Effect of the Apolipoprotein A-I and Surface Lipid Composition of Reconstituted Discoidal HDL on Cholesterol Efflux from Cultured Fibroblasts†

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ABSTRACT: Five series of reconstituted discoidal HDL (LpA-I) particles have been prepared, and their constituents, apolipoprotein A-I (apoA-I), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), unesterified cholesterol (UC), phosphatidylinositol (PI), or sphingomyelin (SM), have been systematically varied to elucidate the relationship between HDL composition and cholesterol efflux from non-cholesterol-loaded human skin fibroblasts. The physical properties, such as hydrodynamic diameters, α-helix contents, and surface potentials, of these LpA-I have been measured and related to the ability of the LpA-I to accept cellular cholesterol. The results show that for LpA-I particles containing 2, 3, or 4 apoA-I per particle, Lp4A-I are the best acceptors of cellular cholesterol, followed by Lp3A-I and then Lp2A-I particles. Discoidal Lp2A-I with variations in POPC content, from 121 to 266 mol/particle, show no difference in their abilities to promote cholesterol efflux. Similarly, inclusion of 7 and 15 mol of free cholesterol to Lp2A-I also does not affect their ability to accept cellular cholesterol. However, increasing the content of either PI or SM, up to 20 mol/particle, is associated with significantly increased abilities of the LpA-I to promote cholesterol efflux. The efflux of cellular cholesterol to discoidal LpA-I particles is independent of specific changes in apoA-I conformation and charge, but appears to be positively related to major changes in the size of the lipoprotein particle. The study suggests that in contrast to interlipoprotein cholesterol transfers, the efflux of cholesterol from cultured fibroblasts is less sensitive to factors that affect the frequency of molecular collisions and more dependent on the ability of an HDL particle to adsorb and retain cholesterol molecules. Since SM and PI appear to modulate this adsorption/desorption of cholesterol to HDL, variations in the concentration of these lipids within HDL would be expected to affect plasma cholesterol homeostasis.

The removal of cholesterol from extrahepatic cells and further transport to the liver for catabolism, a process known as "reverse cholesterol transport" (RCT) (Glomset, 1968), has been suggested to be an important mechanism for the anti-atherogenetic function of high-density lipoprotein (HDL).[1] The initiation of this process, the desorption of cholesterol from the plasma membrane of extrahepatic cells to HDL particles in extracellular compartments, has been reviewed as a limiting step in RCT (Rothblat *et al.*, 1992; Johnson *et al.*, 1991). It has been suggested that the transfer of cholesterol from cell plasma membrane to HDL operates mainly through an aqueous diffusion mechanism (Phillips *et al.*, 1987; Johnson *et al.*, 1991), which does not require the binding of HDL to specific membrane sites (Karlin *et al.*, 1987; Slotte *et al.*, 1987; Johnson *et al.*, 1988; Mendel *et al.*, 1988; Aviram *et al.*, 1989). However, there is also another mechanism which is regulated and dependent on the translocation of cholesterol from intracellular pools to the plasma membrane (Brinton *et al.*, 1986; Slotte *et al.*, 1987; Aviram *et al.*, 1989; Oram *et al.*, 1991).

The great heterogeneity of HDL population in interstitial fluid and lymph as well as in plasma makes it possible that different species of HDL may have the distinct capacity to receive cellular cholesterol. An apoA-I containing preβ migrating HDL has been shown to be the first acceptor for cell-derived cholesterol (Castro & Fielding, 1988), from where the cell-derived cholesterol proceeds further to preβ_2 and then to preβ_3 and to α HDL for esterification (Francone *et al.*, 1989; Francone & Fielding, 1990; Huang *et al.*, 1993). Recently, γ-LpE, a subspecies of HDL containing apoE only, has been reported to be another preferential acceptor for cell-derived cholesterol (Huang *et al.*, 1994).

Using reconstituted discoidal LpA-I particles, an earlier study from this laboratory has suggested that the apoA-I/phospholipid related size of LpA-I was an important contributor to the ability of LpA-I to accept cell-derived cholesterol (Agnani & Marcel, 1993). The larger reconstituted discoidal Lp4A-I have a higher capacity than the smaller Lp3A-I which have a higher capacity than Lp2A-I to accept cell-derived cholesterol from non-cholesterol-loaded human skin fibroblasts. Similar results were also obtained

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[1] Abbreviations: apoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; LpA-I, lipoprotein containing apoA-I; Lp2A-I, LpA-I containing 2 apoA-I per particle; Lp3A-I, LpA-I containing 3 apoA-I per particle; Lp4A-I, LpA-I containing 4 apoA-I per particle; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PC, phosphatidylcholine; UC, unesterified cholesterol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NDGGE, nondenaturing gradient gel electrophoresis.

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by others in different cell lines and using longer incubation times (Jonas *et al.*, 1994; Davidson *et al.*, 1995a). However, in these early studies, reconstituted particles were used with more than one component being varied at a time, preventing a definitive analysis of the effect of size and content of individual constituents on efflux to lipoproteins. Here we have conducted a detailed time- and concentration-dependent study of efflux to discoidal lipoproteins with systematic variation of each component. We prepared five series of reconstituted lipoproteins with varying components, such as apoA-I, PC, PI, SM, and UC, that varied within the range found in native HDL particles. The physico-chemical properties of these discoidal particles have been characterized, and their avidity for binding cellular cholesterol has been analyzed.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), bovine brain sphingomyelin (SM), and bovine liver phosphatidylinositol (PI) were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Essentially fatty-acid free bovine serum albumin and cholesterol (99% grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Guanidine hydrochloride was from Bethesda Research Laboratories (Bethesda, MD). L-α-[Myoinositol-2-^{3}H(N)]-PI (^{3}H-PI), [*cholinemethyl*-^{14}C]-SM (^{14}C-SM), and [1,2-^{3}H]cholesterol were from Du-Pont Canada Inc. (Mississauga, Canada) with specific activities of 11 Ci/mmol, 50 mCi/mol, and 52 Ci/mol, respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, and penicillin−streptomycin used for cell culture were purchased from GIBCO (Grand Island, NY). All other reagents were analytical grade.

