Hyperlipidemia in APOE2 transgenic mice is ameliorated by a truncated apoE variant lacking the C-terminal domain

Gery Gerritsen,* Kyrriakos E. Kypreos,* André van der Zee,* Bas Teusink,† Vassilis I. Zannis,§ Louis M. Havekes,††,*,†† and Ko Willems van Dijk1,*,††

Department of Human Genetics,* Leiden University Medical Center, The Netherlands; TNO Prevention and Health,† Gaubius Laboratory, Leiden, The Netherlands; Boston University School of Medicine,§ Boston, MA; Department of General Internal Medicine,** Leiden University Medical Center, Leiden, The Netherlands; and Department of Cardiology,†† Leiden University Medical Center, Leiden, The Netherlands

Abstract Familial dysbetalipoproteinemia associated with the apolipoprotein E2 (APOE2) genotype is a recessive disorder with low penetrance. We have investigated whether additional expression of full-length APOE3, APOE4, or a truncated variant of APOE4 (APOE4-202) can reduce APOE2-associated hyperlipidemia. This was achieved using adenovirus-mediated gene transfer to mice transgenic for human APOE2 and deficient for endogenous ApoE (APOE2.ApoE−/− mice). The hyperlipidemia of APOE2.ApoE−/− mice was readily aggravated by APOE3 and APOE4 overexpression. Only a very low dose of APOE4 adenovirus was capable of reducing the serum cholesterol and triglyceride (TG) levels. Expression of higher doses of APOE4 was associated with an increased VLDL-TG production rate and the accumulation of TG-rich VLDL in the circulation. In contrast, a high dose of adenovirus carrying APOE4-202 reduced both the cholesterol and TG levels in APOE2.ApoE−/− mice. Despite the absence of the C-terminal lipid-binding domain, APOE4-202 is apparently capable of binding to lipoproteins and mediating hepatic uptake. Moreover, overexpression of APOE4-202 in APOE2.ApoE−/− mice does not aggravate their hypertriglyceridemia. These results extend our previous analyses of APOE4-202 expression in ApoE−/− mice and demonstrate that apoE4-202 functions even in the presence of clearance-defective apoE. Thus, apoE4-202 is a safe and efficient candidate for future therapeutic applications.—Gerritsen, G., K. E. Kypreos, A. van der Zee, B. Teusink, V. I. Zannis, L. M. Havekes, and K. W. van Dijk. Hyperlipidemia in APOE2 transgenic mice is ameliorated by a truncated apoE variant lacking the C-terminal domain. J. Lipid Res. 2003. 44:408–414.

Supplementary key words familial dysbetalipoproteinemia • adenovirus-mediated gene transfer • hypertriglyceridemia • apolipoprotein E

Apolipoprotein E (apoE) plays a central role in the metabolism of chylomicron and VLDL remnants. It functions as a ligand for receptor-mediated endocytosis (1), has an inhibitory effect on the lipolysis of VLDL-triglyceride (TG) (2–5), and stimulates hepatic secretion of VLDL-TG (6, 7). Mutations in APOE are associated with familial dyslipidemia (FD) that is characterized by an accumulation of chylomicron and VLDL remnants in the circulation and results in premature atherosclerosis (8). Most patients with FD are homozygous carriers of APOE2 (Arg158→Cys) (9). ApoE2 is one of the three major isoforms of apoE in the human population. The other two are apoE3 (Cys112; Arg158) and apoE4 (Cys112→Arg) (10). In vitro, apoE2 binds poorly to the LDL receptor (LDLR) (11), which in vivo is a major route for lipoprotein remnant clearance.

