Cholesterol Efflux from Fibroblasts to Discoidal Lipoproteins with Apolipoprotein A-I (LpA-I) Increases with Particle Size but Cholesterol Transfer from LpA-I to Lipoproteins Decreases with Size†

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ABSTRACT: To understand the role of different discoidal lipoproteins in cellular cholesterol efflux, defined discoidal lipoproteins containing 2, 3, or 4 apolipoproteins (apo) A-I per particle (Lp2A-I, Lp3A-I, and Lp4A-I) were prepared from mixtures of apoA-I and phospholipids with or without cholesterol. Each particle had a slow preβ migration on agarose gel electrophoresis which further decreased as the number of apoA-I increased. Incubation of cholesterol-labeled human fibroblasts with the different LpA-I at an equimolar concentration in apoA-I showed that the best acceptors of cellular cholesterol were Lp4A-I, followed by Lp3A-I and Lp2A-I. Cholesterol efflux to these particles was positively correlated to the number of apoA-I, to the ratio of phospholipids to apoA-I, and to the size of particles, three interrelated parameters. To follow the subsequent movement of cellular cholesterol after it became associated with LpA-I, cholesterol- and apoA-I-labeled LpA-I were incubated with plasma which resulted in parallel modifications of each labels electrophoretic migration with time. However, [^{3}H]cholesterol-labeled LpA-I transferred from preβ to α migration with a precursor–product relationship while ^{125}I-LpA-I progressively shifted from preβ to α migration. The change in electrophoretic migration of ^{125}I-LpA-I is independent of cholesterol and appears related only to a modification of apoA-I charge. Lp2A-I was fastest in changing its electrophoretic migration to α, followed by Lp3A-I and then Lp4A-I. The large discoidal particles containing four or three apoA-I thus display a greater stability, and we propose that both this stability and their greater capacity to bind cholesterol can make them the most efficient lipoprotein acceptor of cellular cholesterol in the milieu where they are produced.

Cholesterol movement between cells and high-density lipoprotein (HDL)[1] is a well-studied phenomenon which can occur by nonmediated diffusion across the unstirred water layer (Johnson et al., 1991a). This diffusion is a general property of membrane-associated cholesterol, but the esterification of HDL cholesterol by lecithin–cholesterol acyltransferase can make it an irreversible pathway which forms the basis of reverse cholesterol transport. Although the principle of cholesterol transfer by passive diffusion only requires the existence of a concentration gradient between donor and acceptor structures, the concept of a preferential acceptor also exists because extracellular cholesterol is not a disperse molecule but a part of the multimolecular complexes that are the lipoproteins.

This concept has been recently revived by the interesting and original report of Castro and Fielding (1988) that, upon exposure to very fresh plasma, cellular cholesterol first appears in a small HDL particle with a preβ electrophoretic migration. This particle which contains apoA-I but no apoA-II has a brief existence as a carrier of cellular cholesterol that is soon found in the major HDL class. However, different results may be obtained with different approaches, such as the work of Johnson et al. (1991b), who studied the cholesterol efflux of cells exposed to immunoaffinity-isolated lipoproteins and observed that plasma LpA-I and LpA-I, A-II are equally good acceptors of cellular cholesterol. It is possible that these lipoprotein preparations may have been depleted in these preβ-migrating LpA-I during isolation.

In plasma, the preβ- or α_2-migrating lipoproteins, as we have also proposed to name them (Marcel et al., 1990), are a minor apoA-I containing lipoprotein class or LpA-I which comigrates with or binds to most of plasma CETP and some of plasma LCAT (Francone et al., 1989; Marcel et al., 1990). The slow migration of these LpA-I is typical of the electrophoretic migration of stable discoidal LpA-I particles (Sparks & Phillips, 1992) that differ in size and composition, notably in the number of apoA-I molecules per particle (Jonas et al., 1989; Nichols et al., 1987; Wald et al., 1990). Evidence for the existence of several kinds of discoidal lipoproteins has been obtained in vitro in hepatocyte cultures (McCall et al., 1988, 1989) as well as in vivo in human (Reich, 1990) and dog (Sloop et al., 1987; Lefevre et al., 1988) lymph and in patients with LCAT deficiency (Soutar et al., 1982). This suggests that cells in different tissues may be exposed to different discoidal lipoproteins and these particles, depending on their composition, may vary in their abilities to bind and carry cholesterol away from cells, as they have been shown to differ as substrates for LCAT (Wald et al., 1990). In the present report, we have prepared and characterized defined populations of LpA-I different in size and complement of apoA-I and have studied their abilities, first to serve as

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[1] Abbreviations: HDL, high-density lipoproteins; LCAT, lecithin–cholesterol acyltransferase; LpA-I, apoA-I-containing lipoproteins; POPC, palmitoyloleoylphosphatidylcholine; UC, unesterified cholesterol; TC, total cholesterol; PL, phospholipid; PC, phosphatidylcholine; CETP, cholesteryl ester transfer protein; DTNB, dithiobis(2-nitrobenzoic acid); EYPC, egg yolk phosphatidylcholine.

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acceptors of cellular cholesterol and second to transfer this cholesterol to other lipoproteins. Our aim is to further define the role of discoidal particles in the mediation of cellular cholesterol efflux.