Methods

Isolation of HDL and Purification of Apolipoprotein A-I. HDL was isolated from fresh plasma obtained from normolipidemic blood donors by a standard sequential ultracentrifugation method (Schumaker & Puppione, 1986). The delipidation of HDL and purification of apoA-I were conducted as previously described (Brewer *et al.*, 1986).

Preparation of Reconstituted Discoidal LpA-I. Two methods have been used to prepare reconstituted discoidal LpA-I particles, and in all preparations, POPC was used as a single or major phospholipid component. Reconstituted discoidal LpA-I containing 2, 3, or 4 apoA-I, respectively, were prepared by the sodium cholate dialysis method described by Jonas and colleagues (Jonas, 1986). The molar ratio of POPC/apoA-I/sodium cholate of the initial mixture was 120/1/120. All other discoidal reconstituted LpA-I containing two molecules of apoA-I per particle, and varying levels of POPC and other lipids, were prepared by the cholate dispersion/Bio-bead removal technique previously described by Sparks *et al.* (1992a). For the preparation of discoidal Lp2A-I with varying free cholesterol, PI, or SM contents, these components were mixed and dispersed together with POPC at the beginning of the preparation. Unless indicated specifically, a reconstitution buffer containing 10 mM Tris-HCl, 1 mM NaN_3, 150 mM NaCl, and 1 mM EDTA (pH 8.0) was used in all the preparations.

Characterization of Reconstituted HDL Particles. Nondenaturing gradient polyacrylamide gel electrophoresis (NDGGE) on 8−25% gels from Pharmacia was used to verify the homogeneity of reconstituted LpA-I, and particle diameters were calculated from a quadratic equation fit to the hydrodynamic diameters and the migration distances of five standard proteins: thyroglobulin (17.0 nm), apoferritin (12.2 nm), catalase (10.4 nm), lactate dehydrogenase (8.16 nm), and bovine serum albumin (7.1 nm) (Nichols *et al.*, 1986). The electrophoretic mobilities and surface potentials of LpA-I were measured on 0.6% agarose gels (Beckman, Paragon lipo kit) and calculated as previously described (Sparks & Phillips, 1992). The secondary structure of LpA-I represented by the α-helix content of apoA-I was monitored by circular dichroism (CD) spectroscopy at 222 nm (Sparks *et al.*, 1992b). The number of apoA-I molecules of each reconstituted LpA-I particle was estimated by the DMS cross-linking method (Swaney, 1986). Protein, cholesterol, and phospholipid concentrations were determined by the Lowry method and commercial enzymatic test kits (Boehringer Mannheim GmBH, Mannheim, Germany), respectively. The PI and SM contents of LpA-I were determined in representative preparations by inclusion of either ^{3}H-PI of ^{14}C-SM (purity confirmed by K6 silica gel thin-layer chromatography).

Cell Culture. Normal human skin fibroblasts were purchased from Clonetics Inc. at the ninth passage. The cells were maintained at 5% CO_2, 37 °C in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.4 mM L-glutamine. Cells used in this study were between the 16th and 22th passages.

Cholesterol Efflux from Human Skin Fibroblasts. The procedure for the cellular cholesterol efflux experiments has been described in detail earlier (Zhao & Marcel, 1996). Four days prior to the experiment, cells were seeded in 12 well cell culture plates at a density of 5.5×10^4 cells/well, and grown for 2 days to 70% confluence. The cell monolayers were then washed twice (5 min incubation each) with phosphate-buffered saline (PBS), pH 7.4, containing 0.2% essentially fatty-acid free bovine serum albumin (BSA). Following two other washes with PBS alone, the cells were incubated for 48 h with a labeling medium containing 20 μCi/well (or 50 μCi/well, depending on individual experiments) [1,2-^{3}H]cholesterol, 5% FBS, and other supplements.

For efflux experiments, the [^{3}H]cholesterol prelabeled cells were washed twice with DMEM containing 0.2% BSA and twice with DMEM alone. The washed cells were then incubated at 37 °C with DMEM containing the indicated LpA-I particles. At the indicated time intervals, an aliquot of medium (50 μL) was taken and mixed with 100 μL of PBS containing 2 mg/mL BSA. The mixtures were centrifuged at 10 000 rpm for 5 min to remove any detached cells, and 100 μL of the supernatant was used for radioactivity determination. At the end of the incubation, the remaining medium was removed from wells, and cell monolayers were washed twice with PBS containing 2 mg/mL BSA and twice with PBS alone. The cells were lysed with 0.5 mL of 0.1 N NaOH overnight, and the protein concentration was determined by Lowry assay. The specific activity of cell cholesterol was $(1.0-1.3) \times 10^6$ cpm/μg of cholesterol, and more than 90% of the radioactivity was in the UC fraction as reported by others (Castro & Fielding, 1988). Efflux was

Table 1: Characterization of Reconstituted Discoidal LpA-I with Varying Compositions[a]

LpA-I	composition molar ratios		mol/LpA-I				
	initial[b]	final[c]	apoA-I[d]	T-PL[e]	size[f] (nm)	α-helix[g] (%)	surf pot[h] (−mV)
			Variation in ApoA-I (POPC:ApoA-I)				
Lp2A-I	120:1	78:1	2	155	9.1	72	6.5
Lp3A-I	120:1	111:1	3	332	12.7	58	5.5
Lp4A-I	120:1	131:1	4	522	17.1	56	4.9
			Variation in Phosphatidylcholine (POPC:ApoA-I)				
D1	60:1	61:1	2	122	9.2	69	7.5
D2	100:1	100:1	2	199	9.5	75	6.6
D3	140:1	133:1	2	267	9.7	77	6.2
			Variation in Free Cholesterol (POPC:ApoA-I:UC)				
C1	80:1:0	77:1:0	2	144	9.3	74	6.9
C2	80:1:4	77:1:3.4	2	154	9.4	66	6.7
C3	80:1:8	76:1:7.7	2	152	9.7	65	6.8
			Variation in Sphingomyelin (POPC:ApoA-I:UC:SM)				
SM1	80:1:4:5	72:1:3.6:4.4	2	153	9.5	74	7
SM2	80:1:4:10	67:1:3.4:8.2	2	150	9.9	70	6.6
SM3	80:1:4:20	58:1:3.5:15.1	2	146	10.1	72	6.6
			Variation in Phosphatidylinositol (POPC:ApoA-I:UC:PI)				
PI1	80:1:4:4	72:1:3.6:2.8	2	150	9.1	67	9.6
PI2	80:1:4:8	67:1:3.4:5.6	2	145	8.9	68	11.6