Hepatic overexpression of APOE isoforms in ApoE−/− mice using adenovirus (Ad) mediated gene transfer indicates that moderate levels of APOE3 and APOE4 expression lead to a normalization of the hypercholesterolemia and a dose-dependent but moderate increase in serum TG levels (12–14). Higher levels of hepatic overexpression of apoE3 and apoE4 lead to massive hypertriglyceridemia, even to the extent that the hypercholesterolemia is not reduced any more. A major factor contributing to the hyperlipidemia is the apoE-mediated increase in the VLDL-TG production rate (12, 14–16). Hepatic overexpression of apoE2 does not reduce hypercholesterolemia of ApoE−/− mice at any expression level (15). We have shown recently in ApoE−/− mice that the hypertriglyceridemic effect of APOE overexpression can be abrogated by deletion of the C-terminal lipid binding domain (amino acids 203–299) of apoE (apoE4-202). Injection of a very high dose of up to 1 × 1010 plaque-forming unit (pfu) Ad expressing APOE4-202 in ApoE−/− mice did not result in a rise in the serum triglyceride levels and normalized the hypercho-

Abbreviations: Ad, adenovirus; apo, apolipoprotein; FD, familial dysbetalipoproteinemia; pfu, plaque-forming unit.

1 To whom correspondence should be addressed.

email: kowvd@lumc.nl

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cholesterolemia. These studies demonstrated that the N-terminal 202 amino acids of apoE4 could associate with lipoproteins lacking apoE and mediate hepatic clearance without affecting the VLDL-TG production rate.

Since FD associated with apoE2/E2 phenotype is a recessive disease, it can be anticipated that additional expression of a low amount of an APOE isoform with normal binding affinity to the LDLR will ameliorate the phenotype. However, in FD the steady state serum levels of cholesterol, TG, and apoE2 are elevated, and the adverse effect of APOE overexpression on the VLDL-TG production rate could also overrule the positive effects of increased hepatic clearance. In the present study, the effects of hepatic overexpression of APOE3, APOE4, and APOE4-202 are investigated in a mouse model for FD, the APOE2 transgenic mouse. Similar to the human situation, the APOE2 transgenic mouse model is only hyperlipidemic in the absence of functional apoE, i.e., on the Apoe−/− background (the APOE2.Apoe−/− mouse). Our data indicate that the hyperlipidemia of APOE2.Apoe−/− mice is extremely sensitive to hepatic overexpression of full-length APOE3 and APOE4. Only a very low dose of Ad carrying APOE4 could reduce the hyperlipidemia in these mice, whereas higher doses of Ad-APOE4 aggravated the hyperlipidemia. In contrast, a high dose of Ad-APOE4-202 reduced the lipid levels in APOE2.Apoe−/− mice. Thus, even in the presence of elevated serum apoE levels, apoE4-202 can restore the clearance of VLDL remnants without increasing serum TG levels.

METHODS

Generation and analysis of transgenic mice

Transgenic mice expressing human apoE2(Arg158→Cys) in the absence of endogenous Apoe have been described previously (17). The transgenic status of the breeding offspring was determined by polymerase chain reaction analysis as described. For these experiments, female mice were fed a regular mouse diet (SRM-A; Hope Farms, Woerden, The Netherlands). The mice were housed under standard conditions in conventional cages and given free access to food and water. For Ad-mediated gene transfer experiments, mice were transferred to filter-top cages, placed in a designated room, and allowed to adapt for at least 5 days. All animal experimentation protocols were approved by the Committee on Animal Experimentation of the Leiden University Medical Center.

Ad transfections

The generation and expansion of recombinant Ads, expressing apoE3, apoE4, and apoE4 truncated at amino acid 202 under control of a Cytomegalovirus promoter, have been described (14). For in vivo administration, the virus was purified twice via CsCl gradient centrifugation and dialyzed against dialysis buffer (consisting of 25 mmol/l Tris, 137 mmol/l NaCl, 5 mmol/l KCl, 0.75 mmol/l NaH2PO4, 0.9 mmol/l CaCl2, and 0.5 mmol/l MgCl2, pH 7.45) and finally dialysis buffer supplemented with sucrose (50 g/l). For storage, aliquots of 150 µl virus were frozen at −80°C. Routine titers of the stocks varied from 1 × 108 to 1 × 1011 pfu per milliliter. At least 5 days before Ad injection, basal serum lipid (t = 0) values were measured. At day 0, mice were injected into the tail vein with recombinant Ad diluted with PBS to a total volume of 200 µl. To prevent sequestration of low doses of Ad-APOE by liver Kupffer cells and to achieve a more linear dose-response relation, mice were pre-injected with Ad-LacZ (18). The mice that received the low doses of 2 × 108 and 5 × 108 pfu Ad-APOE4 were pre-injected with, respectively, 8 × 108 and 5 × 108 pfu Ad-LacZ 4 h before administration of the apoE-expressing virus. Up to 10 days after Ad administration, blood samples of 100 µl were drawn from the tail vein of 4 h fasted mice or, when a larger volume of serum was required, blood samples were collected by orbital puncture.

