Heterogeneity of nascent high density lipoproteins secreted by the hepatoma-derived cell line, Hep G2

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Abstract Nondenaturing gradient gel analysis of high density lipoproteins (HDL, d 1.063–1.235 g/ml) isolated from Hep G2 24-hr, serum-free conditioned media shows four distinct, reproducible particle subclasses I, II, III, and IV with apparent Stokes' diameters of 13.3, 12.0, 9.5, and 7.4 nm, respectively. Fractions enriched in lipoproteins from each of these subclasses were isolated by either density gradient ultracentrifugation or gel filtration chromatography and characterized. Size and morphology of the isolated subclasses agreed well regardless of isolation procedure. Electron microscopy revealed subclasses I, II, and III to be disc-shaped, and subclass IV to be spherical. The discoidal subclasses were poor in cholesteryl ester and rich in phospholipid and unesterified cholesterol. The larger-sized subclass I particles were enriched in apolipoprotein (apo) E while subclasses II and III had decreasing amounts of apoE and increasing amounts of apoA-I and A-II. The spherical subclass IV particles contained a higher percentage of protein and had a higher ratio of cholesteryl ester to unesterified cholesterol than that found in the other subclasses. Subclass IV contained predominantly apoA-I. The subclasses isolated from Hep G2 HDL appear to share many similarities with those isolated from patients with lecithin:cholesterol acyltransferase deficiency and are therefore potentially useful in examining the transformation of nascent HDL particles to mature circulating plasma forms. —**McCall, M. R., T. M. Forte, and V. G. Shore.** Heterogeneity of nascent high density lipoproteins secreted by the hepatoma-derived cell line, Hep G2. *J. Lipid Res.* 1988. **29:** 1127–1137.

Supplementary key words apolipoproteins • density gradient ultracentrifugation • electron microscopy • gel filtration chromatography • gradient gel electrophoresis

The intravascular metabolism of high density lipoproteins (HDL) is a dynamic process involving a variety of extremely rapid enzymatic and exchange reactions. High density lipoproteins of hepatic or intestinal origin isolated in the plasma are most likely distinct from their cellular precursors having already been partially or completely remodeled in the extracellular milieu. Recent work with the human hepatoblastoma-derived cell line, Hep G2, has indicated that these cells may be useful as a model for the study of newly secreted HDL that have not been exposed to intravascular processing (1, 2). Although some chromosomal abnormalities have been described (3), the Hep G2 cell line has retained well-differentiated functions, expressing many traits attributed to normal human hepatocytes. In addition to lipoproteins and lipid-poor apolipoproteins, these cells release into the culture media many of the major plasma proteins (4) and also express the receptors for low density lipoproteins (LDL) (5–7), estrogen (8), insulin, and transferrin (9).

Previous studies with the Hep G2 cell line in this laboratory (2) have shown that the HDL harvested from conditioned medium are heterogeneous with respect to particle size and morphology. The present study characterizes this particle heterogeneity and shows that it shares many similarities with HDL isolated from other nascent lipoprotein model systems (10–12) and from the plasma of patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency (13–15).

METHODS

Cell culture

The human hepatoblastoma-derived cell line, Hep G2, was obtained from Dr. Barbara Knowles of the Wistar Institute of Anatomy and Biology (Philadelphia, PA). Cells were grown to confluency and conditioned medium was collected as previously described (2) using 175-cm^2 tissue culture flasks (T 175 flasks, Falcon, Oxnard, CA). Insulin and additional glucose, however, were omitted from the media in these experiments. These omissions had no effects on the parameters studied.

Abbreviations: apo, apolipoprotein; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; proapo, proapolipoprotein; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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HDL isolation and subfractionation

Conditioned serum-free medium samples (1–2 liters) collected from 25 to 50 confluent monolayer cultures were pooled on ice and subsequently centrifuged at 1000 *g* (30 min, 4°C) to remove detached cells and debris. Gentamicin sulfate (0.1 mg/ml), ethylenediaminetetraacetic acid (EDTA)-dipotassium salt (1.0 mg/ml), *p*-hydroxymercuriphenylsulfonic acid (0.25 mM), phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.5 μg/ml), and pepstatin (0.7 μg/ml) were added to protect the lipoproteins from microbial, oxidative, and proteolytic damage. The medium was concentrated approximately 100-fold using Amicon stirred cells (Amicon, Lexington, MA), Amicon PM-30 membranes, and nitrogen pressure.

High density lipoproteins (HDL, d 1.063–1.235 g/ml) were isolated from the concentrated culture medium by sequential ultracentrifugation (16). The majority (60%) of apoA-I in Hep G2-conditioned medium is in the lipid-poor form (2); however, this material is removed from the HDL fraction during ultracentrifugation. Approximately 1.2 μg of total HDL protein was obtained per ml of conditioned medium, i.e., 24 μg/flask. Density adjustments were made with solid NaBr and volumes were adjusted with appropriate NaCl/NaBr solutions.

The lipoproteins isolated within the HDL density range were fractionated by density gradient ultracentrifugation (17) on three separate occasions. A different pool of conditioned media was used for each of the experiments. Two ml of the isolated HDL (d 1.235 g/ml) was sequentially overlayered with 6.5 ml of d 1.125 g/ml NaCl/NaBr solution and 2.5 ml of a d 1.063 g/ml NaCl/NaBr solution. The samples along with salt blanks were ultracentrifuged at 40,000 rpm for 48 hr (15°C) in a Beckman SW41 rotor. Fractions (0.5 ml) were pipeted down the length of the sample tube and dialyzed against 0.05 M NaCl–0.001 M EDTA. Salt blanks prepared by substituting a 1.235 g/ml NaCl/NaBr solution for the HDL sample were used to monitor the density gradient. Density measurements were obtained with a Mettler/Paar density meter.

