

Wild-Type ApoA-I and the Milano Variant Have Similar Abilities to Stimulate Cellular Lipid Mobilization and Efflux

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Objective—The present study is a comparative investigation of cellular lipid mobilization and efflux to lipid-free human apoA-I and apoA-I_{Milano}, reconstituted high-density lipoprotein (rHDL) particles containing these proteins and serum isolated from mice expressing human apoA-I or apoA-I_{Milano}.

Methods and Results—Cholesterol and phospholipid efflux to these acceptors was measured in cell systems designed to assess the contributions of ATP-binding cassette A1 (ABCA1), scavenger receptor type BI (SRBI), and cellular lipid content to cholesterol and phospholipid efflux. Acceptors containing the Milano variant of apoA-I showed no functional increase in lipid efflux in all assays when compared with wild-type apoA-I. In fact, in some systems, acceptors containing the Milano variant of apoA-I promoted significantly less efflux than the acceptors containing wild-type apoA-I (apoA-I_{wt}). Additionally, intracellular cholesterol ester hydrolysis in macrophage foam cells was not different in the presence of either apoA-I_{Milano} or apoA-I_{wt}.

Conclusion—Collectively these studies suggest that if the Milano variant of apoA-I offers greater atheroprotection than wild-type apoA-I, it is not attributable to greater cellular lipid mobilization. (*Arterioscler Thromb Vasc Biol* 2007;27:2022-2029.)

Key Words: apolipoprotein ■ A-I ■ HDL ■ cholesterol efflux ■ apoA-I_{Milano}

Patients presenting with the Milano mutation (R173C) of the apolipoprotein (apo) A-I, have significantly lower plasma HDL levels and paradoxically do not develop premature atherosclerosis.^{1,2} The mechanism by which apoA-I_{Milano} offers atheroprotection is unclear. Studies involving human studies are conflicting with some indicating that expression of apoA-I_{Milano} promotes greater reduction in atherosclerosis, whereas others report the presence of the Milano variant offers no additional protection compared with wild-type apoA-I (apoA-I_{wt}).^{1,3,4} Lipid efflux studies are also contradictory with some reporting apoA-I_{Milano} promotes greater cholesterol efflux,⁵ and others indicating that apoA-I_{Milano} does not enhance cholesterol mobilization.⁶

A number of mechanisms have been identified by which free cholesterol (FC) moves from cells to extracellular acceptors. These pathways include aqueous diffusion,⁷ SRBI-mediated efflux,⁸ ABCA1-mediated efflux,⁹ and ABCG1-mediated efflux.^{10,11} When designing cellular based cholesterol efflux assays the mechanism of efflux, as well as the extracellular acceptor, must be considered.¹² Cholesterol efflux via aqueous diffusion occurs in all cells, and the rate is dependent on the concentration gradient between the cell plasma membrane and the acceptor particles. The ABCA1

receptor is responsive to lipid-free and lipid-poor apolipoproteins, whereas SRBI-mediated efflux occurs to phospholipid-containing acceptors.¹³ The intracellular concentration of cholesterol can lead to alterations in the mechanism of efflux as well as changes in efflux values. Thus, the cholesterol level in the cell has been shown to impact ABCA1^{9,14,15} and SRBI¹⁶ expression. Additionally, increasing the intracellular pool of cholesterol expands the pool of cholesterol available to undergo efflux.^{17,18}

In addition to quantitating the efflux of FC, experiments reported here were also designed to determine whether apoA-I_{Milano} has the ability to enhance the mobilization of cholesterol by stimulating the hydrolysis and clearance of intracellular cholesteryl esters (CE) more efficiently than wild-type human apoA-I.

Materials and Methods

Fetal bovine serum (FBS), calf serum (CS), gentamycin, 9-cis-retinoic acid, 22-hydroxy cholesterol, and 8-(4-chlorophenyl-thio)-cAMP (CTP-cAMP) were purchased from Sigma-Aldrich. Geneticin was obtained from Invitrogen Corporation. [1,2-³H]cholesterol (51 Ci/mmol), [methyl-³H]choline chloride (86 Ci/mmol), and [³H]adenine were from Perkin-Elmer Life Sciences. Organic solvents were purchased from Fisher Scientific. Tissue culture dishes, flasks, and

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plates were from Corning Inc. RPMI 1640, EMEM, and DMEM media and phosphate-buffered saline were obtained from CellGro. MEM buffered with 25 mmol/L HEPES (pH 7.4) was purchased from BioWhittaker Inc. Essentially fatty acid-free bovine serum albumin (BSA) was obtained from Interger Co. The ACAT inhibitor CP-113 818 was provided by Pfizer Pharmaceuticals. Quickchange Site-Directed Mutagenesis Kit was purchased from Stratagene. Human plasma apolipoproteins were obtained by ultracentrifugation and purified by anion-exchange chromatography.¹⁹ Before use, the purified apolipoproteins were solubilized in 6 mol/L guanidine-HCl and dialyzed against Tris buffer (0.01 mol/L Tris, 1.0 mmol/L EDTA, and 0.15 mol/L NaCl). Apolipoprotein AI_{Milano} was supplied by Pfizer Inc.

