Characterization of apoA-I-containing lipoprotein subpopulations secreted by HepG2 cells

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Abstract Recent immunoaffinity studies demonstrate two populations of high density lipoprotein (HDL) particles: one contains both apolipoprotein (apo) A-I and A-II [Lp(A-I w A-II)], and the other contains apoA-I but no A-II [Lp(A-I w/o A-II)]. To investigate whether these two populations are derived from different precursors, we applied sequential immunoaffinity chromatography to study the lipoprotein complexes in HepG2 conditioned serum-free medium. The apparent secretion rates of apoA-I, A-II, E, D, A-IV, and lecithin:cholesterol acyltransferase (LCAT) were 4013 ± 1368, 851 ± 217, 414 ± 64, 171 ± 51, 32 ± 14, and 2.9 ± 0.7 ng/mg cell protein per 24 h, respectively (n = 3–5). Anti-A-II removed all apoA-II but only 39 ± 5% (n = 5) apoA-I from the medium. These HepG2 Lp(A-I w A-II) also contained 31 ± 1% (n = 5) of the apoD and 82 ± 2% (n = 3) of the apoE in the medium. The apoE existed both as E and E-A-II complex. Lipoproteins isolated from the apoA-II-free medium by anti-A-I contained, besides apoA-I, 60 ± 3% of the medium apoD and trace quantities of apoE. The majority of HepG2 apoA-IV (78 ± 4%) (n = 3) and LCAT (85 ± 6%) (n = 3) was not associated with either apoA-I or A-II. HepG2 Lp(A-I w A-II) contained relatively more lipids than Lp(A-I w/o A-II) (45 vs. 37%). The phospholipid, free cholesterol, esterified cholesterol, and triglyceride weight ratios in Lp(A-I w A-II) and Lp(A-I w/o A-II) were 57:27:6:10, and 54:22:7:17, respectively. Electron micrographs showed both discs and spheres in each particle type, but more discs were seen in particles with apoA-II. Upon nondenaturing gradient gel electrophoresis, 66–85% of the Lp(A-I w A-II) was located in the 9.2–17.0 nm Stokes' diameter region. In contrast, 74 ± 14% of the particles without apoA-II were located in the size region below 8.0 nm. These studies showed that 24-h HepG2 conditioned medium contained Lp(A-I w A-II) and Lp(A-I w/o A-II) with physical-chemical characteristics similar to nascent HDL. These nascent apo-specific lipoproteins may be precursors of their plasma counterparts. —**Cheung, M. C., K. D. Lum, C. G. Brouillette, and C. L. Bisgaier.** Characterization of apoA-I-containing lipoprotein subpopulations secreted by HepG2 cells. *J. Lipid Res.* 1989. **30:** 1429–1436.

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The origins of high density lipoprotein (HDL) subpopulations are not well understood. Studies in rats and humans have demonstrated the presence of nascent HDL particles in human mesenteric and thoracic duct lymph, chyluric urine, liver perfusates, and in medium of intestinal and hepatic cell cultures (1–10). Other studies in rats and nonhuman primates have demonstrated the presence of intracellular HDL (11, 12). Taken together, these studies (1–12) indicate an intracellular HDL origin. Although in vitro incubation studies have suggested that some HDL may be derived from surface components of chylomicrons and very low density lipoproteins (VLDL) (13–16), HDL are found when triglyceride-rich lipoproteins are not synthesized, such as in abetalipoproteinemia (17), and from the liver of orotic acid-treated rats (18). Under normal physiological conditions, it is likely that both hepatic and intestinal secretion and intravascular events lead to the creation of plasma HDL. In plasma, the HDL lipid components are further modulated by lipoprotein lipase, hepatic lipase, lecithin:cholesterol acyltransferase (LCAT), the lipid transfer proteins, and HDL conversion factor leading to a heterogeneous HDL population (19–23).

