Isolation and characterization of human apolipoprotein M-containing lipoproteins

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Abstract **Apolipoprotein M (apoM) is a novel apolipoprotein with unknown function. In this study, we established a method for isolating apoM-containing lipoproteins and studied their composition and the effect of apoM on HDL function. ApoM-containing lipoproteins were isolated from human plasma with immunoaffinity chromatography and compared with lipoproteins lacking apoM. The apoM-containing lipoproteins were predominantly of HDL size; ~5% of the total HDL population contained apoM. Mass spectrometry showed that the apoM-containing lipoproteins also contained apoJ, apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, paraoxonase 1, and apoB. ApoM-containing HDL (HDL^{apoM+}) contained significantly more free cholesterol than HDL lacking apoM (HDL^{apoM-}) (5.9 ± 0.7% vs. 3.2 ± 0.5%; $P < 0.005$) and was heterogeneous in size with both small and large particles. HDL^{apoM+} inhibited Cu^{2+}-induced oxidation of LDL and stimulated cholesterol efflux from THP-1 foam cells more efficiently than HDL^{apoM-}. In conclusion, our results suggest that apoM is associated with a small heterogeneous subpopulation of HDL particles. Nevertheless, apoM designates a subpopulation of HDL that protects LDL against oxidation and stimulates cholesterol efflux more efficiently than HDL lacking apoM.—** Christoffersen, C., L. B. Nielsen, O. Axler, A. Andersson, A. H. Johnsen, and B. Dahlbäck. **Isolation and characterization of human apolipoprotein M-containing lipoproteins.** ***J. Lipid Res.*** **2006.** 47: **1833–1843.**

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Atherosclerosis is a major cause of mortality and morbidity in the Western world. The formation of the atherosclerotic plaque is the result of multiple reactions that are not fully characterized. LDL oxidation and reverse cholesterol efflux are both important processes affecting atherogenesis. Oxidation of LDL accelerates the uptake of cholesterol in macrophages and their differentiation to foam cells (1), a key event in intimal lesion formation (2). HDL has the ability to inhibit the oxidation of LDL. Paraoxonase 1 (PON1) is an important mediator in the antioxidant effects of HDL. PON1 has different enzymatic activities, including the ability to hydrolyze oxidized phospholipids (3). PON1 is anchored to the HDL particles by its hydrophobic signal peptide, which is retained in the mature protein (4).

Efflux of cholesterol from macrophages protects the cells from lipid toxicities and attenuates foam cell formation. Cholesterol and phospholipid efflux can be mediated by lipid-free apolipoprotein A-I (apoA-I) or lipid poor preβ-HDL particles in a process that involves the binding of apoA-I to the membrane transporter protein ABCA1 (5, 6). Efflux of cholesterol to spherical HDL particles involves ABCG1 and ABCG4 (7) but also may occur by free diffusion (8). In addition to apoA-I, several other HDL-associated proteins have been reported to affect cholesterol efflux: PON1 enhances HDL-induced cholesterol efflux by increasing the binding affinity of HDL to macrophages and by stimulating ABCA1-mediated cholesterol transport (9), and apoJ, which is associated with lipid-poor HDL, stimulates reverse cholesterol transport by an unknown mechanism (10).

Recently, a novel apolipoprotein, apoM, was described (11). ApoM is predominantly present in HDL and to lesser extent in chylomicrons, VLDL, and LDL. ApoM belongs to the lipocalin family, the members of which share a common tertiary structure of an eight stranded antiparallel β-barrel that forms a hydrophobic ligand binding pocket (12). The lipocalin protein family comprises many proteins with very diverse functions, some of which are antioxidants (13). However, for many lipocalins, including apoM, the endogenous ligand(s) and biological function have not been identified.

Like PON1, apoM lacks a signal peptidase cleaving site (11). The retained signal peptide may serve as a lipid an-

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Abbreviations: apoM, apolipoprotein M; HRP, haptoglobin-related protein; PON1, paraoxonase 1.

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chor for the protein necessary for its association with lipoprotein particles (11). In addition to apoM and PON1, the haptoglobin-related protein (HRP) shares this unusual lipid binding property (14). ApoM has been suggested to play an important role in reverse cholesterol transport (15). Wolfrum, Poy, and Stoffel (15) demonstrated that mice made deficient in apoM using small, interfering RNA had large HDL particles that do not stimulate cholesterol efflux from macrophages to the same extent as HDL from wild-type mice. Moreover, LDL receptor-deficient mice treated with a murine apoM adenovirus construct to increase the apoM expression level developed less extensive atherosclerotic plaques in aorta than LDL receptor-deficient mice treated with a control adenovirus, suggesting that apoM protects against atherosclerosis.

To increase the knowledge of normal human apoM particles, and to better understand the role of apoM in human cholesterol metabolism, we have established a method for purifying apoM-containing lipoproteins from human plasma and explored the protein and lipid composition, size, density, and charge of apoM-containing lipoproteins. Also, we have investigated whether the apoM-containing particles inhibit the oxidation of lipoproteins and stimulate cholesterol efflux from macrophage-derived foam cells.

METHODS

Preseparation of apoM-containing lipoproteins from human plasma with ion-exchange chromatography or ultracentrifugation

ApoM-containing plasma lipoprotein particles were preseparated by either ion-exchange chromatography or sequential fixed-density ultracentrifugation before final purification by immunoaffinity chromatography. Plasma from hyperlipidemic blood donors (typically 250 ml) was diluted 1:3 with 10 mM Tris-HCl, pH 7.5, to decrease the ionic strength and applied at 4 ml/min to a 2.5 × 25 cm column packed with 100 ml of Q-Sepharose (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in 10 mM Tris-HCl, pH 7.5. The column was washed with equilibration buffer until absorbance at 280 nm reached baseline values. Bound proteins were eluted with a 0–0.5 M NaCl gradient (2 × 500 ml), and 10 ml fractions were collected. The fractions were tested for apoM content with an ELISA for apoM and for apoA-I with Western blotting using a monoclonal antibody against apoA-I (M12). Elution profiles of apoA-I and apoM coincided, and two distinct peaks were observed, the first peak eluting relatively early in the gradient (at ~100 mM NaCl) and the second apoA-I/apoM positive peak eluting at ~300 mM NaCl. The peaks were pooled separately and subjected to immunoaffinity chromatography using a monoclonal antibody against apoM (see below). The apoM-containing fractions were dialyzed against 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, before immunoaffinity chromatography.

HDL ($1.063 < d < 1.21$ g/l) and LDL ($1.019 < d < 1.063$ g/l) were isolated by ultracentrifugation of a pool of plasma from 15 normal individuals with a Beckman Ti 50.3 rotor and a Beckman Optima LE-80K ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). The density was adjusted with NaBr. Ultracentrifugation was performed at 50,000 rpm at 4°C for 16 h ($d = 1.019$ and 1.063 g/l) or 24 h ($d = 1.21$ g/l). The purified LDL and HDL were dialyzed extensively against PBS with Na_2EDTA (0.1 mg/ml) at 4°C and stored at −80°C.

Anti-apoM and anti-apoA-I immunoaffinity chromatography

Polyclonal and monoclonal antibodies were raised against truncated (residues 22–188) recombinant human apoM expressed in *Escherichia coli* using the pET-30 Xa/Lic vector (Novagen) as described previously (16) with standard techniques. A monoclonal antibody against human apoA-I (M12) was obtained after immunization of mice with proteins extracted from isolated chylomicrons. The specificity of the M12 antibody was ascertained with apoA-I Western blotting and from its usefulness for the purification of apoA-I-containing HDL from human plasma. Monoclonal antibodies against apoM (M23) or apoA-I (M12) were coupled to 5 ml HiTrap N-hydroxy-succinimide (NHS)-activated columns (Amersham Biosciences) at 10 mg/ml gel, according to the manufacturer's instructions.