EXPERIMENTAL PROCEDURES

Lipoproteins. Plasma was obtained from normolipemic blood donors; HDL (1.063–1.21 g/mL), HDL_2 (1.063–1.125 g/mL), and HDL_3 (1.125–1.21 g/mL) were isolated by a standard sequential ultracentrifugation technique (Havel et al., 1958). Apo A-I was isolated from HDL after delipidation and chromatography on Sephacryl S-200 of apo-HDL resolubilized in Gdn-HCl (3 M) as described by Brewer et al. (1986).

Preparation and Characterization of Reconstituted LpA-I. Reconstituted LpA-I were prepared by the sodium cholate dialysis method (Jonas et al., 1989) using either egg yolk phosphatidylcholine (EYPC) or palmitoyloleoylphosphatidylcholine (POPC) as the source of phospholipids. The molar ratios of PC/cholesterol/apoA-I/sodium cholate used in these preparations were 88:44:1:88 or 120:6:1:120 (Jonas et al., 1989; Nichols et al., 1987; Wald et al., 1990). Similar LpA-I without cholesterol were prepared using the same ratios as above but without cholesterol. All preparations and characterization experiments were done in recombinant buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM NaN_3 unless indicated otherwise. Homogeneous discoidal particles, Lp2A-I, Lp3A-I, and Lp4A-I containing 2, 3, and 4 apoA-I molecules, respectively, were obtained by gel filtration on two serial agarose columns (95 × 2.5 cm) (BioGel 5M, Bio-Rad Labs).

The number of apoA-I molecules per LpA-I particle was determined by cross-linking apoA-I present in each LpA-I fraction with dithiobis(succinimidyl propionate) (Bragg & Hou, 1975) and analyzing the products of the reaction by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels.

Mobility on agarose gels was examined on Paragon Lipogels (Beckman). Electrophoresis, fixing, and lipid staining were performed according to the instructions of the manufacturers. The transfer to nitrocellulose paper and immunodetection have been described earlier (Milthorp et al., 1986). In experiments to determine the mobility of [^{3}H]cholesterol-labeled LpA-I or ^{125}I-labeled LpA-I, small areas of the fixed gel were cut out and counted in scintillation or γ counters (Beckman), respectively. The sizes of isolated particles were measured by nondenaturing gradient gel electrophoresis (GGE) using Pharmacia LKB Biotechnology Inc. PAA 4/30 gels. The concentration of purified apoA-I was determined by Lowry assay (Lowry et al., 1951) and from absorbance measurements at 280 nm considering the extinction coefficient of apoA-I [1.15 mL/(mg·cm)] and that of HDL apoA-I by radioimmunoassay (Marcel et al., 1987). Phospholipids and cholesterol were measured using the enzymatic colorimetric test kits (Boehringer Mannheim GmBH, Mannheim, Germany).

Labeling of LpA-I. Purified LpA-I were labeled either by iodination with Na^{125}I (Amersham Corp.) by the modified MacFarlane procedure (Bilheimer et al., 1972) or with free [^{3}H]cholesterol (60 Ci/mmol, Amersham Corp.) as described previously (Jonas et al., 1989). The labeled LpA-I were stored at 4 °C and used within 1 week of preparation.

Cells. Human skin fibroblasts (strain GM0038, obtained from the human Genetic Mutant Cell Repository, Camden, NJ) were cultured with 10% fetal calf serum in Dulbecco's minimum essential medium (DMEM GIBCO). Cells were seeded in 20-mm-diameter cell culture dishes (at about 40 000 cells/dish) and were grown to near-confluency. They were then washed twice with phosphate-buffered saline (PBS), pH 7.4, and incubated for 48 h in growth medium with 10% fetal calf serum containing unesterified [1,2-^{3}H]cholesterol (50 μCi/100 μL of serum) (Bates & Wissler, 1976).

Incubation Protocol. [^{3}H]Cholesterol-labeled cells were washed twice with PBS and were then incubated with serum-free medium containing 3 mg/mL fatty acid-poor BSA and the indicated lipoprotein particles for 5 min at a concentration of 15 μg/mL apoA-I. The efflux of cell-derived [^{3}H]-cholesterol was determined by counting the radioactivity in the medium. At the end of the efflux period, the remaining medium was removed from the experimental dishes, and cell monolayers were washed 5 times with PBS. The cells were harvested, and the total remaining radioactivity was determined. The total cellular cholesterol specific activity was calculated on the basis of the measured value (4 μg of cholesterol per 100 μg of cellular protein) which was comparable to reported values for fibroblasts under the same conditions. It was also comparable to values established from control cells harvested at zero time. The specific activity of cell cholesterol was (4–8) × 10^6 cpm/μg, and more than 90% of the radioactivity was in the unesterified cholesterol fraction as reported by others (Castro & Fielding, 1988).

Efflux was expressed as cpm of [^{3}H]cholesterol removed per microgram of cellular protein per nanomoles of LpA-I. When the cells were incubated with BSA alone, negligible efflux was observed (less than 0.1 ng/μg of cell protein).

Transfer of Labeled Cholesterol or ApoA-I between LpA-I and Plasma Lipoproteins. Labeled LpA-I and plasma lipoproteins were incubated together at different molar ratios of apoA-I for 1, 5, 10, 15, and 60 min at 37 °C. In some experiments, the incubation was carried out in the presence of 2 mM dithiobis(2-nitrobenzoic acid) (DTNB) to inhibit LCAT activity (Stokke & Norum, 1971). In other experiments, plasma lipoproteins were preincubated at 37 °C for 24 h in the presence of a monoclonal antibody against CETP (TP-2), which has been shown to inhibit neutral lipid transfer (Hesler et al., 1988).

