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Targeted Replacement of Mouse Apolipoprotein A-I with Human ApoA-I or the Mutant ApoA-I_{Milano}

EVIDENCE OF APOA-I_M IMPAIRED HEPATIC SECRETION*

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Cinzia Parolini‡§, Giulia Chiesa‡, Yiwen Zhu¶, Trudy Forte¶, Silvia Caligari‡, Elisabetta Gianazza‡, Maria Grazia Sacco‖, Cesare R. Sirtori‡, and Edward M. Rubin¶

From the ‡Department of Pharmacological Sciences, University of Milan, 20133 Milan, Italy, ‖Istituto di Tecnologie Biomediche-Consiglio Nazionale delle Ricerche, 20090 Segrate, Milan, Italy, the ¶Genome Sciences Department, Lawrence Berkeley National Laboratory, Berkeley, California 94720

Despite a pro-atherogenic profile, individuals carrying the molecular variant (R173C) of apolipoprotein (apo)A-I, named apoA-I_{Milano} (apoA-I_M), appear to be at reduced risk for cardiovascular disease. To develop an *in vivo* system to explore, in a controlled manner, the effects of apoA-I_M on lipid metabolism, we have used the gene targeting technology, or "gene knock-in" (gene k-in), to replace the murine apoA-I gene with either human apoA-I or apoA-I_M genes in embryonic stem cells. As in human carriers, mice expressing apoA-I_M (A-I_M k-in) are characterized by low concentrations of the human apolipoprotein and reduced high density lipoprotein cholesterol levels, compared with A-I k-in animals. The aim of the present study was to investigate the basic mechanisms of hypoalphalipoproteinemia associated with the apoA-I_M mutation. ApoA-I and apoA-I_M mRNA expression, as assessed by Northern blot analysis and quantitative real time reverse transcription-PCR, did not exhibit significant differences in either liver or intestine. Moreover, human apolipoprotein synthesis rates were similar in the k-in lines. When the secretion rate of the human apolipoproteins was assessed in cultured hepatocytes from the mouse lines, secretion from apoA-I_M-expressing cells was markedly reduced (42% for A-I_M k-in and 36% for A-I/A-I_M k-in mice) as compared with that of A-I k-in hepatocytes. These results provide the first evidence that the hypoalphalipoproteinemia in apoA-I_M human carriers may be partially explained by impaired apoA-I_M secretion.

Coronary artery disease is the most common cause of death in developed countries (1), and high density lipoprotein cholesterol (HDL-C)[1] concentrations are a major predictor of risk. Indeed, nearly half of all patients with coronary artery disease have low HDL-C (2, 3). Low HDL-C appears to be associated with, among other factors, an enhanced risk of angioplasty restenosis (4) and with a number of clinical syndromes such as the "metabolic syndrome", which combines low HDL with hypertriglyceridemia and abdominal obesity (5). Raising HDL-C concentrations may have therapeutic value in reducing risk of reinfarction and stroke in coronary patients (6, 7). In agreement with these clinical data, experimental studies indicate that HDL infusions are able to reduce significantly aortic lipid deposition in established atherosclerotic lesions (8–10). The cardio-protective role of HDL is, in part, related to its ability to stimulate cholesterol efflux from cells (11, 12) and by its anti-inflammatory (13) and anti-oxidant properties (14).

Genetic factors play a key role in regulating HDL-C concentrations. Changes in a variety of genes including apolipoprotein A-I/CIII (15), lipoprotein lipase (16), cholesteryl ester transfer protein (17), hepatic lipase (18), scavenger receptor B1 (19), lecithin-cholesterol acyltransferase (20), ATP-binding cassette (A1) transporter gene (21), and others all affect to a variable extent HDL-C concentrations in humans. Several naturally occurring mutations associated with reduced plasma HDL-C and apoA-I concentrations have also been described for human apolipoprotein (apo)A-I (22, 23), the major protein constituent of HDL. Although some of these hypoalphalipoproteinemic states are associated with an increased risk of atherosclerotic vascular disease, others do not seem to predispose to accelerated premature disease (24). One example is the apoA-I_{Milano} (A-I_M) mutant; evaluation of the cardiovascular status in apoA-I_M carriers, compared with control subjects from the same kindred, did not reveal any evidence of increased vascular disease at the preclinical level (25, 26).

ApoA-I_M is the result of a point mutation, with an arginine to cysteine substitution at position 173 (27). The carriers of this mutation are all heterozygotes that exhibit hypertriglyceridemia with markedly reduced HDL and apoA-I levels. The presence of a cysteine residue results in the formation of homodimers and heterodimers with apoA-II.

The kinetic etiology of hypoalphalipoproteinemias associated with apoA-I mutations have been generally related to accelerated catabolism rather than to lower synthesis of apoA-I (28, 29). However, a recent study has shown a reduced secretion

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§ To whom correspondence should be addressed: Dept. of Pharmacological Sciences, University of Milan, via Balzaretti 9, 20133 Milan, Italy. Tel.: 39-02-50318328; Fax: 39-02-50318284; E-mail: cinzia.parolini@unimi.it.

