Cholesterol Mobilization by Free and Lipid-Bound $ApoAI_{Milano}$ and $ApoAI_{Milano}$−ApoAII Heterodimers†

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ABSTRACT: Despite very low plasma levels of HDL, carriers of the apolipoprotein AI Arg173 → Cys mutation $apoAI_{Milano}$ (AIM) have no apparent increase in risk for atherosclerotic vascular disease. HDL apolipoprotein species in AIM carriers include apoAI-AII heterodimers, previously found to confer the enhanced ability of tyrosyl radical-oxidized HDL to mobilize cholesterol for removal from cultured cells. To determine whether enhanced mobilization of cholesterol by apoprotein species in AIM explains a cardioprotective action of this mutation, we examined the ability of lipid-free and lipid-bound AIM and AIM-AII heterodimers to deplete cholesterol from cultured cells. Free AIM and AIM-AII heterodimers showed a decreased capacity to act as acceptors of cholesterol from cholesterol-loaded human fibroblasts compared with native apoAI but similar capacities to deplete fibroblasts of the pool of cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (ACAT). Discoidal reconstituted HDL (rHDL) containing apoAI depleted both of these cholesterol pools more readily than AIM-containing rHDL when compared at equivalent rHDL protein levels, but similar abilities of these rHDL to deplete cell cholesterol were seen when compared at equivalent phospholipid levels. Spherical rHDL generated using the whole lipid fraction of HDL and apoAI or AIM showed similar capacities to deplete total and ACAT-accessible cell cholesterol when compared at similar protein levels, but an increased capacity of AIM-containing particles was seen when compared at equivalent phospholipid levels. Unlike the apoAI-AII heterodimer in tyrosylated HDL, AIM-AII heterodimer-containing spherical rHDL showed no increased capacity to deplete either of these pools of cholesterol. These results suggest a similar or better capacity of native apoAI in lipid-free or lipid-bound form in discoidal rHDL to enhance the mobilization of cellular cholesterol when compared to AIM in its free or lipid-bound forms. Any increase in depletion of cellular cholesterol by lipid-bound AIM in spherical rHDL appears related to altered phospholipid-binding rather than intrinsic cholesterol-mobilizing characteristics of this protein compared to native apoAI. The lack of major differences in these studies in cholesterol mobilization by native apoAI and AIM, or by apoAIM-AII heterodimers, suggests that any protection against atherosclerosis conferred by this mutation is likely related to other beneficial vascular effects of AIM.

The strong inverse correlation between levels of high-density lipoprotein (HDL)[1] in human plasma and atherosclerotic vascular disease has been attributed to several potential protective actions of HDL (*1*, *2*). Among these is the ability of lipid-poor HDL apolipoproteins and nascent HDL to promote the removal of lipids from cells, the first step in the pathway referred to as reverse cholesterol transport (*3*, *4*). In addition to preventing the overaccumulation of cholesterol in cells, recent evidence from patients with Tangier disease and familial hypoalphalipoproteinemia (FHA) suggests that this removal of cellular lipids by nascent HDL and apolipoprotein A-I (apoAI) is the major pathway for the formation of larger HDL particles and therefore a key determinant of circulating HDL levels (*5*−*9*). The recent discovery that mutations in the ATP-binding cassette transporter protein A1 (ABCA1) are present in both Tangier disease and FHA patients (*10*−*14*) provides a new tool to study intracellular cholesterol transport and HDL formation and a potential new target for the prevention and treatment of atherosclerosis.

A low level of circulating HDL is not, however, universally associated with increased risk for atherosclerosis. The Arg173 → Cys mutation of apoAI known as $apoAI_{Milano}$ (AIM) is associated with low levels of HDL (20−30% of that of normal subjects) but absence of increased risk of atherosclerosis or cholesterol deposition in tissues (*15*, *16*). Some studies have suggested that this mutation may in fact confer protection against atherosclerosis. Studies in hypercholesterolemic rabbits showed that treatment with AIM limited the intimal thickening seen in response to balloon

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[1] Abbreviations: HDL, high-density lipoprotein(s); apo, apolipoproteins; AIM, apolipoprotein AI_{Milano}; rHDL, reconstituted HDL; ACAT, acyl-CoA:cholesterol acyltransferase; FC, free cholesterol; BSA, essentially fatty acid-free bovine serum albumin; DTT, dithiothreitol; DTPA, diethylenetriaminepentaacetic acid; PC, phosphatidylcholine; CE, cholesteryl esters; DMEM, Dulbecco's modified Eagle's medium.

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injury or vascular clamping of major arteries, possibly by inhibiting smooth muscle proliferation (*17*, *18*). AIM was also reported to inhibit arterial thrombus formation (*19*) and to prevent atherosclerotic plaque progression and reduce aortic lipid and macrophage content (*20*) in rodent models. None of these studies, however, contained an apoAI control group, so an additional benefit of AIM over wild-type apoAI was not demonstrated.

Studies exploring the ability of AIM to promote cholesterol mobilization from cultured cells have yielded conflicting results. Nascent HDL secreted into the medium of Chinese hamster ovary cells expressing AIM were found to have a significantly lower free cholesterol/phospholipid mole ratio than HDL generated by cells expressing apoAI, suggesting a reduced capacity of AIM to recruit cellular cholesterol (*21*). Another study suggested a similar ability of lipid-free and discoidal apoAI- and AIM-containing reconstituted HDL to deplete macrophage foam cells of free and esterified cholesterol (*22*). A recent study found that serum from human AIM carriers or AIM transgenic mice had a diminished capacity to promote cellular cholesterol efflux, but concluded that the level of efflux was higher per milligram of apoAI (AIM) present in these samples (*23*). The cell line used for these studies (Fu5AH rat hepatoma cells), however, releases lipids poorly to apoAI (*24*−*26*), making apolipoprotein-dependent efflux in these studies difficult to interpret. Another recent study reported that small but not large discoidal reconstituted HDL (rHDL) containing AIM and a single phospholipid species were more efficient than apoAI-containing rHDL at inducing cholesterol mobilization from both Fu5AH and elicited mouse peritoneal macrophages (*27*), suggesting potential enhancement of cholesterol removal by AIM in certain protein−lipid complexes.