Recent immunoaffinity studies demonstrated two populations of HDL particles: one containing both apolipoprotein (apo) A-I and A-II[Lp(A-I w A-II)], and the other containing apoA-I but not A-II[Lp(A-I w/o A-II)] (24). Both populations are found in the operationally defined density subfractions HDL_2 and HDL_3 (25). Current concepts provide some explanations for differences in size, density, and lipid composition of HDL subpopulations, but not for differences in apoA-I and A-II content. Thus the origin of HDL particles that differ in their apoA-II content is unknown. This study was initiated to in-

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; gPAGE, gradient polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

vestigate whether Lp(A-I w A-II) and Lp(A-I w/o A-II) are individually synthesized and secreted. Using immunoaffinity chromatography procedures, both Lp(A-I w A-II) and Lp(A-I w/o A-II) were isolated from the media of cultured human hepatoblastoma G2 (HepG2) cells. This report describes the physical and chemical characteristics of these particles, and their relative concentrations in defined HepG2 conditioned medium.

METHODS

Cell culture

HepG2 cells, originally isolated by Aden et al. (26) and Knowles, Howe, and Aden (27), were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were seeded at 2×10^6 cells per 75-cm^2 culture flask (Falcon Primaria, Becton Dickinson, Lincoln Park, NJ), in 25 ml Dulbecco's modified Eagle's medium with 4.5 g/l glucose (Sigma Chemical Co., St. Louis, MO), supplemented with additional glutamine (8 mM final concentration) (GIBCO Laboratories, Grand Island, NY), 0.1 mM MEM nonessential amino acids, (Sigma), 4 mM sodium bicarbonate, 0.01 M HEPES, and 10% fetal calf serum (HyClone, Logan, UT) (growth medium), and propagated in a humidified atmosphere of 95% air, 5% CO_2. Cells were subcultured weekly at a 1:3 ratio. Confluent cultures were washed three to five times with Hanks' Balanced Salt Solution (Sigma), and then placed in 15 ml of serum-free growth medium supplemented with 20 ng/ml hepatocyte growth factor (glycyl-L-histidyl-L-lysine) (Sigma) (28). After 24 h, the conditioned medium was harvested and pooled for lipoprotein isolation. Cells were treated with trypsin and with sodium hydroxide for cell number and protein determinations.

Isolation of apoA-I containing lipoproteins

ApoA-I-containing lipoproteins with and without apoA-II were isolated from conditioned medium by a previously established two-step immunoaffinity chromatography procedure (24). Between 600 and 1000 ml of pooled conditioned medium was used for each isolation. To isolate particles with apoA-II, 30–40 ml of anti-A-II immunosorbent was mixed and rocked with the pooled medium at 4°C for a minimum of 2 h. The immunosorbent was then sedimented by gravity, and the apoA-II-free supernatant medium was decanted and similarly mixed with 30–40 ml of anti-A-I immunosorbent to isolate the remaining A-I-containing lipoproteins. These sequential steps quantitatively removed apoA-II and A-I from the HepG2 conditioned medium. In each case, to remove nonspecific proteins, the immunosorbent was washed with 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA, and 0.05% NaN_3. Specifically bound lipoproteins were eluted with 3 M NaSCN in 0.02 M sodium phosphate buffer, pH 7.0, and immediately filtered through a Sephadex G-25 (Pharmacia LKB Biotechnology, Piscataway, NJ) column to remove the thiocyanate. All isolated lipoproteins and culture medium recovered from the immunosorbents were concentrated by MicroConFilt concentrators (Bio-Molecular Dynamics, Beaverton, OR) for subsequent analyses.

Particle size determination

Particle size distributions of Lp(A-I w A-II) and Lp(A-I w/o A-II) were determined by nondenaturing gradient polyacrylamide gel electrophoresis (gPAGE), and electron microscopy. Electrophoresis was carried out in 0.09 M Tris, 0.08 M boric acid, 0.003 M EDTA buffer, pH 8.35, at 10°C, 125 V for 24 h using precast 4–30% gels (Pharmacia LKB Biotechnology). Gels were stained overnight for proteins with 0.04% Coomassie Brilliant Blue G-250 dissolved in 3.5% perchloric acid. After destaining the protein to background with 7% acetic acid, the gels were scanned by a laser densitometer (Pharmacia LKB Biotechnology). Particle sizes were calculated with the LKB 2400 Gelscan XL™ software using thyroglobulin, apoferritin, lactate dehydrogenase, and bovine serum albumin as calibration proteins.

