Isolation and characterization of lipoproteins produced by human hepatoma-derived cell lines other than HepG2

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Abstract A total of six established human hepatoma-derived cell lines, including Hep3B, NPLC/PRF/5 (NPLC), Tong/HCC, Hep 10, huH1, and huH2, were screened for their ability to accumulate significant quantities of lipoproteins in serum-free medium. Only two cell lines, Hep3B and NPLC, secreted quantitatively significant amounts of lipoproteins. In a 24-h period the accumulated mass of apolipoproteins (apo) A-I, A-II, B, and E and albumin for Hep3B cells was 1.96, 1.01, 1.96, 1.90, and 53.2 μg/mg cell protein per 24 h, respectively. NPLC cells secreted no detectable albumin but the 24-h accumulated mass for apolipoproteins A-I, A-II, B, and E was 0.45, 0.05, 0.32, and 0.68 μg/mg cell protein per 24 h, respectively. Twenty four-hour serum-free medium of Hep3B cells contained lipoproteins corresponding to the three major density classes of plasma; percent protein distribution among the lipoprotein classes was 4%, 41%, and 56% for very low density lipoprotein ("VLDL"), low density lipoprotein ("LDL"), and high density lipoprotein ("HDL"), respectively. NPLC was unusual since most of the lipoprotein mass was in the d 1.063–1.235 g/ml range. Hep3B "LDL", compared with plasma LDL, contained elevated triglyceride, phospholipid, and free cholesterol. Nondenaturing gradient gel electrophoresis revealed that Hep3B "LDL" possessed a major component at 25.5 nm and a minor one at 18.3 nm. Immunoblots showed that the former contained only apoB while the latter possessed only apoE. Like plasma VLDL, Hep3B "VLDL" particles (30.5 nm diameter) isolated from serum-free medium contained apoB, apoC, and apoE. "HDL" harvested from Hep3B and NPLC medium were enriched in phospholipid and free cholesterol and poor cholesteryl ester which is similar to the composition of HepG2 "HDL." "HDL" from Hep3B and NPLC culture medium on gradient gel electrophoresis had peaks at 7.5, 10, and 11.9 nm which were comparable to major components found in HepG2 cell medium. Hep3B cells, in addition, possessed a particle that banded at 8.2 nm which appeared to be an apoA-II without apoA-I particle by Western blot analysis. The cell line also produced a subpopulation of larger-sized "HDL" not found in HepG2 medium. NPLC "HDL"had a distinct peak at 8.3 nm which by Western blot was an apoE-only particle. Electron microscopy revealed that "HDL" harvested from Hep3B and NPLC medium consisted of discoidal and small, spherical particles like those of HepG2. The "HDL" apolipoprotein content of each cell line was distinct from that of HepG2. ApoA-II at 35% of apolipoprotein distinguishes Hep3B "HDL" from HepG2, which contains only 10%. Unlike Hep3B and HepG2 "HDL", apoE is the major apolipoprotein in NPLC "HDL." Differences in lipoprotein distribution and HDL subclass heterogeneity in Hep3B, NPLC, and HepG2 cell media are probably related to differences in apolipoprotein expression.—**Forte, T. M., M. R. McCall, B. B. Knowles, and V. G. Shore.** Isolation and characterization of lipoproteins produced by human hepatoma-derived cell lines other than HepG2. *J. Lipid Res.* 1989. **30:** 817–829.

Supplementary key words lipoprotein heterogeneity • Hep3B cells • NPLC/PRF/5 cells • gradient gel electrophoresis • electron microscopy

We have previously demonstrated that the human hepatoblastoma-derived cell line, HepG2, accumulated low density lipoproteins (LDL) and high density lipoproteins (HDL) in chemically defined basal medium (1). This has been confirmed by several other laboratories (2, 3). In addition to lipoproteins, it has been demonstrated that this cell line secreted the enzymes lecithin:cholesterol acyltransferase (LCAT) (4) and hepatic lipase (5), and cholesteryl ester transfer activity has been detected in the culture medium (6, 7).

The LDL-like particles isolated from HepG2 medium were compositionally unusual; phospholipid and unesterified cholesterol were elevated while triglyceride formed the particle core (1). Apolipoprotein (apo) B was the sole apolipoprotein associated with the particle. The HDL particles isolated from the medium also differed from normal plasma HDL in that a high proportion of the HepG2 particles were discoidal in morphology (1). Particle mor-

Abbreviations: HDL, high density lipoprotein, d 1.063–1.235 g/ml, isolated from medium; LDL, low density lipoprotein, d 1.006–1.063 g/ml, isolated from medium; VLDL, very low density lipoprotein, isolated from medium; NPLC, NPLC/PRF/5 cell line; apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; HBsAg, hepatitis B virus surface antigen; ELISA, enzyme-linked immunosorbent assay.

phology and the elevated phospholipid and unesterified cholesterol composition reported for HepG2 HDL was, however, similar to HDL from LCAT-deficient plasma (8, 9). Unlike normal plasma HDL, apoA-II constituted only a small percentage of the total HepG2 HDL protein while apoE was relatively abundant (1).

It is not known whether lipoprotein subclass distribution, composition, and morphology previously reported for HepG2 cells is unique for this cell line or whether particles with similar characteristics are produced by other human liver-derived cell lines. In order to determine whether other human hepatoma-derived cell lines secrete lipoproteins, several available lines were screened for their ability to produce lipoprotein complexes. We now describe the particles from two cell lines, Hep3B and NPLC/PRF/5 (NPLC), which accumulated lipoprotein products that differ from those reported for HepG2. These two hepatoma-derived lines, along with HepG2 cells, provide us with cellular models in which hepatic lipoprotein assembly, secretion, and metabolism may be elucidated.

METHODS

Cell cultures

The human hepatoma-derived cell lines, Hep3B (10), NPLC (11), Hep10 (12), Tong/HCC (13), huH1 (14), and huH2 (15), were examined for their ability to secrete lipoproteins. To screen cells, stock cells were subcultured and grown to confluency in 175-cm^2 flasks (Falcon, Oxnard, CA). A minimum of eight flasks was used. All cells except Hep10 and huH2 were grown in Eagles MEM, containing 2 mM glutamine supplemented with 10% fetal bovine serum, at 37°C in a humified incubator with 5% CO_2–95% air; these were the same culture conditions used for HepG2 (16). Hep3B cells were subcultured at a 1:8 split ratio; medium was replaced once a week and cells were confluent within 7 days. NPLC cells were split at a 1:3 ratio, medium was replaced twice weekly, and cells were confluent within 7–10 days. Tong/HCC cells and huH1 cells were subcultured at a 1:2 and 1:4 split ratio, respectively, medium was replaced weekly, and cells were confluent within 7–10 days. Hep10 and huH2 cells were grown on gelatin-coated flasks essentially as described by Simon and Knowles (12) with the exception that HepG2 conditioned medium replaced WI38 conditioned medium.

