Biochemical Characterization of the Three Major Subclasses of Lipoprotein A-I Preparatively Isolated from Human Plasma

Nicolas Duverger,*,†,§ Daniel Rader,‡ Philippe Duchateau,∥ Jean-Charles Fruchart,∥ Graciela Castro,∥ and H. Bryan Brewer, Jr.‡

Molecular Disease Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, and Inserm 325, Institut Pasteur, 1 rue du Professeur Calmette, 59019 Lille, France

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ABSTRACT: Apolipoprotein (apo) A-I is the major protein constituent of plasma high-density lipoproteins (HDL). HDL consist of two major classes of apoA-I-containing lipoproteins: LpA-I and LpA-I:A-II. LpA-I includes heterogeneous lipoprotein particles that differ in size and hydrated density. LpA-I was isolated by immunoaffinity chromatography from the fasting plasma of 24 normal human subjects and separated by gel filtration chromatography. Three major subclasses of LpA-I were eluted: large (Lg-LpA-I), medium (Md-LpA-I), and small LpA-I (Sm-LpA-I). By nondenaturing gradient PAGE, Lg-LpA-I, Md-LpA-I, and Sm-LpA-I had mean Stokes diameters of 10.8 ± 0.5, 8.9 ± 0.5, and 7.5 ± 0.3 nm, respectively. The lipid/protein ratios were 1.25 ± 0.12 for Lg-LpA-I, 0.75 ± 0.10 for Md-LpA-I, and 0.38 ± 0.08 for Sm-LpA-I. Lg-LpA-I was relatively lipid and cholesteryl ester rich compared with Md-LpA-I and Sm-LpA-I. Sm-LpA-I contained phospholipids as the major lipid component. ApoA-I was the major apolipoprotein in all LpA-I subfractions, whereas apoE was present only in Lg-LpA-I and apoA-IV was associated with both Md-LpA-I and Sm-LpA-I. All three LpA-I subclasses exhibited predominantly α mobility on agarose electrophoresis. Lg-LpA-I migrated as a diffuse band in the fast α position, whereas Md-LpA-I and Sm-LpA-I migrated to the slow α position. In addition, both Lg-LpA-I and Sm-LpA-I, but not Md-LpA-I, had components with pre-β electrophoretic mobility. All three LpA-I subclasses bound specifically to Ob 1771 cells and promoted cholesterol efflux. Lg-LpA-I had a significantly higher amount of LCAT and CETP activity per particle than Md-LpA-I and Sm-LpA-I. These data indicate that these LpA-I subclasses have distinctive size, electrophoretic mobility, composition, and metabolic activities and provide new insights into the molecular architecture of LpA-I and HDL.

Plasma levels of high-density lipoprotein (HDL)[1] cholesterol are inversely correlated with the risk of coronary heart disease (CHD) (Gordon & Rifkind, 1989; Gordon et al., 1989); however, the mechanisms by which HDL may be protective against the development of CHD are poorly understood. Human HDL are a heterogeneous population of lipoprotein particles that differ in their physicochemical and metabolic properties. However, relatively little is known about the molecular structure and composition of specific HDL particles.

The major apolipoproteins in HDL are apoA-I and apoA-II. Using immunosorption techniques, HDL particles have been isolated according to their apolipoprotein content (Alaupovic, 1991). It has been shown that HDL consists of two major classes of apoA-I-containing lipoproteins: those which contain both apoA-I and apoA-II (LpA-I:A-II) and lipoproteins which contain apoA-I but no apoA-II (LpA-I) (Cheung & Albers, 1984; Alaupovic et al., 1972). LpA-I has been proposed as a potentially antiatherogenic lipoprotein fraction within HDL (Fruchart & Ailhaud, 1992). LpA-I itself is highly heterogeneous, including subspecies that differ in hydrated density and size (Cheung & Albers, 1984; Ohta et al., 1988). However, detailed studies of the composition, structure, and functional role of specific subclasses of apoA-I-containing lipoproteins have not been reported. We report here the preparative isolation and characterization of the major subclasses of LpA-I in humans.

MATERIALS AND METHODS

Study Subjects. Healthy adult normolipidemic volunteers were placed on a controlled metabolic diet containing 47% carbohydrate, 37% fat, 16% protein, and 200 mg of cholesterol per 1000 kcal for 14 days prior to the study. Then, fresh blood was collected from the fasting volunteers (12 males and 12 females) in tubes containing ethylenediaminetetraacetic acid (EDTA) at a final concentration of 1.5 g/L and cooled on ice immediately. Plasma was collected by low-speed centrifugation for 30 min at 4 °C.

Immunoaffinity Chromatography. Immunosorbents with a mixture of monoclonal antibodies (IgG fraction) to apoA-I (A05–A17–A30) and to apoA-II (G03–G05-G11) (Betard et al., 1987) were prepared by coupling antibodies (300 mg of protein) to CNBr–Sepharose 4B (30 g of dry gel; Pharmacia, Uppsala, Sweden). The final volume of each immunosorbent was approximately 100 mL, and the capacity of the columns was determined to be the equivalent of 8 mL of normal plasma. Immunosorbents were equilibrated with a buffer containing 1 mM KH_2PO_4, 10 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl (pH 7.4), 1 mM EDTA and 0.1% sodium azide. Lipoprotein particles were isolated using a previously described

* To whom correspondence should be addressed at the National Institutes of Health, Building 10, Room 7N117, 9000 Rockville Pike, Bethesda, MD 20892. Phone: 301 496 3460. Fax: 301 402 0190.

‡ National Institutes of Health.

§ Recipient of a postdoctoral fellowship from Rhone-Poulenc-Rorer, 13 quai Jules Guesde, 94403 Vitry sur Seine, France.

∥ Institut Pasteur.

