Cell Cholesterol Efflux to Reconstituted High-Density Lipoproteins Containing the Apolipoprotein A-I_{Milano} Dimer

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ABSTRACT: The apolipoprotein A-I_{Milano} (apoA-I_M) is a molecular variant of apoA-I characterized by the Arg^{173}→Cys substitution, resulting in the formation of homodimers A-I_M/A-I_M. The introduction of the interchain disulfide bridge in the A-I_M dimer limits the apolipoportein conformational flexibility and restricts HDL particle size heterogeneity, thus possibly affecting HDL function in lipid metabolism and atherosclerosis protection. To investigate whether the structural changes in A-I_M/A-I_M affect apoA-I capacity for cell cholesterol uptake, we tested the ability of four reconstituted HDL (rHDL), that contained either apoA-I or A-I_M/A-I_M, to remove cholesterol from Fu5AH hepatoma cells and cholesterol-loaded murine primary macrophages (MPM). As the HDL particle size is known to affect the rHDL capacity for cell cholesterol uptake, the reconstitution conditions were carefully selected to produce two sets of rHDL particles of small and large size (7.8 and 12.5 nm in diameter). The small A-I_M/A-I_M rHDL were more efficient than the corresponding apoA-I particles as acceptors of membrane cholesterol from Fu5AH cells and MPM, and as inhibitors of cholesterol esterification in MPM. The large rHDL and the lipid-free apolipoproteins displayed instead similar capacities for cell cholesterol efflux. These results suggest that cell cholesterol efflux to rHDL particles of different size occurs through different mechanisms. Large HDL accommodate and retain the cholesterol molecules that have desorbed from the cell membrane into the extracellular fluid, in a process that is less sensitive to protein conformation. Small HDL accelerate the desorption of cholesterol from the cell membrane, in a process that is influenced by the conformation of the proteins on the surface of the acceptor particle. The enhanced efficiency of small A-I_M/A-I_M rHDL seems related to the peculiar structure of the protein on the rHDL surface, with a hydrophobic C-terminal domain extending out of the rHDL particle, available for anchoring the acceptor to the plasma membrane.

High-density lipoproteins (HDL)[1] are the major players in reverse cholesterol transport (RCT), the process by which excess cholesterol in peripheral tissues, including the arterial wall, is transported to the liver for excretion from the body (*1*). This function is believed to explain the inverse correlation between plasma HDL levels and risk for cardiovascular disease found in many population studies (*2*). The first step in RCT is the efflux of unesterified cholesterol from peripheral cells to suitable acceptors (*3*). This occurs through two distinct mechanisms (*4*, *5*): (i) nonspecific interaction of lipoprotein acceptors with the cell, and diffusion of cholesterol from the cell membrane into the lipoprotein surface; and (ii) interaction of lipid-free apolipoproteins with a specific site on the cell surface, with membrane microsolubilization, and formation of small pre-β-migrating HDL. Apolipoprotein A-I (apoA-I), the major apolipoprotein in HDL, plays a primary role in cell cholesterol efflux, by favoring cholesterol diffusion from the cell membrane into HDL particles (*6*), and by interacting with specific binding sites on the cell surface (*7*, *8*). Both these mechanisms seem dependent on the peculiar amphipathic helical structure of apoA-I (*9*, *10*).

Most of the HDL activities in RCT, as promotion of cell cholesterol efflux and lecithin:cholesterol acyltransferase (LCAT)-mediated cholesterol esterification, can be mimicked by well-defined, apolipoprotein-specific reconstituted HDL (rHDL), which proved to be a valuable tool in identifying apolipoprotein requirements for specific HDL functions (*11*, *12*). rHDL containing apoA-I as the sole protein are thought to be the most efficient acceptors of cellular cholesterol (*13*, *14*); however, apoA-II or apoC, once incorporated into rHDL, are also able to promote cholesterol efflux, although at a lower extent than apoA-I (*14*−*16*). Furthermore, synthetic peptides structurally unrelated to apoA-I were also reported to stimulate cell cholesterol efflux (*17*, *18*). A common structural feature of these diverse proteins is the presence in the primary sequence of segments able to assume an amphipathic helical conformation (*19*). This evidence has

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[1] Abbreviations: HDL, high-density lipoprotein; RCT, reverse cholesterol transport; apoA-I, apolipoprotein A-I; LCAT, lecithin: cholesterol acyltransferase; rHDL, reconstituted HDL; A-I_M, A-I_{Milano}; A-I_M/A-I_M, A-I_M homodimer; POPC, palmitoyloleoylphosphatidylcholine; GGE, gradient gel electrophoresis; DMS, dimethyl suberimidate; CD, circular dichroism; PBS, phosphate-buffered saline; MPM, mouse peritoneal macrophages; UC, unesterified cholesterol; CE, cholesteryl esters.

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led Rothblat and collaborators to propose a general mechanism to explain the variability among apolipoproteins in promoting cholesterol efflux (*6*). According to this model, segments of the amphipathic helices in apolipoproteins, the so-called "hinged domains" (*19*), interact preferentially with cholesterol-poor lipid domains in the cell membrane, thus increasing the rate of cholesterol desorption. This would explain the variability in the capacity for cell cholesterol of different apolipoproteins, or of the same apolipoprotein on different acceptor particles, due to rearrangements of the common amphipathic motifs, with appearance/disappearance of the hinged domains (*20*). A hinged domain which might be involved in cell cholesterol efflux has been identified in apoA-I, but its precise location is still controversial (*9*).