When cells were approximately confluent, the MEM-fetal bovine serum was removed and cell sheets were carefully washed three times with Hanks balanced salt solution in order to remove residual fetal bovine serum proteins and lipoproteins. Our previous studies on lipoproteins isolated from fetal bovine serum (17) suggest that the latter particles do not significantly contaminate serum-free conditioned medium. The chemical composition and electron microscopic structure of fetal bovine serum lipoproteins were considerably different from that reported for hepatoma-derived lipoproteins in the present study. After washing the cell sheets, 20 ml serum-free MEM with 2 mM glutamine was added to each flask and harvested after 24 h; this procedure was repeated once. The serum-free medium harvested after incubation with cells is referred to as "conditioned medium" throughout the paper. Conditioned medium was placed on ice and gentamicin sulfate (0.1 mg/ml), EDTA (1 mg/ml), *p*-hydroxymercuriphenylsulfonic acid (0.25 mM), phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.5 mg/ml), and pepstatin (0.7 mg/ml) were added to the medium which was then centrifuged at 1000 *g* × 30 min at 4°C to remove cell debris. Media from two 24-h harvests were pooled, concentrated 20- to 50-fold by ultrafiltration (Amicon stirred cell, PM30 membranes) at 4°C under nitrogen, then probed for apolipoproteins A-I, A-II, B, and E by dot-blotting using specific antibodies as outlined below. In order to determine the presence of lipoprotein particles, concentrated conditioned medium was adjusted to d 1.235 g/ml with NaBr and centrifuged at 4°C for 48 h at 40,000 rpm and the total d < 1.235 g/ml fraction was examined by negative staining electron microscopy and by nondenaturing gradient gel electrophoresis on 2–16% and 4–30% gradient gels. Based on these analyses we were able to determine that three lines (Hep3B, NPLC, and Hep10) in addition to HepG2 produced lipoprotein complexes (**Table 1**). Concentrated conditioned medium from Hep10 cells contained trace amounts of apoA-I, apoA-II, apoB, and apoE, and only trace amounts of lipoproteins were noted on 4–30% gradient gels. No identifiable complexes were seen on 2–16% gels. Because of the extremely

TABLE 1. Cell lines tested for their ability to secrete lipoproteins

Cell Line	Reference	Origin	Lipoproteins in Conditioned Medium
Hep3B	10	Hepatocellular Carcinoma	+
NPLC/PRF/5[a]	10, 11	Hepatocellular Carcinoma	+
Hep 10	12	Hepatocellular Carcinoma	±
Tong/HCC[b]	13	Hepatocellular Carcinoma	–
huH-1	14	Hepatocellular Carcinoma	–
huH-2[c]	15	Hepatocellular Carcinoma	–
HepG2	10	Hepatoblastoma	+

[a]Derived from PLC/PRF/5-induced tumor in nude mice.
[b]Cells obtained from Dr. D. Stevenson, Huntington Memorial Hospital, Pasadena, CA.
[c]Cells obtained from Dr. Nambo Huh, University of Tokyo, Tokyo 108, Japan.

low yields, the Hep10 lipoproteins were not further characterized. The Hep3B and NPLC cells were studied in greater detail and their lipoprotein products were characterized. All cell lines in Table 1, except for HepG2, have integrated the hepatitis B virus genome into the cellular genome (10–15); however, infectious virus particles are not formed.

Isolation of lipoproteins from medium

Conditioned medium from confluent Hep3B and NPLC cells (approximately 20 175-cm² flasks) incubated with 20 ml serum-free MEM per flask was harvested from two 24-h incubations and concentrated. Total secreted protein and nondenaturing gradient gel patterns of lipoproteins were compared between the first and second 24-h harvests and they were similar, thus making possible pooling of samples. The average total secreted protein harvested from a 175-cm² flask incubated for 24 h with 20 ml serum-free medium was 2.46 mg/flask for Hep3B cells and 0.42 mg/flask for NPLC cells. Lipoprotein fractions were isolated from Hep3B concentrated conditioned medium by sequential ultracentrifugation at 4°C in a Beckman 50.3 Ti rotor; density was adjusted with NaBr, and samples were spun at 40,000 rpm for 24 h for d < 1.006 g/ml, 36 h for d 1.006–1.063 g/ml, and 48 h for d 1.063–1.235 g/ml fracton. Fractions were harvested by aspiration. Only a single spin, as described above, at d < 1.25 g/ml was carried out on NPLC medium since gradient gel analysis and electron microscopy revealed an absence of particles corresponding to LDL and very low density lipoprotein (VLDL).

Electrophoretic analyses

Particle size distribution of lipoprotein fractions was analyzed on 2–16% and 4–30% nondenaturing gradient gels (Pharmacia Fine Chemicals, Piscataway, NJ) essentially as described by Nichols, Krauss, and Musliner (18). After staining of gels with Coomassie Brilliant Blue G250 (Bio-Rad) the gels were scanned with a Transidyne RFT densitometer (Transidyne Corp., Ann Arbor, MI).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially as described by Weber and Osborn (19) either in 10% polyacrylamide disc or slab gels or in 1.0-mm-thick 3–27% polyacrylamide gradient gels (Isolabs, Akron, OH). The latter were used to separate proteins from NPLC d < 1.235 g/ml fraction. Gels were stained overnight with 0.2% Coomassie Brilliant Blue R250 in 50% methanol–10% acetic acid and destained in 35% methanol–10% acetic acid (gradient gels were destained with 25% methanol–5% acetic acid).

Chemical analysis

Protein was determined by a modified Lowry procedure (20) with bovine serum albumin as the standard. Phospholipid was assayed according to Bartlett (21), while triglyceride was determined by an enzymatic kit (Gilford Diagnostics, Cleveland, OH). Free and esterified cholesterol were determined by gas–liquid chromatography (22).

Immunoassays

Human albumin was quantitated by single radial immunodiffusion on plates (TAGO, Inc., Burlingame, CA) for the concentration range 5–40 mg/dl; standards were validated against a primary standard solution of purified human plasma albumin previously quantitated by amino acid analysis. Apolipoproteins A-I, A-II, and B were quantitated by solid-phase competitive ELISAs (23) in which 96-well plates (Nunc immunoplates, tested version, Alameda Chemical and Scientific, Inc., Oakland, CA) were precoated with purified antibodies. For apoA-II, a high affinity subfraction of goat anti-apoA-II (prepared by P. Blanche of Lawrence Berkeley Laboratory, Donner Laboratory) was used at a concentration of 40–50 ng/well. For apoB, equimolar mixtures of monoclonal antibodies totaling 10–15 ng/well were used. Two antibodies (affinities = 4×10^{10} and 5×10^{10}) from MEDIX Biotech (Foster City, CA) or two antibodies from Chemicon International (El Segundo, CA) were used. For apoA-I, mixtures of two or three monoclonal antibodies (30–40 ng/well) from the same sources as anti-apoB were used. Primary standards included apoA-I and apoA-II, isolated by ion exchange chromatography, and a narrow range density subfraction of low density lipoprotein that contained only apoB plus lipids and was isolated from pooled plasmas. Concentration was established by amino acid analysis. *o*-Phenylenediamine was used as substrate for the peroxidase probe. Antibodies to human apoA-I, A-II, B, and albumin were tested against fetal bovine serum and none of the antibodies cross-reacted with this serum. The latter indicates that apolipoproteins measured in the medium by immunochemical methods were synthesized de novo.

LCAT mass was determined by competitive double radioimmunoassay as described by Albers et al. (24). ApoE quantitation obtained by solid phase radioimmunoassay was kindly provided by Dr. Karl Weisgraber, Gladstone Foundation Laboratories, San Francisco, CA. Western blots and dot blots for apoA-I, A-II, B, and E, and albumin were performed as previously described (16). Antibody used for Western blots for human hepatitis B surface antigen was obtained from Behring Diagnostics, La Jolla, CA.

Electron microscopy

For negative staining, lipoproteins were dialyzed to 0.13 M ammonium acetate containing 0.1 g/l EDTA, pH 7.4. Samples were stained with 1% sodium phosphotungstate

and examined in a JEM 100C electron microscope. Particles were sized as previously described (25).

RESULTS

Apolipoprotein and albumin secretion

The concentrations of apoA-I, A-II, E, and B together with albumin were determined in medium harvested after 24 hr incubation; results are provided in **Table 2**. Nearly equal amounts of apoA-I, B, and E were observed in the medium of Hep3B cells. In contrast to Hep3B cells, NPLC cells produced relatively low levels of apolipoproteins and no albumin; moreover, apoE was the major apolipoprotein harvested from conditioned medium of NPLC cells. For comparison, the apolipoprotein concentrations from HepG2 medium are also provided in Table 2; these values are similar to those previously reported by Thrift et al. (1). Medium from HepG2 cells accumulated more than twofold greater amounts of apoA-I than Hep3B; on the other hand, Hep3B medium contained greater amounts of apoE and apoA-II. The accumulated mass of apolipoproteins in Hep3B cell conditioned medium was similar to that of HepG2 cells (6.8 μg/mg cell protein/per day versus 7.4 μg/mg cell protein per day) while that accumulated by NPLC cells was considerably (80%) less. Hep3B and NPLC conditioned media were similar to that of HepG2 in that 57–67% of secreted apoA-I was in the d > 1.235 g/ml fraction.