The apoA-I/apoM-containing lipoproteins obtained from either the ion-exchange chromatography or the ultracentrifugation were applied first to the M23 anti-apoM column to isolate apoM-containing lipoproteins. The column was washed with TBS followed by TBS with 0.5 mol/l NaCl, and bound particles were eluted with glycine (0.1 mol/l, pH 2.2). One milliliter fractions were collected in tubes containing 50 μl of 1 M Tris (pH 9.0). The protein concentrations were measured with the Pierce BCA Protein Assay Kit (Bie and Berntsen A-S, Rodovre, Denmark) using BSA as a standard. The HDL and LDL preparations were subjected to several rounds of M23 anti-apoM chromatography until all apoM particles had been removed from the respective lipoprotein. In some cases, apoM-containing lipoproteins were concentrated by centrifugation at 10°C for 4 h at 100,000 *g* in a S/N O4U 1660 TLA 100 rotor head with a Beckman Optima™ MAX-E ultracentrifuge (Beckman Coulter). ApoM-free apoA-I-containing particles were isolated from apoM-free HDL with an M12 anti-apoA-I column and a protocol similar to that used for the M23 anti-apoM column.

Characterization of lipoprotein density, size, and charge

The density of apoM-containing lipoproteins was assessed by gradient density ultracentrifugation. A gradient was formed in Ultra-Clear tubes (Beckman Coulter) by layering from the bottom 2 ml of 50% sucrose, 2 ml of 25% sucrose, 5 ml of 12.5% sucrose with 50 μl of apoM-containing lipoproteins, and 3 ml of PBS (17). After centrifugation for 70–71 h at 35,000 rpm and 12°C in a Beckman SW41 Ti rotor using the Beckman Optima LE-80K ultracentrifuge, 12 fractions of 1 ml were collected by aspiration from the top and apoM concentration was measured with an apoM ELISA; the cross-contamination between fractions was <10% (17).

The size of the isolated lipoprotein particles was assessed by nondenaturing gel electrophoresis: lipoproteins and the native high-molecular-weight marker (Amersham Biosciences Europe GmbH, Horsholm, Denmark) were electrophoresed at room temperature for 3 h at 125 V on 4–20% or 3–8% Novex Tris-glycine gels (Invitrogen A/S, Taastrup, Denmark) and stained with SimpleBlue SafeStain (Invitrogen A/S).

Gel-permeation chromatography was performed at 20–24°C using PBS with EDTA on serially connected Superose 6 and Superose 12 10/300 GL fast-protein liquid chromatography columns (Amersham Biosciences Europe). ApoM-containing HDL (HDL^{apoM+}) was analyzed with 1 mg/ml albumin in sample and buffer. The flow rate was 0.2 ml/min, and fractions of 250 μl were collected. Void volume (V_0) and total column volume (V_t) were determined with intralipid and glycerol, respectively, and the

column was calibrated with different molecular markers (high-molecular-weight and low-molecular-weight calibration standards; Amersham Biosciences).

Agarose (0.8% Litex agarose; Medinova Scientific A/S) gel electrophoresis was done in a barbital buffer (pH 8.5). Proteins on the agarose gels were stained with Coomassie Brilliant Blue. Lipid free apoA-I (a gift from Dr. M. H. Andersen, Borean Pharma, Aarhus, Denmark) and human LDL purified by ultracentrifugation were used to identify β- and preβ-migrating particles.

Human apoM ELISA

An ELISA for human apoM based on two monoclonal apoM antibodies (M58 and M42) was used to quantify apoM in fractions. The details of this ELISA will be presented elsewhere. In brief, 96-well Costar plates (Corning BV, Biotech Line A/S, Slangerup, Denmark) were coated with M58, and the plates were quenched and washed using standard techniques. Samples were diluted in TBS with 1% Triton X-100 (Sigma-Aldrich Denmark A/S, Vallensbaek Strand, Denmark) and 1% BSA (Sigma) and added to the wells. After incubation to allow binding of apoM to the immobilized M58, bound apoM was detected with biotinylated M42, streptavidin-avidin-horseradish peroxidase (DAKO A/S, Glostrup, Denmark), and o-phenylenediamine (OPD) (DAKO A/S) according to the manufacturer's instructions.

Protein identification by mass spectrometry

Reduced and nonreduced isolated apoM-containing lipoprotein fractions were separated on 12% SDS-PAGE gels and silver-stained. The protein was identified as described previously (18). In short, the relevant bands were excised from the polyacrylamide gel, reduced, alkylated using iodacetamiden, and digested by trypsin. The resulting fragments were extracted, purified using C18 ZipTip (Millipore), and measured by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry on a Biflex instrument (Bruker, Bremen, Germany). For identification, the mass spectral patterns of the fragments were used to search against several databases.

Western blotting

For Western blotting, proteins were separated on 12% or 16% polyacrylamide gels (Kem En Tec, Copenhagen, Denmark) and transferred to Hybond-P 0.45 μm polyvinylidene difluoride membranes (RPN303F; Amersham Biosciences Europe) using a semi-dry electroblotter (Kem En Tec). The membranes were quenched for 1 h in skim milk (100 mg/ml) followed by incubation for 1 h at room temperature or for 16 h at 4°C with primary antibodies [goat anti-human apoJ (1:1,000; Abcam, Cambridge, UK), rabbit anti-human haptoglobin (1:4,000; DAKO, Copenhagen, Denmark), mouse anti-human PON1 (1:2,000; a gift from Dr. Dragomir Draganov, Michigan Medical School), mouse anti-human apoB (1:6,000; a gift from Dr. Sally McCormick, Otago University, New Zealand), mouse anti-human apoA-I (1:500; Autogen Bioclear, Wiltshire, UK), or rabbit anti-human apoM IgG (2.5 μg/ml)]. The dilutions of antibodies were made in the quenching buffer. The membranes were then washed and incubated with horseradish peroxidase-coupled secondary antibodies (goat anti-rabbit or rabbit anti-goat from DAKO, anti-biotin from Trichem Aps, or goat anti-mouse from BD Biosciences). After another round of washes, binding of antibody was detected with a chemiluminiscence reader (Fujifilm LAS-1000 Intelligent Dark Box II; Fujifilms, Trorod, Denmark) after incubation of the membrane with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemicals, Copenhagen, Denmark).

Lipid analysis

TLC (19) was used to measure the lipid composition of isolated lipoproteins. Briefly, lipids were extracted with chloroform-methanol. The extracts were washed (20) and applied on 20 × 20 cm silica-coated plates (DC-fertigplatten SIL G-25; Macherey-Nagel, Duren, Germany). Lipid extracts and standards were separated in six different solvent systems to enable separation of triglycerides, free cholesterol, esterified cholesterol, and individual phospholipid classes and visualized by heating the plate to 200°C for 2 min before quantification with digital image analysis. Details of the TLC system and intra-assay and interassay variations have been reported previously (20). Cholesterol in gel filtration fractions was measured by an enzymatic method with the CHOD-PAP reagents (Roche A/S, Applied Science, Hvidovre, Denmark).