The apoA-I_{Milano} (apoA-I_M) has been the first described molecular variant of human apolipoproteins (*21*). The A-I_M carriers have a severe hypoalphalipoproteinemia, that suggests a defective RCT, but do not suffer from premature coronary heart disease (*22*). We recently showed that this apparent paradox is explained, at least in part, by an enhanced relative efflux potential of the serum from the A-I_M carriers, as well as from transgenic mice expressing the A-I_M mutant (*23*).

The apoA-I_M differs from wild-type apoA-I by an Arg^{173}→Cys substitution, leading to the formation of disulfide-linked homodimers (A-I_M/A-I_M) and heterodimers with apoA-II. While the structures of monomeric A-I_M and wild-type apoA-I are very close (*24*), the introduction of an interchain disulfide bridge in A-I_M/A-I_M remarkably alters the physicochemical properties of apoA-I (*25*). When compared to apoA-I, the lipid-free A-I_M/A-I_M shows a higher α-helical content and a more folded tertiary structure (*25*). Upon interaction with phospholipids, A-I_M/A-I_M forms only two species of rHDL, despite a wide range of initial lipid-to-protein ratios (*26*); these two rHDL have diameters of 7.8 and 12.5 nm, and contain one or two A-I_M/A-I_M molecules per particle, respectively (*26*). Different from wild-type apoA-I, A-I_M/A-I_M adopts the same conformation in small and large rHDL (*26*), indicating limited spatial rearrangements in relation to the lipid composition and size of rHDL particles (*26*). The disulfide bridge in A-I_M/A-I_M sets a tight constraint to the alignment of the central helical segments of the protein on the rHDL surface; the hinge domain is in the lipid-bound conformation in small and large rHDL, and the C-terminal residues of A-I_M/A-I_M are forced to displace out of the discoidal rHDL (*26*, *27*). The limited structural flexibility and the peculiar conformation of A-I_M/A-I_M likely affect apolipoprotein function. Indeed, once incorporated into small or large rHDL, A-I_M/A-I_M was less effective than apoA-I in activating the LCAT enzyme (*12*). To investigate whether the structural changes in A-I_M/A-I_M also affect apoA-I capacity for cell cholesterol uptake, we have compared the ability of rHDL containing A-I_M/A-I_M or apoA-I, as well as of the lipid-free apolipoproteins, to remove cholesterol from two different cell types, Fu5AH rat hepatoma cells and cholesterol-loaded primary murine macrophages.

METHODS

Preparation and Characterization of rHDL. A-I_M/A-I_M, expressed in *E. coli* and purified by conventional chromatographic procedures (*25*), was a generous gift of Pharmacia&Upjohn, Stockholm, Sweden. ApoA-I was purified from human blood plasma, as previously described (*24*). The recombinant A-I_M/A-I_M was identical to the protein isolated from carriers' plasma (*25*).

Two sets of discoidal rHDL containing A-I_M/A-I_M or apoA-I and palmitoyloleoylphosphatidylcholine (POPC), with a diameter of either 7.8 or 12.5 nm, were prepared by the cholate dialysis technique, as previously described (*26*). The homogeneity and size of rHDL were estimated by nondenaturing gradient gel electrophoresis (GGE) (*28*) on precast 8−25% polyacrylamide gels (Pharmacia Biotec), using the Pharmacia Phast System. After Coomassie Blue staining, the gels were scanned with a Pharmacia Ultroscan XL laser densitometer, and the size of rHDL was calculated with the Pharmacia 2400 Gelscan XL software, using thyroglobulin (17.0 nm), apoferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and bovine serum albumin (7.1 nm) as calibration proteins. The phospholipid content of rHDL was determined by an enzymatic method (*29*), while proteins were measured by the method of Lowry et al. (*30*), using bovine serum albumin as standard. The number of apolipoprotein molecules per rHDL particle was determined by cross-linking with dimethyl suberimidate (DMS) (*25*). Cross-linking was performed by the addition of 1 part DMS solution, 10 mg/mL in 0.3 M triethanolamine, to 10 parts rHDL solution; cross-linked samples were then analyzed by SDS−PAGE on 4−10% acrylamide gradient slab gels, using the Tris−tricine buffer system of Schagger and von Jagou (*31*). The α-helical content of rHDL was calculated by the method of Chang et al. (*32*) from circular dichroism spectra recorded with a Jasco J500A spectropolarimeter at the constant temperature of 25 °C (*25*). Four different preparations of rHDL were made and used for efflux studies.

Cholesterol Efflux from Fu5AH Cells. Cellular cholesterol efflux was determined as described by de la Llera Moya et al. (*33*). Briefly, Fu5AH cells were seeded in Multidish Nunclon plates (35 mm/well) using 100 000 cells per well and were grown in DMEM with 5% FCS for 2 days at 37 °C in a humidified 5% CO_2 atmosphere. Lipids were radiolabeled by adding 2 μCi/mL [1,2-^{3}H]cholesterol (Amersham, Buckingamshire, U.K.) in ethanol to 25% FCS in DMEM, and then diluted to a final concentration of 5% in DMEM. Cells were grown in the presence of radiolabeled cholesterol for 2 additional days to obtain confluent monolayers. The labeling medium was replaced with DMEM containing 1% EFAF−albumin for 18−20 h, to allow equilibration of the label. Cells were washed 2 times with phosphate-buffered saline (PBS) and incubated with control medium or increasing concentrations of acceptors in DMEM with EFAF−albumin 1% for 4 h. At the end of this period, the medium was removed, collected into tubes, and centrifuged for 5 min at 2000 rpm to remove any floating cells; an aliquot was counted for [^{3}H]cholesterol radioactivity (Formula 989, Packard, Groningen, The Netherlands). Cell monolayers were washed with PBS; cellular lipids were extracted with 2-propanol by overnight incubation at room temperature, and radioactivity was measured (Insta-Fluor, Packard, Groningen, The Netherlands). Cholesterol efflux was calculated as the percent of total [^{3}H]cholesterol released to the medium.