Cell Culture

Mouse peritoneal macrophages (MPM) were isolated and prepared as previously described.²⁰ J774 macrophages were grown in RPMI 1640 medium containing 10% FBS + 50 μ g/mL gentamycin. Fu5AH rat hepatoma cells were maintained in EMEM medium containing 5% CS + 50 μ g/mL gentamycin. J774 and Fu5AH cells were seeded in tissue culture plates. The following day wells were washed with MEM-HEPES. The final wash was removed and media containing 1% FBS + [³H]cholesterol or [³H]choline (1 μ Ci/mL) \pm acetylated LDL (acLDL, 100 μ g protein/mL) and \pm CP-113 818 (2 μ g/mL) was added for 24 hours. After this, cells were incubated in media containing 0.2% BSA for 18 hours to allow cellular cholesterol radiolabel to equilibrate. In certain experiments, some incubations contained 0.3 mmol/L CTP-cAMP during equilibration. After equilibration, time zero cells were harvested to determine total cpm, protein, and FC and CE mass in the cells before adding the acceptors.

Preparation and Characterization of Discoidal Reconstituted HDL Particles

Discoidal reconstituted HDL (rHDL) containing human apoA-I and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) were prepared by the cholate dialysis method.²¹ Briefly, POPC in CHCl₃ was added to a 15-mL conical glass tube and dried under nitrogen. The POPC was hydrated in TRIS-buffered saline (TBS), pH 7.4, vortexed to generate multilamellar vesicles, and incubated with sodium cholate at 37°C for 1.5 hour to generate detergent-PC mixed micelles. The solution was mixed every 15 minutes until completely clear. Next, apoA-I freshly dialyzed from 6 mol/L guanidine-HCl in TBS was added to the lipid-detergent mixture at a POPC to apoA-I weight ratio of 1.1:1, POPC/protein (mole ratio 40:1). The same starting weight ratio of apoA-I_{Milano} was used, and because the apoA-I_{Milano} was used in the oxidized state as a dimer, the POPC/AI_{Milano} mole ratio was 20:1. The lipid-detergent-apoA-I complexes were then incubated for 1 hour at 37°C. Sodium cholate was removed by dialysis at 4°C against TBS. Similar size rHDL complexes containing either apoA-I_{wt} or apoA-I_{Milano} were isolated by fractionation on a calibrated gel filtration column (60 \times 1.6-cm Superdex 200 HR column, Amersham Pharmacia Biotech)²² and used within 5 days of preparation. The HDL particles ranged in size from 8 nm to 10 nm with a phospholipid to protein ratio of 0.7/1 to 1.3/1 (w/w).

Preparation of ApoA-I_M Adeno-Associated Virus

ApoA-I_{wt} cDNA was mutated using the Quickchange Site-Directed Mutagenesis Kit by Stratagene. Specific primers were designed to mutate the WT apoA-I cDNA in the pAAVmc8 plasmid.²³ The plasmid containing the WT apoA-I cDNA served as the template for the mutagenic polymerase chain reaction (PCR) reaction. After the PCR reaction the sample was digested with *DpnI*, an endonuclease specific for methylated and hemimethylated DNA, to digest the parental DNA template and select for synthesized DNA containing the apoA-I_{Milano} mutation. The purified mutant plasmid was then transformed into XL1-Blue supercompetent *E. coli*. Several colonies were isolated and used to inoculate Luria-Bertani media containing 100 mg/mL ampicillin. The resulting plasmids were sequenced and restriction digested to confirm the presence of the intended mutation. The plasmid containing the mutant sequence was then submitted to

the University of Pennsylvania Vector Core for use in creating the apoA-I_M adeno-associated virus (AAV).

Preparation and Bleeding of WT ApoA-I and ApoA-I_{Milano} Transgenic Mice

ApoA-I^{-/-} mice (C57 BL/6 background, from Jackson Laboratory, Bar Harbor, Maine) were injected intraperitoneally with 1×10^{12} particles of either apoA-I_M AAV, WT apoA-I AAV, or LacZ AAV with a total of 5 mice per group. Blood was drawn from the retro-orbital plexus 3 and 4 weeks postinjection after 4 hours of daytime fasting and allowed to clot for 1 hour at 4°C. The blood was then spun (10 000 rpm in a tabletop centrifuge, 7 minutes, 4°C) to isolate the serum. Total cholesterol, HDL cholesterol, phospholipid, and human apoA-I levels were measured on a Cobas Fara (Roche Diagnostics Systems Inc) using Sigma Diagnostics reagents.

Isolation of Mouse HDL

Serum was isolated from 5 mice in each group and kept at 4°C. The 0.9 to 1.6 mL sera were adjusted to a density of 1.073 g/mL with KBr and spun at 4°C at 47 000 rpm for 18 hours in a Beckman 70.1Ti rotor. VLDL, IDL, and LDL-containing fractions along with the middle layers were removed. The bottom layers were adjusted to a density of 1.21 g/mL and spun for 24 hours at 50 000 rpm. After removing the top fractions containing total HDL, the purity was checked by agarose gel electrophoresis (Lipoprotein Electrophoresis Kit, Beckman Coulter). Isolation and identification of HDL phospholipid was accomplished using the high-performance liquid chromatography (HPLC) method of Kaduce et al.²⁴ Quantitation of phospholipids was achieved by comparing the UV absorbance of each class of phospholipid to known standards.

