Increased response to cholesterol feeding in apolipoprotein C1-deficient mice

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The function of apolipoprotein (apo) C1 *in vivo* is not well understood. From *in vitro* studies it has been reported that an excess of apoC1 relative to apoE inhibits receptor-mediated uptake of remnant lipoproteins [Sehayek and Eisenberg (1991) J. Biol. Chem. **266**, 22453–22459]. In order to gain a better understanding of the role of apoC1 in lipoprotein metabolism *in vivo*, we have generated apoC1-deficient mice by gene targeting in embryonic stem cells. Homozygous mutant mice are viable and do not show overt abnormalities. Serum triacylglycerol levels are increased by 60% on both a standard mouse diet and a mild hypercholesterolaemic diet compared with controls. Total serum cholesterol levels are similar to controls on the two diets. However, the level of high-density lipoprotein cholesterol in the apoC1-deficient mice fed on the mild hypercholesterolaemic diet is slightly decreased, which is accompanied by a 3-fold increase in very-low-density plus low-density lipoprotein (VLDL + LDL) cholesterol. On a severe atherogenic diet, the homozygous apoC1-deficient mice become hypercholesterolaemic, with a serum cholesterol level of 10.7 ± 3.3 mM compared with 6.7 ± 1.8 mM and 5.1 ± 1.6 mM in heterozygous and control mice respectively. The increase in cholesterol is mainly confined to the VLDL + LDL-sized fractions. Binding experiments revealed that lipoproteins lacking apoC1 with $d < 1.006$ g/ml are poor competitors for ^{125}I-labelled LDL binding to the LDL receptor on HepG2 cells. This suggests that total apoC1 deficiency leads to impaired receptor-mediated clearance of remnant lipoproteins rather than enhanced uptake, as was expected from data reported in the literature.

INTRODUCTION

The human APOC1 gene is located within a gene cluster on chromosome 19q together with APOE, pseudo-APOC1 and APOC2 [1]. Apolipoprotein (apo) C1 is a polypeptide of 57 amino acid residues with a calculated molecular mass of 6.6 kDa [2]. cDNA analysis has indicated that apoC1 is synthesized with a 26-residue signal peptide which is cleaved from the protein during intracellular processing [3]. The major site of production is the liver [4]. In plasma, the concentration of apoC1 is about 6 mg/dl and it principally resides on chylomicrons, very-low-density lipoproteins (VLDLs) and high-density lipoproteins (HDLs) [5].

Although the function of apoC1 *in vivo* is not well understood, *in vitro* studies have shown that it is able to activate the enzyme lecithin–cholesterol acyltransferase [6]. Furthermore, it can block the apoE-mediated binding of apoE-enriched β-VLDLs to the low-density lipoprotein (LDL) receptor [7] and to the LDL receptor-related protein (LRP) [8]. Inhibition of binding by apoC1 can be due to either displacement of apoE from the remnant particle or interaction of apoC1 with apoE on the lipoprotein surface. In summary, these studies suggest that hepatic uptake of remnants is governed by a balance between the amounts of apoE and apoC1 on the lipoprotein particle.

To identify elements controlling the tissue-specific expression of the APOE–APOC1 gene cluster, several transgenic mouse lines from overlapping human APOE and APOC1 genomic fragments have been generated [9]. An APOC1 gene construct containing the entire region between the APOC1 gene and the APOC1′ pseudogene was expressed at high levels in liver of transgenic mice. These animals exhibited slightly elevated plasma cholesterol levels, whereas plasma triacylglycerols were 2- to 3-fold higher than in control mice. In contrast, transgenic mice expressing both the human APOE and APOC1 genes had normal plasma lipid levels. These results are in agreement with *in vitro* experiments mentioned above, showing that an excess of apoC1 relative to apoE on the lipoprotein particle inhibits the binding of these particles to lipoprotein receptors [7,8,10].

Taken together, all insights into the function of apoC1 so far suggest a modulating role of apoC1 in lipoprotein metabolism. A more direct function remains unclear. To date, no impairment of lipoprotein metabolism has been identified that is known to be associated with mutation in the APOC1 gene. Therefore, to clarify the metabolic role of apoC1, we decided to generate apoC1-deficient mice by gene targeting in mouse embryonic stem (ES) cells. Homozygous null mutants are viable and show slightly elevated triacylglycerol levels in animals fed on both a standard mouse diet and a mild hypercholesterolaemic diet. However, on a severe atherogenic diet, the apoC1-deficient mice develop hypercholesterolaemia compared with controls. An accumulation of VLDL + LDL-sized particles is observed on this diet, which could be explained by the decreased binding efficiency of mutant $d < 1.006$ g/ml lipoproteins to the LDL receptor. This suggests that complete apoC1 deficiency leads to impaired receptor-

Abbreviations used: apo, apolipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LRP, LDL receptor-related protein; ES, embryonic stem; hygroB^r, hygromycin B resistance gene; HSV-*tk*, herpes simplex virus thymidine kinase; FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSA, human serum albumin; DMEM, Dulbecco's modified Eagle's medium; HFC diet, mild high-fat/cholesterol diet; HFC0.5% diet, severe high-fat/cholesterol diet.