Fig. 1 shows the accumulation with time of apoA-I and apoB in the medium of Hep3B cells where apolipoproteins could be detected after 1 h incubation. After an initial lag, the appearance of apoA-I and apoB in Hep3B conditioned medium is essentially linear between 3 and 24 h incubation. ApoA-I was quantitated in NPLC conditioned medium after 4 h and 24 h incubation; the amount accumulated per 175-cm² flask was 0.19 μg/flask and 1.24 μg/flask, respectively, indicating that production of apoA-I by this cell line is also linear.

Lipid and protein composition of major lipoprotein classes isolated from conditioned medium

The major lipoprotein density classes were isolated from conditioned medium of Hep3B and NPLC cells by preparative ultracentrifugation; the distribution of protein across the fractions is shown in **Table 3**. Hep3B cells accumulated a small amount (4.0%) of protein in the d < 1.006 g/ml fraction, i.e., "VLDL," while the "LDL" (d 1.006–1.063 g/ml) and "HDL" (d 1.063–1.235 g/ml) accounted for 40% and 55% of the lipoprotein protein mass, respectively. Unlike Hep3B cells, lipoprotein protein of conditioned medium from NPLC cells was confined primarily to the d 1.063–1.235 g/ml fraction. In comparison to Hep3B and NPLC cells, the reported distributions for HepG2 lipoproteins were < 1%, 27%, and 73% for VLDL, LDL, and HDL, respectively (1).

The compositions of Hep3B and NPLC lipoprotein fractions along with those previously reported for HepG2 cells are seen in **Table 4**. The "LDL" fraction from Hep3B cells is enriched in triglyceride and poor in cholesteryl ester, features similar to "LDL" from HepG2 medium. "HDL" particles isolated from Hep3B medium have elevated phospholipid and unesterified cholesterol and low levels of cholesteryl ester as do HepG2 "HDL." Unlike HepG2, however, Hep3B "HDL" contains a somewhat higher percentage of triglyceride (10%). Like Hep3B and HepG2, NPLC "HDL" are also enriched in phospholipid and unesterified cholesterol but poor in core lipids. The percent protein in NPLC "HDL" is overestimated due to the presence of hepatitis B virus surface antigen (HBsAg) protein. This protein co-isolated in the d < 1.235 g/ml fraction (identified by dot blotting with HBsAg antibody) and was not removed by Superose column chromatography. Macnab et al. (26) previously reported that PLC/PRF/5 cells, the line from which NPLC cells were derived, form HBsAg complexes that isolate at d 1.18–1.20 g/ml; thus it is not surprising to find this protein in NPLC conditioned medium. HBsAg, however, was not detected in Hep3B d 1.063–1.235 g/ml fraction; this observation is

TABLE 2. Apolipoprotein and albumin concentrations in conditioned medium harvested after 24-h incubation (mean ± SD)

Cell Line	ApoA-I	ApoA-II	ApoB	ApoE	Albumin
	μg protein/mg cell protein				
Hep3B	1.96 ± 0.25	1.01 ± 0.13	1.96 ± 0.57	1.90 ± 0.3	53.2 ± 6.4
NPLC	0.45 ± 0.05	0.05 ± 0.01	0.32 ± 0.06	0.68 ± 0.11	ND[b]
HepG2[a]	4.6	0.7	2.0	0.1	25.3

Values for Hep3B were obtained from a total of 12 samples representing medium harvested from four different passage numbers; those for NPLC were obtained from 10 samples representing medium harvested from three different passages. Conditioned medium was concentrated 40–50-fold prior to immunoassay.

[a]Thrift et al. (1).

[b]ND, not detected.

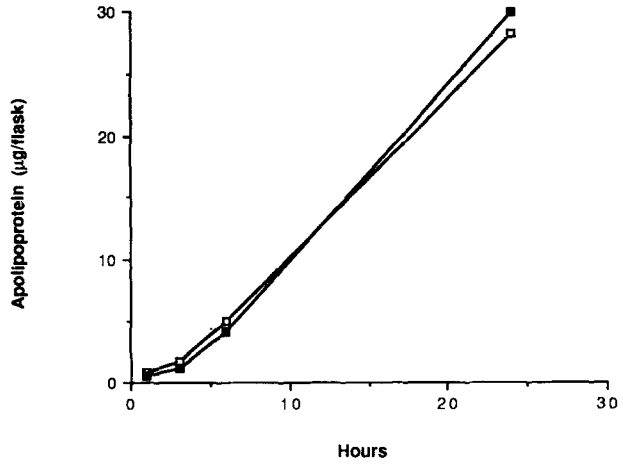

Fig. 1. Accumulation with time of apoA-I (■-■) and apoB (□-□) in Hep3B serum-free medium. Cells were grown to confluency in 175-cm² flasks in the presence of MEM plus 10% fetal bovine serum. To determine secretion in serum-free medium, monolayers were rinsed carefully three times with Hanks balanced salt solution, and 20 ml MEM without fetal bovine serum was introduced into each flask. Serum-free MEM was incubated with cells for 1,3,6, and 24 h. After collection, conditioned medium in all cases was concentrated 90-fold and apoA-I and apoB were determined by ELISA.

consistent with the finding of Aden et al. (27) who showed that Hep3B cells at confluency, under conditions where culture medium is replaced daily, do not secrete HBsAg.

The extremely low levels of "VLDL" made complete compositional analysis difficult. In one experiment the lipid composition of "VLDL" was determined and it was similar to that of "LDL" fraction; "VLDL" triglyceride, unesterified cholesterol, cholesteryl ester, and phospholipid were 35, 17, 9, and 39%, respectively, of total lipids, while Hep3B "LDL" contents were 28, 17, 12, and 43%, respectively.

The apolipoprotein compositions of Hep3B "HDL" and "LDL" compared with that of plasma fractions are presented in **Fig. 2**. This figure indicates that Hep3B "HDL" electrophoresed on 10% SDS-PAGE contains relatively more apoE and apoA-II than plasma HDL; additionally, a large proportion of apoE appears as an apoE-apoA-II heterodimer. Based on densitometry and assuming equal chromagenicity for the proteins, the percent protein composition for unreduced apolipoproteins was: apoE-apoA-II, 14 ± 1%; apoE, 20 ± 1%; apoA-I, 20 ± 3%; apoA-II, 35 ± 3%; apoC, 12 ± 2% (n = 4, ± SD). The percent composition for HepG2 "HDL" apolipoproteins electrophoresed on 10% SDS-PAGE (data not shown) was 8, 13, 65, 10, and 5%, respectively, values similar to those reported by Thrift et al. (1). The data indicate that Hep3B "HDL," unlike that of HepG2, is rich in apoA-II and apoC. "LDL" isolated from Hep3B medium contains a major band corresponding to B-100 (Fig. 2B) and a minor component corresponding to apoE (identification established by dot-blot analyses and by 10% SDS-PAGE). The major "VLDL" apolipoproteins were apoB, apoC, and apoE (data not shown).

Sodium dodecylsulfate polyacrylamide gradient gels (3–27%) were used for determining apolipoprotein composition of NPLC d < 1.235 g/ml fraction. **Fig. 3** shows such a gel; three prominent bands can be seen that correspond with apoE, apoA-I, and HBsAg. The positions of apoA-I and HBsAg were verified by Western blots for these two proteins (data not shown).