Negative staining electron microscopy was performed using carbon-coated Formvar grids, stained with 2% (w/v) potassium phosphotungstate, pH 5.9, and examined with a Phillips EM 400 microscope.

Protein and lipid analysis

ApoA-I, A-IV, and LCAT were quantitated by previously established specific radioimmunoassays (RIA) (29–31). ApoA-II was quantitated by RIA, and apoD by competitive enzyme-linked immunosorbent assay (ELISA). The accuracy of these two immunoassays was verified with respective standardized radial immunodiffusion assays (32, 33). ApoE was quantitated by a RIA similar to the LCAT RIA (31) except samples were diluted in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.2% Tween 20, 1% bovine serum albumin (BSA), 0.02% NaN_3, and 5 mM sodium decyl sulfate. All other reagents in the assay were diluted in the same buffer but without Tween 20 or sodium decyl sulfate. The assay range was 0.2–2 μg/ml. The apoE concentration in normolipidemic plasma measured by this assay was 4.6 ± 1.6 mg/dl (n = 50). Total protein was determined by the method of Lowry et al. using BSA as standard (34). Apolipoprotein composition of the isolated lipoproteins was studied by nonreducing sodium dodecyl sulfate (SDS) PAGE (35). Phospholipid, total cholesterol, unesterified cholesterol, and triglyceride were analyzed by enzymic methods (36). The difference between total and unesterified cholesterol mass was considered as cholesteryl ester.

RESULTS AND DISCUSSION

Protein concentration and distribution

Apolipoproteins and LCAT (mass) were determined in conditioned HepG2 medium after 24 h (**Table 1**). For comparison, the concentrations of these proteins in normolipidemic plasma detected by similar immunoassays (29–33) are also provided. The apparent apoA-I and A-II secretion rates (4013 ± 1368, and 851 ± 217 ng/mg cell protein per 24 h, respectively) were comparable to those previously reported (8, 37). Although the apoE secretion rate (414 ± 64 ng/mg cell protein per 24 h) was similar to that noted by Thrift et al. (8), it was considerably lower than the secretion rate of 104 ± 13 ng/mg cell protein per h reported by Dashti et al (37). This discrepancy is possibly due to differences in cell culture conditions and/or in the methods used for apoE quantitation. Nonetheless, relative to apoA-I, more apoE was detected in HepG2 conditioned medium than in the plasma (Table 1). Similarly, liver perfusate HDL contain a much higher apoE to A-I ratio than plasma HDL (7). Although apoD and A-IV have been detected in HepG2 conditioned medium (37–39), their concentrations have never been reported. As shown in Table 1, the weight ratio of apoA-I:D in the medium (24:1) was comparable to that in plasma (21:1). However, the weight ratio of apoA-I:A-IV in the HepG2 conditioned medium (125:1) was much higher than that in plasma (4:1). This tremendous difference could most likely be explained by observations from other laboratories showing that the intestine may be the only significant source of apoA-IV in humans and that the liver may contribute only minor amounts of this protein to the plasma (40–42). Nonetheless, similar to plasma, the majority of HepG2 apoA-IV (78 ± 4%, n = 3) was consistently found unassociated with either apoA-I or A-II (30). The mean apoA-I:A-II ratio in the culture medium of 4.7 was slightly higher than the plasma ratio of 3.8 (Table 1). When the various preparations of HepG2 conditioned medium were incubated with anti-A-II immunosorbent, all apoA-II was removed but only 39 ± 5% of apoA-I. Hence, 61 ± 5% of the apoA-I in the medium was not associated with apoA-II. The remaining apoA-I was completely absorbed by subsequent incubation with anti-A-I immunosorbent. The presence of two-thirds of apoA-I in Lp(A-I w/o A-II) in the culture medium was different from plasma where approximately one-third of apoA-I was found in these particles (24). This may provide partial explanation for the slightly higher apoA-I:A-II ratio observed in the HepG2 conditioned medium.

