

Characterization of a new mouse model for human apolipoprotein A-I/C-III/A-IV deficiency

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Abstract Human data raised the possibility that coronary heart disease is associated with mutations in the apolipoprotein gene cluster *APOA1/C3/A4* that result in multideficiency of cluster-encoded apolipoproteins and hypoalphalipoproteinemia. To test this hypothesis, we generated a mouse model for human apolipoprotein A-I (apoA-I)/C-III/A-IV deficiency. Homozygous mutants (*Apoa1/c3/a4*^{-/-}) lacking the three cluster-encoded apolipoproteins were viable and fertile. In addition, feeding behavior and growth were apparently normal. Total cholesterol (TC), high density lipoprotein cholesterol (HDLc), and triglyceride levels in the plasma of fasted mutants fed a regular chow were 32% ($P < 0.001$), 17% ($P < 0.001$), and 70% ($P < 0.01$), respectively, those of wild-type mice. When fed a high-fat Western-type (HFW) diet, *Apoa1/c3/a4*^{-/-} mice showed a further decrease in HDLc concentration and a moderate increase in TC, essentially in non-HDL fraction. The capacity of *Apoa1/c3/a4*^{-/-} plasma to promote cholesterol efflux in vitro was decreased to 75% ($P < 0.001$), and LCAT activity was decreased by 38% ($P < 0.01$). Despite the very low total plasma cholesterol, the imbalance in lipoprotein distribution caused small but detectable aortic lesions in one-third of *Apoa1/c3/a4*^{-/-} mice fed a HFW diet. In contrast, none of the wild-type mice had lesions. These results demonstrate that *Apoa1/c3/a4*^{-/-} mice display clinical features similar to human apoA-I/C-III/A-IV deficiency (i.e., marked hypoalphalipoproteinemia) and provide further support for the *apoa1/c3/a4* gene cluster as a minor susceptibility locus for atherosclerosis in mice.—Mezdour, H., G. Larigauderie, G. Castro, G. Torpier, J. Fruchart, M. Nowak, J.-C. Fruchart, M. Rouis, and N. Maeda. **Characterization of a new mouse model for human apolipoprotein A-I/C-III/A-IV deficiency.** *J. Lipid Res.* 2006. 47: 912–920.

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A strong inverse association exists between plasma high density lipoprotein cholesterol (HDLc) levels and the

incidence of coronary artery disease in humans (1). The major locus controlling HDLc levels is *APOA1* (for apolipoprotein A-I), a member of the apolipoprotein gene cluster *APOA1/C3/A4* (2). The cluster genes, *APOA1*, *APOC3*, and *APOA4*, are evolutionarily related and tandemly organized in a region of 17 kb DNA on human chromosome 11, proximal to *APOA5*, a new member of the apolipoprotein gene family (3). These *APOA1/C3/A4* genes code for three apolipoproteins, apoA-I, apoC-III, and apoA-IV, respectively. ApoA-I is a 28 kDa protein synthesized in the liver and small intestine (4). It is the major protein component of HDL. Through its ability to promote cholesterol efflux from cultured cells (5) and to activate LCAT (6), apoA-I plays a key role in reverse cholesterol transport, a mechanism postulated to prevent extrahepatic tissues from accumulating excess cholesterol (7). Apolipoprotein C-III is a 9 kDa protein produced predominantly in the liver. It is a major component of plasma chylomicrons and VLDL. ApoC-III inhibits the hydrolysis of triglyceride by lipoprotein lipase. Apolipoprotein A-IV is a 46 kDa protein associated primarily with chylomicrons and HDL and is present in the lipoprotein-free fraction of plasma (8). It is involved in dietary fat absorption and likely in reverse cholesterol transport (9, 10).

The contribution of the *APOA1/C3/A4* gene cluster to coronary heart disease has been recognized for nearly two decades. Strong support came from rare human patients with apoA-I/C-III or apoA-I/C-III/A-IV deficiencies (11, 12). These patients had very low HDL concentrations in plasma and suffered from premature atherosclerosis. Also, an association of a high risk for premature atherosclerosis

Abbreviations: *APOA1/C3/A4*, apolipoprotein gene cluster (human); *apoa1/c3/a4*, apolipoprotein gene cluster (mouse); *Apoa1/c3/a4*^{+/+} and *Apoa1/c3/a4*^{-/-}, gene cluster wild-type (+/+ or *Aca*+/+) and knockout (-/- or *Aca*-/-) mice, respectively; HDLc, high density lipoprotein cholesterol; HFW, high-fat Western-type; IDL, intermediate density lipoprotein; SR-BI, scavenger receptor class B type I; TC, total cholesterol.

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sis and lower plasma levels of apoA-I has been observed; however, few mutations in the *APOA1/C3/A4* gene cluster that result in reduced levels or absence of apoA-I lead to atherosclerosis (13). In contrast, single deficiencies in apoA-I, apoC-III, and likely apoA-IV are not sufficient to predispose mice to atherosclerosis (14–16), whereas overexpression of human apoA-I (17) and apoA-IV (18) but not apoC-III (19) had been reported to protect mice from genetic or diet-induced atherosclerosis. In addition, simultaneous expression of apoA-I/C-III/A-IV in mice induced marked hypertriglyceridemia and the accumulation of potentially atherogenic lipoproteins but decreased susceptibility to atherosclerosis in an *ApoE*^{-/-} genetic background (20). Together, these observations suggested a complex role of the *apoa1/c3/a4* gene cluster, as an integrated unit, in plasma lipoprotein metabolism and atherosclerosis.