We have recently reported that the markedly enhanced capacity of a modified form of HDL, tyrosyl radical-oxidized HDL (*28*, *29*), to mobilize cellular cholesterol is due specifically to the formation of covalently cross-linked heterodimers of native apoAI and apoAII (*30*). These heterodimers form in the absence of cysteine residues in wild-type apoAI and are not cleaved by disulfide reducing agents (G. Francis, unpublished data). The presence of a cysteine residue in AIM allows the formation of another type of apoAI-AII complex, disulfide-linked AIM-apoAII heterodimers, in AIM carriers (*31*, *32*). This raised the intriguing possibility that an atheroprotective action of AIM might be related to enhanced cellular cholesterol mobilization by AIM-AII heterodimers.

In the current studies, we used lipid-free apoAI, AIM, and AIM-AII heterodimers, as well as discoidal and spherical reconstituted HDL containing these apoproteins, and tested the abilities of these particles to deplete the regulatory pool of cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (ACAT) and to act as acceptors of cholesterol from cultured human fibroblasts. Our results indicate a similar or better capacity of native apoAI in lipid-free or discoidal lipid-bound forms to deplete cells of their regulatory and total pools of cholesterol, when compared to similar complexes containing AIM or AIM-AII heterodimers. Spherical HDL containing AIM showed a modestly increased capacity to mobilize cholesterol relative to those containing apoAI at equivalent phospholipid, but not protein, concentrations.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol (FC), essentially fatty acid-free bovine serum albumin (BSA), and DL-dithiothreitol (DTT) were purchased from Sigma. Egg phosphatidylcholine (PC) was from Avanti Polar Lipids. Recombinant $apoAI_{Milano}$ (AIM) was a generous gift from Pharmacia and Upjohn (Sweden). The recombinant protein was expressed by site-directed mutagenesis, and its purity and sequence were determined as described by Calabresi et al. (*33*). [1-^{14}C]-Oleate (55 mCi/mmol) and [1,2-^{3}H]cholesterol (46 mCi/mmol) were from Amersham Pharmacia Biotech. Tissue culture medium was purchased from Bio-Whittaker and fetal bovine serum from Hyclone. Chloroform, methanol, and 2-propanol were from Fisher. Hexane (85% *n*-hexane) was from J. T. Baker Canada. All organic solvents were analytical grade.

Lipoproteins and Apolipoproteins. HDL_3 ($d = 1.125-1.21$ g/mL, hereafter referred to as HDL) was isolated by standard ultracentrifugation techniques from the pooled plasma of healthy male volunteers (*34*). HDL was subjected to heparin−agarose affinity chromatography to remove apoE- and apoB-containing particles (*35*). The whole protein fraction of HDL was obtained by extraction with ether/acetone (1:3 v/v), and purified apoAI and apoAII were obtained as described (*36*). To generate AIM-apoAII heterodimers, the lipid-free proteins were incubated in a 1:1 mole ratio in phosphate-buffered saline (PBS) containing 10 mM Tris, 0.1 mM diethylenetriaminepentaacetic acid (DTPA), 1 mM EDTA (pH 7.4), and 50 mM DTT at room temperature for 2 h to generate protein monomers. The mixture was then dialyzed exhaustively (6 × 4 L) against 150 mM NaCl and 10 mM Tris containing 0.1 mM DTPA, pH 8.0 (buffer A), over 3 days at 4 °C to remove DTT. Longer dialysis did not result in further protein dimer formation. Lipid-free apoAI and apoAII were cross-linked by tyrosyl radical oxidation as previously described (*30*). The final protein mixtures were stored at 4 °C until use (within 7 days).

Reconstituted HDL. The whole lipid fraction of HDL was isolated by vigorous vortexing of HDL with 60 volumes of hexane/2-propanol (3:2 v/v). Following centrifugation at 2000 rpm for 20 min, the lipid-containing supernatant was transferred to another tube and the protein residue similarly extracted. The pooled extracts were evaporated under nitrogen and resuspended in 7.5 mL of chloroform/methanol/0.74% KCl (8:4:3 v/v/v) (*37*). After brief centrifugation, the upper phase and the interface (containing contaminating proteins) were removed. The chloroform phase was dried, extracted once with the same Folch mixture, and transferred to another tube. By this method recovery of total phospholipids, free cholesterol, cholesteryl esters (CE), and triglycerides was near complete, and no residual intact or fragmented proteins were seen by silver-stained SDS−PAGE of the isolated lipid extract. The isolated lipids were stored at −20 °C in a small volume of chloroform until use. All organic solvents used in isolation and storage contained 0.1% butylated hydroxytoluene to prevent oxidation. The mole ratio of PC:FC:CE in the isolated lipids used to generate reconstituted spherical HDL was 80:33:55.

