Effect of Apolipoprotein M on High Density Lipoprotein Metabolism and Atherosclerosis in Low Density Lipoprotein Receptor Knock-out Mice

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To investigate the role of apoM in high density lipoprotein (HDL) metabolism and atherosclerosis, we generated human apoM transgenic (apoM-Tg) and apoM-deficient (apoM−/−) mice. Plasma apoM was predominantly associated with 10–12-nm α-migrating HDL particles. Human apoM overexpression (11-fold) increased plasma cholesterol concentration by 13–22%, whereas apoM deficiency decreased it by 17–21%. The size and charge of apoA-I-containing HDL in plasma were not changed in apoM-Tg or apoM−/− mice. However, in plasma incubated at 37 °C, lecithin:cholesterol acyltransferase-dependent conversion of α- to pre-α-migrating HDL was delayed in apoM-Tg mice. Moreover, lecithin: cholesterol acyltransferase-independent generation of pre-β-migrating apoA-I-containing particles in plasma was increased in apoM-Tg mice (4.2 ± 1.1%, p = 0.06) and decreased in apoM−/− mice (0.5 ± 0.3%, p = 0.03) versus controls (1.8 ± 0.05%). In the setting of low density lipoprotein receptor deficiency, apoM-Tg mice with ~2-fold increased plasma apoM concentrations developed smaller atherosclerotic lesions than controls. The effect of apoM on atherosclerosis may be facilitated by enzymatic modulation of plasma HDL particles, increased cholesterol efflux from foam cells, and an antioxidative effect of apoM-containing HDL.

Epidemiological studies have demonstrated a strong inverse association between plasma HDL concentrations and risk of premature coronary heart disease (1). However, the molecular heterogeneity of HDL has posed difficulties in understanding HDL metabolism and its effects in atherosclerosis. In 1999, Xu and Dahlbäck (2) discovered apoM, which is mainly associated with HDL, with smaller amounts in LDL and VLDL. ApoM appears to have a novel role in murine HDL metabolism by affecting pre-β-HDL formation (3). Pre-β-HDL is considered antiatherogenic, because it mediates ATP-binding cassette transporter A1 (ABCA1)-dependent efflux of cholesterol from foam cells as part of the reverse cholesterol transport. Pre-β-HDLs, the precursor of mature HDLs, are ~7-nm particles containing apoA-I and phospholipid and can be derived from two sources (4, 5). First, pre-β-HDL can arise from newly synthesized hepatic apoA-I, acquiring free cholesterol and phospholipids through the interaction of apoA-I with ABCA1 (6). The small phospholipid-containing apoA-I particles can act via ABCG1 and remove cholesterol from foam cells (7–9). The free cholesterol in HDL can be esterified by lecithin:cholesterol acyltransferase (LCAT) (10, 11), changing the electrophoretic mobility of HDL from pre-β to α (12–14). In mice, overexpression of LCAT converts α-HDL to pre-α-HDL (15). Second, pre-β-HDL can arise from α-HDL through the action of phospholipid transfer protein (PLTP), which induces fusion of α-HDL particles and concomitant dissociation of small pre-β-HDL particles (16–18). Also, hepatic lipase (HL) can generate pre-β-HDL from α-HDL through its triglyceride lipase activity, either in concert with cholesteryl ester transfer protein (19) or alone (as in mice, which naturally lack cholesteryl ester transfer protein) (20). It is unknown how apoM might affect pre-β-HDL formation.

ApoM (188 amino acids) is 80% identical at the amino acid level in mice and humans and is found in primitive organisms (e.g. fish and frogs) (21). In humans and mice, apoM is...
expressed in hepatocytes and in kidney proximal tubule cells (2, 22). Kidney-derived apoM appears to be secreted into peritoneum and is normally reabsorbed in a megalin-dependent fashion (23). Thus, liver-derived apoM probably is the main source of plasma apoM. ApoM is secreted from the liver without cleavage of the hydrophobic signal peptide, which probably serves as an anchor in the lipid moiety of plasma lipoproteins (2). Structurally, the remaining portion of apoM resembles retinol-binding protein. Thus, apoM was assigned to the lipocalin protein superfamily, whose members bind small lipophilic ligands in a common hydrophobic binding pocket (24). Although the physiological ligand is unknown, recombinant human and mouse apoM can bind retinol and retinoic acid (25). In human plasma, ~5% of the HDL particles contain apoM (26), and the apoM-containing HDL is as heterogenous as the total HDL population in size, density, and protein composition (26). Although apoM is not present on all HDL particles, recent data have shown a positive association between apoM plasma levels and HDL-cholesterol concentrations in humans (27). This supports the possibility that apoM may play a role in HDL metabolism.

To explore roles of apoM in HDL metabolism and atherosclerosis, we generated mice that constitutively lack apoM or overexpress human apoM. The data suggest that plasma apoM is mainly associated with α-HDL and that it affects their conversion into pre-α- and pre-β-HDL particles. Moreover, the data demonstrate that doubling the apoM concentration leads to reduced development of atherosclerosis. This may result from effects of apoM on the enzymatic modulation of plasma HDL, cholestrol efflux from foam cells, and/or an antioxidative effect of apoM-containing HDL.