To begin to explore this issue, we generated a mouse model for human apoA-I/C-III/A-IV deficiency by gene targeting. In homozygous mutants, absence of apoA-I, apoC-III, and apoA-IV from plasma was accompanied by severe hypoalphalipoproteinemia and mild hypotriglyceridemia. In vitro analysis showed a reduced capacity of plasma to promote cholesterol efflux and LCAT activity. However, homozygous mutants displayed a limited susceptibility to atherosclerosis.

METHODS

Generation of apoA-I/C-III/A-IV-deficient mice

Mouse genomic DNA used for targeting vector was cloned from a 129/Ola genomic library. The targeting construct contains a 6 kb *EcoRI-SacI* fragment 5' to the *apoa1* gene as 5' homology and a 3 kb *BamHI-HindIII* fragment 3' of the *apoa4* gene as 3' homology. Neomycin phosphotransferase and thymidine kinase genes were used for positive/negative selection. A subclone (BK4) of the mouse strain 129/Ola embryonic stem cell line, E14TG2a, was cultured and electroporated with the targeting construct, as described previously (21). The embryonic stem cells surviving the positive/negative selection were analyzed by Southern blot after *EcoRI* digestion and hybridization to a 1 kb probe from *apoa4* intron 2. Targeted clones that carry a 13 kb deletion of the *apoa1/C3/a4* gene cluster (*Apoa1/c3/a4* allele) were identified by the presence of a 4.8 kb *EcoRI* fragment, compared with a 15 kb wild-type fragment. Targeted embryonic stem cells were microinjected into C57BL/6 blastocysts, and resulting chimeric mice were mated with C57BL/6 mice to generate F1 heterozygous mutants. Homozygous mutants were F2 generation animals derived from mating F1 *Apoa1/c3/a4*^{+/-} mice. Tail DNA was digested with *EcoRI*, and Southern blots were hybridized with the mouse *apoa4* probe to identify mice carrying the targeted allele. F2 generation mice with a 129/Ola×C57BL/6 mixed genetic background were used for characterization and experiments. Mice (8–10 weeks old) were matched for age and gender, and wild-type littermates were used as controls. Mice were housed in a temperature-controlled room with alternating 12 h light (7 AM–7 PM) and dark periods. The animals had access to diet and water ad libitum. All procedures involving animal handling and care were followed in accordance with institutional guidelines.

Northern blot and real-time RT-PCR analysis

Total RNAs were extracted from tissues (liver and small intestine) with RNA-plus (Quantum), purified, and electrophoresed (20 µg total RNA/lane) on 1% agarose gels containing formaldehyde (22). Samples were blotted onto Hybond-N membranes (Amersham Pharmacia Biotech, Saclay, France) and hybridized with ³²P-labeled cDNA-specific probes. For quantitative PCR, reverse-transcribed apoA-V and apoE cDNA was quantified by real-time PCR on a MX 4000 apparatus (Stratagene) using specific oligonucleotide primers for *apoa5* (5'-CTC TGC CCC ACA AAC TCA CAC G-3' and 5'-AGG TAG GTG TCA TGC CGA AAA G-3') and acidic ribosomal phosphoprotein 36B4 (5'-GGG AAG GTG TAA TCC GTC TCC ACA G-3' and 5'-CAT GCT CAA CAT CTC CCC CTT CTC C-3') to normalize the assay. The forward primer used for mouse apoE was 5'-CCG TGC TGT TGG TCA CAT TGC TGA CAG GAT-3', and the reverse primer was 5'-GTT CTT GTG TGA CTT GGG AGC TCT GCA GCT-3'.

Agarose gel and Western blot analysis

Agarose gel electrophoresis of plasma was performed on 1% agarose gels (Sebia) after transfer of lipoproteins, and nitrocellulose membranes were probed with rabbit polyclonal anti-mouse apoA-I, apoC-III, or apoA-IV antibodies followed by a conjugated goat anti-rabbit antibody (Pasteur Diagnostics). Chemiluminescence (ECL System; Amersham Pharmacia Biotech) was used for detection.

High-fat diet study

Mice (8–10 weeks old) with the indicated genotypes were assigned to three groups. Group 1, or the control group (*Apoa1/c3/a4*^{+/+}; n = 15, 10 males and 5 females) and group 2 (*Apoa1/c3/a4*^{-/-}; n = 15, 10 males and 5 females) were fed a high-fat Western-type (HFW) diet containing 0.2% cholesterol and 21% fat (w/w) (UAR, Epinay sur Orge, France); group 3 (*Apoa1/c3/a4*^{-/-}; n = 8, 4 males and 4 females) was fed a standard chow diet containing <0.03% cholesterol and 5% fat (w/w) (UAR). The HFW diet was continued for 16 weeks.

Plasma lipid and lipoprotein analysis

After a 5 h fast, the mice were anesthetized with avertin and blood was collected from the retro-orbital sinus into microcentrifuge tubes containing EDTA. Plasma total cholesterol (TC) and triglycerides were determined using diagnostic kits (Sigma or Biomerieux) according to the instructions of the manufacturers. HDLc levels were determined in plasma after precipitation of apoB-containing lipoproteins with dextran sulfate-Mg²⁺ (23). TC and HDLc were first determined using homemade standards constituted of pools of mouse plasma. Nevertheless, these standards underestimated both HDL and TC concentrations. Therefore, the final values reported here were obtained after correction with coefficient factors determined after additional calibrations of our initial standards with a commercial standard (Roche). For gel filtration analysis with fast-protein liquid chromatography (Amersham Pharmacia Biotech), equal volumes of plasma samples from the indicated genotypes were pooled, 200 µl was fractionated using two serially linked Superose 6 HR and Superose 12 HR columns, and TC was determined in 100 µl of each collected fraction using an enzymatic method (Sigma).