In a separate experiment, HDL (0.92 mg apoA-I in 0.20 μl) isolated from Hep G2-conditioned media were applied to prepacked Superose 6 and 12 HR 10/30 columns (Pharmacia Fine Chemicals, Piscataway, NJ) connected in series in the Pharmacia FPLC system. Equilibration and elution were with phosphate-buffered saline at a flow rate of 12 ml/hr. Fractions of 0.25 ml were collected; protein elution was monitored by absorbance at 280 nm. Before pooling fractions, aliquots were removed for nondenaturing gradient gel electrophoresis and quantitative immunoassay of apoA-I.

Subfraction characterization

Each 0.5-ml sample recovered from the NaCl/NaBr gradient was analyzed for protein (18) and electrophoresed on a nondenaturing 4–30% polyacrylamide gradient gel (19) which was subsequently stained with Coomassie blue. This procedure was followed so that the migration position of the major protein component within each fraction could be used to determine appropriate fractions for pooling. Fractions containing appreciable quantities of more than one subclass were not included in the major subclass pools. Apolipoprotein composition of each major pool was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Weber and Osborn (20). A 4% stacking gel combined with a 15% running gel was used in a Hoefer Mighty Small electrophoretic apparatus (Hoefer Scientific Instruments, San Francisco, CA). Bio-Rad's low molecular weight standard protein kit and authentic human apolipoprotein (apo) E (a gift from Dr. Karl H. Weisgraber) were used as standards. Quantitation of apoA-I, phospholipid, esterified and unesterified cholesterol, and the electron microscopic assessment of particle size and morphology were carried out as previously described (2).

Fractions from the Superose column were pooled and analyzed in the same manner as those obtained from the density gradient. However, smaller quantities of HDL were available for chromatography and, therefore, characterization of the column pools was not as complete as the characterization of the pools from the density gradient.

Western blotting

The distribution of apoA-I, A-II, B, and E following gradient gel electrophoresis of Hep G2 HDL in 4–30% nondenaturing polyacrylamide gels was determined by Western blotting, as previously reported (2). One μg of protein for apoA-I and A-II and 3 μg of protein for apoB and E were applied to each lane prior to electrophoresis. Completeness of protein transfer to nitrocellulose was assessed by staining the electroeluted gradient gel with Coomassie blue. Blots were developed with biotinylated IgG and avidin:biotin:horseradish peroxidase complex (Vectastain ABC, Vector Laboratories, Burlingame, CA) using 4-chloro-1-napthol and H_2O_2 as substrate. Control blots were developed with the appropriate nonspecific goat or mouse IgG or mouse ascites instead of the specific anti-apolipoprotein. Specific antibodies were used as probes; for apoA-I, a high affinity fraction of anti-A-I prepared by immunoaffinity chromatography of goat anti apoA-I serum on Sepharose-bound HDL (apoA-I) was used. This preparation was generously provided by P. Blanche and A. V. Nichols of the Donner Laboratory. Confirmation was with mouse monoclonal anti-apoA-I antibodies. For apoA-II, goat anti-A-II antibodies, prepared by P. Blanche, were used. For apoB, rabbit anti-apoB antiserum was used, and for confirmation mouse monoclonal anti-apoB antibodies were used. For apoE, a

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mixture of two or three mouse monoclonal anti-apoE antibodies (obtained from Mallinckrodt, Inc., St. Louis, MO, and Chemicon International, Inc., El Segundo, CA) was used. All antibodies were titered against the appropriate purified apolipoprotein and lipoprotein. Specificity of the antibodies was confirmed by dot blotting (sensitivity, 10 ng) known apolipoproteins, lipoproteins, albumin, α_2-macroglobulin, and fibrinogen; e.g., the anti-A-I was tested against HDL, purified apoA-I, apoA-II, apoCs, plasma VLDL, and a narrow density fraction of plasma LDL that contained no apoA-I, as well as the nonlipoprotein proteins.

The electrophoretic mobility of Hep G2 HDL was examined on agarose gels (Paragon, Beckman Instruments, Brea, CA). The HDL could not be visualized by Fat Red 7B staining due to the small amounts of neutral lipids associated with these particles, therefore unstained gels were blotted onto nitrocellulose and probed as above with antibody to apoA-I.

RESULTS

Charge heterogeneity of Hep G2 HDL

The electrophoretic mobility of Hep G2 HDL assessed by agarose gel electrophoresis and subsequent immunoblotting for apoA-I is shown in **Fig. 1.** Hep G2-conditioned medium (lane C) as well as ultracentrifugally isolated Hep G2 HDL (lane B) showed two distinct apoA-I- containing bands. Human plasma (lane E) and ultracentrifugally isolated plasma HDL (lane D) also showed two electrophoretically distinct components. The mobilities of the two plasma components, however, differed from those observed for Hep G2; the first band (nearer the origin) migrated faster and the second band slightly slower than the corresponding Hep G2 material. Interestingly, lipid-poor apoA-I (lane A), isolated from Hep G2-conditioned medium at a density greater than 1.235 g/ml, migrated to the same position as that occupied by the first band in lanes B and C. Purified plasma apoA-I (lane F), in contrast, had a mobility unlike plasma or Hep G2 material. Its charge characteristics were such that it migrated as a single band to a position midway between the Hep G2 and plasma slow-migrating bands.

