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A Unique Protease-sensitive High Density Lipoprotein Particle Containing the Apolipoprotein A-I_{Milano} Dimer Effectively Promotes ATP-binding Cassette A1-mediated Cell Cholesterol Efflux*

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Elda Favari[‡], Monica Gomaraschi[§], Ilaria Zanotti[‡], Franco Bernini[‡], Miriam Lee-Rueckert[¶], Petri T. Kovanen[¶], Cesare R. Sirtori[§], Guido Franceschini[§1], and Laura Calabresi[§]

From the [‡]Department of Pharmacological and Biological Sciences, and Applied Chemistries, University of Parma, Viale delle Scienze 27A, 43100 Parma, Italy, the [§]Center E. Grossi Paoletti, Department of Pharmacological Sciences, University of Milano, Via Balzaretti 9, 20133 Milano, Italy, and the [¶]Wihuri Research Institute, Kalliolinnantie 4, 00140 Helsinki, Finland

Carriers of the apolipoprotein A-I_{Milano} (A-I_M) variant present with severe reductions of plasma HDL levels, not associated with premature coronary heart disease (CHD). Sera from 14 A-I_M carriers and matched controls were compared for their ability to promote ABCA1-driven cholesterol efflux from J774 macrophages and human fibroblasts. When both cell types are stimulated to express ABCA1, the efflux of cholesterol through this pathway is greater with A-I_M than control sera (3.4 ± 1.0% *versus* 2.3 ± 1.0% in macrophages; 5.2 ± 2.4% *versus* 1.9 ± 0.1% in fibroblasts). A-I_M and control sera are instead equally effective in removing cholesterol from unstimulated cells and from fibroblasts not expressing ABCA1. The A-I_M sera contain normal amounts of apoA-I-containing preβ-HDL and varying concentrations of a unique small HDL particle containing a single molecule of the A-I_M dimer; chymase treatment of serum degrades both particles and abolishes ABCA1-mediated cholesterol efflux. The serum content of chymase-sensitive HDL correlates strongly and significantly with ABCA1-mediated cholesterol efflux ($r = 0.542$, $p = 0.004$). The enhanced capacity of A-I_M serum for ABCA1 cholesterol efflux is thus explained by the combined occurrence in serum of normal amounts of apoA-I-containing preβ-HDL, together with a unique protease-sensitive, small HDL particle containing the A-I_M dimer, both effective in removing cell cholesterol via ABCA1.

The efflux of cell cholesterol from the plasma membrane into suitable extracellular acceptors is the first, possibly rate-limiting step in reverse cholesterol transport (1). Interest in studying cell cholesterol efflux has increased with the discovery of a number of membrane proteins that have now been conclusively shown to mediate the movement of cholesterol between cells and extracellular acceptors, like the scavenger receptor BI (SR-BI),[2] and the ABCA1 and ABCG1 transporters (2). Cell cholesterol efflux occurs through several mechanisms (3). Passive diffusion into extracellular acceptors occurs from all cell types but is relatively inefficient; when SR-BI is present in the cell plasma membrane, the efflux is facilitated. Phospholipid-containing HDL particles are the best acceptors of cholesterol through both the diffusion-mediated and SR-BI-facilitated efflux. In both cases, the flux of cholesterol is bidirectional, the direction of net flux depending on the cholesterol gradient between the cell membrane and the acceptor (3).

ABCA1 and ABCG1 are members of a large family of transporters that have common structural motifs and use ATP as an energy source to transport a variety of substrates, including lipids, ions, and cytotoxins across the cell membrane (4). ABCG1 is a half-transporter that might act as a homodimer, mediating the active export of cholesterol and phospholipids to the major forms of plasma HDL, such as HDL_2 and HDL_3 (5). ABCA1, a full transporter comprising two similar halves linked covalently, has been originally discovered as the genetic cause of Tangier disease (TD), which is characterized by severe HDL deficiency, accumulation of cholesterol in tissue macrophages, and accelerated atherosclerosis. This finding highlights the dual role of ABCA1 in HDL formation as well as in cholesterol efflux from macrophages. ABCA1 is distributed both in the plasma membrane and late endosomal compartments, cycling between the two loci (6). ABCA1 expression is regulated by a variety of mechanisms (7). In contrast to passive diffusion and SR-BI-facilitated cholesterol flux, the movement of cholesterol by ABCA1 is unidirectional and, accordingly, activation of this system always results in net efflux of cell cholesterol (3). Also different from SR-BI-facilitated cholesterol efflux, the preferred cholesterol acceptors via ABCA1 are lipid-free/poor apolipoproteins. All of the exchangeable apolipoproteins, such as apoA-I, apoA-II, apoA-IV, apoC, and apoE (8, 9), as well as synthetic apoA-I-mimetic peptides (10) can act as cholesterol

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[1] To whom correspondence should be addressed: Center E. Grossi Paoletti, Dept. of Pharmacological Sciences, Via Balzaretti 9, 20133 Milano, Italy. Tel.: 39-0250319911; Fax: 39-0250319900; E-mail: Guido.Franceschini@unimi.it.

[2] The abbreviations used are: SR-BI, scavenger receptor BI; ABCA1, ATP-binding cassette A1; A-I_M, apolipoprotein A-I_{Milano}; A-I_M/A-I_M, A-I_M homodimer; CHD, coronary heart disease; LCAT, lecithin:cholesterol acyltransferase; POPC, palmitoyloleoylphosphatidylcholine; rHDL, reconstituted HDL; TD, Tangier disease; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HDL, high density lipoprotein; FCS, fetal calf serum; BSA, bovine serum albumin.

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acceptors through ABCA1. In some instances, ABCA1 mediates cholesterol efflux to plasma-derived HDL_3 preparations, this effect being attributable to the presence of preβ-migrating particles within the plasma HDL_3 fraction (11).