Discoidal and spherical reconstituted HDL (rHDL) particles were prepared with the cholate dialysis method (*38*), with some modifications to facilitate incorporation of neutral

lipids into final spherical rHDL. The mole ratio of PC:cholate:apolipoproteins was 80:80:1, with a similar PC content used when preparing discoidal rHDL with egg PC or spherical rHDL with the isolated whole lipid fraction of HDL. In this study, 1 mol of AIM (dimer) was considered equivalent to 2 mol of apoAI in the generation of rHDL (*39*). Lipids were dispersed by sonication in 1 mL of 66 mM KH_2PO_4 buffer containing 0.1 mM DTPA, pH 8.0 (buffer B), for 5 min at room temperature (Ultrasonics sonicator set at 40%, level 4). Cholate (from a 30 mg/mL stock) and protein (1 mg total) were added, and the volume was increased to 2 mL with buffer A. The mixture was stirred for 4 h at room temperature, the volume increased to 3 mL with buffer B, and the mixture centrifuged at 99 000 rpm (Beckman TLA 100.4 rotor, 413000*g*), 4 °C for 8 h without density adjustment. The top protein-poor lipid layer was removed and the density of the remaining mixture adjusted to 1.21 with KBr in a total volume of 3 mL. Following centrifugation at 99 000 rpm in the same rotor, 4 °C for 16 h to sediment unbound apoproteins, the top 1.2 mL containing rHDL was collected and dialyzed exhaustively against buffer A. Final rHDL were stored at 4 °C until use (within 2 weeks). Incorporation of lipids into reconstituted particles was determined enzymatically (*40*), and the conformation of rHDL assessed by negative stain electron microscopy (*41*).

Cell Culture. Normal human skin fibroblasts or human aortic smooth muscle cells [the latter obtained from the medial layer of adult thoracic aorta (*42*)] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described (*43*). Cells were plated at 15 000 cells/16 mm well and grown at 37 °C and 5% CO_2 to confluence (about 7 days). Cells were then washed twice with PBS containing 2 mg/mL BSA (PBS−BSA) and loaded with cholesterol by incubation for 24 h in DMEM containing 2 mg/mL BSA and 30 μg/mL cholesterol (added from a 10 mg/mL solution in ethanol). To allow equilibration of added cholesterol, cell layers were rinsed twice with PBS−BSA and incubated another 24 h in DMEM containing 1 mg/mL BSA (DMEM−BSA).

Cholesterol Depletion Assays. To assess the ability of free apoproteins and rHDL to deplete cellular free cholesterol available for esterification, cholesterol-loaded cells were incubated for 16−20 h in DMEM−BSA and the indicated additions, washed once with PBS, and incubated for 1 h at 37 °C with DMEM containing 9 μM [^{14}C]oleate bound to 3 μM BSA (*30*). Cells were then placed on ice, rinsed twice with ice-cold PBS−BSA and twice with PBS, and stored at −20 °C until extraction. Lipids were extracted with hexane/2-propanol (3:2 v/v) as described (*44*). Neutral lipids were separated by thin-layer chromatography on PE SIL G plastic-backed plates (Whatman) developed in hexane/diethyl ether/acetic acid (130:40:1.5 v/v). Lipid spots were identified by staining in I_2 vapor and comigration with standards. After allowing I_2 stain to evaporate, CE spots were taken for determination of radioactivity.

To assess the ability of apoproteins and rHDL to act as acceptors of cellular cholesterol, cholesterol-loaded cells were washed twice with PBS−BSA and labeled during the equilibration phase with DMEM−BSA containing 0.3 μCi/mL [^{3}H]cholesterol (*29*). Cell layers were then rinsed five times with PBS−BSA prior to incubation with DMEM−BSA plus the indicated additions for another 30 h. The incubation medium was then removed and centrifuged at 2000 rpm for 10 min to remove cell debris, and the supernatant was counted directly for [^{3}H]cholesterol. Cell layers were extracted and cellular FC and CE isolated for determination of [^{3}H]cholesterol radioactivity by thin-layer chromatography as described above. Efflux of cholesterol to the medium was expressed as percent of total medium plus cellular [^{3}H]cholesterol radioactivity (*29*).

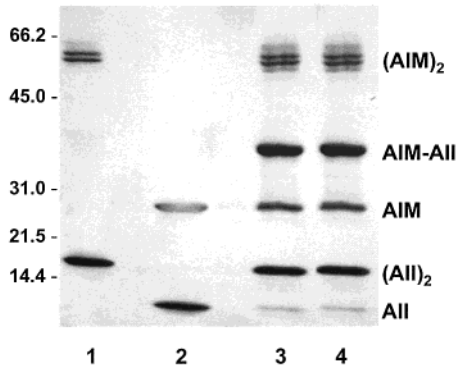

FIGURE 1: SDS−polyacrylamide gradient (7−20%) gel showing nonreduced $apoAI_{Milano}$ (AIM) and apoAII dimers (lane 1), DTT-reduced AIM and apoAII monomers (lane 2), and formation of AIM and apoAII homo- and heterodimers following exhaustive dialysis to remove DTT (lane 3). Lane 4 indicates the apoprotein products formed in HDL reconstituted with AIM and apoAII and the whole lipid fraction of HDL prior to exposure to DTT.

Protein Determination. The protein content of apolipoproteins, HDL and rHDL, was determined by the method of Bradford, due to lack of interference by lipids (*45*), and cell proteins by the method of Lowry et al. (*46*), using BSA as standard.

Gel Electrophoresis. SDS−PAGE was performed using 7−20% polyacrylamide gels under nonreducing conditions (*47*), followed by silver staining (*48*). Nondenaturing gradient gel electrophoresis of rHDL particles was performed using 4−30% polyacrylamide gels (*49*) stained with silver.

Statistics. All figures shown are representative of three or more experiments. Results are expressed as the mean ± standard deviation of three replicates. Differences in cholesterol mobilization by individual apoprotein or rHDL protein or phospholipid concentrations were calculated using paired Student's *t* tests.