Precipitation of ApoB-Containing Lipoproteins From Mouse Serum

Serum was collected from mice and combined with a solution containing 20% polyethylene glycol (PEG) in 200 mmol/L glycine (100 parts serum to 40 parts PEG solution). This mixture was then allowed to incubate at 37°C for 20 minutes and spun in 4°C at 10 000 rpm for 30 minutes. The top layer, containing HDL, was collected and stored on ice at 4°C until use.²⁵

Cholesterol and Phospholipid Efflux

After [³H]cholesterol or [³H]choline labeling the cells, media containing the appropriate acceptor was added for up to 24 hours. In some experiments the ACAT inhibitor CP113 818 (2 μ g/mL) was added to the media as indicated in the figure legends. To determine cholesterol efflux, media were sampled at indicated times, filtered, and counted by liquid scintillation counting to determine [³H] released. [³H] in the media was compared with total [³H] at time zero to determine the percent release of [³H]cholesterol.²⁶ To determine phospholipid efflux, media were sampled and filtered to remove any cellular debris. The media were extracted 3 times by the method of Bligh and Dyer²⁷ to remove any unincorporated [³H]choline.²⁸ Total cpm was determined in the final extract to measure phospholipid efflux.

Cellular Toxicity

Toxicity was measured by the release of [³H]adenine from the cells as previously described.²⁹ Percent adenine release was measured by dividing the counts present in the sample media by the total cellular radioactivity at time zero.

Cholesteryl Ester Hydrolysis

Macrophages were enriched with ³H-CE for 24 hours by incubation with acLDL (100 μ g/mL) containing [³H]-FC (1 μ Ci/ml). Cells were then equilibrated in BSA for 18 hours before the addition of acceptors. After equilibration, cells contained from 40% to 60% of the [³H]cholesterol as CE. The acceptors (see figure legends) were added for 24 hours in media containing the ACAT inhibitor CP113818 (2 μ g/mL). At the end of this incubation, wells were

washed with PBS, allowed to air dry, and extracted overnight with isopropanol. Lipids in the extracts were separated by thin layer chromatography to determine the loss of [3 H] from the CE pool.³⁰

Data Analysis

Data are from representative experiments and are expressed as mean \pm SD for an $n=3$ unless otherwise stated. Statistical test for significance was done using an unpaired t Test with a 95% confidence interval.

Results

The Effect of ApoA-I and A-I_{Milano} on Cholesteryl Ester Hydrolysis in Macrophage Foam Cells

It is well known that macrophages esterify accumulating cholesterol through the action of ACAT and store the resultant CE in cytoplasmic lipid inclusions. An obligatory step in mobilizing cholesterol from these cells is the hydrolysis of the ester by a neutral CE hydrolase (nCEH) as only FC effluxes from the cell.³¹ Although the nCEH does not appear to be tightly regulated, there are several reports indicating intracellular FC levels may affect the rate of hydrolysis of CE.^{32,33} We investigated the ability of extracellular acceptors to promote CE hydrolysis by efficiently removing FC. MPM were enriched and radiolabeled with cholesterol as described in Methods. Hydrolysis of intracellular CE was measured in the presence of an ACAT inhibitor. This allows the assessment of net CE hydrolysis because reesterification by ACAT is prevented.³⁴ Lipid-free apoA-I_{Milano}, apoA-I, rHDL_{Milano}, or rHDL were added to the incubation medium to act as acceptors. Lipid-free apoA-I_{Milano} was added at a concentration of 15 μ g/mL. Because apoA-I_{Milano} exists in solution as a dimer, lipid-free wild-type apoA-I was added at concentrations of 15 and 7.5 μ g/mL so that comparisons could be made on both a mass and molar basis. Table 1 shows results obtained with MPM having basal levels of nCEH ($-cAMP$) and those having nCEH upregulated ($+cAMP$).³⁵ In all groups, there was between 20% and 35% hydrolysis of intracellular CE in 24 hours, which agrees with CE hydrolysis values previously reported.³⁶ The data also indicate that the apoA-I_{Milano} does not affect the rate of CE hydrolysis compared with apoA-I. Pretreatment with cAMP increased the rate of hydrolysis in the presence of both acceptors, but again

there was no difference between apoA-I and apoA-I_{Milano}. Similar results were obtained with J774 macrophages ($39.8 \pm 4.3\%$ apoA-I 7.5 μ g/mL; $41.5 \pm 5.4\%$ apoA-I 15 μ g/mL; $44.3 \pm 2.8\%$ apoA-I_{Milano} 15 μ g/mL).

The Effect of ApoA-I and ApoA-I_{Milano} on Cholesterol Efflux From Macrophages

Because the ability of the Milano variant of apoA-I to promote greater cholesterol efflux than apoA-I_{wt} is controversial,^{6,37} we performed comparative experiments to address the efflux potential of lipid-free and lipid-associated apoA-I_{Milano} (rHDL). In addition, serum and HDL from mice expressing the apoA-I_{Milano} and apoA-I_{wt} were compared.

For the measurement of cholesterol efflux to lipid-free apoprotein, apoA-I_{Milano} was added to the efflux medium at 15 μ g/mL and apoA-I_{wt} was added at 15 or 7.5 μ g/mL to control for the difference in protein molecular weight. ABCA1 expression was upregulated in macrophages by cholesterol-enriching the cells with acLDL as described in Methods. In addition, 18-hour exposure to 0.3 mmol/L CTP-cAMP further increased ABCA1 levels.³⁸ Figure 1A indicates that in J774 macrophages, lipid-free apoA-I_{Milano} was as efficient at promoting ABCA1-mediated efflux as apoA-I_{wt}. Additionally,

