Biochemical Characterization of Pre-β_1 High-Density Lipoprotein from Human Ovarian Follicular Fluid: Evidence for the Presence of a Lipid Core[†,‡]

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ABSTRACT: In order to isolate pre-β_1 HDL, we have focused our interest on a particular model, namely, human preovulatory follicular fluid, which contains only HDL as a lipoprotein class as well as a high proportion of pre-β_1 HDL relative to total HDL (1.5 times more than in homologous plasma) as evidenced by double-dimension gel electrophoresis. Apo A-I in pre-β_1 HDL represented 17.6% of total apo A-I. Stokes' radii corresponded to 3.42 nm in follicular fluid pre-β_1 HDL and 3.48 nm in homologous plasma counterparts. After electroelution from agarose, pre-β_1 HDL were isolated in amounts sufficient to allow characterization by size-exclusion chromatography using HPLC. The estimated apparent molecular mass of these particles is 61.6 kDa. Lipid composition of pre-β_1 HDL evidenced a low lipid content compared to follicular fluid HDL isolated by ultracentrifugation. Phospholipid composition showed a dramatic decrease in phosphatidylcholines (40.5% of total phospholipids), and the presence of lysophosphatidylcholines and of acidic phospholipids such as phosphatidylserine and phosphatidylinositol (13.6 and 13.7%, respectively). Furthermore, cholesteryl ester and triacylglycerol molecules were quantified by gas−liquid chromatography and represented 8−9% of the pre-β_1 HDL total weight. Thus, a lipid core is present in pre-β_1 HDL, which would be compatible with a spherical shape. The follicular fluid appears to be a good model to a better understanding of HDL metabolism.

High-density lipoproteins (HDL)[1] are involved in the reverse cholesterol transport from peripheral cells to the liver (Miller et al., 1985), and this process may involve several HDL subspecies (Castro & Fielding, 1988). Human plasma HDL are heterogeneous in terms of particle size, density, lipid content, and apolipoprotein composition (Eisenberg, 1984). On the basis of their electrophoretic mobility on agarose, HDL have been divided into two main populations: the major subfractions with α mobility, and the others with pre-β mobility (pre-β HDL) (Kunitake et al., 1985; Castro & Fielding 1988; Huang et al., 1993). Most of HDL in plasma are α-HDL, and pre-β HDL represent only 2−10% of total apoA-I, the main apo HDL (Kunitake et al., 1985; Neary et al., 1991). These pre-β HDL are further resolved into pre-β_1, pre-β_2, and pre-β_3 HDL particles according to increasing size, as assessed by two-dimensional gel electrophoresis (Francone et al., 1989). Pre-β_1 HDL are considered to be the first acceptors of cellular cholesterol (Castro & Fielding, 1988; Huang et al., 1993). These pre-β_1 are also present in the interstitial fluid (Asztalos et al., 1993). In contrast to the plasma and the interstitial fluid, the human follicular fluid obtained from preovulatory follicles contains a single class of lipoproteins, HDL (Perret et al., 1985). In the ovarian follicle, granulosa cells are in direct contact with follicular fluid, but are separated from the blood capillaries by the basement membrane (Schreiber & Weinstein, 1986). In this work, we have evidenced the presence of pre-β_1 HDL in the human follicular fluid, which surrounds the preovulatory oocyte. Furthermore, we have isolated and characterized these particles, and their lipid and protein compositions were analyzed.

MATERIALS AND METHODS

Preovulatory follicular fluids and plasma were obtained from patients engaged in an *in vitro* fertilization program as previously described (Parinaud et al. 1993). Hyperstimulation was realized in a two-step procedure. The first step consists of a pituitary blockade using LH−RH analogs, which abolish endogenous secretion of FSH and LH. The second step consists of follicular stimulation achieved by injection of human menopausal gonadotropins. Preovulatory follicles were punctured during laparoscopy. Only the cleanest follicular fluids, with evidence of no blood cell contamination, were retained. Calibration standards for exclusion chromatography and polyacrylamide gel electrophoresis were obtained from Bio-Rad (Ivry sur Seine, France) and Pharmacia (St. Quentin-en-Yvelines, France), respectively. Rabbit anti-human albumin was obtained from Sigma (St. Quentin Fallavier, France). Monoclonal antibodies against human apo A-I, human apo-E (3H1), and apo-AII

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[1] Abbreviations: FF, follicular fluid; GLC, gas−liquid chromatography; apoA-I, apolipoprotein A-I; HDL, high-density lipoprotein(s); TG, triacylglycerol; CE, cholesteryl ester; VHDL, very high density lipoprotein(s).