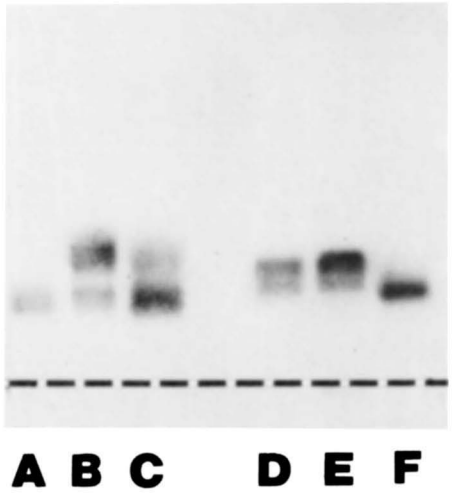

Fig. 1. ApoA-I immunoblot of Hep G2 and human plasma lipoproteins following agarose gel electrophoresis. Lanes A and F contain the d > 1.235 g/ml fraction from Hep G2 conditioned medium and purified apoA-I, respectively. The other lanes are as follows: B, Hep G2 HDL; C, Hep G2 conditioned medium; D, human HDL; E, human plasma. All samples contained approximately 3% human albumin. The dashed line indicates the point of sample application and is opposite the anodal side of the gel.

Hep G2-conditioned medium tended to have a more prominent slow-migrating apoA-I band (lane C) than ultracentrifugally isolated Hep G2 HDL (lane B). This difference is due to the large proportion of lipid-poor apoA-I found in Hep G2-conditioned medium (21). Ultracentrifugation effectively removed the lipid-poor apoA-I from the lipoprotein fractions, resulting in the differences in staining intensities observed in lanes B and C.

Size heterogeneity of Hep G2 HDL

Isolated Hep G2 HDL (d 1.063–1.235 g/ml) was electrophoresed on nondenaturing 4–30% polyacrylamide gels and apolipoproteins A-I, A-II, B, and E were localized by Western blotting. This was done in order to ascertain apolipoprotein distribution relative to the major subclasses localized by protein staining of these gels. Typical Western blots are seen in **Fig. 2.** Lanes A and F show the Coomassie blue G250-stained Hep G2 HDL and protein standards, respectively. Although minor differences from one gel to another are observed, Hep G2 HDL possesses four major subclasses based on particle size, labeled I through IV (Fig. 2), which correspond to standard proteins having Stokes' diameters of 13.3, 12.0, 9.5, and 7.5 nm, respectively. A minor component was occasionally detected between the lactate dehydrogenase and catalase standards. This component, when observed, contained apolipoproteins A-I and A-II. Distribution of apoA-I-containing HDL particles is shown in lane B. This banding pattern closely approximates that observed with the Coomassie blue stain and is similar to that previously reported by Thrift et al. (2). The most prominent apoA-I-containing region corresponds to that of the 7.5-nm particles, subclass IV. Intensely staining regions are also associated with subclasses II, III, and, to a lesser extent, subclass I. The gel immunoblotted for apoA-II (lane C) shows two pronounced bands between the catalase and ferritin standards; both components appear in the gel region occupied by subclass III, although only the smaller-sized component appears unequivocally associated with this subclass. Diffuse apoA-II staining is ob-

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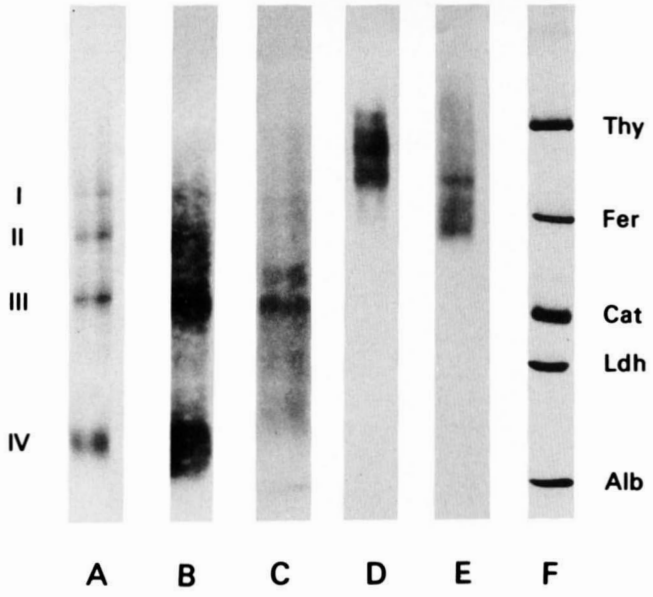

Fig. 2. Nondenaturing 4–30% gradient gel electrophoresis and immunoblots of Hep G2 HDL. Lane A was stained with Coomassie blue G250; the apparent Stokes' diameters of the major subclasses, labeled I through IV, are from top to bottom: 13.5 nm, 11.7 nm, 9.8 nm, and 7.4 nm. Corresponding R_f values are 0.43, 0.49, 0.62, and 0.90, respectively. Lanes B through E were immunoblotted for apolipoproteins A-I, A-II, E, and B, respectively. Lane F contains Coomassie blue G250-stained standard proteins: thyroglobulin, Thy; ferritin, Fer; catalase, Cat; lactate dehydrogenase, Ldh; albumin, Alb.

served in the region between ferritin and thyroglobulin. ApoA-II is also proximate to subclass IV, but is associated with larger diameter components and does not directly overlap with the major apoA-I component. The E and B apolipoproteins, unlike A-I and A-II, are found only in the larger pore regions of the gel (lanes D and E, respectively). The major apoE-containing component (lane D) approaches thyroglobulin in size; in addition, an apoE band appears in the gel region occupied by subclass I. ApoB has a distinct band in the same region as the smaller apoE component (subclass I) and also has a broad, strongly staining band in the subclass II region (lane E). Diffuse apoB staining is also noted between the thyroglobulin standard and subclass I. The increased sensitivity of the immunoblotting technique over standard Coomassie blue staining methods allows for better subclass resolution; however, the significance of finding immunoreactive material in non-Coomassie blue-staining regions cannot be adequately addressed at this time, due to the extremely small amounts of protein involved.