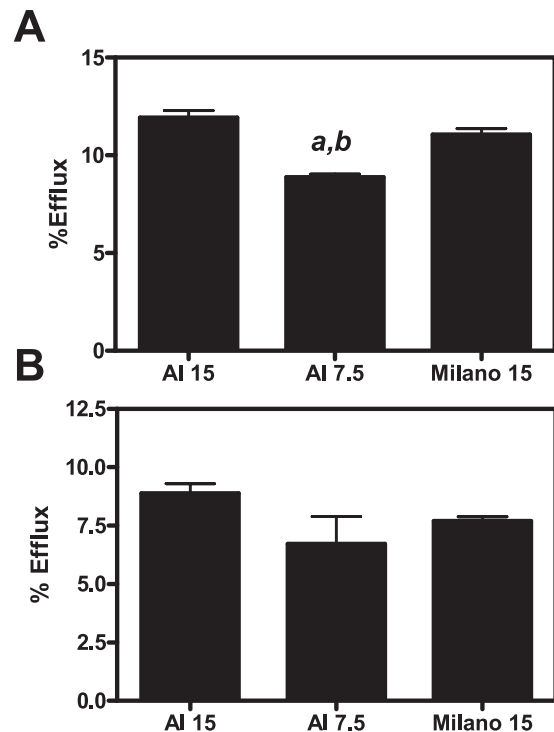


Figure 1. ABCA1-mediated efflux to lipid-free apoA-I or apoA-I_{Milano} from macrophages. J774 (A) or MPM (B) cells were cholesterol-enriched and labeled with [3 H]cholesterol and acLDL (100 μ g/mL) in the presence of 1%FBS. Cells were treated \pm cAMP during the 18-hour equilibration period, and then incubated with 7.5 or 15 μ g/mL apoA-I or 15 μ g/mL apoA-I_{Milano} in the presence of the ACAT inhibitor CP113 818 (2 μ g/mL) for 4 hours. The addition of the ACAT inhibitor allowed for measurement of CE hydrolysis (Table 1). An aliquot of media was removed, filtered, and analyzed by LSC to determine % 3 H release. ABCA1-mediated efflux was calculated by $\% \text{efflux}_{+cAMP} - \% \text{efflux}_{-cAMP}$. Significance is indicated in the figure (**a**=significantly different from apoA-I 15 μ g/mL ($P=0.0016$), **b**=significantly different from apoA-I_{Milano} ($P=0.0035$)).

TABLE 1. CE Hydrolysis in MPM

Acceptor	Concentration	Percent Hydrolysis	
		$-cAMP$	$+cAMP$
ApoA-I	15 μ g/ml	21.1 ± 3.7	35.1 ± 6.8
ApoA-I	7.5 μ g/ml	16.6 ± 0.4	31.4 ± 3.8
ApoA-I _m	15 μ g/ml	20.5 ± 5.1	31.7 ± 6.0
rHDL	15 μ g/ml	30.1 ± 7.5	...
rHDL _m	15 μ g/ml	28.1 ± 0.8	...

MPM were cholesterol-enriched by incubating with acLDL and [3 H]cholesterol. Cells were equilibrated for 18 hours in the presence of 0.2% BSA. Some incubations contained 0.3 mmol/L cAMP during the equilibration phase to increase nCEH activity. Cells were incubated with 7.5 or 15 μ g/mL lipid-free apoA-I, 15 μ g/mL apoA-I_{Milano}, rHDL (15 μ g protein/mL), rHDL_{Milano} (15 μ g protein/mL) in the presence of the ACAT inhibitor CP113 818 (2 μ g/mL) for 24 hours. Percent hydrolysis was measured by determining loss of 3 H from the esterified cholesterol pool.

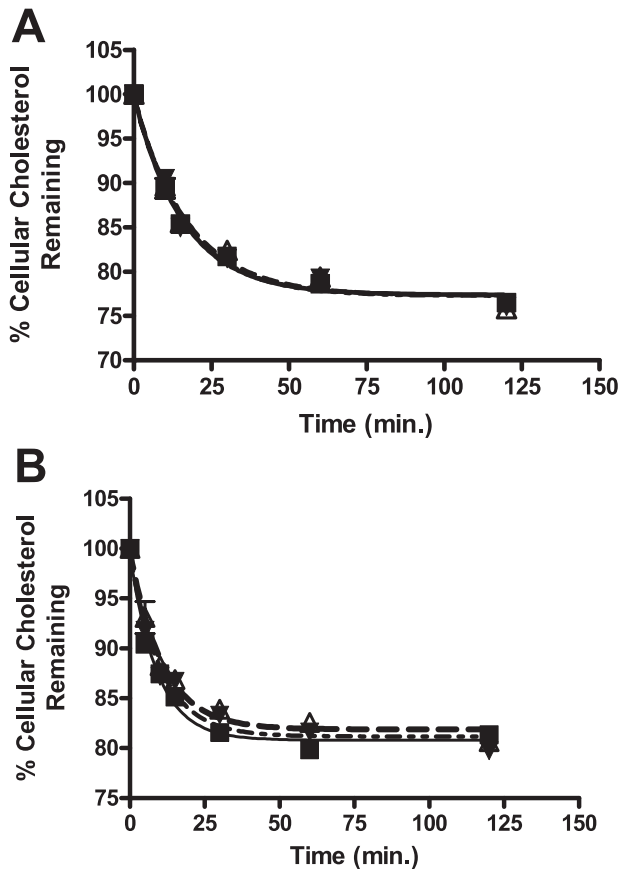


Figure 2. Time course of efflux from cholesterol normal or cholesterol-enriched J774 to rHDL or rHDL_{Milano}. J774 macrophages were labeled with [³H]cholesterol –acLDL (A) or +acLDL (100 μ g/mL; B) for 24 hours. Cells were equilibrated for 18 hours in 0.2% BSA. After equilibration cells were incubated with 15 μ g/mL rHDL (8 nm \blacktriangledown , 10 nm \triangle) or rHDL_{Milano} (\blacksquare) for up to 2 hours. Media was sampled, filtered, and [³H]cholesterol in the media was determined by liquid scintillation counting. The [³H]cholesterol in the media was subtracted from [³H]cholesterol in the cell before the efflux period to determine the percent remaining in the cell.

there was no significant difference in ABCA1-mediated efflux from MPM (Figure 1B) among the acceptors used.