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were kindly provided by Dr. Ross Milne and Dr. Yves Marcel. Goat ^{125}I-labeled F(ab′)$_2$ anti-mouse IgGs were from Amersham (Les Ulis, France). Hydragel apo AI-B were obtained from Sebia (Issy les Moulineaux, France).

Isolation of Follicular Fluid HDL. HDL were obtained from follicular fluid, after the addition of an anti-protease cocktail containing EDTA (0.2 mM), sodium azide (0.01%, w/v), phenylmethanesulfonyl fluoride (0.1 mM), iodoacetamide (1 mM), 1,10-phenanthroline (1 mM), leupeptin (0.1 mM), and pepstatin A (1 μM). HDL were isolated by a single step of follicular fluid ultracentrifugation at a density of 1.21 g/mL, at 120000*g*, during 40 h at 7 °C.

Two-Dimensional Nondenaturing Gel Electrophoresis, Electrotransfer, and Immunoblots. A first dimension was carried out as previously described (Barrans et al., 1994). In the second dimension, polyacrylamide gel electrophoresis was carried out on a 2−15% or 4−30% gradient (Pharmacia, Bromma, Sweden) with a 2% (w/v) stacking gel (2 cm). Two strips of agarose (0.5 × 5 cm) were carefully loaded upon the stacking gel and fixed in place with 0.75% agarose. Migration was realized in Tris (25 mM)/glycine (0.2 M) buffer at pH 8.3 during 1 h, 30 min at 300 V and 3 h, 30 min at 400 V for the 2−15% gradient gels and 16 h at 200 V for the 4−30% gradient. After electrophoresis, the gels were then electrotransferred and immunoblotted against apo A-I according to Barrans et al. (1994), using as first antibody a mixture of monoclonal antibodies raised against human apo A-I and then ^{125}I-labeled goat anti-mouse F(ab′)$_2$ as second antibody. Membranes were exposed overnight to a PhosphorImager screen (Molecular Dynamics, France), and the cassettes were imaged by PhosphorImager. Quantitation of apo A-I was performed using ImageQuant 1.0, and data were expressed as pixel points determined by the computer and were linearly correlated with the dpm of ^{125}I bound to the antigen−antibody complex.

Electroelution of Total Pre-β HDL and α HDL from Agarose Gel Electrophoresis. Follicular fluid electrophoresis onto agarose (3 mm thickness) was carried out at a constant voltage of 150 V in a 50 mM barbital buffer (5,5-diethylbarbituric acid sodium salt, pH 8.6) and at 4 °C. After this electrophoretic migration, a strip of agarose was cut out between pre-β and α HDL, and upstream of α HDL, and electroelution of these particles was realized in barbital buffer (0.1% EDTA was added to these particles). Parts of electroeluted pre-β HDL and α HDL were isolated at a density of 1.25 and 1.21 g/mL, respectively, by ultracentrifugation at 120000*g* during 40 h, at 7 °C. These particles will be further referred to as electroeluted total pre-β HDL and α HDL, in contrast to HDL obtained from a single step of follicular fluid ultracentrifugation (d < 1.21 g.mL), thus named ultracentrifuged HDL.

Size-Exclusion Chromatography. After electroelution of pre-β HDL in barbital buffer as described above, pre-β_1 HDL were further purified by molecular sieving on an HPLC system (Beckman Gold) using a SEC 2000 column (molecular mass range 1−250 kDa, Beckman), and 50 mM Tris-HCl (pH 7.4) as running buffer (0.4 mL/min flow rate, 280 nm wavelength detection). The void volume corresponded to 5.45 mL. Thyroglobulin (670 kDa), γ globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa) were used as standards. The elution volumes were plotted against log MW. Apo A-I-containing fractions were identified using a dot blot immunoassay as previously described (Minnich et al., 1992) and then were pooled for further analysis.