Table 1: Characterization of rHDL

particle	diameter[a] (nm)	apolipoprotein, molecules/particle[b]	POPC:apolipoprotein[c] mass ratio (w/w)	POPC:apolipoprotein[c] mole ratio (mol/mol)	% α-helix[d]
7.8 nm A-I_M/A-I_M rHDL	7.8	1	0.99 ± 0.11:1	73:1	61
12.5 nm A-I_M/A-I_M rHDL	12.5	2	1.99 ± 0.16:1	146:1	62
7.8 nm apoA-I rHDL	7.8	2	1.19 ± 0.11:1	44:1	61
12.7 nm apoA-I rHDL	12.7	3	3.75 ± 0.27:1	138:1	71

[a] Determined by nondenaturing polyacrylamide gradient gel electrophoresis. Values are representative of three independent preparations. [b] Determined by SDS−PAGE of delipidated rHDL after cross-linking with DMS. [c] Data are the means± SD of four different rHDL preparations. [d] Determined from the 190−250 nm CD spectrum by the method of Chang et al. (*32*).

Cholesterol Efflux from Macrophages. Mouse peritoneal macrophages (MPM) were obtained from BALB/c mice (Charles River, Calco, Italy) after intraperitoneal injection of thioglycolate. Cells were plated in 35 mm cell plates at 3 × 10^6 cells/plate and maintained in DMEM with 10% FCS. Unattached cells were removed by washing 3 h after plating. Plasma membrane cholesterol was labeled as described (*16*). MPM were loaded with 50 μg/mL unesterified cholesterol (UC) in DMEM containing 0.2% EFAF−albumin for 24 h at 37 °C, and incubated with 0.5 μCi/mL [^{3}H]UC in ethanol for 8 h. After being washed with PBS, cells were allowed to equilibrate for 18−20 h; the efflux measurements were initiated by the addition of DMEM containing cholesterol acceptors at the appropriate concentration. The medium was removed and centrifuged, and an aliquot was counted for [^{3}H]UC radioactivity (Lipoluma Lumac, Landgraff, The Netherlands). In these experimental conditions, no radioactivity was found in medium cholesteryl esters (CE).

Cellular cholesterol content and esterification was investigated in cholesterol-loaded MPM incubated with appropriate concentrations of acceptors, for different times (*16*). To evaluate cell UC and CE content, the incubation medium was discarded, and cells were washed in PBS; lipids were extracted with hexane/2-propanol (3:2, v/v) and partitioned by TLC (*16*). Cholesterol mass or radioactivity was determined by an enzymatic method (*34*) and by scintillation counting, respectively. Cholesterol esterification was evaluated by measuring CE radioactivity after addition of [1-^{14}C]-oleic acid−albumin complex (0.68 μCi/sample, 54 mCi/mmol) during the last 2 h of incubation (*16*).

Statistical Analyses. Results are reported as means ± SD. Differences in the capacity of rHDL to promote cholesterol efflux and inhibit cholesterol esterification were determined by the two-tailed Student's *t* test.

RESULTS

Characterization of rHDL. The ability of A-I_M/A-I_M and apoA-I to promote cell cholesterol efflux was first compared in experiments with rHDL acceptors containing POPC and either A-I_M/A-I_M or apoA-I. To circumvent the effect of particle size on the acceptor efficiency for cell cholesterol uptake (*35*, *36*), the reconstitution conditions were carefully selected to produce A-I_M/A-I_M and apoA-I rHDL of comparable size (Table 1). All rHDL appeared homogeneous when analyzed by nondenaturing GGE, as previously shown (*26*), and no lipid-free apolipoproteins were present in the various preparations tested (Figure 1) (*37*). Electron microscopy confirmed that the particles were discoidal in shape (data not shown). Protein cross-linking was used to estimate

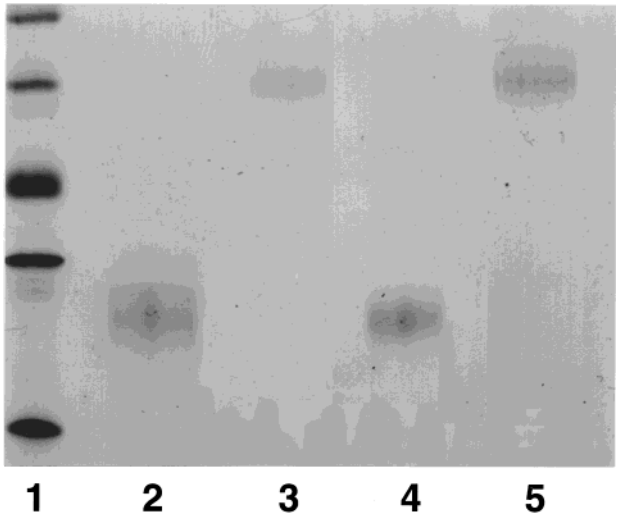

FIGURE 1: Nondenaturing GGE of rHDL containing POPC and either A-I_M/A-I_M or apoA-I. Lane 1: protein standards: thyroglobulin (17.0 nm), ferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and albumin (7.1 nm). Lanes 2 and 3: A-I_M/A-I_M rHDL particles (7.8 and 12.5 nm, respectively). Lanes 4 and 5: apoA-I rHDL particles (7.8 and 12.7 nm, respectively). The lipid-free apolipoproteins would migrate just above the albumin standard (*36*).

