Domains of Apolipoprotein E Contributing to Triglyceride and Cholesterol Homeostasis in Vivo

CARBOXYL-TERMINAL REGION 203–299 PROMOTES HEPATIC VERY LOW DENSITY LIPOPROTEIN-TRIGLYCERIDE SECRETION*

Received for publication, January 17, 2001
Published, JBC Papers in Press, February 9, 2001, DOI 10.1074/jbc.M100418200

Kyriakos E. Kypreos‡§, Ko Willems van Dijk‡§, Andre van der Zee‡, Louis M. Havekes‡, and Vassilis I. Zannis‡‡‡†

From the ‡Whitaker Cardiovascular Institute, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118, the §Department of Human and Clinical Genetics, Leiden University Medical Center, 2333AL Leiden, The Netherlands, ¶TNO-PG Prevention and Health, Gaubius Laboratory, Leiden 2333CK, The Netherlands, and **Department of Biochemistry and Institute of Molecular Biology and Biotechnology, University of Crete, Heraklion, Crete 71110, Greece

Apolipoprotein (apo) E has been implicated in cholesterol and triglyceride homeostasis in humans. At physiological concentration apoE promotes efficient clearance of apoE-containing lipoprotein remnants. However, high apoE plasma levels correlate with high plasma triglyceride levels. We have used adenovirus-mediated gene transfer in apoE-deficient mice (E−/−) to define the domains of apoE required for cholesterol and triglyceride homeostasis in vivo. A dose of 2 × 10⁹ plaque-forming units of apoE-expressing adenovirus reduced slightly the cholesterol levels of E−/− mice and resulted in severe hypertriglyceridemia, due to accumulation of cholesterol and triglyceride-rich very low density lipoprotein particles in plasma. In contrast, the truncated form apoE4–202 resulted in a 90% reduction in the plasma cholesterol levels but did not alter plasma triglyceride levels in the E−/− mice. ApoE secretion by cell cultures, as well as the steady-state hepatic mRNA levels in individual mice expressing apoE4 or apoE4–202, were similar. In contrast, very low density lipoprotein-triglyceride secretion in mice expressing apoE4, but not apoE4–202, was increased 10-fold, as compared with mice infected with a control adenovirus. The findings suggest that the amino-terminal 1–202 region of apoE4 contains the domains required for the in vivo clearance of lipoprotein remnants. Furthermore, the carboxyl-terminal 203–299 residues of apoE promote hepatic very low density lipoprotein-triglyceride secretion and contribute to apoE-induced hypertriglyceridemia.

Apolipoprotein (apo) E is the ligand for several cell receptors and promotes the catalysis of apoE-containing lipoprotein remnants by the liver (1–5). Mutations in apoE that prevent binding of apoE-containing lipoproteins to the LDL receptor and possibly other receptors and hepary sulfat proteoglycans are associated with type III hyperlipoproteinemia and premature atherosclerosis (6–14).

Studies in human patients with type III hyperlipoproteinemia and in animal models with apoE deficiency or defective apoE forms (15–24) showed that apoE is required for the clearance of cholesterol ester-rich lipoprotein remnants found in the VLDL and IDL region (15–27). The accumulation of such remnants in plasma is associated with premature atherosclerosis (15, 17–19). ApoE may also be involved in cholesterol efflux processes (28–31). These functions of apoE contribute to cell and tissue cholesterol homeostasis (28–31) and may explain why, when expressed locally in macrophages or endothelial cells, apoE protects from atherosclerosis (32–34).

In humans, plasma apoE levels correlate with plasma triglyceride levels (26). Animal studies have shown that increased plasma apoE levels inhibit lipolysis of triglyceride-rich lipoproteins in vivo and result in hypertriglyceridemia (35–39). Lipolysis of VLDL in vitro could be partially restored by the addition of apoCII (35, 36). Other studies have shown that overexpression of apoE also stimulates hepatic VLDL-triglyceride production in vivo (39) and in cell cultures (35), possibly by promoting the assembly and/or secretion of apoB-containing lipoproteins. In contrast, lack of apoE is associated with decreased VLDL-triglyceride secretion (40). Such studies provide compelling evidence that apoE contributes both to cholesterol as well as triglyceride homeostasis in vivo.

To identify the domains of apoE that contribute to cholesterol and triglyceride homeostasis in vivo, we used adenovirus-mediated gene transfer in apoE-deficient mice. We have found that overexpression of full-length apoE4 is associated with high cholesterol and triglyceride levels, whereas overexpression of apoE4–202 normalizes the cholesterol levels of apoE-deficient mice and does not trigger hypertriglyceridemia. ApoE secretion by cells, hepatic apoE mRNA expression, and association of apoE with VLDL are not affected by the apoE truncation. In contrast, apoE4, but not apoE4–202, greatly increases hepatic
VLDL-triglyceride secretion. Our findings suggest that truncated forms of apoE may find useful gene therapy applications in the future in correcting remnant removal disorders.