Because there were no differences in cholesterol efflux to the lipid-free apoproteins, we performed a series of experiments to determine the efflux potential of rHDL and rHDL_{Milano}. POPC/apoA-I and POPC/apoA-I_{Milano} rHDL were prepared using the cholate dialysis method,²¹ and the rHDL was analyzed by fast protein liquid (FPLC). The POPC/AI_{Milano} rHDL gave a single population of disks that were approximately 8 nm in diameter with a POPC to protein molar ratio of 12.6:1. The POPC/apoA-I_{wt} rHDL made 2 populations of rHDL: one similar to that of the POPC/AI_{Milano} rHDL rHDL having a diameter of approximately 8 nm and molar ratio of 26:1, whereas the other population had a larger diameter (approximately 9 nm) and a molar ratio of 46:1. The molecular weight used for the Milano protein was 56 000 (dimer form of the protein) and 28 000 for apoA-I_{wt}. FPLC fractions corresponding to each peak were collected and concentrated before use. Isolated fractions were stable for up to 2 weeks at 4°C (data not shown). The efflux potential of these rHDL were then

assessed. Cholesterol-normal J774 macrophages were labeled with [³H]cholesterol as described in Methods and rHDL (15 μ g/mL) was added to the efflux medium for up to 2 hours. The efflux data were fit to a 1-phase exponential decay equation using GraphPad Prism software (GraphPad Software Inc). Figure 2A indicates the rHDL_{Milano} was as efficient an acceptor as rHDL_{wt} regardless of disk size. Because cholesterol enrichment of J774 cells has been shown to modulate the efflux-related proteins,^{9,14–16} we measured the efflux potential of the disks in cholesterol-enriched cells (Figure 2B). The mass of cholesterol in the cells after enrichment was 80 ± 9 μ g cholesterol/mg cell protein (49% as CE). Again, there was no difference in efflux between all acceptors tested (Figure 2B). Half times of cholesterol efflux in cholesterol normal cells were not different among the rHDL (11.1 ± 1.6 minutes rHDL_{Milano}; 12.0 ± 1.9 minutes rHDL_{wt}-9 nm; 11.4 ± 1.7 minutes rHDL_{wt}-12 nm, Figure 2A). Additionally cholesterol efflux half times in cholesterol-enriched cells did not differ among rHDL (6.3 ± 0.9 minutes rHDL_{Milano}; 7.6 ± 1.5 minutes rHDL_{wt}-9 nm; 7.2 ± 1.2 minutes rHDL_{wt}-12 nm, Figure 2B).

Some reports indicate that in animal models apoA-I_{Milano} is more atheroprotective than apoA-I_{wt},³⁹ although this is controversial.⁴⁰ One proposed mechanism for the increased antiatherogenic properties of apoA-I_{Milano} is its ability to stimulate greater cholesterol efflux from peripheral macrophages, thereby increasing reverse cholesterol transport. We therefore determined the efflux potential of serum obtained from apoA-I^{-/-} mice which were infected to express either human apoA-I or apoA-I_{Milano}. For these studies we used cell systems designed to measure ABCA1 and SRBI mediated efflux.

Cholesterol normal MPM were labeled with [³H]cholesterol as described in Methods. After an equilibration period, 2% serum isolated from apoA-I^{-/-} mice expressing either human apoA-I or apoA-I_{Milano} was added for 4 hours. Serum from the apoA-I_{Milano}-expressing mice promoted less cholesterol efflux ($12.0 \pm 1.2\%$ for wild-type, $10.3 \pm 0.9\%$ for Milano, $P=0.03$). When ABCA1 expression was stimulated in the macrophages by the addition of cAMP during the equilibration phase, again there was no difference in the efflux potential of both types of sera ($15.6 \pm 0.7\%$ for wild-type and $14.9 \pm 0.9\%$ for Milano). The ABCA1-mediated efflux (calculated by $\% \text{efflux}_{+\text{cAMP}} - \% \text{efflux}_{-\text{cAMP}}$) was also not significantly different ($4.6 \pm 1.5\%$ for wild-type and $3.6 \pm 0.9\%$ for Milano).