Lipoprotein oxidation

Isolated lipoproteins were dialyzed against PBS, pH 7.4, for 16–20 h at 4°C. Nine hundred fifty microliters was placed in a quartz cuvette (VWR International ApS, Albertslund, Denmark) and mixed with 50 μl of PBS (control) or 50 μl of $CuSO_4$ (final concentration, 2.5 μmol/l). The formation of conjugated dienes at 37°C was measured at 234 nm every minute for 4 h with a Hewlett-Packard 8452A diode array spectrophotometer. The lag time was estimated by drawing a perpendicular line to the *x* axis from the intersection of the two straight lines drawn through the lag and propagation phases of the absorption curve (21). The propagation rate was calculated as the slope of the curve during the linear propagation phase.

Efflux of cholesterol from THP-1 foam cells

Human monocytic THP-1 cells (3.5×10^5) were grown on 24-well plates (Multidishes Nunclon™; VWR International) in 1 ml of medium [RPMI-1640 (−L-glutamine); Sigma-Aldrich Denmark] containing 4 mM L-glutamine (Sigma-Aldrich Denmark), 10% fetal calf serum, and 1% penicillin/streptomycin at 37°C in 5% CO_2. Cells were grown in the presence of phorbol myristate acetate (Sigma-Aldrich Denmark) for 5 days to induce differentiation into macrophages. For the first 24 h, the concentration of phorbol myristate acetate was 50 ng/ml; for the next 4 days, it was 25 ng/ml. The medium was changed on days 1, 3, and 5. On day 5, the same medium as described above without calf serum and with 1.5% BSA, 200,000 dpm/ml [^{3}H]cholesterol [1α,2α(n)-^{3}H-cholesterol; Amersham Bioscience], and 50 μg protein/ml acetylated LDL [prepared according to Basu et al. (22)] was added. This resulted in foam cell formation as judged by light microscopic inspection of Oil Red O-stained cells. After 48 h, cells were washed three times with serum-free medium and incubated with 0.7 ml of serum-free medium containing isolated lipoproteins (25 μg protein/ml) that had been dialyzed against PBS for 16–20 h at 4°C. Twenty-five microliters of medium was collected from each well after 4, 8, 16, 24, and 40 h. At each time point, the cells were inspected by microscopy to ensure that no morphological changes or accumulation of dead cells had occurred. After 40 h, cells were washed twice with PBS and lipids were extracted in 1 ml of hexane-isopropanol (3:2; 20–24°C) for 1 h followed by 0.5 ml of hexane-isopropanol for 15 min. The extracts were dried under N_2 and resuspended in 100 μl of isopropanol with 1% Triton X-100 (Sigma). [^{3}H]cholesterol in cell extracts and media was measured by scintillation counting using OptiPhase Hisafe 3 Scintillation Liquid (Perkin-Elmer Life Sciences, Hvidovre, Denmark) and a Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer Life Sciences). Total cholesterol in cell extracts was measured enzymatically. Cell proteins were hydrolyzed in 0.5 ml of 0.5 M NaOH at 37°C for 24 h and mea-

sured with the Pierce BCA Protein Assay Kit (Bie and Berntsen). Efflux was calculated as the percentage of [^{3}H]cholesterol in medium at a given time compared with the total [^{3}H]cholesterol contents in medium and cells.

RESULTS

Density and size of apoM-containing particles in plasma

To characterize the apoM-containing particles in plasma, a combination of ion-exchange (Q-Sepharose) and monoclonal antibody (M23) immunoaffinity chromatography was used to purify the particles from plasma. All apoM in plasma bound to the Q-Sepharose column and eluted in two distinct peaks, at ~100 mM NaCl (pool 1) and 300 mM NaCl (pool 2) (data not shown). The two pools were separately applied to an anti-apoM monoclonal antibody (M23) column to isolate the apoM-containing particles. The pools were reapplied to the M23 column multiple times until depleted of apoM. ApoM-free apoA-I-containing lipoproteins were then purified using an anti-apoA-I (M12) column. The apoM-containing particles obtained from pools 1 and 2 had densities of 1.07–1.21 g/l, which is comparable to HDL_2 and HDL_3 (**Fig. 1A**). The size of the apoM-containing particles was estimated by nondenaturing polyacrylamide gel electrophoresis (Fig. 1B). Pool 1 demonstrated apoM particles of three different sizes. A minor fraction comigrated with LDL (~23 nm), whereas