**MATERIALS AND METHODS**

**Generation of Human ApoM-Tg Mice and ApoM Gene-targeted Mice**—Human apoM-Tg mice were generated with a 6817-bp genomic DNA fragment spanning the human apoM coding sequences and 2844 bp 5' to the transcription start site and 1729 bp 3' to poly(A) sequences. Upon breeding of hemizygous transgenic mice from both lines with WT mice, ~50% of the offspring were transgenic. To create apoM+/− mice, we cloned an Apom targeting vector designed to delete 39 bp of the endogenous APOM sequences in exon 2 and insert a neomycin resistance-encoding cassette in the APOM locus. An APOM gene-targeting vector was cloned in embryonic stem cells that were subsequently used to make chimeric APOM-targeted mice. Genotyping was done with real time PCR using two sets of primers amplifying the intact APOM and the neomycin resistance-encoding cassette, respectively. Of 103 offspring from crosses of apoM+/− male and females, 26 (25%) were apoM−/−, 57 (55%) were apoM+/−, and 20 (19%) were apoM+/. Breeding of apoM−/− male and females (n = 9 pairs) resulted in litters of 9.4 ± 0.4 pups. For a detailed description of molecular biology methods, please see the supplemental material.

**Animals**—Mice were housed at the Panum Institute (University of Copenhagen) in a temperature-controlled facility with a 12-h dark/light cycle and fed standard chow (Altromin number 1314; Brogaarden) unless otherwise noted. Human apoM-transgenic mice were back-crossed for at least five generations with C57B6/J mice. All apoM-Tg mice studied were hemizygous and compared with littermate controls. Founder apoM+/− mice were back-crossed with C57B6/J background for two generations and intercrossed to obtain apoM−/− and WT apoM+/+ littermate controls.

ApoM-Tg9 male mice (back-crossed five times with C57B6/J mice) were crossed with LDLR−/− female mice on the C57B6/J genetic background (Taconic Europe) to obtain apoM-Tg2/− LDLR−/− and littermate control LDLR−/− mice. Beginning at 4 weeks of age, mice started a cholic acid-free diet containing 0.3% cholesterol (D01061402, Brogaarden) (28). Atherosclerosis was assessed at 20 weeks of age.

Blood samples were taken from the venous plexus in the orbital cavity into Na2EDTA- or heparin-containing tubes and kept on ice. Plasma was isolated by centrifugation at 3000 rpm for 10 min at 4 °C and stored at −80 °C or −20 °C. To inhibit LCAT, 1.5 mm 5,5-dithiobis-2-nitrobenzoic acid (DNTB) (Sigma) was added to plasma immediately after the removal of blood cells. All procedures were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

**mRNA Quantification**—Tissue biopsies were placed in liquid N2 and stored at −80 °C. RNA was isolated with Trizol (Invitrogen) and examined on an RNA Nano LabChip (Agilent Technologies). First-strand cDNA synthesis and quantitative real time PCR analysis with a Lightcycler was performed as described (29) with primers for human APOM (h-apoM-51 and -31), mouse APOM (22), and β-ACTIN (29) or with the following primers (used to evaluate the mRNA expression of genes located in the same chromosomal region as APOM): BAT1 (5'-tctgtcctgttaggctca-3' and 5'-gtgggctcttctctgca-3'), TNT (5'-gaacctgcaagagacct-3' and 5'-agggctgctgacataaat-3'), LST1 (5'-tcctcctctgctcct-3' and 5'-cgatgcaggcatagtc-3'), AIF1 (5'-agcttttgctgctgca-3' and 5'-ctctggcttcacctct-3'), BAT2 (5'-tggaaggccataaacct-3' and 5'-ggccattgattggaat-3'), BAT3 (5'-cgcccataagagcagagag-3' and 5'-ctgctagtaaaagctgaa-3'), and BAT4 (5'-ctggctgactcctatggtg-3' and 5'-gacagacgctccataaag-3').

**Plasma Lipids**—Lipids in plasma and size exclusion chromatography fractions were measured with enzymatic kits (CHOD-PAP from Roche Applied Science, GPO-Trinder from Sigma, phospholipids B R1/R1A from TriChem Aps, and free cholesterol C, COD-PAP, from Wako Chemicals).

**Quantification of ApoM, ApoA-I, and ApoE**—Human apoM was measured with enzyme-linked immunosorbent assay using two monoclonal anti-apoM antibodies (M58 and M42), as described (26, 27). To evaluate the relative concentrations of apoM in human and mouse plasma, we performed Western blotting of mouse and human plasma (see below) with a rabbit antiserum (anti-h/m-apoM) that recognizes human and mouse apoM with similar efficacies; rabbits were immunized with an apoM peptide (LRTERGPDMDKT) coupled to keyhole limpet hemocyanin by m-maleimidobenzoic acid N-hydroxysuccinimide ester (Cambridge Research Biochemicals). The peptide has 100% homology with both mouse and human apoM. Mouse apoA-I and apoE were quantitated by an enzyme-linked immunosorbent assay (30, 31).