Statistical Analysis. Linear regressions and correlations were calculated using the 1990 Graph PAD software, Version 1.11a (San Diego, CA).

RESULTS

Characterization of Discoidal LpA-I. We prepared several reconstituted discoidal LpA-I with different ratios of phospholipids (either EYPC or POPC), cholesterol, and apoA-I which have been shown to constitute stable particles (Jonas et al., 1989; Nichols et al., 1987; Wald et al., 1990). The Lp2A-I, Lp3A-I, and Lp4A-I particles isolated from the mixture with the initial molar ratio of POPC or EYPC/UC/apoA-I, 88:44:1, had the respective sizes of 7.8, 10.8, and 17 nm. Those obtained from the initial molar ratio POPC/UC/apoA-I, 120:6:1, had the respective sizes of 9.7, 13.4, and 17 nm. The LpA-I prepared without cholesterol had the same size as the corresponding LpA-I with cholesterol.

Electrophoresis of these LpA-I particles on agarose gels demonstrated their slow mobility as compared to plasma HDL (Figure 1). In freshly isolated plasma, most of apoA-I is contained in α-migrating lipoproteins, but small amounts are also present in slow α-, or preβ-migrating lipoproteins (Kunitake et al., 1985; Ishida et al., 1987; Marcel et al., 1990). Here we show for the first time that the electrophoretic migration decreases as the number of apoA-I per particle

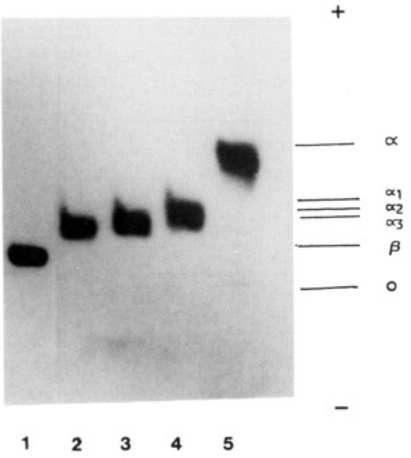

FIGURE 1: LpA-I electrophoretic migration on an agarose gel. Plasma and LpA-I samples were submitted to electrophoresis on a 0.5% agarose gel, followed by immunoblots with antibodies against apoB (4G3) and apoA-I (4H1). Lane 1, plasma (4G3); 2, Lp2A-I; 3, Lp3A-I; 4, Lp4A-I; 5, plasma (4H1). The LpA-I particles were isolated from the initial molar ratio POPC/UC/apoA-I = 120:6:1. The same electrophoretic patterns were obtained with the particles without cholesterol.

increases. Recently, Sparks and Phillips (1992) have shown that neither particle size nor cholesterol content seemed to affect electrophoretic migration of LpA-I. We also observed similar differences in the electrophoretic migration of Lp2A-I, Lp3A-I, and Lp4A-I independently of the presence of cholesterol in the particle (not illustrated). Therefore, since the phosphatidylcholine does not contribute a net charge to the particle, these differences in electrophoretic migration reflect the charge of apoA-I. This suggests that the different charge of apoA-I in discoidal compared to spherical particles may be due to a different conformation although it remains to be established that each apoA-I molecule has a similar conformation in these particles. The respective mobilities of Lp4A-I, Lp3A-I, and Lp2A-I have been called here α_3, α_2, and α_1 migrations.

Transfer of Cellular [^{3}H]Cholesterol to LpA-I. These particles have been tested for their ability to promote the efflux of [^{3}H]cholesterol from the plasma membrane pool of fibroblasts. Efflux was monitored by the appearance in the medium of [^{3}H]cholesterol after a 5-min incubation with LpA-I. Since efflux from cells to small discoidal particles occurs rapidly (Castro & Fielding, 1988), we tested several incubation times ranging from 1 to 60 min and selected a short incubation time that afforded both accurate determination of efflux rates and discrimination among the different LpA-I for their respective abilities to serve as cholesterol acceptors. Under these conditions, we observed that Lp4A-I were 2–3 times more efficient in removing cholesterol from cells than Lp3A-I, which were themselves about twice as efficient as Lp2A-I (Table I). These differences in efflux between the LpA-I were maintained at different times of incubation, but were greatest at early time (not illustrated).

LpA-I without cholesterol were able to promote an efflux 2- or 3-fold greater than the LpA-I with cholesterol, in agreement with the notion that the cholesterol gradient between donors and acceptors is the major driving force of diffusion. With the cholesterol-containing particles, there was a very highly significant correlation between efflux and phospholipid concentration in the particles (Table I, $r = 0.9989$, $p < 0.02$), which did not reach significance in the cholesterol-free particles ($r = 0.9773$). These results are in agreement with earlier studies using lipid vesicles (Stein et al., 1976) and provide additional evidence for the prominent role of phospholipids in the clearance of cellular cholesterol. As should be expected, the presence of cholesterol in LpA-I, by decreasing the cholesterol gradient from cells to medium, seemed to act as an inhibitor of efflux, but the negative correlation between the ratio of phospholipids to cholesterol in these LpA-I and the rate of efflux did not reach significance ($r = -0.993$, $p = 0.07$).

With this pooled set of efflux experiments, a significant relationship ($p < 0.03$) existed between the size of particles and efflux, a relationship which is related to both the number of apoA-I and the phospholipid concentration since these two parameters are reflected in the size of particles. When the efflux calculated per particle was normalized per apoA-I, the efflux still increased with particle size, but became a constant when normalized for both apoA-I and phospholipid content per particle (Table I). We also tested the ability of plasma HDL to promote cellular cholesterol removal, and we observed a difference of cellular cholesterol efflux in relation to the size of HDL_2 versus HDL_3; efflux, phospholipid content, and size were greater in HDL_2 than HDL_3 (Table II).