The apolipoprotein A-I_{Milano} (A-I_M) mutation was originally described in a family originating from Limone sul Garda, in Northern Italy (12). This apoA-I variant shows a single amino acid substitution, arginine 173 to cysteine, which leads to the formation of homodimers (A-I_M/A-I_M) and heterodimers with apoA-II (13). Forty-four carriers have been identified up to now, all heterozygous for the mutation and sharing a lipoprotein phenotype characterized by very low plasma levels of HDL cholesterol associated with moderate hypertriglyceridemia (14), a condition that has been repeatedly linked to a high risk of premature coronary heart disease (CHD). Surprisingly, the A-I_M carrier status was originally associated with a reduced cardiovascular risk (15), a finding now supported by a recent clinical study showing the A-I_M carriers do not present with any clear evidence of vascular disease at the preclinical level (16). The apparent A-I_M paradox of severe HDL deficiency with vascular health may be explained by an enhanced capacity of the carrier HDL to remove cholesterol from the arterial wall and drive it to the liver for excretion. Such hypothesis was initially investigated by comparing the capacity of serum from A-I_M carriers and from control subjects to extract cholesterol from Fu5AH cells, which express high levels of SR-BI in the plasma membrane, and thus efflux cholesterol mainly via SR-BI-facilitated diffusion. SR-BI-mediated cholesterol efflux to A-I_M sera was found to be only slightly reduced when compared with control sera, despite the remarkable decrease in the content of mature HDL, the preferential acceptors of SR-BI cholesterol (17), suggesting a higher efficiency of A-I_M HDL for cholesterol uptake via this pathway. These previous investigations are complemented in the present study, where we evaluated the ability of sera from A-I_M carriers and matched controls to promote ABCA1-mediated cholesterol efflux from macrophages and fibroblasts.

EXPERIMENTAL PROCEDURES

Subjects—Fourteen adult A-I_M carriers (7 females, 7 males), belonging to the previously described A-I_M kindred (15) volunteered for this study. Fourteen age- and sex-matched noncarriers were selected among close relatives in the same families. All participating subjects were healthy, were taking no medications, and consumed a typical Mediterranean diet. All subjects were fully informed of the modalities and end points of the study. Fasting blood was collected into empty plastic tubes and serum prepared by low speed centrifugation at 4 °C. Aliquots were immediately frozen and stored at −80 °C until assayed.

Reconstituted HDL—ApoA-I and A-I_M/A-I_M were purified from human plasma, and discoidal reconstituted HDL (rHDL) containing palmitoyloleoylphosphatidylcholine (POPC) and either A-I_M/A-I_M or apoA-I, with a diameter of 7.8 nm, were prepared by the cholate dialysis technique, as described (18). In the final preparation, all the protein was incorporated into stable rHDL, with no lipid-free apolipoprotein remaining. The apoA-I rHDL contained two apoA-I molecules, with a POPC: apolipoprotein molar ratio of 44:1. The A-I_M/A-I_M rHDL contained only one A-I_M/A-I_M molecule, with a POPC:apolipoprotein molar ratio of 73:1.

Analyses—Serum total and HDL cholesterol, triglyceride, and phospholipid levels were determined with standard enzymatic techniques by using a Roche Diagnostics Integra 400 autoanalyzer. LDL cholesterol was calculated with the Friedewald formula. Serum apolipoprotein A-I, A-II, and B levels were determined by immunoturbidimetry, using the Integra 400 analyzer with commercially available polyclonal antibodies. The sheep anti-human apoA-I antibody recognizes all forms of A-I_M (monomer, homodimer, and heterodimer); therefore the apoA-I concentration determined in sera of A-I_M carriers, who are heterozygotes for the mutation, is the sum of mutant and wild-type apoA-I.

Agarose Gel Electrophoresis—Agarose gel electrophoresis of lipid-free apolipoproteins and rHDL was performed using the Beckman Paragon system; proteins were stained with Coomassie Blue G-250 (19).

Two-dimensional Electrophoresis and Immunoblotting—Serum HDL subclasses were separated by two-dimensional electrophoresis, in which agarose gel electrophoresis was followed by nondenaturing polyacrylamide gradient gel electrophoresis and subsequent immunoblotting (11). In the first dimension, serum (5 μl) was run on a 0.5% agarose gel; agarose gel strips containing the separated lipoproteins were then transferred to a 3–20% polyacrylamide gradient gel. Separation in the second dimension was performed at 30 mA for 4 h. Fractionated HDL were then electroblotted onto a nitrocellulose membrane and detected with a sheep antihuman apoA-I antibody, which recognizes all forms of A-I_M (monomer, homodimer, and heterodimer). Lipoproteins containing A-I_M/A-I_M were detected with a mouse monoclonal antibody raised against A-I_M/A-I_M, which does not recognize either other A-I_M forms (monomer, heterodimer) or wild-type apoA-I. The relative content of distinct HDL subclasses was calculated by using the Bio-Rad Multi-Analyst/PC Software, and expressed as percentage of total immunoreactivity.

Isolation of Mast Cell Granule Remnants—Serosal mast cells were isolated from the peritoneal and pleural cavities of rats. Degranulation was induced with compound 48/80 (Sigma) and the exocytosed chymase-containing granules, *i.e.* granule remnants, were isolated from the released material by centrifugation, as described (20). The heparin-bound chymase present in granule remnants is partly resistant to inhibition by the physiological antiproteases found in human serum, and, therefore, addition of the remnants to serum results in progressive proteolysis of the various chymase-sensitive proteins present in serum (21).

Proteolysis of Serum by Granule Remnant Chymase—500 μl of serum from 4 A-I_M carriers and 4 control subjects were incubated in the absence or presence of granule remnants (30 μg/ml of granule remnant total protein, equal to 40 BTEE units/ml) for 2 h at 37 °C. After incubation, tubes were cooled down rapidly by placing them on ice. Tubes were centrifuged at 4 °C, 15,000 rpm for 5 min to remove the granule remnant-bound chymase, and the chymase-free supernatants were collected and used for HDL subclass separation by two-dimensional electrophoresis and cell cholesterol efflux experiments. The HDL

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digestion products were analyzed by SDS-PAGE on 10–16% acrylamide gradient slab gels, using the Tris-Tricine buffer system, and then electrophoretically transferred to nitrocellulose membranes (22). ApoA-I peptides were detected by the use of the sheep antihuman apoA-I antibody, and A-I$_M$/A-I$_M$ fragments were detected by the use of the mouse anti A-I$_M$/A-I$_M$ monoclonal antibody.