RESULTS

Generation of AIM-ApoAII Heterodimers. HDL apoprotein species in AIM carriers include AIM dimers, AIM-AII heterodimers, and native apoAI monomers and AII dimers (*16*). To generate AIM-AII heterodimers in vitro, recombinant AIM and human apoAII were reduced in the presence of DTT to produce pure populations of monomeric AIM and apoAII (Figure 1). Removal of the reducing agent by dialysis resulted in formation of AIM-AII heterodimers and reformation of AIM and apoAII homodimers. Some AIM remained in the monomeric form despite exhaustive dialysis to remove DTT. The pattern of proteins regenerated was identical when the apoproteins were lipid-free or reconstituted with lipids prior to exposure to reducing agent and dialysis (Figure 1, lanes 3 and 4, respectively).

Characterization of rHDL. Native apoAI and recombinant AIM were reconstituted with egg PC and sodium cholate at

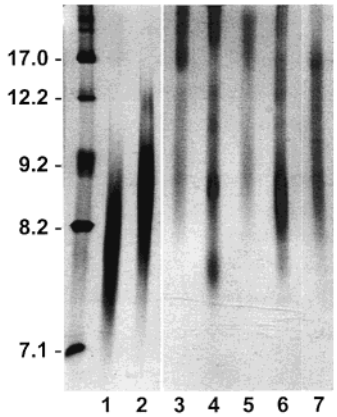

FIGURE 2: Nondenaturing polyacrylamide gradient (4−30%) gel electrophoresis stained with silver showing rHDL containing apoAI/PC (lane 1), AIM/PC (lane 2), and rHDL generated with the whole lipid fraction of HDL and apoAI (lane 3), AIM (lane 4), apoAI and apoAII (lane 5), AIM and apoAII (not cross-linked, lane 6), and the apoprotein mixture containing cross-linked AIM-AII heterodimers (lane 7). Protein standards on the left are thyroglobulin (17.0 nm), ferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and albumin (7.1 nm).

an initial molar ratio of 80:80:1 PC:cholate:protein to generate discoidal rHDL (*22*, *30*). rHDL containing the whole lipid fraction of HDL were also generated, using a mole ratio of the whole lipid fraction PC:cholate:protein of 80:80:1 to assess the capacity of these proteins, with or without apoAII, to promote cholesterol mobilization when present on a spherical rHDL surface (*30*). Nondenaturing gradient gels stained with silver to increase sensitivity of detection of minor species showed apoAI:PC rHDL in a size range of ∼7.3−9.0 nm, while AIM:PC rHDL species were slightly larger at ∼7.5−9.5 nm (Figure 2, lanes 1 and 2). The AIM:PC product also contained a small population of 12.2 nm particles. Reconstitution of apoAI ± apoAII with the whole lipid fraction of HDL resulted in variable-sized particles, with a large proportion ≥17.0 nm and a smaller peak at ∼9.2 nm (Figure 2, lanes 3 and 5). AIM-containing rHDL exhibited a variety of particle sizes, with peaks at 7.5, 8.8, and >17 nm for AIM-only particles, 8.2−9.0 nm and lesser amounts of larger particles for AIM/apoAII-containing particles, and a range of particles between 8.2 and 17.0 nm for particles containing cross-linked AIM-apoAII heterodimers (Figure 2, lanes 4, 6, and 7, respectively). The size range of HDL isolated by immunoaffinity chromatography from sera of AIM carriers is mainly in the 7.7−10.1 nm range (*50*). The formation of a population of larger AIM-containing rHDL using our method may have been due to residual monomeric AIM, which, unlike AIM dimers, has been reported to allow conversion of small to large HDL particles (*51*). Electron micrographs of rHDL generated with the whole lipid fraction of HDL and the protein mixture containing AIM-apoAII heterodimers showed a variety of species, including small numbers of disks, but confirmed mainly spherical particles in the 8−17 nm range (Figure 3). Electron micrographs of other rHDL generated with the whole lipid fraction of HDL also showed predominantly spherical particles, while those generated with egg PC alone were discoidal.

The PC:protein ratios of rHDL generated with the different apoprotein and lipid components are shown in Table 1. AIM in both discoidal and spherical rHDL showed lower PC:protein ratios than the same particles generated with apoAI.

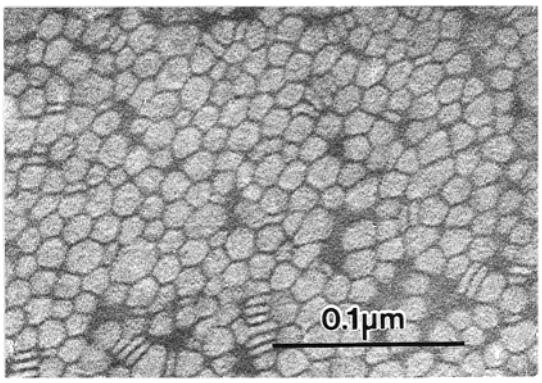

FIGURE 3: Negative staining electron micrograph of rHDL prepared as in ref *30*, using the whole lipid fraction of HDL and the protein species generated by reduction with DTT and subsequent dialysis of a 1:1 molar ratio of AIM and apoAII. The mole ratio in the reconstitution was 80:80:1 whole lipid fraction phosphatidylcholine:sodium cholate:protein. Magnification, 276000×. Bar represents 100 nm.

Table 1: Phosphatidylcholine:Protein Ratios of rHDL

particle[a]	PC:protein mass ratio (w/w)[b]
AI:PC rHDL	6.02 ± 0.10:1
AIM:PC rHDL	3.40 ± 0.15:1
AI:WL rHDL	2.16 ± 0.07:1
AIM:WL rHDL	1.55 ± 0.06:1
AI/AII:WL rHDL	3.08 ± 0.15:1
AIM/AII:WL rHDL	2.05 ± 0.06:1
AIM-AII:WL rHDL	2.14 ± 0.24:1

[a] Abbreviations: PC, egg phosphatidylcholine; rHDL, reconstituted HDL; WL, whole lipid fraction isolated from HDL; AI/AII and AIM/AII, apoAI or AIM present with but not cross-linked to apoAII in rHDL; AIM-AII, rHDL containing AIM-apoAII cross-links. [b] Values are the mean ± SD of three different rHDL preparations. Mass ratios for rHDL generated with the whole lipid fraction of HDL are based on PC content of these particles.