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[1] Abbreviations: HDL, high-density lipoprotein(s); apo, apolipoprotein(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LCAT, lecithin–cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; TC, total cholesterol; FC, free cholesterol; P, phospholipid(s); TG, triglyceride(s); PBS, phosphate-buffered saline; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

immunoaffinity chromatography procedure (Duverger et al., 1993). All lipoprotein isolations were performed at 4 °C. Plasma aliquots (6 mL) were applied to the anti-apoA-II immunosorbent at a flow rate of 10 mL/h in order to remove all apoA-II-containing lipoproteins (LpA-I:A-II). The fraction not retained by the anti-apoA-II immunosorbent was then applied to the anti-apoA-I immunosorbent at a flow rate of 60 mL/h in order to retain all remaining lipoproteins containing apoA-I (LpA-I). Lipoproteins bound to the immunosorbent were eluted with 3 M NaSCN at a flow rate of 60 mL/h and immediately desalted over Sephadex G25 (200 mL; Pharmacia). All isolated lipoprotein particles were concentrated and dialyzed against PBS/0.01% EDTA in a Micro-Prodicon (Spectrum, Houston, TX) and filtered using a 0.22-μm Millipore filter. Total protein, apoA-I, and apoA-II recoveries from the sequential immunochromatography were 90 ± 7%, 95 ± 5%, and 91 ± 6%, respectively.

Gel Filtration Chromatography. Individual LpA-I subclasses were isolated by gel filtration chromatography using fast protein liquid chromatography (FPLC) with three Superose 12 HR 10/30 prepacked columns and one Superose 6 HR 10/30 prepacked column (Pharmacia) in series equilibrated in PBS at a flow rate of 9 mL/h. Total LpA-I (0.5 mg of protein) was injected into the chromatographic system, and 0.2-mL fractions were collected. The system was calibrated for particle molecular weight and size using low and high molecular weight gel filtration kits (Pharmacia). The molecular weight and size of the LpA-I particles were determined from their elution volume based on calibration with globular protein standards (thyroglobulin, ferritin, catalase, aldolase, albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A). The chromatographic system was recalibrated with globular protein standards every four runs (standard procedure). Each peak was detected and integrated by a UV-monitor (280 nm). A liquid chromatography LCC-500 plus controller (Pharmacia) has been used for quantitative evaluation and reporting of chromatographic results. LpA-I subclasses were isolated on two separate occasions from plasma of each of four different study subjects in order to examine the reproducibility of the method. Total protein and apoA-I recoveries from the gel filtration were 95 ± 6% and 96 ± 5%, respectively.

Analytical Methods. Particle Stokes diameters were independently determined by nondenaturing gradient PAGE using precast 4–30% gels (Pharmacia) as described (Nichols et al., 1986); recently, we used precast 4–30% GC-9 gels (Isolab Inc., Akron, OH) and got similar results. Total cholesterol, free cholesterol, triglyceride, and phospholipid were measured using commercial kits (Wako, Osaka, Japan). The protein content of particles was determined by the method of Lowry (Lowry et al., 1951). Molecular volumes of the lipid components of the particles (Shen et al., 1977) and a median specific molecular volume of 2.32 Å/dalton for proteins (Matthews, 1968) were used to calculated total lipoprotein particle volumes. ApoA-I and apoA-II were quantitated by competitive ELISA (Bojanovski et al., 1988; Stein et al., 1986), apoE was determined by radioimmunoassay (Gregg et al., 1983), and LpA-I was quantitated by differential electroimmunoassay (Parra et al., 1990). The specific protein composition of particles was analyzed by 4–20% acrylamide gradient SDS–PAGE electrophoresis (Laemmli, 1970). Two-dimensional gel electrophoresis was performed as previously described (Sprecher et al., 1984). Chemical cross-linking studies were performed with dimethyl suberate according to the method of Swaney and O'Brien (1978). We used the lowest protein mass from cross-linking experiments, i.e., 125, 90, and 50 kDa for Lg-LpA-I, Md-LpA-I, and Sm-LpA-I, respectively, divided by the apoA-I mass to estimate the number of apoA-I molecules present at the surface of each LpA-I subclass. Protein bands were visualized by staining with a solution of 0.03% Coomassie blue dissolved in MeOH/acetic acid/water (25/10/45 v/v) as well as by Western blotting (Towbin et al., 1979). Gels were scanned with a laser densitometer (Pharmacia). Lipoprotein particles were radioiodinated as previously described (Rader et al., 1991) with approximately 0.5 mol of iodine/mol of particle. The electrophoretic mobility of the radiolabeled lipoprotein particles was analyzed by agarose gel electrophoresis using the Titan Gel system (Helena Laboratories, Wako, TX). Lipoproteins were isolated from normolipemic human plasma by sequential flotation ultracentrifugation (Havel et al., 1955). A Student's t test was used to compare the means of groups.

Cell Culture Studies. The characterization of the Ob 1771 mouse preadipocyte cell line has been reported previously (Doglio et al., 1986). Competitive inhibition of binding of ^{125}I-HDL$_3$ to intact Ob 1771 cells was performed for 2 h at 4 °C as described previously (Steinmetz et al., 1990). Nonspecific binding was determined using a 20-fold excess of HDL$_3$.

For cholesterol efflux studies, LDL was labeled with [^{3}H]-cholesteryl linoleate by the method of Craig (Craig et al., 1982). Differentiated Ob 1771 cells were maintained for 48 h in 7% lipoprotein-deficient serum and then exposed to [^{3}H]-cholesteryl linoleate labeled LDL for 48 h (0.15 mg of LDL cholesterol/mL) in the same buffer. Cells were then rinsed in 0.1 M PBS, maintained in serum-free medium, and incubated at 37 °C for 1–6 h with lipoprotein particles or with dimyristoylphosphatidylcholine (50 ng/mL) as a control. Cells were then washed with PBS and solubilized in 0.1 N NaOH. The labeled cholesterol in the media and cells was quantitated by liquid scintillation counting on a β counter (Beckman, Gagny, France).