Analysis of plasma metabolic activity

Rat Fu5AH hepatoma cells, which have a high expression level of scavenger receptor class B type I (SR-BI), and J774 mouse

macrophages, which express ABCA1 and SR-BI at very low levels, were used to assess cholesterol efflux, as described previously (24), except that J774 cells were not pretreated with cAMP, a molecule that upregulates ABCA1 gene expression. [³H]cholesterol-loaded cells were incubated for 3 h with 2.5% diluted plasma. All efflux values are reported as averages of at least three determinations in different wells. Isolated human HDL samples were included in each assay as positive controls. LCAT activity was determined using the exogenous proteoliposome substrate method with 25 μ l of plasma from female mice (20). Experiments were repeated twice with three different pools of samples for controls and mutants (coefficient of variation <5%).

Histological analysis

Mice were euthanized, and the hearts were perfused from the apex with a cold Krebs-Ringer buffer and fixed in a fixative containing 4% paraformaldehyde in PBS, pH 7.4. The hearts, containing the aortic origin, and other tissues (liver, lung, etc.) were carefully removed and fixed in paraformaldehyde overnight. Serial cryosections were obtained from tissues and stained using Oil Red O and hematoxylin (25).

Statistical analysis

Results are expressed as means \pm SD. In all experiments, non-transgenic littermates of each line were used as controls. Statistical differences in lipoprotein and lipid levels between different groups of animals were evaluated using Student's two-tailed *t*-test, unless indicated otherwise. Group differences or correlations with *P* < 0.05 were considered statistically significant.

RESULTS

ApoA-I/C-III/A-IV-deficient mouse

To ensure total ablation of the *apoa1/c3/a4* gene cluster, a gene-targeting vector was constructed such that homologous recombination would delete 13 kb of the mouse's endogenous cluster, including the three *apoa1* coding exons, the entire *apoc3* gene, intergenic regions, and the

first two exons of *apoa4* (Fig. 1A, B). Loss of expression of the cluster genes was demonstrated by Northern blot analysis of total liver and intestine mRNA (Fig. 1C), and the absence of apoA-I, apoC-III, and apoA-IV proteins from plasma was demonstrated by Western blot analysis using specific anti-mouse apolipoprotein antisera (Fig. 2B). Therefore, we consider the *Apoa1/c3/a4*^{-/-} a functional null. Animals homozygous for the ablated cluster genes appeared healthy and have apparently normal growth and feeding behavior.

ApoA-I/C-III/A-IV deficiency causes dyslipidemia

On chow diet, total plasma TC and HDLc concentrations were lower than those of wild-type littermates by 68% and 83%, respectively. Their triglyceride concentration was lower by 29% compared with normal controls, but the decrease was statistically significant only in females (Table 1). The mean ratio of TC to HDLc was 2.6 in *Apoa1/c3/a4*^{-/-} versus 1.4 in *Apoa1/c3/a4*^{+/+}, reflecting a severe reduction of HDLc. Severe hypoalphalipoproteinemia was also demonstrated by agarose gel electrophoresis showing reduced α fraction of lipoproteins (Fig. 2A). Plasma lipid levels did not change significantly with age or gender (data not shown).

Plasma samples from several mice of each *apoa1/c3/a4* genotype were pooled and applied to the Superose 6 and 12 columns to fractionate the lipoproteins by size (Fig. 3A). In wild-type mice fed regular chow diet, the major lipoprotein eluted in the region corresponding to HDL. Only a small amount of cholesterol was found in regions corresponding to VLDL, intermediate density lipoprotein (IDL), and LDL. Both male and female homozygous mutants had similar lipoprotein profiles, which demonstrates a dramatically reduced amount of cholesterol in the HDL fraction (Fig. 3A).