the number of protein molecules per rHDL particle; the two A-I_M/A-I_M rHDL with diameters of 7.8 and 12.5 nm contained 1 and 2 A-I_M/A-I_M molecules/particle, respectively. As expected, the 7.8 and 12.7 nm apoA-I rHDL contained 2 and 3 apoA-I molecules/particle. Although the four rHDL particles were simultaneously prepared by using identical reconstitution procedures, and consistent with previous results (*26*), rHDL generated with A-I_M/A-I_M had a slightly lower POPC content compared with those containing apoA-I (Table 1). Circular dichroism in the far-ultraviolet region was used to monitor the secondary structure of apoA-I and A-I_M/A-I_M in the various rHDL particles (*25*) (Table 1). The α-helix content of apoA-I was higher in the small than large rHDL. In contrast, the α-helix content of A-I_M/A-I_M in small and large rHDL was identical and similar to that of apoA-I in the 7.8 nm rHDL particles (Table 1), consistent with the concept that the conformation of apoA-I changes in response to increases in rHDL particle size (*38*, *39*), whereas A-I_M/A-I_M adopts an identical conformation in small and large rHDL (*26*).

Nondenaturing GGE of cell medium before and after a 6 h efflux experiment showed that the size of the rHDL particles remained the same, indicating that they are stable and do not precipitate out of solution during the assay (*35*, *40*). Moreover, no lipid-free apolipoproteins were detected

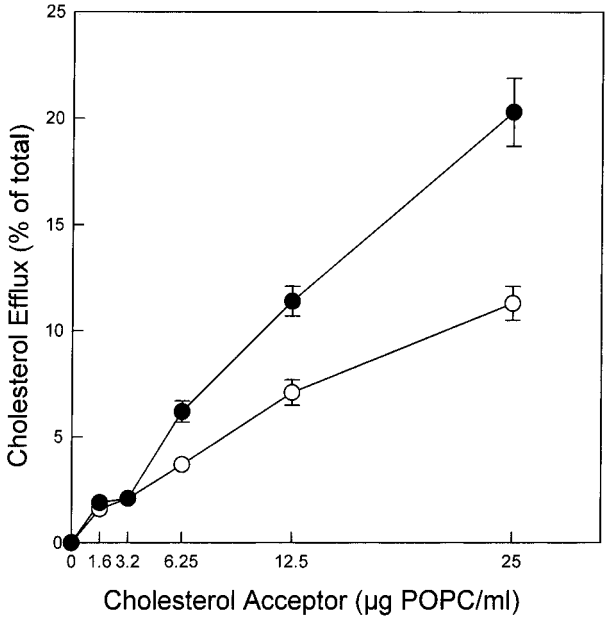

FIGURE 2: Concentration dependence of cholesterol efflux from Fu5AH cells to small (7.8 nm) A-I_M/A-I_M and apoA-I rHDL. Cells were incubated with increasing concentrations of A-I_M/A-I_M (●) or apoA-I (○) rHDL for 4 h in DMEM containing 1% EFAF−albumin. Cholesterol efflux is expressed as the percent of total [^{3}H]-cholesterol appearing in the medium. Data are the means ± SD of triplicate samples from a representative experiment performed 4 times with different rHDL preparations. Error bars within the limits of the symbols are not shown.

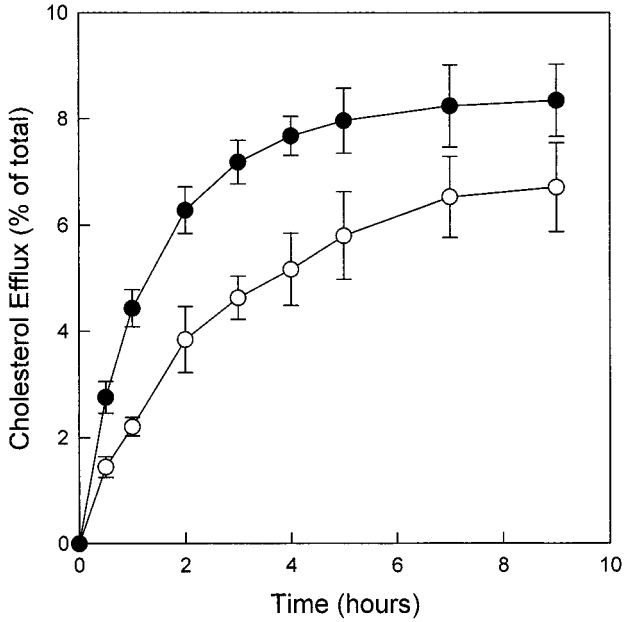

FIGURE 3: Time course of cholesterol efflux from Fu5AH cells to small (7.8 nm) A-I_M/A-I_M and apoA-I rHDL. Cells were incubated with 12.5 μg/mL A-I_M/A-I_M (●) or apoA-I (○) rHDL for increasing time in DMEM containing 1% EFAF−albumin. Cholesterol efflux is expressed as the percent of total [^{3}H]cholesterol appearing in the medium. Data are the means ± SD of triplicate samples from a representative experiment performed 4 times with different rHDL preparations.

in the medium after incubation, implying that in this particular experimental setting we were evaluating lipoprotein-mediated cholesterol efflux (*4*). Cholesterol efflux to lipid-free apolipoproteins (*5*) was analyzed in a separate set of experiments.