LCAT and CETP Analysis. The LCAT activity in plasma and isolated lipoproteins was determined by the apoA-I/lecithin/[^{14}C]cholesterol proteoliposome substrate method of Albers (Chen & Albers, 1982) as previously described (Cheung et al., 1986), with the modification that the esterified cholesterol and the unesterified cholesterol were separated by thin-layer chromatography. The CETP activity in plasma and isolated lipoproteins was determined by the method of Albers (Albers et al., 1984). Briefly, lipoprotein samples were mixed with 0.1 mg of a [^{14}C]cholesteryl ester–HDL$_3$ donor and 0.1 mg of LDL acceptor, followed by incubation at 37 °C in a shaking water bath for 5 h. The reaction was stopped by chilling the tubes on wet ice. Donor and acceptor lipoproteins were separated using the dextran sulfate/magnesium chloride precipitation procedure, and radioactivity was quantitated in both. These LCAT and CETP activity assays using exogenous substrates in excess reflect the mass of these two proteins (Chen & Albers, 1982; Albers et al., 1984).

The LCAT and CETP activities in plasma and isolated lipoprotein fractions (LpA-I, LpA-I:A-II, and plasma without apoA-I- or apoA-II-containing lipoproteins) were normalized to the total protein concentration. Recovery of LCAT and CETP activities after immunoaffinity isolation was determined to be 71 ± 10% and 79 ± 10%, respectively. Recovery of LCAT and CETP activities after gel filtration chromatography was 90 ± 7% and 91 ± 5%, respectively. Calculation of the

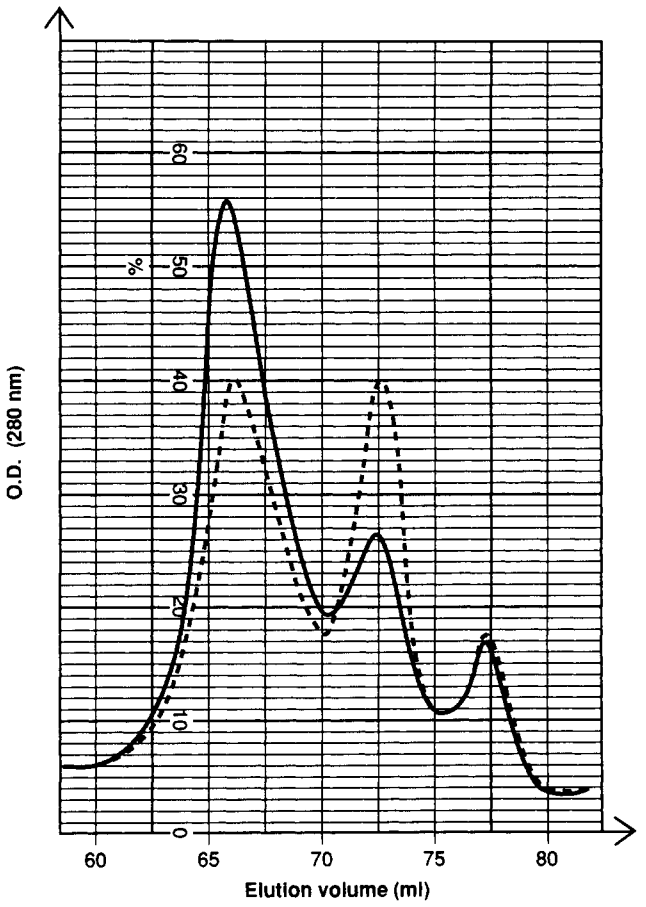

FIGURE 1: Representative examples of gel filtration chromatographic separation of LpA-I subclasses isolated from one male (dashed line) and one female subject (solid line). 0.5 mg of LpA-I protein was applied to a series of three Superose 12 HR 10/30 and one Superose 6 HR 10/30 prepacked columns and eluted at a flow rate 9 mL/h in PBS.

distribution of LCAT and CETP activities among isolated lipoprotein fractions was performed as previously described (Cheung & Albers, 1984).

Antisera against sequence-specific peptides (residues 1–20 of LCAT and residues 1–18 and 290–306 of CETP) were prepared in rabbits, affinity-purified, and used for immunoblotting to detect LCAT and CETP in the isolated lipoprotein particles as described (Francone et al., 1989).

RESULTS

Particle Size and Distribution. Three subclasses of LpA-I particles were separated by gel filtration chromatography and designated large LpA-I (Lg-LpA-I), medium LpA-I (Md-LpA-I), and small LpA-I (Sm-LpA-I). Representative gel filtration profiles from one male and one female subject are illustrated in Figure 1. The distribution (Figure 1) of LpA-I subclasses was different between males and females ($p < 0.001$): LpA-I in males ($n = 12$) consisted of 48 ± 6% Lg-LpA-I, 45 ± 5% Md-LpA-I, and 7 ± 2% Sm-LpA-I, whereas LpA-I in females ($n = 12$) consisted of 68 ± 7% Lg-LpA-I, 26 ± 5% Md-LpA-I, and 6 ± 3% Sm-LpA-I. In contrast, elution volumes of the LpA-I subclasses were similar in males and females, indicating that there were no sex differences with regard to particle size. Using the gel filtration calibrators, the mean molecular masses and Stokes diameters were 365 ± 70 kDa and 12.0 ± 0.9 nm for Lg-LpA-I, 215 ± 45 kDa and 10.3 ± 0.7 nm for Md-LpA-I, and 95 ± 35 kDa and 8.7 ± 0.6 nm for Sm-LpA-I. Gradient nondenaturing PAGE was performed on the LpA-I subclasses in order to independently determine the Stokes diameters and to evaluate the purity of the individual fractions (Figure 2). By this method, Lg-LpA-I, Md-LpA-I, and Sm-LpA-I had mean Stokes diameters of 10.8 ± 0.5, 8.9 ± 0.5, and 7.5 ± 0.3 nm, respectively. The purity of the subclasses was high (<7% contamination between Lg-LpA-I and Md-LpA-I and <4% between Md-LpA-I and Sm-LpA-I). In order to examine the reproducibility of the immunoaffinity isolation and gel filtration chromatography procedures employed in this study, LpA-I subclasses were isolated on two separate occasions from two male and two female subjects. The lipoproteins separated by repeat isolation had a mean difference of less than 2% in distribution and sizes of the lipoproteins.