[a] Results are the averages of at least three preparations. [b] Composition molar ratios of initial mixture for the preparation of reconstituted LpA-I. [c] Composition molar ratios of reconstituted LpA-I after reisolation. [d] Determined by protein cross-linking with DMS and subsequent SDS/PAGE. [e] Total phospholipid content of each reconstituted LpA-I particle, intersample variation <10%. [f] Hydrodynamic diameter from nondenaturing gradient gel electrophoresis, ±0.5 nm (SD). [g] Determined from molar ellipticities at 222 nm in CD spectra, ±4% (SD). [h] Calculated from the electrophoretic migration of LpA-I on agarose gel, ±0.2 mV (SD).

expressed in cpm of [^{3}H]cholesterol in the medium per microgram of cellular protein.

RESULTS

Characterization of the Homogeneity, Composition, and Size of Discoidal LpA-I Particles. The discoidal LpA-I particles prepared at 4 °C from an initial POPC/apoA-I mixture of 120/1 (molar ratio) show three subspecies which were reisolated by size-exclusion chromatography (Jonas *et al.*, 1989; Nichols *et al.*, 1987). The homogeneity of these discoidal LpA-I is confirmed by NDGGE. As summarized in Table 1, the isolated LpA-I particles contain 2, 3, and 4 apoA-I per particle, respectively, as determined by DMS cross-linking. Their molar ratios of POPC/apoA-I are 78/1, 111/1, and 130/1, and hydrodynamic diameters are 9.1, 12.7, and 17.1 nm, respectively. It is apparent that both apoA-I and POPC molecules contribute to the size difference among these particles since LpA-I particles with more apoA-I have higher POPC levels.

In contrast to the heterogeneous population of LpA-I particles generated at 4 °C, dispersions of POPC and apoA-I with sodium cholate at 37 °C produce homogeneous particles when the POPC/apoA-I molar ratios of initial mixtures are kept within the optimal range from 40/1 to 140/1. Each of these particles contains two apoA-I, and those prepared from the initial molar ratios of 60/1, 100/1, and 140/1 (POPC/apoA-I) are named D1, D2, and D3. The POPC/apoA-I final molar ratios of these LpA-I are 61/1, 100/1, and 133/1, respectively, demonstrating a greater than 95% incorporation of POPC into LpA-I. Varying the phospholipid content of Lp2A-I is associated with small changes in particle size, with the hydrated diameters increased by approximately 5% following the increase of POPC content from 61/1 to 100/1 and to 133/1 (9.2, 9.5, and 9.7 nm, respectively, for D1, D2, and D3) (Table 1). When POPC/apoA-I reaches a molar ratio above 140/1, a larger subspecies of LpA-I appears migrating to a position on NDGGE corresponding to Lp3A-I in agreement with a previous report (Davidson *et al.*, 1995a). It is therefore apparent that the apoA-I number is the key component for the total level of phospholipid incorporated in reconstituted LpA-I and their resulting sizes. Addition of 4 or 8 mol of free cholesterol does not affect either the homogeneity of Lp2A-I or the amount of POPC incorporated per particle. The presence of UC at the above-mentioned ratios also slightly increases the hydrated diameter of these Lp2A-I from 9.3 to 9.7 nm (Table 1), in agreement with a previous report (Sparks *et al.*, 1993).

In this study, we show that up to 30 mol of SM (15 mol/apoA-I) can be incorporated into reconstituted discoidal Lp2A-I particles without affecting their homogeneity. When the starting SM to POPC ratios are progressively increased while maintaining a constant POPC concentration, the resulting LpA-I contains increasing amounts of SM but reduced POPC (Table 1). This suggests that SM has, not only as reported earlier, a higher affinity for cholesterol (Lund-Katz *et al.*, 1988; Yeagle & Young, 1986; Fugler *et al.*, 1985), but may also compete with POPC to bind to apoA-I (Swaney, 1983). The increased total phospholipid content per particle with increasing SM is accompanied by a slight increment in particle size, which increases from 9.4 to 10.1 nm as SM increases from 0 to 15.1 mol (Table 1). Discoidal Lp2A-I particles containing PI have also been prepared and characterized (Table 1). The yield of incorporation of PI into LpA-I is about 70%, and the increase in PI content to 2.8 and 5.6 mol per apoA-I results in a small nonsignificant decrease in particle size (9.4 to 9.1 and 8.9 nm).

Effect of Composition on the Physical Properties of Discoidal LpA-I. Variation in the composition of discoidal LpA-I is associated with changes in apoA-I conformation which are accompanied by variation in several representative physical parameters, such as the α-helix content, the elec-

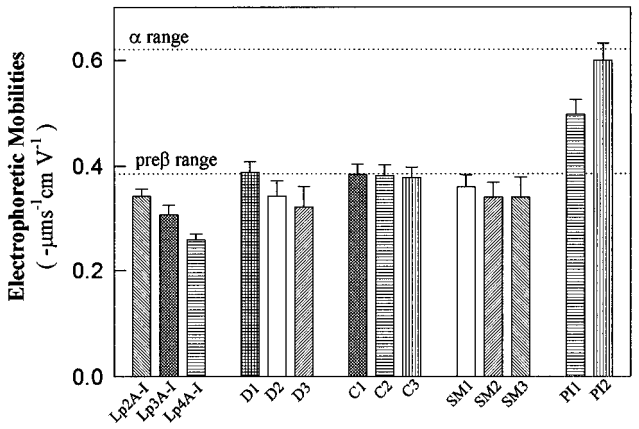

FIGURE 1: Electrophoretic mobilities of reconstituted discoidal LpA-I. Reconstituted discoidal LpA-I particles were electrophoresed on 0.6% agarose gels at 100 V for 30 min. The electrophoretic mobilities were calculated from the migration distance of these particles as previously described (Sparks *et al.*, 1992b). The standard preβ and α migration positions were calculated from the migration distance of the corresponding lipoproteins from fresh plasma electrophoresed in the same conditions. The data are the average of at least four determinations.