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant Adenoviruses Expressing ApoE4 and ApoE4–202**—The construction of pUC-apoE4 cDNA has been described previously (41). pUC-apoE4–202 was generated by overlap-extension PCR that resulted in mutagenesis of codon 203 (GTA) to a stop TAA (Table I) using pUC-apoE4 as a template and the four sets of oligonucleotides indicated in Table I as primers. The set of external primers OUTPR1-S (sense) and OUTPR2-A (antisense) correspond to nucleotides encoding amino acids 103–111 and 208–215 of apoE, respectively, and contain the restriction sites NcoI and BstEII, respectively. The set of mutagenic oligonucleotides extending 10 residues 5′ and 10 residues 3′ of codon 203 has been altered in its sequence to a stop TAA codon (Table I). The PCR-based mutagenesis of codon 203 involved two separate amplification reactions. The first reactions used the 5′ external primer and the antisense mutagenic primer (Internal-S, Table I) covering codon 203. An aliquot of 4% of the volume of each PCR mix was mixed, and the sample was amplified by the 5′ and the 3′ external primers. The amplified fragment was then digested with NcoI and BstEII and was used to replace the WT sequence of the pUC-E4 plasmid. To incorporate the PCR-generate

**TABLE I**

Oligonucleotides used in overlap extension PCR

| Oligo name | Oligo sequence |
|------------|----------------|
| OUTPR1-S   | 5'-GCT GGG TGC AGA CAC TCT GTG AGC-3' (contains a 5' NcoI site) |
| OUTPR2-A   | 5'-CGC AGC GGC TCG CCG CAC GAG GCC TT-3' (contains a 3' BstEII site) |
| Internal-S | 5'-C GGC CAG CCG TGA CAG GAG CCG G-3' |
| Internal-A | 5'-C GCC CTC TCT TCA CGG CTC GCG G-3' |

Bold underlined bases represent the mutated codon.

cytoma) grown to confluence in medium containing 10% fetal calf serum were infected with AdGFP-E4 or AdGFP-E4–202, at a multiplicity of infection of 20. Twenty four hours post-infection, cells were washed twice with phosphate-buffered saline (PBS) and preincubated in serum-free medium for 2 h. Following an additional wash with PBS, fresh serum-free medium was added. After 24 h of incubation, medium was collected and analyzed by enzyme linked immunosorbent assay (ELISA) and SDS-PAGE for apoE expression. In some experiments, 60-mm diameter cultures were labeled metabolically with 0.5 mCi of [35S]methionine for 2 h, in which case medium was collected 2 h after addition of the radiolabeled amino acid for further analysis.

**Association of ApoE Secreted into the Culture Medium of Cells with Plasma Lipoproteins following Density Gradient Ultracentrifugation**—For this analysis, following the labeling of cells with [35S]methionine, 1 ml of medium from one 100-mm diameter dish was mixed with 1 ml of lipoprotein fractions of densities (d) in the range of 1.006–1.21 g/ml, which were previously separated from plasma by density gradient ultracentrifugation, the mixture was adjusted to density 1.24 with KBr solution and spun with 4 ml of KBr solution of d = 1.20, 1.16, and 2 ml of KBr solution of d = 1.019 g/ml. The mixture was centrifuged for 24 h in a SW-41 rotor at 32,000 rpm. Following ultracentrifugation, 12 ml fractions were collected and analyzed by SDS-PAGE and autoradiography. This analysis showed that both apoE4 and apoE4–202 can associate with exogenous lipoproteins that float in the IDL to HDL region.

**Isolation of VLDL by Density Gradient Ultracentrifugation**—One ml of serum sample obtained from apoE- and LDLR-deficient mice were overlaid on a KBr density gradient of 1.019–1.21 g/ml, which were previously separated from plasma by density gradient ultracentrifugation, the mixture was adjusted to density 1.006 with KBr solution and spun at 37 °C for 30 min and then subjected to density gradient centrifugation to separate free apoE, as described above, and VLDL, purifying the step above. Then the apoE-enriched VLDL and free apoE fractions were isolated and analyzed for apoE concentration by SDS-PAGE and immunoblotting.

**Animal Studies**—Female apoE-deficient mice 20–25 weeks old were used in these studies (44). Groups of mice were formed based on their plasma cholesterol and triglyceride levels before initiation of the experiments to ensure similar mean cholesterol and triglyceride levels in each group. The mice were injected intravenously through the tail vein with doses ranging from 5 × 10⁸ to 1 × 10¹⁰ pfu of AdGFP (control adenovirus), AdGFP-E4, or AdGFP-E4–202 virus, as indicated. Each group contained 8–10 mice. Blood was obtained from the tail vein or retro-orbital plexus after a 4-h fast preceding adenoviral injection. On the indicated days after injection (0, 4, 5, 8, and 12), blood was collected into a CB300 or CB1000 blood collection tube (Sarstedt). Aliquots of plasma were stored at 4 and -20 °C. One or more animals from each group was sacrificed on each of the indicated days so that mRNA expression in the mouse liver could be analyzed.