These results are consistent with previous findings of greater phospholipid affinity of AIM compared to apoAI and less protein per particle in apoAI-containing than AIM-containing rHDL (*27*, *52*). Addition of apoAII tended to reduce the difference in PC:protein ratios between apoAI- and AIM-containing spherical rHDL, likely due to the high lipid affinity of apoAII.

Depletion of Cellular Cholesterol by Lipid-Free ApoAI and AIM. HDL apoproteins are believed to promote cellular lipid mobilization through both active and passive mechanisms, with free or lipid-poor proteins playing a more active role compared to phospholipid-rich particles, that can also accept cholesterol passively from the cell surface (*4*). Some apoAI exists in vivo in a lipid-poor if not lipid-free state (reviewed in ref *3*), and some AIM may also be present in a lipid-poor form. The abilities of lipid-free apoAI and AIM to deplete cholesterol from fibroblasts labeled with [^{3}H]cholesterol during growth and then loaded with non-lipoprotein cholesterol were compared. We found a statistically greater capacity of apoAI to deplete cellular cholesterol than AIM at higher concentrations in the range tested (Figure 4A). ApoAI also showed a greater but not significantly different ability to deplete human aortic smooth muscle cells of [^{3}H]cholesterol when compared to lipid-free AIM (data not shown). The depletion of cellular cholesterol available for esterification by acyl-CoA:cholesterol acyltransferase (ACAT) is also stimulated by apoAI, either directly or as a consequence of

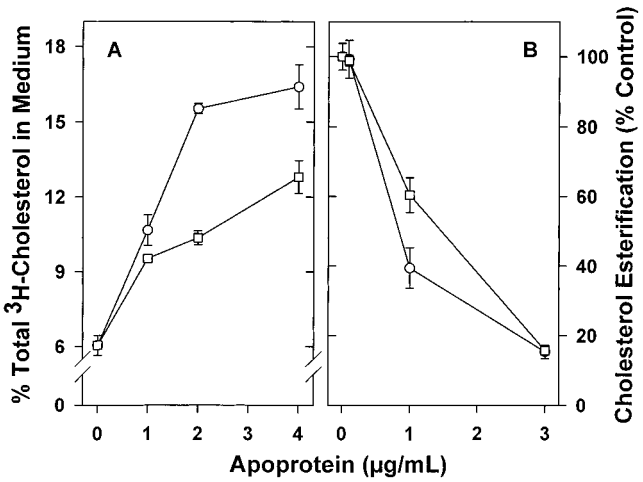

FIGURE 4: Depletion of cellular cholesterol by lipid-free apoAI and AIM. Normal human fibroblasts were grown to confluence in the presence of [^{3}H]cholesterol (A) or serum-supplemented medium alone (B), loaded with non-lipoprotein cholesterol, and incubated with DMEM−BSA and the indicated concentration of lipid-free apoAI (○) or AIM (□). (A) [^{3}H]Cholesterol released into the medium during a 30 h incubation with apoproteins, expressed as percent of total medium and cellular [^{3}H]cholesterol. (B) Incorporation of [^{14}C]oleate into cholesteryl esters in 1 h following a 16 h preincubation with apoproteins, expressed as percent of picomoles of [^{14}C]oleate incorporated into cholesteryl ester per milligram of cell protein per hour in cells treated with DMEM−BSA alone (control). Results for apoAI are significantly greater than AIM at 2 and 4 μg/mL protein in panel A and at 1 μg/mL in panel B ($p < 0.01$ for all).

depletion of plasma membrane cholesterol (*4*). We found a decreased ability of AIM to deplete this pool of cholesterol at 1 μg of protein/mL compared to apoAI but similar abilities of the two proteins at 3 μg/mL (Figure 4B). Lipid-free AIM-AII heterodimers showed a similar capacity to AIM to promote [^{3}H]cholesterol efflux and depletion of the ACAT substrate pool. These results suggest lipid-free AIM and AIM-AII heterodimers have either a lower or similar, but not enhanced, capacity to promote the depletion of total and regulatory pools of cellular cholesterol when compared to apoAI.

Depletion of Cellular Cholesterol by Lipid-Bound ApoAI and AIM. HDL apoproteins may exist in vivo complexed with small amounts of lipid as discoidal particles, or in larger, spherical (neutral lipid-containing) particles (*3*). We tested the ability of apoAI and AIM in discoidal or spherical rHDL to mobilize cellular cholesterol pools. When compared on an equivalent protein level, discoidal apoAI:PC rHDL showed a slightly higher ability than AIM:PC rHDL to deplete the ACAT substrate pool of cholesterol for esterification, but the two proteins showed a similar V_{max} for this effect (Figure 5A). The higher PC:protein mass ratio in apoAI:PC rHDL means less protein per particle than in AIM:PC rHDL and therefore more apoAI:PC particles to achieve similar protein mass. When the same data were replotted according to levels of PC in the acceptor rHDL pool, and therefore roughly the same particle number based on sizing gels, the small difference between the two preparations in mobilizing ACAT-accessible cholesterol disappeared (Figure 5B). Expression of our data either by protein or phospholipid in the acceptor medium, therefore, did not indicate a higher capacity of AIM-containing particles to deplete the ACAT pool of cholesterol from these cells. The moderately increased ability of apoAI:PC rHDL expressed on a protein basis in acceptor medium to deplete cholesterol-loaded fibroblasts of [^{3}H]cholesterol was also lost when these results were normalized to phospholipid content of the apoAI and AIM discoidal rHDL (Figure 6).