Lipid, lipoprotein, and apoE measurements

Total serum cholesterol and TG levels were measured enzymatically with commercially available kits, respectively Boehringer Mannheim 236691 and Sigma Chemical Co 337-B.

Lipoprotein fractions were separated using density gradient ultracentrifugation. Prior to, and 4 days after, infection with Ad-ApoE4 or Ad-apoE4-202, sera of individual mice were pooled to a volume of 100 µl. After centrifugation, the gradient was fractionated in fractions of 0.5 ml. The fractions were analyzed enzymatically for cholesterol and TG, as described above. Human apoE concentrations were determined by sandwich ELISA as described previously (19).

Characterization of VLDL

The VLDL fractions (d < 1.006 g/ml) were analyzed for protein content by the method of Lowry et al. (20). Furthermore, total and free cholesterol, TG, and phospholipid content were determined enzymatically, using commercially available kits (236691 and 310328, Boehringer-Mannheim; 337-B, Sigma Chemicals; and 99054099, Wako Chemicals).

Hepatic VLDL-TG production

At day 6 after infection with Ad, the mice were fasted for 4 h and then intravenously injected with 500 mg/kg Triton WR 1339 (Sigma) as described (21). Blood samples of Ad-APOE4- and Ad-APOE4-202-infected mice were collected 1 and 60 min after Triton injection. Serum TG concentrations were determined enzymatically, as described above. The hepatic VLDL-TG production rate was measured as the accumulation of serum TG after Triton injection and expressed as mg/dl/min.

RESULTS

Serum lipid, lipoprotein, and apoE levels after Ad-mediated gene transfer of full-length and truncated APOE in APOE2.Apoe−/− transgenic mice

Full-length APOE3, APOE4, and the truncated variant APOE4-202 were expressed in female APOE2.Apoe−/− mice using Ad-mediated gene transfer. Serum cholesterol and TG levels were followed for up to 10 days after virus injection. Upon administration of a high dose of 2 × 108 pfu of Ad expressing full-length apoE3 (Fig. 1A), the serum cholesterol levels did not change (20.0 ± 3.7 to 20.7 ± 5.6 mM, P = 0.84). Serum TG level rose from 2.3 ± 0.7 to 24.8 ± 11.0 mM (P < 0.01) at day 5, but progressively decreased at days 7 and 9 to the level of day 0 (Fig. 1D). Injection of lower doses of Ad (5 × 108 or 1 × 109 pfu) expressing full-length apoE4 (Fig. 1B, E) resulted in hypertriglyceridemia, combined with nonsignificant changes in cholesterol levels at day 5 after injection. Only the lowest dose of 2 × 108 pfu of Ad expressing full-length apoE4 re-
resulted in improvement of the hyperlipidemia at day 5 after injection (cholesterol from 13.8 ± 1.7 to 2.2 ± 0.7 mM (P < 0.01); TG from 3.1 ± 2.2 to 0.7 ± 0.3 mM (P < 0.05)). In contrast, expression of 2 × 10^9 pfu of Ad expressing the truncated apoE4-202 variant resulted in a significant reduction of serum cholesterol levels from 13.1 ± 3.2 to 3.8 ± 1.1 mM (P < 0.01) and did not raise the serum TG level in these mice at any time point after injection (Fig. 1C, F).

To determine the effects of the apoE variants on the individual lipoprotein classes, serum samples were subjected to density gradient ultracentrifugation. After fractionation, the lipid levels in the fractions were measured. The lipoprotein profile of untreated APOE2/ApoE−/− mice shows that cholesterol is mainly distributed over the VLDL and IDL/LDL fractions (Fig. 2A). The tremendous increase in serum TG levels after Ad-mediated expression of APOE4 is mainly confined to the VLDL fraction (Fig. 2B). The ratio of VLDL cholesterol (VLDL-C) to HDL-C decreased about five times as compared with the non-infected mice.