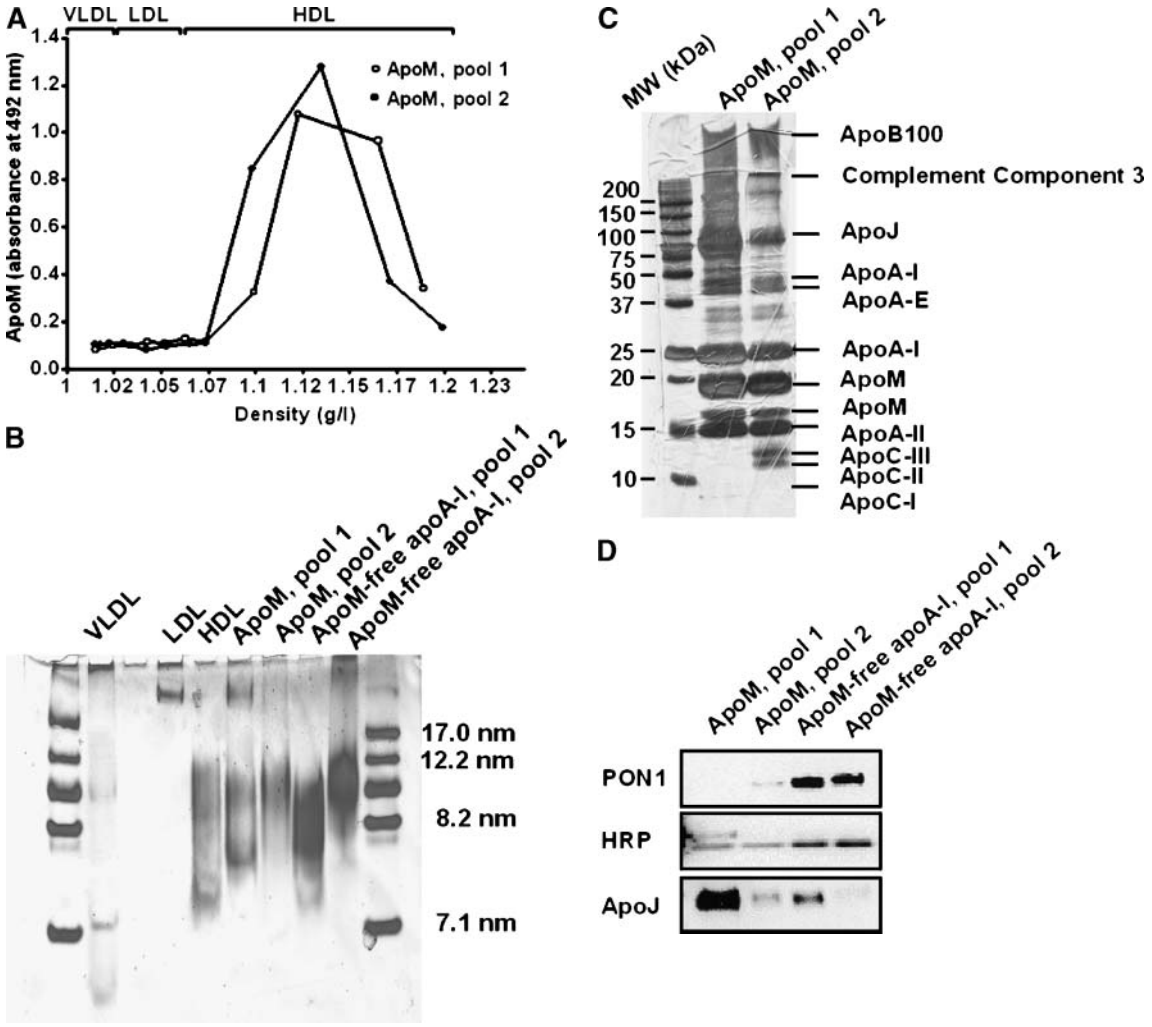

Fig. 1. Characterization of apolipoprotein M (apoM)-containing lipoprotein particles isolated from human plasma. A: The apoM-containing particles from pools 1 and 2 (~15 μg of total protein) were subjected to sucrose gradient ultracentrifugation to estimate their density. Human apoM in fractions was measured by ELISA; the absorbance at 492 nm is shown. Open circles represent apoM in pool 1, and closed circles represent apoM in pool 2. B: The sizes of the apoM-containing and apoM-free apoA-I-containing particles of pools 1 and 2 were evaluated by 4–20% nondenaturing gradient gel electrophoresis by loading 5–10 μg of protein. For comparison, VLDL, LDL, and HDL (isolated by ultracentrifugation) were also analyzed. The gel was stained with Coomassie Brilliant Blue. C: Proteins in apoM-containing particles from pools 1 and 2 (~10 μg in each lane) were separated on a 12% SDS-PAGE gel under nonreducing conditions and silver-stained. Each band was excised and trypsinized for mass spectrometry analysis. The identified proteins are indicated. The 37 kDa band contained apoA-I in addition to apoE. This probably reflects contamination from the abundant ~26 kDa apoA-I band. A week ~10 kDa band that cannot be seen here contained apoC-I. MW, molecular mass. D: Western blot analysis of paraoxonase 1 (PON1), haptoglobin-related protein (HRP), and apoJ in apoM-containing and apoM-free apoA-I-containing particles of pools 1 and 2.

the two major fractions migrated corresponding to ~8.0 nm particles or ~10–12 nm particles. The apoM-containing particles of pool 2 were all of HDL size (i.e., they migrated similarly to apoM-free apoA-I-containing particles and to total HDL; the migration corresponded to ~12 nm particles). This indicated that the isolated plasma apoM was located mainly in HDL and to a minor extent in LDL. However, we cannot exclude the possibility that a minor fraction of the isolated apoM particles were larger than LDL, as VLDL did not enter the gel. Also, because the nondenaturing gels were not to equilibrium, the actual size of the apoM-containing particles should be judged with caution.

Protein composition of apoM-containing particles

To explore the apolipoprotein composition of the apoM-containing particles, the proteins were separated by SDS-PAGE and identified by mass spectrometry (Fig. 1C, **Table 1**). The apoM-containing particles of pools 1 and 2 were composed of multiple proteins. In pool 1, apoB, apoJ, apoA-I, apoA-II, apoC-I, and apoM were identified. Pool 2 contained the same proteins except for apoC-I, which was replaced by apoC-II and apoC-III. There were two apoM bands, migrating at apparent masses of 20 and 17 kDa on the unreduced gels. The two bands represent glycosylated and nonglycosylated apoM, respectively (12). The intensities of the different protein bands varied between the two pools. ApoM-containing particles from pool 1 contained more apoJ than those isolated from pool 2 and apoM-free apoA-I-containing particles from both pools (Fig. 1D).

ApoM retains its signal peptide in plasma because of the lack of a signal peptidase cleavage site, a unique property shared with the HDL-associated PON1 and HRP (4, 14). We used Western blotting to determine whether the apoM-containing particles also contained PON1 and HRP (Fig. 1D). ApoM-containing particles from both pools contained less HRP than apoM-free apoA-I-containing particles. PON1 was detected in apoM-containing particles from pool 2, albeit at a much lower level than in the apoM-free apoA-I-containing particles, but could only be detected after extensive exposure in those from pool 1.

TABLE 1. Number of peptide sequences and percentage sequence coverage in mass spectrometry analyses of proteins in apoM-containing particles

Protein	Number of Peptides Identified	Sequence Coverage
		%
ApoB-100	37	13
Complement component 3	29	22
ApoJ	11	27
ApoA-I (dimer)	13	50
ApoE	10	33
ApoA-I (monomer)	20	58
ApoM	8	45
ApoA-II	2	31
ApoC-III	6	90
ApoC-II	6	90
ApoC-I	7	60

ApoM, apolipoprotein M. Bands were excised from a SDS-polyacrylamide gel (Fig. 1C) for matrix-assisted laser-desorption ionization time-of-flight mass spectrometry. Sequences were identified by searching the NCBInr and SwissProt databases.

Basic characterization of apoM-containing HDL and LDL particles from plasma

As apoM was associated with both HDL and LDL, we purified apoM particles from HDL and LDL that had been isolated by ultracentrifugation. ApoM-containing HDL and LDL were compared with HDL and LDL lacking apoM. Purification of apoM-containing particles from both HDL and LDL was effectively performed using multiple rounds of purification on a HiTrap column with a monoclonal antibody (M23) against apoM (**Fig. 2A**), resulting in preparations of apoM-containing HDL^{apoM+} and LDL^{apoM+} and apoM-free HDL^{apoM-} and LDL^{apoM-} particles (Fig. 2B, C). Analysis using several techniques including Western blotting against apoA-I, apoB, and apoM demonstrated good separation of lipoprotein fractions: LDL and HDL contained no detectable apoA-I and apoB, respectively (Fig. 2C). ApoM could not be detected in LDL^{apoM-} and HDL^{apoM-} (Fig. 2C). On analysis of HDL, HDL^{apoM-}, and HDL^{apoM+} with nondenaturing gradient gels, the major portion of HDL^{apoM+} migrated similarly to HDL but a minor portion migrated corresponding to ~7.2 nm particles (**Fig. 3A**). Small apoM-containing particles were also seen on gel filtration analysis of HDL^{apoM+} (Fig. 3B). The band corresponding to the smaller particles in HDL^{apoM+} contained both apoA-I and apoM: apoA-I was seen on Western blots of nondenaturing gels with an apoA-I antibody, and apoM was detected in the smaller band when the portion of the nondenaturing gel with ~7.2 nm particles was cut out and incubated with buffer followed by quantification of apoM in the eluate with ELISA (data not shown). Of note, the small apoM-containing particles in HDL^{apoM+} were not seen in plasma on gel filtration analyses (Fig. 3B). Thus, they probably arose during the ultracentrifugation or column purification procedures. LDL^{apoM+} tended to be slightly smaller than total LDL and LDL^{apoM-} (Fig. 3A).

On SDS-PAGE, HDL^{apoM+} mainly contained apoA-I and apoM, whereas other apolipoproteins appeared to be less abundant than in total HDL. However, HDL^{apoM+} contained detectable amounts of apoA-II, apoC-II, and apoC-III (**Fig. 4A**). HDL that was prepared by ultracentrifugation contained less apoJ than HDL isolated by ion-exchange and affinity chromatography (data not shown).

On charge-separating agarose gel electrophoresis, both total HDL and HDL^{apoM+} displayed α- and preβ-migrating bands (Fig. 4B), whereas apoM was predominantly in α-migrating particles (Fig. 4C). Compared with total HDL, HDL^{apoM+} contained similar amounts of phosphatidylcholine, triglyceride, and cholesteryl esters but slightly more free cholesterol (Fig. 4C).