HDL subfractionation by density gradient ultracentrifugation

Density gradient ultracentrifugation of Hep G2 HDL resulted in the protein and density distributions shown in **Fig. 3.** The fractions that were subsequently combined are marked I through IV on the abscissa. Comparison of gradient gel electrophoretic patterns obtained from unfractionated Hep G2 HDL and the various density gradient fractions permitted the pooling of fractions with electrophoretic mobilities characteristic of the major Hep G2 HDL subclasses (i.e., fractions enriched with lipoproteins with apparent Stokes' diameters of 13.3, 12.0, 9.5, and 7.5 nm). The effectiveness of this procedure for isolating the Hep G2 HDL subclasses is illustrated in **Fig. 4.** The five electrophoretograms shown were obtained from Coomassie blue-stained gradient gels of the following: (A) Hep G2 HDL; (B) pool I, subclass I; (C) pool II, subclass II; (D) pool III, subclass III; (E) pool IV, subclass IV. It is apparent in the scans that subclasses I and II are still quite heterogeneous, although the major protein component in each pool has the appropriate particle size. Subclasses III and IV, on the other hand, are much more homogeneous in particle size. Similar results were obtained on each of the other two density gradient fractionations. Mean Stokes' diameters of the major subclass peaks for the three separations expressed as the mean ± SD were: 13.7 ± 0.3, 12.1 ± 0.3, 9.8 ± 0.1, and 7.5 ± 0.1. The four major subclasses isolated by density gradient ultracentrifugation accounted for 65 ± 10% (mean ± SD of three separations) of the total HDL protein. The distri-

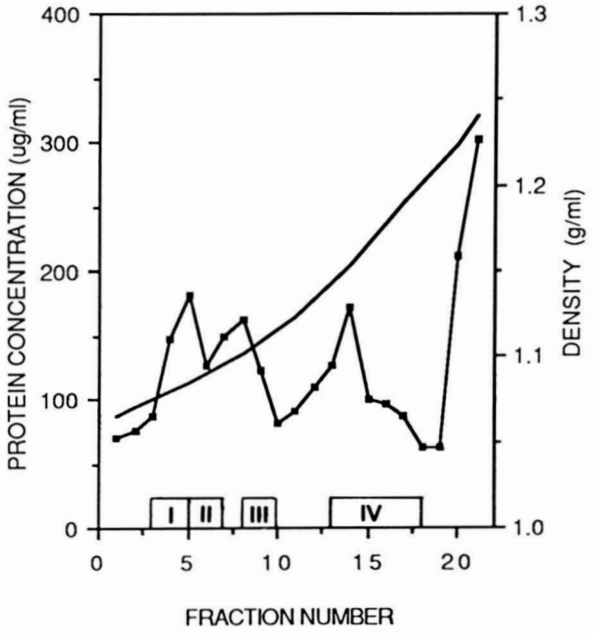

Fig. 3. Protein (■—■) and density (—) distribution of Hep G2 HDL (d 1.063–1.235 g/ml) after density gradient ultracentrifugation. Fractions of 0.5 ml were aspirated down the length of the tube following the 48-hr density gradient run (Beckman SW41 rotor, 40,000 rpm; 15°C). Regions marked by Roman numerals indicate fractions that were pooled. Pooling was based on gradient gel mobility and not on the protein distribution shown.

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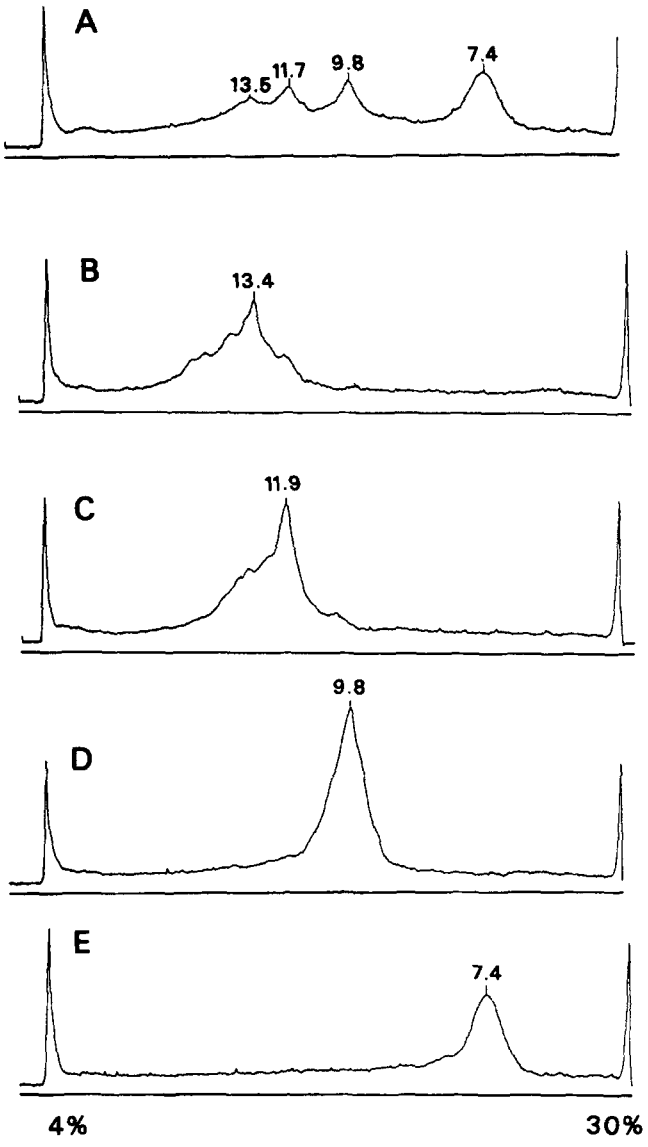

Fig. 4. Densitometric scans of Coomassie blue G250-stained 4–30% polyacrylamide gradient gels of Hep G2 HDL and HDL subclasses. Profile A shows the characteristic pattern of Hep G2 HDL; peak positions in nm are indicated above each peak. The subclasses (I–IV) obtained after pooling density gradient ultracentrifugation aspirates are shown in profiles B through E, respectively. Size estimates were based on gradient gel electrophoresis of standard globular proteins. Subclass I–III lipoproteins are discoidal (Fig. 6); therefore, their peak positions can only be used as a relative size determinant.

bution of protein among the subclasses was 10 ± 2%, 18 ± 5%, 15 ± 4%, and 22 ± 2% for subclasses I, II, III, and IV, respectively.