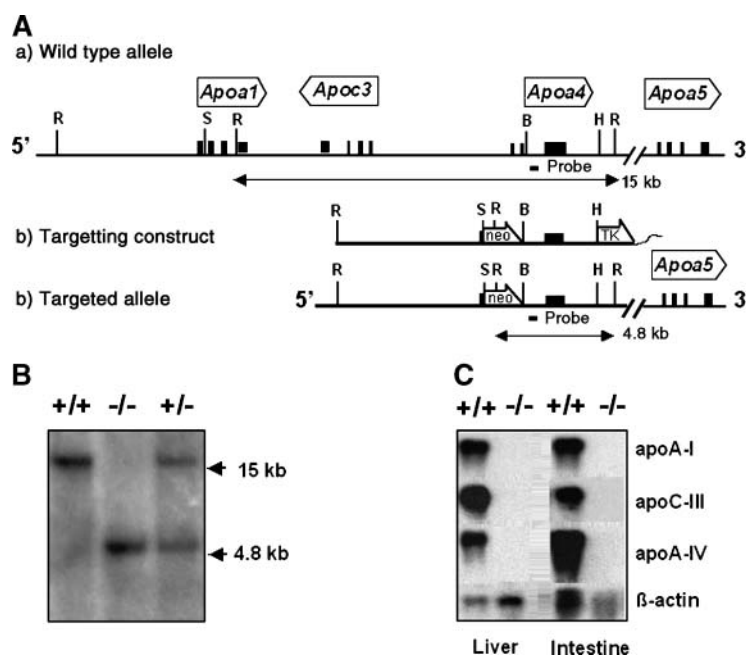


Fig. 1. Targeted disruption of the mouse apolipoprotein gene cluster *apoa1/c3/a4*. **A:** Schematic representation of the gene-targeting strategy. **a:** The mouse *apoa1/c3/a4* gene cluster (wild-type allele). **b:** Targeting construct containing genes for neomycin phosphotransferase (*neo*) and herpes simplex virus thymidine kinase (*TK*) used for selection, and 5' and 3' regions of homology. **c:** The resulting targeted allele. The sizes of diagnostic fragments are shown between the *EcoRI* sites. B, BamHI; H, HindIII; R, EcoRI; S, SacI. **B:** Southern blot analysis of tail DNA digested with *EcoRI* and hybridized with the mouse *apoa4* probe to identify F2 generation mice carrying the targeted allele. The 15 kb hybridizing *EcoRI* fragment indicates the wild-type allele, and the 4.8 kb fragment indicates the mutated allele. **C:** Northern blot analysis. Total RNA from liver and intestine of each genotype was subjected to electrophoresis, and expression of the cluster genes was examined using specific cDNA probes for mouse *apoa1*, *apoc3*, and *apoa4*. Mouse β -actin was used as the loading control.

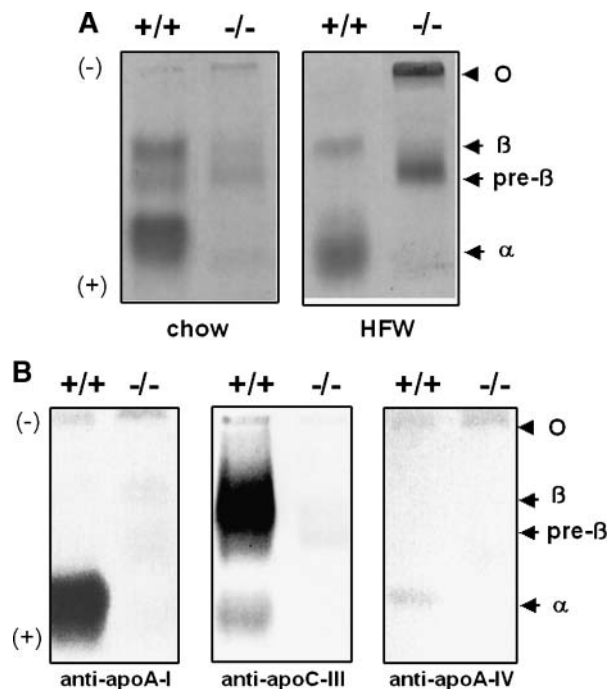


Fig. 2. Agarose gel electrophoresis and Western blot analysis. **A:** Plasma samples from mice fed a chow diet or a high-fat Western-type (HFW) diet were electrophoresed on 1% agarose gels. Gels were stained with Sudan black after drying. The positions α , β , pre β , and O correspond to HDL, LDL, VLDL, and origin, respectively. **B:** Immunoblots of lipoprotein fractions after transfer from agarose gels using specific antibodies to mouse apolipoprotein A-I (apoA-I), apoC-III, and apoA-IV.

Response to the high-fat diet

When fed a HFH diet for 16 weeks, wild-type and homozygous mutant animals responded differently (**Table 2**). In *Apoa1/c3/a4*^{-/-} males and females, TC increased by 52% and 39%, whereas HDLc decreased by 7- and 2.5-fold, respectively. In *Apoa1/c3/a4*^{+/+} males and females, TC increased by 72% and 50%, with increases in HDLc of 19% and 9%, respectively. The TC/HDLc ratio increased to 28.6 in *Apoa1/c3/a4*^{-/-} males compared with 1.7 in *Apoa1/c3/a4*^{+/+} males and increased to 8.6 in *Apoa1/c3/a4*^{-/-} females versus 2.2 in *Apoa1/c3/a4*^{+/+} females, suggesting a further imbalance of plasma cholesterol distribution in *Apoa1/c3/a4*^{-/-} mice. In contrast, plasma triglyceride levels

were less affected by the high-fat diet in either normal or mutant animals.

A 16 week high-fat diet resulted in an increase in the lipoprotein fractions eluting in the VLDL/IDL region and a further decrease in the HDL fraction in *Apoa1/c3/a4*^{-/-} mice (**Fig. 3B**). The increase in TC, therefore, was mainly the result of increased VLDL/IDL cholesterol, as confirmed by the increase in the pre β fraction of lipoproteins on agarose gel electrophoresis (**Fig. 2A**). Mice tolerated high fat without obvious distress.

Impaired plasma metabolic activity

In the Fu5AH cell model, plasma from *Apoa1/c3/a4*^{-/-} mice promoted cholesterol efflux, a pathway that involves SR-BI, 4-fold less efficiently than *Apoa1/c3/a4*^{+/+} mouse plasma ($4.1 \pm 1.2\%$ vs. $16.3 \pm 3.4\%$ in males and $7.1 \pm 2.6\%$ vs. $15.4 \pm 2.2\%$ in females; $P < 0.001$ and 0.01 , respectively) (**Fig. 4A**). In the J774 macrophage model, the capacity of *Apoa1/c3/a4*^{-/-} plasma for cholesterol efflux, which is mediated mostly through ABCA1 and to a less extent by SR-BI, was 65% that of wild-type plasma ($6.3 \pm 0.8\%$ vs. $9.6 \pm 1.1\%$ in males and $6.5 \pm 1.1\%$ vs. $9.2 \pm 0.9\%$ in females; $P < 0.01$) (**Fig. 4B**). Similarly, up to a 45% decrease in LCAT activity was observed in *Apoa1/c3/a4*^{-/-} mice compared with *Apoa1/c3/a4*^{+/+} mice ($11.2 \pm 2.2\%$ vs. $18.1 \pm 1.7\%$ in males and $8.9 \pm 2.3\%$ vs. $19.9 \pm 1.8\%$ in females; $P < 0.01$) (**Fig. 4C**).