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mediated clearance of remnant lipoproteins rather than enhanced uptake, as was expected from data reported in the literature.

MATERIALS AND METHODS

Construction of targeting vector

A replacement-type targeting vector was derived from an 8.8 kb *Eco*RI fragment spanning the entire *Apoc1* gene, subcloned from an *Apoe–c1–c2* cluster carrying 129 Sv/Ev mouse cosmid clone [11]. It was designed to disrupt the endogenous *Apoc1* gene by replacing a 0.7 kb *Bam*HI–*Sam*I fragment, containing exons 1 and 2 (including the translational start site [12]) and part of exon 3, with a 2.0 kb hygromycin B-resistance (hygroB^r) gene [13]. The resulting construct had segments of 3.3 and 4.8 kb with 5′ and 3′ homology to the endogenous *Apoc1* locus. A herpes simplex virus thymidine kinase (*HSV-tk*) gene [14] was placed at the 3′ end of the vector, to enable the application of a positive–negative-selection strategy [15] (Figure 1). Both selectable genes were placed in the same transcriptional orientation as the *Apoc1* gene.

ES cell culture and transfection

E14 ES cells [16], kindly provided by Dr. Plump, Rockefeller University, New York, NY, U.S.A., were cultured and subsequently selected on hygroB^r mouse embryonic fibroblasts derived from mice deficient in muscle creatine kinase [17], as described by Robertson [18]. Batches of about 4×10^6–8×10^6 ES cells were electroporated in 0.4 ml of electroporation buffer (10 mM potassium phosphate, pH 7.2, 0.25 M sucrose, 1 mM $MgCl_2$, 200 μg/ml BSA) in the presence of 10 μg/ml linearized vector DNA at 4.0 kV/cm in a TA750 transfection apparatus (Krüss G.m.b.H., Hamburg, Germany). Selection with 250 μg/ml hygromycin B (ICN Biochemicals, Cleveland, OH, U.S.A.) and 0.2 μM (1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil) (FIAU) (Bristol Myers, New York, NY, U.S.A.) in ES cell culture medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM 2-mercaptoethanol] was applied 24 h after transfection. To determine the enrichment factor of the negative selection, a few plates were cultured with selection medium without FIAU. Individual double-resistant colonies were picked in 96-well plates at day 10 and expanded to 24-well plates. Each clone in a confluent well was split into two equal parts for storage and Southern-blot analysis.

Generation of chimaeric and apoC1-deficient mice

Targeted clones were injected into C57BL/6 recipient blastocysts, and embryos were transferred to the uterine horns of (C57BL/6 × CBA/Ca)F1 pseudopregnant females [19]. The extent of chimaerism was determined by the degree of agouti coat colour contribution. Male chimaeras were mated with C57BL/6 females, and germline transmission was scored by the presence of agouti fur in the offspring. Transmission of the apoC1 mutation was assessed by genomic Southern-blot analysis of tail-tip DNA. Heterozygous mutants were interbred to obtain homozygous apoC1-deficient mice. All mice used in this study were littermates derived from matings of heterozygotes, and were 9–13 weeks of age at the onset of the study. Animals were bred and housed under standard conditions in the Transgenic Animal Facilities of the Central Animal Laboratory of the Medical Faculty, Nijmegen University.

Genomic Southern-blot analysis

ES cells were lysed in 0.5 ml of 0.5 M Tris/HCl, pH 9.0, containing 20 mM EDTA, 10 mM NaCl, 1% SDS and 100 μg/ml proteinase K at 55 °C overnight. DNA was purified by phenol extraction and ethanol precipitation. Tail-tip DNA was prepared as previously described [20].

Approx. 5 μg portions of DNA were digested with *Hin*dIII, and DNA fragments were resolved on 0.7% (w/v) agarose gels, transferred to Biotrace HP nylon membranes (Gelman Sciences, Ann Arbor, MI, U.S.A.) and hybridized to probe A, which is located 5′ to the targeting vector (see Figure 1). Genomic probes were isolated by random subcloning of a *Sau*3A-digested cosmid, carrying the *Apoe–c1–c2* cluster.

Northern-blot analysis

Total RNA was isolated from liver using the RNAZOL procedure (Cinna/Biotecx, Houston, TX, U.S.A.). RNA samples (10 μg per lane) were size-separated by electrophoresis through a denaturing agarose gel (1.2%, w/v) containing 7.5% formaldehyde and transferred to a nylon membrane (Hybond-N$^+$; Amersham, Bucks., U.K.) according to the manufacturer's recommendations. Blots were subsequently hybridized with a ^{32}P-labelled mouse *Apoc1* cDNA probe (derived from mAPOC1c16, see ref. [12]) and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe at 55 °C in a solution containing 55% formamide [21]. The intensity of the hybridization signal was quantified with a Phosphor Imager (Molecular Dynamics), using the software program Imagequant (Molecular Dynamics). The level of expression in the heterozygotes and homozygotes was compared with wild-types and related to the level of internal standard (GAPDH).