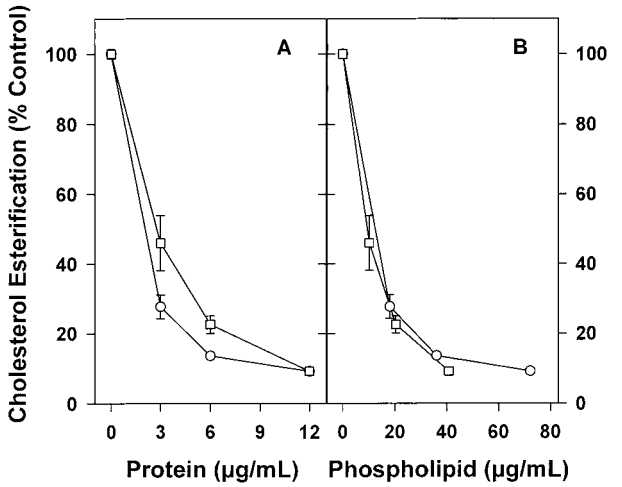

FIGURE 5: Depletion of cholesterol available for esterification in human fibroblasts by discoidal apoAI and AIM-containing rHDL. Cells grown to confluence and loaded with non-lipoprotein cholesterol were incubated with the indicated concentration of apoAI (○) or AIM (□) rHDL protein (A) or phospholipid (B) for 16 h and then with [^{14}C]oleate for 1 h, and cellular cholesteryl [^{14}C]-oleate formed was determined as described under Experimental Procedures. Results are expressed as in Figure 4B. Depletion of ACAT-accessible cholesterol was significantly greater for apoAI than AIM at 3 and 6 μg/mL in panel A ($p < 0.01$); no differences were seen in panel B.

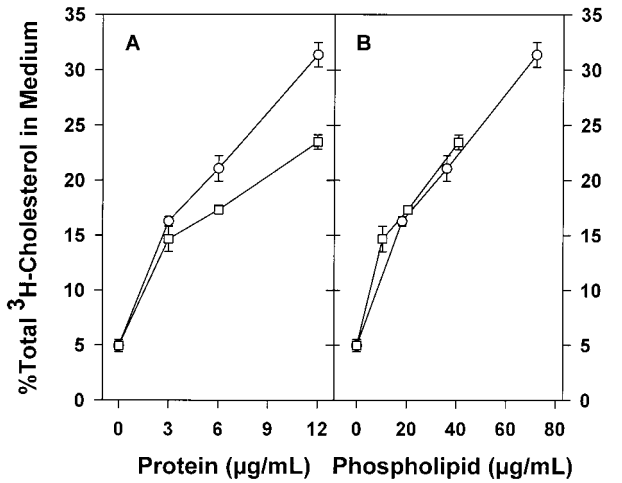

FIGURE 6: Cholesterol efflux to discoidal apoAI and AIM-containing rHDL. [^{3}H]Cholesterol-labeled and cholesterol-loaded fibroblasts were incubated with apoAI (○) and AIM (□) rHDL at the indicated concentration of protein (A) or phospholipid (B) for 30 h, and efflux of cholesterol was measured as indicated in Figure 4A. Efflux to medium containing apoAI rHDL was significantly higher than AIM rHDL at 6 and 12 μg/mL protein in panel A ($p < 0.001$); no significant differences were seen in panel B.

The presence of AIM-apoAII heterodimers in AIM carriers could potentially explain a cardioprotective effect of this mutation. We previously found that the enhanced cholesterol mobilization conferred by the cross-linked apoAI-AII heterodimer in tyrosylated HDL requires the apoproteins of tyrosylated HDL to be reconstituted with lipid into spherical rHDL and was not seen with the lipid-free apoproteins of

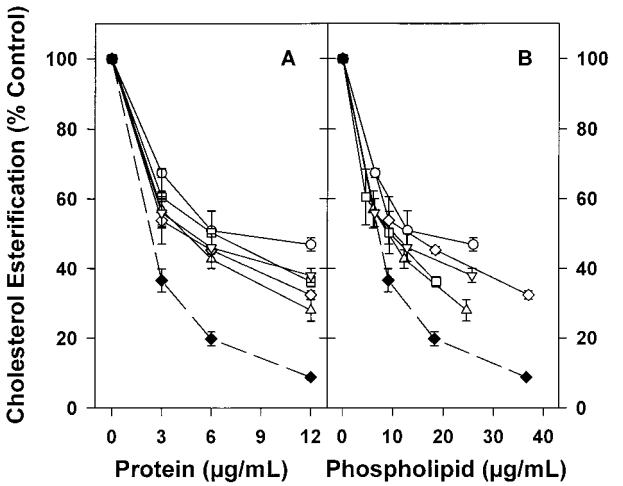

FIGURE 7: Depletion of cholesterol available for esterification in fibroblasts by spherical rHDL containing apoAI or AIM, with or without apoAII. Cholesterol-loaded cells were incubated with the indicated concentration of protein (A) or phospholipid (B) of rHDL generated by sonication and cholate dialysis of the whole lipid fraction of HDL and apoAI alone (○), AIM alone (□), apoAI and apoAII (non-cross-linked, ◇), AIM and apoAII (non-cross-linked, △), apoproteins containing the AIM-apoAII heterodimer (cross-linked as in Experimental Procedures, ▽), or apoproteins containing the apoAI-AII heterodimer generated by tyrosyl radical oxidation of an apoAI, apoAII mixture (◆), for 16 h, and [^{14}C]oleate incorporation into cholesteryl esters was determined and expressed as in Figure 4B. Depletion of ACAT-accessible cholesterol was significantly greater for rHDL containing tyrosyl radical-cross-linked apoAI-apoAII than for all other particles ($p < 0.001$) in panel A; no significant differences were seen between the other rHDL particles based on protein concentration. With results normalized to 20 μg/mL phospholipid in panel B, rHDL containing AIM or AIM/apoAII (non-cross-linked) showed a significantly higher capacity to deplete ACAT-accessible cholesterol than rHDL containing apoAI, apoAI/apoAII, or AIM cross-linked to apoAII ($p < 0.01$ for all). rHDL containing tyrosyl radical-cross-linked apoAI-apoAII still showed a greater capacity to deplete this cholesterol pool than all other rHDL ($p < 0.01$ for all).