Histology

No atherosclerotic lesions were observed in *Apoa1/c3/a4*^{-/-} mice maintained on regular chow ($n = 8$), even in two homozygotes older than 10 months of age. In contrast, small atherosclerotic lesions were present in the aortas of 4 males out of 10 and 1 female out of 5 *Apoa1/c3/a4*^{-/-} mice fed a HFH diet for 16 weeks. These lesions were limited to fatty streaks with small populations of foam cells near the aortic valve attachment sites (**Fig. 5A, B**). No lesion was observed in wild-type controls fed the same high-fat diet ($n = 15$). A complete necropsy of *Apoa1/c3/a4*^{-/-} and *Apoa1/c3/a4*^{+/+} mice was performed to determine other morphologic changes secondary to ablation of the *apoa1/c3/a4* gene cluster. No gross histopathological differences from normal animals were found in tissues of *Apoa1/c3/a4*^{-/-} animals fed a chow diet, such as the lung,

TABLE 1. Concentration of plasma lipids in mice fed a normal chow diet

Genotype	Gender	TC	HDLc	Triglycerides	TC/HDLc
<i>Apoa1/c3/a4</i> ^{+/+}	Male	69.3 \pm 11.9 (9)	56.3 \pm 15.2 (8)	53.5 \pm 9.2 (5)	1.2
	Female	82.5 \pm 21.5 (4)	51.0 \pm 8.5 (2)	68.8 \pm 17.7 (4)	1.6
<i>Apoa1/c3/a4</i> ^{-/-}	Male	24.4 \pm 6.0 ^a (9)	8.8 \pm 2.3s ^a (8)	45.8 \pm 8.6 (9)	2.8
	Female	23.4 \pm 7.7 ^a (5)	9.4 \pm 3.3 ^a (5)	37.6 \pm 4.2 ^b (5)	2.4

HDLc, high density lipoprotein cholesterol; TC, total cholesterol. Data are means \pm SD. Numbers of mice are indicated in parentheses.

^a $P < 0.001$, *Apoa1/c3/a4*^{-/-} versus *Apoa1/c3/a4*^{+/+} matched for gender.

^b $P < 0.01$, *Apoa1/c3/a4*^{-/-} versus *Apoa1/c3/a4*^{+/+} matched for gender.

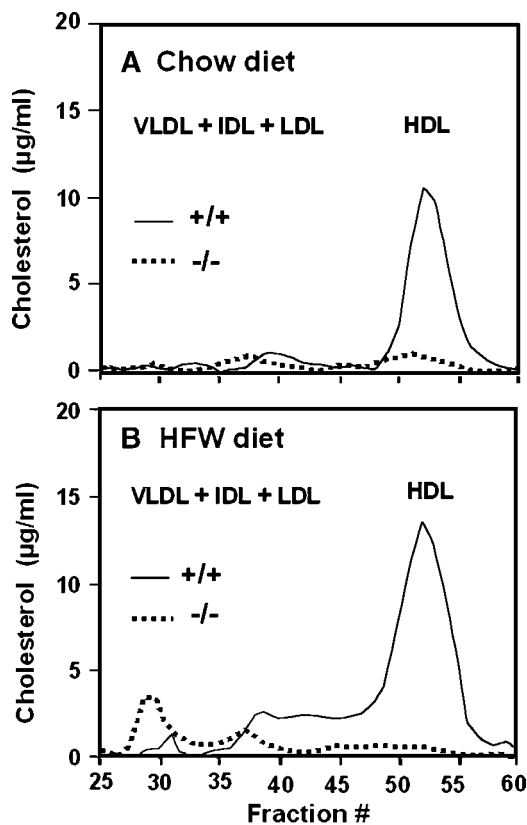


Fig. 3. Lipoprotein profiles. Pooled plasma (200 μ l) from homozygous male mutants and wild-type male controls fed a normal chow diet (A) or a HFW diet (B) was fractionated by gel filtration chromatography on Superose 6B columns. Fractions were collected, and total cholesterol was measured by an enzymatic assay in each fraction. IDL, intermediate density lipoprotein.

kidney, spleen, and liver (Fig. 5C, D). Feeding a high-fat diet to wild-type mice for a long period caused severe fatty changes in their livers (Fig. 5E). Thus, wild-type livers were enlarged and pale. The hepatocytes in the wild-type livers were enlarged with microvesicular lipid deposits. In contrast, *Apoa1/c3/a4*^{-/-} mice fed the same diet have relatively normal livers. Histologically, the *Apoa1/c3/a4*^{-/-} liver had very few lipid-laden cells (presumably Kupffer cells) in the sinusoidal spaces surrounding hepatocytes, with essentially normal appearance (Fig. 5D). In marked contrast, most of the *Apoa1/c3/a4*^{-/-} male and female mutants had enlarged spleens, and severe splenomegaly was

observed in 4 out of 15 mice (3 males and 1 female) fed a HFW diet (Fig. 5F).