Extraction and Analysis of HDL Neutral Lipids by Gas−Liquid Chromatography. Lipids of the different particles were extracted according to Bligh and Dyer (1959) after acidification of the aqueous phase with formic acid (12 μL/mL). Before extraction, four internal standards were added on the basis of 20 nmol of free cholesterol extracted: 3 μg of stigmasterol, 6 μg of 1,3-dimyristoyl-*sn*-glycerol (DG14:0), 6 μg of heptadecanoylcholesterol (CE 17:0), and 1 μg of triheptadecanoylglycerol (TG 17:0), obtained from Sigma (Saint Quentin Fallavier, France). After evaporation to dryness of the chloroform phase, samples were dissolved in 100 μL of ethyl acetate. Lipids were analyzed by gas−liquid chromatography (Intersmat, Model120 DFL), using an Ultra 1 Hewlett Packard fused silica capillary column (5 m × 0.31 m i.d.) coated with cross-linked methylsilicon. The oven temperature was programmed from 205 to 345 °C at a rate of 4 °C/min, and the carrier gas was hydrogen (0.6 bar). Profiles of neutral lipid molecular species were determined according to total acyl carbon number (C_n). The response factors for the different lipid classes were determined using a mixture of internal standards. The variation coefficients of intra- and interassays represented less than 6%.

Other Analytical Methods. Measurement of apo A-I in HDL subfractions was realized from dot blot immunoassay by densitometric analysis of autoradiograms, using purified apo A-I as a standard for the establishment of calibration curves. Hydragel apo A-I/B were also used for the quantification of apo A-I based on an electroimmunodiffusion technique in an agarose gel, using a pool of human sera as a standard of apo A-I/B. Measurement of proteins was realized according to Lowry et al. (1951) using bovine serum albumin as a standard. Eventually, follicular fluid and plasma proteins were separated on cellulose acetate followed by Ponceau red staining and densitometric analysis. Lipid extraction was performed as described by Bligh and Dyer (1959), after acidification of the aqueous phase by formic acid. Phospholipids were measured according to their phosphorus content (Böttcher et al., 1961) following lipid extraction. Different classes of lipids were separated by thin-layer chromatography (TLC) on Silicagel G60. For phospholipids, the separation was realized by two-dimensional TLC as previously described (Fourcade et al., 1995) using for the first dimension a mixture of chloroform, methanol, and ammonia (65/25/5) and for the second dimension a mixture of chloroform, methanol, acetic acid, and water (45/20/6/1). Results are expressed as means ± SE. Statistical comparisons were performed using the Student's *t*-test, for unpaired samples.

RESULTS

Characterization of Pre-β_1 HDL from Follicular Fluid Compared to Plasma Counterparts Using Two-Dimensional Gel Electrophoresis. In this study, follicular fluid was used as a source of pre-β_1 HDL. The presence of HDL as the single class of lipoprotein in the follicular fluid during the preovulatory period makes it an interesting model to study HDL metabolism without the contribution of any other lipoprotein (i.e., VLDL and LDL). We analyzed the apo A-I distribution in follicular fluid and homologous plasma by two-dimensional gel electrophoresis (2−15%). After

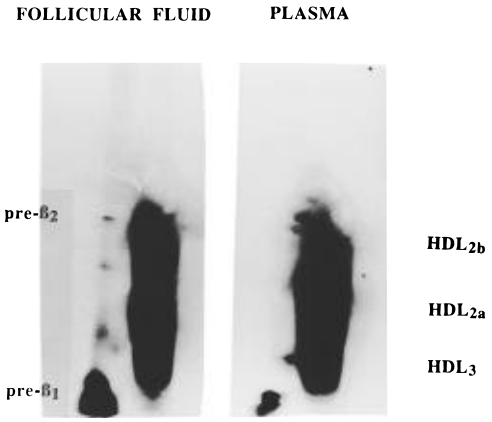

FIGURE 1: Apo A-I distribution in follicular fluid and homologous plasma lipoproteins. Immunodetection of apoA-I containing HDL subpopulations of human follicular fluid (left panel) and homologous plasma (right panel), by two-dimensional nondenaturing gel electrophoresis (2/15%). Gels were transferred to nitrocellulose and blotted first with a mixture of monoclonal antibodies raised against human Apo A-I and then with ^{125}I-labeled goat anti-mouse F(ab′)$_2$ as second antibody.