Cell Culture—J774 mouse macrophages were cultured in RPMI with 10% FCS. Human control and TD fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% FCS. All cells seeded in 12-well plates and incubated at 37 °C, in 5% CO_2, and utilized when cultures have reached 80–90% of confluence.

CPT-cAMP Stimulation—J774 monolayers were washed with phosphate-buffered saline and incubated for 24 h in RPMI containing [1,2-^{3}H]cholesterol (4 μCi/ml), as described (23). The labeling medium contained 1% FCS and 2 μg/ml of an ACAT inhibitor to ensure that all labeled cholesterol was present as unesterified cholesterol. After the labeling period, cells were washed and incubated in RPMI with 0.2% BSA, with or without 0.3 mM CPT-cAMP for 18 h. After this incubation, some wells were washed with PBS, dried, and extracted with 2-propyl alcohol. These cells provide baseline (time 0) values for total [1,2-^{3}H]cholesterol content. Human control and TD fibroblasts were treated like J774 cells.

Measurement of Cell Cholesterol Efflux—CPT-cAMP-stimulated and unstimulated monolayers containing [1,2-^{3}H]cholesterol were washed with phosphate-buffered saline and incubated for efflux time (4 h) in the presence of 5% serum, or with rHDL at the protein concentrations of 12.5 and 25.0 μg/ml. Cell media were centrifuged to remove floating cells, and radioactivity in the supernatant was determined by liquid scintillation counting. Cholesterol efflux was calculated as: ((cpm in medium at 4 h/cpm at time 0) $\times$ 100). In some experiments, efflux was evaluated after incubation of cells for 2 h with 10 μM probucol (24).

Statistical Analyses—Results are expressed as mean ± S.D. Differences among groups were evaluated by analysis of variance (one-way analysis of variance). Simple regression analyses were performed to assess the association between parameters, and the significance of the correlations was determined by the F parameter and by the correlation coefficient. Group differences or correlations with $p < 0.05$ were considered statistically significant.

RESULTS

Characteristics of the Population—The fasting serum lipid and lipoprotein concentrations in the examined A-I$_M$ carriers and controls are given in Table 1. Similar to previous studies (14), the A-I$_M$ carriers had remarkably lower serum HDL-C, apoA-I, and apoA-II levels and higher serum triglycerides than did controls.

Cholesterol Efflux to Serum—Previous studies using Fu5AH cells, which express high levels of SR-BI, have shown that the A-I$_M$ serum contains lipoprotein particles with high efficiency for cholesterol uptake via SR-BI-facilitated passive diffusion (17). In the present study, the capacity of A-I$_M$ and control serum to extract cell cholesterol via ABCA1 was initially tested in J774 murine macrophages. Under basal conditions, the J774 macrophages express SR-BI, but not ABCA1, and release membrane cholesterol to extracellular acceptors mostly by passive diffusion (25, 26). In such condition, cholesterol efflux to A-I$_M$ sera was similar to efflux induced by control sera (10.8 ± 1.9% *versus* 11.9 ± 1.2%). Stimulation of J774 macrophages with CPT-cAMP induces ABCA1 expression and ABCA1-mediated cholesterol efflux to lipid-free/-poor apolipoproteins and preβ-HDL (11). Indeed, when stimulated with CPT-cAMP, the J774 cells released more cholesterol to serum than did unstimulated J774 cells (14.2 ± 1.6% to A-I$_M$ sera and 14.2 ± 1.0% to control sera). The ABCA1-mediated cholesterol efflux was calculated as the percentage efflux from stimulated J774 cells minus the percentage efflux from unstimulated cells. There was a biological variability within the two groups; however, on average, ABCA1-dependent cholesterol efflux to serum from A-I$_M$ carriers was significantly greater (3.4 ± 1.0%) than efflux to serum from controls (2.3 ± 1.0%, p = 0.013) (Fig. 1).

To demonstrate further that A-I$_M$ serum is more effective than control serum in promoting cell cholesterol efflux through

TABLE 1
Serum lipid/lipoprotein levels in A-I$_M$ carriers and controls

	A-I$_M$ carriers	Controls	p
Gender (M/F)	7/7	7/7	
Age (y)	41.1 ± 17.4[a]	39.0 ± 14.3	NS[b]
BMI (kg/m^2)	24.4 ± 4.3	24.0 ± 3.6	NS
Total cholesterol (mg/dl)	184.3 ± 46.1	210.6 ± 50.8	NS
LDL cholesterol (mg/dl)	130.0 ± 13.3	138.6 ± 43.7	NS
HDL cholesterol (mg/dl)	21.9 ± 11.0	49.7 ± 20.6	<0.001
Triglycerides (mg/dl)	155.6 ± 65.1	111.9 ± 37.3	0.023
Phospholipids (mg/dl)	222.8 ± 83.4	247.8 ± 60.5	NS
ApoA-I (mg/dl)	83.1 ± 31.6	132.6 ± 21.5	<0.001
ApoA-II (mg/dl)	17.8 ± 5.5	40.3 ± 7.1	<0.001
ApoB (mg/dl)	99.2 ± 30.2	86.6 ± 24.8	NS
Preβ-HDL (%)	15.6 ± 5.1	15.5 ± 3.3	NS

[a] Data are reported as mean ± S.D.
[b] NS, not significant.