Cholesterol Efflux to rHDL. The ability of acceptor particles to promote cell cholesterol efflux may vary widely depending on cell type (*6*). In the present studies, we used two different cellular systems: Fu5AH hepatoma cells and primary mouse peritoneal macrophages. The Fu5AH cells provide a well-established, easy to use, and reproducible model for cholesterol efflux from the plasma membrane, and indeed have been widely employed to explore the capacity of serum and of isolated acceptors to remove cholesterol from the cell membrane (*33*, *35*, *41*−*43*). The cholesterol-loaded murine macrophages represent instead an extensively studied in vitro model for human foam cells (*44*, *45*), and were used here to confirm the Fu5AH findings in a nonhepatic peripheral cell model of relevance for atherogenesis.

A-I_M/A-I_M and apoA-I rHDL with an identical size of 7.8 nm were compared for their ability to promote cholesterol efflux from the Fu5AH hepatoma cells. To determine the efflux over a range of acceptor particle concentrations, cells were incubated with rHDL at each of five concentrations, ranging from 1.6 to 25 μg of POPC/mL. Both rHDL particles promoted cholesterol efflux from the membrane of Fu5AH cells, but A-I_M/A-I_M rHDL were substantially more efficient than apoA-I rHDL (Figure 2). At the highest concentration tested, the A-I_M/A-I_M rHDL gave 2 times more efflux than apoA-I rHDL. Figure 3 shows the time-dependent release of cholesterol from Fu5AH cells to the two small rHDL particles at a concentration of 12.5 μg of POPC/mL of medium; the initial rate of cholesterol removal was higher with A-I_M/A-I_M than apoA-I rHDL.

Since the phospholipid content differed among the four rHDL particles, the number of particles in the medium at the same POPC concentration also differed. Therefore, the cholesterol efflux data were replotted against particle number. Again, at the same particle concentration, the small A-I_M/A-I_M rHDL were more efficient than those containing apoA-I in promoting cholesterol release from Fu5AH cells (not shown).

To investigate whether the higher efficiency of A-I_M/A-I_M vs apoA-I rHDL for Fu5AH cholesterol paralleled a higher ability to remove excess intracellular cholesterol, the two rHDL particles were compared for their ability to inhibit cholesterol esterification in MPM. This reaction is catalyzed by microsomal acylCoA:cholesterol acyltransferase (ACAT), the activity of which critically depends on the size of the intracellular UC pools. Therefore, the measurement of ACAT activity provides a easy tool to monitor the ability of acceptor particles to promote intracellular cholesterol efflux (*16*). When tested over a range of particle concentrations (Figure 4), or in time-dependent experiments at fixed particle concentrations (Figure 5), A-I_M/A-I_M rHDL were consistently more efficient in inhibiting cholesterol esterification than apoA-I rHDL. The two particles were also compared for their ability to promote cholesterol efflux from the plasma membrane of MPM. As shown by others (*6*), macrophages released less cholesterol to the rHDL particles than Fu5AH cells (compare Figure 6 with Figure 2); nevertheless, A-I_M/A-I_M rHDL were again more efficient than apoA-I rHDL. These results indicate that the higher ability of 7.8 nm A-I_M/A-I_M vs apoA-I rHDL to promote cell cholesterol efflux is independent of the particular cell type used.

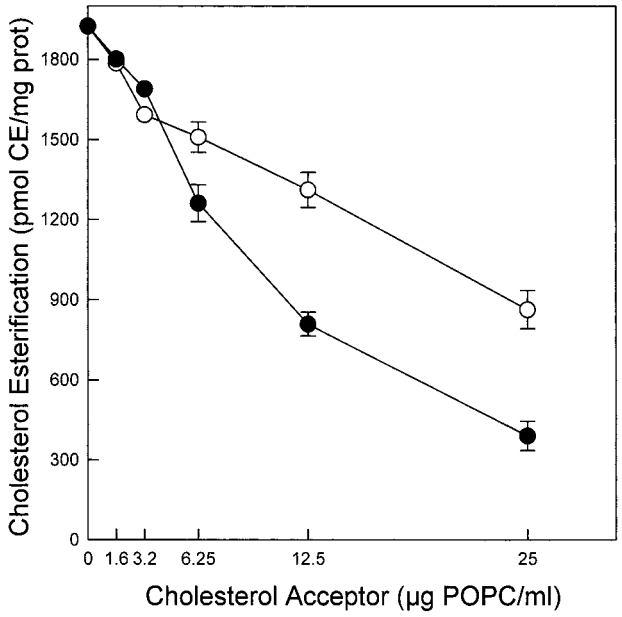

FIGURE 4: Concentration dependence of inhibition of cholesterol esterification in MPM by small (7.8 nm) A-I_M/A-I_M and apoA-I rHDL. Cells were incubated for 24 h in DMEM containing 0.2% EFAF−albumin and increasing concentrations of A-I_M/A-I_M (●) or apoA-I (○) rHDL. Cholesterol esterification was determined as described under Methods. Data are the means ± SD of triplicate samples from a representative experiment performed 4 times with different rHDL preparations. Error bars within the limits of the symbols are not shown.