**FPLC Analysis**—For FPLC analysis of serum samples, 12 ml of serum were diluted 1:5 with PBS. Then sample was loaded onto a Sepharose CL-4B column in a SMART micro FPLC system (Amersham Pharmacia Biotech) and eluted with PBS. A total of 25 fractions of 50-ml volume each were collected for further analysis.

**Triglyceride and Cholesterol Analysis**—Ten ml of serum sample were diluted with 40 ml of phosphate-buffered saline (PBS), and 7.5 ml of the dilute sample were analyzed for triglycerides and cholesterol using the GPO-Trinder Kit (Sigma) and CHOL-PRK3 kit (Roche Molecular Biochemicals), according to the manufacturer’s instructions.
and cholesterol concentrations were determined spectrophotometrically at 540 and 492 nm, respectively. Triglyceride analysis of the FPLC fractions was performed using the TG Buffer (Sigma), and concentrations were determined spectrophotometrically at 492 nm according to the manufacturer’s instructions. Cholesterol analysis of the FPLC fractions was performed as described above for the serum samples.

Quantification of Human ApoE—Serum human apoE4 concentrations were measured by using sandwich ELISA (45). Affinity-purified polyclonal goat anti-human apoE antibodies were used for coating microtiter plates, and polyclonal goat anti-human apoE coupled to horseradish peroxidase was used as the secondary antibody. The immunoperoxidase procedure was employed for the colorimetric detection of apoE at 450 nm, using tetramethylbenzidine as substrate. Pooled plasma from healthy human subjects with known apoE level was used as a standard.

RNA Isolation and Hybridization Analysis—Total RNA was isolated from livers of mice five days post infection using the RNA Easy solution (RNA Insta-Pure, Eurogentec Pure) according to the manufacturer’s instructions. For Northern blot analysis, RNA samples (15 μg) were denatured and separated by electrophoresis on 1.0% formaldehydeagarose gels. RNA was stained with ethidium bromide to verify integrity and equal loading and then transferred to GeneScreen Plus (PerkinElmer Life Sciences). RNA was cross-linked to the membrane by UV irradiation (Stratalinker, Stratagene) at 0.12 J/cm² for 30 s. Probes, prepared by random priming, were used as described previously, and 2.0 × 10⁶ cpm/ml 32P-labeled DNA was employed. Quantitation by scanning densitometry was performed using a Molecular Dynamics PhosphorImager (model 400B). ApoE mRNA expression was normalized for GAPDH mRNA levels and reported in the form of a bar graph. Experiments were performed in triplicate, and data are reported as mean ± S.D.

Rate of VLDL-Triglyceride Production in Mice Injected with Different ApoE Forms—To determine the effects of the apoE truncations on hepatic VLDL-triglyceride secretion, four apoE-deficient mice for each group were infected with a dose of 2 × 10⁹ pfu of AdGFP-E4 and 1 × 10⁶ pfu of the AdGFP-E4–202 or the control AdGFP viruses, respectively. Four days post-infection, mice were fasted for 4 h and then injected with Triton WR1339 at a dose of 500 mg/kg of body weight, using a 15% solution (w/v) in 0.9% NaCl (Triton WR1339 has been shown to inhibit completely VLDL catabolism (46)). Serum samples were isolated 5, 10, 20, 30, 40, 50, and 60 min after injection with Triton WR1339. As control, serum samples were isolated 1 min immediately after the injection with the detergent. Serum triglyceride levels were determined as described under the “Experimental Procedures,” and a linear graph of serum triglyceride versus time was generated. The rate of VLDL-triglyceride secretion expressed in mg/dl/min was calculated from the slope of the linear graph for each individual mouse. Then slopes were grouped together and reported as mean ± S.D. in the form of a bar graph.