electrotransfer to nitrocellulose, the distribution of apo A-I was detected with specific antibodies. Total follicular fluid and homologous plasma apo A-I concentrations were 0.95 ± 0.07 and 1.60 ± 0.10 g/L, respectively ($n = 5$), which is significantly different ($p < 0.001$). Follicular fluid and plasma were loaded onto the gel at an equal volume, and pre-β migrating HDL were evidenced in follicular fluid and plasma (Figure 1). The proportion of pre-β_1 HDL in follicular fluid (estimated as described under Materials and Methods) was significantly greater than in plasma ($p < 0.001$), about 17.6 ± 1.2% and 10.9 ± 1.3% ($n = 5$) of total HDL, respectively. We also evidenced some spots having pre-β migration which might correspond to pre-β_2 HDL (Figure 1). The presence of pre-β_2 HDL was very variable between individuals, whereas pre-β_1 HDL were always present. Using specific antibodies against apo E and apo A-II, we observed that these apolipoproteins are completely absent in preβ_1 HDL. The apparent Stokes radius of pre-β_1 HDL was determined (Figure 2) by comparison with protein standards using two-dimensional gel eletrophoresis at equilibrium (4−30%). They corresponded to 3.42 and 3.48 nm in follicular fluid and homologous plasma, respectively. We also estimated the Stokes radius of the very high density lipoproteins (VHDL) in follicular fluid and plasma (3.61 and 3.29 nm, respectively).

Isolation of Follicular Fluid Pre-β_1 HDL by HPLC. Pre-β_1 HDL were isolated by a two-step procedure. First, after follicular fluid electrophoretic migration onto an agarose gel, total pre-β HDL were electroeluted in barbital buffer. Then, pre-β_1 HDL were further separated from the bulk of pre-β HDL by HPLC using size-exclusion chromatography. Figure 3 shows a HPLC profile obtained after elution of pre-β HDL. After immunodetection, only fractions containing apo A-I were pooled. A very low amount of apo A-I was present in the void volume corresponding probably to pre-β_2 HDL. The apparent molecular mass of pre-β_1 HDL was estimated at 61.6 ± 0.6 kDa ($n = 9$) from the elution volumes. When the same procedure was used for electroeluted alpha HDL from an agarose gel, a small peak of VHDL was detected at an apparent molecular mass of 80.6 ± 5.0 kDa ($n = 3$) (not shown), whereas the bulk of alpha HDL remained in the void volume. We also tested the effect of a mixture of monoclonal antibodies raised against apo A-I on the elution pattern of pre-β HDL obtained from an agarose gel. After incubation with these antibodies for 30 min at room temperature, pre-β_1 HDL were eluted on a SEC 2000 column with a retention volume corresponding to a higher molecular mass, at the column exclusion limit (Figure 3). This size shift evidenced a complex between the antibodies and apo A-I containing pre-β_1 HDL.

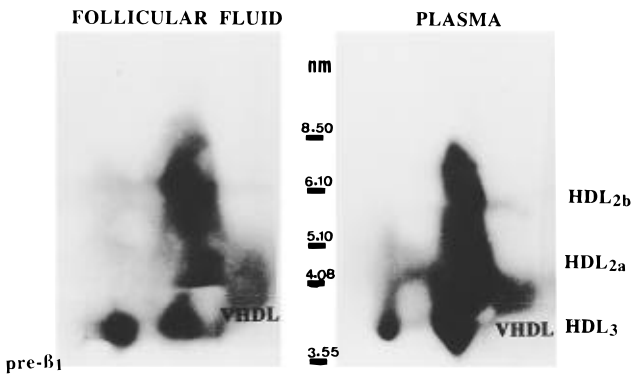

FIGURE 2: Determination of follicular fluid and plasma pre-β_1 HDL Stokes radii. Immunodetection of apoA-I containing HDL subpopulations of human follicular fluid (left panel) and homologous plasma (right panel), in two-dimensional nondenaturing gel electrophoresis 4/30% at equilibrium. Gels were transferred to nitrocellulose and blotted first with a mixture of monoclonal antibodies raised against human apo A-I and then with ^{125}I-labeled goat anti-mouse F(ab′)$_2$ as second antibody. Hydrodynamic Stokes radii of standards: thyroglobulin (8.50 nm), ferritin (6.10 nm), catalase (5.10 nm), lactate dehydrogenase (4.08 nm), and bovine albumin (3.55 nm).