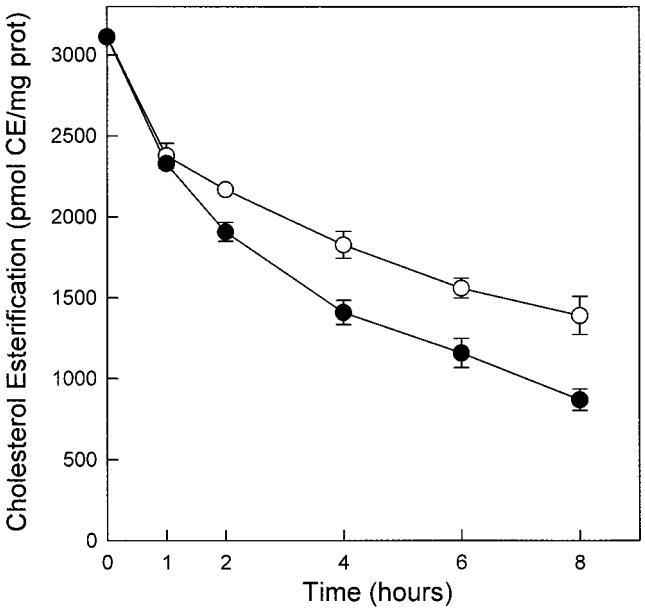

FIGURE 5: Time course of inhibition of cholesterol esterification in MPM by small (7.8 nm) A-I_M/A-I_M and apoA-I rHDL. Cells were incubated with 12.5 μg/mL A-I_M/A-I_M (●) or apoA-I (○) rHDL for increasing time in DMEM containing 0.2% EFAF−albumin. Cholesterol esterification was determined as described under Methods. Data are the means ± SD of triplicate samples from a representative experiment performed 4 times with different rHDL preparations. Error bars within the limits of the symbols are not shown.

Previous studies have shown that cholesterol efflux from various cell types to apoA-I-containing rHDL is proportional to the acceptor particle size (*35*, *36*). To test whether particle size also influences the relative capacity of A-I_M/A-I_M vs apoA-I rHDL to promote cell cholesterol efflux, larger rHDL

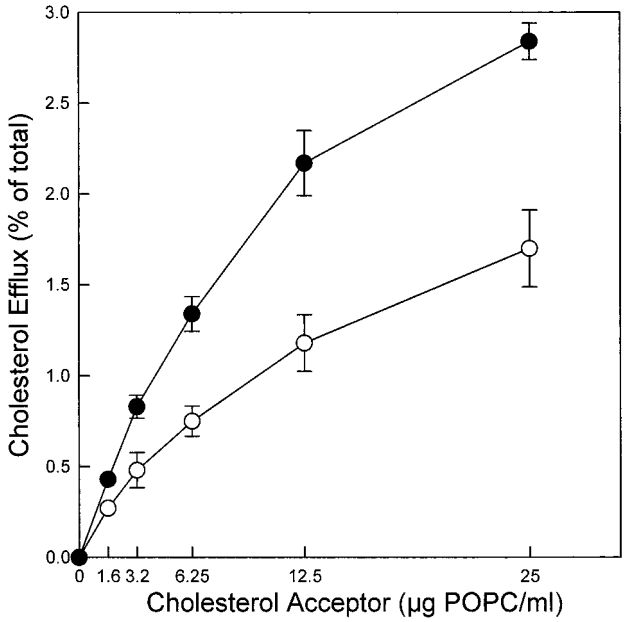

FIGURE 6: Concentration dependence of cholesterol efflux from MPM to small (7.8 nm) A-I_M/A-I_M and apoA-I rHDL. Plasma membrane cholesterol was labeled as described under Methods; cells were then incubated with increasing concentrations of A-I_M/A-I_M (●) or apoA-I (○) rHDL for 4 h, and radioactivity in the medium was evaluated by liquid scintillation counting. Data are the means ± SD of triplicate samples from a representative experiment. Error bars within the limits of the symbols are not shown.

particles, 12.5 nm in diameter, were compared for their ability to promote cholesterol efflux from the plasma membrane of Fu5AH cells. Cholesterol efflux was measured for each particle over the same range of concentrations (1.6−25 μg of POPC/mL) used for the 7.8 nm rHDL particles. Since the phospholipid and protein contents differed among the four rHDL particles, the number of particles in the medium at the same POPC concentration also differed. Therefore, the Fu5AH efflux data for the four rHDL were plotted against particle concentration (Figure 7). These results are consistent with those reported by others (*35*, *36*, *46*) in showing that large particles are more efficient than the small ones in promoting cholesterol efflux. Again, the small A-I_M/A-I_M rHDL were more efficient than those containing apoA-I, but no difference was detectable between the larger rHDL.