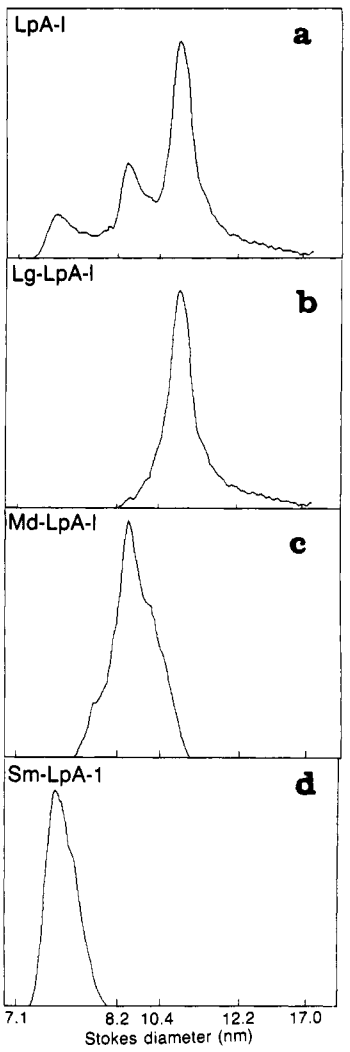

FIGURE 2: Densitometer scans of 4–30% nondenaturing gradient gel electrophoresis of LpA-I subclasses. (a) Total LpA-I; (b) Lg-LpA-I; (c) Md-LpA-I; (d) Sm-LpA-I.

Table I: Lipid Composition of LpA-I Subclasses

	lipid composition (mass %)			
particle, $n = 24$	TC	FC	P	TG
Lg-LpA-I	46.5 ± 1.9	15.1 ± 1.4	43.8 ± 1.9	9.7 ± 0.5
Md-LPA-I	41.1 ± 2.2	11.5 ± 2.0	45.9 ± 1.6	13.0 ± 0.7
Sm-LpA-I	15.9 ± 2.3	9.1 ± 2.0	80.6 ± 2.2	3.6 ± 1.8

Lipoprotein Particle Composition. The lipid composition of the LpA-I subclasses is summarized in Table I. Because there were no significant differences in composition between

Table II: Molecular Composition of LpA-I Particle Subclasses[a]

particles	Lg-LpA-I	Md-LpA-I	Sm-LpA-I
amino acid	1650	1250	720
phospholipid	114	53	29
free cholesterol	78	27	7
cholesteryl ester	96	41	3
triglyceride	22	14	1

[a] Molecules per particle using the following molecular weights for the calculations: amino acid, 100; phospholipid, 775; cholesterol, 387; cholesteryl ester, 650; triglyceride, 850.

Table III: LpA-I Subspecies Volume Calculations[a]

parameter	Lg-LpA-I	Md-LpA-I	Sm-LpA-I
volume ($\times 10^3$ Å^3)	714	440	208
Stokes diameter (nm), calculated	11.1	9.4	7.4
Stokes diameter (nm), from nondenaturing gradient PAGE	10.8 ± 0.5	8.9 ± 0.5	7.5 ± 0.3

[a] Using the following molecular volumes for calculations: amino acid, 2.32 Å^3/dalton; phospholipid, 1270 Å^3; cholesterol, 600 Å^3; cholesteryl ester, 1090 Å^3; triglyceride, 1600 Å^3.

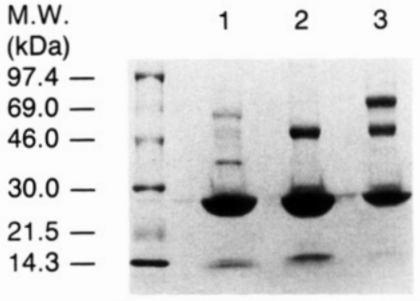

FIGURE 3: Representative SDS–PAGE of LpA-I subclasses. The samples (30 μg) were reduced prior to gel separation. Lane 1, Lg-LpA-I; lane 2, Md-LpA-I; lane 3, Sm-LpA-I.

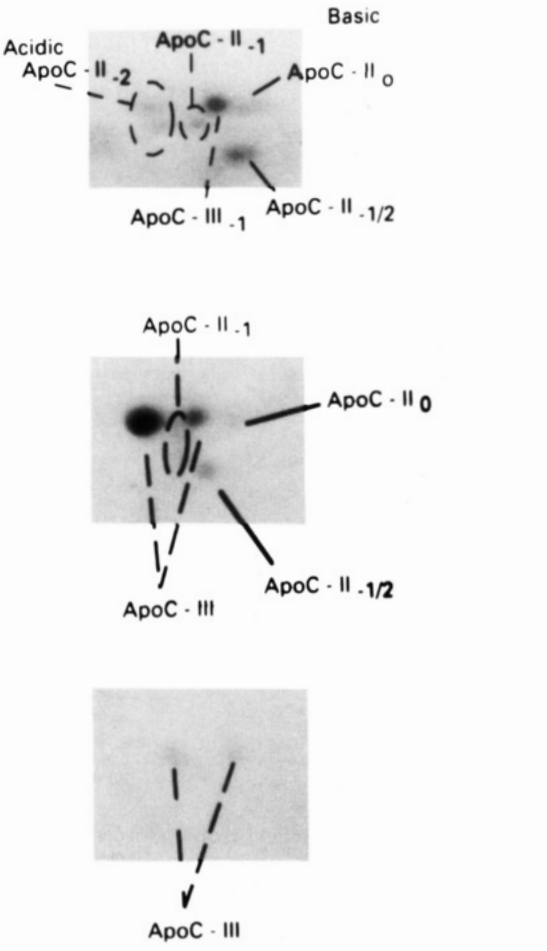

FIGURE 4: Representative two-dimensional electrophoretograms of LpA-I subclasses (apoC region only). Three hundred micrograms of each LpA-I subclass was applied to the gel. (Top) Lg-LpA-I; (middle) Md-LpA-I; (bottom) Sm-LpA-I.