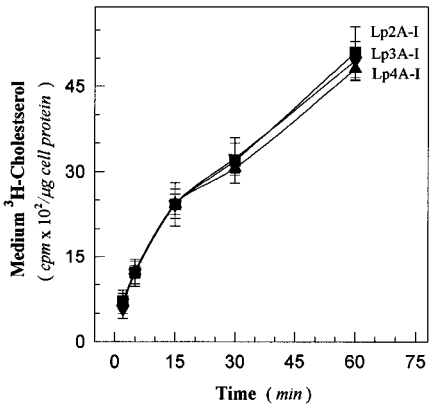

FIGURE 2: Cholesterol efflux to LpA-I with varying apoA-I number, added to the medium at the same protein concentration. Fibroblasts were seeded in 12 well plates at a density of 5.5×10^4 cells/well and grown at 37 °C in the presence of 10% FBS for 48 h to reach 80% confluence. After washing, the cell monolayers were then labeled by incubation in a medium containing [^{3}H]cholesterol (50 μCi/mL) and 5% FBS for 48 h. For the efflux study, the cells were washed and then incubated with DMEM containing Lp2A-I, Lp3A-I, or Lp4A-I at a concentration of 45 μg of apoA-I protein/mL. Aliquots of medium were taken at the indicated times between 2 and 60 min for the determination of radioactivity. Efflux is expressed as medium cpm/μg of cell protein (mean ± SD, $n = 4$ replicate dishes).

trophoretic migration, and the surface potential. Since the predominant phospholipid components of LpA-I, such as PC and SM, are uncharged, the change in the electrophoretic migration or surface potential of LpA-I accompanying a variation in the content of these lipids will represent an alteration in the secondary and/or tertiary structure of apoA-I (Sparks *et al.*, 1992a,b). All the reconstituted discoidal LpA-I particles prepared with the exception of those containing PI exhibit surface potentials corresponding to the preβ migration range on an agarose gel (Table 1, Figure 1). The surface potentials are negatively related to the apoA-I number, the POPC content, and the SM content of these particles. At the ratio studied, the presence of free cholesterol does not affect significantly the particles' surface charge. As expected, a progressive increase in the surface potential of Lp2A-I containing PI is observed reflecting the negative charge of this phospholipid (Figure 1 and Table 1). The presence of 5.6 mol of PI per particle shifts the migration of the Lp2A-I particle from a position intermediate between the preβ and α position to an α position (Figure 1).

Although apoA-I, POPC, and SM all appear to reduce the surface potentials of reconstituted discoidal LpA-I, they affect the structure of LpA-I differently. Circular dichroic measurements show that apoA-I on the Lp2A-I particles has an average α-helix content of 72%, while those on Lp3A-I and Lp4A-I are 58% and 56%, respectively. Also, the increase in POPC content of Lp2A-I is accompanied by increases in α-helix content in contrast to the decreased surface potentials observed for the same particles. Addition of UC reduces the α-helix contents of the lipid-bound apoA-I (Table 1). A slight increase in α-helicity but no change on surface potential is observed (Table 1). In Lp2A-I particles containing a constant level of UC, addition of 4.4 mol of SM significantly increases the α-helix content from 66% to 74% (intersample SD < 4%); however, further increase in SM causes a minor decrease in the α-helix content of LpA-I. The presence of PI does not affect the α-helix content of Lp2A-I (Table 1).

Effect of LpA-I Composition on Cellular Cholesterol Efflux. A previous study in this laboratory has shown that in a 5 min incubation with fibroblasts, Lp4A-I could promote more cholesterol efflux than Lp3A-I or Lp2A-I (Agnani & Marcel, 1993). Here, we have investigated in detail the time- and concentration-dependent efflux of cholesterol from fibroblasts to these defined and homogeneous discoidal particles. As showed in Figure 2, when Lp2A-I, Lp3A-I, or Lp4A-I at the same protein concentration (45 μg/mL) are incubated with [^{3}H]cholesterol-labeled, non-cholesterol-loaded fibroblasts, each has a similar ability to promote cholesterol efflux during an incubation period of 2−60 min. However, since these lipoproteins contain different numbers of apoA-I per particle, a more meaningful comparison of these particles requires that their protein concentration in the incubation media be adjusted to provide the same particle density for each type of LpA-I. As shown in Figure 3, when Lp2A-I, Lp3A-I, or Lp4A-I are added to the incubation medium of fibroblasts at the same particle density, the efflux to Lp4A-I is significantly higher than that to Lp3A-I and to Lp2A-I. The difference in cellular cholesterol efflux is also statistically significant between Lp2A-I and Lp3A-I ($p < 0.05$). Concentration-dependent cholesterol efflux to Lp2A-I or Lp4A-I has also been studied. Again, at the same protein concentration of LpA-I in the medium, similar efflux rates to Lp2A-I and Lp4A-I are observed (Figure 4A). However, at the same particle density in the medium, cholesterol efflux to Lp4A-I is again significantly higher than that to Lp2A-I (Figure 4B). As observed with native HDL (Johnson *et al.*, 1986, 1990), the efflux promoted by these particles does not saturate up to 200 μg of protein/mL.