[1] The abbreviations used are: HDL, high density lipoproteins; C, cholesterol; apo, apolipoprotein; A-I_M, A-I_{Milano}; k-in, knock-in; GGE, gradient gel electrophoresis; RT, reverse transcription; FIAU, 1(1-2-deoxy-2-fluoro-β-Darabinofuransyl)-5-iodouracil.

rate for the apoA-I variant known as apoA-I_{FIN}, in addition to its enhanced clearance from plasma (30). ApoA-I turnover in apoA-I_M carriers was investigated in two different studies (31, 32). Both studies showed that low apoA-I levels are consequent to the rapid catabolism of apoA-I and apoA-I_M, whereas results on the production rate of both the normal and mutant forms of apoA-I have remained controversial.

Although mice expressing an A-I_M transgene were previously generated and studied (33, 34), the technical limitations of microinjection (unpredictability of chromosomal location and copy number of the transgene) did not allow definitive establishment of the molecular mechanisms of the phenotypic expression of the mutation. In the present study, a gene targeting replacement strategy (gene knock-in (k-in)) was used to obtain comparable mouse lines expressing either human apoA-I (A-I k-in) or human apoA-I_M (A-I_M k-in). Using these mouse models we present evidence that the dominant negative effects on HDL-C concentrations resulting from the apoA-I_M mutation can, in part, be explained by reduced apoA-I_M production. The expression of this mutation does not appear to affect transcription or mRNA stability but causes impaired hepatic secretion of the human apolipoprotein by primary hepatocytes.

EXPERIMENTAL PROCEDURES

Gene Targeting Replacement—A 3-kb *Not*I-*Kpn*I strain 129 mouse genomic fragment containing sequences upstream of the mouse apoA-I gene (2,633 bp) and including exon 1 and the 5′ part of intron 1 (104 bp) (see Fig. 1*B*) was generated by PCR using a pV-90 plasmid, kindly provided by Dr. N. Maeda (35), as a template. A 4.8-kb *Eco*RI strain 129 mouse genomic fragment, containing the 3′ half of exon 4 (419 bp) and 3′-flanking sequences (4,381 bp) of the mouse apoA-I gene was also derived from pV-90 (see Fig. 1*B*). Human genomic fragments, containing the 3′ part of intron 1 (116 bp) and exons 2–4 of the human apoA-I gene or the human apoA-I_M gene were isolated by sequential digestion with *Kpn*I and *Sal*I from pApoA-Ig (36) and pBS_M plasmids (34), respectively. To obtain the targeting constructs, the 3-kb mouse genomic fragment and the human genomic fragments were inserted into a pPN2T vector (37) upstream of the neomycin-resistant gene, whereas the 4.8-kb genomic fragment was inserted downstream of the neomycin-resistant gene (see Fig. 1*B*). The targeting constructs were linearized by *Not*I digestion, purified, and redissolved in TE (10mM Tris-Hcl, pH 8, 1 mM EDTA), pH 7.4, for electroporation. A subclone of mouse strain 129 embryonic stem cell line, ESVJ (Go Germline, GenomeSystems, Inc.), was cultured on neomycin-resistant mouse fibroblast feeder layers and electroporated with 20 μg of the linearized human apoA-I or apoA-I_M targeting vectors as described previously (38). Stable integrants were selected by positive-negative selection, using neomycin (G418-Geneticin; Invitrogen) at a final concentration of 200 μg/ml and gancyclovir (FIAU, Moravek Biochemicals, Brea, CA) at a final concentration of 2 μM. After 10–12 days, the colonies were transferred into 96-well plates and tested for successful targeting by Southern blotting using conventional procedures. Approximately 10–15 embryonic stem-targeted cells were injected into the blastocele cavity of C57BL/6J embryos. Surviving blastocysts were transferred into the pseudopregnant CD-1 females. Animals chimeric by coat color were bred to C57BL/6J animals to determine their germ line competency. Heterozygous mutants were identified by Southern blotting of DNA isolated from the tail, and brother-sister mating was carried out to generate homozygous k-in mutant mouse lines expressing human apoA-I (A-I k-in) or human apoA-I_M (A-I_M k-in). Homozygous A-I k-in and A-I_M k-in mice were then crossed to create the heterozygous human apoA-I/A-I_M mouse line (A-I/A-I_M k-in).

Lipid/Lipoprotein Analyses—Lipid and apolipoprotein analyses were performed on A-I k-in, A-I_M k-in, A-I/A-I_M k-in, and C57BL/6J/129 control mice of both sexes, aged 12–16 weeks. Blood was collected after an overnight fast from the retro-orbital plexus into tubes containing 0.1% (w/v) EDTA and centrifuged in a microcentrifuge for 10 min at 8000 rpm at 4 °C. Serum total and unesterified cholesterol were measured by enzymatic methods (Hoffmann La Roche, Basel, Switzerland and Roche Molecular Biochemicals) (39). Triglyceride concentrations were corrected for the free glycerol present in serum as described (Sigma-Aldrich) (40). HDL cholesterol levels were measured after precipitation of apoB-containing lipoproteins with PEG 8000 (20% w/v) in 0.2 M glycine, pH 10 (40). Human apolipoprotein concentrations were determined by immunoturbidimetric assays, using a sheep antiserum specific for human apoA-I (Hoffmann La Roche) that also recognizes apoA-I_M (34).