Transfer of Cholesterol and ApoA-I from LpA-I to Plasma Lipoproteins. Having defined the relative efficiency of different LpA-I as acceptors of cellular cholesterol, we sought to examine the fate of the LpA-I cholesterol in the presence of plasma lipoproteins. Because apoA-I has been shown to equilibrate between apoA-I-containing lipoproteins, we also studied the transfer of apoA-I from reconstituted LpA-I to plasma lipoproteins to understand whether these transfers were comparable and could imply either independent transfer of constituents or transformation of the LpA-I particle itself. When [^{3}H]cholesterol-labeled LpA-I migrating on an agarose gel in α_2 or α_1 were incubated with plasma lipoproteins, there was a rapid movement of label first to an α- and then to a β-migrating position (Figure 2). This movement or transfer rate was dependent on the relative concentration of LpA-I present in the reaction mixture (Table II). The cholesterol of Lp2A-I transferred more rapidly to plasma HDL than that of Lp3A-I, itself more rapidly than Lp4A-I. After an incubation of 1 min, half of the label initially in Lp2A-I (at an apoA-I molar ratio in Lp2A-I/plasma of 1) migrated with α mobility while, at such a time, no change in migration was seen for labeled Lp3A-I (Table III). [^{3}H]Cholesterol from labeled Lp4A-I transferred to α migration only after a 60-min incubation at an apoA-I molar ratio of 0.1 (LpA-I/plasma). For Lp2A-I and Lp3A-I, complete transfer of labeled cholesterol to particles with α and β migration was already reached at 60 min.

^{125}I-Labeled LpA-I had a preβ migration in the agarose gel similar to that of [^{3}H]cholesterol-labeled LpA-I, but after incubation with plasma, the ^{125}I label moved only to an α position (Figure 2), reflecting the expected lack of transfer of apoA-I to the β-lipoprotein particles. We also observed that, like their cholesterol-labeled counterparts, the ^{125}I-Lp2A-I were converted more rapidly to α-migrating particles than were ^{125}I-Lp3A-I or ^{125}I-Lp4A-I. We can exclude the possibility that apoA-I of LpA-I exchanges with other apoA-I in HDL since the level of radioactivity of the ^{125}I-LpA-I remains constant and gradually moves to the α position. Thus, in the transfer reactions taking place at 37 °C between LpA-I and plasma lipoproteins, two components of LpA-I, apoA-I and cholesterol, migrated first and with similar rates of change from preβ to α positions, suggesting that the modified particle itself had migrated to this position. However, the radioactivity of LpA-I cholesterol transferred from preβ to α with a

Table I: LpA-I-Mediated Cholesterol Efflux from Human Skin Fibroblasts[a]

LpA-I	size (nm)	POPC:UC:apoA-I[b] molar ratio initial	POPC:UC:apoA-I[b] molar ratio final	POPC:UC ratio	efflux per[c] particle	efflux normalized per apoA-I	efflux normalized per apoA-I and phospholipid
Lp2A-I	9.6	120:6:1	118:2.4:1	49.2	15 ± 0.5[d]	7.5	64
Lp3A-I	13.0	120:6:1	136:3.1:1	43.9	26.2 ± 0.7	8.8	64
Lp4A-I	17.0	120:6:1	210:8.9:1	23.6	50.6 ± 0.3	12.7	60
Lp2A-I	9.6	120:0:1	112:0:1		28.7 ± 0.6	14.3	128
Lp3A-I	13.0	120:0:1	131:0:1		72.5 ± 0.6	24.2	184
Lp4A-I	17.0	120:0:1	144:0:1		100.4 ± 1.9	25.1	174

[a] The cells were prelabeled with medium containing [1,2-^{3}H]cholesterol and incubated with LpA-I (15 μg of apoA-I/mL) for 5 min as described under Experimental Procedures. [b] Initial molar ratio of LpA-I components represents the composition in the starting mixture; the final ratio is the composition of LpA-I after dialysis and purification of agarose columns. [c] Efflux is expressed as cpm of[^{3}H]UC ($\times 10^{-3}$) of (μg cellular protein)$^{-1}$ (particle of LpA-I)$^{-1}$ ($n = 3$). In the last two columns, the efflux is normalized first per cpm of [^{3}H]UC (μg of cellular protein)$^{-1}$ (nmol of apoA-I)$^{-1}$ and second per cpm of [^{3}H]UC (μg of cellular protein)$^{-1}$ (nmol of apoA-I)$^{-1}$ and (nmol of phospholipid in LpA-I)$^{-1}$. [d] Values are means ± standard deviations of triplicate analysis of separate dishes.