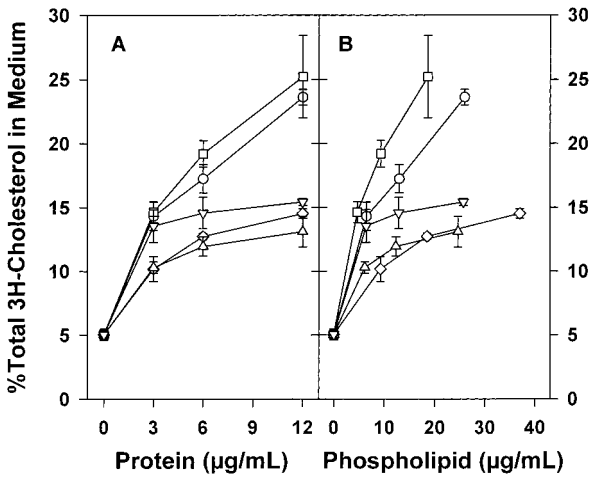

FIGURE 8: Cholesterol efflux from fibroblasts by spherical rHDL containing apoAI, AIM, with or without apoAII. [^{3}H]Cholesterol-labeled and cholesterol-loaded cells were incubated with the rHDL particles indicated in Figure 7, and cholesterol efflux was determined as in Figure 4A. Cholesterol efflux was significantly greater to media containing apoAI and AIM-containing rHDL than all other rHDL at 6 and 12 μg of protein/mL ($p < 0.005$ for all) (A). With results normalized to 20 μg/mL of phospholipid in panel B, rHDL containing AIM showed a greater capacity to promote cholesterol efflux than all other particles ($p < 0.05$ for all); apoAI-containing rHDL also promoted cholesterol efflux better than all particles containing apoAII when normalized this way ($p < 0.01$ for all).

this form of HDL (*30*). We therefore assessed the ability of spherical rHDL containing apoAI, apoAIM, and these two proteins present but not cross-linked to apoAII on the rHDL surface, or cross-linked to apoAII on the particle surface, to mobilize cell cholesterol. Compared as equivalent protein concentrations in the incubation medium, we found no significantly different capacity of rHDL containing apoAI, AIM, apoAI/AII, AIM/AII, or AIM-AII (cross-linked proteins) to deplete the cellular pool of ACAT-accessible cholesterol (Figure 7). As shown previously (*30*), rHDL containing the apoprotein species generated by tyrosyl radical oxidation of a mixture of native apoAI and apoAII (which includes apoAI-AII heterodimers) showed a markedly enhanced capacity to deplete this cholesterol pool. When the same data were replotted according to phospholipid mass in the acceptor particles, rHDL containing AIM alone or with (but not cross-linked to) apoAII showed a modest and intermediate increase in depletion of this pool compared to the other rHDL and rHDL containing tyrosyl radical cross-linked apoAI and apoAII. When normalized to 20 μg/mL phospholipid in the medium, the AIM and AIM/AII rHDL showed a significantly higher capacity to deplete ACAT-accessible cholesterol than rHDL containing apoAI, apoAI/AII, and AIM cross-linked to apoAII. Differences between these particles were not significant at lower phospholipid concentrations. Plotted either way, rHDL containing AIM-AII heterodimers did not reproduce the enhanced capacity of rHDL containing apoAI-AII heterodimers generated by tyrosyl radical oxidation to enhance depletion of this regulatory pool of cholesterol.

Efflux of [^{3}H]cholesterol to the medium by apoAI and AIM-containing spherical rHDL was significantly higher than all rHDL particles containing apoAII when compared on an equivalent protein basis (Figure 8A). Normalization of these data to phospholipid concentration resulted in a similar pattern but also suggested a greater ability of AIM-containing spherical rHDL to deplete cellular cholesterol to the medium than spherical rHDL containing apoAI above a phospholipid concentration of 7 μg/mL (Figure 8B). rHDL containing tyrosyl radical-cross-linked apoAI-AII heterodimers were previously found to have a similar capacity to non-cross-linked apoAI- and AII-containing rHDL in promoting cholesterol efflux by this assay (data not shown). These data suggest a modestly increased capacity of AIM on spherical rHDL to deplete cellular cholesterol based on altered phospholipid affinity of AIM, rather than intrinsic cholesterol-mobilizing characteristics of the protein, compared to apoAI.

DISCUSSION

These studies provide evidence that $apoAI_{Milano}$ has a diminished or similar capacity compared to native apoAI to promote depletion of the regulatory and total pools of cholesterol from cells, depending on its state of lipidation. Lipid-free AIM was less efficient or similar as an acceptor of plasma membrane cholesterol compared with free apoAI, depending on the cell type assessed. The two proteins showed similar high capacities to deplete the regulatory pool of free cholesterol available for esterification ("ACAT-accessible" cholesterol). This is consistent with previous reports of avid depletion of ACAT-accessible cholesterol by several lipid-

free exchangeable α-helical proteins, including apoAI, apoAII, apoE, and apolipophorin III (*4*).