Lipoprotein isolation

The individual lipoprotein fractions (VLDL, $d < 1.006$; intermediate-density lipoprotein + LDL, $d = 1.006$–1.063; HDL, $d < 1.063$–1.21 g/ml) were isolated from pooled serum, composed of sera of at least nine starved mice per group. Isolation was achieved by sequential ultracentrifugation at the respective densities at 84000 *g* (40000 rev./min) overnight, using a Ti-50 fixed-angle rotor (Beckman, Geneva, Switzerland), followed by dialysis at 4 °C overnight against PBS, pH 7.4. The amount of protein was determined by the method of Lowry et al. [22]. Human LDLs were isolated as described by Redgrave et al. [23].

Western-blot analysis

From each lipoprotein fraction, samples of 5 μg of protein were analysed by SDS/PAGE using 4–20% gradient gels. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) followed by incubation with polyclonal rabbit anti-(mouse apoA1), -apoC1, -apoC3 or -apoE (kindly provided by Dr. Weisgraber, Gladstone Foundation Laboratories for Cardiovascular Disease, San Francisco, CA, U.S.A.). Goat anti-rabbit IgG conjugated to peroxidase (Nordic Immunology, Tilburg, The Netherlands) was used as secondary antibody, and detection was by the immunoperoxidase procedure using 4-chloro-1-naphthol as substrate.

Analysis of lipid and lipoprotein

After an overnight period without food (16–17 h), approx. 200 μl of whole blood was obtained from each mouse via tail bleeding. Levels of total serum cholesterol and triacylglycerol (without

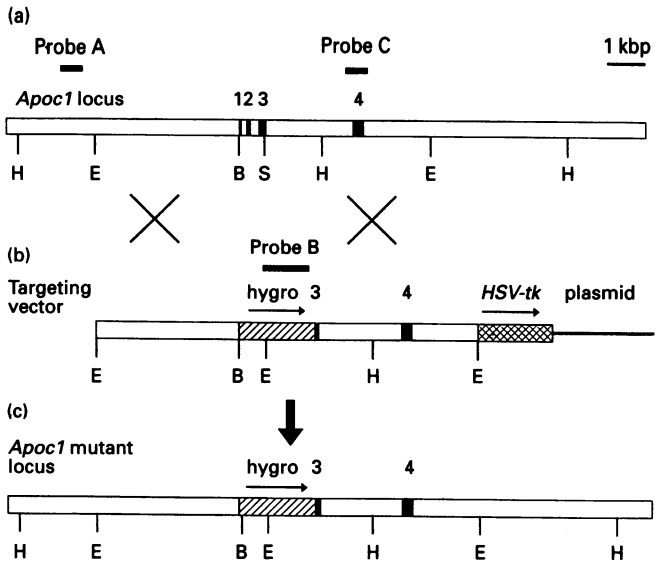

Figure 1 Strategy for the disruption of the mouse *Apoc1* gene

Schematic diagram showing (**a**) the structure of the endogenous *Apoc1* locus, (**b**) the targeting vector and (**c**) the predicted structure of the *Apoc1* locus after homologous recombination. Numbers 1–4 and the closed boxes denote exon sequences, and the bars indicate the positions of probes A, B and C. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

measuring free glycerol) were determined using Boehringer-Mannheim enzymic assay kit no. 236691 and Sigma GPO-Trinder kit no. 337-B respectively. Triacylglycerols, free cholesterol and phospholipids in the individual lipoprotein fractions were measured with Boehringer-Mannheim enzymic assay kits nos. 701904 and 310328 and an analytical kit (B) from Wako Chemicals G.m.b.H. (Neuss, Germany) respectively. Cholesterol esters are total cholesterol minus free cholesterol.

For f.p.l.c. size fractionation of lipoproteins, 200 μl of pooled serum from at least nine starved mice per group was injected on to a 25 ml of Superose 6 preparation-grade column (Pharmacia, Uppsala, Sweden), and eluted at a constant flow rate of 0.5 ml/min with PBS, pH 7.4. The effluent was collected in 0.5 ml fractions, and cholesterol and triacylglycerol concentrations were measured enzymically in each fraction, as described above. Lipoproteins were identified on the basis of the elution profile of human serum lipoproteins. The relative distribution of cholesterol among the VLDL + LDL- and the HDL-sized fractions was calculated from the area under the curve in f.p.l.c. fractions 14–23 and 24–33 respectively.

To evaluate the size distribution of VLDL-sized particles more specifically, 200 μl of pooled serum from at least nine starved mice per group was injected on to a Bio-Gel A 150 m column (operating range 10^6 to 150×10^6 kDa) (Bio-Rad, Richmond, CA, U.S.A.), and eluted at a constant flow rate of 0.1 ml/min with PBS, pH 7.4.