Since LpA-I particles with different number of apoA-I also vary in POPC content, we have studied cellular cholesterol efflux to LpA-I with constant 2 apoA-I per particle but varying POPC levels to determine whether the POPC/apoA-I ratio in itself contributes to the ability of LpA-I to receive cellular cholesterol. As shown in Figure 5A, varying the phospholipid content of Lp2A-I (D1, D2, and D3) does not significantly alter their ability to promote cellular cholesterol efflux when they are added to the medium at the same

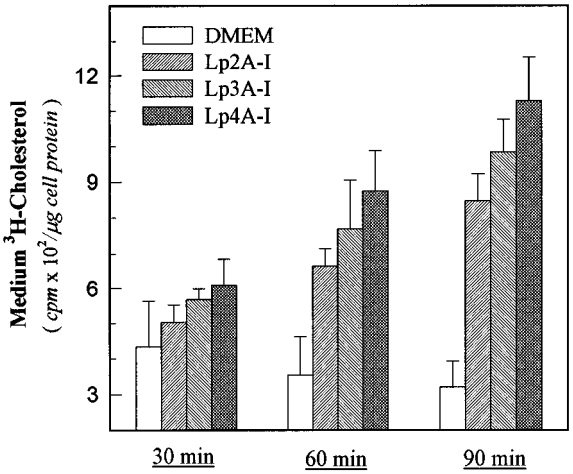

FIGURE 3: Cholesterol efflux to LpA-I with varying apoA-I number, added to the medium at the same particle concentration. Fibroblasts prelabeled with [^{3}H]cholesterol (20 μCi/mL) were incubated with medium containing 22.5, 33.75, or 45.0 μg of protein/mL of Lp2A-I, Lp3A-I, or Lp4A-I, respectively, under the same conditions as for Figure 2. Medium aliquots were taken at 30, 60, and 90 min of incubation for radioactivity counting. Efflux is expressed as medium cpm/μg of cell protein ($n = 4$).

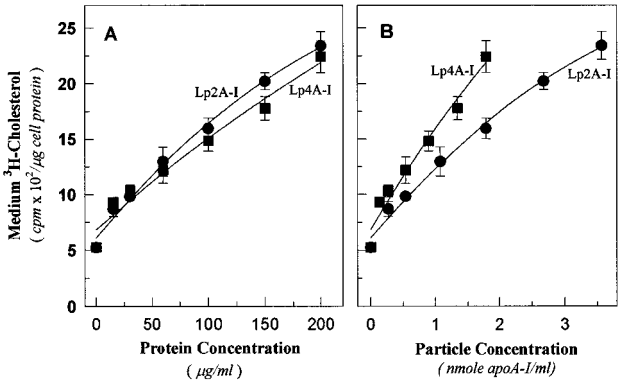

FIGURE 4: Effect of lipoprotein concentration on cholesterol efflux to Lp2A-I and Lp4A-I. Fibroblasts prelabeled with [^{3}H]cholesterol (20 μCi/mL) were incubated in medium containing either Lp2A-I or Lp4A-I at concentrations of 15, 30, 60, 100, 150, and 200 μg/mL apoA-I protein, respectively. Medium samples were taken out after 90 min of incubation for radioactivity counting and measurement of efflux ($n = 4$). Panel A represents the efflux (radioactivity of medium) plotted as a function of the protein concentrations of these different LpA-I particles. Panel B represents the efflux plotted as a function of the different particle concentrations in the medium.

concentration of 45 μg of protein/mL. Therefore, taken together with previous experiments, this result suggests that apoA-I number, which is the major determinant of both the level of phospholipid incorporation and the size of LpA-I, is the most important component dictating the ability of LpA-I to promote cellular cholesterol efflux to discoidal LpA-I.

The incorporation of cholesterol into LpA-I is limited by the reconstitution technique to the maximal level of 15.4 mol per particle. As showed in Figure 5B, addition of 6.8 or 15.4 mol of UC into Lp2A-I particles does not affect their ability to promote cellular cholesterol efflux.

The time-dependent efflux of UC from non-cholesterol-loaded human skin fibroblasts to reconstituted discoidal LpA-I exhibits a biphasic pattern (Figures 2, 5A,B, and 6A). An early rapid efflux phase is observed during the first 15 min of incubation, followed by a plateau up to about 30 min, and a second linear phase starts from about 30 to 60 min.

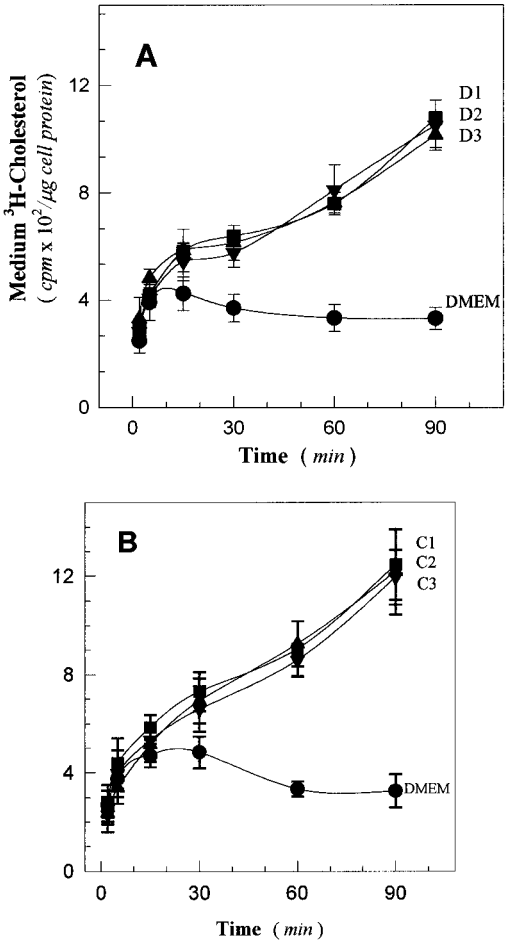

FIGURE 5: Cholesterol efflux to Lp2A-I with varying ratios of POPC (panel A) or UC (panel B). Fibroblasts prelabeled with [^{3}H]-cholesterol (20 μCi/mL) were incubated with discoidal Lp2A-I particles (45 μg/mL) containing either (panel A) varying ratios of POPC/apoA-I identified as D1, D2, and D3, respectively (described in Table 1), or (panel B) varying ratios of UC identified as C1, C2, and C3, respectively (described in Table 1). Aliquots of medium were taken between 2 and 90 min of incubation for the measurement of efflux as described for Figure 2 ($n = 4$).