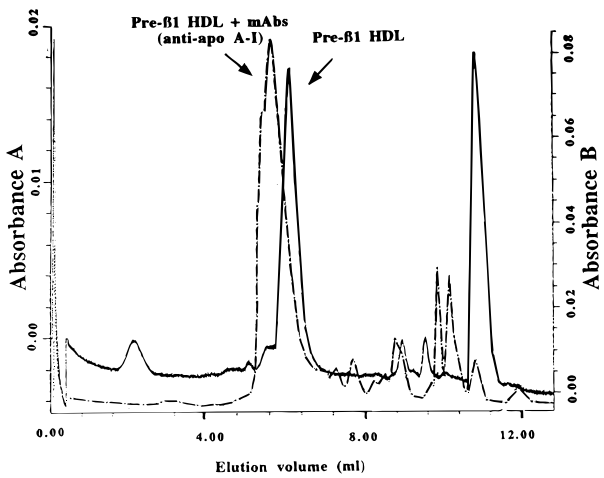

FIGURE 3: Size-exclusion chromatography of follicular fluid pre-β_1 HDL using HPLC. After electroelution of follicular fluid pre-β HDL from agarose gel, as described under Materials and Methods, pre-β_1 HDL were further purified by molecular sieving on an HPLC system (Beckman Gold) using a SEC 2000 column (exclusion limit 250 kDa, Beckman), and 50 mM Tris-HCl (pH 7.4) as running buffer (0.4 mL/min flow rate, 280 nm wavelength detection). Optical density (280 nm) is plotted against elution volume (mL). The elution profile of isolated pre-β_1 HDL control (absorbance A, —) is compared to the elution profile of pre-β_1 HDL (absorbance B, —·—) following a prior incubation with a mixture of monoclonal antibodies (mAbs) raised against human apo A-I. In the elution of control pre-β_1 HDL (A), all the apo A-I is present in the major peak eluting at 6.4 mL, while the peak eluting at 10.8 mL corresponds to barbital salts. The amplitude of this salt peak seems different between the two profiles due to change in scale. Thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa) were used as standards. The elution volumes were plotted against the log MW.

Table 1: Lipid Composition of Pre-β_1 HDL Compared to Pre-β HDL and α HDL[a]

	FC/A-I (μmol/mg)	TC/A-I (μmol/mg)	(TG/A-I (μmol/mg)	PL/A-I (μmol/mg)	FC/PL molar ratio
pre-β_1 HDL, $n = 6$	0.05 ± 0.01	0.21 ± 0.04	0.02 ± 0.01	0.43 ± 0.07	0.13 ± 0.04
total pre-β HDL, $n = 4$	0.08 ± 0.01	1.00 ± 0.09	0.02 ± 0.01	0.45 ± 0.09	0.26 ± 0.09
α HDL, $n = 4$	0.14 ± 0.04	1.22 ± 0.17	0.04 ± 0.01	0.66 ± 0.17	0.17 ± 0.06

[a] Results are expressed as means ± SE. Electroeluted pre-β_1 HDL from agarose were isolated by HPLC. Total pre-β HDL and alpha HDL were isolated by ultracentrifugation ($d < 1.25$ and $d < 1.21$ g/mL, respectively), following a prior electroelution on agarose gel, as described under Materials and Methods. Lipids were analyzed by gas−liquid chromatography, and apo A-I was measured as described under Materials and Methods. Abbreviations: PL, phospholipid; TG, triacylglycerol; FC, free cholesterol; TC, total cholesterol.

Table 2: Phospholipid Composition[a]

PL (mol %)	LPC	SM	PC	PS-PI	LPA	PE
pre-β_1 HDL, $n = 5$	13.57 ± 2.93[b]	16.03 ± 2.09	40.51 ± 7.99[b]	13.70 ± 1.02[c]	5.19 ± 2.42[c]	11.08 ± 3.96
HDL, $n = 3$	2.79 ± 0.86	13.76 ± 1.07	76.01 ± 2.03	3.08 ± 0.25	ND	4.36 ± 0.73
follicular fluids, $n = 7$	22.35 ± 1.24	11.11 ± 0.78	45.43 ± 1.00	6.70 ± 0.70	8.07 ± 0.49	6.30 ± 0.45

[a] Results are expressed as means ± SE. Follicular fluids were obtained from seven patients engaged in an *in vitro* fertilization program. Phospholipid of pre-β_1 HDL isolated by HPLC and HDL isolated from a single-step procedure of follicular fluid ultracentrifugation ($d < 1.21$ g/mL) were extracted according to Bligh and Dyer (1959) and separated as described under Materials and Methods. Abbreviations: LPC, lysophosphatidylcholine; SM, sphingomeylin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; LPA, lysophosphatidic acid; PE, phosphatidylethanolamine; ND, not detectable. Statistical comparison between pre-β_1 HDL and ultracentrifuged HDL. [b] $p < 0.05$. [c] $p < 0.001$.