We found no increased capacity of AIM-containing compared to apoAI-containing discoidal rHDL generated with egg PC to deplete either plasma membrane cholesterol or ACAT-accessible cholesterol. Cholesterol mobilization by spherical rHDL normalized for apoAI or AIM protein content was similar, whereas normalization for phospholipid content suggested a modestly enhanced capacity of spherical AIM-containing particles to deplete cholesterol pools. Normalization for phospholipid rather than particle number in these experiments would favor efflux to AIM-containing particles, due to their lower phospholipid content per particle, and therefore larger number of particles at equivalent phospholipid levels in the incubation medium. Had we normalized for particle number, any increase in cholesterol mobilization by AIM-containing rHDL would likely have been lost due to the higher phospholipid per particle in the apoAI-containing rHDL. Overall, our results suggest no enhancement of cholesterol mobilization by AIM related directly to intrinsic properties of the protein. Any potential increase in cholesterol mobilization by AIM-containing rHDL is likely related to increased phospholipid affinity of the protein and thus an enhanced passive acceptor function compared to equivalent phospholipid concentrations in apoAI rHDL.

Previous reports reached conflicting conclusions about the cholesterol mobilizing potential of AIM-containing rHDL (*22*, *27*). Westman et al. reported no difference in the capacity of similar protein concentrations of unfractionated apoAI- and AIM-containing discoidal rHDL, prepared as in our studies using egg PC, to deplete cholesterol from human macrophage foam cells (*22*). Calabresi et al., using isolated subfractions of rHDL generated using pure palmitoyloleoyl-PC (POPC), found an increased capacity of small (7.8 nm) rHDL but not larger sized (12.5 nm) particles containing AIM to deplete plasma membrane and ACAT-accessible cholesterol from Fu5AH rat hepatoma cells and elicited mouse macrophages, compared to similar sized apoAI-containing rHDL (*27*). POPC is the major but not sole PC species in egg PC, and human PC species also contain significant amounts of stearic, linoleic, and arachidonic acids (with linoleate being twice as abundant in total human plasma and HDL phospholipids as oleate) (*53*). The increased heterogeneity of discoidal and spherical rHDL of particles generated with egg PC and the isolated whole lipid fraction of HDL (Figure 2) is likely a consequence of the presence of these different lipid species. The range of discoidal particles generated in our system is more likely to represent the range of particles generated in vivo, and nondenaturing gradient gels did not suggest segregation of discoidal particles into discrete fractions as reported for particles generated with pure POPC (*27*). Although a particular subfraction of AIM rHDL generated in vitro using an isolated lipid species may exhibit enhanced cholesterol removal capacity, our data suggest no increased capacity of the mixture of discoidal AIM rHDL generated with egg PC to mobilize cell cholesterol when expressed by content of protein or phospholipid, compared to apoAI rHDL disks.

The cholesterol-mobilizing effects of AIM-containing spherical rHDL generated for our studies need to be interpreted in light of the particle sizes seen in human AIM carriers and transgenic animals. Previous studies reported that HDL_3-sized particles predominate in these carriers, with the HDL_2 fraction being nearly depleted (*50*, *54*). Bielicki and colleagues reported a restriction in the size of, and reduced cholesterol mobilization by, HDL particles generated by AIM-expressing Chinese hamster ovary cells, with AIM HDL mainly in the 7.4 nm range (*21*). Mice expressing AIM as well as human apoAI and apoAII, to attempt to recreate the HDL apoprotein milieu seen in human AIM carriers, showed a slight shift in HDL to smaller particles (*55*). These results suggest that smaller (yet spherical) HDL are present in carriers and that larger spherical HDL are present in lesser or low amounts. The modest increase in cholesterol mobilization by the spectrum of AIM spherical rHDL particles generated in our system (which include small LDL-sized particles), therefore, seem unlikely to explain the antiatherogenic actions of AIM in vivo or to explain only a minor component of the protective actions of this protein. The low total HDL and apoAI/AIM concentrations in AIM carriers and the shortened residence time of AIM in plasma (*56*) also suggest that minor differences in cholesterol mobilization capacity by isolated species of AIM-containing particles are unlikely to account for the potential cardioprotective effects of this mutation.

In contrast to our finding that the presence of apoAI-AII heterodimers generated by tyrosyl radical oxidation markedly enhance the ability of HDL to mobilize cellular cholesterol (*30*), we found no increased capacity of lipid-free apoproteins or rHDL containing AIM-AII disulfide-linked heterodimers to promote the mobilization of ACAT-accessible or total cholesterol. The reason for the increased cholesterol removal by apoAI-AII heterodimers in tyrosylated HDL is not yet known but is presumed to involve an enhanced interaction of this complex on spherical HDL with cell surface receptors that initiate a cascade for cellular lipid mobilization. The lack of enhanced cholesterol mobilization by spherical rHDL containing AIM-AII heterodimers suggests that these complexes assume a different conformation than apoAI and apoAII cross-linked by tyrosyl radical oxidation. We are currently investigating the sites of cross-linking involved in the heterodimer formed upon tyrosyl radical oxidation of HDL. The current findings suggest that protection against atherosclerosis in AIM carriers is not related to the presence of apoAIM-AII heterodimers in these individuals.

In conclusion, our results suggest no overall enhancement of cellular cholesterol mobilization by $apoAI_{Milano}$ compared to apoAI in their varying states of lipidation and suggest that any anti-atherogenic actions of the mutant apolipoprotein may be related to other protective actions in the artery wall. Further in vitro and in vivo animal studies, as well as the planned use of AIM in human clinical trials, will hopefully provide further insights into additional cardioprotective actions of this apolipoprotein as compared to native apoAI.

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