Fu5AH rat hepatoma cells have been extensively used to measure SRBI-mediated cholesterol efflux.^{41–43} Figure 3A indicates that serum obtained from the apoA-I_{Milano}-expressing mice promoted less cholesterol efflux compared with the mice expressing the wild-type human apoA-I when both sera were added to the efflux media at 2%. Similar data were obtained if the apoB-containing lipoproteins were precipitated from the sera and this “HDL fraction”²⁵ of the serum was added to the efflux medium (data not shown). However, HDL is the primary acceptor for SRBI-mediated efflux in vivo and the serum HDL-cholesterol values from the 2 sets of experimental mice was significantly different (Table 2). One explanation for the lower apoA-I_{Milano} stimulated efflux is that the serum HDL-cholesterol and HDL-protein values in these

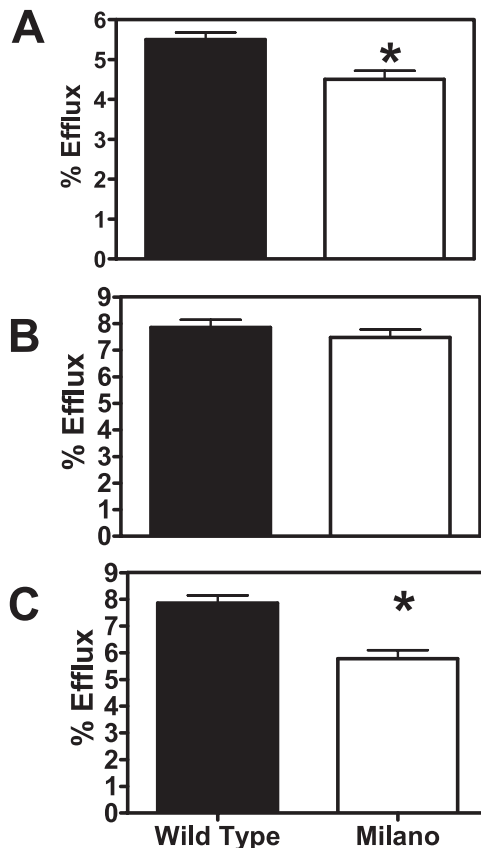


Figure 3. Efflux to mouse serum, HDL, and HDL_{Milano}. Fu5AH were labeled with [³H]cholesterol in the presence of 1%FBS. The cells were then incubated for 4 hours with 2% serum isolated from apoA-I^{-/-} mice expressing either human apoA-I or apoA-I_{Milano} (A), HDL or HDL_{Milano} isolated from the serum of these mice at 25 μ g protein/mL (B), or 23 μ g phospholipid/mL (C). An aliquot of media was removed, filtered, and analyzed by LSC to determine % ³H release. *Indicates significance $P<0.05$.

mice were less than the levels of HDL in the mice expressing human apoA-I (Table 2). It should also be noted that the Milano-expressing mice had significantly lower serum phospholipid concentrations (Table 2). For valid comparison, equivalent amounts of HDL should be used in each group. To address this problem we compared the HDL from both types of mice on the basis of equivalent protein and equivalent phospholipid. Figure 3B and 3C summarizes the data for

efflux from Fu5AH cells to HDL and HDL_{Milano}; the HDL were added at 25 μ g/mL protein (Figure 3B) and at 23 μ g/mL phospholipid (Figure 3C). When the HDL was added at equivalent protein (Figure 3B) there was no significant difference in efflux despite the HDL_{Milano} having a greater phospholipid to protein weight ratio than wild-type HDL (HDL_{wt}). In addition, if the HDL were added to the efflux medium at equivalent phospholipid concentrations, there was significantly less efflux to the HDL_{Milano} compared with HDL_{wt} (Figure 3C). Previously we have shown that modulating the phospholipid content of HDL effects SRBI mediated efflux.⁴⁴ The phospholipids present on the HDL isolated from the apoA-I and apoA-I_{Milano} transgenic mice were identified using an HPLC assay as described in Methods. Both HDL_{wt} and HDL_{Milano} had similar levels of phosphatidylserine and phosphatidylethanolamine. However there was a significant difference in phosphatidylcholine (PC) and sphingomyelin (SM) content of the HDL. Wild-type HDL had a PC to SM ratio of 8.7:1 (w/w) and HDL_{Milano} had a PC to SM ratio of 2.0:1 (w/w), indicating that the HDL_{Milano} was depleted in PC relative to sphingomyelin (Table 2).

The Effect of ApoA-I and ApoA-I_{Milano} on Phospholipid Efflux From Macrophages

Apolipoproteins have been shown to stimulate not only ABCA1-mediated cholesterol efflux but also ABCA1-stimulated phospholipid efflux.²⁸ To determine whether apoA-I_{Milano} has an advantage over apoA-I_{wt} in promoting phospholipid efflux we labeled J774 macrophages with [³H]choline chloride as described in Methods. Some cells were stimulated with cAMP to increase ABCA1 expression. ApoA-I_{Milano} was added to the efflux medium at 7.5 μ g/mL and apoA-I_{wt} was added at 15 or 7.5 μ g/mL for 4 hours. In all experimental groups we did not observe any significant differences in phospholipid efflux between lipid-free apoA-I_{Milano} and apoA-I (data not shown). Additionally, the ABCA1-mediated efflux (calculated by %efflux_{+cAMP} – %efflux_{-cAMP}) was not significantly different ($0.08\pm0.03\%$ for apoA-I 7.5 μ g/mL, $0.08\pm0.02\%$ for apoA-I 15 μ g/mL, and $0.07\pm0.03\%$ for apoA-I_{Milano} 15 μ g/mL).