To determine HDL particle size distribution, total lipoproteins ($d <$ 1.215 g/ml) were isolated by salt gradient ultracentrifugation (41). Plasma from five fasting mice of each genotype was pooled and adjusted to a density of 1.215 g/ml with solid KBr and centrifuged for 6 h at 4 °C at 100,000 rpm in a Beckman TL100 ultracentrifuge equipped with a Beckman TL100.3 rotor. HDL particle size distribution was determined by nondenaturing polyacrylamide gradient gel electrophoresis (GGE) essentially as described by Nichols *et al.* (42). Aliquots (20 μl) of the total lipoprotein fraction were loaded onto a nondenaturing 4–30% polyacrylamide gradient gel and electrophoresed for 25 h at 125 V at 4 °C. The proteins were stained with Coomassie R-250, and HDL particle size was determined by densitometry, as previously described (42).

Two-dimensional electrophoretic maps of mouse sera were obtained by immobilized pH gradient gel-Da (43). Serum was prepared by low speed centrifugation of blood collected from six fasting mice for each line. The sample load was 15 μl of serum diluted to 50 μl with either double distilled water for nonreducing conditions or with 2% β mercaptoethanol for reducing conditions. The proteins were first resolved according to charge on a nonlinear pH 4–10 immobilized pH gradient gel-Da (44) in the presence of 8 M urea and 0.5% carrier ampholytes. The focused proteins were then fractionated according to size by SDS-PAGE on 7.5–17.5% polyacrylamide gradients in the discontinuous buffer system of Laemmli (45). Interfacing between the first and second dimension occurred after equilibration with 2% SDS for nonreducing conditions or after protein carboxymethylation in the presence of 2% SDS (46) for reducing conditions. The anode to cathode distance was 11 cm in the immobilized pH gradient gel-Da gel; the anodal 8 cm were mounted head to tail on 16 × 14-cm^2 SDS-PAGE slabs. The proteins were stained with Coomassie R-250.

Northern Blot Analysis—Total RNA was extracted from mouse liver according to the method of Chomczynski and Sacchi (47), using Ultra-Pure™ Trizol Reagent (Invitrogen). For Northern blot analysis, 15 μg of denatured RNA was separated by formaldehyde-agarose gel electrophoresis, transferred to nylon membrane, and hybridized with a human apoA-I probe that spans the majority of human exon 4; β-actin mRNA (Ambion) was used as an internal standard for normalizing total RNA loads.

Quantitative Real Time RT-PCR—Total RNA was extracted from the liver and intestine of 6 mice from each transgenic line using Ultra-Pure™ Trizol Reagent (Invitrogen). About 2 μg of total RNA from each sample was treated with Promega RQ1 RNase-free DNase. About 0.5 μg of DNase-treated total RNA was reverse-transcribed using TaqMan reverse transcription reagents from Applied Biosystems. PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7700 sequence detector. The selected primers used for amplification of human apoA-I cDNA were AGCTTGCTGAAGGTGGAGGT (in exon 4) and ATCGAGTGAAGGACCTGGC (in exon 3). The primers amplify a 154-bp product. The 18 S internal standard control was from Ambion (315 bp product). A ratio of 1:1.5 of 18 S primer pair:18 S competimers was used. All of the procedures and calculation of the results were carried out according to manufacturer's recommendations.

Hepatic Human apoA-I or apoA-I_M Synthesis and Secretion Rates—Apolipoprotein synthesis rate was determined in primary hepatocytes isolated from A-I_M k-in, A-I/A-I_M k-in, and A-I k-in mice. The animals were fasted for 5 h and anesthetized with 5% sodium pentobarbital, and the hepatocytes were prepared with slight modifications of a method described previously (48). Cell viability was assessed by trypan blue staining, and 500,000 live cells were plated on 35-mm plates. The culture medium (Williams' medium; Sigma) was changed after 4 h of incubation at 37 °C. The next day, to assess the human apolipoprotein synthesis rates, the cells were washed once with phosphate-buffered saline, preincubated for 1 h in leucine-free Dulbecco's modified Eagle's medium without serum, and then incubated for different time points up to 10 min with 1 ml of the same medium containing 200 μCi/ml [^{3}H]leucine (PerkinElmer Life Sciences). After incubation, the cells were washed three times with ice-cold phosphate-buffered saline and subsequently lysed in cold lysis buffer containing protease inhibitors (phosphate-buffered saline, 1% Triton X-100, 0.01% phenylmethylsulfonyl fluoride, and 0.005% aprotinin). As a control, the incorporation of radioactivity into total protein was determined after trichloroacetic acid precipitation of cell lysates and was found to be similar among the k-in lines (data not shown). Radiolabeled human apolipoproteins were quantitatively isolated from cell lysates by immunoprecipitation using a rabbit polyclonal anti-human apoA-I antibody (DAKO, Glostrup,