Table 3: Ponderal Composition of Pre-β_1 HDL and Electroeluted α HDL[a]

	% FC	% EC	% TG	% PL	% A-I
pre-β_1 HDL, $n = 6$	1.28 ± 0.36	7.16 ± 1.53	1.46 ± 0.80	22.46 ± 3.06	67.64 ± 2.33
α HDL, $n = 4$	1.57 ± 0.33	28.53 ± 2.75	1.82 ± 0.60	20.58 ± 4.43	47.50 ± 2.03

[a] All results are expressed as the mean ± SE. Pre-β_1 HDL were isolated by HPLC, and α HDL were isolated by ultracentrifugation ($d < 1.21$ g.mL), following a prior electroelution on agarose gel, as described under Materials and Methods. These calculations are based on the molecular weights of apo A-I (28 000), free cholesterol (FC) (386), esterified cholesterol (EC) (645), triacylglycerol (TG) (880), and phospholipids (PL) (775).

Lipid Composition of Pre-β_1 HDL Isolated from Follicular Fluid. Pre-β_1 HDL were isolated in amounts sufficient to allow characterization (Table 1). Neutral lipids of pre-β_1 HDL isolated by size-exclusion chromatography, electroeluted total pre-β HDL, and alpha HDL were analyzed by gas−liquid chromatography (GLC). The high sensitivity of GLC in lipid analysis enabled us to evidence for the first time the presence of a lipid core (esterified cholesterol and triacylglycerols) in pre-β_1 HDL (Table 1). The ratio of unesterified cholesterol to apo A-I expressed as micromoles per milligram was lower in pre-β_1 HDL (0.05 ± 0.01) than in total pre-β HDL (0.08 ± 0.01) and in unfractionated alpha HDL (0.14 ± 0.01). Free cholesterol represented 24.46 ± 5.13% of total cholesterol in pre-β_1 HDL compared to 8.70 ± 1.81% in total pre-β HDL and 11.74 ± 2.64% in follicular fluid HDL. The ratio of phospholipids/A-I expressed as micromoles per milligram was 0.43 ± 0.07 in pre-β_1 HDL compared to 0.66 ± 0.17 in alpha HDL. This value is very close to that previously reported in total follicular fluid HDL isolated by a single step of ultracentrifugation (Perret et al., 1985). Finally, the molar ratio of free cholesterol to phospholipids in pre-β_1 HDL corresponded to 0.13 ± 0.04 compared to 0.17 ± 0.06 in alpha HDL. The distribution of phospholipid classes in pre-β_1 HDL evidenced a high content of lysophosphatidylcholines, 13.57 ± 2.93% of total phospholipids compared to 2.79 ± 0.86% in follicular fluid total HDL (Table 2). It is noteworthy that lysophosphatidylcholines are abundant in total follicular fluid (22.35 ± 1.24%) but most of it was found associated with albumin (Perret et al., 1985). The proportion of phosphatidylcholine (40.51 ± 7.99%) was significantly lower than in HDL (76.01 ± 2.03%), whereas the contributions of phosphatidylethanolamine and phosphatidylserine−phosphatidylinositol were much higher: 11.08 ± 3.96% and 13.70 ± 1.02% compared to 4.36 ± 0.73% and 3.08 ± 0.25%, respectively. We also evidenced the presence of another minor phospholipid species in pre-β_1 which was tentatively identified as lysophosphatidic acid and represented about 5.19 ± 2.42%. The same compound was evident among total follicular fluid phospholipids but was undetectable in ultracentrifuged HDL.

DISCUSSION

Follicular fluid is an interesting model of extravascular fluid which contains, in the preovulatory period, a single class of lipoprotein, the HDL (Perret et al., 1985). The further resolution of the HDL subfractions in this fluid, by double-dimension electrophoresis, revealed a high proportion of pre-β HDL. Indeed, pre-β_1 HDL in this model represented 17.6 ± 1.2% of total HDL apo A-I compared to 10.9 ± 1.3% in plasma ($n = 5$), which is significantly different ($p < 0.001$). However, when referred to total apo A-I concentration, we have estimated that the apo A-I concentration in follicular fluid pre-β_1 HDL was 0.17 ± 0.02 g/L, which is identical to their concentration in the plasma (0.17 ± 0.01 g/L). While the total protein concentration was significantly lower in the follicular fluid than in plasma, the estimated albumin concentration was not (35.7 ± 2.9 and 43.4 ± 1.5 g/L in the follicular fluid and the plasma, respectively). This may be the reflection of a selective filtration of plasma molecules through the follicle barrier, and the smallest (apo A-I containing) HDL subfractions would thus go through too. Accordingly, it was reported that follicular fluid HDL

would be mostly of small size compared to plasma HDL (Le Goff, 1994). These data suggest that follicular fluid pre-β_1 HDL may merely proceed from plasma through follicle filtration. However, due to their particular composition, this process may involve some local modification of these particles.