DISCUSSION

In this study, we investigated the role of the *apoa1/c3/a4* gene cluster in plasma lipoprotein metabolism in mouse. Like humans with apoA-I/C-III/A-IV deficiency, the *Apoa1/c3/a4*^{-/-} mutants showed severe hypoalphalipoproteinemia, mild hypotriglyceridemia, and an apparent decrease in non-HDL particles corresponding to β - and pre β fractions of lipoproteins (Fig. 2A). In contrast to cluster-deficient patients, however, combined deficiencies of apoA-I, apoC-III, and apoA-IV did not predispose mice to severe atherosclerosis. It is well established that mice are naturally resistant to spontaneous atherosclerosis. The most susceptible strain is C57BL/6, which develops fatty lesions only when fed a high-fat diet (15% fat, 1.5% cholesterol, with 0.5% cholate) (25). In this study, *Apoa1/c3/a4*^{-/-} mice were fed a less toxic and more physiological "Western-type" diet containing 21% fat and 0.2% cholesterol without cholate. This diet is not atherogenic in wild-type mice even in a susceptible C57BL/6 strain, although it accelerates lesion formation in genetically predisposed mice such as LDL receptor-deficient mice and apoE-deficient mice (26). Interestingly, despite having only 30 mg/dl total plasma cholesterol, one-third of *Apoa1/c3/a4*^{-/-} mice fed a HFW diet developed small but detectable aortic lesions. Although this heterogeneity may be attributable to their genetic background (F2 between C57BL/6 and 129/Ola), this suggests that the increased TC/HDLc ratio contributes to the atherosclerosis in *Apoa1/c3/a4*^{-/-} mice. Although additional studies are clearly necessary, these observations suggest a possible contribution of the *apoa1/c3/a4* gene cluster to atheroprotection.

When fed a HFW diet, plasma cholesterol levels in *Apoa1/c3/a4*^{-/-} mice were less affected than in *Apoa1/c3/a4*^{+/+} normal controls. Although cholesterol levels in normal male and female mice increased by 72% and 50%, respectively, mostly in VLDL, IDL, and LDL fractions and less so in HDL, these levels increased in *Apoa1/c3/a4*^{-/-} mice by only 52% and 39%, respectively, exclusively in the VLDL/LDL range of lipoproteins. One possible explanation for the smaller increase in non-HDLc in the mutants fed a HFW diet is that mice lacking apoA-I/C-III/A-IV could have a poorer absorption of fat compared with nor-

TABLE 2. Concentration of plasma lipids in mice fed a HFW diet

Genotype	Gender	TC	mg/dl			TC/HDLc
			HDLc	Triglycerides		
<i>Apoa1/c3/a4</i> ^{+/+}	Male	119.3 \pm 57.1 (8)	69.2 ^a	28.0 \pm 10.1 (8)	1.7	
	Female	124.2 \pm 39.8 (4)	55.8 ^a	26.1 \pm 11.3 (4)	2.2	
<i>Apoa1/c3/a4</i> ^{-/-}	Male	37.2 \pm 15.0 ^b (10)	1.3 ^a	24.9 \pm 8.4 (10)	28.6	
	Female	32.6 \pm 11.4 ^b (5)	3.8 ^a	28.4 \pm 3.7 (5)	8.6	

HFW, high-fat Western-type. Data are means \pm SD. Numbers of mice are indicated in parentheses.

^a Measured in pooled samples.

^b $P < 0.001$, *Apoa1/c3/a4*^{-/-} versus *Apoa1/c3/a4*^{+/+} matched for gender and diet.

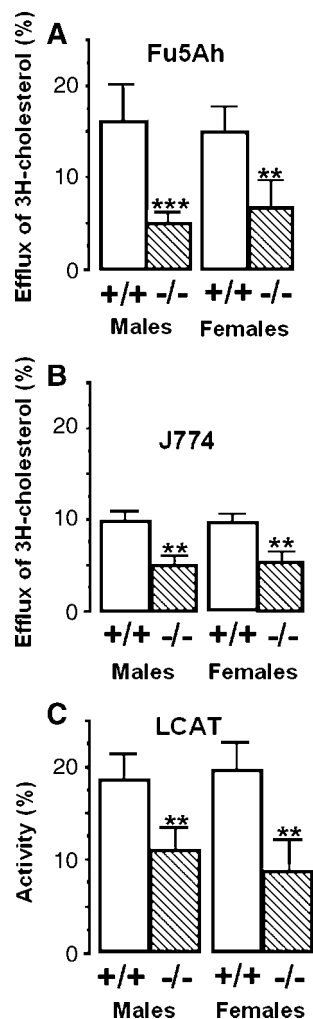


Fig. 4. Plasma metabolic activities. A, B: Plasma capacity to induce cellular cholesterol efflux was determined using Fu5AH hepatoma cells (A) and J774 cells (B). A 2.5% dilution of plasma from *Apoa1/c3/a4*^{+/+} controls (open bars) and *Apoa1/c3/a4*^{-/-} mutants (hatched bars) fed a HFW diet for 16 weeks was incubated for 3 h with [³H]cholesterol-labeled cells. Data are from representative experiments with triplicate wells. C: LCAT activity was determined in triplicate according to an exogenous substrate method. Values are expressed as means \pm SD. ** $P < 0.001$, *** $P < 0.01$ as determined by the Mann-Whitney nonparametric test.

mal mice. Support for the possible involvement of apoA-IV in the intestinal absorption of fat comes from the observation that the secretion of apoA-IV is increased during fat absorption (27) and in patients with apoA-I/C-III/A-IV deficiency, in whom an abnormal absorption of fatty acids was observed (12). In contrast, patients with apoA-I/C-III deficiencies have normal lipid absorption (11). Thus, despite apparently normal feeding behavior and growth, *Apoa1/c3/a4*^{-/-} mice have a defective metabolism in the non-HDL fraction of lipoproteins, which suggests either a faster catabolism of apoC-III-deficient lipoproteins and/or abnormal lipid absorption and lipoprotein synthesis.