Labelling of human LDL with ^{125}I

Immediately after isolation, human LDLs were labelled by the ^{125}ICl method as described by Bilheimer et al. [24], followed by dialysis against PBS (4×500 ml) at 4 °C overnight. They were then stabilized by adding human serum albumin (HSA) (1%, w/v). The ^{125}I-labelled LDLs were stored at 4 °C, and used within 1 week. Specific radioactivity was about 200 c.p.m/ng of lipoprotein protein. When not labelled with ^{125}I, lipoproteins were stabilized immediately by the addition of 1% HSA and then extensively dialysed against culture medium [DMEM supplemented with 20 mM Hepes buffer (pH 7.4) and 10 mM $NaHCO_3$].

Measurement of competition of lipoproteins with ^{125}I-labelled LDL for binding to HepG2 cells

HepG2 cells were cultured in 24-well plates as previously described [25]. About 24 h before the start of the experiment, DMEM supplemented with 1% HSA instead of fetal calf serum was added to the cells. Competition experiments were performed by incubating HepG2 cells for a period of 4 h at 0 °C with ^{125}I-labelled LDL (10 μg/ml of protein) in the presence or absence of increasing amounts of unlabelled lipoproteins, as indicated. After removal of the medium, cells were washed three times with ice-cold PBS containing 0.1% (w/v) BSA, followed by one wash with PBS without BSA. Cells were then dissolved in 1 ml of 0.2 M NaOH. Protein content was measured by the method of Lowry et al. [22]. The radioactivity in a portion of the sample represents the binding.

Diets

Mice were given free access to water and food. Before starting the dietary treatment, mice were fed a regular breeding chow diet (RMH-B) containing 6.2% fat. The two semisynthetic diets were made up essentially as described by Nishina and co-workers [26], and were purchased from Hope Farms, Woerden, The Netherlands. Between 2 and 3 months of age, mice were put on a mild high-fat/cholesterol (HFC) diet for 3 weeks. This diet contained 15% cocoa butter, 0.25% cholesterol, 40.5% sucrose, 10% cornstarch, 1% corn oil and 6% cellulose. Afterwards, they were fed a severe high-fat/cholesterol diet (HFC0.5%) for at least 3 weeks, containing 15% cocoa butter, 1% cholesterol, 0.5% cholate, 40.5% sucrose, 10% cornstarch, 1% corn oil and 4.7% cellulose (all percentages are by weight).

RESULTS

Generation of apoC1-deficient mice

A replacement-type targeting vector was prepared as described in the Materials and methods section, and the selection strategy is shown in Figure 1. This construct was transfected into E14 ES cells [16]. Targeted clones were identified by hybridizing Southern blots of *Hin*dIII-digested DNA with probe A, which is located just upstream of the *Eco*RI site flanking the 5′ border of the targeting construct (Figure 1). Correctly targeted clones, yielding a new diagnostic 9.3 kb fragment together with the endogenous 8.0 kb band (Figure 2a), were observed at fairly high frequency (about 1:5, with an enrichment factor of approx. 6 gained by anti-*tk* selection). To obtain further evidence that the targeting vector had integrated via a genuine homologous recombination event, *Hin*dIII- and *Eco*RI-digested blots were also hybridized with a hygroB^r-specific probe (B) and a probe (C) in the 3′ arm of the targeting construct (see Figure 1; blotting results not shown). No abnormal targeting events were scored.

A total of eight targeted E14 ES cell lines were injected into C57BL/6 host blastocysts, and embryos were reimplanted into foster mothers. Chimaeric males were bred to C57BL/6 females, and males derived from one clone were found to transmit the mutation through the germline. Heterozygous mutants were interbred to generate mice deficient in apoC1 with a mixed C57BL/6 and 129/Ola background. Figure 2(b) shows an example of the genotype of these animals. The mutation segregated in a completely normal Mendelian fashion: from a

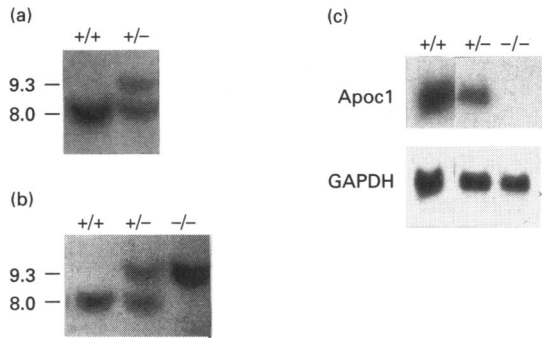

Figure 2 Identification of targeted ES cells and apoC1-deficient mice

Southern-blot analysis of genomic DNA isolated from ES cells (**a**) and mouse tail tips (**b**) digested with *Hin*dIII and hybridized with probe A (see Figure 1). DNA size (kb) is indicated. (**c**) Northern-blot analysis of 10 μg of RNA per lane prepared from mouse livers hybridized with an *apoc1* cDNA probe and a GAPDH cDNA probe. +/+, normal ES cell DNA or control mice; +/−, targeted ES cell DNA or heterozygous apoC1-deficient mice; −/−, homozygous apoC1-deficient mice.

total of 98 offspring, 20 were wild-types, 51 heterozygotes and 27 null mutants.