Composition of density gradient fractions

The compositions of the major Hep G2 HDL subclasses isolated by density gradient ultracentrifugation are presented in **Table 1.** Increases in particle density corresponded with increases in the percentage protein and decreases in the percentage of unesterified cholesterol and phospholipid. Subclass IV had a considerably higher protein-to-lipid ratio and contained a higher percentage of cholesteryl ester than subclasses I–III. The cholesteryl ester-to-unesterified cholesterol ratio of 6.1 for subclass IV was remarkably higher than the ratios calculated for subclasses I–III. The ratio was less than 0.2 for the latter three subclasses. Triglyceride determinations were not carried out because of the relatively small amounts of sample available and the low triglyceride concentrations found in Hep G2 HDL.

The apolipoproteins associated with total Hep G2 HDL and isolated subclasses were separated by SDS-PAGE; a typical distribution is shown in **Fig. 5.** Apolipoprotein distribution in unfractionated HDL (lane 8) was similar to that previously reported by our laboratory (2), where apoA-I is the major protein followed by apoA-II dimer, apoE, apoE-A-II heterodimer (apoE-apoA-II monomer), and the apoCs. Equal amounts of protein were loaded on the gel for the isolated subclasses, permitting the estimation of relative apolipoprotein distribution. Apolipoprotein E and the heterodimer of apoE and apoA-II decreased with decreasing particle size (lanes 4–7). The smallest size particles, those isolated as subclass IV (lane 7), had no detectable apoE or apoE-A-II heterodimer. The predominant HDL apolipoprotein, apoA-I, increased with decreasing particle size. Similarly, the dimeric form of apoA-II tended to increase with decreasing particle size. The relative apoA-I distribution among subclasses suggested by the Coomassie-stained gel was supported by quantitation of apoA-I concentrations by radial immunodiffusion. ApoA-I values expressed as a percentage of total protein (Lowry) for each subfraction were as follows: I, 38 ± 3%; II, 53 ± 3%; III, 73 ± 5%; and IV, 74 ± 4%.

Electron microscopic structure of density gradient fractions

Morphological characteristics of the four Hep G2 HDL subclasses are shown in **Fig. 6.** Subclasses I–III, as might be inferred from the compositional data (large amounts of phospholipid and unesterified cholesterol relative to neutral lipid), appear to be mainly discoidal structures in rouleaux. The mean diameter of the discs decreases with increasing density; the mean diameters for subclasses I, II, and III are 21.5 ± 2.6 nm, 18.7 ± 2.1 nm and 15.1 ± 1.9 nm, respectively. Subclass IV lipoproteins are small, round particles 7.8 ± 1.6 nm in diameter. The discrepancy between the size of particles estimated by electron microscopy and gradient gel electrophoresis (Fig. 4) relates to the nonspherical nature of the lipoproteins isolated in subclasses I through III; globular proteins were used to calibrate the gradient gels. The two methods were in good agreement with the spherical particles isolated as subclass IV.

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TABLE 1. Composition of the major Hep G2 HDL subclasses

Subclass	Composition (% by Weight)				d	GGE[a]
	Protein	Cholesteryl Ester	Unesterified Cholesterol	Phospholipid		
					g/ml	*nm*
I	24.6 ± 2.3	1.3 ± 1.2	23.0 ± 3.4	51.3 ± 5.5	1.084	13.7
II	28.3 ± 3.1	1.5 ± 0.4	22.7 ± 1.0	47.3 ± 4.2	1.096	11.9
III	38.0 ± 3.6	1.9 ± 0.7	17.7 ± 1.2	42.3 ± 4.0	1.116	9.8
IV	63.6 ± 5.8	4.9 ± 1.2	0.8 ± 0.4	30.7 ± 6.4	1.171	7.6
Unfractionated HDL	41.3 ± 2.1	2.7 ± 1.5	17.1 ± 1.0	38.7 ± 3.8	1.063–1.235	

Each value represents the average of three experiments ± standard deviation.
[a]Size estimates are based on GGE (gradient gel electrophoresis) of standard globular proteins.

Characterization of subclasses isolated by Superose column chromatography

Hep G2 HDL isolated by ultracentrifugation were separated into the four major subclasses by gel filtration. This procedure reduced the centrifugation time required for the isolation of subclasses and permitted the comparison of subclass particles isolated solely on the basis of size with those isolated on the basis of density and size. The apoA-I elution profile from the single Superose column fractionation is shown in **Fig. 7.** The fractions that were eventually combined are marked I through IV on the abscissa. In general, there was good agreement between the two isolation strategies. Particle diameter and morphology as assessed by electron microscopy were similar (subclass I, 21.5 ± 2.8 nm, disc; subclass II, 20.3 ± 2.9 nm, disc; subclass III, 17.3 ± 2.6 nm, disc; subclass IV, 7.9 ± 1.4 nm, round) as were the compositions of subclasses II–IV (data not shown). Sufficient material for compositional analysis of subclass I was not obtained.