Efflux and Free Cholesterol-Induced Cellular Toxicity

We have previously shown that excess cytotoxic FC generated from the hydrolysis of macrophage foam cell CE is

TABLE 2. Serum and HDL Composition

Serum Type	Cholesterol mg/dl	HDL mg/dl	Triglyceride mg/dl	Phospholipid mg/dl	ApoAI mg/dl
Wild-type	68.0 \pm 4.5	48.4 \pm 4.2	40.8 \pm 7.7	133.6 \pm 8.9	99.4 \pm 3.2
AI Milano	43.2 \pm 6.0*	21.8 \pm 6.2*	59.4 \pm 10.6*	102.8 \pm 35.8	74.6 \pm 19.1*
HDL Type	Phospholipid:Protein (w:w)		Cholesterol:Protein (w:w)	Phosphatidylcholine:Sphingomyelin (w:w)	
Wild-type	0.92:1		0.32:1	8.7:1	
Milano	1.33:1		0.40:1	2.0:1	

ApoA-I^{-/-} mice were adenovirus infected to express human apoA-I or apoA-I_{Milano}. Serum was collected at 4 weeks after infection, analyzed as described in Methods, and the data are presented above. The serum from each set of mice was then pooled and the HDL was isolated from the pool using ultracentrifugation as described in Methods. The HDL was subsequently analyzed, and the data are presented above. Serum data are reported as mean \pm SD with an n=5 for each group. *Significantly different from wild-type mice, $P<0.05$.

Toxicity Vs Efflux in J774 Macrophage Foam Cells -24h

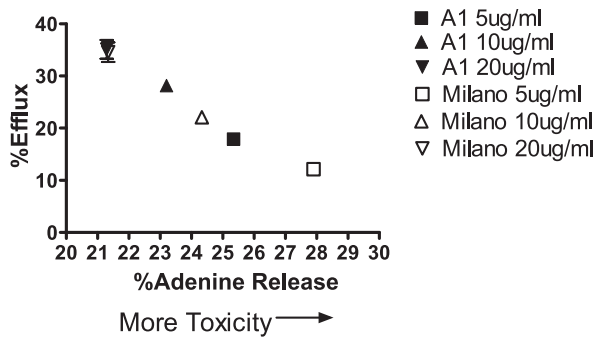


Figure 4. Effectiveness of apoA-I or apoA-I_{Milano} in preventing FC-induced cytotoxicity by acting as an extracellular acceptor. J774 macrophages were cholesterol-enriched and labeled by incubation with acLDL (100 µg/mL) and [³H]cholesterol. Cytotoxicity was measured by labeling parallel sets of cells with [³H]adenine for 2 hours after the enrichment period. After equilibration, both sets of cells were exposed to media containing CP-113 818 and apoA-I (5 (■), 10 (▲) or 20 (▼) µg protein/mL), or apoA-I_{Milano} (5 (□), 10 (△), or 20 µg phospholipid/mL) for 24 hours. Media was sampled to determine [³H]cholesterol or [³H]adenine released.

selectively removed from the cell via ABCA1.³⁸ Treatment with an ACAT inhibitor, in the absence of extracellular acceptors, produces hydrolysis of stored CE causing the accumulation of cellular FC while resulting in FC-induced cytotoxicity.^{29,45} Incubation of cells with ACAT inhibitor plus an acceptor results in FC efflux, thereby reducing intracellular cholesterol concentrations and saving the cell from FC-induced cytotoxicity. Thus, we compared the ability of apoA-I_{Milano} and apoA-I in removing cytotoxic FC from ACAT-inhibited J774 foam cells (Figure 4). At all concentrations measured, apoA-I_{Milano} was as or less effective in removing cytotoxic cholesterol than apoA-I_{wt} indicating that this is not an atheroprotective mechanism for apoA-I_{Milano}.

Discussion

Carriers of the Milano variant of apoA-I have dramatically reduced circulating HDL levels and yet do not develop premature atherosclerosis. One explanation for this inconsistency is that expression of the apoA-I_{Milano} promotes greater reverse cholesterol transport, presumably by creating a more efficient HDL. The objective of this investigation was to compare the effects of wild-type human apoA-I to apoA-I_{Milano} on various aspects of cellular cholesterol metabolism.

Our studies compared the wild-type and Milano apoproteins presented to cells in three different forms: (1) as lipid-free molecules, (2) in reconstituted phospholipid/protein rHDL, and (3) as native particles either in the serum or isolated the serum of apoA-I^{-/-} mice adenovirus-infected to express human wild-type or Milano apoA-I. The aspects of cellular cholesterol metabolism that were measured were CE hydrolysis rates using J774 and MPM. In both cases we used untreated cells and cells exposed to cAMP to enhance CE hydrolysis.³⁵ This approach not only quantifies CE hydrolysis but also measures ABCA1-mediated efflux. The third type of cell we used was the Fu5AH rat hepatoma that expresses high

levels of SRBI, thereby providing a comparative estimate of the impact of the 2 apoA-I variants on SRBI-mediated efflux.

Modulation of ABCA1 in vivo has been shown to affect the initiation and progression of atherosclerosis.⁴⁶ It is generally accepted that lipid-free apoprotein stimulates cholesterol and phospholipid efflux via ABCA1.^{47–49} We compared lipid efflux to lipid-free apoA-I_{Milano} and apoA-I_{wt} and found that apoA-I_{Milano} does not promote greater cholesterol (Figure 1) or phospholipid efflux from macrophages than apoA-I_{wt}. These observations were consistent regardless of whether the apoproteins were compared on a mass or molar basis.