To confirm that the targeted mutation had indeed generated the desired apoC1 deficiency, a Northern-blot analysis of RNA prepared from liver was hybridized with a mouse *Apoc1* cDNA probe. No *Apoc1* mRNA was detectable in homozygous apoC1-deficient mice, and heterozygotes had a reduced level of expression compared with controls (Figure 2c).

Alteration of serum lipid levels in heterozygous and homozygous apoC1-deficient mice in response to diet

Total serum triacylglycerols were slightly increased in heterozygous and homozygous apoC1-deficient mice on a chow diet, whereas serum cholesterol levels did not show any significant change (Table 1). The increase in triacylglycerols was found in the VLDL + LDL-sized fractions, as determined by f.p.l.c. analysis (Figure 3a). The distribution of cholesterol among the VLDL + LDL- and HDL-sized fractions is presented in Table 1, and appears to be comparable for all groups on a chow diet.

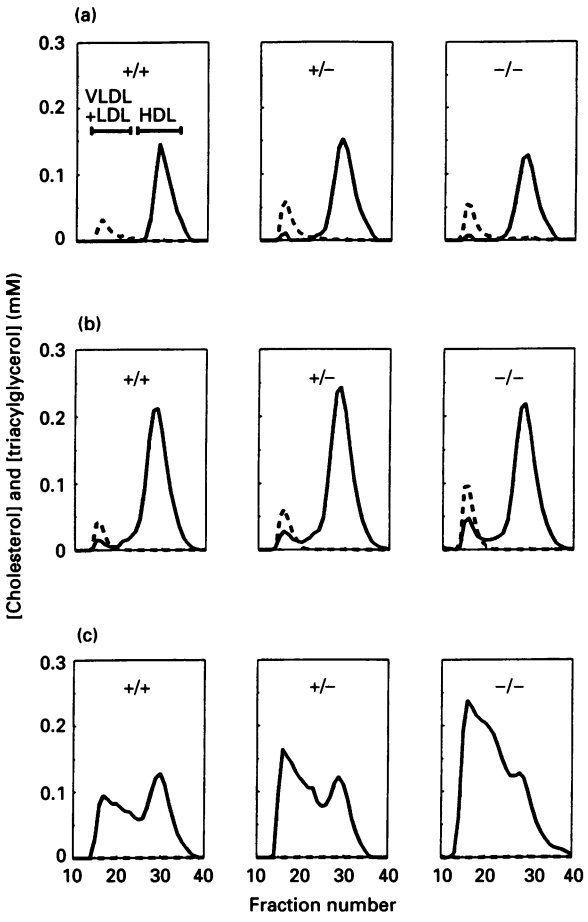

Figure 3 Lipoprotein profiles of apoC1-deficient and control mice on chow and two different hypercholesterolaemic diets

Sera from at least nine starved controls, heterozygous and homozygous apoC1-deficient mice were pooled and separated on the basis of size by f.p.l.c. Mice had been fed on a standard mouse chow (**a**), a mild hypercholesterolaemic diet (HFC, containing 0.25% cholesterol) (**b**) or a severe hypercholesterolaemic diet (HFC0.5%, containing 1% cholesterol and 0.5% cholate) (**c**). Fractions 14–23 represent VLDL + LDL and fractions 24–33 represent HDL (based on the elution profile of human serum lipoproteins). ——, Cholesterol; – – – –, triacylglycerol. On the HFC0.5% diet, triacylglycerol levels were too low to be detected.

Table 1 Serum cholesterol and triacylglycerol levels in apoC1-deficient mice

Results in parentheses are percentage of total serum cholesterol. +/+, Control mice; +/−, heterozygous apoC1-deficient mice; −/−, homozygous apoC1-deficient mice; HFC, mild high-fat/cholesterol diet with 0.25% cholesterol; HFC0.5%, severe high-fat/cholesterol diet, containing 1% cholesterol and 0.5% cholate (see the Materials and methods section); N.D., not detectable. *$P < 0.05$ compared with +/+ mice on the same diet using the non-parametric Mann–Whitney U test; †$P < 0.05$ compared with +/− mice on the same diet using non-parametric Mann–Whitney U test.

Genotype	No. of mice analysed	Diet	Serum lipid concentration (mM)		Lipoprotein cholesterol (mM)	
			Total cholesterol	Triacylglycerols	VLDL + LDL	HDL
+/+	10	Chow	3.0 ± 0.6	0.2 ± 0.1	0.0 (< 1)	3.0 (> 99)
+/−	10	Chow	3.0 ± 0.4	0.4 ± 0.1*	0.1 (3)	3.0 (97)
−/−	10	Chow	2.4 ± 0.3	0.4 ± 0.2*	0.1 (3)	2.3 (97)
+/+	10	HFC	3.9 ± 1.0	0.6 ± 0.4	0.3 (8)	3.6 (92)
+/−	10	HFC	3.9 ± 0.6	0.7 ± 0.3	0.5 (13)	3.4 (87)
−/−	9	HFC	4.0 ± 0.5	1.1 ± 0.4*†	0.6 (16)	3.4 (84)
+/+	10	HFC0.5%	5.1 ± .1.6	N.D.	2.2 (43)	2.9 (57)
+/−	10	HFC0.5%	6.7 ± 1.8	N.D.	3.8 (56)	2.9 (44)
−/−	9	HFC0.5%	10.7 ± 3.3*†	N.D.	6.8 (64)	3.9 (36)

Hardly any cholesterol was detectable in the VLDL-LDL-sized fractions.