The subclass IV lipoproteins isolated by gel filtration contained only apoA-I as determined by SDS-PAGE (gel not shown). The Western blot analyses of unfractionated Hep G2 HDL (Fig. 2) also suggested that subclass IV particles contained only apoA-I. The discrepancy between these data and those obtained by density gradient ultracentrifugation, where subclass IV possessed both apoA-I and apoA-II, may reflect differences between lipoproteins obtained by the two isolation strategies. Subclasses isolated on the basis of size and density (ultracentrifugation) may be similar, but not identical to subclasses isolated on the basis of size alone (chromatography). It is also possible that the differences resulted from cross-contamination

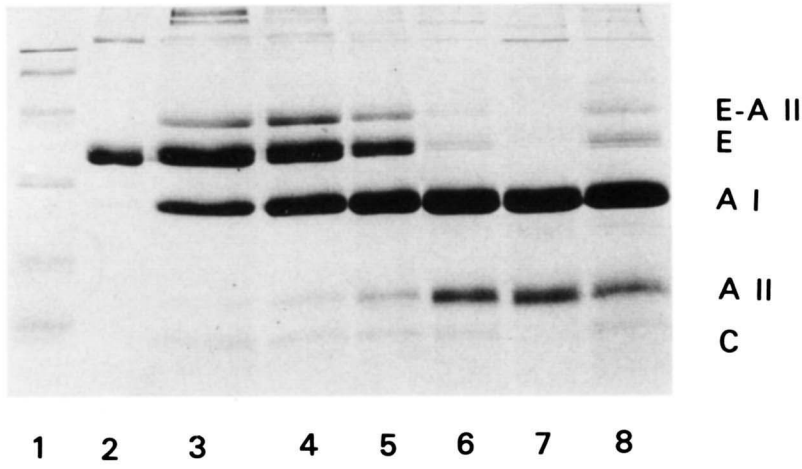

Fig. 5. SDS polyacrylamide gel (15%) electrophoresis of unfractionated Hep G2 HDL and HDL subclasses. Standard proteins were electrophoresed in lane 1 (from top to bottom; phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme) and authentic human apoE in lane 2. Lane 3 contains material aspirated from the top 1.5 ml of the density gradient. Subclasses I–IV were electrophoresed in lanes 4–7, respectively, (15 μg of protein per lane). Unfractionated HDL (20 μg of protein) was run in lane 8. Samples were not reduced prior to electrophoresis. Apolipoprotein bands, identified on the right, were confirmed by Western blot analysis.

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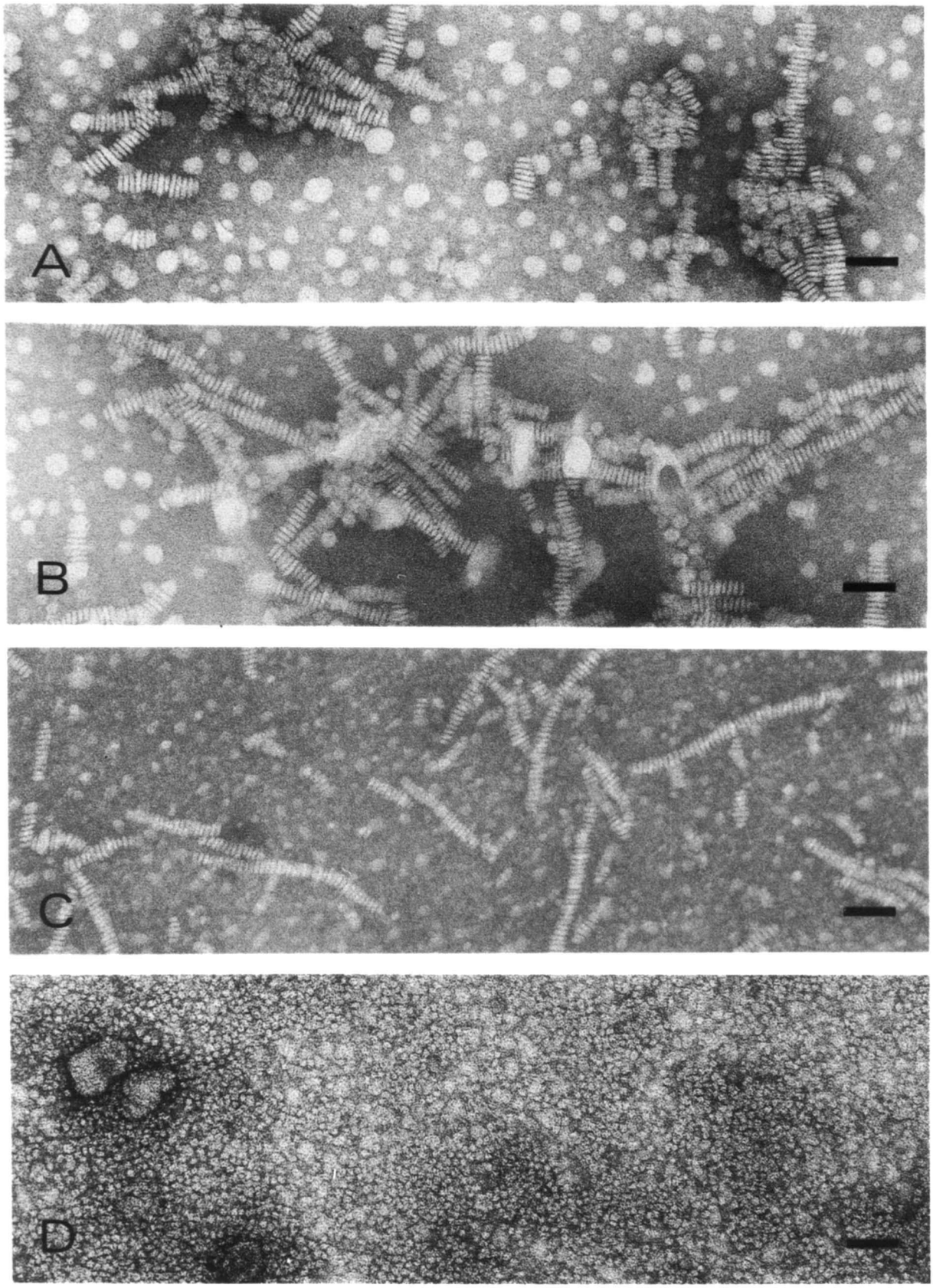

Fig. 6. Electron micrographs of negatively stained lipoproteins: (A) subclass I; (B) subclass II; (C) subclass III; (D) subclass IV. The large, round particles in panel A may represent contaminating LDL. The bar marker in the lower right-hand corner of each micrograph indicates 50 nm.