**Isolation and Separation of Plasma Lipoproteins**—HDL (1.063 < d < 1.21 g/liter), VLDL/LDL (d < 1.063 g/liter), and
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d > 1.21 g/liter proteins were isolated by ultracentrifugation of 350–675 μl of mouse plasma at 100,000 rpm and 10 °C for 5–16 h (d = 1.063 g/liter) or 16–24 h (d = 1.21 g/liter) with a Beckman TLA 110 rotor and a Beckman Optima MAX-E ultracentrifuge (Beckman Coulter). In addition, the d > 1.063 g/ml fraction was isolated from 50 μl of mouse plasma using 200-μl ultracentrifugation tubes and a TLA 100 rotor at 100,000 rpm and 15 °C for 4 h. The densities were adjusted with NaBr. Purified VLDL/LDL and HDL were extensively dialyzed against 10 mM PBS with 0.1 g/liter Na2EDTA (PBS-EDTA) at 4 °C and stored at −20 °C. Human HDL3 (1.125 < d < 1.21 g/ml) was prepared as described (32).

Size exclusion chromatography of mouse plasma (200 μl) was performed at 20–24 °C using PBS-EDTA and Superose 6 and Superose 12/300 GL fast protein liquid chromatography (FPLC) columns (GE Healthcare). The flow rate was 0.2–0.4 ml/min. Fractions of ∼250 μl were collected and stored at −20 °C.

For agarose gel electrophoresis, Litex agarose (0.8%) (Medinova) was dissolved in a Tris/barbital buffer (365 mmol/liter Tris and 123 mmol/liter barbital, pH 8.5) and cast on glass plates (0.23 ml/cm2). Plasma (2–4 μl) or HDL (9 μg of total protein in Tris/barbital buffer) was subjected to electrophoresis at 20 °C and 300 V until the dye front had migrated 5.5 cm. For Western blotting, proteins were transferred to 0.45 μm nitrocellulose membranes (Invitrogen) by semidyed electroblotting. For protein staining, gels were fixed in 16:4:1 ethanol/H2O/acetic acid for 45 min, stained for 20 min in Coomassie Brilliant Blue, and destained in 77:25:8 H2O/ethanol/acetic acid.

For two-dimensional gel electrophoresis, HDL was separated with 0.8% Litex agarose gels. After electrophoresis, the lipoprotein-containing portion of each lane was cut into eight consecutive 3-mm slices and placed in 40 mM PBS with 0.1 g/liter Na2EDTA (PBS-EDTA) at 4 °C and stored at −20 °C. Human HDL3 (1.125 < d < 1.21 g/ml) was prepared as described (32).

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For two-dimensional gel electrophoresis, HDL was separated with 0.8% Litex agarose gels. After electrophoresis, the lipoprotein-containing portion of each lane was cut into eight consecutive 3-mm slices and placed in 40 mM nondenaturing sample buffer for 12–16 h at 4 °C to elute proteins. The eluted proteins were separated with nondenaturing 4–20% Novex Tris-glycine gels (Invitrogen) followed by silver staining or were denatured and reduced before electrophoresis in 12% SDS-polyacrylamide gels and Western blotting.

Western blotting of SDS-polyacrylamide gels was done as described (26) using rabbit anti-human apoM IgG (2.5 μg/ml), rabbit anti-mouse apoM IgG (290 ng/ml), monoclonal mouse anti-human apoM antibody (M58; 1:1000), rabbit anti-mouse apoA-I (1:2000 or 1:5000) (Nordic Biosite AB), anti h/m-apoM antiserum (1:200), goat anti-human apoC-I (1:750) (Academy Bio-medical Co.), rabbit anti-mouse apoA-II (1:2000), and rabbit anti-mouse apoE (1:2000) (produced at the National Public Health Institute (Helsinki) by immunizing rabbits with recombinant human apoA-I or purified mouse plasma apoE, respectively).

Crossed immunoelectrophoresis to quantitate pre-β-HDL was performed as described (31), using 2 μl of DNTB-containing mouse plasma that had been preincubated for 4 h at 4 or 37 °C. The first dimension electrophoresis was done in a native 1.0% agarose gel at 270 V until the dye front had migrated 4 cm. The agarose plug was cut from the gel and attached to a gel bond film with an anti-mouse apoA-I antisemum-containing agarose gel; the separated proteins and a control plasma sample (1 μl) were subjected to second dimension electrophoresis into the antiserum-containing gel (50 V for 16 h). After drying, gels were stained with Coomassie Brilliant Blue. The areas of the pre-β- and α-HDL peaks were calculated as the width of the peak at 50% of the peak height multiplied by the peak height.

Plasma Activity of LCAT, PLTP, and HL—Mouse plasma activities of LCAT, PLTP, and HL on exogenous substrates were measured using radiometric assays, as described (18, 33–35).

Atherosclerosis—To analyze atherosclerosis, the aorta was perfused with 0.9% NaCl through a cannula in the left ventricle. The heart with 2–3 mm of the aortic arch was immersed in 4% paraformaldehyde and embedded in tissue-Tek OCT Compound (Sakura Finetek). Aortic cross-sections (10 μm) from the aortic valve area were cut with a Microm HM560 microtome (Brock & Michelsen). Series of 12 sections were collected on three slides with four sections on each slide and 30 μm between sections on a slide. Two consecutive slides from a series were stained with Oil Red O (O-0625; Sigma) to visualize neutral lipids; the third slide was stained with Picro Sirius red (Direct Red 80; Sigma) to visualize collagen. Stained areas were measured with a Leica imaging program IM50 (Leica Microsystems Imaging Solutions). Total en face plaque area fraction in the thoracic aorta (from the heart to the 6th rib) was measured as described (36). Subsequently, aortic lipids were extracted with chloroform/methanol and quantified with a thin layer chromatography-based method (37).