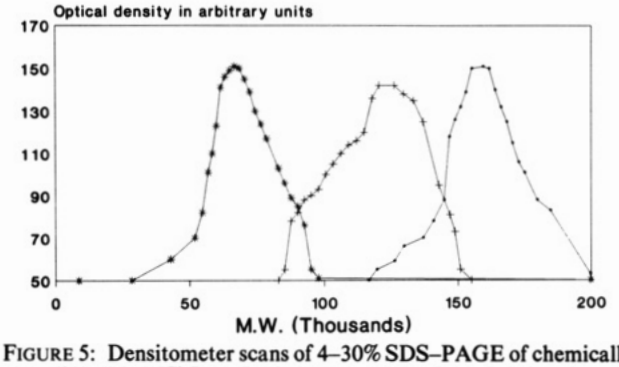

FIGURE 5: Densitometer scans of 4–30% SDS–PAGE of chemically cross-linked (DMS) LpA-I subclasses. (■) Lg-LpA-I; (+) Md-LpA-I; (*) Sm-LpA-I.

males and females, the data were pooled. The lipid/protein ratios, similar between males and females, were 1.25 ± 0.12 for Lg-LpA-I, 0.75 ± 0.10 for Md-LpA-I, and 0.38 ± 0.08 for Sm-LpA-I. Lg-LpA-I was relatively lipid and cholesteryl ester rich compared with Md-LpA-I and Sm-LpA-I. Sm-LpA-I contained phospholipids as the major lipid component. The fraction of unesterified cholesterol/total cholesterol was 0.32 in Lg-LpA-I, 0.28 in Md-LpA-I, and 0.60 in Sm-LpA-I. The calculated molar composition per particle is summarized in Table II. The cholesteryl ester and triglyceride components present in these lipoproteins could only be accommodated in a spherical core, indicating that the majority of the particles are likely to be spherical. The calculated particle volumes based on their molar composition are presented in Table III. These volumes correlated well with the particle diameters determined by nondenaturing gradient gel electrophoresis.

The apolipoprotein content of the LpA-I subclasses was analyzed by SDS–PAGE (Figure 3). ApoA-I was the major apolipoprotein in all LpA-I subfractions. ApoE was present only in Lg-LpA-I. ApoA-IV was associated with both Md-LpA-I and Sm-LpA-I. Sm-LpA-I also contained some albumin. When total LpA-I was chromatographed on an anti-human serum albumin immunosorbent prior to gel filtration, the fraction of Sm-LpA-I was decreased relative to the other two particles, suggesting that the albumin may be a component of some Sm-LpA-I particles and not simply a contaminant.

The content of the C apolipoproteins in LpA-I subclasses was determined by two-dimensional gel electrophoresis. As illustrated in Figure 4, the content of the C apolipoproteins differed among the LpA-I subclasses. ApoC-II was found in Lg-LpA-I and Md-LpA-I but not Sm-LpA-I. In contrast, apoC-III$_2$, the most abundant isoform of apoC-III, was found in Md-LpA-I and Sm-LpA-I but not Lg-LpA-I. The relative proportion of proapoA-I to mature apoA-I in the different subclasses was ascertained using the intensity of apoA-I-stained bands on the 2-D gels. No differences in the apoA-I isoform content were observed among the three LpA-I subclasses.

By cross-linking experiments, the mean protein content of each LpA-I subclass was 165 kDa for Lg-LpA-I, 125 kDa for Md-LpA-I, and 70 kDa for Sm-LpA-I. Assuming that apoA-I is the major protein in each LpA-I subclass, it was determined that, at least four, three, and two molecules of apoA-I are

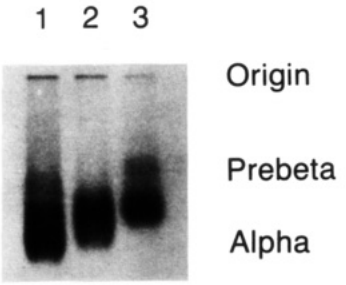

FIGURE 6: Representative agarose electrophoresis of LpA-I subclasses. Radiolabeled LpA-I subclasses were electrophoresed in agarose. Lane 1, Lg-LpA-I; lane 2, Md-LpA-I; lane 3, Sm-LpA-I. Pre-β and α regions are labeled. Individual labeled bands were cut out and identified using an autogradiograph, and the radioactivity was quantited in a γ-radiation spectrometer (see text for values).

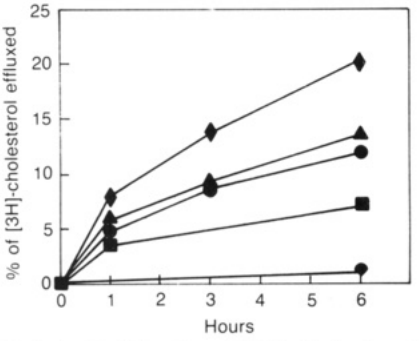

FIGURE 7: Cholesterol efflux from [^{3}H]cholesterol-preloaded Ob 1771 cells. The radioactivity appearing in the medium was expressed as the percent of initial cell-associated [^{3}H]cholesterol. Measurements were performed in triplicate on parallel dishes. The initial cholesterol content (mean ± SD) of cholesterol-preloaded cells was 50 ± 5 μg/mg of cell protein. (▼) DMPC; (▲) total LpA-I; (●) Lg-LpA-I; (♦) Md-LpA-I; (■) Sm-LpA-I.

present at the surface of the Lg-LpA-I, Md-LpA-I, and Sm-LpA-I, respectively.