The lipid composition of follicular fluid pre-β_1 HDL evidenced low free cholesterol/apo A-I (micromoles per milligram) and free cholesterol/phospholipid molar ratios as compared to alpha HDL, making them a good candidate for the removal of the cellular cholesterol as emphasized earlier (Phillips et al., 1987). Indeed, pre-β_1 HDL have been demonstrated to play a major role as first cholesterol acceptors (Castro & Fielding, 1988; Huang et al., 1993). Using the high sensitivity of gas−liquid chromatography, we determined for the first time the presence of a lipid core in pre-β_1 HDL, containing esterified cholesterol and triacylglycerol, and representing about 8.5% of the total particle weight. On the basis of an apparent molecular mass of 61 600 Da for pre-β_1 HDL as estimated by HPLC, the predicted molecular composition of this particle would be 1−2 apo A-I (1.5), 18 phospholipids, 2 free cholesterol, 7 esterified cholesterol, and 1 triacylglycerol molecules per particle. HDL particles are heterogeneous, and even after a separation procedure, HDL subfractions consist of particles different in size and apolipoprotein/lipid contents (see for instance HDL_2/HDL_3 or LpA-I/LpAI−AII). However, in most cases HDL subfractions appear as a single peak upon gel filtration. In the case of follicular fluid pre-β_1 HDL, the peak limits on a SEC 2000 column ranged between 6.10 and 6.83 mL, corresponding to an interval of 40 433 and 86 800 daltons as molecular mass. Such an interval could be largely accounted for by the presence/absence of one or two apo A-I copies, associated with lipids. Due to the probable heterogeneity of pre-β_1 HDL, only an average composition can be estimated, like with other kinds of HDL subfractions. As regards alpha HDL, on the basis of a molecular mass of 180 000 Da, the predicted molecular composition would be 3 apo A-I, 48 phospholipid, 7 free cholesterol, 80 esterified cholesterol, and 4 triacylglycerol molecules. These estimations were calculated from the molecular mass values of 28 000 Da for apo A-I, 386 Da for free cholesterol, 645 Da for esterified cholesterol, 880 Da for triacylglycerol, and 775 Da for phospholipids. This molecular composition of pre-β_1 HDL contrasts considerably with that described by Castro et al. (Castro & Fielding, 1988) for plasma pre-β_1 HDL, who reported the absence of esterified cholesterol.

As already discussed, pre-β_1 HDL may result from plasma filtration through the follicle barrier. However, their composition seems to be the reflection of particular metabolic events occurring in the follicular fluid, which is supported by the presence of lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) in this medium. In plasma, the high instability of pre-β_1 HDL, in the absence of cell cholesterol donors, has been attributed to the continuous action of LCAT, acting on larger pre-β_3 and alpha HDL, since LCAT inhibition prevented pre-β_1 HDL disappearance (Ishida et al., 1990; Miida et al., 1992; Kawano et al., 1993). But, in follicular fluids, LCAT activity is at a twice lower level than in plasma, and CETP activity is at a comparable level (B. Jaspard, ongoing studies). However, in follicular fluids, the CETP-mediated transfer of esterified cholesterol cannot be direct toward low-density lipoproteins, but only within the HDL class. Hence, CETP may act as an esterified cholesterol shuttle, between alpha HDL and pre-β_1 HDL, thus favoring the formation of a neutral lipid core.

With a Stokes radius estimated at 3.42 nm for follicular pre-β_1 HDL (compared to 3.48 nm in plasma pre-β_1 HDL), we can calculate a total particle volume of 168 nm^3 and an internal volume of 12 nm^3, considering the thickness of the amphipathic layer at about 2.0 nm (Kédzy, 1978). Thus, the ratio of internal to total volume represents 7.2%, which is in good agreement with the mass proportion of lipid core measured in the pre-β_1 HDL, and could be compatible with a spherical shape. But, because the particles are so protein-rich there are other possibilities such as amphipathic protein-stabilized lipid domains. That such structure may actually be present is supported by evidence adduced by Kunitake et al. (1990), who demonstrate conformational differences between the apo A-I in plasma pre-β HDL and α species.