The LCAT concentration in the HepG2 conditioned medium was 2.9 ± 0.7 ng/mg cell protein per 24 h. This was only 15% of its relative concentration in plasma (Table 1). Likewise, the level of LCAT in primate liver perfusate was also very low (12). Most of the HepG2 LCAT (85 ± 6%) (n = 3) was not associated with either type of the apoA-I-containing lipoproteins. This was different from plasma where approximately 90% of LCAT was associated with apoA-I-containing lipoproteins (43). Our observation that most LCAT secreted by HepG2 was not associated with apoA-I and/or A-II was in agreement with a preliminary report showing that on nondenaturing agarose gels, the migration of LCAT secreted by HepG2 cells differed from that of HepG2-secreted apoA-I and A-II particles (44). Possibly lipoprotein composition and conformation, or factors not secreted by HepG2 cells, are necessary to induce LCAT to associate with HDL. However, other studies have shown that HepG2-secreted LCAT co-elutes with fractions having a mean apparent molecular weight of 200,000 (45). Whether HepG2 LCAT was associated with HDL or was poorly resolved from 200,000 mol wt proteins during high performance gel filtration is unclear. Of the 15% HepG2 LCAT that was associated with apoA-I, 12% was located in particles without apoA-II. Similarly, plasma Lp(A-I w/o A-II) contained relatively more LCAT than Lp(A-I w A-II) (43).

TABLE 1.

	A-I	A-II	D	E	A-IV	LCAT
A. Concentration of proteins in 24 h HepG2 conditioned medium and normolipidemic plasma						
HepG2 (ng/mg cell protein per 24 h)						
Mean ± SD	4013 ± 1368	851 ± 217	175 ± 51	414 ± 64	32 ± 14	2.9 ± 0.7
(n)	(5)	(5)	(5)	(3)	(5)	(4)
Plasma[a] (mg/dl)						
Mean ± SD	128 ± 22	34 ± 6	6.1 ± 1.2	4.6 ± 1.6	37 ± 4	0.62 ± 0.09
(n)	(360)	(360)	(74)	(50)	(5)	(66)
B. Ratios of apoA-I to other proteins in 24 h HepG2 conditioned medium and normolipidemic plasma calculated from data in A						
HepG2	1	4.7	23.5	9.7	125	1384
Plasma	1	3.8	21.0	27.8	3.5	206

[a]Concentrations in normolipidemic plasma are reported in refs. 30–33. These same immunoassays were used for quantitating the respective proteins in HepG2 conditioned medium.

With regard to apoD, we found that 31 ± 2% (n = 5) was associated with Lp(A-I w A-II), 60 ± 3% with Lp(A-I w/o A-II), and 9 ± 2% was not associated with either apoA-I or A-II. In plasma, most apoD is also apoA-I-associated (24). That relatively more HepG2 apoD was associated with Lp(A-I w/o A-II) as compared to plasma may be a result of the predominance of this particle in the medium.

Most of the apoE (82 ± 2%) (n = 3) in the HepG2 medium was associated with Lp(A-I w A-II). No more than 2% was associated with Lp(A-I w/o A-II), and 16% was not associated with either apoA-I or A-II. Similarly, the recent studies of Nichols et al. (46) have also shown that HepG2 Lp(A-I w/o A-II) contained only a trace of apoE and that HepG2 conditioned medium contained an apoE population not bound by antibodies to apoA-II or A-I. The relative distribution of HepG2 apoE among the various lipoprotein particles, however, was not reported.

Lipid composition

Based on five experiments, HepG2-secreted Lp(A-I w A-II) contained 55 ± 2.4% (weight) protein and 45 ± 2.4% lipid, while Lp(A-I w/o A-II) contained 63 ± 8.8% protein and 37 ± 8.8% lipid. Thus HepG2 Lp(A-I w/o A-II) appeared to be denser particles. The lipid composition of these particles was also different. The phospholipid, free cholesterol, esterified cholesterol, and triglyceride weight ratios in Lp(A-I w A-II) and Lp(A-I w/o A-II) were 57:27:6:10 and 54:22:7:17, respectively. Thus, consistent with nascent HDL particles, HepG2 apoA-I-containing particles were poor in esterified cholesterol (8, 47), a condition due possibly to the low level of LCAT in the medium. HepG2 Lp(A-I w/o A-II), however, contained relatively more core lipid than Lp(A-I w A-II). These lipid and protein compositions were different from their plasma counterparts.