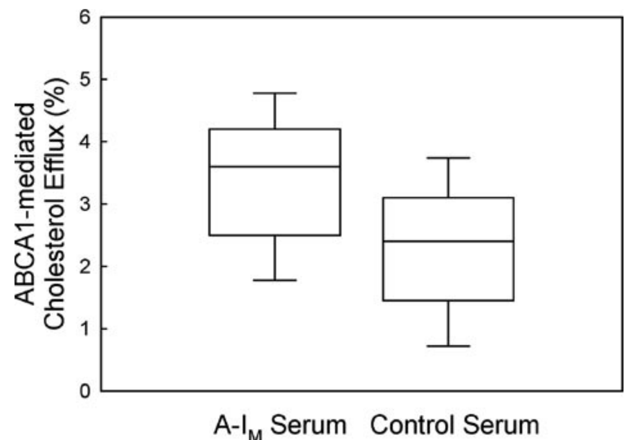

FIGURE 1. **ABCA1-mediated cholesterol efflux from J774 macrophages to sera from A-I$_M$ carriers and controls.** Macrophages were labeled with 4 μCi/ml [^{3}H]cholesterol for 24 h in RPMI medium with 1% FCS and 2 μg/ml of an ACAT inhibitor. Cells were then incubated for 18 h with 0.2% BSA in the absence or presence of 0.3 mM CPT-cAMP, washed and incubated for 4 h with 5% serum. Each sample was run in triplicate. ABCA1-mediated cholesterol efflux was calculated as the percentage efflux from CPT-cAMP-stimulated cells minus the percentage efflux from unstimulated cells. The *box plot* displays median values with the 25th and 75th percentiles; *capped bars* indicate the 10th and 90th percentiles. Each group consists of 14 subjects.

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ABCA1, two sets of experiments were performed. First, before assessment of cell cholesterol efflux, unstimulated and cAMP-stimulated J774 cells were treated with probucol, a selective inhibitor of ABCA1-mediated lipid efflux (24). Probucol completely abolished the cAMP-induced increase in cholesterol efflux to both A-I_M serum (11.6 ± 0.8% from cAMP-stimulated cells *versus* 11.5 ± 2.2% from unstimulated cells) and control serum (13.5 ± 0.3% from cAMP-stimulated cells *versus* 13.0 ± 1.7% from unstimulated cells). Second, cell cholesterol efflux to A-I_M and control sera was assessed by using skin fibroblasts from a healthy individual and from a patient with TD. A-I_M and control sera displayed the same capacity to extract cholesterol from unstimulated control fibroblasts (Fig. 2); cAMP stimulation of these cells enhanced cholesterol efflux to serum, the enhancement being significantly greater to sera from A-I_M carriers (5.2 ± 2.4%) than to sera from controls (1.8 ± 0.1%, $p < 0.001$) (Fig. 2). In sharp contrast, when sera were added to stimulated TD fibroblasts, which do not express ABCA1, no cAMP-induced increase in cholesterol efflux was observed with either type of serum (Fig. 2). These findings provide definite evidence that serum from A-I_M carriers is more effective than control serum in removing cell cholesterol via ABCA1.

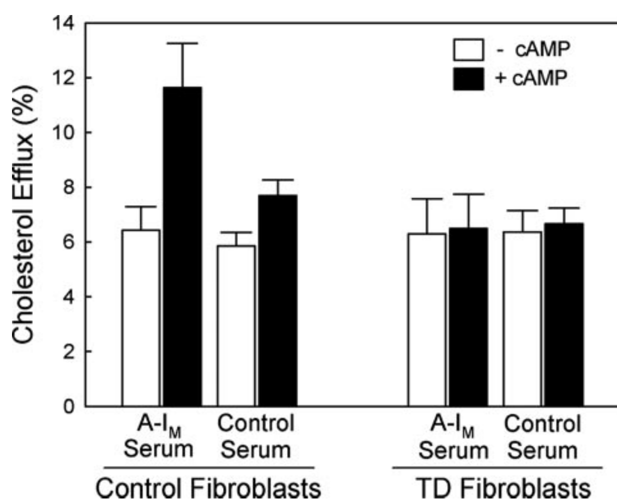

FIGURE 2. **Cholesterol efflux from human control and TD fibroblasts to sera from A-I_M carriers and controls.** Cell monolayers were labeled with 4 μCi/ml [^{3}H]cholesterol for 24 h in Dulbecco's modified Eagle's medium with 1% FCS and 2 μg/ml of an ACAT inhibitor. Cells were then incubated for 18 h with 0.2% BSA in the absence (*open bars*) or presence (*filled bars*) of 0.3 mM CPT-cAMP, washed and incubated for 4 h with 5% serum. Cholesterol efflux was calculated as: ((cpm in medium at 4 h/cpm at time 0) × 100). Each sample was run in triplicate. Data are mean ± S.D.; $n = 4$.