To investigate the response of apoC1-deficient mice on a hypercholesterolaemic diet, mice were fed on two types of high-fat diet. The HFC and HFC0.5% diets were considered to be mildly and severely hypercholesterolaemic, respectively (see the Materials and methods section). The latter diet contained 0.5% cholate, which facilitates intestinal uptake of fat and cholesterol. After 3 weeks on the HFC diet, total serum levels of cholesterol and triacylglycerol were increased among all groups compared on the chow diet (Table 1). At this time, plasma lipid levels had reached a plateau level (not shown). Again, the serum triacylglycerols in the homozygotes were slightly elevated (1.6-fold) compared with wild-type mice. This increment was confined to the VLDL+LDL-sized fractions, as is shown by the f.p.l.c. patterns (Figure 3b). There was no significant difference in serum triacylglycerol level among controls and heterozygotes. Total cholesterol levels were similar for all groups on the HFC diet. However, on this diet the relative amount of cholesterol in the VLDL+LDL-sized fractions of the apoC1-deficient mice had doubled compared with wild-type animals (from 8 to 16%; see Table 1). Thus, in the absence of apoC1 protein, the HFC diet causes a shift from HDL cholesterol towards VLDL+LDL cholesterol.

When the animals were fed on the HFC0.5% diet, this shift was even more pronounced. Remarkably, serum cholesterol levels in homozygotes were then increased to 10.7 ± 3.3 mM compared with 6.7 ± 1.8 and 5.1 ± 1.6 mM in heterozygous and control mice respectively, whereas serum triacylglycerols had declined below detectable level (Table 1). Lowering of triacylglycerol levels concomitant with an increase in serum cholesterol in mice on a severe hypercholesterolaemic diet is a common phenomenon, and has previously been observed by us [27] and others [28,29]. The lipoprotein profile had changed for all animals fed on the HFC0.5% diet (Figure 3c). In homozygotes in particular a dramatic shift of cholesterol from HDL- to VLDL+LDL-sized particles occurred (64% compared with 43% in controls). This accumulation of VLDL+LDL-sized particles in homozygotes might even be underestimated, because the LDL and HDL fractions were not completely separated (Figure 3c). Thus the homozygous apoC1-deficient mice are more susceptible to a severe hypercholesterolaemic diet than heterozygous and wild-type mice, as demonstrated by an increased accumulation of VLDL+LDL-sized particles.

Table 2 Lipid composition of serum lipoproteins in apoC1-deficient mice

Lipid levels were measured in concentrated lipoprotein fractions, derived from pooled serum of at least nine starved mice per group on HFC0.5% diet, as described in the Materials and methods section. Because the samples are concentrated, triacylglycerols are now detectable (not detectable in Table 1). TG, Triacylglycerols; FC, free cholesterol; CE, cholesterol ester; PPL, phospholipids. +/+, control mice; +/−, heterozygous apoC1-deficient mice; −/−, homozygous apoC1-deficient mice.

Zygosity	Serum density fraction (g/ml)	Lipid composition (% of total by weight) TG	FC	CE	PPL	(PPL+FC)/(TG+CE) (w/w)
+/+	$d < 1.006$	26	5	60	8	0.16
+/+	$d < 1.006$–1.063	9	10	71	11	0.26
+/+	$d < 1.063$–1.21	12	4	64	19	0.31
+/−	$d < 1.006$	9	8	74	9	0.20
+/−	$d < 1.006$–1.063	6	10	72	12	0.28
+/−	$d < 1.063$–1.21	6	5	73	16	0.27*
−/−	$d < 1.006$	18	8	66	9	0.20
−/−	$d < 1.006$–1.063	6	12	70	12	0.31
−/−	$d < 1.063$–1.21	15	7	63	15	0.28*

* Low ratio may be due to contamination with LDL.

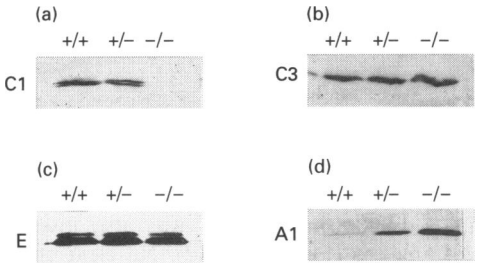

Figure 4 Western-blot analysis of mouse lipoproteins

Lipoproteins of $d < 1.006$ g/ml from controls (+/+), heterozygous (+/−) and homozygous (−/−) apoC1-deficient mice were isolated by ultracentrifugation and subjected to SDS/PAGE (4–20% gradient gels) and transferred to a nitrocellulose membrane (5 μg of protein per lane). The filter was incubated with polyclonal rabbit anti-(mouse apoC1) (**a**), anti-(mouse apoC3) (**b**), anti-(mouse apoE) (**c**) and anti-(mouse apoA1) (**d**) antibodies.