Subclass distribution and morphology of HDL

Particle size heterogeneity of "HDL" fractions harvested from Hep3B and NPLC medium was compared to that of HepG2 "HDL" by nondenaturing gradient gel electrophoresis. Representative scans of Hep3B and NPLC "HDL" distribution are shown in **Fig 4A and 4B**, respectively. A scan of HepG2 "HDL" is provided for comparison (Fig. 4C). The banding positions of the major peaks and shoulders of Hep3B "HDL" from five separate experiments are 15.2 ± 0.4 nm; 13.2 ± 0.3 nm; 12.3 ± 0.4 nm; 10.8 ± 0.2 nm; 10.0 ± 0.2 nm; 8.1 ± 0.1 nm; and 7.5 ± 0.1 nm. The average positions for "HDL" components from NPLC cells are 14.1, 11.9, 10.0, 8.3, and 7.4 nm. Hep3B is distinguished by the presence of a well-defined peak at 8.2 nm and by virtue of the fact that the particle size extends a greater distance into the large-pore region of the gel. The latter is clearly indicated by the presence of a peak at 15.2 nm. All three cell lines appear to have common components at approximately 7.4, 10.0, and 12.0 nm. NPLC contains a prominent band near the top of the gel; Western blots of electroeluted samples probed with anti-HBsAg showed a strong positive reaction in this region (data not shown), indicating that this peak corresponds with HBsAg complexes.

In order to determine whether there is extensive redistribution of "HDL" particles during 24 h incubation, Hep3B d < 1.235 g/ml fractions obtained after 3-, 6-, and 24-h incubations were electrophoresed on 4–30% nondenaturing gradient gels. Scans of Coomassie-stained "HDL" components from this experiment are provided in **Fig. 5**. They indicate that after only 3 h incubation one

TABLE 3. Percent protein distribution among lipoprotein classes

	Density Fraction		
Cell Line	d < 1.006	1.006–1.063	1.063–1.235
		% protein	
Hep3B[a]	3.9 ± 0.9	40.8 ± 4.5	55.7 ± 4.2
NPLC	ND[b]	ND	100
HepG2[c]	<1	27	73

[a]Represents mean and standard deviation of three separate experiments.
[b]ND, not detected.
[c]From Thrift et al. (1).

TABLE 4. Composition of major lipoprotein fractions isolated from Hep3B and NPLC conditioned medium

Fraction	Cell Line	Protein	PL[a]	CE	UC	TG	N
				% total weight			
"LDL"	Hep3B	22.9 ± 3.1	33.2 ± 6.8	9.1 ± 4.5	12.9 ± 3.4	21.8 ± 3.2	4
"LDL"	HepG2[b]	27	28	6	14	25	
"HDL"	Hep3B	34.1 ± 3.5	40.2 ± 6.2	4.2 ± 1.1	11.8 ± 2.3	9.8 ± 3.0	4
"HDL"	NPLC	49.9 ± 6.5	32.0 ± 6.0	3.3 ± 0.1	14.9 ± 3.5	ND[c]	3
"HDL"	HepG2[b]	38	40	3	16	2	

[a]PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol; TG, triglyceride.
[b]HepG2 composition data reported by Thrift et al. (1) is provided for comparison.
[c]ND, not detected.

can discern lipoproteins banding at 7.6 nm and at 15.1 nm, as well as a somewhat diffuse staining region between 15 and 7.6 nm. The pattern, as well as that for 6-h incubation, is similar to 24-h incubated medium. Since NPLC cells secrete relatively small quantities of lipoproteins compared with Hep3B cells, the d < 1.235 g/ml fractions isolated after 4- and 24-h incubations were first electrophoresed on 4–30% gels and then immunoblotted for apoA-I; in both cases blots revealed apoA-I-reacting material in the region of 7.5 nm and a diffuse band between catalase and ferritin (data not shown). Similarities of nondenaturing gradient gel profiles between short and long periods of incubation suggest that modification of lipoprotein particles, if it occurs in the culture medium, is not extensive.

The electron microscopic morphology (**Fig. 6**) corroborated "HDL" particle heterogeneity. Hep3B "HDL" contained numerous discoidal particles 15.5 ± 2.8 nm long axis and 4.2 ± 0.3 nm short axis as well as round profiles that ranged from 6.4 to 11.8 nm diameter. The d < 1.235 g/ml fraction from NPLC medium contains discoidal particles 16.6 ± 3.3 nm by 4.3 ± 0.2 nm, round profiles 10.5 ± 1.8 nm, and large 24.4 ± 4.9 nm particles. The size and morphology of the latter particles are compatible with that previously reported by Macnab et al. (26) for HBsAg particles isolated at d 1.18–1.20 g/ml.

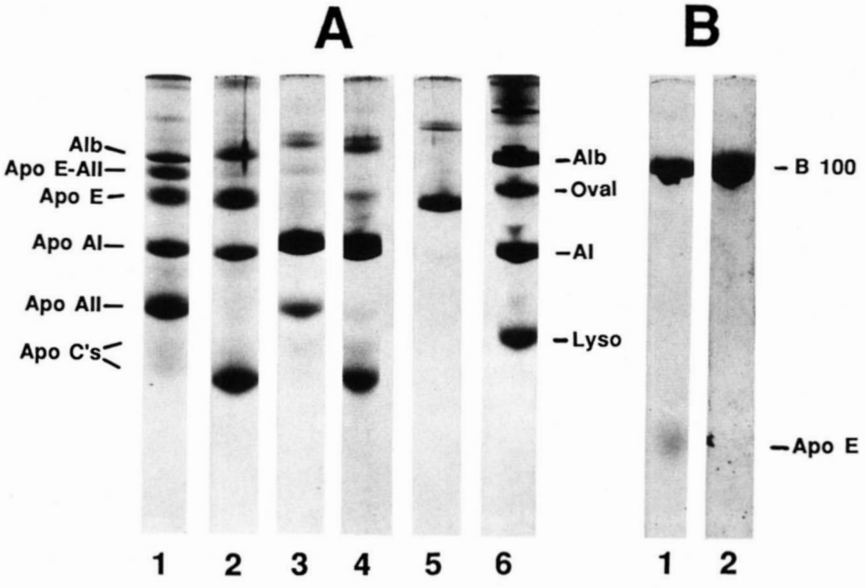

Fig. 2. SDS-polyacrylamide gel electrophoresis of Hep3B "HDL" and "LDL" proteins. Panel A exhibits 10% SDS-polyacrylamide tube gels stained with Coomassie blue R250: 1) unreduced Hep3B "HDL"; 2) β-mercaptoethanol-reduced Hep3B "HDL"; 3) unreduced plasma HDL; 4) β-mercaptoethanol-reduced plasma HDL; 5) human apoE standard; 6) small molecular weight standard including bovine albumin (Alb), ovalbumin (Oval), bovine apoA-I (AI), and lysozyme (Lyso). The apolipoprotein bands are identified on the left side of panel A: apoE-apoA-II heterodimer (E-AII); apoE (E); apo A-I(AI); apoA-II (AII); and apoC's. Panel B exhibits 3% SDS-polyacrylamide gels of 1) Hep3B "LDL" and 2) plasma LDL which were electrophoresed under reduced conditions.

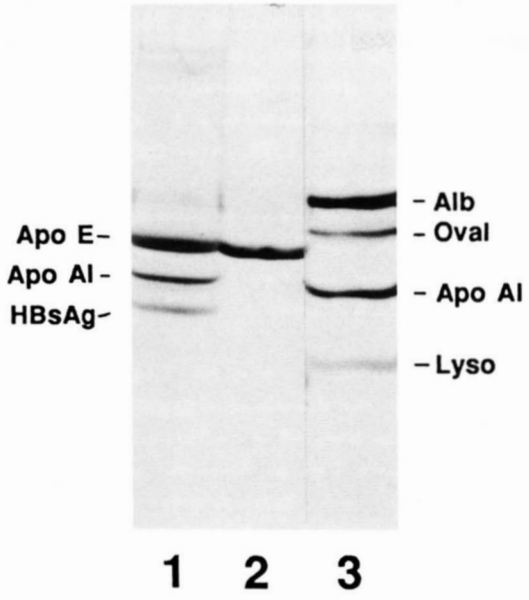

Fig. 3. NPLC d < 1.235 g/ml proteins electrophoresed on 3–27% SDS-polyacrylamide gradient gels and stained with Coomassie blue R250. 1) NPLC reduced proteins are indicated as apolipoprotein (apo)E, apolipoprotein (apo)A-I, and hepatitis B surface antigen (HBsAg); the positions of the latter two proteins were identified by immunoblotting for the respective proteins. 2) Human apoE, reduced. 3) Small molecular weight standard; same as that in Fig. 2.