Cholesterol Efflux to Lipid-Free Apolipoproteins. In another set of experiments, we evaluated cholesterol efflux from Fu5AH cells and cholesterol-loaded MPM to lipid-free apolipoproteins. As reported by others (*47*, *48*), apolipoprotein-mediated cholesterol efflux varied significant with cell type, being almost undetectable when Fu5AH cells were exposed to either apoA-I or A-I_M/A-I_M. The lipid-free apolipoproteins were instead highly effective in promoting cell cholesterol efflux (not shown), and inhibiting cellular cholesterol esterification in cholesterol-loaded MPM (Figure 8). No significant differences were observed between A-I_M/A-I_M and apoA-I, when tested over a range of particle concentrations (Figure 8), or in time-dependent experiments at fixed particle concentrations (not shown).

DISCUSSION

The present results demonstrate that small HDL containing the disulfide-linked A-I_M dimer are more efficient than small

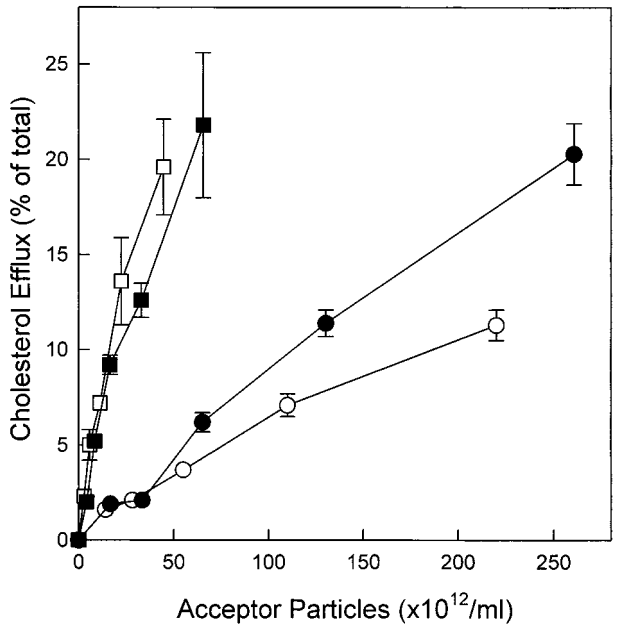

FIGURE 7: Concentration dependence of cholesterol efflux from Fu5AH cells to large (12.5 nm) and small (7.8 nm) A-I_M/A-I_M and apoA-I rHDL. Cells were incubated with increasing concentrations of 7.8 nm A-I_M/A-I_M (●) and apoA-I (○) rHDL, and of 12.5 nm A-I_M/A-I_M (■) and apoA-I (□) rHDL for 4 h in DMEM containing 1% EFAF−albumin. Cholesterol efflux is expressed as the percent of total [^{3}H]cholesterol appearing in the medium. Data are the means ± SD of triplicate samples from a representative experiment performed 3 times with different rHDL preparations. Error bars within the limits of the symbols are not shown.

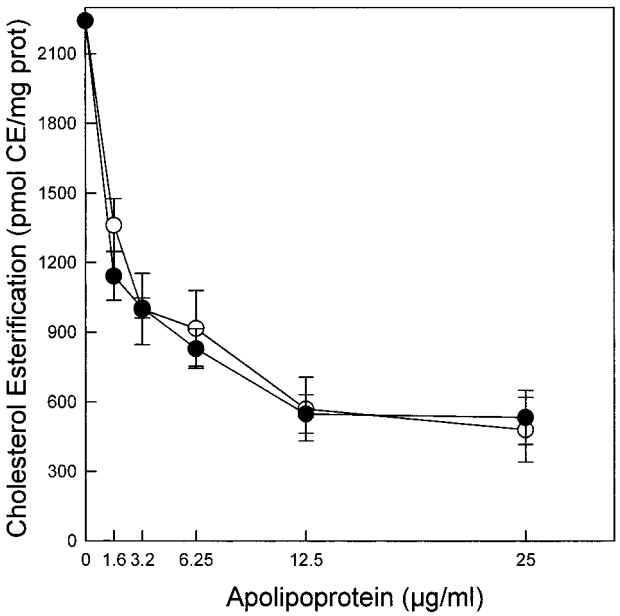

FIGURE 8: Concentration dependence of inhibition of cholesterol esterification in MPM by lipid-free A-I_M/A-I_M and apoA-I. Cells were incubated for 24 h in DMEM containing 0.2% EFAF−albumin and increasing concentrations of A-I_M/A-I_M (●) or apoA-I (○). Cholesterol esterification was determined as described under Methods. Data are the means ± SD of triplicate samples from a representative experiment performed 4 times. Error bars within the limits of the symbols are not shown.

HDL with wild-type apoA-I in removing cholesterol from the plasma membrane of Fu5AH cells. The same small A-I_M/A-I_M HDL displayed an enhanced uptake of membrane and intracellular cholesterol from lipid-laden macrophages. No differences were observed when large HDL containing either A-I_M/A-I_M or apoA-I, or the lipid-free apolipoproteins, were used as cell cholesterol acceptors. It is noteworthy that A-I_M carriers have HDL that are smaller in size than control HDL (*49*, *50*); therefore, they accumulate in plasma particles with improved efficiency for cell cholesterol uptake, that may account for the enhanced cholesterol efflux potential of the A-I_M serum (*23*).