Effect of apoC1 deficiency on lipid composition of the individual lipoprotein fractions

To investigate whether the effect of apoC1 deficiency on serum lipid levels was accompanied by a change in the lipid composition of the different lipoprotein fractions, the respective lipoproteins were isolated by sequential ultracentrifugation. Lipoprotein particles from mice fed on the HFC0.5% diet were chosen because the striking hypercholesterolaemia in the apoC1-deficient mice was only observed on this diet. The relative lipid compositions of the individual lipoprotein fractions are shown in Table 2. The mass ratio of phospholipids+free cholesterol over triacylglycerols+cholesterol esters was calculated and taken as a measure of mean particle size. No clear difference was found between the ratios of the homozygous mice and the respective ratios observed for heterozygous and control mice fed on the HFC0.5% diet. VLDL and LDL particles isolated from wild-type mice on chow are, as expected, a lot richer in triacylglycerol and poorer in cholesterol than the particles that accumulate in mice on the HFC0.5% diet, and are smaller in size than after the HFC0.5% diet (results not shown).

Serum of mutant mice and controls was also applied to a Bio-Gel A 150 m column, which enables measurement of the size distribution of the VLDL fraction. No differences in size distribution between VLDL from homozygous, heterozygous and wild-type mice could be identified (results not shown).

Effects of apoC1 deficiency on apolipoprotein composition

To evaluate whether the effect of apoC1 deficiency could be explained by a change in apolipoprotein distribution, VLDLs ($d < 1.006$ g/ml) were isolated from starved heterozygous, homozygous and control animals fed on the HFC0.5% diet and subjected to SDS/PAGE. Western-blot analysis of $d < 1.006$ g/ml particles using an antibody against mouse apoC1, as expected, showed that apoC1 protein was completely absent from the homozygous mutant animals and was reduced in heterozygotes compared with control mice (Figure 4a). The effect of apoC1 deficiency on the distribution of apoC3, apoE and

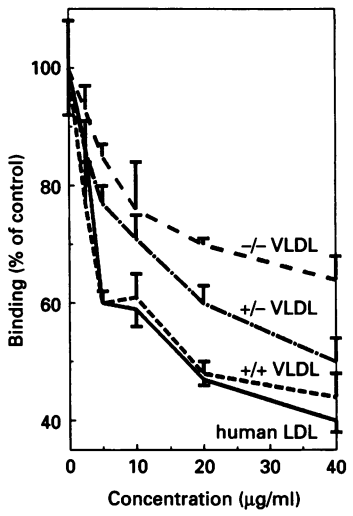

Figure 5 Competition by normal and apoC1-deficient VLDL and human LDL for ^{125}I-labelled LDL binding to HepG2 cells

HEPG2 cells were grown to confluence in 24-well plates. Measurement of the ability of VLDL ($d < 1.006$ g/ml) control (+/+; ----), heterozygous (+/−; —·—·) and homozygous (−/−; ——) apoC1-deficient mice as well as human LDL (——) to compete with ^{125}I-labelled human LDL for receptor binding to HepG2 cells is described in the Materials and methods section. Values representing binding as a percentage of the binding in the absence of unlabelled lipoprotein (100%). Binding of each lipoprotein sample was carried out in triplicate. Each value represents the mean ± S.D. The non-saturable component, measured as a 20-fold excess of unlabelled lipoproteins, was 23 ± 1, 31 ± 2, 34 ± 1 and 37 ± 2% for LDL, control, heterozygous and homozygous VLDL respectively. Comparable results were obtained when the lipoproteins were added on the basis of amount of cholesterol.

apoA1 on $d < 1.006$ g/ml particles was also investigated by Western-blot analysis (Figures 4b–4d). No clear changes were observed in the amount of apoC3 and apoE present on these particles. However, apoA1 appeared in the $d < 1.006$ fraction of mutant mice, and about twice the amount of apoA1 was found in homozygotes compared with heterozygous apoC1-deficient mice (Figure 4d). When a Western blot of total lipoproteins ($d < 1.21$ g/ml or $d < 1.063$–1.21 g/ml) was incubated with antibodies against mouse apoA1, apoC3 and apoE, no overt changes were measured in these apolipoprotein concentrations in null mutants compared with controls (results not shown).

Impaired binding of apoC1-deficient VLDL to the LDL receptor

To find out whether the accumulation of VLDL + LDL-sized particles on the HFC0.5% diet is due to disturbed binding of lipoprotein remnants to the LDL receptor, the ability of isolated $d < 1.006$ g/ml lipoproteins from mice fed on the HFC0.5% diet to compete with human ^{125}I-labelled LDL for binding to the LDL receptor on HepG2 cells was determined. Figure 5 shows that control mouse VLDL competed as efficiently as human LDL with ^{125}I-labelled LDL. Strikingly, the $d < 1.006$ g/ml particles from homozygous apoC1-deficient mice were poor competitors for ^{125}I-labelled LDL binding to the receptor. Heterozygous VLDL was an intermediate competitor.