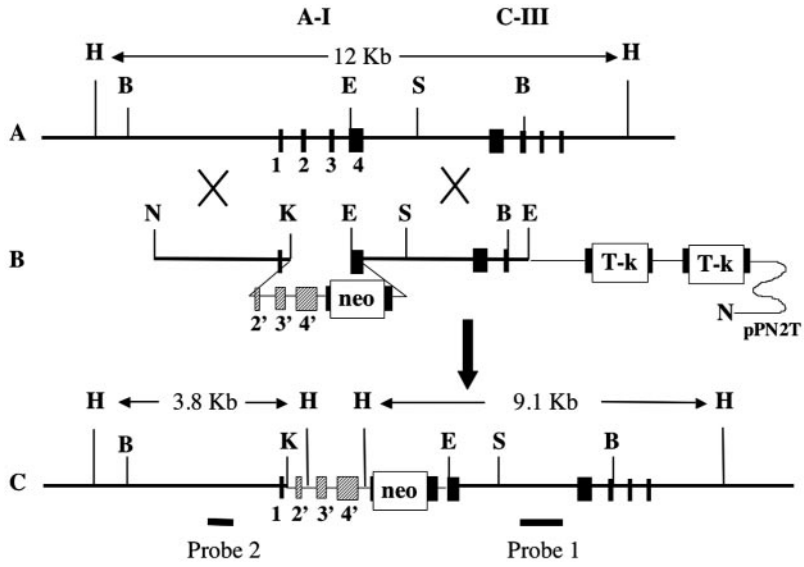

FIG. 1. **Strategy for targeted replacement of the mouse apoA-I gene with the human apoA-I or apoA-I_M gene.** *A*, endogenous mouse apoA-I and apoC-III loci, each with four exons (*black boxes*). *B*, the targeting construct containing the 5′ and 3′ arms of mouse homology (*black lines* and *boxes*) interrupted by the human apoA-I or apoA-I_M gene (*hatched boxes 2′*, *3′*, and *4′*); the neomycin-resistant (*neo*) and thymidine kinase (*T-k*) genes are for positive-negative selection of the targeted cells, and pPN2T is the plasmid vector. *C*, the resulting chimeric gene after homologous cross-over now coding human apoA-I or apoA-I_M. The sizes of diagnostic fragments are indicated. *Probes 1* and *2* are a 800-bp *Sac*I-*Xba*I fragment and a 350-bp *Xba*I-*Sph*I fragment, respectively. Restriction sites are as follows: *H*, *Hin*dIII; *B*, *Bam*HI; *E*, *Eco*RI; *S*, *Sac*I; *N*, *Not*I; *K*, *Kpn*I.

Denmark) that recognizes both human apoA-I and apoA-I_M. The immunoprecipitate was further purified by SDS-PAGE under reducing conditions. A band corresponding to human apoA-I was excised from the gel; the label was extracted with Solvable (Packard Instruments Co., Inc., Meriden, CI) and counted (49). The results were normalized to cellular protein content of each plate determined by the method of Bradford (50). The data presented are the means of triplicate measurements and are representative of three independent experiments.

To determine apoA-I secretion rate, the cells were isolated, plated, and incubated essentially as described above except that conditioned medium was collected after 30, 60, 90, and 120 min. The medium was centrifuged at 12,000 × *g* at 4 °C for 5 min to remove cell debris. The human apolipoproteins were quantitatively isolated from the medium by immunoprecipitation and then purified by SDS-PAGE under reducing conditions. A band corresponding to human apoA-I was excised from the gel and counted (49). The results were normalized to the cellular protein content of each plate determined by the method of Bradford (50). The data presented are the means of triplicate measurements and are representative of three independent experiments.

Statistical Analysis—Differences among groups were evaluated using a one-way analysis of variance followed by a Bonferroni's post-hoc test. Differences in the synthesis and secretion rate of human apolipoproteins were evaluated by linear regression.

RESULTS

Replacement of the Mouse ApoA-I Gene with the Human ApoA-I or ApoA-I_M Gene—The targeting strategy used to replace the mouse apoA-I coding exons 2–4 with the human counterpart is illustrated in Fig. 1. Homologous recombination between the endogenous mouse apoA-I locus (Fig. 1*A*) and the targeting construct (Fig. 1*B*) results in a chimeric gene (Fig. 1*C*) where all of the mouse coding sequences have been replaced with sequences coding for human apoA-I or apoA-I_M. This chimeric locus retains all of the normal mouse regulatory elements in addition to the noncoding mouse exon 1.

Embryonic stem cell DNA, digested with *Hin*dIII and hybridized with probe 1 (Fig. 1), revealed a 12-kb endogenous band and a 9.1-kb targeted band, resulting from the novel restriction site, demonstrating the correct location of the targeted gene in the 3′ region (Fig. 2*A*). Similarly, hybridization with probe 2 (Fig. 1) confirmed correct modification of the 5′ region (data not shown). The modified locus (apoA-I or apoA-I_M) was transmitted to the F1 generation from chimeras that were made from one of the targeted cell lines. Genotypes of F2 animals were determined using Southern blotting analysis of tail DNA digested with *Hin*dIII and hybridized with probe 2, revealing a 12-kb endogenous band and a 3.8-kb targeted band (Fig. 2*B*).

Serum Distribution of Human Apolipoproteins—The expression of human apoA-I or apoA-I_M was assessed by two-dimensional electrophoretic analysis on mouse serum (Fig. 3). A 28-kDa spot was observed in each serum analyzed, corresponding either to murine apoA-I (only in control serum), human apoA-I, or the monomeric form of apoA-I_M. From their sequence, the pI of murine apoA-I is computed at 5.42, the pI of human apoA-I at 5.27, and the pI of human apoA-I_M at 5.19, as indicated in the Fig. 3. The spots marked with superscript *−1* correspond to a post-translationally modified form that differs from the *0* superscript form for a Asn → Asp deamidation event (51). Because the charge difference at pH = pI is 1 unit both between apoA-I and apoA-I_M and between A-I^0 and A-I^{-1}, A-$I_M{}^0$ and A-I^{-1} do overlap (see in Fig. 3 the spot indicated as *A-I^{-1}+A-$I_M{}^0$*). As expected, in A-I_M k-in and A-I/A-I_M k-in serum, an additional spot (see *circles* in Fig. 3, *upper panel*) corresponding to the dimeric form of apoA-I_M (56 kDa), is visible and disappears upon sample reduction (Fig. 3, *lower panel*).

