Isolation of human serum HDL₁ by zonal ultracentrifugation

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Abstract
High density lipoprotein subfraction-1 (HDL₁) is thought to interact with the high-affinity apoprotein B, E receptors of peripheral cells and may act as a modulator of LDL binding and uptake. In the present study the concentration and composition of HDL₁ in normal and hypercholesterolemic sera were studied using zonal ultracentrifugation. To permit separation of the HDL₁ from VLDL, LDL, and Lp(a), the apoB-containing lipoproteins were first precipitated from serum using the phosphotungstic acid/magnesium chloride (PTA/MgCl₂) method after which the supernatant fraction was subjected to zonal ultracentrifugation. It could be demonstrated that following PTA/MgCl₂ precipitation HDL₁ floats as a single peak at d 1.06-1.09 g/ml (NaBr) and is sufficiently separated from high density lipoprotein-2 (HDL₂) and high density lipoprotein-3 (HDL₃). The HDL₂/HDL₃ subfraction pattern was not affected by the precipitation method. As previously described, in vitro incubation of serum leads to the LCAT-dependent interconversion of HDL₃ or HDL₂. Using the technique described here, it was discovered that a simultaneous elevation in HDL₁ occurred. This increase in HDL₁ concentration could not be observed when LCAT was inhibited by heat inactivation or addition of Ellman’s reagent. In normal fresh serum only a small HDL₁ peak could be detected, but in patients with familial hypercholesterolemia (apoB, E receptor deficiency) HDL₁ was elevated five to tenfold compared to normal values and further increased in concentration upon incubation of serum. On the other hand, in sera of patients with familial HDL deficiency (Tangier disease), HDL₁ was undetectable. Analysis of the HDL fractions in serum of a patient with abetalipoproteinemia revealed that following in vitro incubation there was formation of HDL₃, despite the lack of apoprotein B-containing lipoproteins. These data support the concept that HDL₁ formation occurs during LCAT-mediated HDL₃/HDL₂ interconversion in vitro.——Schmitz, G., and G. Assmann. Isolation of human serum HDL₁ by zonal ultracentrifugation. J. Lipid Res. 1982. 23: 903–910.

Supplementary key words
LCAT • HDL₂ • HDL₃ • apoE

Despite present understanding of the role of various lipoproteins in transporting cholesterol in blood, little is known about the factors that regulate the flux of cholesterol to and from the peripheral tissues. The homeostatic control of these two pathways may be critical in restricting cholesterol accumulation in tissues and thus of importance in preventing atherosclerosis. The bulk of the plasma cholesterol is transported in the LDL fraction. LDL transfers cholesterol to the peripheral tissues, where it is recognized by apoB, E receptor cells and, after cellular uptake, is degraded (1). Transfer from peripheral tissues to the liver is thought, on the other hand, to be mediated by high density lipoproteins (2, 3). Thus, the deposition of cholesterol in tissues theoretically may result from enhanced transfer by LDL or from a failure to eliminate cellular cholesterol via the HDL pathway.

The precise mechanism by which HDL removes cholesterol from peripheral cells is not yet known. In addition to the possible role of circulating HDL₃ as a cholesterol-acceptor macromolecule, tissue cholesterol may be alternatively removed by HDL precursors that are interconverted to HDL₂ by the activity of lecithin:cholesterol acyltransferase (LCAT) (4). These particles may then be taken up by the liver as intact particles (5), or the cholesterol ester moiety may be transferred to the hepatic cells with subsequent reconstitution to form an HDL₃ particle (6). A further HDL subfraction potentially involved in the transport of cholesterol is the apoprotein E-containing HDL₁ fraction. The present study provides evidence that HDL₁ is formed during the LCAT-mediated HDL₃/HDL₂ interconversion and that in the serum of patients with apoB, E receptor deficiency, HDL₁ is increased in concentration. It is suggested that the concentration of HDL₁ may be physiologically important in regulating LDL uptake by apoB, E receptor cells.

MATERIAL AND METHODS

Incubation studies
All incubations were performed in a shaking waterbath at 37°C with serum obtained from freshly drawn blood. The phosphotungstic acid/magnesium chloride

Abbreviations: HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; HPTLC, high performance thin-layer chromatography.
(PTA/MgCl2) method (7) was used to precipitate the apoprotein B-containing lipoproteins from fresh and incubated samples. Using the PTA/MgCl2 precipitation method, the supernatant is free of apoB-containing lipoproteins, while the concentration of HDL is not affected (7). Coprecipitation studies of 125I-labeled HDL1 added to normal serum before PTA/MgCl2 precipitation revealed a 90–95% recovery of the radioactivity in the supernatant. LCAT was inhibited either with Ellman’s reagent (1.4 mmol/l) (2) or by heat-inactivation at 56°C for 30 min (9). Addition of Ellman’s reagent resulted in 95% inhibition of LCAT activity, whereas heat-inactivation led to total inhibition.