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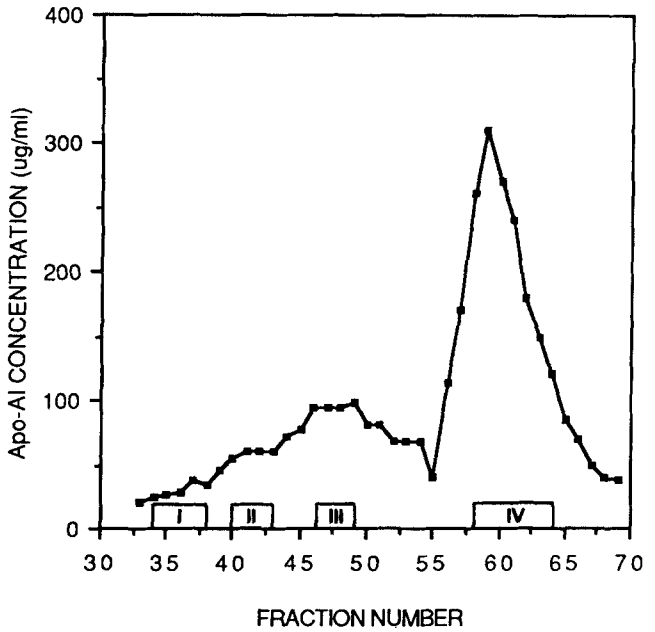

Fig. 7. ApoA-I distribution after fractionation of Hep G2 HDL (d 1.063–1.235 g/ml) by gel filtration chromatography. Regions marked by Roman numerals indicate fractions that were pooled. Pooling was based on gradient gel mobility and not on the apoA-I elution profile shown.

during the aspiration procedure employed with the density gradient technique.

DISCUSSION

Recent work with the Hep G2 cell line in this laboratory has focused on the characterization of the major lipoprotein classes produced under defined basal conditions (2). It was evident in these studies that the HDL class was heterogeneous with respect to particle size and structure and that this heterogeneity was distinct from that observed in normal human plasma HDL. In the present study we have found that Hep G2 HDL possess two apoA-I-containing populations that can be separated by agarose gel electrophoresis. These two particle populations differ somewhat in charge from two similarly separated plasma HDL populations. It is striking when considering the morphological and compositional differences of plasma HDL and Hep G2 HDL that both are separated on agarose into only two apoA-I-containing components. The morphology (which may influence exposed charge) and apolipoprotein composition of these two particle classes are presently under investigation in our laboratory. We speculate that the large proportion of proapoA-I in Hep G2-conditioned medium may, in part, contribute to the observed mobility differences in Hep G2 and plasma. The additional positive charge associated with proapoA-I would tend to reduce eletrophoretic mobility.

We have isolated, by density gradient ultracentrifugation and by gel filtration, the major Hep G2 HDL subclasses that are consistently found in media harvested after 24 hr of culture. The relative enrichment of each subclass that we were able to obtain by the density gradient procedure suggests a close relationship between Hep G2 HDL hydrated density and particle size. These lipoproteins may reflect: *1*) nascent particles as released by the cell; *2*) thermodynamically stable structures formed extracellularly by the association of free apolipoproteins and lipid; or *3*) particles formed by either of these mechanisms and subsequently modified by enzymes, transfer proteins, or re-uptake and re-secretion. We have reported previously that Hep G2 cells release LCAT into the culture medium (22), although the activity of this enzyme appears to be inhibited under the present culture conditions. Since most of the cholesteryl ester content of plasma HDL is thought to be the result of LCAT activity, it is not surprising that the Hep G2 HDL subfractions I–III contained relatively small amounts of cholesteryl ester. Hepatic lipase (23), as well as cholesteryl ester transfer activity (24), have been reported to be present in Hep G2 cultures. The activities of these proteins, however, were determined using artificial substrates. It is unclear whether these latter mechanisms contribute to lipoprotein remodeling in the culture media. We have found, however, that after only 3 hr of culture, the medium-derived HDL have the same subclass distribution in the same relative proportions as 24-hr products (**Fig. 8**), indicating that changes, if they occur, are rapid and that the resulting particles are stable.

The HDL subclasses that we have isolated from the conditioned media of Hep G2 cells share morphological and compositional similarities with HDL obtained from patients with LCAT deficiency (13–15) and from other systems in which LCAT activity is naturally low (12) or has been inhibited (10, 11). **Table 2** summarizes published information on the composition, isolation density, and particle size of lipoproteins analogous to our Hep G2 subclass IV. In all cases, the particles are relatively poor in core neutral lipids and rich in protein and phospholipid. It appears that, at least in the case of the rat, an analogous particle may originate in the intestine (11). The predominant apolipoprotein associated with these particles is apoA-I. Our immunoblot and SDS-PAGE analyses of subclass IV apolipoproteins suggest that, at least in the case of particles isolated by gel filtration, the sole apolipoprotein present is A-I. Preliminary cross-linking experiments with dimethylsuberimidate (25) indicate that subclass IV particles contain two A-I molecules per particle. An analogous lipoprotein isolated by Chen et al. (15) from the plasma of patients with familial LCAT deficiency was calculated to contain two A-I molecules per particle. Normal human plasma may also contain small quantities of a subclass IV analogue; Cheung and Albers (26) have ob-

