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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF DISCRETE APOLIPOPROTEIN A-CONTAINING LIPOPROTEIN PARTICLES SECRETED BY HUMAN HEPATOMA CELL LINE HepG2

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SUMMARY: The purpose of this study was to identify the apolipoprotein A-containing lipoprotein particles produced by HepG2 cells. The apolipoprotein A-containing lipoproteins separated from apolipoprotein B-containing lipoproteins by affinity chromatography of culture medium on concanavalin A were fractionated on an immunosorber with monoclonal antibodies to apolipoprotein A-II. The retained fraction contained apolipoproteins A-I, A-II and E, while the unretained fraction contained apolipoproteins A-I and E. Both fractions were characterized by free cholesterol as the major and triglycerides and cholesterol esters as the minor neutral lipids. Further chromatography of both fractions on an immunosorber with monoclonal antibodies to apolipoprotein A-I showed that 1) apolipoprotein A-II only occurs in association with apolipoprotein A-I, 2) apolipoprotein A-IV is only present as part of a separate lipoprotein family (lipoprotein A-IV), and 3) apolipoprotein E-enriched lipoprotein A-I:A-II and lipoprotein A-I are the main apolipoprotein A-containing lipoproteins secreted by HepG2 cells.

A number of studies have shown that apolipoprotein A-I (ApoA-I) and apolipoprotein B (ApoB) occur as major protein constituents of several, distinct lipoprotein families differentiated on the basis of their apolipoprotein composition (1,2). The major ApoB-containing lipoproteins include lipoprotein B (LP-B), lipoprotein B:E (LP-B:E), lipoprotein B:C:E (LP-B:C:E) and lipoprotein B:C (LP-B:C) (1-3), whereas major ApoA-I-containing lipoproteins are lipoprotein A-I (LP-A-I) and lipoprotein A-I:A-II (LP-A-I:A-II) (4-6). Some discrete lipoprotein particles may be secreted directly from the liver and intestine and some may be generated within the plasma compartment (7,8). To distinguish between these alternative routes, we have utilized human hepatocarcinoma cell line, HepG2, as a model for studying the chemical nature of human hepatic lipoproteins (9). We have shown previously that

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The abbreviations used are: Apo, apolipoprotein; LP, lipoprotein; ConA, concanavalin A; MEM, minimum essential medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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polydisperse, triglyceride-rich LP-B and LP-B:E comprise two major ApoB-containing lipoproteins secreted by HepG2 cells (9).

In this paper, we are presenting evidence that HepG2 cells secrete apolipoprotein E (ApoE)-enriched LP-A-I and LP-A-I:A-II as the major, discrete ApoA-I-containing lipoprotein families of characteristic neutral lipid composition. Evidence is also presented that ApoA-IV occurs in the HepG2 cell culture medium as a distinct lipoprotein, lipoprotein A-IV (LP-A-IV). Preliminary results of this study have been reported in an abstract form (10).

MATERIALS AND METHODS

Cell culture. The human hepatocarcinoma HepG2 cells were obtained from American Type Culture Collection (Rockville, MD). The cells were plated in 100 mm Falcon petri dishes (15 dishes/experiment) and grown in minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 10% (v/v) fetal bovine serum (FBS) at 37° in a 95% air, 5% CO_2 atmosphere as described previously (9,11); the medium was changed every two days. After 4 days in culture, when cells were near confluence, the maintenance medium was removed, monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.4, to remove traces of FBS, and serum-free MEM was added. After an 18-hour incubation, the medium was removed and preservatives including EDTA (1 mg/ml), glutathione (0.2 mg/ml), ε-amino caproic acid (1.3 mg/ml), penicillin G (500 units/ml), and chloramphenicol (0.02 mg/ml) were added. The medium was centrifuged at 2000 rpm for 20 minutes at 4°C to remove cells and debris and the supernatant fraction was concentrated 10-15-fold with polyvinylpyrrolidone and sucrose placed outside the dialysis bag (Mr 5000 cutoff).