Agarose Gel Electrophoresis. Radiolabeled LpA-I particle subclasses were applied to nondenaturing agarose gel electrophoresis to determine the relative mobility of these individual particles (Figure 6). All three LpA-I subclasses exhibited predominantly α mobility. Lg-LpA-I migrated as a diffuse band in the fast α position, whereas Md-LpA-I and Sm-LpA-I migrated to the slow α position. In addition, both Lg-LpA-I and Sm-LpA-I, but not Md-LpA-I, had components with pre-β electrophoretic mobility. A relative quantitation of the pre-β versus α migrating LpA-I subclasses was determined by γ spectrometry. The Lg-LpA-I pre-β fraction represented 3% of the total Lg-LpA-I, and the Sm-LpA-I pre-β fraction represented 7% of the total Sm-LpA-I.

Cell Binding and Cholesterol Efflux Promotion. The specificity of the binding of LpA-I subclasses to the Ob 1771 mouse adipose cells was examined by competition studies. ^{125}I-HDL$_3$ were effectively competed by total LpA-I and HDL$_3$. Total LpA-I had an affinity for the HDL$_3$ binding sites that was nearly identical to that of HDL$_3$, indicating that competition with labeled HDL$_3$ is a valid method for assessing the binding of LpA-I subclasses. Lg-LpA-I, Md-LpA-I, and Sm-LpA-I competed with ^{125}I-HDL$_3$ with high affinity, and the differences between their binding were not significant (data not shown).

All three LpA-I subclasses promoted cholesterol efflux from the Ob 1771 cells, whereas no efflux was promoted by the control DMPC liposomes (Figure 7). Md-LpA-I promoted the most cholesterol efflux on a per milligrams of protein basis ($p < 0.01$).

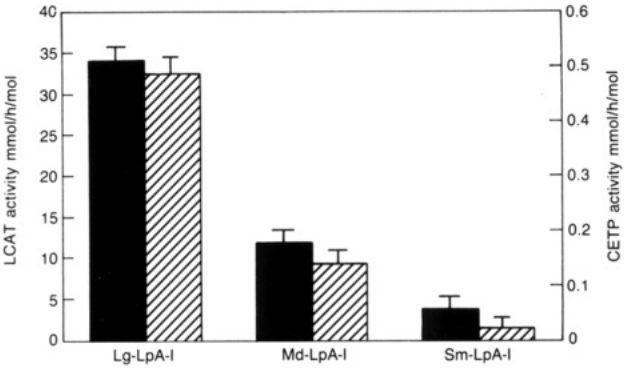

FIGURE 8: Molar quantitation of LCAT (solid bars) and CETP (hatched bars) in LpA-I subclasses. LCAT was assayed by proteoliposome assay (results expressed as nanomoles of cholesteryl ester formed per hour per mole of particle) and CETP by radiolabeled cholesteryl ester transfer assay (results expressed as moles of cholesteryl ester transferred from HDL$_3$ to LDL per hour per mole of particle).

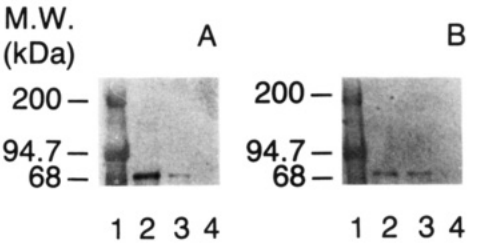

FIGURE 9: Representative immunoblot of LCAT (A) and CETP (B) in LpA-I subclasses. Particles were separated by 4–20% SDS–PAGE, proteins were transferred onto nitrocellulose, and LCAT and CETP were visualized using specific antibodies.

Table IV: Distribution of LCAT and CETP Activity Associated with Isolated Lipoprotein Particles

particle	% of LCAT plasma activity	% of CETP plasma activity
LpA-I	72 ± 15	83 ± 10
Lg-LpA-I	54 ± 8	49 ± 7
Md-LpA-I	16 ± 4	33 ± 15
Sm-LpA-I	2 ± 1	1 ± 1
LpA-I:A-II	13 ± 8	8 ± 5
Lp without apoA-I or apoA-II	15 ± 9	9 ± 5

LCAT and CETP Activity. The LCAT activity of the LpA-I subclasses, expressed as millimoles of cholesteryl ester formed per hour per mole of particle, is shown in Figure 8. Lg-LpA-I had a significantly higher amount of LCAT per particle than Md-LpA-I and Sm-LpA-I ($p < 0.001$). The CETP activity of the LpA-I subclasses, expressed as millimoles of cholesteryl ester transferred per hour per mole of particle, is also shown in Figure 8. Lg-LpA-I also had a significantly higher amount of CETP per particle than Md-LpA-I and Sm-LpA-I ($p < 0.001$). These data were confirmed by immunoblot analysis of the SDS–PAGE of the LpA-I subclasses using antibodies specific for LCAT and CETP (Figure 9). LCAT and CETP were found primarily in Lg-LpA-I, less in Md-LpA-I, and very little in Sm-LpA-I.

The distribution of LCAT and CETP among the isolated lipoprotein particles as a percent of total plasma activities was determined (Table IV). The majority of plasma LCAT and CETP were present in LpA-I rather than LpA-I:A-II, and specifically in the Lg-LpA-I particles.