ApoE levels in these fractions were measured by sandwich ELISA. In APOE2/ApoE−/− mice (Fig. 2D), apoE was mostly present in the VLDL and IDL/LDL fractions. Expression of APOE4 resulted in a considerable amount of apoE in the HDL and bottom fractions (Fig. 2E), the latter indicating the presence of unbound apoE. Furthermore, abundant apoE was present in the VLDL fractions. Expression of apoE-202 resulted in a significant amount of apoE in the bottom and HDL fractions (Fig. 2F), but apoE was also clearly present in the VLDL fractions.

**VLDL composition**

The lipid composition of the VLDL fractions isolated by density gradient ultracentrifugation was determined (Fig. 3). Expression of apoE4 in APOE2/ApoE−/− mice resulted in an increase in TG content of circulating VLDL from 46% to 71% (Fig. 3A, B). This latter level approaches the TG content of nascent VLDL (76%, Fig. 3D). After expression of apoE4-202, the TG content of circulating VLDL increased from 46% to 61% (Fig. 3A, C).
Hepatic VLDL production

The VLDL-TG production rate after apoE4 and apoE4-202 overexpression in APOE2.Apoε−/− mice was determined after injection with Triton WR1339 (Fig. 4). Triton WR1339 blocks VLDL-TG lipolysis and VLDL remnant clearance. Expression of apoE4 in APOE2.Apoε−/− mice resulted in a 10-fold increase in the VLDL-TG production rate, whereas expression of apoE4-202 did not affect the VLDL-TG production rate.

DISCUSSION

FD is characterized by elevated levels of VLDL and chyomicron remnants in the serum of patients and an increased risk for premature atherosclerosis. The majority of patients with FD are homozygous for the E2 allele of apoE. The association of the APOE2/E2 phenotype with FD is at least partly explained by the observation that apoE2 binds poorly to the LDLR in vitro, which is a major route of lipoprotein remnant uptake in vivo. Thus, it can be anticipated that additional expression of apoE with normal binding affinity to the LDLR will reduce the hyperlipidemia associated with the recessive FD disease. We have previously generated a transgenic mouse line expressing the human APOE2 gene (17). In the presence of endogenous mouse apoE, APOE2 mice do not develop hyperlipidemia. However, in the absence of mouse apoE, VLDL size remnants accumulate in the serum of these APOE2.Apoε−/− mice, and serum lipid and apolipoprotein levels are highly reminiscent of the FD phenotype. Using this mouse model for FD, we now show that hepatic overexpression of full length APOE3 or APOE4 leads to a dose-dependent induction of hypertriglyceridemia (Fig. 1). Only a very low level of APOE4 expression reduced the hyperlipidemia of the APOE2.Apoε−/− mouse. Higher expression levels induced hypertriglyceridemia, without reducing serum cholesterol levels. In contrast, the hyperlipidemia of APOE2.Apoε−/− mice is reduced significantly by high levels of APOE4-202 expression.

We have shown previously in Apoe−/− mice that Ad-mediated overexpression of full-length apoE4 resulted in hypertriglyceridemia at high doses of Ad and resulted in rescue of the hyperlipidemia at moderate doses of Ad (14, 16). Similar to the observations in the APOE2.Apoε−/− mice, the truncated apoE4-202 variant reduced the hyperlipidemia in Apoe−/− mice at high expression levels. Thus, APOE4-202 does not have a hypertriglyceridemic effect at high expression levels in either mouse model. Moreover, apoE4-202 can associate with lipoproteins both in the absence of endogenous mouse apoE and in the presence of