Production and characterization of monoclonal antibodies. Monoclonal antibodies (anti) to apolipoproteins A-I, A-II and B were produced and characterized as described in previous studies from this laboratory (12-14). Briefly, after immunization of mice with purified apolipoproteins, mouse spleen cells were fused with mouse myeloma cells (Sp 2/0) using the polyethylene glycol method. Resulting hybridomas were cloned by the limiting dilution method. Clones were screened for the reactivity with chylomicrons, very low-, low-, and high-density lipoproteins and apolipoproteins A-I, A-II, B, C-I, C-II, C-III, D and E using enzyme-linked immunosorbent assays (ELISA) on microtiter plates. Antibodies selected for this study were those that recognized all lipoprotein and apolipoprotein forms of the antigens with closely comparable affinities and were able to completely remove the respective apolipoproteins from the plasma. These antibodies are referred to as monoclonal "pan" antibodies. The "pan" antibodies to ApoA-II (14) and ApoB (12,13) were developed in this laboratory, whereas "pan" antibody to ApoA-I was described by Bekaert et al. (15).

Immunoaffinity chromatography. Monoclonal "pan" antibodies to apolipoproteins A-I, A-II, and B were purified from ascites by the use of protein A column and coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Affi-Gels coupled with respective antibodies were used to prepare immunoaffinity columns as previously described (9). HepG2 cell culture medium or fractions thereof were applied to immunoaffinity columns and incubated for 15-18 hr at 4°C to allow binding of lipoprotein particles to immobilized antibodies. This was followed by elution of unretained material and washing of columns. The retained lipoproteins were eluted with 3 M NaSCN, pH 7.4, as previously described (9). The structural integrity of ApoB- and ApoA-I-containing lipoprotein particles isolated by this procedure has been verified and documented in previous studies from this laboratory (16).

Determination of neutral lipids and apolipoproteins. The concentrations of triglycerides, free cholesterol and cholesterol esters were determined by gas-liquid chromatography (17). The concentrations of apolipoproteins A-I, A-II, B, C-I, C-II, C-III, D and E were measured by electroimmunoassays developed in this laboratory as previously described (18).

Polyacrylamide gel electrophoresis and immunoblotting. Electrophoresis of lipoprotein particles was carried out in an SDS containing 12% polyacrylamide slab gel using the Laemmli buffer system (19). The proteins were transferred to nitrocellulose by electroblotting using the Bio-Rad minitransblot electrophoretic transfer cell. The membranes were immunoblotted with polyclonal antibodies to ApoA-I, ApoA-II, and ApoA-IV as described previously (20).

RESULTS AND DISCUSSION

As shown previously (9,11), ApoB was the major apolipoprotein in the culture medium followed, in the order of decreasing concentrations, by apolipoproteins A-I, E, A-II, and C-III. The weight percentage of ApoA-II was approximately one-half of that of ApoA-I, while ApoC-III was a minor apolipoprotein. Although detectable in some culture media, the concentrations of apolipoproteins C-I, C-II and D were too small to be quantified accurately by the electroimmunoassay. ApoA-IV was detected qualitatively by immunoblotting.

To separate the ApoA- from ApoB-containing lipoproteins, concentrated HepG2 cell culture medium was fractionated either by affinity chromatography on concanavalin A (ConA) (21) or by immunoaffinity chromatography on an anti-ApoB immunosorber as described previously (9). As shown in Table 1, both fractionation procedures were equally effective in separating the ApoB-containing lipoproteins (the retained fractions) from ApoA-containing

Table 1. Distribution of Neutral Lipids and Apolipoproteins Between Subfractions of HepG2 Cell Culture Medium

	Neutral Lipids			Apolipoproteins		
Fraction	Triglyceride	Free cholesterol	Cholesterol ester	A-I	A-II	B
		%			%	
ConA-R[a]	60.5 ± 3.9	24.8 ± 2.8	42.6 ± 3.3	2.7 ± 0.6	0	100
ConA-U[a]	39.5 ± 3.9	75.2 ± 2.8	57.4 ± 3.3	97.3 ± 0.6	100.0	0
"Pan"B-R[b]	60.1 ± 7.2	24.3 ± 1.6	42.3 ± 3.5	1.0 ± 0.4	0	100
"Pan"B-U[b]	39.9 ± 7.2	75.7 ± 1.6	57.7 ± 3.5	99.0 ± 0.4	100.0	0

After an 18 hr incubation, conditioned medium was removed, concentrated 15-fold, and applied to either concanavalin A (ConA) or to an anti-ApoB immunosorber ("pan"B). The concentrations of neutral lipids and apolipoproteins were measured in both the retained (R) and unretained (U) fractions. Values are mean ± SEM of a) 7 experiments and b) 2 experiments.

lipoproteins (the unretained fractions). However, due to its higher binding capacity, ConA was used in most of the experiments. The unretained fractions contained 75% of the total free cholesterol and all the ApoA-I and ApoA-II (Table 1). Negligible amounts of ApoA-I detected in retained fractions were most probably due to a nonspecific absorption of this apolipoprotein to column matrices. The unretained fractions contained 80% of the total ApoE.