Oxidation of Lipoproteins—Lipoproteins were incubated with Cu2+, and oxidation was assessed from the formation of conjugated dienes as described (26).

Cholesterol Efflux—Peritoneal macrophages were isolated from C57B6/J mice 5 days after an intraperitoneal injection of 3% BBL thioglycollate (BD Biosciences) and cultured in 24-well plates (5 × 105 cells/well) in 0.5 ml of serum-free medium with 30 μg/ml cholesterol (C8667; Sigma) and 200,000 dpm/ml [3H]cholesterol ([1α,2α,3H]cholesterol; GE Healthcare) for 24 h. Foam cell formation was also induced by loading the cells with acetylated LDL, as previously described (26). After washing and equilibration in 0.2% fatty acid-free bovine serum albumin-containing (Sigma) and serum-free medium for 24 h, the cells were incubated with total HDL (12.5 μg of protein/well) from WT, apoM-TgN, or apoM-TgH mice. [3H]Cholesterol in medium was measured after 0, 8, 16, and 24 h to assess cholesterol efflux (26).

Statistics—Differences between groups were analyzed with t tests. Welch’s correction for unequal variances was used whenever appropriate. Results are expressed as mean ± S.E.

RESULTS

Generating Human ApoM Transgenic and ApoM−/−Mice—Five human apoM transgenic (apoM-Tg) founders were generated by pronuclear injection of a ~6.8-kb genomic fragment with the apoM-coding sequences and 2.8 kb of 5’ and 1.7 kb of 3’ sequences. A founder with ~38 copies of the human APOM (apoM-TgN) and one with ~22 copies (apoM-TgH), as determined by real time PCR, were bred for further investigations. The tissue-specific expression of human apoM mRNA in liver and kidney (supplemental Fig. 1A) was similar to that of the endogenous apoM gene (22). Overexpression of human apoM
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did not affect the expression of mouse apoM mRNA in either tissue (not shown). In apoM-Tg\textsuperscript{H} and apoM-Tg\textsuperscript{N} mice, human apoM was detectable in plasma (supplemental Fig. 1B) at \(\approx 100\) and \(\sim 1000\) of the concentration in pooled plasma from healthy blood donors (supplemental Fig. 1C). Western blot analyses with a rabbit antiserum against a common peptide epitope in human and mouse apoM showed that the average apoM concentration was similar in human and WT mouse plasma (not shown). Thus, the apoM-Tg\textsuperscript{H} and apoM-Tg\textsuperscript{N} lines have \(\sim 11\)-fold and \(\sim 2\)-fold more apoM than WT mice.

ApoM\textsuperscript{−/−} mice were generated with an APOM targeting vector (supplemental Fig. 2A). Homologous recombination of the vector with chromosomal DNA in embryonic stem cells was verified by Southern blotting with an external probe (supplemental Fig. 2, A and B) and long range PCR (supplemental Fig. 2, A and C). Southern blotting with an internal probe confirmed that the vector had integrated only in the APOM locus (not shown).

In apoM\textsuperscript{+/−} mice, plasma apoM concentrations were reduced by \(\sim 42\)% (as judged from Western blots); apoM\textsuperscript{−/−} mice had no detectable apoM in plasma (supplemental Fig. 2D). Accordingly, apoM mRNA could not be amplified from apoM\textsuperscript{−/−} mouse liver cDNA with primers in exons 2 and 3 of APOM (supplemental Fig. 2E). In apoM\textsuperscript{−/−} mice, hepatic mRNA expression of genes in the same chromosomal region as APOM (BAT1, TNF, LST, AIF3, BAT2, BAT3, and BAT4), measured with real time PCR, were 95 \(\pm\) 8%, 79 \(\pm\) 13%, 98 \(\pm\) 25%, 122 \(\pm\) 14%, 98 \(\pm\) 8%, 88 \(\pm\) 20%, and 99 \(\pm\) 10% \(\left(n = 7\right)\), respectively, of the average level in apoM\textsuperscript{+/+} (\(n = 9\)) livers.

**Plasma Lipids and Lipoproteins in ApoM-Tg and ApoM\textsuperscript{−/−} Mice (supplemental Table I)**—In apoM-Tg\textsuperscript{H} mice, total plasma cholesterol was increased by \(13\)% \(\left(p < 0.005\right)\) in males and 22% \(\left(p < 0.001\right)\) in females. Cholesterol levels were not affected in apoM-Tg\textsuperscript{N} mice but were \(17\)% lower in male \(\left(p = 0.06\right)\) and 21% lower in female \(\left(p < 0.05\right)\) apoM\textsuperscript{−/−} mice than in gender-matched apoM\textsuperscript{+/+} littermates. Free cholesterol constituted \(\sim 20\)% of total plasma cholesterol in apoM-Tg\textsuperscript{H}, apoM-Tg\textsuperscript{N}, apoM\textsuperscript{−/−}, and WT mice (not shown). Plasma triglycerides were increased by \(25\)% in male \(\left(p < 0.05\right)\) and \(41\)% in female \(\left(p < 0.05\right)\) apoM-Tg\textsuperscript{N} mice and decreased by \(34\)% \(\left(p < 0.05\right)\) in female apoM\textsuperscript{−/−} mice but were not affected in male apoM\textsuperscript{−/−} or apoM-Tg\textsuperscript{N} mice. Plasma phospholipids were increased by 11% \(\left(p < 0.05\right)\) and 25% \(\left(p < 0.001\right)\) in apoM-Tg\textsuperscript{N} males and females but were unaffected in apoM\textsuperscript{−/−} and apoM-Tg\textsuperscript{N} mice.