We compared the efflux potential of lipid-associated apoA-I or apoA-I_{Milano}. Cholesterol-free discoidal HDL has been shown to be an effective cholesterol acceptor without the complications of cholesterol influx or the presence of lipid-free apoprotein. We therefore generated purified discoidal rHDL containing wild-type and Milano apoA-I and tested them in our macrophage system. Cholesterol enrichment of J774 macrophages has been shown to increase ABCA1 expression.³⁸ There was no difference in efflux potential between rHDL and rHDL_{Milano} (Figure 2). These findings agree with studies by Wang et al⁵⁰ who looked at efflux to rHDL and rHDL_{Milano} in fibroblasts. Our data also agrees, in part, with a study by Favari et al where they reported no increase in ABCA1 efflux to rHDL_{Milano} disks but did see a small difference in unstimulated cells.³⁷ In the present study we saw no difference in unstimulated cells (Figure 2).

A wide variety of extracellular cholesterol acceptors are available for use in cholesterol efflux studies. Lipid-free apoprotein and reconstituted discoidal HDL promote unidirectional flux of cholesterol. Whole serum is a more physiological, more complex acceptor. The milieu of whole serum contains many factors that can influence cholesterol flux such as CE and phospholipid transfer proteins, and a spectrum of apoproteins and lipoproteins.¹² We used Fu5AH hepatoma cells to evaluate the efflux potential of serum isolated from apoA-I^{-/-} mice expressing human apoA-I or apoA-I_{Milano} and found that serum from mice expressing apoA-I_{Milano} was less efficient in promoting cholesterol efflux (Figure 3A). Franceschini et al⁵¹ demonstrated that whole serum collected from transgenic mice expressing human apoA-I was more efficient in promoting efflux from Fu5AH cells than serum from transgenic mice expressing apoA-I_{Milano}. However the serum HDL-cholesterol values in these studies was significantly lower in the apoA-I_{Milano} transgenic mice. When the efflux values were corrected for serum apoA-I concentrations, efflux promoted by serum from apoA-I_{Milano} mice showed a small but statistically significant increase over efflux to serum from apoA-I_{wt} mice. If we also normalized our data to protein, we see a small difference (data not shown). Because of the small differences seen with whole serum we extended these studies by directly comparing HDL isolated from animals expressing apoA-I_{wt} and apoA-I_{Milano}. In the present study, Table 2 indicates that the serum HDL-cholesterol concentration is about half in the apoA-I_{Milano} adenovirus-infected mice compared with the human apoA-I adenovirus-infected mice. This is likely to impact greatly on SRBI-mediated efflux, the major efflux pathway in Fu5AH cells.⁵² Because SRBI efflux is responsive to phospholipid-containing acceptors,⁵³ it should be noted that the serum phospholipid concentrations in

the apoA-I_{Milano} mice, while not significantly different, have the trend to less serum phospholipid. Thus, to make a valid comparison of the relative efficiencies of cholesterol efflux mediated by HDL_{wt} or HDL_{Milano}, we isolated the HDL from the mice by ultracentrifugation and added the HDL at equal protein or phospholipid concentrations (Figure 3B and 3C). Because HDL_{Milano} had a greater protein to phospholipid ratio (Table 2), we predicted that the HDL_{Milano} would show greater SRBI-mediated efflux. However, when added at the same protein concentration, the HDL_{Milano} exhibited equal SRBI-mediated efflux compared with HDL_{wt} (Figure 3B) even though there was more phospholipid present in the incubation containing the HDL_{Milano}. This observation would seem to be contradictory to our current understanding of the nature of SRBI acceptors.⁴⁴ We therefore added the HDL at the same phospholipid concentration (Figure 3C) and found that HDL_{Milano} was less efficient in promoting SRBI-mediated efflux. Therefore, although HDL_{Milano} contains more phospholipid compared with the HDL_{wt}, the phospholipid present on the HDL_{Milano} is less efficient in promoting SRBI-mediated efflux. Analysis of the phospholipid composition of the HDL indicated that the HDL_{Milano} is enriched in SM and poor in PC compared with HDL_{wt} (Table 2). Yancey et al have shown that depleting HDL in PC resulted in reduced SRBI-mediated efflux whereas enriching in PC increased SRBI-mediated efflux.⁴⁴ The data in our studies are consistent with these findings.

Deficient cellular lipid efflux via ABCA1 has been implicated in promoting atherosclerosis. It is possible that apoA-I_{Milano} may offer atheroprotection not through cholesterol efflux but rather increased phospholipid efflux. To address this we measured ABCA1-mediated phospholipid efflux to wild-type and Milano apoA-I and found no significant difference.

Macrophage foam cells present in atherosclerotic plaques are not only enriched in CE but also have a large amount of FC. This FC is cytotoxic^{38,54} and may play an integral role in plaque progression.⁵⁵ We have previously shown that excess cytotoxic cholesterol is removed from macrophage foam cells by apoA-I via ABCA1.³⁸ ApoA-I_{Milano} may offer atheroprotection through increased removal of cytotoxic cholesterol. However, we found that apoA-I_{Milano} and apoA-I_{wt} were equally effective at removing accumulating cholesterol (Figure 4).

Human carriers of the Milano variant of apoA-I offer an interesting paradox: significantly lower HDL and no apparent increase in coronary artery disease.⁴ The mechanism by which apoA-I_{Milano} prevents increased atherosclerosis remains unclear. One theory is that apoA-I_{Milano} creates a more efficient HDL. The present studies made direct comparisons of the ability of lipid-free and lipid-associated apoA-I_{Milano} and apoA-I_{wt} to mobilize lipids from cells. We found that apoA-I_{Milano} did not provide any increase in function compared with apoA-I_{wt} and conclude that the atheroprotection offered by apoA-I_{Milano} is not attributable to increased cellular lipid mobilization but rather other cardiovascular benefits.

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Disclosures

None.

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