefully acknowledged. Dr. Marten Hofker is an Established Investigator of the Netherlands Heart Foundation.

Manuscript received 25 June 1998 and in revised form 28 September 1998.

REFERENCES

1. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
2. 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 ed. Chapter 47. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1195–1213.
3. 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.
4. Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setala, 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.
5. 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.
6. van Ree, J. H., W. J. van den Broek, V. E. Dahlmans, P. H. Groot, M. Vidgeon Hart, R. R. Frants, B. Wieringa, L. M. Havekes, and M. H. Hofker. 1994. Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis.* **111:** 25–37.
7. Ishibashi, S., S. Perrey, Z. Chen, J. Osuga, M. Shimada, K. Ohashi, K. Harada, Y. Yazaki, and N. Yamada. 1996. Role of the low density lipoprotein (LDL) receptor pathway in the metabolism of chylomicron remnants. A quantitative study in knockout mice lacking the LDL receptor, apolipoprotein E, or both. *J. Biol. Chem.* **271:** 22422–22427.
8. Windler, E. E., J. Greeve, W. H. Daerr, and H. Greten. 1988. Binding of rat chylomicrons and their remnants to the hepatic low-density-lipoprotein receptor and its role in remnant removal. *Biochem. J.* **252:** 553–561.
9. Choi, S. Y., L. G. Fong, M. J. Kirven, and A. D. Cooper. 1991. Use of an anti-low density lipoprotein receptor antibody to quantify the role of the LDL receptor in the removal of chylomicron remnants in the mouse in vivo. *J. Clin. Invest.* **88:** 1173–1181.
10. Mahley, R. W., and M. M. Hussain. 1991. Chylomicron and chylomicron remnant metabolism. *Curr. Opin. Lipidol.* **2:** 170–176.
11. Ishibashi, S., J. Herz, N. Maeda, J. L. Goldstein, and M. S. Brown. 1994. The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc. Natl. Acad. Sci. USA.* **91:** 4431–4435.
12. Herz, J., and T. E. Willnow. 1995. Lipoprotein and receptor interactions in vivo. *Curr. Opin. Lipidol.* **6:** 97–103.
13. Beisiegel, U., A. Krapp, W. Weber, and G. Olivecrona. 1994. The role of alpha 2M receptor/LRP in chylomicron remnant metabolism. *Ann. NY Acad. Sci.* **737:** 53–69.
14. Herz, J., J. L. Goldstein, D. K. Strickland, Y. K. Ho, and M. S. Brown. 1991. 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J. Biol. Chem.* **266:** 21232–21238.
15. Willnow, T. E., Z. Sheng, S. Ishibashi, and J. Herz. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. *Science.* **264:** 1471–1474.
16. Rohlmann, A., M. Gotthardt, R. E. Hammer, and J. Herz. 1998. Inducible inactivation of hepatic LRP gene by Cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J. Clin. Invest.* **101:** 689–695.
17. Ji, Z. S., W. J. Brecht, R. D. Miranda, M. M. Hussain, T. L. Innerarity, and R. W. Mahley. 1993. Role of heparan sulfate proteoglycans in the binding and uptake of apolipoprotein E-enriched remnant lipoproteins by cultured cells. *J. Biol. Chem.* **268:** 10160–10167.
18. van Dijk, M. C., J. K. Kruijt, W. Boers, C. Linthorst, and T. J. van Berkel. 1992. Distinct properties of the recognition sites for beta-very low density lipoprotein (remnant receptor) and alpha 2-macroglobulin (low density lipoprotein receptor-related protein) on rat parenchymal cells. *J. Biol. Chem.* **267:** 17732–17737.
19. Yen, F. T., C. J. Mann, L. M. Guermani, N. F. Hannouche, N. Hubert, C. A. Hornick, V. N. Bordeau, G. Agnani, and B. E. Bihain. 1994. Identification of a lipolysis-stimulated receptor that is distinct from the LDL receptor and the LDL receptor-related protein. *Biochemistry.* **33:** 1172–1180.