The unretained fractions separated by affinity chromatography of culture medium on ConA column were fractionated on an anti-ApoA-II immunosorber. The retained fraction contained apolipoproteins A-I, A-II and E, whereas the unretained fraction only contained apolipoproteins A-I and E (Table 2). These results suggested that the former fraction consisted mainly, if not exclusively, of LP-A-I:A-II particles and the latter fraction of LP-A-I particles. Approximately 53% of the total ApoA-I was present in LP-A-I:A-II and 47% in LP-A-I particles; the corresponding distribution of neutral lipids and ApoE was 54-60% in LP-A-I:A-II and 40-45% in LP-A-I (Table 2).

The protein moiety of LP-A-I consisted of 60% ApoA-I and 40% ApoE (Table 3). The LP-A-I:A-II particles also contained a substantial amount of ApoE which accounted for 33% of the total apolipoprotein content (Table 3). The molar ratio of ApoA-I/ApoA-II was close to unity. The lipid composition of both LP-A-I and LP-A-I:A-II was characterized by free cholesterol as the major and triglycerides and cholesterol esters as the minor constituents.

To exclude the possible presence of lipoprotein A-II particles (LP-A-II), the anti-ApoA-II retained fraction was chromatographed on an anti-ApoA-I immunosorber. The near complete recovery of apolipoproteins A-I and A-II in the retained fraction indicated that ApoA-II was only present in association with ApoA-I. To verify the presence of LP-A-I:E, the anti-ApoA-II unretained

Table 2. Distribution of Neutral Lipids and Apolipoproteins Between ApoA-Containing Lipoprotein Particles Secreted by HepG2 Cells

Lipoprotein Fractions	Neutral Lipids			Apolipoproteins		
	TG	FC	CE	A-I	A-II	E
	%			%		
ApoA-I-Containing Particles	40.0 ± 5.0	45.8 ± 2.9	39.4 ± 3.0	47.3 ± 2.1	0	41.2 ± 2.1
ApoA-I:A-II-Containing Particles	60.0 ± 5.0	54.2 ± 2.9	60.3 ± 3.0	52.7 ± 2.1	100.0	58.8 ± 2.1

Cells were cultured under conditions described in Table 1. The unretained fraction from ConA column was fractionated on an immunosorber with monoclonal antibodies to ApoA-II. Values are mean ± SEM of 9 experiments and represent the percent of total neutral lipids and apolipoproteins recovered in the unretained (ApoA-I-containing) and retained (ApoA-I:A-II-containing) fractions of anti-ApoA-II immunosorber. TG, triglycerides; FC, free cholesterol; CE, cholesterol esters.

Table 3. The Neutral Lipid and Apolipoprotein Composition of ApoA-Containing Lipoprotein Particles Secreted by HepG2 Cells

Lipoprotein Fractions	Neutral Lipids			Apolipoproteins		
	TG	FC	CE	A-I	A-II	E
		%			%	
ApoA-I Containing Particles	23.5 ± 2.2	61.8 ± 3.3	14.6 ± 1.7	59.5 ± 2.5	0	40.1 ± 2.6
ApoA-I:A-II Containing Particles	30.6 ± 4.4	53.3 ± 5.6	16.1 ± 1.5	39.6 ± 1.7	27.5 ± 2.9	32.9 ± 2.9

Cells were cultured under conditions described in Table 1. The unretained (ApoA-I-containing) and retained (ApoA-I:A-II-containing) fractions of anti-ApoA-II immunosorber were analyzed for neutral lipids and apolipoproteins. Values are means ± SEM of 9 experiments. TG, triglycerides; FC, free cholesterol; CE, cholesterol esters.

fraction was chromatographed on an anti-ApoA-I immunosorber. The protein moiety of the retained fraction consisted of ApoA-I (71%) and ApoE (29%) demonstrating the presence of LP-A-I:E and possibly LP-A-I. Although it appears that most of ApoE is associated with LP-A-I and LP-A-I:A-II, the exact proportions of these two lipoproteins with and without ApoE and the possible presence of lipoprotein E (LP-E) remain to be determined.