Serum HDL Subclasses and ABCA1-mediated Cell Cholesterol Efflux—To identify the component of A-I_M serum responsible for the improved cholesterol efflux capacity via the ABCA1 transporter, sera from A-I_M and control subjects were analyzed for their apoA-I-containing subpopulations by two-dimensional electrophoresis and immunoblotting. We have previously shown that control small preβ-migrating HDL, uniquely sensitive to proteolytic degradation by chymase, are a very efficient acceptor for ABCA1-mediated cholesterol efflux (11). A similar small preβ-migrating HDL is detectable in A-I_M sera; it contains wild-type apoA-I, consistent with the fact that the A-I_M carriers are all heterozygous for the mutation, but not the A-I_M/A-I_M, as no signal was detected in the pre-β region by using an antibody specific for the homodimeric form of A-I_M (Fig. 3). This particle is therefore identical to apoA-I-containing small preβ-HDL found in control serum. We then determined the relative proportion of preβ-HDL in the sera from A-I_M carriers and controls. No difference was observed between the two groups of subjects (Table 1), suggesting that components of A-I_M serum other than the small apoA-I-containing preβ-HDL were responsible for the enhanced ABCA1-mediated cholesterol efflux to A-I_M sera. Interestingly, immunodetection of A-I_M HDL subclasses with the anti A-I_M/A-I_M antibody identified two sets of particles: a series of α-migrating HDL particles ranging in diameter from 8.5 to 13 nm, *i.e.* in the range of control α-HDL, and a small HDL particle, with an estimated diameter of 7.8 nm, migrating in a position intermediate between the preβ and the α regions (Fig. 3). Such particle has an apparent molecular mass of 92 kDa, and thus likely contains one A-I_M/A-I_M molecule (56 kDa) as the sole protein component with ~45–50 phospholipid molecules. No signal for apoA-II was detectable in this region, indicating that this particle contains neither apoA-II nor the heterodimer A-I_M/A-II (not shown). According to densitometric analysis, these particles accounted for 22.2 ± 6.5% of total A-I_M/A-I_M immunoreactivity. To investigate whether this unique A-I_M/A-I_M-containing HDL particle acts as cell cholesterol acceptor via ABCA1, sera from A-I_M carriers and controls were treated with chymase, previously shown to specifically and completely abolish the ABCA1-mediated component of cell cholesterol efflux to control HDL through degradation of preβ-HDL, without affecting cell cholesterol efflux through other pathways, *e.g.* SR-BI or passive diffusion (11). As expected, chymase treatment of control serum resulted in the complete degradation of preβ-HDL particles (Fig. 3). Similarly, in chymase-treated A-I_M sera, apoA-I-

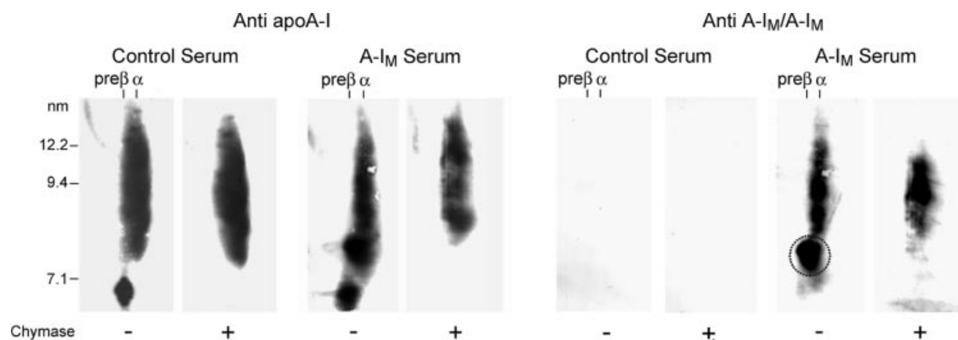

FIGURE 3. **Effect of chymase treatment on HDL subclasses in serum from A-I_M carriers and controls.** Serum was incubated with chymase-containing granule remnants for 2 h at 37 °C. At the end of the incubation, HDL subclasses were separated by two-dimensional electrophoresis and transferred onto a nitrocellulose membrane, on which lipoproteins were detected with a sheep anti-human apoA-I antibody, which recognizes also all forms of A-I_M (monomer, homodimer, and heterodimer), and with a mouse monoclonal antibody against A-I_M/A-I_M, which does not recognize either other A-I_M forms (monomer, heterodimer) or wild-type apoA-I. The small HDL containing A-I_M/A-I_M and migrating in a position intermediate between the preβ and the α regions is indicated with a *circle*.

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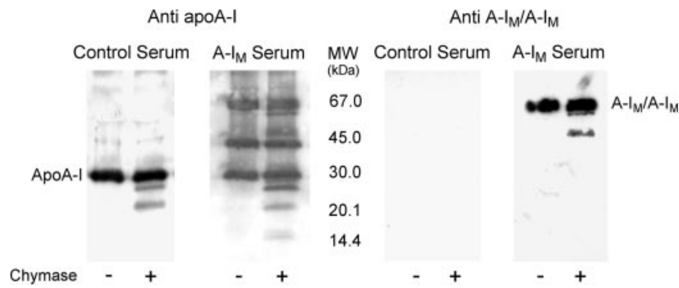

FIGURE 4. **Effect of chymase treatment on HDL apolipoproteins in serum from A-I$_M$ carriers and controls.** Serum was incubated with chymase-containing granule remnants for 2 h at 37 °C. At the end of the incubation, HDL apolipoproteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane on which apolipoproteins were detected with a sheep anti-human apoA-I antibody, which recognizes also all forms of A-I$_M$ (monomer, homodimer, and heterodimer), and with a mouse monoclonal antibody against A-I$_M$/A-I$_M$, which does not recognize either other A-I$_M$ forms (monomer, heterodimer) or wild-type apoA-I.

containing preβ-HDL were fully degraded (Fig. 3). In addition, after chymase treatment of A-I$_M$ serum, the unique A-I$_M$/A-I$_M$-containing HDL particle was no more detectable by either anti apoA-I or anti A-I$_M$/A-I$_M$ antibody (Fig. 3). Analysis of the various chymase-treated sera by SDS-PAGE and immunodetection of apoA-I and A-I$_M$/A-I$_M$, showed fragmentation of these proteins by chymase (Fig. 4). Chymase-treated sera were added to unstimulated and cAMP-stimulated J774 macrophages to measure ABCA1-independent and -dependent cell cholesterol efflux. As expected, chymase-treated sera were able to remove cell cholesterol through pathways other than ABCA1, *e.g.* SR-BI and passive diffusion (11), with no significant difference between A-I$_M$ and control sera (Fig. 5); this is consistent with findings from previous studies (17), in which we looked at the ability of A-I$_M$ sera to remove cell cholesterol from Fu5AH cells, which express SR-BI but not ABCA1 (27). No cAMP-induced increase in cholesterol efflux was observed with either control or A-I$_M$ sera (Fig. 5). This clearly indicates that simultaneous depletion of both the preβ-HDL and the unique A-I$_M$/A-I$_M$-containing HDL abolishes ABCA1-dependent efflux induced by A-I$_M$ serum, a finding compatible with the idea that both these particles can remove macrophage cholesterol via ABCA1. To prove that this is indeed the case, we investigated the association between the serum content of chymase-sensitive HDL subclasses, *i.e.* preβ-HDL plus the unique A-I$_M$/A-I$_M$-containing HDL in A-I$_M$ serum and preβ-HDL in control serum, and the serum capacity to promote macrophage cholesterol efflux via ABCA1. The serum content of chymase-sensitive HDL ranged from 23.7 to 47.6% (33.3 ± 6.8%) in A-I$_M$ carriers, and from 11.7 to 22.0% in controls (15.5 ± 3.3%). In the whole series of sera, the content of chymase-sensitive HDL was strongly and significantly correlated with ABCA1-mediated cholesterol efflux to serum ($r = 0.542$, $p = 0.004$) (Fig. 6). Altogether, these findings allow us to conclude that the unique A-I$_M$/A-I$_M$-containing HDL particle is an efficient acceptor of cell cholesterol exported via the ABCA1 transporter like the control preβ-HDL, and that the simultaneous presence of the two particles explains the enhanced capacity of the A-I$_M$ sera to induce ABCA1-mediated cell cholesterol efflux.