Table II: Cellular [^{3}H]Cholesterol Efflux to HDL[a]

lipoproteins	size (nm)	PL:TC:UC:apoA-I molar ratio	cellular [^{3}H]UC efflux[b] a	cellular [^{3}H]UC efflux[b] b
HDL_2	9.7–12.9	35.0:11.3:2.8:1	4.44 ± 0.12[c]	25.37 ± 2.06[c]
HDL_3	7.2–8.8	13.9:4.1:0.6:1	4.32 ± 0.18	12.00 ± 0.66

[a] [^{3}H]Cholesterol-labeled fibroblasts were incubated with HDL_2 or HDL_3 (15 μg of apoA-I HDL/mL) for 5 min at 37 °C. [b] Cellular [^{3}H]UC efflux is expressed as (a) cpm of [^{3}H]UC ($\times 10^{-3}$)/μg of cell protein or (b) cpm of [^{3}H]UC (μg of cell protein)$^{-1}$ (μmol of HDL)$^{-1}$. [c] Values are mean ± standard deviation of triplicate assays.

precursor–product relationship, whereas the radioactivity of ^{125}I-LpA-I shifted gradually from preβ to α without change in the level. Therefore, it is clear that the transfers of these two constituents of LpA-I follow different paths and may be independent of one another.

In order to establish whether the change in migration of the LpA-I constituent was due to the esterification of LpA-I cholesterol by plasma LCAT, we prepared ^{125}I-labeled Lp2A-I without cholesterol and incubated them with plasma for the same periods. The results showed that incubation of these ^{125}I-Lp2A-I with plasma also transformed their migration to α mobility at the same rate as that of Lp2A-I-containing cholesterol (data not shown). Furthermore, [^{3}H]cholesterol-labeled Lp2A-I incubated with plasma in the presence of DTNB (an inhibitor of LCAT activity) exhibited a similar change in mobility to the α position. These experiments indicated that the increase in electrophoretic mobility of LpA-I was not dependent on LCAT activity.

In order to exclude the possibility that the change in mobility may be due to a transfer of CE from HDL to Lp2A-I or Lp3A-I, we tested the capacity of an anti-CETP antibody, TP-2, to inhibit the modification to α migration of the particles. Incubation for 2 h at 37 °C of ^{125}I-Lp2A-I and ^{125}I-Lp3A-I-containing cholesterol with HDL or plasma preincubated with TP-2 did not prevent the change in electrophoretic migration of the particles (data not shown). Negative controls were carried out with the same components incubated at 4 °C or with ^{125}I-labeled LpA-I alone incubated at 37 °C for 2 h. In addition, the simultaneous inhibition of LCAT by DTNB and of CETP by TP-2 did not prevent the change in the migration of LpA-I. Therefore, the modification of ^{125}I-LpA-I migration did not seem to be due to transfer of CE from HDL. We also incubated ^{125}I-LpA-I with VLDL and LDL, but in the presence of these lipoproteins, no change in the migration of labeled apoA-I (comparatively to its initial migration) was observed. This indicates that the transfers of cholesterol and phosphatidylcholine known to take place between LDL and LpA-I are not sufficient to modify ^{125}I-LpA-I electrophoretic migration.

The effect of incubation of ^{125}I-labeled Lp2A-I or Lp3A-I with plasma on their size distribution was studied by gradient gel electrophoresis and autoradiography. It was found that their change to α migration was accompanied by a decrease in the Stokes diameter of these particles (Figure 3). After a 5-min incubation with plasma, the labeled Lp2A-I with an initial homogenous size of 9.7 nm had already changed into a 7.2-nm particle, which corresponds to the smallest size reported for these discoidal LpA-I. Incubation of the labeled Lp3A-I (13.4 nm) with plasma for 5 min induced a diminution of the particle size to 9.6–9.8 nm while a fraction of the initial particles remained unchanged. After a 15-min incubation of these same components, we obtained ^{125}I-Lp2A-I particle sizes of 9.7 and 7.2 nm. Finally, after 1 h of incubation, most of the particles were converted to particles with a 7.2-nm diameter. These decreases in the sizes of LpA-I upon interaction with plasma are consistent with those of HDL observed by others (Nichols et al., 1985, 1987, 1989; Clay et al., 1992).

DISCUSSION

In the present study, our aim was to identify which of the known stable discoidal reconstituted particles has the greatest affinity for cellular cholesterol and constitutes the preferential acceptor identified as preβ lipoproteins by Castro and Fielding (1988). Two considerations support the design of this investigation. First, as mentioned earlier, different discoidal lipoproteins with slow electrophoretic migration have been shown to exist in physiologic conditions (McCall et al., 1988; Soutar et al., 1982), and their size and composition change from plasma to lymph and/or interstitial fluid (Reich, 1990; Lefevre et al., 1988), supporting the idea that cells in different tissues may be exposed to different discoidal particles. Second, the preβ lipoproteins of plasma remain to be completely characterized. For example, the apparent molecular mass reported for the small LpA-I ranged from 71 kDa (Castro & Fielding, 1988; Ishida et al., 1987) to 80 kDa (Kunitake et al., 1985) and is much smaller than the apparent molecular mass of the smallest identified Lp2A-I, which is 123 kDa (Nichols et al., 1987). The concentration of apoA-I in these small LpA-I also varies from 47.5% (Castro & Fielding, 1988) to 91% (Kunitake et al., 1985), raising the possibility that different particles may exist. Fielding and colleagues (Castro & Fielding, 1988; Ishida et al., 1987) concluded, only on the basis of the size and composition of their preβ_1 LpA-I species, that this particle contained only one apoA-I. Although such a type of Lp1A-I has never been observed in synthetically reconstituted lipoproteins, it is possible that it may be transiently generated during the transformation of discoidal Lp2A-I into spherical Lp3A-I by action of LCAT (Nichols