This biphasic efflux may represent the transition of the cholesterol efflux process from the pool represented by only cell plasma membrane to that also including mobilization of cholesterol from intracellular pools (Mahlberg & Rothblat, 1992).

In contrast to the efflux to reconstituted discoidal Lp2A-I containing varying POPC or UC content, the addition of only 2.8 mol of PI per apoA-I (PI1) does translate into a significant increase in cholesterol efflux (Figure 6A). This enhancement is rapid, occurs in the first phase of cholesterol efflux, and remains constant during the lag period and the second phase. It may represent a specific ability of this PI-containing particle to release UC from the plasma membrane. Incorporation of more PI into Lp2A-I has no further effect on efflux (Figure 6A).

The time-dependent efflux to SM-containing Lp2A-I is increased significantly with 4.4 and 8.2 mol of SM per apoA-I, and appears to plateau at the same level (Figure 6B). In contrast to the efflux to PI-containing Lp2A-I, the increase in efflux to SM-containing Lp2A-I is greater in the second phase than that in the initial phase of efflux.

In summary, there are apparent relationships between cholesterol efflux and the physicochemical parameters of

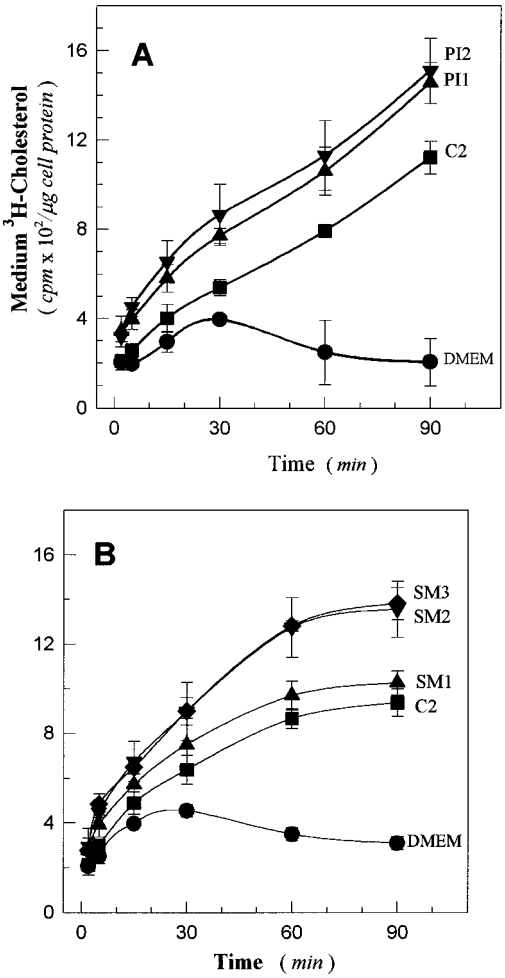

FIGURE 6: Cholesterol efflux to Lp2A-I with varying levels of PI (panel A) or SM (panel B). Fibroblasts prelabeled with [^{3}H]-cholesterol (20 μCi/mL) were incubated with discoidal Lp2A-I particles (45 μg/mL) containing varying levels of PI (panel A) identified as PI1 and PI2, respectively (described in Table 1), or containing varying ratios of SM (panel B) identified as SM1, SM2, and SM3, respectively (described in Table 1). Aliquots were taken between 2 and 90 min of incubation, and efflux was measured as described for Figure 2 ($n = 4$).

LpA-I containing 2−4 molecules of apoA-I, surface potentials, α-helix contents, and hydrated diameters. In contrast, no such relationships are apparent in Lp2A-I with varying POPC and UC levels. Therefore, the efflux of cellular cholesterol to discoidal LpA-I particles with varying apoA-I is independent of their POPC or cholesterol contents, but appears associated with both the number of apoA-I molecules per particle, the accompanying change in particle size, and other corresponding physicochemical parameters.

DISCUSSION

Discoidal HDL particles are thought to be a subspecies of HDL that are present in interstitial fluid/peripheral lymph as well as in the plasma of humans (Reichl *et al.*, 1985, 1990) and animals (Sloop *et al.*, 1983a,b; Dory *et al.*, 1985; Forte *et al.*, 1983; Lefevre *et al.*, 1988). Increases in plasma and interstitial fluid discoidal HDL appear to accompany several alterations of cholesterol metabolism, such as in LCAT deficiency (Jonas *et al.*, 1993; Ohta *et al.*, 1994), in the hypercholesterolemic and hypothyroid state (Sloop *et al.*, 1983a), and in normal animals fed a high-cholesterol chow (Dory *et al.*, 1985). Like other HDL, discoidal HDL represent a group of heterogeneous particles differing in composition and size. Larger discoidal LpA-I particles containing several apoA-I molecules have been identified as the nascent HDL secreted by the liver (McCall *et al.*, 1988), in the incubation of lipid-free apoA-I with nontransfected CHO-C19 cells (Forte *et al.*, 1993), and in interstitial fluid/peripheral lymph (Reichl *et al.*, 1990; Sloop *et al.*, 1987; Lefevre *et al.*, 1988). Their relatively high concentration in interstitial fluid/peripheral lymph, as compared to plasma (Sloop *et al.*, 1983a,b; Forte *et al.*, 1983; Reichl *et al.*, 1985; Dory *et al.*, 1985), suggests that these particles may have a greater chance to interact with extrahepatic cells, and could play an important function in the efflux of cellular cholesterol, thus explaining their cholesterol enrichment in certain conditions as reviewed above.