RESULTS

Expression and Flotation Properties of ApoE Secreted by HTB-13 Cell Cultures Infected with Control and Recombinant Adenovirus Containing ApoE4 or ApoE4–202—HTB-13 cells that do not synthesize endogenous apoE were infected with recombinant adenoviruses expressing apoE4 or apoE4–202, designated AdGFP-E4 and AdGFP-E4–202, respectively, at a multiplicity of infection of 20. Analysis of the culture medium by SDS-PAGE and sandwich ELISA showed that both apoE4 and apoE4–202 were secreted efficiently at comparable levels in the range of 60–80 μg of apoE per ml 24 h after infection with AdGFP-E4 or AdGFP-E4–202. To study the flotation properties of apoE4 and apoE4–202, 100-mm diameter dishes of HTB-13 cultures that were infected with AdGFP-E4 or AdGFP-E4–202, respectively, were metabolically labeled with 0.5 mCi of [35S]methionine for 2 h, and then 1 ml of medium from each dish was mixed with ~1 mg of lipoprotein fractions of densities (d) in the range of 1.006–1.21 g/ml, which were previously separated from plasma by density gradient ultracentrifugation. The mixture was subjected again to density gradient ultracentrifugation, and 12 fractions were collected and fractionated on SDS-PAGE, followed by autoradiography. This analysis showed that the majority of apoE4 and a considerable fraction of apoE4–202 floats in the d > 1.21 g/ml lipoprotein fraction, although a large quantity of apoE–202 was also found in the lipid-poor or lipid-free fraction (Fig. 1A). To establish further the ability of apoE4 and apoE4–202 to associate with VLDL, 15 μg of apoE4 or apoE4–202 were mixed with VLDL fractions isolated from the plasma of apoE- and LDLR-deficient mice by ultracentrifugation, and the mixtures were incubated at 37 °C for 30 min. The mixture was then subjected to density gradient ultracentrifugation. The amounts of the free and lipoprotein-associated apoE were assessed by fractionation on SDS-PAGE followed by Western blot analysis for apoE. As shown in Fig. 1B, both the full-length apoE4 and the truncated apoE4–202 associate with particles with densities in the VLDL to LDL region. ApoE4 in this analysis is found in the VLDL to HDL3 region. ApoE4–202 is found in the VLDL to LDL region. ApoE4–202 appears to associate with particles in the VLDL to HDL3 region. The data indicate that both the wild-type apoE4 as well as the truncated apoE4 form, apoE4–202, have the ability to associate with pre-existing lipoprotein particles, a process that is required for receptor-mediated lipoprotein clearance.

The Carboxyl-terminal 203–299 Segment of ApoE Contributes to Hypertriglyceridemia in ApoE-deficient Mice—To assess the effects of apoE4 and apoE4–202 on hyperlipidemia in vivo, apoE-deficient mice (E−/−) were infected with the recombinant adenoviruses AdGFP-E4 or AdGFP-E4–202, respectively. To
assess potential nonspecific effects of virus infection, some mice were infected with the control AdGFP virus. Analysis of plasma lipid levels showed that the infection of mice with $2 \times 10^9$ pfu of the apoE4-adenovirus did not result in a significant reduction in the plasma cholesterol levels, as compared with the cholesterol levels of mice infected with the control virus and non-infected mice (Fig. 2A). In addition, infection with $2 \times 10^9$ pfu of the apoE4 adenovirus induced severe hypertriglyceridemia (Fig. 2B). When expression of apoE4 on day 8 post-infection was reduced or when the dose used for infection was decreased to $5 \times 10^8$ pfu, hypertriglyceridemia was also reduced or eliminated (Fig. 2, A and B, right side). In contrast to the mice infected with the adenovirus expressing the wild-type E4, mice that were infected with $2 \times 10^9$ or even $10^{10}$ pfu of the AdGFP-E4–202 adenovirus did not result in a significant reduction in plasma cholesterol levels. Approximately 70% of cholesterol was distributed in VLDL and 20% in HDL. On day 8 post-infection, the ratio of VLDL/HDL was similar in infected mice, under conditions of apoE4-induced hypertriglyceridemia. Therefore, the different effects of apoE4 and apoE4–202 on hypertriglyceridemia are most likely not due to different levels of expression of these two apoE forms.

**Fig. 2.** A and B, cholesterol (A) and triglyceride (B) levels of E<sup>−/−</sup> mice infected with the control adenovirus AdGFP or recombinant adenoviruses expressing apoE4 or apoE4–202. Mice were infected in triplicate with the indicated doses of recombinant virus, and serum samples were isolated and analyzed for cholesterol (A) and triglyceride levels (B) on the indicated days after infection as described under “Experimental Procedures.”

**The Hepatic ApoE4 and ApoE4–202 mRNA Levels Are Similar in Infected Mice, under Conditions of ApoE4-induced Hypertriglyceridemia—**To assess the expression of apoE4 and apoE4–202 in the mice infected with AdGFP-E4 and AdGFP-E4–202, respectively, at least three infected mice from each group were sacrificed on day 5 post-infection, and their livers were collected. Total RNA was isolated from these livers and analyzed for apoE mRNA levels by Northern blot analysis. In agreement with cell culture data, where we see similar levels of apoE4 and apoE4–202 protein expression following adenovirus infection, the apoE mRNA levels in mice infected with a dose of $2 \times 10^9$ pfu AdGFP-E4 are similar to those in mice infected with either $2 \times 10^9$ pfu or $1 \times 10^{10}$ pfu AdGFP-E4–202 (Fig. 3, A and B). However, apoE4–202 clears efficiently the cholesterol from the plasma of E<sup>−/−</sup> mice without causing hypertriglyceridemia, whereas the full-length apoE4 does not clear cholesterol from the plasma of E<sup>−/−</sup> mice and causes hypertriglyceridemia (Fig. 3, C and D). Thus, the different effects of apoE4 and apoE4–202 on hypertriglyceridemia most likely are not due to different levels of expression of these two apoE forms.