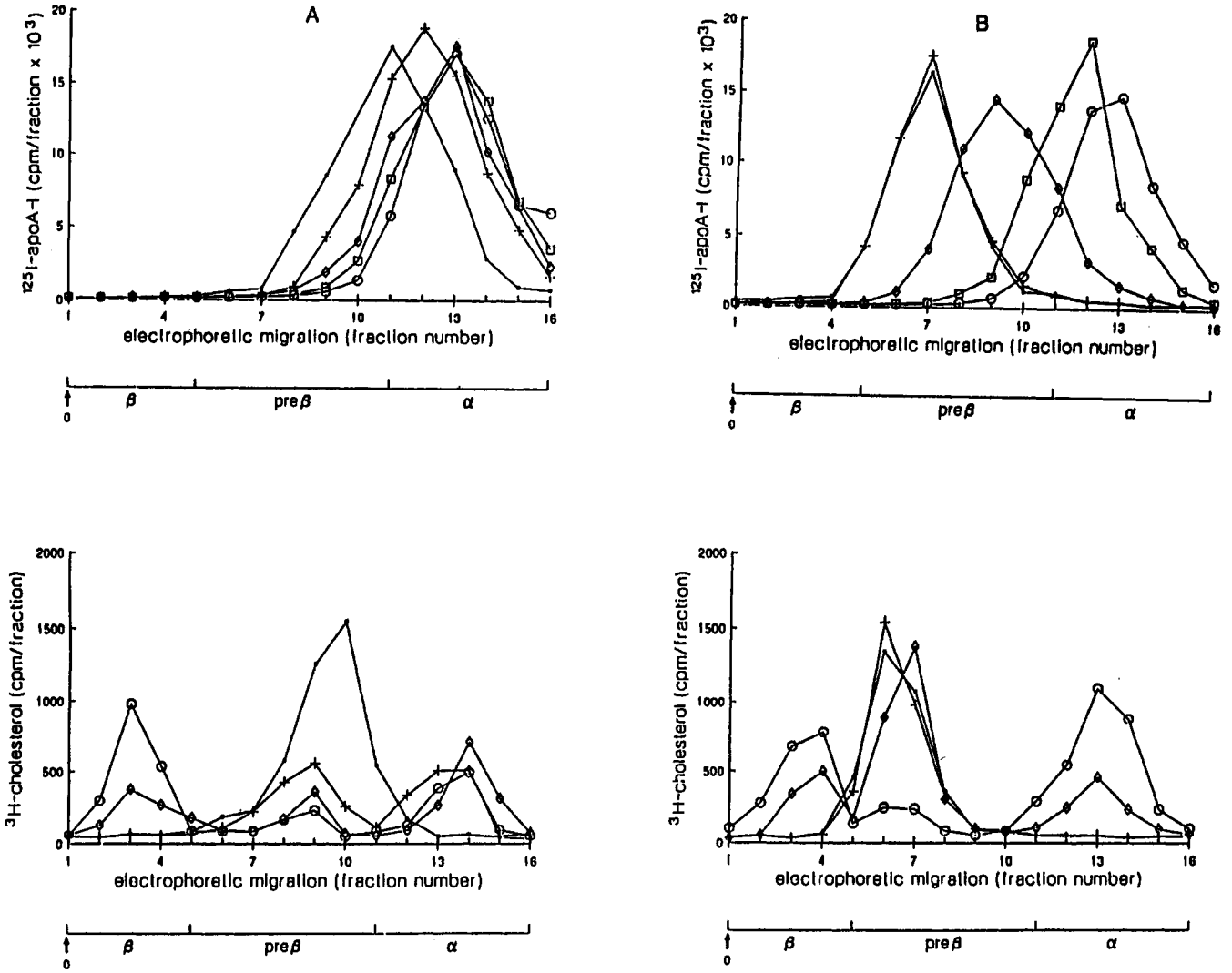

FIGURE 2: Change in electrophoretic mobility of labeled LpA-I upon incubation with plasma. ^{125}I-Labeled LpA-I (top) or [^{3}H]cholesterol-labeled LpA-I (bottom) were incubated at 37 °C with plasma lipoproteins at an apoA-I molar ratio of LpA-I/plasma = 0.1. Aliquots were electrophoresed in an agarose gel as described under Experimental Procedures. The areas of the gel from the origin to the α-migrating region were cut out and counted in a scintillation or a γ counter. Panels A and B represent the migration of ^{125}I-labeled Lp2A-I and ^{125}I-labeled Lp3A-I, respectively, after incubation with plasma for 0, 1, 5, 15, and 60 min (●, +, ◇, □, and ○, respectively). The initial electrophoretic position of Lp2A-I and Lp3A-I were in pre-β, more precisely in α_1 and α_2, respectively. These results are representative of three experiments carried out using three different preparations.

Table III: Change in Electrophoretic Mobility of [^{3}H]Cholesterol-Labeled LpA-I upon Incubation with Plasma[a]

LpA-I:plasma apoA-I molar ratio	incubation time (min)	lipoprotein electrophoretic migration								
		Lp2A-I			Lp3A-I			Lp4A-I		
		preβ	α	β	preβ	α	β	preβ	α	β
1	1	50.1	49.9		100					
	5	20.5	49.0	30.5	61.4	22.0	16.0			
	60	11.4	32.3	56.2	9.4	37.0	53.5			
0.3	1	66.6	24.5	8.6	74.5	8.1	17.4			
	5	20.6	51.5	27.9	65.4	19.1	15.5			
	60	6.7	35.5	57.8	5.7	38.9	55.5			
0.1	1	71.2	16.3	12.5	83.5	8.0	8.6	100		
	5	8.6	35.4	56.0	55.3	31.8	12.9	100		
	60	8.9	35.4	55.7	9.1	40.1	58.8	57	43	