Morphology and size distribution

Negative staining electron microscopy shows that the Lp(A-I w A-II) and Lp(A-I w/o A-II) particles in the HepG2 conditioned medium consisted of both discoidal and spherical particles (**Fig. 1**). However, considerably more discoidal particles were seen in Lp(A-I w A-II). This is consistent with their differences in lipid composition (i.e., increased surface to core lipid ratio) described above. When Lp(A-I w A-II) particles were separated by nondenaturing gPAGE, stained for protein, and scanned by a densitometer, multiple size subpopulations could be detected (**Fig. 2**). Several subpopulations were often found in the 9.2–17.0 nm region. These large particles constituted 66–85% of all Lp(A-I w A-II) in five experiments. Means of 14% and 11% of the particles were located in molecular size regions of 7.1–9.2 nm and less than 7.1 nm. Such size distributions were quite different from those of plasma Lp(A-I w A-II), where at least two-thirds of the size subpopulations were normally located in the 7.1–9.2 nm region, with no more than trace quantities of other proteins present in regions below the size of BSA (21, 48).

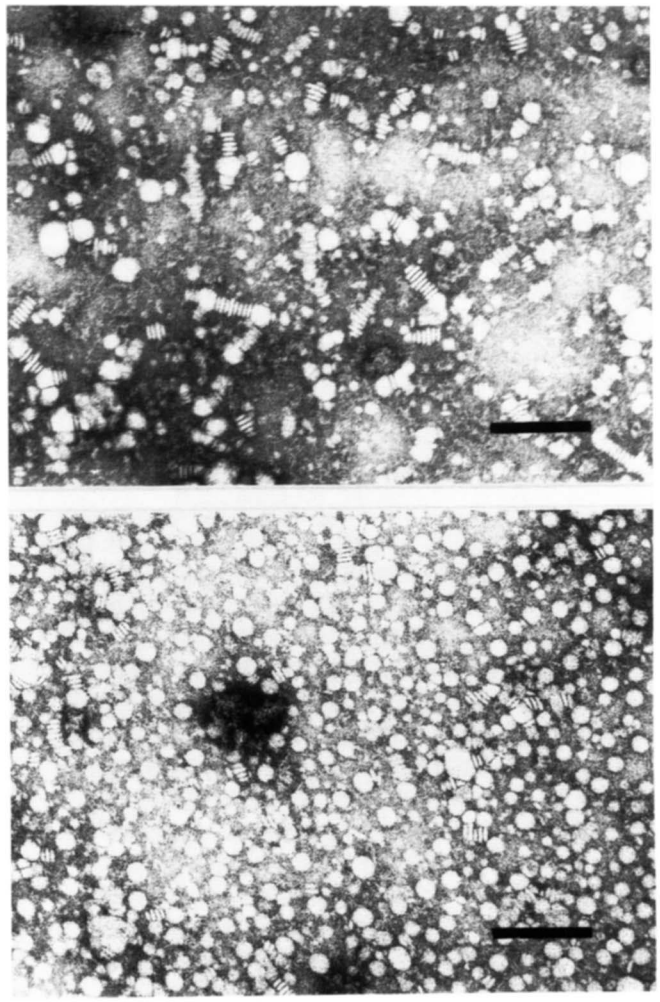

Fig. 1. Electron micrographs of Lp(A-I w A-II) (top) and Lp(A-I w/o A-II) (bottom) isolated from 24 h serum- free HepG2 conditioned medium. Sizing bar in the lower right corner represents 1000 Å.

Scans of the nondenaturing gradient gels demonstrated that HepG2 Lp(A-I w/o A-II) particles were very different in size from those of HepG2 Lp(A-I w A-II) and from plasma Lp(A-I w/o A-II) (Fig. 2). Most noticeably, 74 ± 14% (n = 5) of all the particles was located in the size region below 8.0 nm. Two modes of particles with peak positions around 7.4–7.6 and 6.9–7.0 nm were consistently observed. The remaining 26% of particles was found scattered throughout the 8.0–17.0 nm interval. Overall, the morphology and size of HepG2 Lp(A-I w A-II) and Lp(A-I w/o A-II) were consistent with those reported for nascent HDL (5, 7, 8, 16, 47). Small spherical and large discoidal HDL particles were also found in the plasma of LCAT-deficient subjects (49).