The effect of apoM overexpression or deficiency on plasma lipoprotein size distribution was determined by size exclusion chromatography. The cholesterol and phospholipid elution profiles did not differ in apoM-Tg\textsuperscript{H}, apoM-Tg\textsuperscript{N}, and WT mice (Fig. 1A; not shown). Also, ApoM deficiency did not alter cholesterol or phospholipid elution profiles (Fig. 1B; not shown), and the size distribution of apoA-I-containing particles was similar in apoM\textsuperscript{−/−} and control mice (Fig. 1B). Similar results were obtained in apoM\textsuperscript{−/−} mice generated from two independent embryonic stem cell lines. Mice lacking the hepatic transcription factor HNF-1α have almost no apoM (22) and large HDL1 particles in plasma (3, 38). This result was confirmed in the present study (Fig. 1B). However, apoM\textsuperscript{−/−} mice did not have enlarged HDL (Fig. 1B).

**FIGURE 1. Plasma lipoproteins in apoM-Tg and apoM\textsuperscript{−/−} mice.** A, size-exclusion chromatography profile of plasma cholesterol in apoM-Tg mice. Pooled plasma from six WT, six apoM-Tg\textsuperscript{H}, and 10 apoM-Tg\textsuperscript{N} mice were separated on a Superose 6 and 12 FPLC columns. The elution positions of VLDL, LDL, and HDL were determined using human lipoproteins. B, size exclusion chromatography profile of plasma cholesterol in apoM\textsuperscript{−/−} and HNF-1α\textsuperscript{−/−} mice. Pooled plasma from 10 WT (apoM\textsuperscript{−/−}), seven apoM\textsuperscript{−/−}, or two HNF-1α\textsuperscript{−/−} mice was separated on a Superose 6 column. The elution positions of VLDL, LDL, and HDL were determined with human lipoproteins. ApoA-I and apoM in gel filtration fractions were identified after separation of proteins from pools of three fractions with 12% SDS-polyacrylamide gels followed by Western blotting with polyclonal rabbit anti-mouse apoA-I and apoM antibodies.

**Lipoprotein Association of ApoM**—Upon size exclusion chromatography of plasma from apoM-Tg\textsuperscript{H} and apoM-Tg\textsuperscript{N} mice, human apoM eluted with HDL (supplemental Fig. 3A), like mouse apoM in WT mice (22). After ultracentrifugation of plasma from apoM-Tg\textsuperscript{H} and apoM-Tg\textsuperscript{N} mice, the vast majority of human apoM was recovered in the HDL density fraction (supplemental Fig. 3B). Both mouse and human apoM were located in α-migrating particles, as revealed by agarose gel electrophoresis of plasma and Western blot analysis (supplemental Fig. 3C).

To further examine apoM-containing lipoproteins, we isolated HDL from plasma of apoM-Tg and WT mice by ultracentrifugation. In Coomassie-stained nonreducing SDS-polyacrylamide gels, human apoM could be seen in both the apoM-Tg\textsuperscript{N} and apoM-Tg\textsuperscript{H} mouse HDL fractions as an \(\sim 25\) kDa band (Fig. 2A).
On agarose gel electrophoresis and Western blotting analyses, both human apoM and mouse apoM were located in α-migrating particles, whereas apoA-I migrated in both α- and pre-β-positions (Fig. 2B). Native two-dimensional gel electrophoresis of HDL from apoM-TgH mice showed that human and mouse apoM were in 10–12-nm α-migrating particles (Fig. 2C). ApoA-I was detected both in pre-β- and α-migrating HDL. Similar results for mouse apoM and apoA-I were obtained from analysis of HDL from WT mice (not shown). As expected, size distribution analysis of apoM and apoA-I in human HDL3 particles (1.12 g/liter < d < 1.21 g/liter) exposed to PLTP showed human apoA-I in 8–12-nm particles (α-migrating) (5) and in <7-nm particles (pre-β-migrating) (5) (Fig. 2D). Human apoM was exclusively located in 10–12-nm particles (Fig. 2D), although apoA-I-containing pre-β-particles formed in the presence of PLTP.

The apolipoprotein composition of the isolated HDL was assessed by Western blotting (Fig. 3A). ApoM overexpression was associated with an increase in HDL-associated apoE, whereas the contents of apoA-I, apoA-II, and apoC-I in HDL were not affected by changing the apoM content. ApoA-IV was not present in isolated HDL, presumably due to loss during ultracentrifugation. To verify the increase in apoE, we isolated the d > 1.063 g/ml plasma fraction and measured apoE and apoA-I with enzyme-linked immunosorbent assay (Fig. 3B). ApoM overexpression was associated with a doubling of the apoE concentration in the d > 1.063 g/ml fraction both in apoM-TgN and apoM-TgH mice and, as such, was not dependent on the extent of apoM overexpression. ApoM deficiency did not affect the concentration of apoE in the d > 1.063 g/ml fraction.