DISCUSSION

HDL consists of two major classes of apoA-I-containing lipoproteins: LpA-I and LpA-I:A-II. Evidence is accumu-

lating that these two types of HDL particles have different physiologic properties and *in vivo* metabolism (Brewer & Rader, 1991). ApoA-I is synthesized by both liver and intestine, but apoA-II is made only in the liver (Eggerman et al., 1991). The majority of plasma LCAT and CETP was found in association with LpA-I, even though its plasma concentration is lower than that of LpA-I:A-II (Cheung et al., 1986). LpA-I:A-II appears to be a more effective substrate for hepatic lipase than LpA-I (Mowri et al., 1992) and to be the main acceptor of apoC-II, apoC-III, apoD, and apoE within HDL (Bekaert et al., 1991). Finally, apoA-I on LpA-I is catabolized at a faster rate than apoA-I on LpA-I:A-II, suggesting a greater "flux" of LpA-I particles (Rader et al., 1991).

It has been proposed that LpA-I may be more directly protective against the development of atherosclerosis than LpA-I:A-II (Fruchart & Ailhaud, 1992). Although this has been controversial (Cheung et al., 1991), recent epidemiologic data suggest that LpA-I is a better predictor of coronary heart disease risk than LpA-I:A-II (Parra et al., 1992). In addition, an apoA-I-containing subfraction with pre-β mobility has been proposed as a primary acceptor for the cholesterol derived from cells (Castro & Fielding, 1988). LpA-I promotes cholesterol efflux more effectively than LpA-I:A-II from some types of cells (Barbaras et al., 1987), but not from others (Johnson et al., 1991; Ohta et al., 1992). Overexpression of human apoA-I in transgenic mice protects the development of atherosclerotic lesions (Rubin et al., 1991), whereas overexpression of both human apoA-I and apoA-II does not prevent atherogenesis, despite similar plasma levels of HDL and apoA-I (Schultz et al., 1992).

LpA-I itself is highly heterogeneous and includes lipoprotein particles of different hydrated densities and sizes (Cheung & Albers, 1984; James et al., 1988). In order to gain greater understanding of the molecular architecture of HDL subpopulations, we preparatively isolated the major subclasses of LpA-I on the basis of their size differences and analyzed their chemical composition and structure. Using gel filtration chromatography, we isolated three major LpA-I subclasses. The size determinations by gel filtration gave slightly higher values for the Stokes diameters compared to nondenaturing gradient gel electrophoresis. Similar differences between these two methods have been described (Cheung et al., 1986; Kilsdonk et al., 1990). The sizes are consistent with the reported sizes of HDL_2, HDL_3, and VHDL (HDL_4) (Nichols et al., 1986; Shen et al., 1977). The calculated particle volumes based on the molecular composition correlated well with the size determinations by nondenaturing gradient gel electrophoresis.

LpA-I concentrations have been reported to be higher in women than in men (Ohta et al., 1988; Bekaert et al., 1991), which we confirm here. We found that the distribution of LpA-I subclasses was different between males and females, in that females have a significantly higher concentration of Lg-LpA-I. Therefore, the increased levels of apoA-I and LpA-I in women may be due specifically to high concentrations of the Lg-LpA-I particle. There were, however, no differences between males and females in the composition of individual LpA-I subclasses.

The lipid/protein ratios of 1.25 for Lg-LpA-I, 0.75 for Md-LpA-I, and 0.38 for Sm-LpA-I are consistent with the mean lipid/protein ratio of LpA-I of 0.96 ± 0.21 previously reported (Cheung & Wolf, 1988). Using the mean protein content (from the cross-linking experiments) and the molecular weight of each LpA-I subclass, we independently calculated the lipid/protein ratios to be 1.21 for Lg-LpA-I, 0.72 for Md-LpA-I, and 0.35 for Sm-LpA-I. Thus, two independent methods of determining lipid/protein ratios produced very similar results.

There were several differences in apolipoprotein composition among the LpA-I subclasses. ApoA-IV was associated with Md-LpA-I and Sm-LpA-I but not Lg-LpA-I. This finding is in agreement with our data (Duverger et al., 1993) showing that the sizes of immunoaffinity-isolated apoA-IV-containing lipoproteins were in a range corresponding to Md-LpA-I and Sm-LpA-I. The binding of apoA-IV to these two LpA-I subclasses could be explained by its low surface activity compared to other apolipoproteins. Weinberg et al. (1992) analyzed apoA-IV adsorption to a lipid monolayer and found that at pressures >28–29 mN/m apoA-IV could not penetrate the lipid monolayer. Using a similar mathematical approach with the physical parameters and molar composition of the LpA-I subclasses, we calculated that the surface pressures of Lg-LpA-I and Md-LpA-I are 30–35 and 25–30 mN/m, respectively. The value for Md-LpA-I is in agreement with that calculated for HDL_3 by Idbah et al. (1989) (25 mN/m). Therefore, the surface pressure of Lg-LpA-I may be too high to allow the binding of apoA-IV whereas the surface pressure of Md-LpA-I (and probably of Sm-LpA-I) is lower and permits the binding of apoA-IV. Several studies have indicated that apoE was found preferentially in LpA-I:A-II compared with LpA-I (Cheung & Albers, 1984; Ohta et al., 1988; Bekaert et al., 1991), often as an apoE/apoA-II heterodimer (Weisgraber & Mahley, 1978; Ohta et al., 1988). However, apoE has also been found associated with LpA-I (Cheung & Albers, 1984; Ohta et al., 1988; Bekaert et al., 1991). We found that apoE is associated only with Lg-LpA-I and not with Md-LpA-I or Sm-LpA-I. An increase in the HDL core lipid has been shown to be accompanied by an increase in the apoE content of the particle (Gordon et al., 1983). The failure of apoE to bind to Md-LpA-I and Sm-LpA-I might be related to steric effects, surface stoichiometry, or surface curvature. Alternatively, the presence of apoE in Lg-LpA-I may be due to a direct interaction of Lg-LpA-I with TG-rich lipoproteins during the CE and TG exchange mediated by CETP. The presence of apoE not covalently linked to apoA-II in Lg-LpA-I may facilitate the binding of this lipoprotein to the apoB/E receptor (Innerarity et al., 1978). ApoE has also been reported to increase the hydrolysis of PL and TG by hepatic lipase (Thuren et al., 1991); perhaps Lg-LpA-I-containing apoE is a preferred substrate for hepatic lipase.