On the other hand, it has been proposed that pre-β HDL correspond to nascent HDL with a discoidal structure (Francone & Fielding, 1990). Numerous studies performed on synthetic HDL (Sparks et al., 1992a,b; Dalton & Swaney, 1993) containing two to four apo A-I per particle (Lp2A-I, -3A-I, and -4A-I) and palmitoyloleylphosphatidylcholine with or without esterified cholesterol have shown that particles devoid of apolar lipids present a discoidal structure, whereas they are spherical in the presence of esterified cholesterol. In addition, the smallest Lp2A-I discoidal particle (4.65 nm) migrates on agarose close to the position of alpha HDL, whereas apo A-I in synthetic spherical Lp2A-I (3.7 nm) have a pre-β migration on agarose. This is in good agreement with our observations, since our particles possess about two A-I and a lipid core.

The phospholipid composition of pre-β_1 HDL was completely different from that found in ultracentrifuged HDL, and may partly contribute to their slower migration on agarose. A drastic decrease of phosphatidylcholines was noted in pre-β_1 HDL, compared to ultracentrifuged HDL, 40.5% vs 76%. Surprisingly, the amounts of phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were higher in pre-β than in alpha HDL, whereas sphingomyelin was present in similar proportions. In order to verify that this unusual phospholipid composition did not reflect a low contamination by human albumin having trapped lysophospholipids and acidic phospholipids, albumin was immunoprecipitated using specific antibodies. Following this procedure, a similar phospholipid composition of pre-β_1 HDL was observed (not shown). This demonstrates that acidic phospholipids and lysophospholipids are integral components of these particles. In order to prevent oxidation of lipids during the experiments, all the samples were preserved in the presence of EDTA (0.1%). Furthermore, the presence of cholesteryl arachidonate in pre-β_1 HDL ($6.7 \pm 0.3\%$ of total esterified cholesterol, $n = 6$) showed clearly that no oxidation process occurs during the isolation procedure (not shown), since arachidonic acid is more prone to oxidation than other fatty acids. These values are closed to that found in plasma HDL ($7.3 \pm 0.5\%$, $n = 5$) (unpublished observations).

The pre-β_1 HDL remain stable in the follicular fluid even after a 6 h incubation at 37 °C (not shown). This contrasts with the reported instability of pre-β_1 HDL in plasma (Ishida et al., 1990; Miida et al., 1992; Kawano et al., 1993), for which the composition differs from that of follicular fluid

pre-β_1 HDL. The presence of a neutral lipid core may contribute to stabilize these particles. This postulate is further supported by different studies on synthetic particles (Jonas et al., 1990; Sparks et al., 1992a) which showed that apo A-I in lipid core containing spherical particles is more resistant to guanidine hydrochloride or thermal denaturation than in discoidal particles. This probably reflects a different conformation of apo A-I according to particle composition and structure (Jonas et al., 1990; Sparks et al., 1992a,b). Indeed, Sparks et al. (1992a) proposed that in discoidal Lp2A-I with a low phospholipid/A-I ratio, apo A-I undergoes a condensation which may bring in close contact the negatively charged residues, leading to charge repulsion and exposure of the helical segment more sensitive toward denaturating reagents. By contrast, in spherical Lp2A-I with a comparable phospholipid/A-I ratio, apo A-I is less negative which is indicative of minor charge repulsions on a sphere. This suggests a more flexible structure of apo A-I in spherical particles, and a lower susceptibility to denaturation. Moreover, if we compare the molecular composition of pre-β_1 HDL with that reported for synthetic spherical Lp2A-I, the latter display a twice higher ratio of phospholipid/A-I, for a calculated molecular mass of about 100 kDa. Thus, a low phospholipid/A-I ratio in our pre-β_1 HDL may still favor a flexible conformation of apo A-I. Furthermore, we and others (Perret et al., 1987; Clay et al., 1990) have observed that even after treatment of HDL by phospholipase A_2, leading to a 90% phospholipid depletion, the modified HDL remain stable and do not lose apo A-I. In these treated HDL, the apo A-I conformation was modified (Collet et al., 1991). However, further research on synthetic model particles is required to study the effects of acidic phospholipids on apo A-I stability.

In conclusion, we provided the characterization of pre-β_1 HDL in a particular extravascular fluid. The most important finding concerns the presence of a neutral lipid core evidenced for the first time in those particles and a particular phospholipid composition. Although follicular fluid is a particular model for extravascular fluid, these findings may open new questions about the metabolism of HDL subfractions.

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