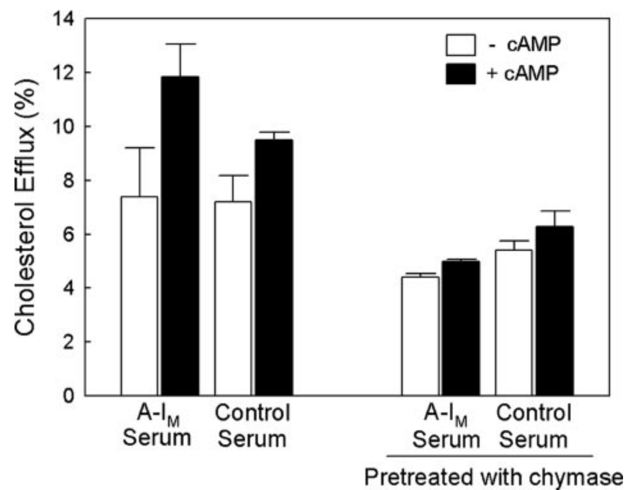

FIGURE 5. **Effect of chymase treatment of sera from A-I$_M$ carriers and controls on serum-induced cholesterol efflux from J774 macrophages.** Sera were pretreated with chymase-containing granule remnants for 2 h at 37 °C. Macrophages were labeled with 4 μCi/ml [^{3}H]cholesterol for 24 h in RPMI medium with 1% FCS, incubated for 18 h with 0.2% BSA in the absence (*open bars*) or presence (*filled bars*) of 0.3 mM CPT-cAMP, washed and incubated for 4 h with 5% of either untreated (*left*) or chymase-treated (*right*) serum. Cholesterol efflux was calculated as: ((cpm in medium at 4 h/cpm at time 0) × 100). Each sample was run in triplicate. Data are mean ± S.D.; *n* = 4.

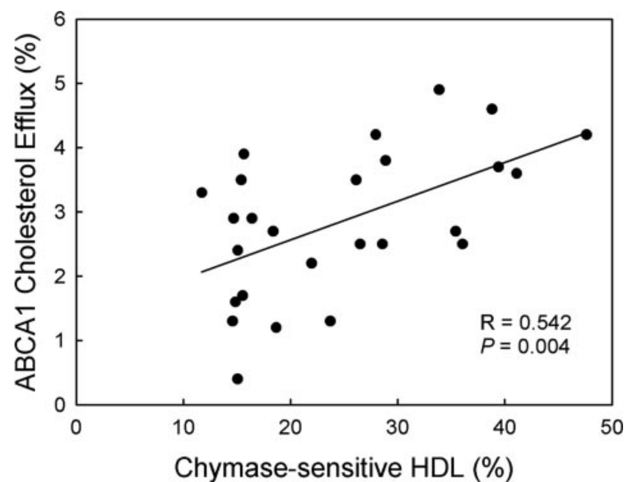

FIGURE 6. **Correlation between ABCA1-mediated cholesterol efflux to serum and chymase-sensitive HDL subclasses.** The serum capacity to promote ABCA1-mediated cholesterol efflux, calculated as the percentage efflux from CPT-cAMP-stimulated J774 macrophages minus the percentage efflux from unstimulated J774 cells, was plotted *versus* the serum content of HDL particles sensitive to chymase degradation, expressed as percentage of total apoA-I.

Cholesterol Efflux from J774 Macrophages to rHDL Containing A-I$_M$/A-I$_M$ or apoA-I—To investigate the impact of the distinct protein composition of the unique A-I$_M$/A-I$_M$-containing HDL particle and the apoA-I-containing preβ-HDL on their capacity to remove macrophage cholesterol via ABCA1, rHDL containing exclusively A-I$_M$/A-I$_M$ or apoA-I were prepared and tested for their ability to induce cholesterol efflux from J774 macrophages. The two rHDL particles have the same size (7.8 nm) and phospholipid content, and contain 1 A-I$_M$/A-I$_M$ or 2 apoA-I molecules per particle (18). While apoA-I rHDL migrate in the preβ position on agarose gels, the A-I$_M$/A-I$_M$ rHDL migrate faster, so reaching a position intermediate between the preβ and the α regions, like the unique A-I$_M$/A-

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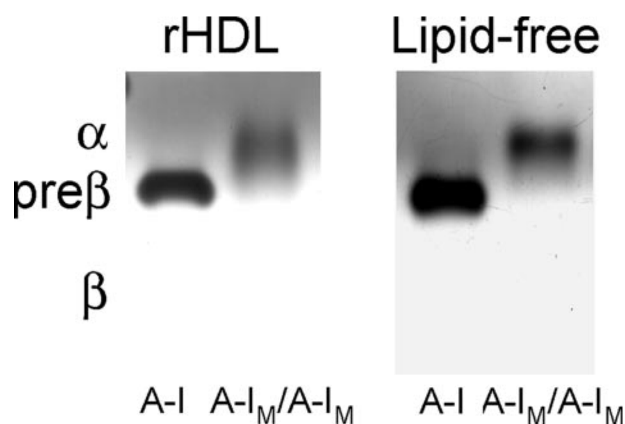

FIGURE 7. **Agarose gel electrophoresis of rHDL containing apoA-I or A-I_M/A-I_M, and of the lipid-free apolipoproteins.** rHDL containing apoA-I or A-I_M/A-I_M and POPC (*left*), and lipid-free apoA-I and A-I_M/A-I_M (*right*) were run on agarose gels and stained with Coomassie Blue G-250.