20. Windler, E., J. Greeve, S. Jackle, F. Rinninger, C. Pox, D. Puchta, D. Petkova, H. Robenek, W. Daerr, and H. Greten. 1996. Endocytic mechanisms for uptake and metabolism of chylomicron remnants in the liver. *Z. Gastroenterol.* **34 Suppl. 3:** 103–104.
21. Rensen, P. C., N. Herijger, M. H. Netscher, M. C. Meskers, M. van Eck, and T. J. van Berkel. 1997. Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus the hepatic remnant receptor in vivo. *J. Lipid Res.* **38:** 1070–1084.
22. Kowal, R. C., J. Herz, K. H. Weisgraber, R. W. Mahley, M. S. Brown, and J. L. Goldstein. 1990. Opposing effects of apolipoproteins E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J. Biol. Chem.* **265:** 10771–10779.
23. Hussain, M. M., F. R. Maxfield, J. Mas Oliva, I. Tabas, Z. S. Ji, T. L. Innerarity, and R. W. Mahley. 1991. Clearance of chylomicron remnants by the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J. Biol. Chem.* **266:** 13936–13940.
24. Shimano, H., Y. Namba, J. Ohsuga, M. Kawamura, K. Yamamoto, M. Shimada, T. Gotoda, K. Harada, Y. Yazaki, and N. Yamada. 1994. Secretion–recapture process of apolipoprotein E in hepatic uptake of chylomicron remnants in transgenic mice. *J. Clin. Invest.* **93:** 2215–2223.
25. Ji, Z. S., S. Fazio, Y. L. Lee, and R. W. Mahley. 1994. Secretion–capture role for apolipoprotein E in remnant lipoprotein metabolism involving cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **269:** 2764–2772.
26. Ishibashi, S., M. S. Brown, J. L. Goldstein, R. D. Gerard, R. E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery [see Comments]. *J. Clin. Invest.* **92:** 883–893.
27. Kashyap, V. S., S. Santamarina-Fojo, D. R. Brown, C. L. Parrott, D. Applebaum-Bowden, S. Meyn, G. Talley, B. Paigen, N. Maeda, and H. B. Brewer, Jr. 1995. Apolipoprotein E deficiency in mice: gene replacement and prevention of atherosclerosis using adenovirus vectors. *J. Clin. Invest.* **96:** 1612–1620.
28. Fallaux, F. J., O. Kranenburg, S. J. Cramer, A. Houweling, H. Van

Ormondt, R. C. Hoeben, and A. J. Van Der Eb. 1996. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* **7:** 215–222.
29. Redgrave, T. G., D. C. Roberts, and C. E. West. 1975. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* **65:** 42–49.
30. van Vlijmen, B. J., H. B. van't Hof, M. J. Mol, H. van der Boom, A. van der Zee, R. R. Frants, M. H. Hofker, and L. M. Havekes. 1996. Modulation of very low density lipoprotein production and clearance contributes to age- and gender-dependent hyperlipoproteinemia in apolipoprotein E3-Leiden transgenic mice. *J. Clin. Invest.* **97:** 1184–1192.
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
32. Otway, S., and D. S. Robinson. 1967. The use of a non-ionic detergent (Triton WR 1339) to determine rates of triglyceride entry into the circulation of the rat under different physiological conditions. *J. Physiol. London.* **190:** 321–332.
33. van Vlijmen, B. J., K. W. van Dijk, H. B. van't Hof, P. J. van Gorp, A. van der Zee, H. van der Boom, M. L. Breuer, M. H. Hofker, and L. M. Havekes. 1996. 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. *J. Biol. Chem.* **271:** 30595–30602.
34. Jong, M. C., V. E. Dahlmans, P. J. van Gorp, K. W. van Dijk, M. L. Breuer, M. H. Hofker, and L. M. Havekes. 1996. In the absence of the low density lipoprotein receptor, human apolipoprotein C-I overexpression in transgenic mice inhibits the hepatic uptake of very low density lipoprotein via a receptor-associated protein-sensitive pathway. *J. Clin. Invest.* **98:** 2259–2267.