Fig. 2. Distribution of cholesterol, TG, and apoE in lipoprotein fractions after Ad-mediated gene transfer of APOE4 and APOE4-202 to APOE2.Apoε−/− transgenic mice. Female mice (n = 5 per group) were left untreated (A, D), injected with 2 × 10⁹ pfu Ad-APOE4 (B, E), or injected with 2 × 10⁹ pfu Ad-APOE4-202 (C, F). Four days post-injection, sera of five fasted mice per group were pooled and lipoproteins were separated by density gradient ultracentrifugation. After fractionation, the individual fractions were analyzed for cholesterol and TG (A–C) and apoE content (D–F).
binding-defective human apoE2 and mediate hepatic lipoprotein uptake. Interestingly, the lowest dose of Ad-APOE4 that rescues the hyperlipidemia of Apoe<sup>-/-</sup> mice (5 × 10<sup>8</sup> pfu) (14) does result in hypertriglyceridemia in APOE2.Apoe<sup>-/-</sup> mice. The higher sensitivity of the APOE2.Apoe<sup>-/-</sup> mice is in line with the fact that the hypertriglyceridemic effect of APOE4 is gene dose dependent and that the starting level of APOE gene expression in the APOE2 mice is higher.

It is interesting to note that the reduction of hyperlipidemia in the APOE2.Apoe<sup>-/-</sup> mice achieved with a very low dose (2 × 10<sup>8</sup> pfu) of Ad-APOE4 combined with pre-injection of Ad-LacZ (1.8 × 10<sup>9</sup> pfu). Without pre-injection of Ad, doses below 5 × 10<sup>8</sup> pfu give extremely low levels of gene expression, a dose of 5 × 10<sup>8</sup> pfu gives low levels of gene expression, and a dose of 2 × 10<sup>9</sup> pfu gives very high levels of gene expression. Tao et al. have reported that this nonlinear Ad dose response is caused by the initial sequestration and nonfunctional infection of Ad vectors by the kupffer cells in the liver (18). It was shown that pre-injection with a control Ad vector rendered the Ad dose response linear, a finding we have confirmed (data not shown). The data presented in Fig. 1 also show that by pre-injection with Ad, even very low doses of Ad, result in productive gene expression.

In the Apoe<sup>-/-</sup> mice, APOE4 overexpression had a major impact on the VLDL-TG production rate (14, 15). The dramatically increased VLDL-TG production rate in the APOE2.Apoe<sup>-/-</sup> mouse (Fig. 4) is obviously in line with this observation. Neither in the Apoe<sup>-/-</sup> mice, nor in the APOE2.Apoe<sup>-/-</sup> mice did APOE4-202 overexpression result in an increase in the VLDL-TG production rate. The mechanism of the increase in the VLDL-TG production rate, which is apparently mediated via the C-terminal 97 amino acids of apoE, remains to be determined. It has been speculated that apoE is involved in one of the last steps of de novo VLDL synthesis, the intracellular addition of TG to the nascent VLDL particle (22). It is possible that the C-terminal lipid binding domain of apoE stabilizes the TG content of the nascent VLDL particle and that overexpression of full-length apoE thus enhances VLDL-TG secretion.

As compared with nascent VLDL, the VLDL sized particles that accumulate in untreated APOE2.Apoe<sup>-/-</sup> mice are relatively depleted of TG (76% vs. 46%) (Fig. 3). The TG content of circulating VLDL is affected by both hepatic VLDL production and uptake, but also by peripheral VLDL-TG lipolysis. In APOE2.Apoe<sup>-/-</sup> mice, VLDL is poorly cleared, resulting in a prolonged circulation time and thus prolonged exposure to lipoprotein lipase (LPL). This is believed to result in the observed relatively low TG content of circulating VLDL in APOE2.Apoe<sup>-/-</sup> mice. Overexpression of APOE4 in the APOE2.Apoe<sup>-/-</sup> mice resulted in an accumulation of circulating VLDL that had a relatively high TG content (71%). This could be explained by the increased VLDL-TG secretion rate upon APOE4 overexpression. However, it has also been demonstrated in vitro that the rate of LPL-mediated VLDL-TG lipolysis is inhibited by apoE in a dose-dependent manner (2, 4, 5). At high levels of hepatic APOE4 expression, the

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**Fig. 3.** VLDL composition following Ad-mediated gene transfer of APOE4 and APOE4-202 to APOE2.Apoe<sup>-/-</sup> transgenic mice. Female mice (n = 5 per group) were left untreated (A) or injected with 2 × 10<sup>8</sup> pfu Ad-APOE4 (B) or 2 × 10<sup>9</sup> pfu Ad-APOE4-202 (C, D). For the analysis of circulating VLDL (A–C), sera were collected from fasted mice four days post-injection and pooled. For the analysis of nascent VLDL (D), on day 6 following injection with Ad-APOE4-202, fasted mice were injected with Triton WR 1339, and sera were collected 1 h after Triton injection. VLDL was isolated by density gradient ultracentrifugation. Lipid composition was determined in w/w percentage of total lipids (FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid).