[a] [^{3}H]Cholesterol-labeled LpA-I and plasma were incubated together at 37 °C for 1, 5, and 60 min at LpA-I/plasma apoA-I molar ratios = 0.1, 0.3, or 1. An aliquot of each mixture was electrophoresed on an agarose gel. After being fixed, the gel was cut in small fractions, and the radioactivity in each fraction was counted. The radioactivity present in preβ-, α-, and β-migrating lipoproteins was calculated from the total as a percent of the total radioactivity. At zero time, all the radioactivity was contained in the preβ migration.

et al., 1987) or following modification of HDL by CETP and hepatic lipase (Clay et al., 1992). It is therefore important to understand how the different discoidal particles which can exist in vivo may perform as acceptors of cellular cholesterol efflux.

The lipoproteins used here are the reconstituted LpA-I prepared in vitro (Nichols et al., 1987; Jonas et al., 1989; Wald et al., 1990; Sparks & Phillips, 1992) which have been shown to exist as stable lipoproteins of discrete sizes defined by a stoichiometry of apoA-I, phospholipids, and cholesterol

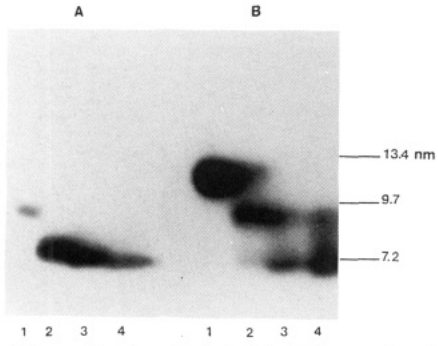

FIGURE 3: Change in size of labeled LpA-I upon incubation with plasma. Plasma was incubated with ^{125}I-Lp2A-I (A) or ^{125}I-Lp3A-I (B) at an apoA-I molar ratio of LpA-I/plasma = 0.1. Samples of these mixtures were separated by electrophoresis on a nondenaturing polyacrylamide gradient gel. Autoradiograms represent ^{125}I-LpA-I incubated alone for 60 min (lane 1) and ^{125}I-LpA-I incubated with plasma for 5 (lane 2), 15 (lane 3), and 60 min (lane 4).

molecules and are believed to be the precursors of the intravascular HDL particles (Nichols et al., 1987; McCall et al., 1989). Although the cholate dialysis technique used in their preparation (Nichols et al., 1983) probably bears little resemblance to the still undefined mechanism or pathway by which such HDL particles are assembled in vivo during secretion by liver or intestine or during metabolic remodeling of HDL (Sloop et al., 1987; Lefevre et al., 1988; McCall et al., 1988, 1989; Reich, 1990), these defined LpA-I both are stable and readily reactive with LCAT, making them good candidates to be tested as acceptors of cellular cholesterol.

The relationship between cellular cholesterol efflux and size of the acceptor particles has also been studied by others (Rothblat & Phillips, 1982; Delamatre et al., 1986). However, in these earlier studies, which concluded to a faster efflux in the presence of small particles, heterologous preparations of reconstituted lipoprotein structures were used. Rothblat and Phillips (1982) compared efflux to unilamellar spheres made with EYPC, to a mixture of spheres with some disks made with EYPC and apo-HDL, and to disks made with EYPC and taurocholate. DeLamatre and colleagues (1986) compared the efflux obtained with small unilamellar vesicles and with disks made with EYPC and of apo-HDL, apoA-I, or apoC. Our study is the first to compare homogeneous preparations of homologous reconstituted discoidal particles of different sizes and therefore should not be compared to those cited above. It is clear here that efflux is faster to large particles and that it is driven by the interrelated parameters of size, apoA-I number, and phospholipid to apoA-I ratio. The present study does not differentiate between these parameters, and other experiments are necessary to understand if one of them is preponderant. Reconstituted LpA-I with a constant number of apoA-I per particle, but with a varying ratio of phospholipids to apo-I, needs to be tested at different absolute concentrations in order to elucidate whether the size of particles, determined by both apoA-I and phospholipid concentrations, is the dominant characteristic of efficiency for the acceptor of cellular cholesterol, or whether the ratio of phospholipids to apoA-I in a given LpA-I class is also an important parameter. The variation in the ratio of phospholipids to apoA-I may affect the binding of apoA-I to lipids and the packing of the amphipathic α helices on the edge of the disk, which in turn would control the ability of lipid to bind to LpA-I. In the lymph of healthy subjects, apoA-I is associated with lipoproteins larger than those in plasma (Reichl & Pflug, 1982); likewise, in the lymph of hypercholesteremic pigs, there is an increase in the concentration of large apoA-I-containing lipoproteins under conditions where, presumably, reverse cholesterol transport is increased (Lefevre et al., 1988). Thus, the large apoA-I-containing lipoproteins, shown here to be the most efficient acceptors of cellular cholesterol, are also found in lymph and may have an important physiological role.