Lipid and Apolipoprotein Concentrations—Mouse plasma lipid and apolipoprotein concentrations are shown in Table I. The apoA-I_M concentrations in A-I_M k-in mice was ~50% of apoA-I in A-I k-in mice; the concentration of the human apolipoproteins in the apoA-I/A-I_M k-in mice was the same as in A-I_M k-in. Total cholesterol in A-I_M k-in mice was significantly lower than that measured in every other group. The heterozygotes (A-I/A-I_M k-in) had total cholesterol values intermediate to A-I_M and A-I k-in mice. Plasma HDL cholesterol concentrations in A-I_M k-in mice were substantially lower than those observed in A-I k-in and A-I/A-I_M k-in mice (−63% and −50%, respectively). In addition, a significant increase in plasma unesterified to esterified cholesterol ratio was observed in A-I_M k-in compared with A-I k-in mice (0.69 ± 0.13 *versus* 0.41 ± 0.02; $p < 0.001$), suggesting impaired cholesterol esterification in the former. In contrast to changes in cholesterol concentrations, plasma triglyceride levels were similar in all of the mouse lines analyzed.

HDL particle size distribution of k-in mice was investigated by nondenaturing GGE. As seen in Fig. 4, HDL from A-I k-in mice has a homogeneous population of large particles (10.79 nm). Control mice had a similar HDL size distribution (data not shown). The GGE profile of A-I_M k-in mice is heterogeneous, exhibiting a major HDL subpopulation of smaller particles (8.96 nm) and minor populations at 9.81 and 10.79 nm. In contrast, the GGE profile of A-I/A-I_M k-in mice is characterized by a bimodal size distribution with a major population at 10.79 nm and a minor one at 8.96 nm.

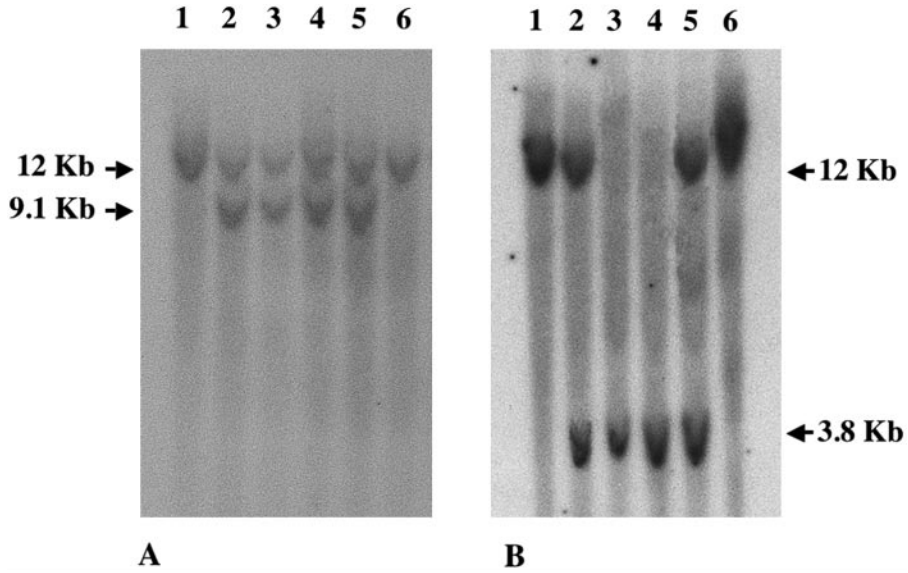

FIG. 2. **Southern blot analysis.** *A*, Southern blot of genomic DNA from six embryonic stem cell colonies digested with *Hin*dIII and hybridized to probe 1 (see Fig. 1). Parental cell DNA is in *lanes 1* and *6*; the 12-kb band indicates the presence of the unmodified apoA-I allele. *Lanes 2* and *3* and *lanes 4* and *5* contain DNA from human apoA-I and apoA-I_M colonies, respectively, that have been correctly targeted; a 9.1-kb band is present in addition to the 12-kb band. *B*, Southern blot analysis of tail DNA digested with *Hin*dIII and hybridized to probe 2 (see Fig. 1) to identify F2 mice carrying the targeted allele. A 3.8-kb band indicates the presence of the human apoA-I allele. Shown are two controls (*lanes 1* and *6*), two heterozygous (*lanes 2* and *5*), and two homozygous (*lanes 3* and *4*) k-in mice. *Lanes 2* and *3* and *lanes 4* and *5* contain tail DNA from A-I and A-I_M k-in mice, respectively.