Antioxidative effect of apoM-containing HDL and LDL

To measure the antioxidant effect of HDL^{apoM+} and LDL^{apoM+}, we monitored Cu^{2+}-induced development of

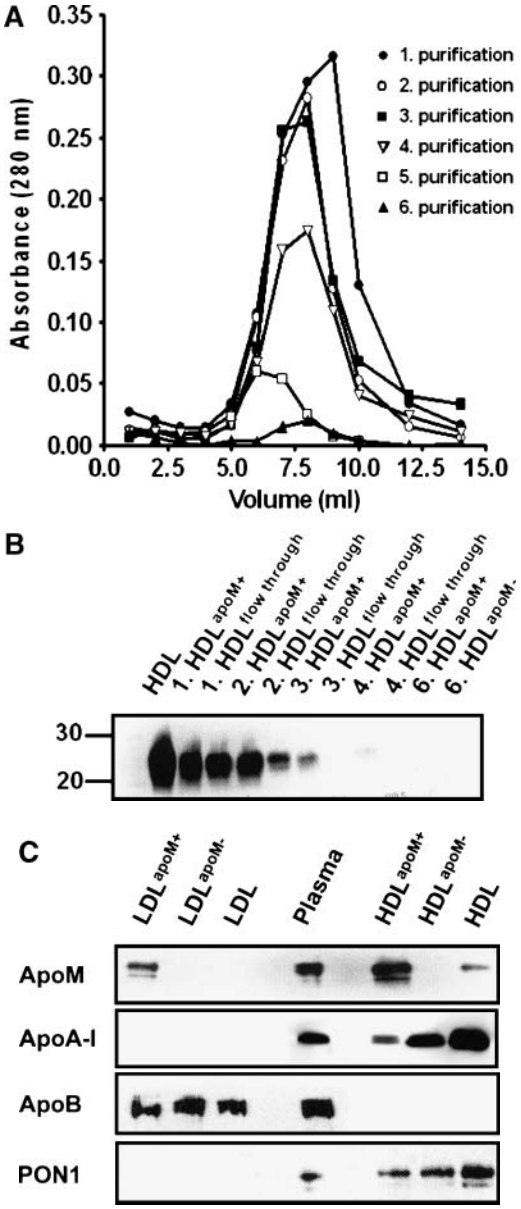

Fig. 2. Purification of apoM particles from HDL and LDL. A: HDL that had been isolated by ultracentrifugation (10 ml containing 120 mg total protein) was passed over an apoM immunoaffinity column to purify apoM-containing HDL. Bound particles were eluted and collected in 1 ml fractions before the flow-through was passed over the column again. The procedure was repeated six times. The absorbance at 280 nm was measured in each fraction. The purification of HDL^{apoM+} yielded ~3.9 mg of protein as judged from the absorbance measurements. B: Western blot analysis of apoM in HDL (starting material), the eluted apoM-containing HDL fractions from each purification round, and the corresponding flow-through fraction. One microliter of each fraction was applied to the gel. HDL^{apoM+} was detected only in the eluate in the first three purification rounds. HDL that was completely devoid of apoM (HDL^{apoM-}) was obtained after six purification rounds. C: Western blot analysis of apoM, apoA-I, apoB, and PON1 in purified lipoprotein fractions. LDL and HDL particles were adjusted to concentrations of 0.5 and 1.3 μg/μl, respectively. The volumes of LDL and HDL particles analyzed were, respectively, 1.5 and 0.5 μl for apoM, 1 and 0.05 μl for apoA-I, and 1 and 3 μl for apoB and PON1. ApoM could be detected in total LDL on long exposures of the Western blot (and when more protein was loaded on the gels).

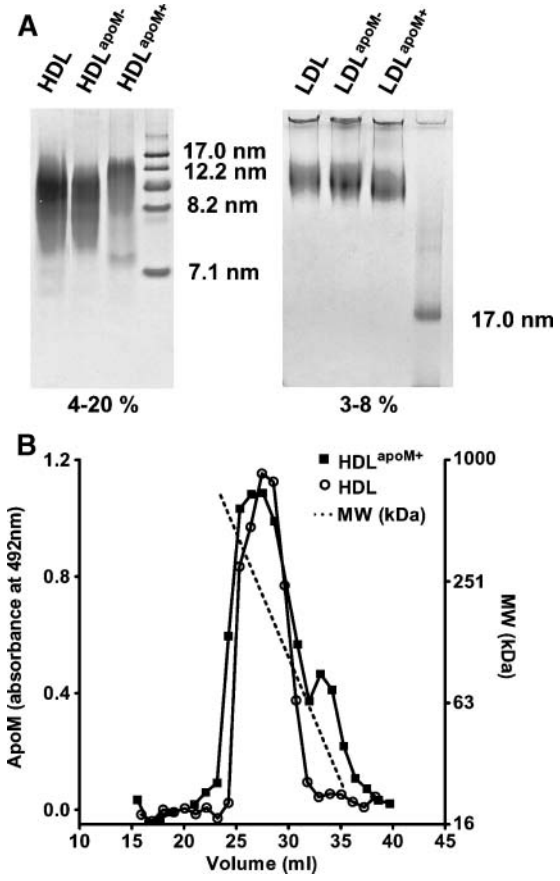

Fig. 3. Sizes of apoM-containing HDL and LDL. A: HDL particles (13 μg of protein) and LDL particles (5.4 μg of protein) were analyzed on 4–20% and 3–8% nondenaturing gradient gels, respectively. B: HDL and HDL^{apoM+} were subjected to gel filtration chromatography using Superose 6HR and 12HR columns in serial connection. ApoM in HDL^{apoM+} (closed squares) and total HDL (open circles) was measured with ELISA; the absorbance at 492 nm is shown (left *y* axis). The dotted line indicates the relation between elution volume and molecular size (right *y* axis) and is derived from calibration with six different molecular mass (MW) markers (kDa). Void volume (V_0) = 15.5 ml and total column volume (V_t) = 41.5 ml.

dienes at 234 nm (**Fig. 5**, **Table 2**). The oxidation curve obtained for HDL^{apoM+} demonstrated increased lag time (see Methods for a definition) after the addition of Cu^{2+} compared with corresponding curves obtained with total HDL and HDL^{apoM-}. This suggests that the apoM-containing HDL particles were more resistant to oxidation than the other HDL particles (Fig. 5A). Moreover, HDL^{apoM+} was able to prolong the lag time and reduce the propagation rate when added to LDL (Fig. 5B, Table 2) or HDL (Fig. 5C) at a level identical to albumin, thus appearing to protect both LDL and HDL against Cu^{2+}-induced oxidation. Finally, LDL^{apoM+} demonstrated a slightly shorter lag time than did total LDL or LDL^{apoM-}, but the maximal amount of Cu^{2+}-induced diene formation was decreased (Fig. 5D, Table 2). To ensure that these results were not attributable to the purification of HDL^{apoM+} by immunoaffinity chromatography, HDL^{apoM-} was further purified by chromatography with immobilized