FIGURE 2. ApoM is mainly associated with large α-migrating HDL. A, proteins in HDL (10 μg of protein) from WT, apoM-TgN, and apoM-TgH mice were separated with 12% nonreducing SDS-polyacrylamide gels and stained with Coomassie Blue. B, HDL from WT and apoM-TgH mice (9 μg of protein) was separated with 0.8% agarose gels followed by Coomassie Blue staining to visualize protein or Western blotting with polyclonal antibodies against human apoM, mouse apoM, or mouse apoA-I. C, two-dimensional gel electrophoresis of HDL (7 μg of protein) from apoM-TgH mice, performed as described under “Experimental Procedures.” D, human HDL3 (8.2 μg of apoA-I protein) was mixed with PLTP (100 nmol/h), incubated at 37 °C for 27 h to induce pre-β-HDL formation, and size-separated for 20 h at 150 V with 4–30% nondenaturing gels. Proteins were transferred to Hybond nitrocellulose membrane by wet blotting at 380 mA for 2 h at 4 °C after a 2-h incubation in 0.1% SDS solution. Membranes were exposed to monoclonal mouse anti-human apoM (M58 1:3000) or apoA-I (monoclonal antibody 8G8, 1:1000) antibodies, followed by peroxidase-labeled goat anti-mouse IgG. Bands were visualized with an ECL system.

FIGURE 3. Increased apoE in HDL from apoM-transgenic mice. A, Western blot of isolated HDL from WT, ApoM+/−, and apoM-TgH mice. The amounts of HDL protein in each lane were 1.00, 0.50, 0.40, 0.20, and 0.20 μg for blotting against apoE, apoA-IV, apoC-I, apoA-I, and apoA-II, respectively. Although apoA-IV was easily detected in total mouse plasma (not shown), it was not detected in the isolated HDL fraction, suggesting that it had been lost during ultracentrifugation. B, the d > 1.063 g/ml fraction was isolated from 50 μl of mouse plasma by ultracentrifugation. The HDL-containing infranatant was used for enzyme-linked immunosorbent assay measurements of mouse apoE and apoA-I. The indicated concentrations are per ml of plasma. Values are mean ± S.E., n = 5 in each group. The p values are from two-group comparisons with Student’s t test.
ApoM affects LCAT-dependent interconversion of α-HDL to pre-α-HDL. A and B, freshly drawn plasma (4 μl) pooled from four WT and four apoM-TgH mice was incubated at 37 °C for 0, 20, or 60 min with or without the LCAT inhibitor DNTB and separated on homemade 0.8% agarose gels before Western blotting with antibodies against mouse apoA-I (A) and mouse apoM (B). Similar results were obtained in three independent experiments. C, plasma LCAT activity on a discoidal HDL substrate, measured from the esterification rate of labeled free cholesterol upon incubation with plasma from 21 WT (control), 12 apoM-Tg, or 10 apoM−/− mice. Values (mean ± S.E.) from apoM-TgH and apoM−/− mice were similar, as were those from apoM-Tg and apoM−/− littermate controls.

Ex Vivo Interconversion of HDL Subfractions in Plasma from ApoM-Tg and ApoM−/− Mice—Incubation of mouse plasma at 37 °C causes interconversion of HDL from α- to pre-α-migrating particles by LCAT (12, 15). To determine if LCAT-dependent conversion of α- to pre-α-HDL is affected in apoM-TgH mice, we incubated plasma from apoM-TgH and WT mice at 37 or 4 °C (control) for 20 or 60 min in the presence or absence of the LCAT inhibitor DNTB (final concentration, 1.5 mM). The electrophoretic mobility of apoA-I- and apoM-containing lipoproteins was assessed with agarose gels and Western blotting (Fig. 4, A and B). In WT mouse plasma, HDL particles were converted from α- to pre-α-migrating particles after 60 min; this conversion was inhibited by DNTB (Fig. 4, A and B). However, LCAT-dependent conversion was markedly delayed in plasma from apoM-TgH mice relative to WT plasma (Fig. 4, A and B). When the esterification rate of labeled free cholesterol in a discoidal HDL substrate was measured during incubation with mouse plasma, plasma LCAT activity did not differ in apoM-Tg, apoM−/−, and WT mice (see Fig. 6C).

Pre-β-HDL constitutes 1–5% of total HDL in fresh mouse plasma but is formed upon prolonged incubation of plasma with an LCAT inhibitor (12, 16). To quantitate the effect of apoM on LCAT-independent formation of pre-β-HDL, we incubated mouse plasma with DNTB at 37 °C and used crossed immunoelectrophoresis with a polyclonal anti-mouse apoA-I antiserum to measure pre-β-HDL (Fig. 5A). Before incubation, the amounts of pre-β-migrating apoA-I-containing particles in plasma were similar in WT (1.2 ± 0.5%), apoM-Tg (0.6 ± 0.2%), and apoM−/− (0.6 ± 0.2%) mice (Fig. 5B). After incubation, the amount of pre-β-HDL in plasma was higher in apoM-Tg mice and lower in apoM−/− mice than in WT mice (Fig. 5B). PLTP and HL both facilitate the conversion of α-HDL to pre-β-HDL (16–18, 20). Plasma PLTP activity was significantly higher in apoM-Tg mice than in WT mice but was similar in apoM−/− and WT mice (supplemental Fig. 4A). Plasma HL activities did not differ in apoM-Tg, apoM−/−, and WT mice (supplemental Fig. 4B).