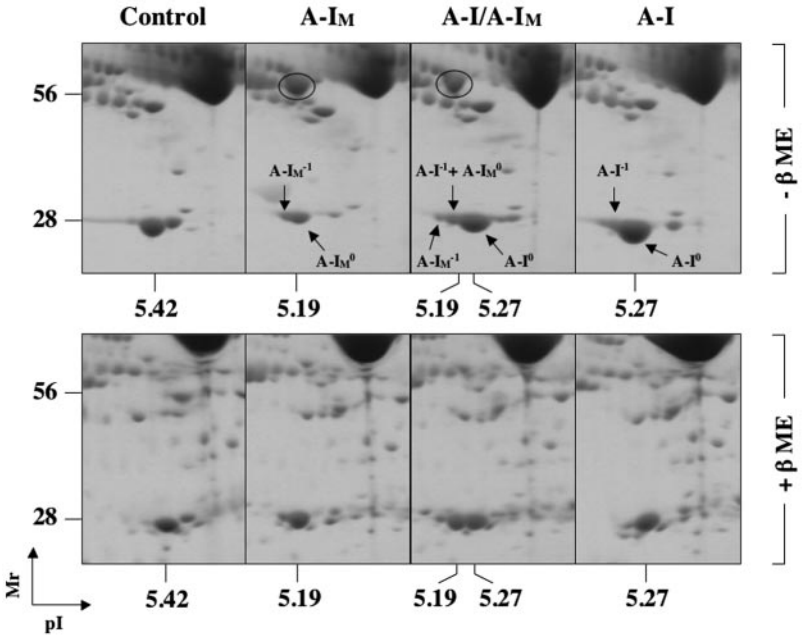

FIG. 3. **Close-up views of two-dimensional electrophoretic maps of mouse sera pools obtained from control, A-I_M k-in, A-I/A-I_M k-in, and A-I k-in mice under nonreducing (*upper panel*, *−β ME*) and reducing (*lower panel*, *+β ME*) conditions.** The proteins were first resolved according to charge on a nonlinear pH 4–10 IPG and then fractionated according to size by SDS-PAGE and stained with Coomassie R-250. The *circles* indicate the spot corresponding to the dimeric form of human apoA-I_M.

TABLE I

Plasma lipid profiles and human apolipoprotein concentration in k-in and control mice

The results are expressed as the means ± S.D. TC, total cholesterol; FC, free cholesterol; TG, triglyceride.

Mice	*n*	TC	FC	HDL-C	TG	apoA-I or apoA-I_M
		mg/dl	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*
A-I_Mk-in	9	52.89 ± 5.87[a,b]	21.21 ± 2.32[c,d]	28.08 ± 5.00[e]	33.08 ± 24.12	100.68 ± 19.43[c]
A-I/A-I_M k-in	8	78.07 ± 5.46	19.29 ± 1.45	56.68 ± 1.96	29.41 ± 14.34	107.78 ± 30.18[c]
A-I k-in	8	132.04 ± 34.58	38.26 ± 9.09	75.46 ± 14.85	31.78 ± 21.75	213.70 ± 41.76
Controls	5	106.80 ± 7.76	29.37 ± 2.30	57.29 ± 3.00	57.61 ± 20.30	

[a] $p < 0.001$ *versus* A-I k-in, and control mice.
[b] $p < 0.05$ *versus* A-I/A-I_M k-in mice.
[c] $p < 0.001$ *versus* A-Ik-in mice.
[d] $p < 0.05$ *versus* control mice.
[e] $p < 0.001$ *versus* A-I k-in, A-I/A-I_M k-in, and control mice.

Human Apolipoprotein Expression—Apolipoprotein A-I or A-I_M gene expression was assessed by Northern blot analysis on livers of six mice, matched for age and sex, from each one of the three lines (A-I_M k-in, A-I/A-I_M k-in, and A-I k-in). The data were normalized to the constitutively expressed β-actin, and the average values are shown in Fig. 5. No significant differences were observed between human apoA-I and apoA-I_M mRNA levels in the three k-in lines. This lack of difference in the human apolipoprotein expression was confirmed by quantitative real time RT-PCR. In fact, the apoA-I/A-I_M mRNA expression (normalized to the endogenous control 18 S) was 0.633 ± 0.159 for A-I k-in mice and 0.593 ± 0.244 for A-I_M k-in mice ($p = 0.778$).

Quantitative real time RT-PCR was also performed on total

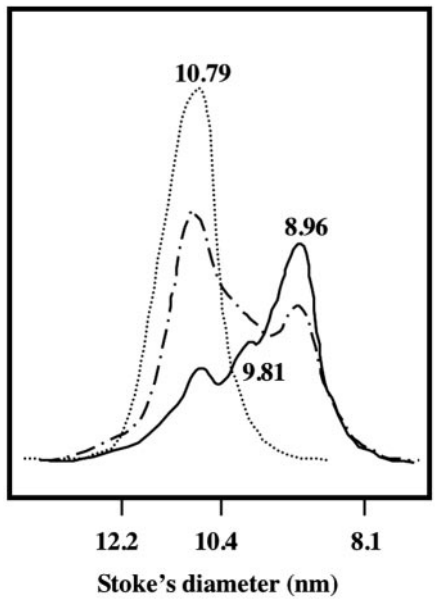

FIG. 4. **Nondenaturing GGE of mouse lipoproteins.** Total lipoproteins ($d < 1.215$ g/ml) were loaded onto a nondenaturing 4–30% polyacrylamide gradient gel, and the proteins were stained with Coomassie R-250. Computer-assisted scanning densitometry of the gel was used to determine electrophoretic pattern and particle size as described under "Experimental Procedures." HDL particle size distribution of A-I_M k-in (*solid line*), A-I/A-I_M k-in (*dashed line*), and A-I k-in (*dotted line*) mice.