In this investigation, we have characterized the effect of variations in discoidal LpA-I composition on the ability of these lipoproteins to receive cholesterol from fibroblasts. These studies are designed to measure and compare the rates of cholesterol efflux after short-term incubations (<90 min). The efflux protocol of these studies attempts to elucidate initial rates of cholesterol transfer between cells and intact LpA-I particles. It should be noted that as with all series of LpA-I, no particle fusion or major structural rearrangement of the LpA-I particles was evident after the efflux incubation. In the more common long-term (6−48 h) transfer studies of others (Davidson *et al.*, 1995a; Jonas *et al.*, 1994; Oram *et al.*, 1991), it is possible that other factors that may be secreted or generated by the cultured cells during the long incubations alter lipoprotein composition and structure and subsequently modify lipoprotein and lipid equilibrations.

In short-term incubation studies with discoidal LpA-I, we have routinely observed a unique two-phase pattern of cholesterol efflux, with the exception of efflux to SM-containing Lp2A-I. This biphasic profile appears to be a characteristic of human skin fibroblasts and is not evident in other studies with smooth muscle cells or endothelial cells.[2] The rapid first phase can be best observed with an incubation procedure that includes gentle shaking of the culture dishes (Zhao & Marcel, 1996). With the exception of LpA-I varying in apoA-I and in PI, minimal differences were seen for the initial rates of efflux to most discoidal LpA-I, suggesting that it may depend mostly on the ability of the cell plasma membrane to release cholesterol into medium. The second phase, which is linear and starts between 30 and 60 min, appears to represent mostly the collisional properties and/or the ability of the acceptor lipoprotein to bind and retain cholesterol molecules. It is mainly during this second linear phase that we could demonstrate the most significant differences in cholesterol adsorption capacity between the different acceptor lipoproteins. The enhanced ability of specific LpA-I particles to accept cholesterol molecules in this study could reflect modified collisional properties of the LpA-I or the enhanced ability of the particles to bind and retain cholesterol.

This investigation has elucidated the abilities of discoidal Lp2A-I, Lp3A-I, and Lp4A-I to promote cholesterol efflux and confirmed our earlier observations (Agnani & Marcel, 1993). When efflux rates are expressed on a per particle basis, it is evident that the larger Lp4A-I have the highest

[2] Marcel *et al.*, unpublished observation.

ability to promote cholesterol efflux from non-cholesterol-loaded fibroblasts. Lp2A-I are the poorest acceptors of cellular cholesterol, and Lp3A-I are somewhat intermediate between Lp4A-I and Lp2A-I. This conclusion is consistent with the data from an earlier report showing that, on a per particle basis, larger reconstituted discoidal Lp3A-I (10.8 nm) and Lp2A-I (9.6 nm) have a higher ability to promote cholesterol efflux from adipocytes than do small Lp2A-I (7.8 nm). However, the authors of this report concluded that smaller particles had the higher ability to accept cholesterol, based on efflux rates that were corrected to a constant phospholipid concentration (Jonas *et al.*, 1994). This differential interpretation reflects a common problem encountered when comparing different cholesterol efflux protocols. Indeed, our results also appear to be inconsistent with another recent report (Davidson *et al.*, 1995a) which suggests that Lp2A-I with high POPC content and larger hydrodynamic diameters promote a greater cellular cholesterol efflux than smaller Lp2A-I particles. While in this study comparisons were made at the same particle concentration, the primary difference is that a longer efflux period (1−6 h) was used rather than our short period of efflux (2−90 min). Thus, short-term studies suggest that variation in the phospholipid content of LpA-I does not affect the ability of the lipoprotein to accept cholesterol. This observation may indicate that variation in the amount of phospholipid in a discoidal Lp2A-I particle has no effect on the ability of the lipoprotein to adsorb and/or retain cholesterol molecules. Neither hypothesis could be confirmed without simultaneously measuring the rate of cholesterol adsorption and desorption from the LpA-I surface. It is possible that the constant rates of efflux observed when LpA-I POPC contents are varied may be a function of concomitant changes in adsorption and desorption of cholesterol. This is consistent with what has been observed in transfers of cholesterol between LDL and LpA-I (Meng *et al.*, 1995), which showed that increasing the POPC/apoA-I ratio in similar discoidal LpA-I particles increases the ability of the lipoprotein to both accept and also donate cholesterol molecules to LDL.

Previous studies have suggested that a concentration gradient of cholesterol, usually represented by the UC/phospholipid ratio of the cell plasma membrane and HDL, determines the direction of the cholesterol exchange (Phillips *et al.*, 1987). Since this UC/PC ratio is relatively constant for a given cell species, the ratio of UC/PC in HDL may be critical to whether HDL acts as either a donor or an acceptor of cholesterol. Experiments were performed to characterize the specific effect of UC on the ability of HDL to accept cellular cholesterol, and it was shown that the incorporation of up to 16 molecules of UC into an Lp2A-I has no effect on cholesterol efflux. This may indicate that cholesterol has no effect on the ability of HDL to accept cholesterol or that this lipid simultaneously modifies both the adsorption and desorption of cholesterol. In transfers between LDL and LpA-I, we showed that increases in cholesterol in an LpA-I particle also affected the adsorption and desorption of cholesterol (Meng *et al.*, 1995). In addition, inclusion of a high level of cholesterol also appeared to modify the kinetics of cholesterol transfer between LpA-I and LDL, from being independent of acceptor concentration to a first-order reaction that is dependent on LpA-I concentration. This suggested that changes in LpA-I cholesterol content may affect the rate of cholesterol exchange by changing the frequency of collisions between LDL and LpA-I particles. Since this same compositional modification had no effect on the efflux of cellular cholesterol, it follows that cholesterol exchange between cells and HDL particles may be less sensitive to the collisional properties of discoidal LpA-I particles.