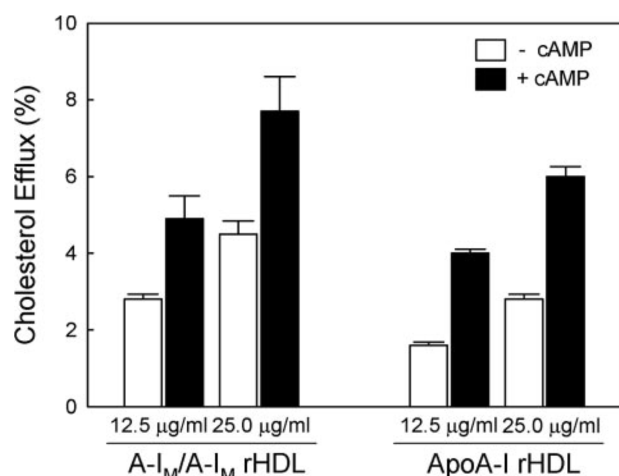

FIGURE 8. **Cholesterol efflux from J774 macrophages to rHDL containing A-I_M/A-I_M or apoA-I.** Macrophages were labeled with 4 μCi/ml [^{3}H]cholesterol for 24 h in RPMI medium with 1% FCS, incubated for 18 h with 0.2% BSA in the absence (*open bars*) or presence (*filled bars*) of 0.3 mM CPT-cAMP, washed and incubated for 4 h with rHDL containing A-I_M/A-I_M (*left*), or apoA-I (*right*) at the protein concentration of 12.5 and 25 μg/ml. Data are mean ± S.D. of a representative experiment performed in triplicate.

I_M-containing HDL found in the A-I_M serum (Fig. 7). This migration likely reflects a different surface charge of the mutant *versus* wild-type protein; indeed, the purified lipid-free A-I_M/A-I_M also migrates in the same position as A-I_M/A-I_M rHDL (Fig. 7). Consistent with previous findings in Fu5AH cells (28), unstimulated J774 macrophages released more cholesterol to A-I_M/A-I_M rHDL than apoA-I rHDL (Fig. 8); as expected, very little cholesterol efflux occurred to the lipid-free apolipoproteins (apoA-I: 0.59 ± 0.16%; A-I_M/A-I_M: 0.40 ± 0.02%; both at 12.5 μg/ml). Stimulation of cells with CPT-cAMP enhanced cell cholesterol efflux to rHDL (Fig. 8). When ABCA1-mediated cholesterol efflux was calculated as the percentage efflux from stimulated J774 cells minus the percentage efflux from unstimulated cells, no difference was observed between A-I_M/A-I_M and apoA-I rHDL (2.2 ± 0.3% *versus* 2.4 ± 0.1% at 12.5 μg/ml and 3.2 ± 0.3% *versus* 3.1 ± 0.1% at 25.0 μg/ml). Consistent with previous data (11), the lipid-free apolipoproteins were more effective than rHDL in removing cell cholesterol via ABCA1, again with no significant difference between A-I_M/A-I_M and apoA-I (4.8 ± 0.4% *versus* 5.5 ± 0.2%; both at 12.5 μg/ml).

DISCUSSION

The present study demonstrates that the serum from A-I_M carriers is more efficient than the serum from control subjects in promoting cell cholesterol efflux through the ABCA1 pathway. In cholesterol efflux studies with whole serum, the efficiency of cholesterol efflux is likely determined by individual variations in the serum content of a variety of potential acceptors (29). We have previously demonstrated that the ABCA1-dependent cholesterol efflux to control HDL has small apoA-I-containing preβ-HDL as preferential acceptors (11). Here we show that sera from various A-I_M carriers contain normal amounts of such small apoA-I-containing preβ-HDL, together with varying concentrations of a unique HDL particle, a small A-I_M/A-I_M-containing HDL with electrophoretic migration on agarose gel intermediate between the preβ and the α regions. Limited proteolysis of A-I_M serum with chymase degrades the small A-I_M/A-I_M-containing HDL particle, as well as apoA-I-containing preβ-HDL, and completely abolishes ABCA1-mediated cholesterol efflux. Moreover, rHDL made with A-I_M/A-I_M, resembling the small A-I_M/A-I_M-containing HDL found in A-I_M serum, are as effective as rHDL containing apoA-I, which resemble serum preβ-HDL, as cholesterol acceptors via ABCA1. Finally, in the whole series of examined samples, the serum content of chymase-sensitive HDL is strongly correlated with ABCA1-dependent cholesterol efflux to whole serum. Altogether these findings indicate the enhanced efficiency of A-I_M serum in promoting ABCA1-mediated cell cholesterol efflux is caused by the simultaneous presence of the unique small A-I_M/A-I_M-containing HDL particles and the preβ-HDL, all equally effective in inducing efflux of cholesterol via ABCA1.