Western blot analysis of Hep3B and NPLC HDL apolipoproteins

In order to determine the distribution of apoA-I, apoA-II, and apoE among HDL subpopulations, Western blots were carried out on "HDL" electrophoresed on 4–30% non-denaturing gels. **Fig. 7** demonstrates the pattern of apolipoprotein distribution of NPLC and Hep3B "HDL" as compared with that of HepG2. The apoA-I distribution is quite similar in Hep3B and HepG2 blots; both have a major component (corresponds to 7.4–7.6 nm peak) between albumin and lactate dehydrogenase markers, as well as several distinct bands between the catalase and thyroglobulin markers. In NPLC, the major apoA-I-staining component is the 7.4 nm particle and only faint bands are seen between the catalase and apoferritin markers. In all three cell lines the small molecular weight component at approximately 7.4–7.6 nm appears to be an apoA-I-only particle. The immunoreactivity for apoA-II of Hep3B "HDL" is much more pronounced than that for HepG2, which is consistent with the increased apoA-II mass relative to apoA-I in Hep3B "HDL." In Hep3B the apoA-II pattern shows a very distinct band just below the lactate dehydrogenase marker that corresponds to the 8.2 nm peak on Coomassie-stained gels. In addition, apoA-II staining is much more intense in the large pore region of the gel than its HepG2 counterpart. NPLC "HDL" react weakly to apoA-II in the region between lactate dehydrogenase and apoferritin. ApoE from all three cell lines is associated with particles that have distinct bands in the large pore region of the gel (between apoferritin and thyroglobulin). Additionally, apoE from NPLC forms a discrete complex near the lactate dehydrogenase marker that corresponds to the 8.3 nm subpopulation on gradient gels; this HDL component appears to be an apoE-only particle.

Subclass distribution and morphology of LDL and VLDL

Heterogeneity in the d < 1.006 g/ml ("VLDL") and d 1.006–1.063 g/ml ("LDL") fractions from Hep3B cells was assessed by electophoresis on 2–16% gradient gels. The results are seen in **Fig. 8**. The d < 1.006 g/ml fraction has a well-defined peak at 30.5 nm (mean 30.7 ± 0.3 nm, n = 3, Fig. 8A) and, occasionally, a slight peak of larger diameter particles is also in evidence but this was not a consistent observation. The d 1.006–1.063 g/ml fraction contains a very pronounced peak at 25.3 nm (25.5 ± 0.3, n = 5) with a shoulder at 24.5 nm (Fig. 8B). The isolated "LDL" fraction also consistently reveals a minor broad peak at approximately 18.3 nm (18.7 ± 0.3 nm, n = 3).

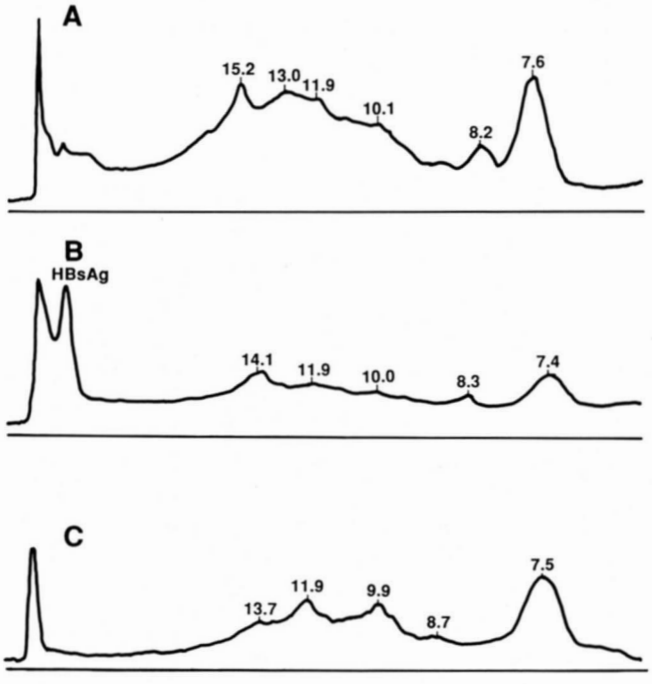

Fig. 4. Representative scans of Coomassie blue G250-stained gels after electrophoresis of "HDL" on 4–30% nondenaturing gradient gels. (A) Hep3B "HDL" (d 1.063–1.235 g/ml); numbers over the peaks indicate banding position in nm ascertained from standards consisting of globular proteins with known diameters. (B) NPLC "HDL". The pronounced peak in the large pore region of the gel has been identified as HBsAg by Western blotting. (C) Scan of HepG2 "HDL" provided for comparison.

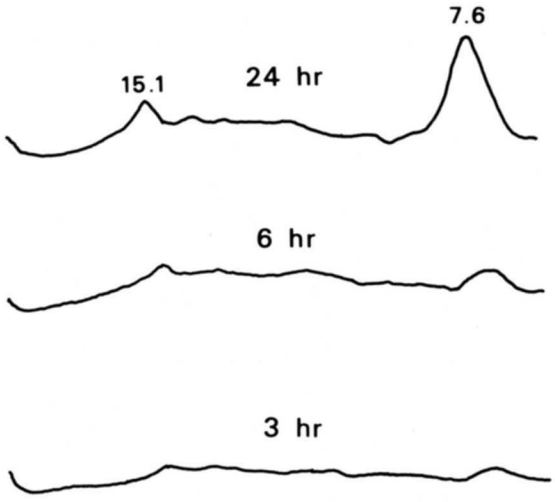

Fig. 5. Nondenaturing polyacrylamide gradient gel (4–30%) scans of "HDL" components of Hep3B culture medium after 3-,6-, and 24 h incubations. Cells were incubated in serum-free medium for the time indicated and the medium was concentrated 90-fold. The d < 1.235 g/ml fraction was subsequently isolated from each sample and 25 μl was applied to each sample lane. The gel was stained with Coomassie G250 before scanning. The numbers over the peaks indicate peak position in nm.

Since SDS-PAGE indicated that Hep3B "LDL" contained apoE, an experiment was carried out to determine whether apoE was present throughout the particle size spectrum or whether it was localized to a specific subpopulation. To achieve this, "LDL" were first electrophoresed on 2–16% gels and then electroeluted to nitrocellulose. Transferred lipoproteins were probed with antibodies to apoB and apoE. As shown in **Fig. 9**, apoB was associated mainly with the large-sized particles while apoE immunostaining corresponded with the broad peak around 18.8 nm.

Electron microscopy of Hep3B "LDL" and "VLDL" (**Fig. 10**) confirms the distribution noted on gradient gels. The "VLDL" particles (Fig. 10B) are heterogeneous, round particles with a mean diameter of 31.5 ± 7.8 nm. Isolated "LDL" are more homogeneous (Fig. 10A) with a mean particle size of 25.7 ± 2.7 nm that agrees well with the major peak at 25.3 nm on gradient gels. Inspection of the micrographs indicates that an occasional particle smaller (13.6–18.9 nm) than "LDL" is present in this fraction; this is not unexpected since gradient gels demonstrated the presence of smaller-sized components that contain apoE.