The protective mechanism of HDL with respect to coronary heart disease is believed to reside in its ability to pro-

move cholesterol efflux from peripheral tissues, a key process in reverse cholesterol transport, a mechanism postulated to prevent extrahepatic tissues from accumulating excess cholesterol (7, 28). Efflux of cholesterol occurs via multiple mechanisms: aqueous diffusion, SR-BI, or ABCA1. Both diffusion-mediated and SR-BI-mediated efflux occur to phospholipid-containing acceptors. In both cases, the flux of cholesterol is bidirectional, with the direction of net flux depending on the cholesterol gradient. In contrast, efflux via ABCA1 is unidirectional and mediates the cellular efflux of phospholipids and cholesterol to lipid-poor apolipoproteins. The ABCA1-mediated process plays a significant role in the formation of nascent HDLs and facilitates the removal of additional excess cellular cholesterol, which is esterified by LCAT. The relative importance of the SR-BI and ABCA1 efflux pathways in preventing the development of atherosclerotic plaque is not known but will depend on the expression levels of the two proteins and on the type of cholesterol acceptors available. A number of apolipoproteins have been implicated in the process of promoting the efflux of free cholesterol from the macrophages, including apoA-I, apoA-IV, and apoE. Cholesterol efflux is significantly impaired in cluster knockout mice but had only a limited effect on atherosclerosis susceptibility. These results suggest that, in addition to apoA-I/C-III/A-IV deficiency, a further imbalance in lipoprotein distribution (i.e., an increase in atherogenic lipoprotein level) is the primary factor affecting cholesterol homeostasis in peripheral tissues leading to foam cell formation. One can also speculate that a limitation in atherosclerosis susceptibility in *Apoa1/c3/a4*^{-/-} mice could be related to apoE. It is well known that lipid transport in mice is highly dependent on apoE (26, 29), a protein that is produced by various tissues (30). There were no alterations in the apoE levels in *Apoa1/c3/a4*^{-/-} mice compared with wild-type mice (data not shown). It was observed that macrophage-derived apoE exerts antiatherogenic properties largely independent of its concentration in plasma and effects on plasma lipoproteins, likely through its contribution to cholesterol efflux from macrophages. Indeed, transplantation of wild-type bone marrow or the selective expression of a human apoE transgene in macrophages decreased atherosclerosis in apoE-deficient mice (31). Therefore, decreased cholesterol influx caused low total and LDL-cholesterol, and an efficient apoE-mediated cholesterol efflux may limit atherogenesis in apoA-I/C-III/A-IV deficiency.

Other known genetic determinants associated with apoA-I deficiency and hypoalphalipoproteinemia are *Lcat* and *Abca1* (1, 32). Indeed, like *Apoa1/c3/a4*^{-/-} and *Apoa1*^{-/-} mice, *Lcat*^{-/-} and *Abca1*^{-/-} mice display marked decreases in plasma levels of TC ($\sim 70\%$) and HDLc ($\sim 80\text{--}90\%$) in homozygous mutants (33–35), but a decrease in triglyceride level was observed only in apoC-III deficiency, secondary to *apoc3* gene deletion in *Apoa1/c3/a4*^{-/-} mice or downexpression in *Apoa1*^{-/-} mice (Table 3). Downexpression of *apoc3*, but not *apoa4*, results from interference by the nearby neomycin resistance cassette (14, 21). In contrast, *apoa5* gene expression was found to be normal in *Apoa1/c3/a4*^{-/-}