**Cholesterol, Triglyceride, and ApoE FPLC Profiles of Plasma Isolated from Mice Infected with AdGFP-E4, AdGFP-E4–202, or the Control Virus AdGFP—**FPLC analysis of plasma from adenovirus-infected mice showed that in mice expressing apoE4 5 days post-infection, cholesterol levels were high. Approximately 70% of cholesterol was distributed in VLDL and ~20% in HDL. On day 8 post-infection, the ratio of VLDL/HDL...
C-terminal Domain of ApoE Contributes to Hypertriglyceridemia

cholesterol was −1:1 to 2:1 (Fig. 4A, upper panel). In mice infected with AdGFP-E4–202, cholesterol was distributed in the VLDL and HDL, and the ratio VLDL cholesterol to HDL cholesterol was −1:1 on either day, when the dose of adenovirus used was either $2 \times 10^9$ or $10^{10}$ pfu (Fig. 4A, lower panels). In mice infected with AdGFP-E4, as expected, triglyceride levels were very high in the VLDL fractions and barely detectable in the rest of the lipoprotein fractions, whereas in mice infected with AdGFP-E4–202 triglyceride levels were very low in all the lipoprotein fractions either 5 or 8 days post-infection (Fig. 4B). As an additional control, infection with 2 $\times 10^9$ pfu of the control virus AdGFP, did not result in any change in the cholesterol and triglyceride profiles of the apoE-deficient mice (data not shown).

Analysis of FPLC fractions by sandwich ELISA showed that in mice infected with $2 \times 10^9$ pfu AdGFP-E4, 5 days post-infection −50% of the total apoE was distributed in HDL and 25% in VLDL, and the remaining apoE was distributed across the other FPLC fractions. In contrast, in mice infected with $10^{10}$ pfu AdGFP-E4–202, apoE was uniformly distributed in all lipoprotein fractions (Fig. 5). The levels of apoE4 and apoE4–202 are similar in fractions 22–25, which represent the lipid-free apoE form (Fig. 5). This indicates that similar steady-state levels of lipid-free apoE4 and apoE4–202 exist in the plasma of mice infected with $2 \times 10^9$ pfu AdGFP-E4 and $10^{10}$ pfu AdGFP-E4–202, respectively. The average total plasma apoE levels, based on a pool of plasma of five mice, were 315 $\mu$g/ml for apoE4 and 13 $\mu$g/ml for apoE4–202. The apparent lower concentration of apoE4–202 in plasma and in the lipoprotein-containing fractions most likely reflects the efficient catabolism of the apoE4–202-containing lipoproteins.

**Wild-type ApoE4 Increases Significantly the Rate of Hepatic VLDL-triglyceride Production as Compared to the Truncated Form ApoE4–202 and the Control AdGFP Virus—**The rate of VLDL-triglyceride secretion in the plasma was determined following injection of Triton WR1339 5 days after the infection with the recombinant adenoviruses. It was found that the rate of triglyceride secretion decreased −50% in mice infected with $1 \times 10^{10}$ pfu of AdGFP-E4–202 and increased 10-fold in mice infected with $2 \times 10^9$ pfu of AdGFP-E4, as compared with mice infected with $1 \times 10^{10}$ pfu of the control AdGFP adenovirus (Fig. 6). The findings suggest that the carboxyl-terminal region of apoE influences the rate of VLDL-triglyceride secretion and contributes to apoE-induced hypertriglyceridemia.

**DISCUSSION**

ApoE promotes receptor-mediated catabolism of apoE-containing lipoproteins by cell receptors (1–5). Early studies showed that mutation in apoE within the vicinity of amino acids 130–160 affected the recognition of this protein by the LDL receptor and resulted in dominant or recessive forms of type III hyperlipoproteinemia and premature atherosclerosis (6–13, 47–49). Two additional functions of apoE pertinent to triglyceride homeostasis were suggested by the analysis of human subjects and transgenic animals expressing different levels of apoE. The first function of apoE is related to secretion of VLDL-triglycerides (35, 39, 40, 50), and the second is related to the inhibition of lipolysis (25, 27, 35–38, 51). Both processes are expected to increase the plasma triglyceride levels in humans and in experimental animals (25–27, 35–38, 51). To dissect these apoE functions, we have used adenovirus-mediated gene transfer to express at similar levels either the full-length apoE4 or the truncated apoE4–202 form in apoE-deficient mice. The mice were analyzed 4–8 days post-infection for apoE expression, plasma lipid and lipoprotein profiles, and mechanisms that impede the clearance of plasma cholesterol and triglycerides when the full-length apoE4 is overexpressed.