In HDL, SM and PI represent about 10% and 4% of total HDL phospholipid mass and as such are phospholipids secondary only to PC in abundance (Skipski, 1972). SM has been reported to have a higher affinity toward cholesterol in small unilamellar vesicles than other phospholipids (Lund-Katz *et al.*, 1988; Yeagle & Young, 1986; Fugler *et al.*, 1985). A positive subcellular codistribution of SM with cholesterol has been well demonstrated in intact cells (Lange *et al.*, 1989; Wattenberg & Silbert, 1983; van Blittersuijk *et al.*, 1987). The important function of SM in the maintenance of cellular cholesterol homeostasis has become evident in the past decade. For example, addition of exogenous SM to cultured cells resulted in a marked increase of cholesterol biosynthesis and a reduction of LDL binding and degradation (Gatt & Bierman, 1980). Depletion of SM content of cell plasma membrane by SMase stimulates an extensive redistribution of cholesterol between plasma membrane and intracellular pools (Slotte *et al.*, 1989, 1990; Pörn *et al.*, 1990, 1991). Since addition of as little as 4 mol of SM per mole of apoA-I significantly increases the ability of Lp2A-I to accept cellular cholesterol, it may be this increased ability of SM to associate with cholesterol that allows the particle to bind and retain more cholesterol molecules.

Increased cholesterol efflux to particles containing SM appears to be specifically related to SM content; variation of either POPC or cholesterol contents in the same Lp2A-I has no effect on cholesterol efflux. The effect plateaus at about 16 mol of SM per particle where cholesterol efflux is nearly doubled as compared to the control (Figure 6B). This observation appears consistent with a previous study, which also reported elevated cholesterol efflux rates (when corrected per LpA-I particle) from both mouse L-cell fibroblasts and rat Fu5AH hepatoma cells to discoidal LpA-I particles prepared from SM (Davidson *et al.*, 1995b). In an earlier study, cholesterol efflux from human skin fibroblasts has been shown to correlate positively with the SM content of a series of sonicated SM-liposomes (Stein *et al.*, 1988). In addition, preβ_1 HDL, a subspecies of HDL that has been shown to have higher ability to accept cellular cholesterol, may also contain higher levels of SM than other HDL subclasses (Fielding & Fielding, 1995). The increased efflux-promoting ability of SM-containing Lp2A-I may be due to the influence of this phospholipid on the packing of the phospholipid monolayer of HDL. SM may create packing defects on the surface of an HDL particle much like apoA-I is thought to (Davidson *et al.*, 1995b; Letizia & Phillips, 1991). If interfacial packing defects promote the adsorption of cholesterol molecules, as proposed by Phillips and colleagues, SM may stimulate cholesterol efflux by actually creating spaces for incoming cholesterol molecules. Increasing the number of molecules of apoA-I on the surface of the discoidal particle (e.g., in Lp3A-I and Lp4A-I) may similarly increase surface packing defects and thereby stimulate cholesterol adsorption.

Although our results indicate that introduction of PI into Lp2A-I significantly stimulates cholesterol efflux to these particles, the enhanced surface charge due to the phospholipid does not appear to be associated with this phenomenon. We

have observed no consistent relationship between particle surface potentials and the rates of cholesterol efflux for all series of LpA-I particles. The effect of PI on cholesterol transfers could thus be indirect and may result from a combined effect of charge and surface lipid packing, and may affect interaction with the cell membrane. While little is known about the effect of PI on lipid packing in the surface of a lipoprotein, it appears possible that this lipid, much like phosphatidylserine, may have a propensity to segregate into local charged regions in the presence of divalent cations (Paphadjopoulos *et al.*, 1978). These regions may form crystalline nucleation points that would be expected to disrupt the surface packing and may actually promote fusion with the surface lipids of other lipoproteins and/or cells. Increased negative charges at the surface of an HDL particle would also be expected to affect and perhaps repel negative charged residues in apoA-I. In this respect, PI may modify apoA-I conformation in a manner that may indirectly affect surface lipid packing and the ability of the LpA-I particle to adsorb cholesterol molecules.

We have previously shown that changes in discoidal LpA-I composition can have major effects on the ability of these lipoproteins to receive cholesterol from LDL particles (Meng *et al.*, 1995). The study suggested that variations in the composition of LpA-I particles affect interlipoprotein cholesterol movement in a manner that is not consistent with a mechanism based purely upon aqueous diffusion of cholesterol (Phillips *et al.*, 1987; Johnson *et al.*, 1991). There is now mounting evidence that cholesterol exchange occurs by a more complex process that involves the desorption of UC from the plasma membrane, followed by collision with and incorporation into the acceptor particle (Thomas & Poznansky, 1988; Steck *et al.*, 1988; Davidson *et al.*, 1995a). Investigations by Fielding and colleagues (Miida *et al.*, 1990) have further shown that cholesterol transfers between lipoproteins and between lipoproteins and cells may be regulated very differently. Evidence from the present and previous studies in this laboratory is in agreement with these observations and suggests that while factors that affect the frequency of collisions between desorbed cholesterol and acceptor lipoproteins may govern interlipoprotein cholesterol transfers (Meng *et al.*, 1995), cellular cholesterol efflux may be more dependent on factors that affect the ability of an HDL particle to bind and retain this lipid.

In conclusion, we have shown that when additional molecules of apoA-I, SM, or PI are incorporated into a discoidal LpA-I particle, these constituents can affect the ability of these lipoproteins to adsorb and retain cholesterol molecules from cultured fibroblasts. It is of note that the efflux of cellular cholesterol to discoidal LpA-I particles does not appear to be affected by specific changes in apoA-I conformation and charge, but does appear to be related to major changes in the size of the lipoprotein particle. Further experiments are needed to characterize how changes in the physical properties/packing of surface lipids in an HDL particle may correlate with the adsorption/desorption of cholesterol to the surface. Notwithstanding, the observation that SM modulates cholesterol efflux is significant in that it appears to parallel the observation that an increased content of SM in a unique HDL subspecies, preβ_1 HDL, is also associated with an increased ability to promote cholesterol efflux (Fielding & Fielding, 1995). Other studies have shown that the amount of these preβ HDL particles may increase in the plasma of hyperlipidemic subjects (Ishida *et al.*, 1987). The concentrations of SM and PI in various classes of lipoproteins may therefore play important roles in governing lipid transfer processes and if modified in hyperlipidemic subjects may significantly affect plasma cholesterol homeostasis.

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