There is clear evidence that lipid-free apolipoproteins are the preferred lipid acceptors for ABCA1-mediated efflux; all of the exchangeable apolipoproteins, such as apoA-I, apoA-II, and apoA-IV, can act as cholesterol and phospholipid acceptors for ABCA1 (8, 9). Apolipoprotein lipidation through ABCA1 results in the formation of discoidal, nascent HDL which than mature into spherical HDL through the action of the lecithin: cholesterol acyltransferase (LCAT) enzyme (30); such mature HDL remove further cell cholesterol through either SR-BI or ABCG1 (31). We have earlier shown that lipid-free A-I_M/A-I_M is as effective as apoA-I in removing cholesterol from cholesterol-loaded macrophages (28), a process now known to be mostly dependent on ABCA1 (2). Moreover, CHO cells transfected with the A-I_M gene secrete A-I_M/A-I_M, which then generates small discoidal HDL particles (32), likely through interaction with ABCA1 (30). These nascent HDL contain one molecule of A-I_M/A-I_M (32), thus resembling the A-I_M/A-I_M-HDL found here in A-I_M serum, being however somewhat smaller in size. Therefore, lipid-free A-I_M/A-I_M is fully active in generating nascent HDL particles via ABCA1. Nascent A-I_M/A-I_M-HDL slowly remodel into larger, spherical HDL because of a high intrinsic stability conferred by the disulfide-linked dimer (33), and a reduced ability of the dimer to interact with, and activate LCAT (34). Indeed, kinetic studies in A-I_M carriers

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showed a normal A-I_M/A-I_M production rate together with a remarkable delay in A-I_M/A-I_M catabolism (35, 36).

In addition to lipid-free apolipoproteins, ultracentrifugally isolated plasma HDL can promote cholesterol efflux via ABCA1, this effect being attributed to small preβ-HDL commonly found in the serum HDL fraction (11). These small preβ-HDL account for ~10% of total HDL in normal serum, and their serum concentration increases in pathological conditions, like primary biliary cirrhosis (37) or LCAT deficiency (38). Notably, the serum content of small preβ-HDL has been reported to correlate with ABCA1-mediated efflux to whole serum in a number of studies (37, 39). Moreover, interventions leading to enrichment of control HDL fraction or whole plasma with small preβ-HDL result in parallel increases in ABCA1-dependent cell lipid release (11, 40). These small preβ-HDL are likely discoidal in shape, and reportedly contain only apoA-I with a few phospholipid molecules per particle (41). The role of HDL phospholipids in ABCA1-mediated efflux has not been carefully examined, but there is evidence that the addition of a certain amount of phospholipids to apoA-I does not impair its capacity to interact with ABCA1 and promote cell cholesterol efflux (42). This concept is further supported by the present experiment with small apoA-I-containing rHDL, which are able to promote cell cholesterol efflux through ABCA1, although less effectively than the lipid-free apolipoprotein. Small A-I_M/A-I_M-containing rHDL are as good as apoA-I-containing rHDL in removing cell cholesterol through ABCA1. Notably, the C terminus of both apoA-I and A-I_M/A-I_M is distinctively exposed in discoidal small HDL (22), possibly facilitating the interaction with the cell membrane prior to ABCA1 binding to promote cell cholesterol efflux (43, 44).

The results of the present study integrate those of earlier investigations in providing a plausible explanation for the apparent A-I_M paradox, where subjects with severe reductions in the plasma concentration of antiatherogenic HDL do not present with preclinical atherosclerosis and premature CHD (16). We have previously shown that the SR-BI-mediated cholesterol efflux to sera from A-I_M carriers is only slightly reduced compared with efflux to control sera (17). SR-BI-mediated cholesterol efflux to serum is strongly correlated to the serum apoA-I and HDL cholesterol levels (17), which likely reflect the content of mature HDL particles, the best acceptors for cell cholesterol through SR-BI (2). The slight reduction of SR-BI mediated cholesterol efflux to A-I_M serum despite the severe reduction in serum apoA-I and HDL-cholesterol levels was thus interpreted as being due to an enhanced efficiency of mature A-I_M-containing HDL particles in accepting cell cholesterol through SR-BI (17). Indeed, rHDL containing the dimeric form of A-I_M were more efficient than rHDL containing apoA-I of comparable size in promoting cholesterol efflux from Fu5AH cells (28), which express SR-BI but not ABCA1 (27). Consistent with these previous findings, we show here that the A-I_M/A-I_M-containing rHDL are more effective than apoA-I-containing rHDL in removing cholesterol from unstimulated J774 macrophages (Fig. 8) which also likely express SR-BI (25) but not ABCA1 (26).

Here we now demonstrate that serum from A-I_M carriers has a greater cholesterol efflux capacity through ABCA1 than serum from control subjects. Although the ABCA1-mediated efflux to serum is lower than efflux from unstimulated cells, likely due to the presence in serum of a variety of acceptors for cell cholesterol via SR-BI and ABCG1 (45, 46), it is a unidirectional process, resulting in net cholesterol removal from donor cells. The enhanced capacity of A-I_M serum for ABCA1 cholesterol is due to the presence of a unique small A-I_M/A-I_M-containing HDL particle with the same efficiency of common apoA-I-containing preβ-HDL particles in removing cell cholesterol. Notably, the direct infusion of small A-I_M/A-I_M-containing synthetic HDL in animals has shown these particles are able to penetrate into the atherosclerotic plaque, remove cholesterol from macrophages and cause a rapid regression of the atherosclerotic lesion (47). Even more strikingly, a short term treatment with the same synthetic HDL caused a regression of atherosclerotic lesions in coronary patients (48). If, as generally believed, ABCA1 is the most significant factor in removing cholesterol from macrophages, thus preventing atherosclerosis development (49), the present findings would provide mechanistic support to further development of A-I_M/A-I_M-containing synthetic HDL for the treatment of CHD. Whether the enhanced capacity of A-I_M serum for ABCA1-mediated efflux contributes to the lack of preclinical atherosclerosis and premature CHD in the carriers is presently difficult to predict, considering the still unknown relative contributions of the various HDL functions, *e.g.* cell cholesterol removal, anti-inflammation, and anti-oxidant, in HDL-mediated atheroprotection (50), and the peculiar anti-inflammatory and anti-oxidant properties of the A-I_M mutant *versus* wild-type apoA-I (51, 52).

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