**The Amino-terminal Segment 1–202 of ApoE Can Associate with Lipoproteins and Direct Their Catabolism in Vivo**

Receptor-mediated catabolism of apoE requires association of apoE with lipoprotein particles, whereas lipid-free apoE does not bind to lipoprotein receptors (1–6). Heparan sulfate proteoglycans may also be involved in apoE binding on the cell surface (52–55). Domains of apoE involved in receptor binding (47–49, 56), heparin binding (57–58), and lipid and lipoprotein binding (59, 60) have been identified in vitro. The receptor binding domain is found between residues 136 and 152, whereas neighboring residues may also indirectly affect receptor binding (48, 49). The amino acids 142–147 of the receptor binding domain are also involved in heparin binding. Two other heparin binding domains were found between residues 211 and 218 and 243 and 272 (57, 58). It has been proposed that binding of apoE-containing lipoproteins to heparan sulfate proteoglycans contributes to their subsequent internalization with or without the participation of LRP (2, 6, 55). Other studies suggested that the region of apoE between residues 244 and 299 contributes to the binding of apoE to lipids and lipoproteins, whereas the amino-terminal region of apoE lacks the determinants required for association with lipoproteins (60). In addi-
C-terminal Domain of ApoE Contributes to Hypertriglyceridemia

Fig. 4. A and B, FPLC profiles of cholesterol (A) and triglycerides (B) of mice infected with apoE4- or apoE4–202-expressing adenoviruses. Serum samples obtained from mice infected with $2 \times 10^5$ of the control virus AdGFP, or the recombinant adenoviruses expressing AdGFP-E4 or AdGFP-E4–202 on days 5 and 8 post-infection, were fractionated by FPLC, and then the cholesterol and triglycerides levels of each FPLC fraction were determined as described under “Experimental Procedures.”
tion, residues 267–299 were found to be important for the tetramerization of apoE (60).

The present study refines the domains of apoE required for its association with lipoproteins, a process required for their in vivo clearance. Our findings establish that the amino-terminal residues 1–202 of apoE contain the domains necessary for the clearance of cholesteryl ester-rich lipoprotein remnants in vivo. This implies that the region 1–202 of apoE contains the necessary determinants for the association of apoE with these lipoprotein remnants in vitro. This conclusion is supported by in vitro data in the present study, showing that apoE4 and apoE4–202 remain associated with VLDL and LDL particles following ultracentrifugation. The clearance of lipoprotein remnants by apoE4–202, which contains only the 142–147 heparin binding domains, is extremely efficient. Previous studies showed that the 243–272 heparin binding domain of apoE is shielded when apoE is bound to lipids and lipoproteins and thus is inactive as a ligand for heparan sulfate proteoglycans (57). It is possible that the major route of clearance of the lipoproteins containing apoE4–202, which contains only the 142–147 heparin binding domain, is via the LDL receptor and LRP pathways without the involvement of the heparan sulfate proteoglycan pathway. The potential participation of the LRP and the LDL receptor in the clearance of apoE4–202-containing remnants may make their uptake and the subsequent cholesterol clearance more efficient and thus may account for the observed efficiency of apoE4–202 in cholesterol clearance. This hypothesis is currently under investigation.

The Carboxyl-terminal Domain of ApoE Contributes to Hypertriglyceridemia

A very important finding of this study is that removal of the carboxyl-terminal region 203–299 of apoE4 prevents the development of hypertriglyceridemia following adenoviral infection. Several control experiments were performed to confirm that the different properties of apoE4 and apoE4–202 are not the result of differences in expression, secretion, or association with VLDL. Northern blot analysis of total RNA has established unequivocally that the steady-state apoE mRNA levels in mice overexpressing apoE4 that result in hypertriglyceridemia and the mRNA levels in mice overexpressing apoE4–202 that do not cause hypertriglyceridemia are very similar (Fig. 3, A–D). This analysis indicates that it is unlikely that decreased expression of the truncated apoE forms is responsible for this effect. In addition, cell culture experiments showed that C127 (mouse mammary tumor) cell lines stably transfected and expressing apoE4 or apoE4–202 (data not shown), or HTB-13 cell cultures infected with recombinant adenovirus, secrete similar amounts of apoE4 and apoE4–202. These observations indicate that the truncated apoE4–202 form is stable and is secreted as efficiently as its wild-type apoE4 counterpart.
Mechanisms of ApoE-induced Hypertriglyceridemia

Aside from differences in synthesis, secretion, and VLDL association between apoE4 and apoE4–202, other factors that could have contributed to the apoE-induced hypertriglyceridemia are increased hepatic VLDL-triglyceride synthesis, decreased catabolism of apoE4 as compared with apoE4–202-containing particles, and a combination of both processes.