Effect of ApoM Overexpression on Atherogenesis in LDLR−/− Mice—Next, we assessed the effect of a 2-fold increase in plasma apoM concentration on the development of atherosclerosis. apoM−/− mice were bred with LDLR−/− mice to obtain females hemizygous for the human apoM transgene and homozygous for the LDLR null mutation; littermate LDLR−/− females served as controls. To induce atherosclerosis, the mice were fed 0.3% cholesterol from 4 weeks of age (28). ApoM
expression did not affect the total plasma cholesterol concentration (supplemental Table 2), and the distribution of cholesterol between lipoproteins did not differ in apoM-Tg\(^N\)/LDLR \(^{-/-}\) mice (Fig. 6A).

The mice were sacrificed at 20 weeks of age. On histological analyses of the aortic root, the average plaque area was smaller in apoM-Tg\(^N\)/LDLR \(^{-/-}\) mice than in LDLR \(^{-/-}\) controls (Fig. 6B). This decrease in lesion size was accompanied by reduction in both neutral lipid-stained and collagen-stained lesion areas that were, respectively, 39% \((p = 0.008)\) and 30% \((p = 0.04)\) smaller in apoM-Tg\(^N\)/LDLR \(^{-/-}\) mice than in LDLR \(^{-/-}\) controls (not shown). The fraction of the plaque area staining for neutral lipids was reduced (Fig. 6B, C). Percentage of lesion area staining for neutral lipids with oil-red O. The lines indicate mean values. \(p\) values are from two-group comparisons with Student’s \(t\) test.

![FIGURE 6. Plasma lipoproteins and aortic atherosclerosis in apoM-Tg\(^N\)/LDLR \(^{-/-}\) mice. A, size exclusion chromato-}

ApoM-containing HDL from human plasma is more effective in mediating cholesterol efflux from foam cells and inhibiting oxidation of LDL than apoM-free HDL (26). Hence, to further examine putative mechanisms for the antiatherogenic effect of apoM overexpression in mice, we measured the ability of HDL to mediate cholesterol efflux from \(^{3}\)Hcholesterol-labeled macrophages and to protect lipoproteins against \(\text{Cu}^{2+}\)-induced oxidation. HDL from apoM-Tg\(^H\) and apoM-Tg\(^N\) mice facilitated more \(^{3}\)Hcholesterol efflux from cholesterol-loaded macrophages than HDL from WT mice (Fig. 7A). Also, when the \(^{3}\)Hcholesterol-labeled macrophages had been converted into foam cells with acetylated LDL prior to the incubation with HDL, the efflux of \(^{3}\)Hcholesterol during 24 h was 32 ± 1 and 25 ± 1% \((p = 0.01, n = 2 \times 4)\) when using HDL from apoM-Tg\(^N\) and WT mice, respectively.

\(\text{Cu}^{2+}\)-induced oxidation of HDL from apoM-Tg\(^H\) and apoM-Tg\(^N\) mice was delayed 35 ± 2 min (Fig. 7B, \(p < 0.001\)) and 13 ± 1 min \((p < 0.00; \text{not shown})\), respectively, when compared with HDL from WT mice. HDL reportedly protects LDL from oxidation (39). When HDL was mixed with LDL before the addition of \(\text{Cu}^{2+}\), HDL from apoM-Tg\(^H\) mice prolonged the lag time for oxidation of LDL more than HDL from WT mice.
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(94 ± 25 versus 75 ± 20 min, p < 0.04) (Fig. 7B), whereas HDL from apoM-TgN had the same effect as HDL from WT mice (not shown). Of note, the lag times for oxidation of LDL and HDL cannot be directly comparable.

DISCUSSION

The studies of mouse models with constitutively modified apoM gene expression provide novel insights into the role of apoM in lipoprotein metabolism and atherosclerosis. Western blotting with an apoM peptide antiserum suggested that the plasma concentration of apoM is similar in mice and humans (i.e. ~20–50 mg/liter) (22, 40). It was previously suggested that a large fraction of plasma ApoM resides in the pre-β-migrating HDL pool (3). However, in the present study, apoM was almost exclusively present in ~10–12-nm α-migrating HDL particles in mice, as shown by analyses of size, charge, and density of apoM in plasma and HDL from WT and human apoM-Tg mice. This result was consistent with the analyses of human plasma in this and previous studies (26). Although apoM is robustly expressed during embryonic life (22), apoMα− mice bred normally, suggesting that apoM is not essential for normal reproduction or development. Importantly, the apoM gene-targeting vector did not change the expression of neighboring inflammatory genes in the ApoM locus.

ApoA-Iα− mice have substantially reduced plasma apoM levels, suggesting a connection between apoM and apoA-I metabolism (22). However, since expression in apoM-Tg mice or the absence of apoM in apoA-Iα− mice only had minor effects on HDL and plasma apoA-I levels, apoM is not a major determinant of murine HDL formation. ApoM deficiency did not affect the plasma lipoprotein profile determined by size exclusion chromatography. This finding was surprising, because small interfering RNA-induced reduction of apoM expression by ~90% in mice results in the formation of large HDL1 particles (3). Mice lacking the transcription factor HNF-1α also have large HDL1 particles, which we confirmed in this study, and very low plasma levels of apoA, as demonstrated previously (3, 22). It has been speculated that the formation of large HDL1 particles in HNF-1α− mice was due to the reduced level of apoM, but this study suggests that formation of large HDL1 particles cannot exclusively be attributed to low level expression of apoM.