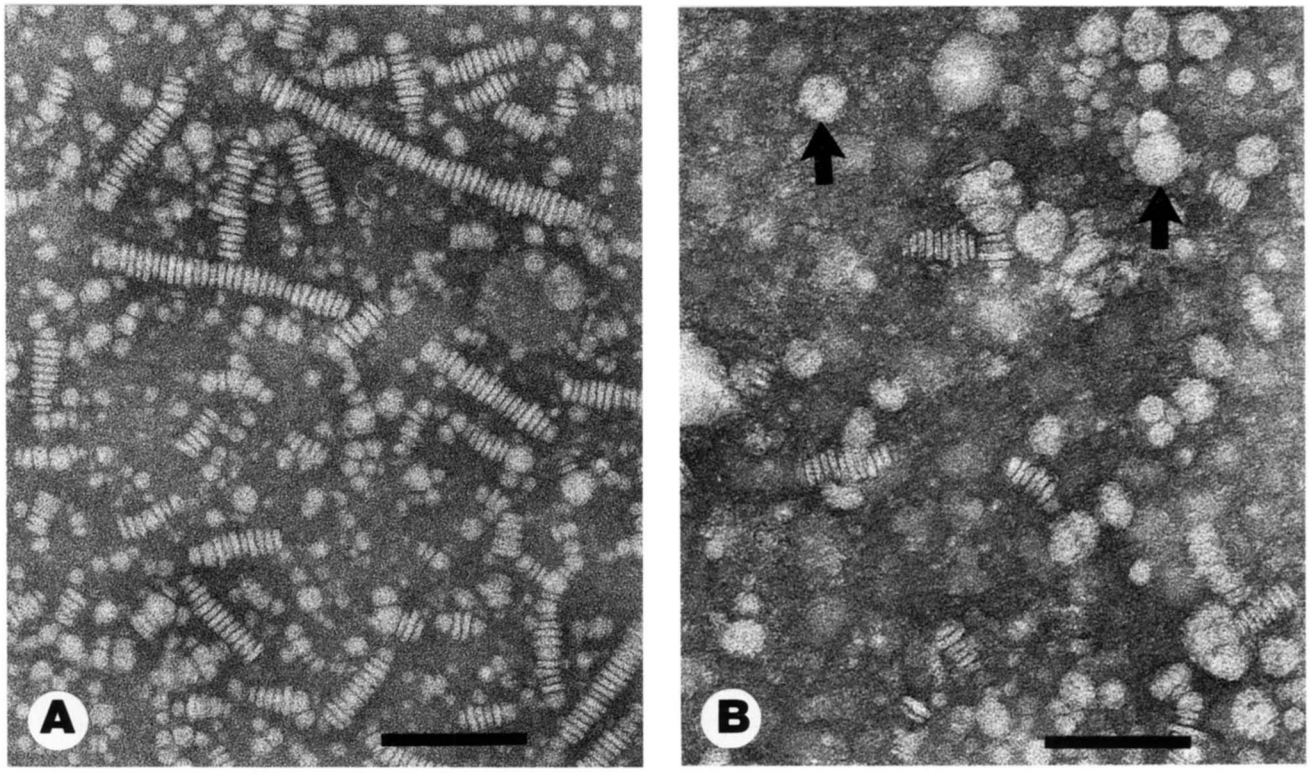

Fig. 6. Electron micrographs of negatively stained Hep3B "HDL" (A) and NPLC "HDL" (B). Hep3B particles are a mixture of discoidal and small round particles. NPLC possess discoidal particles and small, round particles as well as large particles, denoted by arrows, that are probably HBsAg. Bar marker indicates 100 nm.

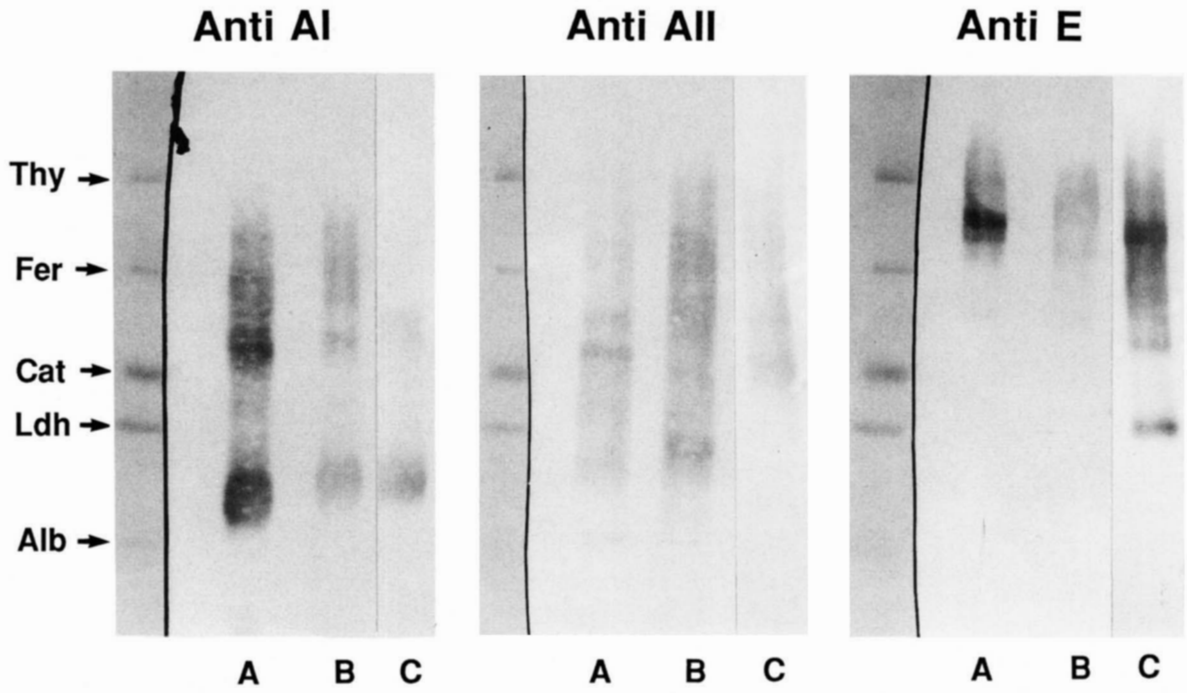

Fig. 7. Western blots for apoA-I, apoA-II and apoE of medium "HDL" isolated from HepG2 (A), Hep3B (B), and NPLC (C) cells. Samples were electrophoresed on 4–30% gels under nondenaturing conditions. HepG2 and Hep3B samples were electrophoresed on the same gel while those of NPLC were electrophoresed in a separate experiment. Standards are indicated on the left: thyroglobulin (Thy); apoferritin (Fer); catalase (Cat); lactate dehydrogenase (Ldh), and albumin (Alb). Amounts of protein applied to gels were 1.5, 1.5, and 3 μg for apoA-I, A-II, and E, respectively, for Hep3B and HepG2, and 5 μg each for apoA-I, A-II, and E for NPLC samples.

DISCUSSION

Hepatocyte cultures can be extremely useful for elucidating the processes regulating synthesis and secretion of nascent lipoproteins. Indeed, the human hepatoma-derived HepG2 cell line has proven particularly attractive for studies on lipoprotein metabolism. Although an early report of Zannis et al. (28) indicated that the Hep3B cell line also secreted the major plasma apolipoproteins and that the PLC/PRF/5 line (NPLC in the present study) secreted apoE, it was not known whether these and other hepatoma-derived cell lines assembled lipoproteins and, if they did, whether such particles had characteristics in common with HepG2. Of the cell lines surveyed, only Hep3B cells proved similar to HepG2 in the rate of total apolipoprotein accumulation in conditioned medium; however, the level of expression of specific apolipoproteins was quite different. Under basal serum-free conditions, Hep3B cells produced greater amounts of apoA-II and apoE, and less apoA-I, than HepG2 cells. Unlike either Hep3B or HepG2, NPLC clearly expresses mainly apoE.

Apolipoprotein production by Hep3B cells, after an initial lag, was essentially linear between 3 and 24 h incubation. These results suggest that during incubation with serum-free medium, the lipoproteins harvested from the medium do not reflect massive release of preformed lipoproteins and apolipoproteins. The concentrations of apolipoproteins accumulating in the medium, even for short incubation periods, probably reflect minimal values since one cannot account for lipoproteins that may have been degraded in the medium, bound to the cell surface, or internalized. Both Hep3B and NPLC cells possess receptors that bind and internalize gold-labeled plasma LDL, thus suggesting the presence of LDL receptors on these cell lines (T. M. Forte, unpublished observation). One cannot conclude from this observation, however, that LDL-receptors bind newly secreted lipoproteins. It still remains to be demonstrated that medium-derived lipoproteins which have physical–chemical properties different from mature plasma lipoproteins (29, and present studies) are readily recognized by the LDL receptor.