Increased Hepatic VLDL-Triglyceride Secretion Promoted by the Carboxyl-terminal 203–299 Residues of ApoE4—The present study shows that the hepatic VLDL-triglyceride secretion, following injection of Triton WR1339, decreases by 50% in mice expressing apoE4–202 and increases 10-fold in mice expressing apoE4, as compared with the mice infected with the control virus AdGFP (Fig. 6). This implies that apoE4 contributes to hypertriglyceridemia, at least partially, by increasing VLDL-triglyceride secretion. The hypertriglyceridemic VLDL may be a poor ligand for apoE-recognition receptors, despite the fact that it is enriched with apoE. A previous study also showed that VLDL-triglyceride secretion is reduced by 50% in E−/− mice (40). The findings imply that the carboxyl-terminal residues 203–299 of apoE may be directly involved in intracellular assembly and secretion of VLDL-triglycerides.

Increased VLDL Catabolism Is Mediated by Truncated ApoE Forms and Impediment of Triglyceride Hydrolisis Is Caused by Overexpression of ApoE—Work by others (60) has shown that injection of two 125I-truncated apoE forms extending from residues 203–299 of apoE is directly involved in intracellular assembly and secretion of VLDL-triglycerides.

Aside from differences in synthesis, secretion, and VLDL association between apoE4 and apoE4–202, other factors that could have contributed to the apoE-induced hypertriglyceridemia are increased hepatic VLDL-triglyceride synthesis, decreased catabolism of apoE4 as compared with apoE4–202-containing particles, and a combination of both processes.

Expression of apoE within a physiological range clears lipoprotein remnants, whereas overexpression results in hypertriglyceridemia. The undesirable side effect resulting from apoE overexpression diminishes significantly the therapeutic value of apoE. The inability of the truncated apoE form that lacks the carboxyl-terminal 203–299 region to induce hypertriglyceridemia, as described in this research, coupled with its intact ability to clear cholesterol, makes it an attractive candidate in future gene therapy approaches to correct remnant removal disorders.

Acknowledgments—We thank Markella Zanni for editorial suggestions and comments, Anne Flunkett for secretarial assistance, and Pamela Moran and Hans van der Boom for technical assistance.

REFERENCES

1. Innerarity, T. L., and Mahley, R. W. (1978) Biochemistry 17, 1440–1447
2. Herz, J., and Willnow, T. E. (1995)Curr. Opin. Lipidol. 6, 97–103
3. Wolf, B. B., Lopes, M. B., VandenBerg, S. R., and Gionis, S. L. (1992)Am. J. Pathol. 141, 37–42
4. Kim, D.-H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H.-J., Suzuki, H., Kondo, H., Sueki, S., and Yamamoto, T. (1996) J. Biol. Chem. 271, 8373–8380
5. Takahashi, S., Kawarabayasi, Y., Nakai, T., Sakai, J., and Yamamoto, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9252–9256
6. Mahley, R. W., and Huang, Y. (1999)Curr. Opin. Lipidol. 10, 207–217
7. Dong, L.-M., Parkin, S., Trakhanov, S. D., Rupp, B., Simmons, T., Arnold, K. S., Newhouse, Y. M., Innerarity, T. L., and Weisgraber, K. H. (1996)Nat. Struct. Biol. 3, 718–722
8. Wardell, M. R., Brennan, S. O., Janus, E. D., Fraser, R., and Carrell, R. W. (1987) J. Clin. Invest. 80, 483–490
9. Rall, S. C., Jr., Newhouse, Y. M., Clarke, H. R., Goege, Weisgraber, K. H., McCarthy, B. J., Mahley, R. W., and Bernst, P. F. (1989)J. Clin. Invest. 83, 1095–1101
10. Mann, W. A., Gregg, R. E., Sprecher, D. L., and Brewer, H. B., Jr. (1989)Biochem. Biophys. Acta 1005, 239–244
11. Wardell, M. R., Weisgraber, K. H., Havekes, L. M., and Rall, S. C. Jr. (1989)J. Biol. Chem. 264, 21205–21210
12. van den Maagdenberg, A. M. de Knijff, P., Stalenhoef, A. P., Gevers Leuven, J. A., Havekes, L. M. and Frants, R. R. (1989)Biochem. Biophys. Res. Commun. 165, 851–857
13. Smit, M. P., de Knijff, P., van der Kooij-Meijis, E., Groeneveld, C., van den Maagdenberg, A. M., Gevers Leuven, J. A., Stalenhoef, A. P., Stuyt, P. M., Frants, R. R., and Havekes, L. M. (1990)J. Lipid Res. 31, 45–53
14. Gihselli, G., Schaefer, E. J., Gascon, P., Brewer, H. B., Jr. (1981)Science 214, 1239–1241
15. Schaefer, E. J., Gregg, R. E., Gihselli, G., Forte, T. M., Ordovas, J. M., Zech, L. A., and Brewer, H. B. (1986)J. Clin. Invest. 78, 1206–1219
16. Clarasus, C., Hadjopoulo-Clarasus, M., Felker, B. K., Pavlikis, G., and Zannis, V. I. (1987)J. Clin. Invest. 79, 2310–2315
17. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setala, K., Walsch, A., Verstayft, J. D., Rubin, E. M., and Breslow, J. L. (1992)Cell 71, 343–353
18. Zhang, S. H., Reddick, R. L., Piedrahita, J. A., and Maagdenberg, A. M., Gevers Leuven, J. A., Havekes, L. M. and Frants, R. R. (1989)Biochem. Biophys. Res. Commun. 165, 851–857
19. Reddick, R. L., Zang, S. H., and Maeda, N. (1994)Arterioscler. Thromb. Vasc. Biol. 14, 141–147
20. Van den Maagdenberg, A. M. J., Hofker, M. H., Krimpenfort, P. J. A., DeBruijn, I., Van Vlijmen B., van der Boom, H., Havekes, L. M., and Frants, R. R. (1993)J. Biol. Chem. 268, 10540–10545
21. Fazio, S., Lee, Y., Sheng, X., and Rall, S. C. Jr., (1993)J. Clin. Invest. 92, 1497–1503
22. Fazio, S., Horie, Y., Simonet, W. S., Weisgraber, K. H., Taylor, J. M., and Rall, S. C. Jr., (1994)J. Lipid Res. 35, 408–416
C-terminal Domain of ApoE Contributes to Hypertriglyceridemia