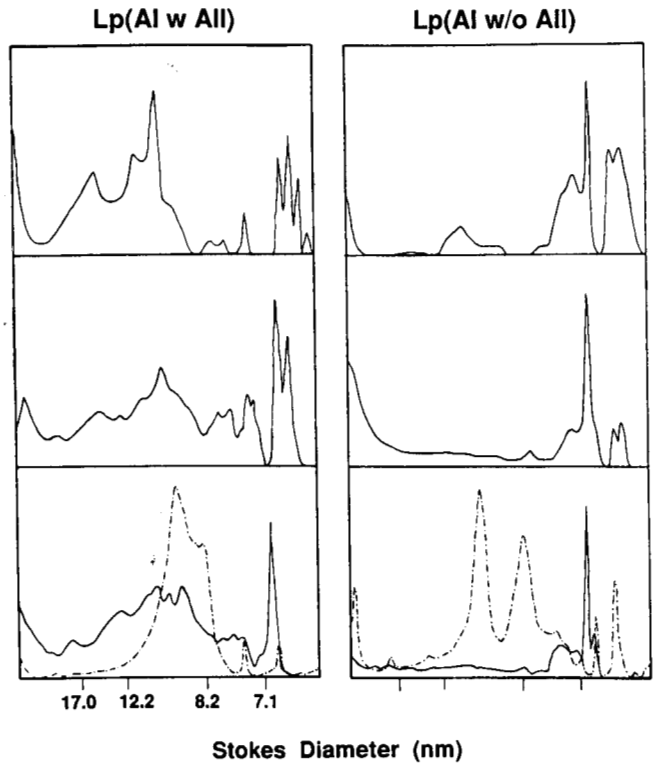

Fig. 2. Densitometric scans of nondenaturing gPAGE (4–30%) of HepG2 Lp(A-I w A-II) (left panel) and Lp(A-I w/o A-II) (right panel) from three experiments (——). Representative scans of Lp(A-I w A-II) and Lp(A-I w/o A-II) from normolipidemic plasma (·····) are shown in the bottom frames for comparison. Indicated in the bottom scale are positions and sizes of the calibration proteins: thyroglobulin (17 nm), apoferritin (12.2 nm), lactate dehydrogenase (8.2 nm), and bovine serum albumin (7.1 nm).

HepG2 Lp(A-I w A-II) and Lp(A-I w/o A-II) from two experiments were further subfractionated by Superose 6B fast protein liquid chromatography (FPLC) (Pharmacia LKB Biotechnology) and fractions were pooled for gPAGE, electron microscopy, lipid and protein determinations. In both populations of apoA-I-containing lipoproteins, more discoidal particles were observed in pool I

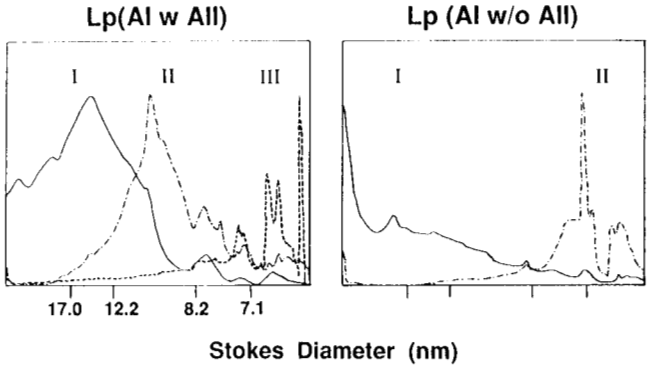

Fig. 3. Densitometric scans of nondenaturing gPAGE (4–30%) of FPLC pools I (——), II (·····), and III (---) of Lp(A-I w A-II) (left) and FPLC pools I (——), II (·····) of Lp(A-I w/o A-II) (right).