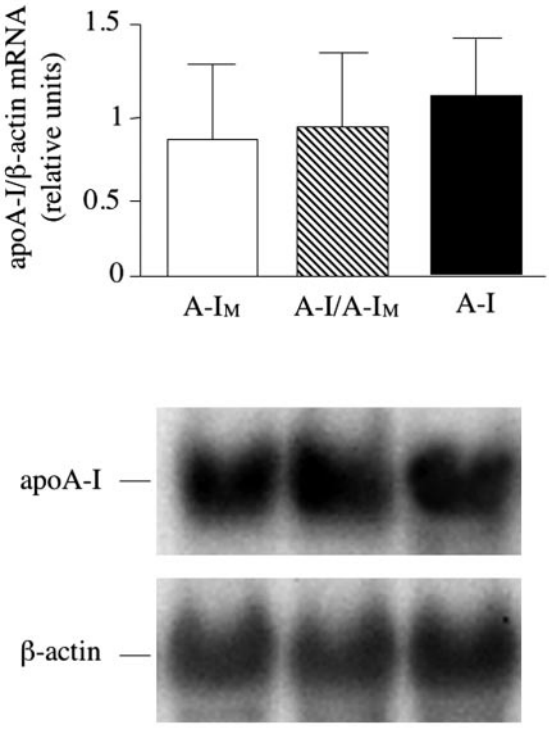

FIG. 5. **Quantitative Northern blot analysis of apoA-I gene expression in liver.** RNA was prepared from livers of A-I_M, A-I/A-I_M and A-I k-in mice, Northern blotted, and hybridized with a human apoA-I probe that spans the majority of human exon 4. The *bar graph* in the *upper panel* shows the amount of apoA-I mRNA corrected for the amount of constitutively expressed β-actin mRNA. In the *lower panel*, representative blots from each of the three mouse genotypes are shown. The *bar graphs* represent the means ± S.D.

RNA extracted from mouse intestine; in each mouse line, intestinal expression of the human apolipoproteins contributed for 28–32% of the total amount expressed. Similarly to what

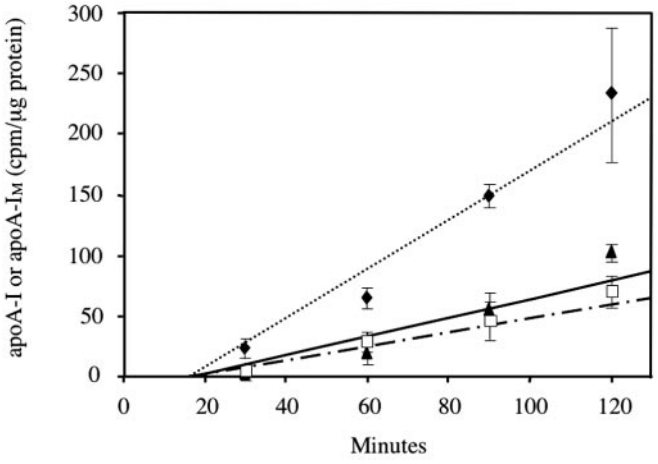

FIG. 6. ***In vitro*** **analysis of apoA-I or apoA-I_M secretion from primary hepatocytes.** The hepatocytes were prepared from A-I_M k-in, A-I/A-I_M k-in, and A-I k-in mice, and the presence of the human apolipoproteins in the medium (normalized to cell protein content) was determined at 30, 60, 90, and 120 min after addition of [^{3}H]leucine. ▲, apoA-I_M secretion; □, apoA-I/A-I_M secretion; ♦, apoA-I secretion. The data are representative of three separate experiments; each point is the mean ± S.D. of triplicate measurements.

was observed in livers, no differences were detected among the lines ($p > 0.05$).

Analysis of Human ApoA-I or ApoA-I_M Production—Synthesis and secretion rates of the human apolipoproteins were assessed in cultured primary hepatocytes isolated from A-I_M k-in, A-I/A-I_M k-in, and A-I k-in mice. For the evaluation of synthesis rate, the appearance of intracellular radiolabeled human apolipoproteins was measured at different time points and was found to be linear along the experimental period. The synthesis rates, calculated as the slope of the regression lines, were not different among the k-in lines (2.92 cpm/μg/min for A-I_M k-in, 2.78 cpm/μg/min for A-I/A-I_M k-in, and 2.89 cpm/μg/min for A-I k-in mice). For the secretion rate, radiolabeled apoA-I or apoA-I_M was quantified in culture medium at 30, 60, 90, and 120 min after the addition of [^{3}H]leucine. As shown in Fig. 6, secretion of apoA-I/A-I_M was linear for all of the k-in mouse lines ($r = 0.906$ for A-I k-in mice, $r = 0.934$ for A-I/A-I_M k-in mice, and $r = 0.964$ for A-I_M k-in mice $p < 0.01$). Secretion rates, however, differed dramatically between A-I k-in mice and the other mouse lines, where secretion by apoA-I_M and apoA-I/A-I_M cells was 58 and 64% ($p < 0.05$), respectively, of that calculated for apoA-I hepatocytes.

DISCUSSION

In contrast to classical transgenic approaches, gene targeting replacement strategies (52) for manipulating the mouse genome allow precise location of the transgene, thus permitting direct comparisons between different genes at the same chromosomal location. This procedure has allowed, for the first time, the generation of two animal models that differ only in the biochemical nature of the apoA-I, *i.e.* carrying in one case the human wild type apoA-I gene (A-I k-in) and, in the other, the apoA-I_M gene (A-I_M k-in). These mice provide a means to study the molecular mechanisms responsible for the lipoprotein abnormalities noted in apoA-I_M human carriers.