35. Medh, J. D., G. L. Fry, S. L. Bowen, M. W. Pladet, D. K. Strickland, and D. A. Chappell. 1995. The 39-kDa receptor-associated protein modulates lipoprotein catabolism by binding to LDL receptors. *J. Biol. Chem.* **270:** 536–540.
36. Herz, J., S. Q. Qiu, A. Oesterle, H. V. DeSilva, S. Shafi, and R. J. Havel. 1995. Initial hepatic removal of chylomicron remnants is unaffected but endocytosis is delayed in mice lacking the low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA.* **92:** 4611–4615.
37. Kuipers, F., M. C. Jong, Y. Lin, M. van Eck, R. Havinga, V. Bloks, H. J. Verkade, M. H. Hofker, H. Moshage, T. J. C. van Berkel, R. J. Vonk, and L. M. Havekes. 1997. Impaired production of very low density lipoprotein-triglycerides by apolipoprotein E-deficient mouse hepatocytes. *J. Clin. Invest.* **100:** 2915–2922.
38. Mulder, M., H. van der Boom, P. de Knijff, C. Braam, A. van den Maagdenberg, J. A. Leuven, and L. M. Havekes. 1994. Triglyceride-rich lipoproteins of subjects heterozygous for apolipoprotein E2(Lys146→Gln) are inefficiently converted to cholesterol-rich lipoproteins. *Atherosclerosis.* **108:** 183–192.
39. Jong, M. C., V. E. Dahlmans, P. J. van Gorp, M. L. Breuer, M. J. Mol, A. van der Zee, R. R. Frants, M. H. Hofker, and L. M. Havekes. 1996. Both lipolysis and hepatic uptake of VLDL are impaired in transgenic mice coexpressing human apolipoprotein E*3Leiden and human apolipoprotein C-I. *Arterioscler. Thromb. Vasc. Biol.* **16:** 934–940.
40. Huang Y., X. Q. Liu, S. C. Rall, Jr., and R. W. Mahley. 1998. Apolipoprotein E2 reduces the low density lipoprotein level in transgenic mice by impairing lipoprotein lipase-mediated lipolysis of triglyceride-rich lipoproteins. *J. Biol. Chem.* **273:** 17483–17490.
41. Rensen, P. C. N., and T. J. C. van Berkel. 1996. Apolipoprotein E effectively inhibits lipoprotein lipase-mediated lipolysis of chylomicron-like triglyceride-rich lipid emulsions in vitro and in vivo. *J. Biol. Chem.* **271:** 14791–14799.
42. Gomez Coronado, D., G. T. Saez, M. A. Lasuncion, and E. Herrera. 1993. Different hydrolytic efficiencies of adipose tissue lipoprotein lipase on very-low-density lipoprotein subfractions separated by heparin-Sepharose chromatography. *Biochim. Biophys. Acta.* **1167:** 70–78.
43. Jong, M. C., V. E. Dahlmans, M. H. Hofker, and L. M. Havekes. 1997. Nascent very-low-density lipoprotein triacylglycerol hydrolysis by lipoprotein lipase is inhibited by apolipoprotein E in a dose-dependent manner. *Biochem. J.* **328:** 745–750.
44. Havel, R. J., C. J. Fielding, T. Olivecrona, V. G. Shore, P. E. Fielding, and T. Egelrud. 1973. Cofactor activity of protein components of human very low density lipoproteins in the hydrolysis of triglycerides by lipoproteins lipase from different sources. *Biochemistry.* **12:** 1828–1833.
45. LaRosa, J. C., R. I. Levy, P. Herbert, S. E. Lux, and D. S. Fredrickson. 1970. A specific apoprotein activator for lipoprotein lipase. *Biochem. Biophys. Res Commun.* **41:** 57–62.
46. Huang Y., S. W. Schwendner, and S. C. Rall, Jr., and R. W. Mahley. 1996. Hypolipidemic and hyperlipidemic phenotypes in transgenic mice expressing human apolipoprotein E2. *J. Biol. Chem.* **271:** 29146–29151.
47. Huang Y., S. C. Rall, Jr., and R. W. Mahley. 1997. Genetic factors precipitating type III hyperlipoproteinemia in hypolipidemic transgenic mice expressing human apolipoprotein E2. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2817–2824.