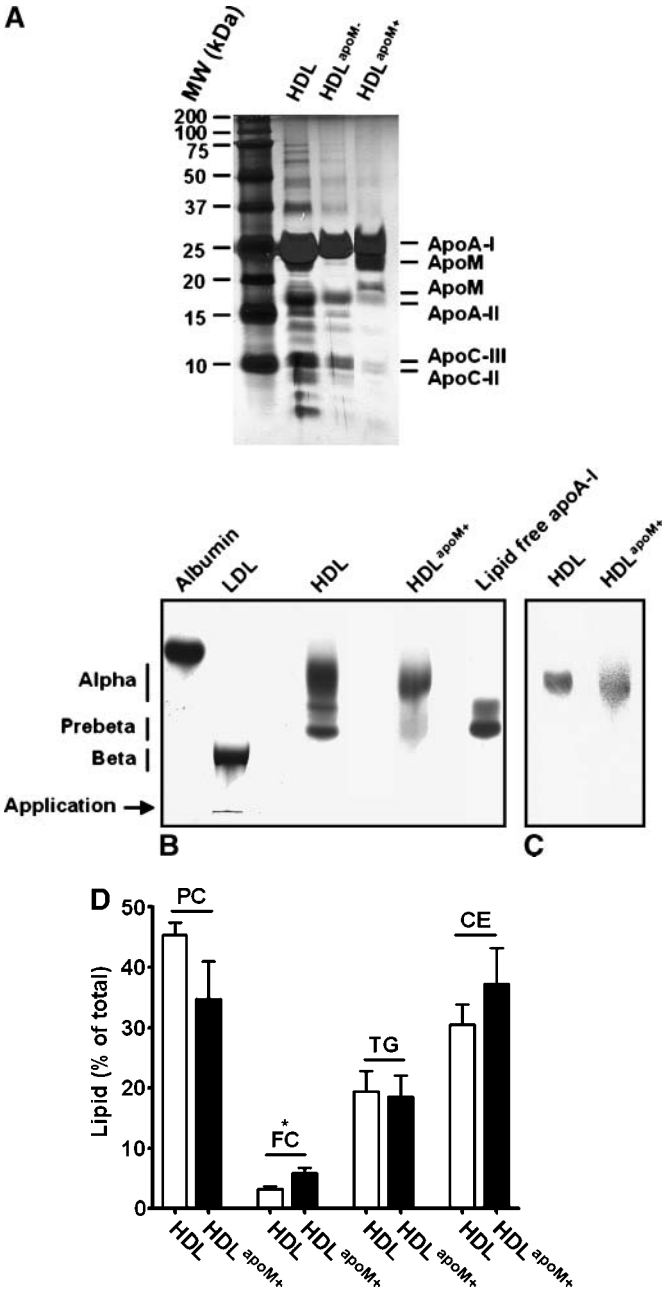

Fig. 4. Protein and lipid composition of apoM-containing HDL particles. A: Proteins in total HDL (~6 μg), HDL^{apoM-} (~1 μg), and HDL^{apoM+} (~1 μg) were reduced, separated by 12% SDS-PAGE, and silver-stained. The identity of the apolipoproteins was deduced from comparison with Fig. 1C. Note that apoM and apoA-I are less well separated under reducing compared with nonreducing conditions. MW, molecular mass. B: Agarose gel electrophoresis of lipoprotein fractions, lipid-free apoA-I, and albumin (10 μg of protein each); proteins were stained with Coomassie Brilliant Blue. C: Western blot of apoM in 5 μg of HDL and 0.5 μg of HDL^{apoM+} after separation by agarose gel electrophoresis. D: Lipid contents (percentage of total lipid mass) of HDL^{apoM+} (closed bars; n = 11) and HDL (open bars; n = 19) (data for total HDL and HDL^{apoM-} were similar and thus combined). CE, cholesteryl ester; FC, free cholesterol; PC, phosphatidylcholine; TG, triglyceride. * $P < 0.005$ by Student's *t*-test . Values are means ± SEM.

antibody against apoA-I (M12) using the same protocol. However, the results obtained with HDL^{apoM-} with or without immunoaffinity chromatography purification were essentially identical (data not shown).

ApoM and efflux of cholesterol from THP-1 foam cells

To explore the cholesterol efflux-stimulating capacity of HDL^{apoM+} and LDL^{apoM+}, we added them to THP-1 foam cells. HDL^{apoM+} caused more efflux of [^{3}H]cholesterol to the medium than did either total HDL or HDL^{apoM-} (**Fig. 6A**). There was no detectable difference in the cholesterol efflux-stimulating capacity of total HDL and HDL^{apoM-}. Moreover, the total amount of cholesterol remaining in THP-1 foam cells after 40 h was lower in cells incubated with HDL^{apoM+} than in cells incubated with total HDL or HDL^{apoM-} (Fig. 6B).

Incubation of THP-1 foam cells with LDL^{apoM+} resulted in higher net efflux of [^{3}H]cholesterol from the THP-1 foam cells than incubation with total LDL or LDL^{apoM-} (**Fig. 7A**). Incubation with LDL for 40 h increased the cellular cholesterol content above that seen in cells incubated with medium alone (indicating net uptake of cholesterol from LDL). Interestingly, this effect was less pronounced for LDL^{apoM+} than for total LDL and LDL^{apoM-} (Fig. 7B).

DISCUSSION

In this investigation, we used ion-exchange and immunoaffinity chromatography to isolate apoM-containing particles from human plasma. The apoM particles had densities similar to both HDL_2 (1.063–1.125 g/l) and HDL_3 (1.125–1.210 g/l). In addition, apoM particles were isolated by immunoaffinity chromatography from HDL and LDL that had been prepared with traditional ultracentrifugation. From the amounts of HDL and LDL recovered in apoM immunoaffinity chromatography (Fig. 2A and data not shown), we estimate that apoM is present in ~5% of HDL particles and in <2% of LDL particles.

The apoM-containing particles contained both HDL-related (apoA-I, apoA-II, apoC-I, apoC-II, apoC-III, and apoJ) and LDL-related (apoB) apolipoproteins. ApoJ-containing HDL particles are known to be heterogeneous in size and composition, and apoJ has been found in both HDL_2 and HDL_3 (23). However, apoJ could not be identified in the HDL^{apoM+} that was isolated by the combination of ultracentrifugation and immunoaffinity chromatography. Indeed, control experiments showed that apoJ was lost from HDL during ultracentrifugation of plasma for 24 h at d = 1.21 g/l (data not shown). A similar phenomenon has been reported for other lipoproteins, such as LCAT (24).

ApoM contains no signal peptidase cleaving site, and the signal peptide is retained in the mature apoM in plasma (11, 12). Although both PON1 and HRP also contain retained signal peptides, they were present only in small amounts in the isolated preparations of apoM-containing particles. This suggests that although the hy-