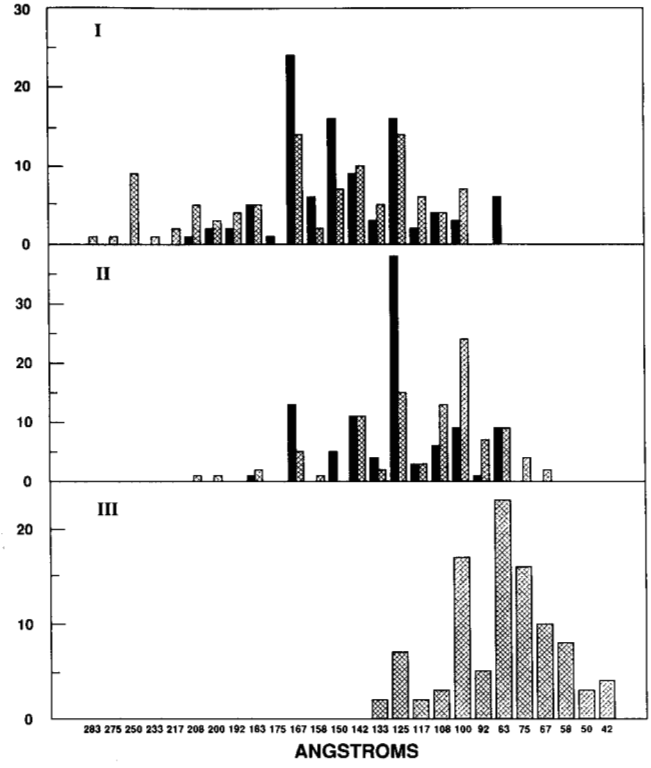

Fig. 4. Morphographic analysis of particle diameters of FPLC pools I, II, and III of Lp(A-I w A-II). The solid and hatched bars represent discoidal and spherical particles, respectively. For each particle type, n = 100.

than in the subsequent pool(s). Pool III of Lp(A-I w A-II) and pool II of Lp(A-I w/o A-II) contained essentially only spherical particles (data not shown). Their particle size distribution studied by gPAGE (**Fig. 3**) and electron

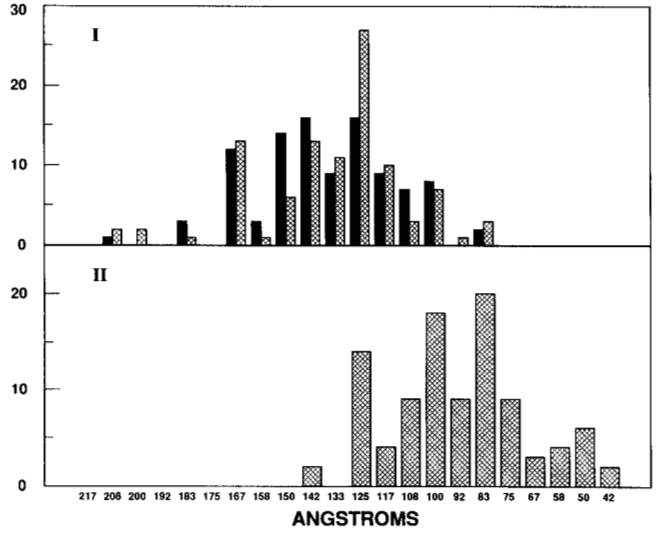

Fig. 5. Morphographic analysis of particle diameters of FPLC pools I and II of Lp(A-I w/o A-II). The solid and hatched bars represent discoidal and spherical particles, respectively. For each particle type, n = 100.

microscopy (**Fig. 4** and **Fig. 5**) are reasonably comparable.

The protein composition of these FPLC pools was studied by SDS-PAGE (**Fig. 6**). Larger particles (i.e., pool I) contained more proteins in the 30–50 kDa region than the smaller particles (pools II and III). ApoA-II and apoE immunoblotting confirmed the presence of apoE and apoE-A-II complex in Lp(A-I w A-II), and apoE in Lp(A-I w/o A-II) in these pooled fractions (data not shown). The occurrence of large A-I-containing lipoproteins rich in apoE and E/A-II complex is consistent with previous observations (8, 50). In pool I of Lp(A-I w A-II) where most of the apoE and E-A-II complex were found, the mean A-I:A-II:E:E-A-II ratio based on densitometry was 1:0.15:0.62:0.36. Several unidentified proteins with molecular weights larger than 50,000, and proteins with molecular weights similar to the apoCs were also present. These proteins have also been found in plasma Lp(A-I w A-II) and Lp(A-I w/o A-II) (48, 51). **Table 2** shows the lipid and protein composition of the various FPLC pools. In general, larger particles contained more lipid than smaller particles. The weight ratio of cholesteryl ester to free cholesterol increased as particle size decreased. Furthermore, the smallest Lp(A-I w A-II) particles were relatively enriched with triglyceride. In the plasma of hypertriglyceridemic subjects, small triglyceride-rich, cholesteryl ester-poor HDL with Stokes diameter of 7.8 nm have also been detected (52).