HDL consists of subpopulations of particles with distinctive structure, composition, and function. The heterogeneity results from continuous remodeling of HDL by, for example, LCAT, cholesteryl ester transfer protein, PLTP, and lipases and affects metabolism and atheroprotective functions of HDL (41). The plasma activities of LCAT and HL, as measured toward exogenous substrates were not affected in apoM-Tg or apoMα− mice. Also, the plasma PLTP activity was not affected in apoMα− mice despite a modest increase in apoM-Tg mice. Nevertheless, our findings suggest that the presence of apoM in the α-HDL particle affects its modulation. First, the LCAT-dependent interconversion of α to pre-α-migrating HDL was delayed in plasma from apoM-Tgα mice compared with controls. LCAT activity on free cholesterol in HDL is enhanced by the interaction with apoA-I and is affected by the conformational state of apoA-I (42, 43). The addition of apoE or apoC-I to apoA-I-containing HDL reduces the ability of LCAT to esterify cholesterol in the particles, possibly through effects of apoE and apoC-I on the conformation of apoA-I (44–46). Interestingly, the content of apoE in HDL was increased in apoM-transgenic mice, which, as such, may explain the delayed conversion of α to pre-α-migrating HDL. It remains to be determined, however, if apoM in HDL also affects the conformation of apoA-I or sterically hinders the interaction between LCAT and apoA-I. Second, apoM affected the LCAT-independent interconversion of α-HDL to pre-β-HDL ex vivo. Upon incubation with an LCAT inhibitor at 37°C, plasma from apoM-Tg mice formed more pre-β-HDL than WT plasma, and plasma from apoM−/− mice formed less. Both PLTP and HL can induce formation of pre-β-HDL from α-HDL (16–20). The effect of apoM expression levels on pre-β-HDL formation, however, was not paralleled by alterations of plasma PLTP or HL activities. Although the apoE content in HDL was increased in apoM-transgenic mice, it was not affected by apoM deficiency. Thus, it is likely that the apoM in HDL particles affects the ability of PLTP or HL to produce pre-β-HDL from α-HDL.

ApoM-containing HDL particles purified from human plasma protect LDL against Cu2+-induced oxidation and stimulate cholesterol efflux from macrophage foam cells more efficiently than HDL particles lacking apoM (26), and treatment with an apoM-encoding adenovirus reduces atherosclerosis in mice (3). Thus, it was of interest to study whether constitutive ~2-fold overexpression of apoM in mice was atheroprotective. In apoM-TgN/LDLR−/− mice, the lesion area in the aortic root and the fraction of the plaques staining for neutral lipids were reduced. Interestingly, these effects could be observed in the setting of unaltered total plasma lipoprotein levels. The present study points to putative mechanisms for the antiatherogenic effect of apoM. First, although plasma levels of pre-β-HDL did not differ in apoM-Tg and control mice, apoM overexpression increased pre-β-HDL formation ex vivo. Thus, if apoM overexpression increases pre-β-HDL formation in vivo in or near the arterial wall, it could increase cholesterol efflux from foam cells. However, it is still unclear what role pre-β-HDL plays in cholesterol efflux and reverse cholesterol transport (47, 48). Second, HDL from apoM-Tg mice caused significantly more cholesterol efflux from macrophage foam cells than HDL from WT mice. The observation may to some extent reflect an effect of apoM on conversion of α-HDL to pre-β-HDL. However, other possibilities may also be considered. ApoM belongs to the lipocalin protein superfamily (24). Several lipocalins bind and transport lipophilic compounds, such as vitamins and steroids (49–51). Recent data suggest that ApoM can bind retinol and retinoic acid (25). Retinoids exhibit multiple and diverse biological functions, including regulation of the nuclear hormone receptors LXR and RXR (52). The ABCA1 and ABCG1 genes control phospholipid and cholesterol efflux from macrophage foam cells and are regulated by liver X receptor and retinoid X receptor (53). Thus, it will be interesting to determine if apoM in concert with retinoids might affect expression of ABCA1 and ABCG1. Nevertheless, the importance of apoM for reverse cholesterol transport should be further tested in vivo. Third, the data suggest that HDL from apoM-Tg mice is more resistant to oxidation by Cu2+ than HDL from WT mice and more effective...
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in protecting LDL from oxidation. This observation agrees with previous studies (26). Thus, the presence of apoM in HDL particles may enhance their antioxidative effects and thereby decrease atherosclerosis (54).

In conclusion, the present study of mice with constitutively altered apoM expression complements a previous seminal study of mice with transiently changed apoM expression (3). Unlike the previous study, our results do not support the notion that apoM resides in pre-β-mobie pool of HDL or that apoM deficiency has a major effect on plasma LDL size or plasma pre-β-HDL concentrations in mice in vivo. We did find, however, that pre-β-HDL formation in vitro was affected by the presence of apoM in the α-mobie HDL pool. Moreover, in accordance with the antiatherogenic effect of adenovirus-mediated apoM overexpression (3), we found that a modest overexpression (3) of human apoM reduced development of early atherosclerotic lesions in LDLR−/− mice. The data suggest that apoM-mediated atheroprotection could be due to several factors, including increased pre-β-HDL formation, increased ability of HDL to accelerate cholesterol efflux from macrophage foam cells, and/or protection of LDL against oxidation.

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