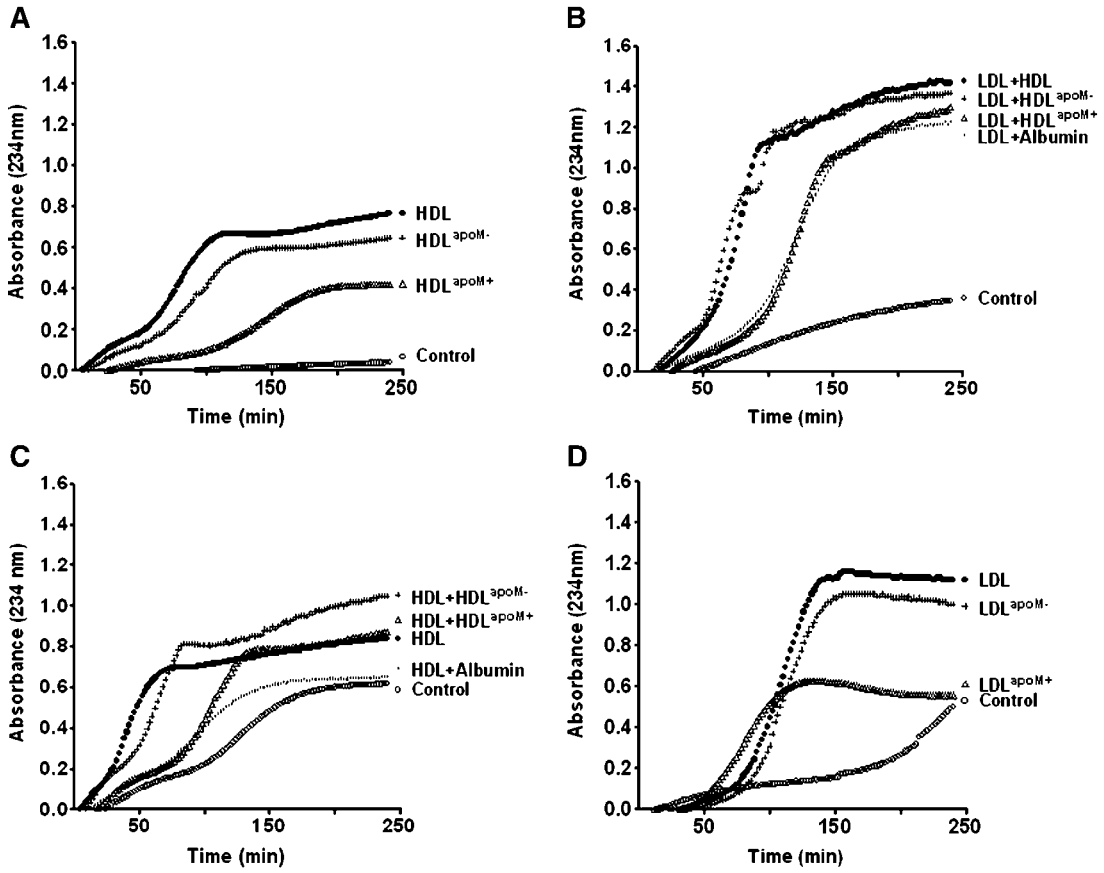

Fig. 5. Cu^{2+}-induced oxidation of apoM-containing HDL and LDL. Oxidation was induced by adding 2.5 μM $CuSO_4$ (final concentration) to 950 μl of lipoprotein solution. Diene formation was followed by absorbance measurement at 234 nm every minute for 4 h. A: Analyses of 75 μg of total protein of HDL, HDL^{apoM-}, or HDL^{apoM+}. B: Analyses of 100 μg of total protein of LDL mixed with 100 μg of total protein of HDL, HDL^{apoM-}, HDL^{apoM+}, or albumin. C: Analyses of 100 μg of total protein of HDL mixed with 100 μg of total protein of HDL, HDL^{apoM-}, HDL^{apoM+}, or albumin. D: Analyses of 50 μg of total protein of LDL, LDL^{apoM-}, or LDL^{apoM+}. Closed circles, HDL or LDL; crosses, HDL^{apoM-} or LDL^{apoM-}; triangles, HDL^{apoM+} or LDL^{apoM+}, dots, albumin; open circles, controls (HDL and LDL without $CuSO_4$). Data are representative of two to four independent experiments.

drophobic signal peptides probably serve to anchor the respective apolipoproteins in the lipid moiety of the lipoprotein particle, the signal peptide per se does not confer any specificity for association with a certain subpopulation of HDL.

Based on experiments performed in mice, it was recently proposed that apoM is a constitutive component of preβ HDL and is required for preβ HDL formation (15). In this study, the size and charge patterns of the isolated HDL^{apoM+} particles suggest them to be as extensively heterogeneous as regular HDL. The main part of HDL^{apoM+} particles were α-migrating. Interestingly, our analyses suggest that the content of free cholesterol relative to other lipids is increased in HDL^{apoM+} compared with total HDL particles.

We explored the function of the HDL particles containing apoM and found that HDL^{apoM+} could protect itself, as well as other HDL and LDL particles, from Cu^{2+}-induced oxidation. The HDL-associated proteins PON1 and apoJ have previously been reported to have antioxidative effects (25–27). Although these proteins were present in some apoM-containing particles, it is important to stress that the antioxidative effects of HDL^{apoM+} were not attributable to increased levels of PON1 or apoJ. Indeed, LDL^{apoM+} also seemed more resistant to oxidation than LDL^{apoM-}. The mechanism for the antioxidative effect of apoM is still unknown, and it is interesting that several other lipocalins have been described to have similar antioxidant properties (13).

The HDL^{apoM+} particles were more efficient than total HDL or HDL^{apoM-} in stimulating cholesterol efflux from THP-1 foam cells and reducing total levels of intracellular cholesterol. The results obtained with the THP-1 cells and apoM-containing particles are in agreement with observations reported by Wolfrum, Poy, and Stoffel (15) suggesting that apoM can stimulate cholesterol efflux from murine Raw cells. It is possible that the increased efflux capacity of the apoM-containing HDL simply is a reflection of them being enriched in small preβ-migrating HDL. Thus, whether apoM contributes to apoA-I-mediated cholesterol efflux or uses other unknown pathways remains to be investigated. However, the data suggest unique proper-

TABLE 2. Lag time, propagation rate, and maximal absorbance (234 nm) upon Cu^{2+}-induced oxidation of apoM-containing HDL and LDL

Lipoprotein	Lag Time	Propagation Rate	Maximal Absorbance	N
	min	*absorbance units$_{234nm}$/min × 10^{-2}*	*absorbance units$_{234nm}$*	
LDL	90 ± 8	2.1 ± 0.2[a]	1.4 ± 0.2[b]	3
LDL^{apoM-}	104 ± 16[b]	1.6 ± 0.3	1.3 ± 0.1	3
LDL^{apoM+}	50 ± 13	1.1 ± 0.2	0.7 ± 0.1	3
LDL + HDL	61 ± 7[c]	2.5 ± 0.3[d]	1.3 ± 0.1	4
LDL + HDL^{apoM-}	60 ± 18[d]	2.0 ± 0.2[c]	1.4 ± 0.0[d]	4
LDL + HDL^{apoM+}	99 ± 8	1.8 ± 0.2	1.3 ± 0.0	4

Oxidation was induced by adding 2.5 μM $CuSO_4$ (final concentration) to 950 μl of lipoprotein solution. For analysis of LDL, LDL^{apoM-}, and LDL^{apoM+}, 50 μg of total protein mass was used, whereas 100 μg of total protein mass of each component was analyzed by adding HDL, HDL^{apoM-}, or HDL^{apoM+} to LDL. Diene formation was followed by absorbance measurement at 234 nm every minute for 4 h. Lag time, propagation rate, and maximal absorbance were calculated as described in Methods. Two-group comparisons were performed using Student's *t*-test. Values are means ± SEM. N = number independent experiments.

[a] $P < 0.001$ compared with LDL^{apoM+}.
[b] $P < 0.05$ compared with LDL^{apoM+}.
[c] $P < 0.001$ compared with LDL + HDL^{apoM+}.
[d] $P < 0.05$ compared with LDL + HDL^{apoM+}.

ties of apoM in relation to cholesterol accumulation in foam cells. Thus, the protein composition of LDL^{apoM+} and LDL^{apoM-} was mainly distinguished by the presence of apoM, and LDL^{apoM+} mediated more [^{3}H]cholesterol net efflux and induced less cholesterol accumulation than LDL^{apoM-}.