**Fig. 4.** Hepatic VLDL-TG production in APOE2.Apoe<sup>-/-</sup> mice injected with Ad-APOE4 or Ad-APOE4-202. Female mice were left untreated (n = 5) or injected with 2 × 10<sup>8</sup> pfu Ad-APOE4 (n = 1) or 2 × 10<sup>9</sup> pfu Ad-APOE4-202 (n = 5). VLDL-TG production was measured 6 days post-injection with the virus expressing the APOE4 or APOE4-202. Fasted serum TG level was determined 1 and 60 min after Triton WR 1339 injection. The VLDL-TG secretion rate was calculated by the increase in TG content as a function of time and expressed as mg/dl/min. Values are represented as mean ± SD.
relative apoE content of the circulating VLDL is high (Fig. 2E). Thus, it cannot be excluded that inhibition of VLDL-TG lipolysis contributes to the high TG level of the circulating VLDL particles in APOE2 Apoe−/− mice overexpressing APOE4.

The TG content of circulating VLDL after overexpression of APOE4-202 (61%) is increased as compared with circulating VLDL from untreated APOE2 Apoe−/− mice (46%) (Fig. 3), but is below that of circulating VLDL from wild-type mice (69%) (21). As shown in Fig. 4, the hepatic VLDL-TG production rate does not contribute to this effect, since it remains unchanged after overexpression of APOE4-202. The relative TG enrichment of the circulating VLDL in APOE2 Apoe−/− mice after overexpression of APOE4-202 is likely caused by decreased residence times in the circulation (and thus shorter exposure to LPL) as a result of enhanced VLDL particle uptake. The enhanced VLDL particle uptake is demonstrated by the lowering in serum lipid levels (Figs. 1, 2). However, APOE2 Apoe−/− mice overexpressing APOE4-202 do accumulate a modest amount of VLDL in serum (Fig. 2C) and the VLDL-TG content is below that of wild-type level. Thus it can be concluded that the residence time of VLDL containing both APOE2 and APOE4-202 is somewhat prolonged as compared with wild-type VLDL.

At high hepatic expression levels of APOE4, the circulating VLDL contains abundant apoE4 (Fig. 2B, E), which in principle would be an excellent substrate for LDLR-mediated uptake. A tiny amount of APOE expression can result in the hyperlipidaemia of diabetics that are characterized by increased serum levels of VLDL and VLDL remnants, such as FD. The data presented here in APOE2 Apoe−/− mice and previously in Apoe−/− mice indicate that apoE affects lipoprotein metabolism in a dose-dependent manner. Low levels of hepatic APOE expression are associated with reduction of the hyperlipidaemia, whereas higher levels are associated with serious aggravation of the hyperlipidaemia. Our data indicate that in a dyslipidemic situation, such as in APOE2-associated FD, additional expression of full-length APOE may readily aggravate the hyperlipidaemia. The relatively small therapeutic window makes additional or induced expression of full-length APOE a risky treatment. However, the insight that the hyperlipidemic effect can be circumvented by deletion of the C-terminal domain, combined with the observation that this truncated apoE variant is functional in the presence of APOE2, provides novel avenues for the application of apoE variants as therapeutic proteins.

This research was conducted in the framework of the "Leiden Center for Cardiovascular Research LUMC-TNO" and supported by the Netherlands Heart Foundation (project 2000.099), the EU fifth framework Program (MCIF 2000.02051) to K.E.K., and National Institutes of Health Grant HL-68216 to V.I.Z. The authors thank Dr. Patrick Rensen for helpful discussions.

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