ApoA-I-containing lipoproteins with pre-β electrophoretic mobility have been described and isolated from human plasma (Kunitake et al., 1985). This pre-β fraction, which does not contain apoA-II, has been proposed to be an initial acceptor of cellular cholesterol (Castro & Fielding, 1988). Pre-β-migrating LpA-I is rapidly transformed to α-migrating lipoprotein (Francone & Fielding, 1990). Some studies using immunoaffinity chromatography to isolate LpA-I have not reported a pre-β component (Cheung & Albers, 1984; Ohta et al., 1988); however, only lipid staining was used to visualize lipoproteins. When we analyzed our radioiodinated isolated LpA-I subclasses by agarose electrophoresis, we consistently observed distinct pre-β-migrating fractions in Sm-LpA-I and Lg-LpA-I, but not in Md-LpA-I. The migration distance on an agarose gel of the Lg-LpA-I pre-β fraction and the Sm-LpA-I pre-β fraction corresponded to the migration distance of $LpA\text{-}I_{pre\beta 2\text{-}3}$ and $LpA\text{-}I_{pre\beta 1}$, respectively, previously described (Castro & Fielding, 1988; Francone et al., 1989). These results indicate that our purification procedure preserves the structure of these potentially instable pre-β particles. On

the basis of their molecular masses, Sm-LpA-I may correspond to pre-β_1 (Castro & Fielding, 1988) and Lg-LpA-I to pre-β_2 and pre-β_3 (Francone et al., 1989). Although these pre-β lipoproteins are in low concentration relative to α-migrating LpA-I, approximately 30% of plasma LCAT and CETP was estimated to be associated with the pre-β_3-migrating LpA-I fraction (Francone et al., 1989). Therefore, much of the LCAT and CETP we found in Lg-LpA-I may be associated with the pre-β fraction of Lg-LpA-I. This would be consistent with the fact that pre-β LpA-I may be discoidal (Sparks & Phillips, 1992) and that synthetic discoidal bilayer apoA-I-containing particles have considerably higher affinity for both LCAT and CETP than spherical vesicles (Nishida et al., 1990).

In order to gain insight into the potential roles of these LpA-I subclasses in reverse cholesterol transport, we studied the individual steps in this process: cell binding, cholesterol efflux promotion, cholesterol esterification, and transfer of CE to apoB-containing lipoproteins. LpA-I has been shown to bind to and promote cholesterol efflux from cholesterol-preloaded Ob 1771 mouse adipose cells (Barbaras et al., 1987) as well as other cell types (Johnson et al., 1991; Oram et al., 1981). LpA-I:A-II has been reported to promote cholesterol efflux from most other types of cells but not from Ob 1771 cells (Barbaras et al., 1987). Ob 1771 cells are unusual in that they are unable to esterify cholesterol (Barbaras et al., 1985), and therefore results obtained using these cells should be interpreted cautiously. Nevertheless, this model is useful to compare differences among lipoprotein particles in their ability to promote cholesterol efflux. All LpA-I subclasses bound specifically to Ob 1771 cells and promoted cholesterol efflux. On a molar basis, Md-LpA-I was the most effective in promoting cholesterol efflux, despite the fact that it is the only one of the three subclasses which does not contain a pre-β component. Hence, pre-β lipoproteins do not appear to be required for promotion of cholesterol efflux from Ob 1771 cells. It has been reported that HDL_3 is more effective in promoting cholesterol efflux than HDL_2 (Barbaras et al., 1986). This is in agreement with the observation that Md-LpA-I (which corresponds in size to HDL_3) is a better efflux promoter than Lg-LpA-I (which corresponds in size to HDL_2). Moreover, apoA-IV/PL complexes have been shown to be more efficient than apoA-I/PL complexes in removing cholesterol from mouse adipose cells (Steinmetz et al., 1990). Hence, the presence of apoA-IV may enhance the ability of Md-LpA-I to promote cholesterol efflux from Ob 1771 cells.

The affinity of LCAT and CETP for various native or reconstituted lipoproteins and their interaction at the lipid/water interface have been investigated (Yamazaki et al., 1983; Nishida et al., 1990; Morton, 1985). LCAT and CETP activity assays using exogenous substrates in excess reflect the mass of these two proteins. In the present study, LCAT and CETP were also preferentially associated with LpA-I rather than LpA-I:A-II, consistent with a previous report (Cheung et al., 1986). LCAT and CETP were primarily associated with Lg-LpA-I and Md-LpA-I as assessed both by activity assays and also by direct immunoblotting of both proteins. LCAT and CETP have been shown to bind to both HDL_2 and HDL_3 (Yamazaki et al., 1983; Nishida et al., 1990). Nishida et al. (1990) showed that LCAT and CETP probably directly interact with each other at the surface of HDL_3 but not of HDL_2 and speculated that this interaction may result in greater activity. Therefore, even though Lg-LpA-I contains more LCAT and CETP mass per mole of particle, these proteins may be more active on smaller Md-LpA-I particles where they may be more likely to directly interact.

In summary, 3 discrete subclasses of LpA-I have been preparatively isolated from the plasma of 24 normolipidemic human subjects and characterized. Our results indicate that these lipoprotein particles have distinctive size, electrophoretic mobility, composition, and metabolic activities, and thus provide new insights into the molecular architecture of HDL and the potential roles of LpA-I subclasses in reverse cholesterol transport.

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