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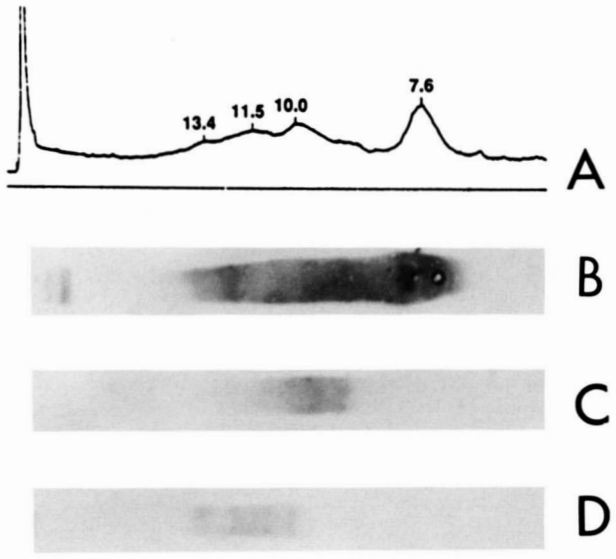

Fig. 8. Nondenaturing 4–30% gradient gels of Hep G2 HDL isolated from 3-hr conditioned medium that have been stained and scanned or immunoblotted. Panel A shows a densitometric scan of a Coomassie blue G250-stained gel. The apparent Stokes' diameters of the major subclasses measured from this gel are shown, and they correspond closely to the major peaks seen after a 24-hr harvest (cf. Fig. 4A). Panels B through D are from gels immunoblotted for apolipoproteins A-I, A-II, and E, respectively; the profiles of these blots are similar to those obtained from 24-hr conditioned medium.

served that an apoA-I without apoA-II lipoprotein fraction isolated from normal human plasma by immunoaffinity chromatography contains a minor subpopulation that has a particle diameter about 7.6 nm.

Similarly, disc-shaped lipoproteins isolated as subclasses I through III appear to resemble the disc-shaped HDL isolated from LCAT-deficient systems (12–14). The most appropriate comparison appears to be with the study of Soutar, Knight, and Myant (14) in which plasma from patients with familial LCAT deficiency was fractionated on the basis of particle size. These investigators isolated three disc-shaped HDL subclasses using a Sephacryl S-300 column; particle diameter, assessed by electron microscopy, ranged between 30 and 35 nm for the largest particles, 16–24 nm for the next largest particles, and 11–18 nm for the smallest particles. The two smaller-diameter constituents from LCAT-deficient plasma are similar in size to our subclasses II and III. In addition, the compositional data from their smaller-size subclasses indicates, as does ours, that the percentage of cholesterol, phospholipid, and apoE decreases with decreasing disc diameter, while the percentage of total protein, apoA-I, and apoC increases.

Mitchell et al. (13) have reported that the large disc-shaped HDL observed in LCAT deficiency are composed of a population of particles rich in apoE and a population of particles containing apoA-I and apoA-II but no apoE. The apoE-rich population of discoidal particles is, in general, larger in diameter than the apoA-I and A-II particle population; there is, however, some overlap in particle size. In the case of Hep G2, our immunoblot data provide suggestive evidence for the existence of apoE-containing complexes relatively free of apoA-I and A-II at the larger end of the particle size spectrum.

It is of interest that, in immunoblots of nondenaturing gels of Hep G2 HDL, some apoB appears to be associated with particles in the size range of subclasses I and II. Immunoblots of Hep G2 HDL electrophoresed on 4–30% SDS-polyacrylamide gels indicated that immunoreactive apoB protein was composed of B-100 and fragments larger than 200,000 Daltons (McCall, M. R., T. M. Forte, and V. G. Shore, unpublished data). The B protein could not be seen on simultaneously run gels stained with Coomassie blue, suggesting that it was present in very small quantities. The fragments represent either partially degraded apoB or incomplete apoB translation products. The apoB-containing material is probably lipid-associated, since it was isolated between the densities of 1.063 and 1.235 g/ml. ApoB has been detected by less sensitive techniques than immunoblotting in HDL from perfused rat livers (27) and cultured rat hepatocytes (28). The metabolic significance of apoB in HDL has yet to be elucidated.

In normal human plasma HDL, two populations of apoA-I-containing lipoprotein particles have been

TABLE 2. Composition of Hep G2 subclass IV particles with analogous particles from plasma, lymph, and liver perfusate

	Composition (% by Weight)						
	PR[a]	PL	UC	CE	TG	d	EM Size
						g/ml	*nm*
Hep G2 fraction IV	63.6	30.7	0.8	4.9		1.17	7.8
LCAT deficiency (14)	59.2	26.8	14.1				7.5
LCAT deficiency (15)	57.9	35.5	4.3	0.7	1.5		
Liver perfusate (monkey) (12)	51.7	38.8	3.5	4.4	1.6	1.11–1.135	7.7
						1.135–1.21	6.7
Mesenteric lymph (rat) (11)	61	29	2	7	1	1.13–1.18	8.0

[a]PR, protein; PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride; EM, electron microscopy.

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identified (22); a population containing apoA-I with A-II and a population containing apoA-I without A-II. There is considerable overlap between these populations when isolation is based on size or hydrated density (22). Hep G2 HDL possess an apoA-I without A-II particle (subclass IV isolated by gel filtration chromatography) as well as particles containing both apoA-I and apoA-II. Isolation strategies that differ from the ones used (e.g., immunoaffinity chromatography) should yield more information on the distribution of specific apolipoprotein-lipid complexes within the Hep G2 HDL density range.

Physical-chemical characteristics of nascent HDL subclasses isolated from Hep G2 conditioned medium suggest that discrete particles are formed, some of which may contain a single apolipoprotein species. That the isolated Hep G2 subclasses are physiologically meaningful entities is borne out by the fact that, in many respects, they are analogous to HDL isolated from patients with LCAT deficiency. It is also likely that the normal liver secretes an apoA-I-only particle analogous to our subclass IV. The distinct molecularity of the HDL subclasses isolated in this study suggests that these particles represent valuable models for the conversion of nascent HDL to mature plasma forms.

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