DISCUSSION

Very little is known about the *in vivo* function of apoC1 in lipoprotein metabolism. To clarify its metabolic role, we have generated mice deficient in apoC1 by gene targeting in ES cells. The effect of the null mutation on lipid levels of mice fed on chow or a mild hypercholesterolaemic diet was rather subtle. However, when the animals were fed on the severe atherogenic diet HFC0.5%, the homozygous apoC1-deficient mice became hypercholesterolaemic compared with wild-type mice.

A mild hypertriglyceridaemia was observed in the null mutants on chow and the HFC diet. This weak phenotype could possibly be explained by a decreased suitability of apoC1-deficient chylomicrons and VLDL as substrate for lipoprotein lipase, and is the subject of further investigation.

The mild hypertriglyceridaemia was measured in serum of starved mice. The duration of starvation is of importance, as recent data from LeBoeuf and co-workers [30] have demonstrated that plasma triacylglycerol levels paradoxically increase in mice during starvation and the level of increase is related to the period of starvation. Furthermore, triacylglycerol levels fall significantly with time since blood samples were taken, presumably because of an active lipase present in mouse plasma [31]. Because of the relatively minimal metabolic consequences under mild dietary conditions, we have standardized as much as possible analysis of triacylglycerols with respect to period of food deprivation and time of analysis after sampling. However, at present it cannot be ruled out that, even though control samples have been treated in exactly the same way, the mild hypertriglyceridaemia observed in homozygous apoC1-deficient mice on mild diets is caused by a difference in lipase activity in serum samples of apoC1-deficient mice and controls.

When the animals were challenged with a severe atherogenic diet, the homozygous apoC1-deficient mice clearly developed hypercholesterolaemia. This phenotype is not as severe as observed in, for example, apoE-deficient mice [20,32], indicating that the function of apoC1 in lipoprotein metabolism is not as critical as that of apoE. However, the hypercholesterolaemia in the *Apoc1* knock-out mice is observed when compared with wild-type littermates treated in exactly the same way, demonstrating that the high serum cholesterol levels are caused either directly or indirectly by deficiency in apoC1.

The observed hypercholesterolaemia after dietary treatment was opposite to what one would expect from the literature. *In vitro* studies have shown that apoE can be displaced from β-VLDL by apoC1, resulting in a reduced binding efficiency of the particles to the LDL receptor [7] and the LRP [8]. In addition, apoC1 can inhibit the uptake of triacylglycerol emulsions or chylomicron remnants by the perfused rat liver [10,33]. These results suggest that the plasma clearance of remnants is retarded by an excess of apoC1 on the surface of the particle. This is consistent with results from *in vivo* experiments demonstrating that transgenic mice overexpressing human apoC1 exhibit moderate increases in total plasma cholesterol and triacylglycerol levels compared with control animals [9].

Similarly, excess amounts of apoC3 can interfere with the apoE-mediated clearance of remnant lipoproteins. Both *in vitro* experiments [7,10] and *in vivo* studies with transgenic mice overexpressing human apoC3 [34,35] have shown that an excess of apoC3 can also inhibit the clearance of remnant lipoproteins, although less efficiently than apoC1. It has been suggested that apoC1 and apoC3 either displace apoE from the particle or interact directly or indirectly with apoE on the lipoprotein surface [7,8,35].

In line with this reasoning, we expected that apoC1 deficiency would lead to an acceleration of remnant lipoprotein removal via apoE-mediated processes, resulting in normal or even reduced serum levels of cholesterol and triacylglycerol. Paradoxically, instead of a decrease, a moderate to strong increase in levels of VLDL + LDL-sized lipoproteins was found, depending on the diet administered. Binding-competition experiments revealed

that apoC1-deficient $d < 1.006$ g/ml lipoproteins do not bind efficiently to the LDL receptor (see Figure 5), suggesting a retarded plasma clearance of lipoprotein remnants in mutant mice. The reduced binding efficiency could be due to changes in the apolipoprotein composition of these particles. The apoE concentration of the $d < 1.006$ lipoproteins of the mutant mice was not reduced, but was similar to controls (see Figure 4). Also apoC3, which can displace apoE and cause hypertriglyceridaemia when over-expressed in mice [34,35], did not substitute for the deficient apoC1 on the lipoproteins. However, apoA1 was present in the $d < 1.006$ g/ml fraction of *Apoc1* null mutants. The appearance of apoA1 on $d < 1.006$ g/ml lipoproteins has also been observed in apoE-deficient mice [20,32], suggesting that apoA1 replaces lacking apolipoproteins on VLDL and LDL particles in general. Whether the presence of apoA1 can modulate the apoE-mediated receptor binding is the subject of further investigation.

In summary, from our results we conclude that the complete absence of apoC1 on the chylomicron and VLDL particles leads to impaired receptor-mediated clearance of remnant lipoproteins rather than an enhanced uptake, as expected from *in vitro* and *in vivo* studies reported in the literature [7–10]. Obviously, the amount of apoC1 relative to apoE influences the efficiency of hepatic uptake of remnant lipoproteins in a discontinuous way.

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