In conclusion, 24-h HepG2 conditioned medium contained Lp(A-I w A-II) and Lp(A-I w/o A-II) with physical and chemical characteristics comparable to nascent HDL obtained from liver perfusates and hepatic cell cultures. It is possible that these particles are partially modified by other factors present in the medium prior to their isolation. These nascent apo-specific lipoproteins may be precursors of those in plasma. The observation that HepG2 conditioned medium contained more Lp(A-I w/o A-II) than Lp(A-I w A-II) was not predicted from current knowledge of their relative concentration in plasma. Possibly, the intestinal contribution of apoA-I-containing lipoproteins may markedly alter the relative proportion of these particles in plasma. In addition, unpublished observations from our laboratory (M. Cheung, D. Foster, and R. Knopp) show that Lp(A-I w/o A-II) are catabolized faster than Lp(A-I w A-II). Cell culture conditions may also affect the relative amounts of these two apo-specific HDL subpopulations in the HepG2 conditioned medium. Experiments are now underway to determine the various factors which may determine the rate of formation and the physical–chemical characteristics of these two apoA-I-containing lipoprotein subpopulations.

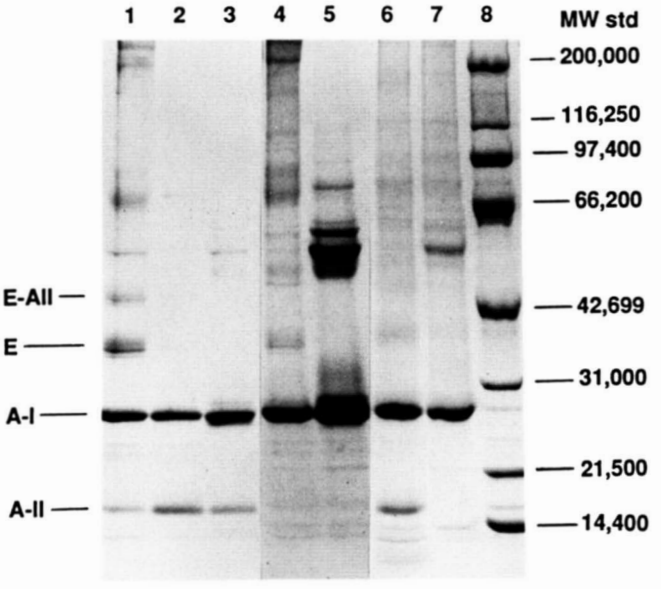

Fig. 6. SDS PAGE pattern of Lp(A-I w A-II) and Lp(A-I w/o A-II) before and after subfractionation by FPLC. Samples applied on lanes 1, 2, and 3 were: FPLC pools I, II, and III, respectively, of Lp(A-I w A-II); lanes 4 and 5: FPLC pools I and II, respectively, of Lp(A-I w/o A-II); lane 6, Lp(A-I w A-II) before FPLC; lane 7: Lp(A-I w/o A-II) before FPLC; and lane 8: molecular weight standards (Bio-Rad Laboratory).

TABLE 2. Composition of pooled FPLC fractions of HepG2 Lp(A-I w A-II) and Lp(A-I w/o A-II)

	Protein	Phospholipid	Free Cholesterol	Cholesteryl Ester	Triglyceride
			% by weight		
Lp(A-I w A-II)					
Pool I	35.0	38.7	16.3	3.0	7.0
Pool II	46.7	34.2	10.2	3.3	5.6
Pool III	70.7	13.5	1.9	2.2	11.7
Lp(A-I w/o A-II)					
Pool I	49.9	26.0	10.3	4.8	9.0
Pool II	70.1	17.9	2.7	2.3	7.0

Data represent mean of two experiments.

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