The possible association of ApoA-IV with LP-A-I and LP-A-I:A-II particles was studied by immunoblotting technique. Testing of LP-A-I:A-II and LP-A-I isolated from HepG2 cell culture medium with antisera to ApoA-I and ApoA-II showed the presence of ApoA-I and ApoA-II bands in the former (Fig. 1A, lane 2) and ApoA-I band in the latter (Fig. 1A, lane 4) particles. However, testing of these lipoprotein particles (Fig. 1B, lanes 1 and 2) with a

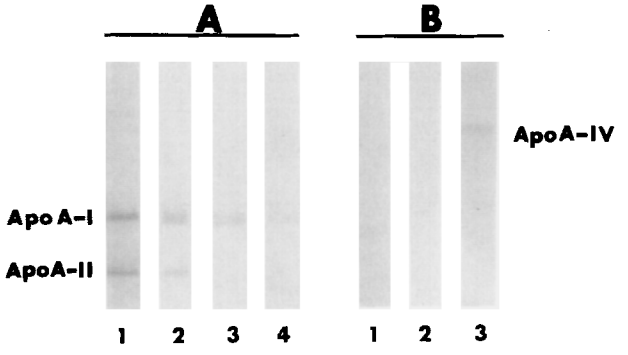

Figure 1. Analysis of lipoprotein particles by electrophoresis and immunoblotting. The lipoprotein fractions were analyzed by 12% polyacrylamide gel slab electrophoresis in the presence of SDS. The proteins were transferred to nitrocellulose and immunoblotted with polyclonal antisera to ApoA-I and ApoA-II (Figure 1A) and ApoA-IV (Figure 1B). Samples in Figure 1A are: lane 1, LP-A-I:A-II from plasma; lane 2, LP-A-I:A-II from HepG2; lane 3, LP-A-I from plasma; lane 4, LP-A-I from HepG2. Samples in Figure 1B are: lane 1, LP-A-I:A-II; lane 2, LP-A-I; lane 3, anti-ApoA-II unretained-anti-ApoA-I unretained (LP-A-IV). All samples in Figure 1B were isolated from HepG2 cell culture medium.

polyclonal antiserum to ApoA-IV failed to reveal the presence of ApoA-IV band. The ApoA-IV band was only identified in a fraction (Fig. 1B, lane 3) that was first unretained on the anti-ApoA-II and then on the anti-ApoA-I immunosorber (anti-ApoA-II unretained-anti-ApoA-I unretained fraction). This finding has indicated that ApoA-IV exists in the HepG2 cell culture medium as a separate lipoprotein family, LP-A-IV.

Results of this study show that HepG2 cells secrete LP-A-I and LP-A-I:A-II as two distinct ApoA-I-containing lipoprotein families. These two lipoproteins share some but differ in other properties from those of corresponding lipoprotein families in plasma. They both contain apolipoproteins A-I and A-II as the characteristic protein constituents (Fig. 1A) and their percent distribution seems to be similar in HepG2 cell culture medium (Table 2) and plasma (22,23). However, LP-A-I and LP-A-I:A-II particles from HepG2 cell culture medium have a higher content of free cholesterol and a lower content of cholesterol esters that the corresponding lipoprotein families from plasma. This difference in free cholesterol/cholesterol ester ratios is most probably due to low activity of lecithin:cholesterol acyltransferase (LCAT) in the cell medium as compared to that in plasma (24). Furthermore, LP-A-I and LP-A-I:A-II particles from the cell culture medium have a substantially higher content of ApoE but negligible contents of ApoC-peptides and ApoD than LP-A-I and LP-A-I:A-II particles from plasma (6,25). The present results are consistent with our previous study (11) demonstrating that the HDL particles secreted by HepG2 cells were deficient in cholesterol esters but enriched in free cholesterol and ApoE. The production of HDL particles with similar properties has been demonstrated in rat liver perfusate in the absence of LCAT activity (26) and in LCAT deficiency (27). These marked compositional differences suggest that nascent hepatic LP-A-I and LP-A-I:A-II particles undergo substantial modifications in the plasma compartment consistent with their putative roles as a source or donors of ApoE (27,28) on one hand, and as receptors of ApoC-peptides during the lipolytic degradation of triglyceride-rich lipoproteins (29) on the other.

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