Although these results suggest unique properties of apoM-containing HDL and LDL, it is important to note that these results suggest that apoM is present in only ~5% of HDL particles and <2% of LDL particles. Hence, the bulk cholesterol efflux and antioxidative capacity of HDL resides with apoM-free HDL. Accordingly, there was no difference between total HDL and HDL^{apoM-} in the oxidation and cholesterol efflux evaluations. These investigations of the biological effects of purified HDL^{apoM+} should be interpreted with caution, because purification by ultracentrifugation leads to a loss of apolipoproteins that may have physiologically important effects (e.g., apoJ). Also, the purification of HDL^{apoM+} resulted in the formation of small apoA-I- and apoM-containing particles that could not be seen in plasma and that may have effects in the oxidation and cholesterol efflux assays. Nevertheless, these particles constituted <10% of the total HDL^{apoM+}. Moreover, multiple passages of apoM-free HDL over the anti-apoM column did not change its protein composition (data not shown), and the effects of HDL^{apoM-} (which had passed over the affinity column multiple times) and total HDL were similar both in the oxidation and cholesterol efflux assays. Finally, there was no effect of immunoaffinity purification of apoM-free apoA-I-containing HDL on its ability to protect LDL from oxidation. These results suggest that the immunoaffinity purification procedures did not confer major changes in the biological properties of the HDL subclasses.

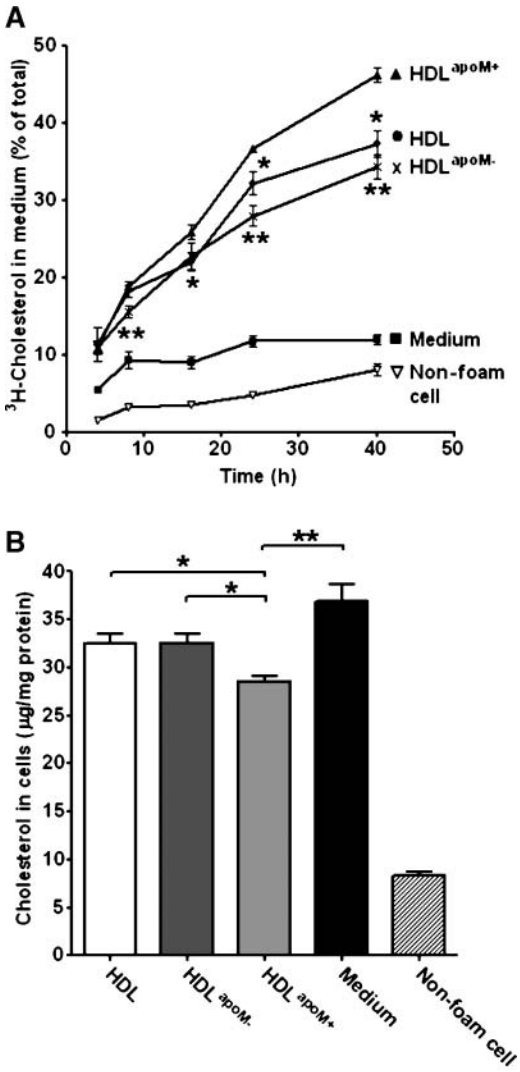

Fig. 6. Effect of apoM-containing HDL on cholesterol efflux from THP-1 cells. THP-1 cells were differentiated to foam cells with acetylated LDL and labeled with [^{3}H]cholesterol. The labeled foam cells were incubated with 25 μg total protein/ml HDL^{apoM+}, HDL^{apoM-}, total HDL, or serum-free medium (0.7 ml). Undifferentiated THP-1 (nonfoam) cells were incubated with the medium as an additional control. A: [^{3}H]cholesterol in aliquots of the medium (25 μl) was measured at 4, 8, 16, 24, and 40 h after the addition of acceptor lipoproteins and compared with [^{3}H]cholesterol in the cells after 40 h. $P < 0.05$ when HDL^{apoM+} was compared with HDL (*) or HDL^{apoM-} (**) using Student's *t*-test. Importantly, after 8 h of stimulation, the cholesterol efflux was higher from cells incubated with HDL^{apoM+} than from cells incubated with HDL^{apoM-}. B: Total cholesterol in foam cells after incubation with total HDL, HDL^{apoM-}, HDL^{apoM+}, or medium for 40 h. Undifferentiated (nonfoam) cells were included as controls. * $P < 0.01$ and ** $P < 0.005$ for the indicated two-group comparisons using Student's *t*-test. All data represent means ± SEM of four individual wells and were confirmed in an independent experiment.

In conclusion, human apoM is associated with plasma lipoproteins that are heterogeneous in size and charge as well as in protein and lipid composition. Nevertheless, as judged from the in vitro investigations of lipoprotein oxidation and cholesterol efflux, apoM appears to designate a small subpopulation of HDL (and LDL) that potentially

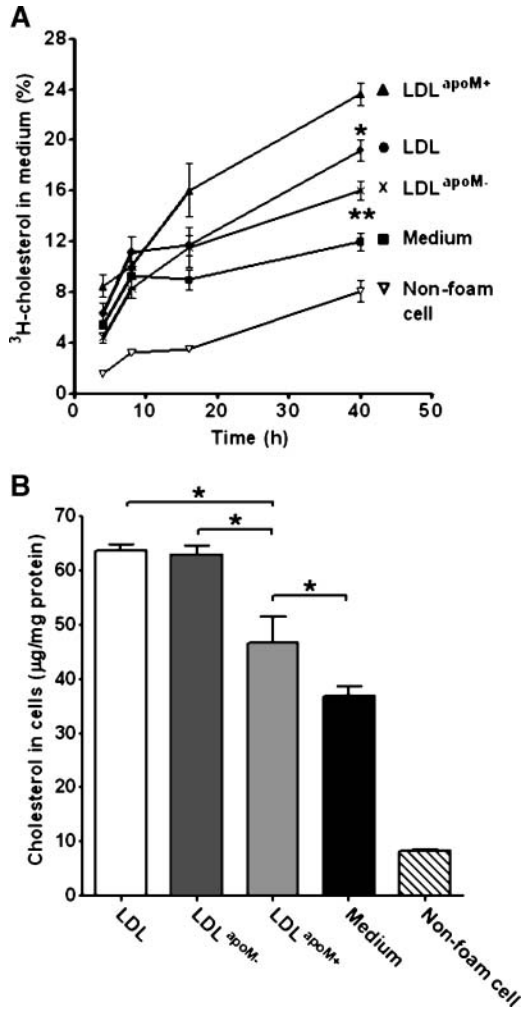

Fig. 7. Effect of apoM-containing LDL on cholesterol efflux from THP-1 cells. THP-1 cells were differentiated to foam cells with acetylated LDL and labeled with [^{3}H]cholesterol. The labeled foam cells were incubated with 25 μg total protein/ml LDL^{apoM+}, LDL^{apoM-}, total LDL, or serum-free medium (0.7 ml). Undifferentiated THP-1 (nonfoam) cells were incubated with the medium as an additional control. A: [^{3}H]cholesterol in aliquots of the medium (25 μl) was measured at 4, 8, 16, 24, and 40 h after the addition of acceptor lipoproteins and compared with [^{3}H]cholesterol in the cells after 40 h. $P < 0.005$ when LDL^{apoM+} was compared with LDL (*) or LDL^{apoM-} (**) using Student's *t*-test. B: Total cholesterol in foam cells after incubation with total LDL, LDL^{apoM-}, LDL^{apoM+}, or medium for 40 h. Undifferentiated (nonfoam) cells were included as controls. * $P < 0.01$ and ** $P < 0.005$ for the indicated two-group comparisons using Student's *t*-test. All data represent means ± SEM of four individual wells and were confirmed in an independent experiment.

may be relatively more antiatherogenic than the HDL population as a whole.

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