It is generally accepted that HDL and their apolipoproteins induce cellular cholesterol efflux by two distinct mechanisms (*4*, *5*, *51*). Lipidated apoA-I particles, as circulating HDL, remove cholesterol by an aqueous diffusion mechanism through a relatively nonspecific interaction of the apolipoproteins with the plasma membrane (*52*, *53*). Lipid-free (*48*) or lipid-poor (*54*) apolipoproteins act through a membrane microsolubilization pathway that involves the interaction with a specific binding site(s) on the cell surface, followed by the removal of lipids with generation of small pre-*β*-migrating HDL (*55*). In the present experiments, the lipid-free A-I_M/A-I_M and apoA-I did not promote cholesterol efflux from Fu5AH cells, and were equally effective in removing cholesterol and inhibiting ACAT in lipid-laden macrophages. These results support the concept that a specific interaction between lipid-free apolipoproteins and the plasma membrane is crucial for cholesterol efflux from macrophages but not from hepatoma cells (*56*, *57*). Moreover, they provide indirect evidence that lipid-free A-I_M/A-I_M and apoA-I, two proteins with distinct secondary and tertiary structure (*25*), display a similar affinity for binding site(s) on the cell surface, consistent with a relatively broad apolipoprotein specificity of the postulated HDL receptor(s) (*58*).

In the experiments with reconstituted, phospholipid-containing HDL, the various rHDL preparations did not contain lipid-free apolipoproteins, nor were apolipoproteins released from rHDL into the culture media. Therefore, in this particular setting, we were exclusively evaluating lipoprotein-mediated cholesterol efflux. Within small rHDL, those containing A-I_M/A-I_M were more efficient than apoA-I-containing rHDL in promoting cholesterol efflux from both the plasma membrane and the intracellular stores. Large rHDL had instead the same capacity for cell cholesterol uptake.

Cholesterol efflux to lipoprotein acceptors occurs in two steps: desorption of cholesterol from the cell membrane into small acceptors, followed by diffusion into the extracellular fluid, where it is taken up by large, phospholipid-containing particles (*4*, *59*). Previous studies with rHDL agree with the present finding that apoA-I-containing rHDL of varying size exhibit a linear correlation between the cholesterol acceptor capacity and particle diameter, number of apoA-I molecules per particle, and phospholipid to apoA-I ratio (*35*, *36*, *46*), three interrelated parameters. The higher capacity of large vs small apoA-I rHDL for cell cholesterol has been attributed to differences in phospholipid packing, resulting in a different ability of colliding cholesterol molecules to incorporate into the acceptor particle (*35*), or differences in the stability of the acceptor particle, affecting its capacity to retain the incorporated cholesterol molecules (*36*). Therefore, cholesterol efflux to large rHDL appears to be dependent on the capacity of the acceptor to accommodate and retain large amounts of cholesterol, in a process that is only partially sensitive to the structure of the protein on the acceptor surface

(*35*). Indeed, large rHDL containing apoA-I or A-I_M/A-I_M had a similar capacity for cell cholesterol uptake, despite a diverse conformation of the protein on the rHDL particle.

Cholesterol desorption from the cell membrane seems instead particularly sensitive to the structure/conformation of the protein on the acceptor surface (*6*). Some portions of peptides/apolipoproteins bound to the small lipoprotein acceptors can transiently displace from the particle surface, and anchor the acceptor to the cell by interacting with specific lipid domains in the plasma membrane (*60*), increasing substantially the rate of cholesterol desorption from the cell membrane (*6*). Alternatively, apolipoproteins can change the arrangement of phospholipids on the particle surface (*61*), therefore affecting the interaction of the acceptor with phospholipid-sensitive receptors involved in cell cholesterol efflux, like the scavenger receptor BI (*43*, *62*).

Studies with monoclonal antibodies have identified a hinge domain in the central portion of apoA-I that is critical for apoA-I-facilitated cholesterol efflux (*63*−*66*). This domain, consisting of a pair of helical segments, can exist either bound to the rHDL surface or "looping out" of the disk edge (*67*). In the large apoA-I rHDL, the hinge domain is in the lipid-bound conformation, where it extends away from the particle surface in the small rHDL (*20*). When looping out of the surface of small rHDL, this domain can facilitate the interaction of the particle with the plasma membrane, in turn increasing cholesterol acceptor efficiency. The small apoA-I and A-I_M/A-I_M rHDL have the same α-helix content, the same total number of helical segments at the disk edge, and the same number of residues (approximately 90) looping out of the rHDL surface. However, the disulfide bridge in A-I_M/A-I_M sets a tight constraint to the alignment of the central helical segments of the protein on the rHDL surface; the hinge domain is in the lipid-bound conformation in small and large rHDL, and the C-terminal residues of A-I_M/A-I_M are forced to displace out of the disk (*26*, *27*). Therefore, apoA-I and A-I_M/A-I_M assume similar conformations in the small rHDL, the only difference being in the portion of the molecule extending out of the rHDL surface: the hinge domain in apoA-I rHDL, and a novel C-terminal domain in A-I_M/A-I_M rHDL. It is noteworthy that the C-terminal portion of A-I_M/A-I_M, which has a high affinity for lipid surfaces and has been involved in the initial binding of the apolipoprotein to the phospholipid bilayer (*68*−*70*), seems to be more efficient than the hinge domain of apoA-I in facilitating cell cholesterol efflux to small rHDL. It thus appears that the hydrophobicity and the affinity for lipids rather than the primary sequence of the apoA-I domain, which is able to transiently dissociate from the HDL surface and interact with cell surfaces, can efficiently modulate cell cholesterol efflux to a small apoA-I-containing particle.

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