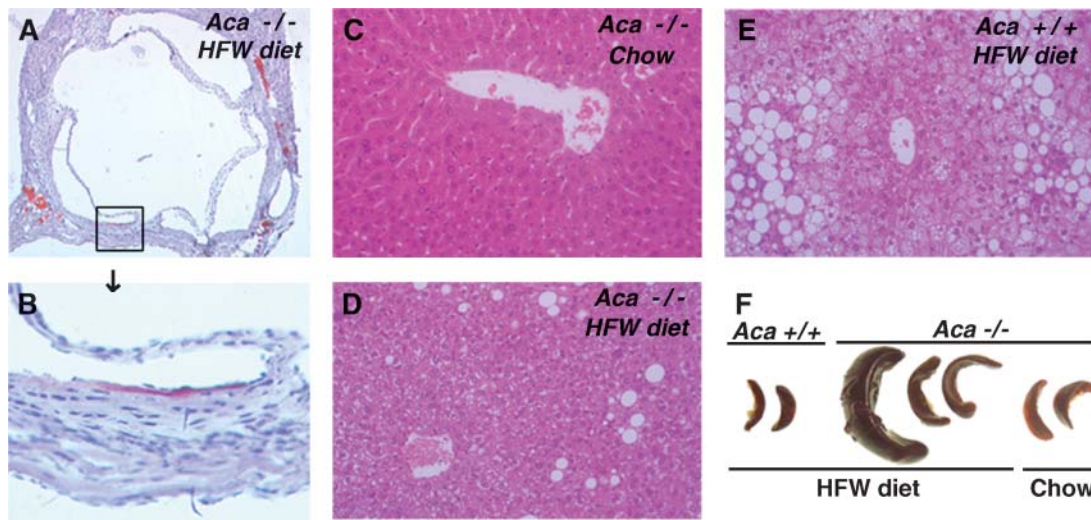


Fig. 5. Morphological and histological evaluations. All mice were F2 between the C57BL/6 and 129/Ola genetic backgrounds. Lesion occurrence was determined in sections from the proximal aorta near the aortic valve attachment sites. Sections of tissues are stained with Oil Red O and counterstained with hematoxylin. A: Cross-section of an aortic sinus. The micrograph is from a 7 month old mouse fed a HFW diet for 16 weeks. B: Small lesions, consisting mainly of foam cells, are observed in a *Apoa1/c3/a4^{-/-}* (*Aca^{-/-}*) mouse fed a HFW diet. C: Liver section from a wild-type (*Aca^{+/+}*) male fed a chow diet showing normal hepatocytes. D: Liver section from an *Apoa1/c3/a4^{-/-}* male mouse fed a HFW diet for 16 weeks, showing mild fatty changes of hepatocytes. E: Liver section from a wild-type male mouse fed a HFW diet for 16 weeks, showing severe fatty changes of hepatocytes. F: Splenomegaly was observed in *Apoa1/c3/a4^{-/-}* mice and was aggravated by a HFW diet.

mice (data not shown) and so likely in *ApoA1^{-/-}* mutants, consistent with transgenic studies showing that the apoC-III enhancer regulates the expression of apoA-I, apoC-III, and apoA-IV but not apoA-V in vivo (36). These data demonstrate that *ApoA1^{-/-}* mice are apoA-I/C-III-deficient and differ from *Apoa1/c3/a4^{-/-}* mice mostly by the presence of apoA-IV. However, like *Abca1^{-/-}* and *Lcat^{-/-}* mice, *ApoA1^{-/-}* mutants displayed susceptibility to atherosclerosis only in severe hypercholesterolemia (i.e., in the *ApoE^{-/-}* or *Ldlr^{-/-}* background) (37–39), whereas *Apoa1/c3/a4^{-/-}* mutants developed small atherosclerotic lesions in less atherogenic conditions. These results suggest that, despite a minor contribution to plasma HDLc variation, apoA-IV may play a significant role in atheroprotection. Further comparative studies of these hypoalphalipoproteinemia models (i.e., *Apoa1/c3/a4^{-/-}* mice vs. *ApoA1^{-/-}* mice) will likely provide better insight into the specific roles of apoA-IV in lipid metabolism and atherosclerosis.


HDL particles have been shown to protect LDL from oxidative modification (40, 41), to suppress adhesion mol-

ecule expression, and to induce cyclooxygenase-2 in vascular endothelium (42), properties that are believed to be protective against atherosclerosis. The lower level of HDL and the absence of apoA-I- and apoA-IV-containing particles in *Apoa1/c3/a4^{-/-}* mice fed a HFW diet may increase the oxidative modifications of apoB-containing lipoproteins and the physiological dysfunctions of vascular endothelium. Moreover, these modified particles could be cleared through extrahepatic tissues. Thus, it is interesting that morphological examination of mice at necropsy revealed protection from fatty changes of hepatocytes but enlarged spleens in *Apoa1/c3/a4^{-/-}* mice. Prolonged exposure to a high-fat diet increased the occurrence of splenomegaly, an abnormality that was also reported in patients with Tangier disease (32) and in ABCA1-deficient mice (34), although not yet in human apoA-I/C-III/A-IV deficiency. Although the mechanism leading to splenomegaly-associated hypoalphalipoproteinemia has not been identified, one hypothesis involves a dysfunction in cholesterol efflux from macrophages.

TABLE 3. Phenotypes of mouse models for hypoalphalipoproteinemia

Genotype	ApoA-I	ApoC-III	ApoA-IV	TC	HDLc	Triglycerides	Aortic Lesions	Ref.
<i>Apoa1/c3/a4^{-/-}</i>	0	0	0	32%	17%	70%	HFW diet: ±	
<i>ApoA1^{-/-}</i>	0	↓	≈	33%	20%	45%	HFC diet: ≈ <i>Ldlr^{-/-}</i> : +	(14, 21, 37)
<i>Lcat^{-/-}</i>	↓	n.r.	n.r.	<30%	<7%	~100%	<i>Ldlr^{-/-}</i> or <i>ApoE^{-/-}</i> : +	(33, 38)
<i>Abca1^{-/-}</i>	↓	n.r.	n.r.	<30%	<5%	~100%	<i>Ldlr^{-/-}</i> or <i>ApoE^{-/-}</i> : ≈ <i>Abca1^{-BM/-BM}</i> , <i>ApoE^{-/-}</i> : +	(34, 35, 39)

ApoA-I, apolipoprotein A-I. Values shown are relative concentrations (percentage of wild-type controls); arrows indicate effects on plasma apolipoproteins. Lesion formation was determined in mutants with the indicated genetic background, in bone marrow transplantation in the *ApoE^{-/-}* background (*Abca1^{-BM/-BM}*, *ApoE^{-/-}*), or in diet-induced hypercholesterolemia (HFC, high-fat and high-cholesterol diet containing cholate). n.r., not reported; +, increased susceptibility; ±, low susceptibility; ≈, no effect on susceptibility.

In summary, our findings demonstrate that *Apoa1/c3/a4^{-/-}* mice display marked hypoalphalipoproteinemia and that apoA-I/C-III/A-IV deficiency has a profound impact on plasma lipoprotein metabolism. However, in contrast to humans, our results in mice suggest that hypoalphalipoproteinemia secondary to apoA-I/C-III/A-IV deficiency requires additional genetic or environmental risk factors that increase LDL-cholesterol level for the initiation of atherosclerosis. 

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