The lipid/lipoprotein profile of A-I_M k-in and A-I/A-I_M k-in mice is, in many respects, similar to that of human carriers, *i.e.* characterized by low plasma total and HDL-C levels compared with A-I k-in mice. Reduction in HDL-C concentrations are also associated with the appearance of a heterogeneous population of HDL particles not present in control and A-I k-in mice. Nevertheless, most noteworthy are the differences observed

between A-I_M k-in and A-I/A-I_M k-in mouse lines. We found that the expression of apoA-I in the A-I_M k-in mouse background did not increase the plasma apolipoprotein concentrations but did increase plasma total and HDL-C levels and altered HDL size distribution. This difference could be explained by the fact that the absence of apoA-I, a better cofactor for LCAT activity (53, 54), may impair cholesterol esterification in A-I_M k-in mice, as suggested by the unesterified/esterified cholesterol ratio measured in this mouse line (0.69 ± 0.13 *versus* 0.33 ± 0.04 in A-I/A-I_M k-in mice), allowing the formation of cholesterol-poor HDL particles (34). In addition, the decreased formation of cholesteryl esters may result in a diminished core of HDL particles and hence in a reduced HDL particle size.

Differently from the apoA-I_M clinical condition (55) and the transgenic model previously generated, expressing both human apoA-I_M and apoA-II (33, 34), a clear rise of triglycerides in the A-I_M k-in model was not observed. In the A-I_M k-in line, triglyceride elevation was, in fact, of minimal degree and did not attain statistical significance. Moreover, A-I_M k-in mice displayed an HDL size distribution that lacks a subpopulation of very small particles present in both human carriers (55) and in the previously generated A-I_M/A-II transgenic mice (33, 34). A possible explanation for these differences may reside in the absence of human apoA-II in A-I_M k-in mice. Overexpression of human apoA-II has been shown, in fact, to be associated with hypertriglyceridemia and small HDL particles (56). Moreover, in human carriers and in A-I_M/A-II transgenic mice the presence of human apoA-II allows the formation of A-I_M/A-II heterodimers, because the human apoA-II contains a free cysteine residue not present in the murine apoA-II. Franceschini *et al.* (55) found a correlation between hypertriglyceridemia and abundance of small HDL particles (HDL_{3b}), enriched in A-I_M/A-II heterodimers. Furthermore, experimental data have shown that the expression of human apoA-II in apoA-I transgenic mice increased plasma triglyceride levels and restricted HDL particle size (40). In summary, although speculative, in the absence of human apoA-II, *i.e.* in the present k-in mouse model, triglyceride metabolism is not affected by the presence of the apoA-I mutant, thus accounting for normal triglyceride levels in A-I_M k-in and A-I/A-I_M k-in mice.

A major objective of the present study was to utilize the k-in mice to explore the possibility that hypoalphalipoproteinemia associated with apoA-I_M is due to defective expression of the human apoA-I_M gene. Quantitative real time RT-PCR on liver mRNA, coherent with Northern blot analysis, did not show any significant difference in the apoA-I and apoA-I_M gene expression, revealing that neither transcription nor mRNA stability is responsible for the low apoA-I_M plasma levels. Moreover, because the intestine contributes significantly to the apoA-I expression, we have also performed quantitative real time RT-PCR on mouse intestine, and having obtained similar results among the mouse lines, we could demonstrate that differences between the plasma levels of apoA-I and A-I_M are not a consequence of a lower intestinal apoA-I_M mRNA expression. Differences in apolipoprotein plasma levels cannot also be attributed to an altered apoA-I_M synthesis rate, because experiments performed in primary hepatocytes demonstrated comparable results among the k-in lines. In contrast, secretion of human apolipoproteins into the medium was reduced in both apoA-I_M and apoA-I/A-I_M hepatic cells compared with apoA-I hepatocytes, reflecting the apolipoprotein levels detected in mouse plasma. These data suggest that an impaired apoA-I_M hepatic secretion contributes to the reduction of apoA-I_M plasma levels observed in human carriers. Although speculative, reduced apoA-I_M secretion may be related to a different intracellular processing (*i.e.* dimerization) and/or transport of the mutant apolipoprotein.

The possible kinetic basis for the decreased plasma apoA-I and A-I_M levels in apoA-I_M carriers was previously examined by radiolabeling normal and mutant apoA-I and injecting them into normal and apoA-I_M subjects (31). This study has shown that the hypoalphalipoproteinemia in apoA-I_M carriers is apparently caused by the rapid catabolism of both apoA-I and apoA-I_M, with a normal production rate of the normal and mutant forms of apoA-I. ApoA-I_M also appeared to be catabolized more rapidly as a monomer than as a dimer. The clinical study was thus not wholly consistent with our observation that apoA-I_M secretion is impaired. Further, a more recent study by Perez-Mendez *et al.* (32) evaluating the turnover kinetics of apoA-I- and apoA-I_M-specific subclasses using stable isotope techniques also corroborated these earlier findings by indicating that hypercatabolism of apoA-I and apoA-I_M accounted for a major reduction in apoA-I and HDL in human carriers, whereas the total apoA-I production rate appeared not to be altered. However, detailed examination of the data also indicates that production rates for apoA-I_M monomers and apoA-I_M dimers are considerably lower than for normal apoA-I. These observations are consistent with our finding that the hepatic secretion of apoA-I_M is impaired. In conclusion, we suggest that both factors, *i.e.* reduced secretion of apoA-I_M and rapid catabolism of normal and mutant apoA-I, are major contributors to the hypoalphalipoproteinemia found in human apoA-I_M carriers.

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