Lipoproteins accumulating in the medium during a 24-h period do not necessarily reflect nascent, unaltered particles released into the medium. One cannot exclude the possibility that such particles have undergone modification by lipases, proteinases, lipid exchange proteins, and other factors likely to be secreted by the hepatoma lines. The presence and potential role of such factors in Hep3B

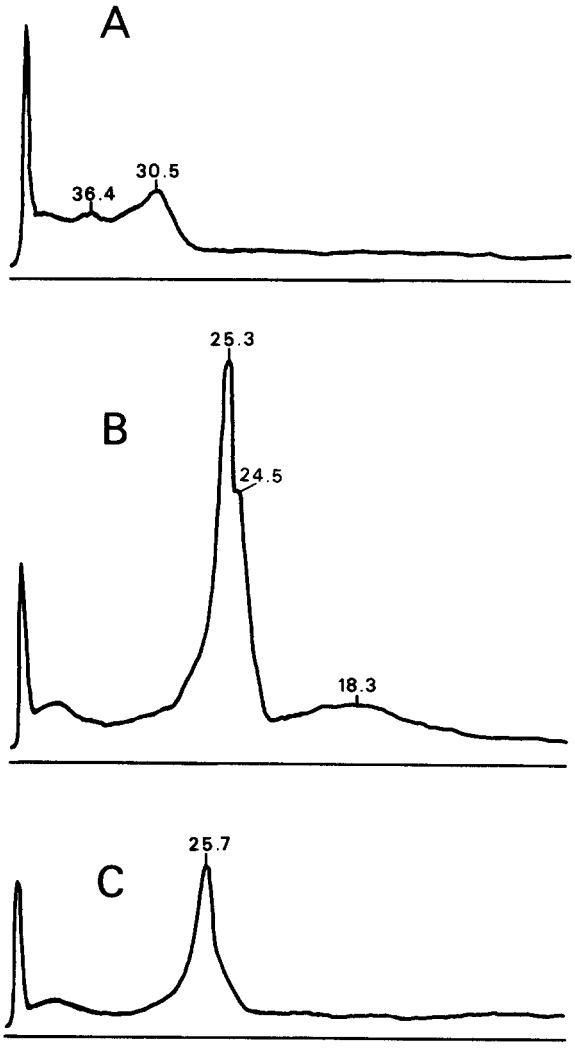

Fig. 8. Representative scans of Hep3B "VLDL" and "LDL" electrophoresed on nondenaturing 2–16% gels and stained with Coomassie blue G250; numbers over peaks indicate particle diameters in nm. (A) "VLDL" fraction; (B) "LDL" fraction; (C) HepG2 d < 1.063 g/ml fraction shown for comparison.

and NPLC medium are yet to be explored. However, nondenaturing gradient gel patterns of Hep3B "HDL" harvested from medium after short incubations suggested that modification and redistribution of lipoprotein products was minimal, since subclass distributions and relative proportions of subclasses for short incubations were similar to those from 24-h incubations.

Relatively large amounts (57-67%) of apoA-I in both Hep3B and NPLC medium were in the lipid-poor fraction, i.e., d > 1.235 g/ml fraction. Similar quantities of lipid-poor apoA-I have been noted in HepG2 medium (30); moreover, the presence of lipid-poor apoA-I has also been described for rhesus monkey liver perfusate (31) and for primary rat hepatocyte medium (32). For HepG2 lipid-poor apoA-I, it has been shown that incubation of d > 1.21 g/ml fraction with dimyristoylphosphatidylcholine yielded a phospholipid-apoA-I discoidal complex that floated at d < 1.21 g/ml (30). Because lipid-poor apoA-I in the case of HepG2 cells readily formed "in vitro" lipoprotein complexes in the presence of phospholipid, we cannot rule out the possibility that hepatoma cells incubated in serum-free culture medium may secrete primarily lipid-poor apoA-I that assembles discoidal particles extracellularly through interaction with plasma membranes and/or other lipoprotein particles.

The present study showed that Hep3B cells accumulate "LDL" comparable in composition and morphology to those of HepG2 (1). Unlike normal plasma LDL that contain a core of cholesteryl ester, triglyceride was the major core lipid in "LDL" from both Hep3B and HepG2; moreover, both contained increased percentages of phospholipid and unesterified cholesterol compared with plasma LDL. It has been suggested that triglyceride-rich "LDL" harvested from HepG2 medium may represent direct secretion of "LDL," since little or no "VLDL" particles were produced by this cell line (1). Since "VLDL"are present in Hep3B conditioned medium, it is unclear whether de novo synthesis and secretion account for the accu-

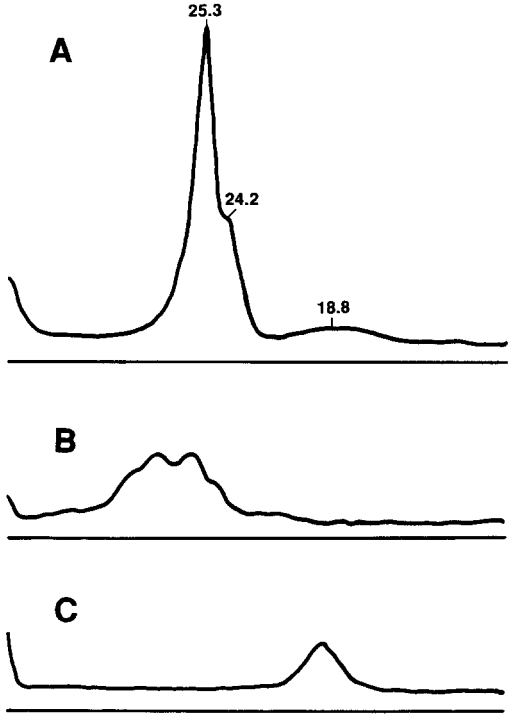

Fig. 9. Hep3B "LDL" electrophoresed on 2–16% gels and subsequently immunoblotted for apoB and apoE. (A) Coomassie blue G250-stained profile of "LDL." A major peak is present at 25.3 nm while a broad minor peak is visible at 18.8 nm. (B) and (C) are reflectance scans of apoB and apoE immunoblots, respectively. Reaction of anti-apoB in the region where there is little Coomassie blue G250 stain may result from distortion of the very porous region of the gel after it is sandwiched for electrophoretic transfer.