23. Fazio, S., Sanan, D. A., Lee, Y.-L., Ji, Z.-S., Mahley, R. W., and Rall, S. C., Jr. (1994) Arterioscler. Thromb. 14, 1873–1879
24. van Vlijmen, B. J., van Dijk, K. W., van't Hof, H. B., van Gorp, P. J., van der Zee, A., van der Boom, H., Breuer, M. L., Hofker, M. H., and Havekes, L. M. (1996) J. Biol. Chem. 271, 30595–30602
25. Cohn, J. S., Tremblay, M., Amisl, M., Bouthillier, D., Roy, M., and Genes, J. Jr. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 149–159
26. Chait, A., Hazzard, W. R., Albers, J. J., Kishwaha, R. P., and Brunzell, J. D. (1979) Metabolism 28, 1055–1066
27. Ehnholm, C., Mahley, R. W., Chappell, D. A., Weisgraber, K. H., Ludwig, E., and Witzlum, J. L. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5566–5570
28. Huang, Y., von Eckardstein, A., Wu, S., Maeda, N., and Assmann, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1834–1838
29. Huang, Y., Ji, Z.-S., Brecht, W. J., Rall, S. C., Jr., Taylor, J. M., and Mahley, R. W. (1998) J. Biol. Chem. 273, 26388–26393
30. Kulpers, F., Jong, M. C., Lin, Y., van Eck, M., Havinga, R., and Bloke, V. (1997) J. Clin. Invest. 100, 2915–2922
31. Aleshkov, S. A., Abraham, C. R., and Zannis, V. I. (1998) Biochemistry 36, 10571–10580
32. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2509–2514
33. Shimano, H., Ohsuga, J., Shimada, M., Namba, Y., Gotoda, T., Harada, K., Katsuki, M., Yazaki, Y., and Yamada, N. (1995) J. Lipid Res. 36, 583–592
34. Dong, L. M., Wilson, C., Wardell, M. R., Simmons, T., Mahley, R. W., and Agard, D. A. (1994) J. Biol. Chem. 268, 12348–12354
35. Levanon, A. Z., Boyles, J. K., Amid, B., Gorecki, M., and Mahley, R. W. (1983) J. Biol. Chem. 258, 14791–14799
36. Katsuki, M., Yazaki, Y., and Yamada, N. (1995) J. Biol. Chem. 270, 17483–17490
37. Rensen, P. C. N., and van Berkel, T. J. C. (1998) J. Biol. Chem. 273, 17483–17490
38. Rall, S. C., Jr., Weisgraber, K. H., Innerarity, T. L., and Mahley, R. W. (1982) J. Biol. Chem. 257, 258, 12756–12763
39. Wilson, C., Mau, T., Weisgraber, K. H., Wardell, M. R., Mahley, R. W., and Agard, D. A. (1994) Science 262, 1817–1822