In the second part of our study, we observed that the large LpA-I, which are the best acceptors of cellular cholesterol, also exhibit the most stable electrophoretic migration when incubated with plasma. Therefore, this suggests that the cholesterol remains associated with Lp4A-I longer than it does with Lp3A-I or Lp2A-I. Indeed, the rate of change of migration of labeled cholesterol initially present in α_3-migrating (or slow preβ) Lp4A-I to plasma lipoproteins and specifically to HDL is much slower than that in α_2-migrating Lp3A-I, itself 2–3-fold slower than that in α_1-migrating Lp2A-I. The extremely slow rate of transfer of cholesterol from Lp4A-I to other plasma lipoproteins compared to Lp3A-I and Lp2A-I cannot be explained by the sole difference of their phospholipid content, since the change in the ratio of Lp4A-I to plasma lipoproteins over a 10-fold range does not change significantly the rate of transfer of cholesterol. The number of apoA-I per particle may thus be the most important factor which determines the particle's ability to exchange cholesterol with other lipoproteins. Then, Lp4A-I which retain their cholesterol for the longest time and are also the slowest of the preβ particles to be converted to α particles represent the most efficient intermediates for cellular cholesterol efflux. Their stability as cholesterol-containing particles may translate into longevity in vivo and in physiological importance. Such a hypothesis is again consistent with the presence of large discoidal particles in lymph and interstitial fluid which are enriched in phospholipid and free cholesterol (Reich, 1990; Sloop et al., 1987; Lefevre et al., 1988). Under our experimental conditions with an apoA-I molar ratio for LpA-I/HDL of 0.1, close to the plasma ratio (Ishida et al., 1987), we did not observe any inhibition of the change in electrophoretic migration of the particles in the presence of an anti-CETP antibody (TP-2) and/or when LCAT is inactive. Thus, the modification of the electrophoretic migration of the cholesterol and apoA-I components of LpA-I from preβ to α does not depend on the building of a hydrophobic core in the LpA-I. This is a puzzling observation for apoA-I given our other results showing that the presence or absence of cholesterol does not alter the preβ migration of LpA-I and since phosphatidylcholine does not by itself contribute any charge to the particle. Because the electrophoretic migration of ^{125}I-LpA-I does not change upon incubation with VLDL and LDL, we propose that the modification of apoA-I charge requires the interaction of apoA-I in LpA-I with a component of HDL, maybe by association with an HDL-bound apoA-I. The change in folding from a disk- to a sphere-bound structure is such that it results in an increase in the negative charge on apoA-I. The respective α_3, α_2, and α_1 migrations of Lp4A-I, Lp3A-I, and Lp2A-I are consistent with the hypothesis that each disk-bound apoA-I is less negatively charged than each sphere-bound apoA-I. It remains to be understood how the apoA-I conformation changes, and which residues become charged which were not before.

Considering the change in size of these discoidal particles upon incubation with plasma, we have shown that the large Lp4A-I are more slowly converted than are Lp3A-I, which in turn are also more slowly converted than Lp2A-I. However, under these conditions, the end product seems to be the same for all LpA-I, in the form of a particle of 7.2 nm or the smallest

Lp2A-I. These results could explain why the only preβ-migrating particles found in small amounts in plasma are those with an Lp2A-I size, and this despite the marked size heterogeneity of nascent HDL (McCall et al., 1988, 1989; Reich, 1990). The very rapid change in size of Lp2A-I components which we observed is in agreement with the earlier work of Castro and Fielding (1988), who reported a very short half-life for their small preβ-migrating HDL. However, the time course of the size modification of discoidal LpA-I upon incubation with plasma differs in the experiments of Nichols et al. (1987, 1989). This is probably related, first, to the longer time of incubation and, second, to the different apoA-I molar ratio of LpA-I/HDL used.

In conclusion, the present study shows that the efflux of cholesterol from normal human fibroblasts to defined and homogeneous preparations of discoidal LpA-I added at the same concentration of apoA-I is proportional to the number of apoA-I per particle, to the phospholipid to apoA-I ratio, and to the size of the particles, three interrelated parameters. As expected from the literature, similar results are obtained with cholesterol-containing LpA-I, but the initial cholesterol content of LpA-I reduces cellular cholesterol efflux by reducing the cholesterol gradient (Johnson et al., 1991a). Therefore, the most efficient acceptors of cellular cholesterol are, respectively, Lp4A-I, Lp3A-I, and Lp2A-I, an order which reflects the capacity of each particle to bind cholesterol. We propose that this order also reflects the respective stability of these LpA-I in the presence of other plasma lipoproteins. Our studies demonstrate that the large discoidal particles containing several apoA-I molecules which have been found in lymph (Reich, 1990; Sloop et al., 1987) may be the best acceptors of excess cellular cholesterol, a property which may also explain why these lymph lipoproteins are also rich in cholesterol. However, we cannot rule out that the combination of slow uptake of cellular cholesterol by Lp2A-I combined with the very rapid equilibration or transfer of Lp2A-I cholesterol to HDL may represent a very efficient pathway for egress of cellular cholesterol. Although Lp2A-I particles have not been characterized in vivo, evidence has also been presented that lipid-free apoA-I may be generated under specific conditions and can constitute an efficient cholesterol acceptor (Hara & Yokoyama, 1991, 1992). The combination of lipid-free apoA-I with phospholipids and cholesterol may constitute a physiological pathway which generates Lp2A-I or a very unstable LpA-I as a short-lived intermediate. Continued investigations of the pathways which control the genesis and recycling of apoA-I-containing lipoproteins, not only in the intravascular but also in the extravascular compartments, are essential to our understanding of reverse cholesterol transport. We have shown that cholesterol fluxes through LpA-I can vary greatly, but it remains to be understood whether particle size and phospholipid to apoA-I ratio are distinct determinants of efflux, and how and where different LpA-I particles are being generated.

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