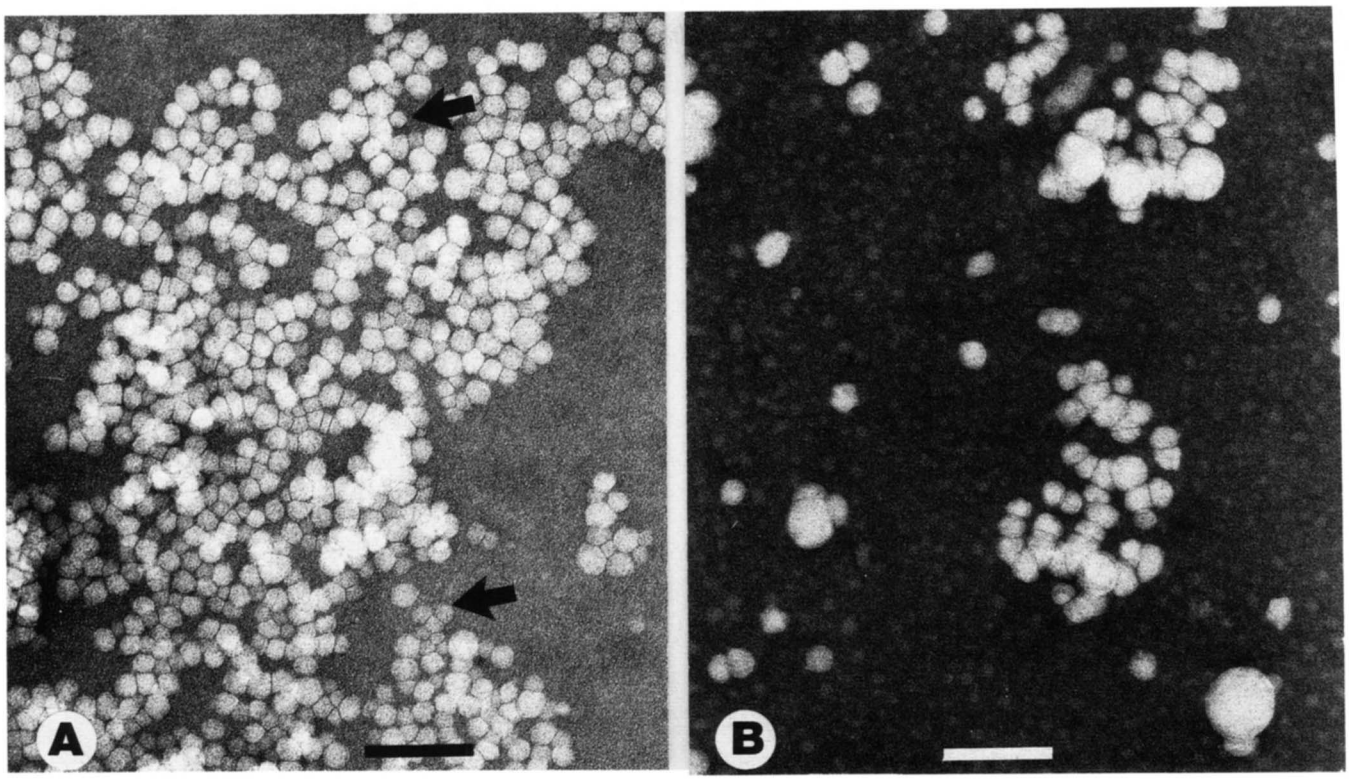

Fig. 10. Electron micrographs of negatively stained d 1.006–1.063 g/ml (A) and d < 1.006 g/ml (B) fractions isolated from Hep3B conditioned medium. The arrows in micrograph (A) indicate particles that are smaller than "LDL." Bar marker represents 100 nm.

mulation of "LDL" particles. A precursor–product relationship between "VLDL" and "LDL" cannot be ruled out; however, the establishment of the metabolic relationship between these two particles awaits identification of lipase activity and/or isolation of Golgi-derived particles.

Hep3B cells incubated under serum-free conditions produced a minor subpopulation of particles within the "LDL" fraction that contained apoE rather than apoB and the particles were considerably smaller (13.6–18.9 nm spherical particles) by electron microscopy than the bulk of the "LDL." The spherical morphology suggests that these small particles probably possessed nonpolar lipid cores, although the exact nature of the core awaits further characterization. The production of core-containing apoE-only particles by cultured cells has not been previously described, but apoE-only discoidal particles have been isolated from several systems including macrophage-conditioned medium (33) and HepG2 serum-free conditioned medium (34). An apoE-lipid complex floating at d 1.09 g/ml has been recovered from medium of mouse L cells transfected with the human apoE gene; no information, however, is available on the morphology or composition of such particles (35).

"HDL" recovered from Hep3B and NPLC media contained numerous discoidal particles. Discoidal "HDL" appear to be a common feature of the so-called "nascent HDL" derived from liver, for in addition to those found in Hep3B, NPLC, and HepG2 medium (1), they were also isolated from the perfusates of LCAT-inhibited rat liver (36) and African green monkey liver (37). The morphology of "HDL" harvested from hepatoma-derived conditioned medium is similar to that previously reported for plasma HDL from LCAT-deficient patients (8). The presence of discoidal "HDL" in the Hep3B and NPLC medium suggests that LCAT is absent or nonfunctional. We have previously noted that HepG2 cells that produced discoidal "HDL" also secreted LCAT (7.9 ng/flask per 24 h) (4), suggesting that in this case LCAT is nonfunctional. The same may be true for Hep3B cells where LCAT is also secreted into the medium (7.1 ng/flask per 24 h). In the case of NPLC cells, no LCAT was detected in the medium, thus suggesting a true LCAT deficiency.

Nondenaturing gradient gel analysis revealed that each cell line had its own unique HDL subclass distribution that is undoubtedly related to differences in apolipoprotein expression. At least three subclasses were, albeit in different proportions, common to all lines including particles banding at 7.5, 10, and 12 nm. The 7.5-nm peak in all cases was the predominant one. This peak from HepG2 "HDL" was shown to consist of small spherical particles that contained cholesteryl ester (16) and apoA-I without apoA-II or apoE (16, 38). Although not isolated

from Hep3B and NPLC conditioned medium, the 7.5-nm particle contained only apoA-I as indicated by immunoblots of gradient gels. Immunoblots suggested that the 10-nm and 12-nm components were hybrids containing apoA-I, apoA-II, and apoE. The presence of subclasses that are unique for a particular hepatoma line may be governed by the proportions of apolipoproteins secreted. It is interesting that NPLC, a high expressor of apoE, formed an 8.3-nm apoE-without apoA-I and apoA-II particle; such a small, presumably discoidal apoE-only particle has not been previously described.

The apoA-II signal in immunoblots of Hep3B "HDL" gradient gels was extremely broad and was localized, for the most part, with particles that contained apoA-I and apoE. However, a prominent Coomassie-stained peak was noted at 8.2 nm which coincided with the discrete apoA-II band found just below the lactate dehydrogenase standard in the immunoblot. This band may represent an apoA-II without apoA-I or apoE particle, although conclusive evidence awaits its isolation. The in vivo appearance of apoA-II particles without apoA-I has been documented in HDL from patients with apoA-I and apoC-III deficiency (39). In this genetic disorder, one of the major HDL subclasses banded at approximately 8.0 nm, a position similar to that of the apoA-II without apoA-I immunostaining band of Hep3B "HDL." Unlike Hep3B, HepG2 cells secrete only a small quantity of apoA-II, which was recently demonstrated by immunoaffinity chromatography to be associated with apoA-I and apoE (34).

A distinguishing difference between Hep3B cells and HepG2 cells is the ability of the former to accumulate small quantities of "VLDL" in serum-free medium. In our hands, HepG2 cells, grown under identical conditions as those of Hep3B cells, do not accumulate "VLDL" in the medium. The Hep3B "VLDL" particles provide supporting evidence that, in the human, newly secreted VLDL most likely are associated with apolipoproteins B, C, and E. These apolipoproteins are also the major ones associated with fasting plasma VLDL. Nascent VLDL transporting apolipoproteins B, C, and E have been isolated from nonrecirculating rat liver perfusate (40), primary rat hepatocyte cultures (41), and nonhuman primate liver perfusate (37). In an interesting study with sucrose-fed rat livers, Nestruck and Rubinstein (42) showed that under this dietary condition which enhances VLDL secretion, nascent VLDL isolated from the Golgi contained apoB and apoE but little or no apoC. In the presence of colchicine, which slows or inhibits VLDL secretion, however, they found that nascent VLDL isolated from Golgi had acquired apoC. These findings suggest that where there is rapid transit of nascent VLDL through the hepatocyte one might expect particles with primarily apoB and apoE, while where transit time is slow, as would be the case in Hep3B cells grown under basal, serum-free conditions, the nascent particle acquires apoC during the later stages of VLDL assembly.

In summary, we have shown diversity in lipoproteins produced by several readily available human hepatoma-derived cell lines grown in serum-free medium. Such differences are potentially extremely useful for examining modulation of lipoprotein subclasses. One can take advantage of unique "HDL" subclasses produced by Hep3B and NPLC cells to elucidate the metabolic transformation of specific apoE-rich or apoA-II-rich particles such as are found in familial LCAT deficiency, abetalipoproteinemia, and apoA-I-apoC-III deficiency.

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