Isolation of lipoproteins from incubated samples

Zonal ultracentrifugation. Two-ml aliquots of the samples before and after 12 hr incubation at 37°C were subjected to zonal ultracentrifugation for analysis of HDL subclasses. Ultracentrifugation was performed with a Beckman L8-70 ultracentrifuge and a Beckman Z-60 rotor at 59,000 rpm for 18 hr at 4°C. All zonal ultracentrifugations were carried out at constant ω2 t values. A nonlinear three-step NaBr gradient modified for the Z-60 rotor was used for the separation of lipoproteins (10). With this procedure, lipoproteins of d 1.063 g/ml emerge in the first 30–40 ml of the rotor effluent, while the HDL range is maximally expanded. The sera were adjusted to d 1.4 g/ml NaBr prior to injection. After centrifugation the rotor was deloaded by displacing the content with a d 1.4 g/ml NaBr solution (flow rate of 10 ml/min) from the rotor periphery. The effluent was continuously monitored by absorbance at 280 nm, and collected in 5-ml fractions. The gradient of each 5-ml fraction was measured by sodium determination in a Beckman K-Li-Na-flame photometer and the density was calculated from the following formula: at 20°C d- NaBr (g/ml) = 2.307 × 10–3 × (Na+ (mmol/l)) + 1.00155, corrected for density at 4°C (11).

Isopycnic ultracentrifugation. For preparative purposes, HDL1 was obtained from serum after phosphotungstic acid/magnesium chloride precipitation followed by ultracentrifugation for 24 hr at d 1.075 g/ml KBr, 4°C in a Beckman 70 Ti-rotor. The supernatant was dialyzed against 0.005 M Tris buffer, 0.15 M NaCl, pH 7.4. The final dialysis was performed in 0.005 M Tris buffer (pH 7.4) containing 0.005 M NaCl and 0.025 M MnCl2. The dialyzed fractions were subsequently applied to a 0.8 × 40 cm heparin-Sepharose affinity column for separation of residual HDL-A (consisting primarily of apoA-I and apoA-II) from HDL-E (consisting of apoE in addition to apoA-I and apoA-II) (Fig. 8) according to the method described by Mahley and Weisgraber (12), using Sigma analytical-grade heparin.

Labeling of HDL-E-containing particles

HDL particles containing apoE were isolated either as described for the isopycnic ultracentrifugation of HDL1 or from the HDL3 subfraction derived from normal serum after zonal ultracentrifugation and subsequent heparin-Sepharose chromatography (12). The column effluent was concentrated in an Amicon micro-cell (XM-100 membrane) up to 15 mg protein/ml and labeled by the McFarlane method (13). The labeled lipoprotein was separated from excess iodine by dialysis against 0.005 M Tris-HCl, 0.15 M NaCl, pH 7.4. Radioactivity was measured in a Kontron gamma-counter Mod. MR 480. Four to six % of the radioactivity was found in the lipid fraction; the remainder was associated with the apoproteins. As demonstrated by SDS-polyacrylamide gel electrophoresis (14), 55% of the protein-associated activity comigrated to apoA-I and 28% to apoE when β-mercaptoethanol and 1% decylsulfate (Eastman Kodak) were added to the samples.

Analysis of lipoproteins

The zonal rotor fractions were pooled and analyzed for lipids and apoproteins. Each pool was dialyzed exhaustively against normal saline, pH 7.4. After dialysis the fractions were concentrated to a volume of 0.5–1 ml of Amicon multiconcentration cells using XM-100 membranes. Free and esterified cholesterol, triglycerides, and phosphatidylcholine were analyzed either by enzymatic procedures (Boehringer Lot No.: 124 087; 274 119) or by quantitative high performance thin-layer chromatography (HPTLC) (15). Protein was measured by the procedure of Lowry et al. (16). Lipoproteins were delipidated with ethanol–ether 3:1 at −20°C as previously described (17). Apoprotein analysis was performed either by flat bed SDS polyacrylamide gel electrophoresis using the Neville system (14) in the one- or two-dimensional technique or by polyacrylamide gel isoelectric focusing in the pH range between 3.5 and 7 (18). Apoproteins were visualized by staining with Coomassie blue G-250 and quantitated by gel densitometry in a Shimadzu CS-910 Scanner. With respect to individual chromogenicity, calibration curves (four points) were prepared for each apoprotein on the same gel. The individual masses of polymorphic forms, as derived from the curves, were added to yield the total mass for each apoprotein. Identification of the apoprotein bands in isoelectric focusing and SDS gels were performed using the comigration technique of purified apoproteins in one- and two-dimensional SDS gels. Electron microscopy was carried out with a Hitachi H 500 electron microscope. The lipoprotein particles were visualized by negative staining using either phosphotungstic acid or uranyl acetate.
Patients

The normal sera were derived from ten normolipidemic male students. Other sera were obtained from two patients with the homozygous or heterozygous form of familial hypercholesterolemia (FH). Patient P.D. was a 12-year-old girl (total cholesterol, 21.98 mmol/l; triglyceride, 0.84 mmol/l) homozygous for FH; and patient G. Sch. was a 35-year-old woman (total cholesterol, 9.05 mmol/l; triglyceride, 0.49 mmol/l) heterozygous for FH. Both patients were known to have a deficiency of apoB, E receptors in fibroblasts. The patients affected with abetalipoproteinemia (M.K., 20 years old, male: total cholesterol, 0.70 mmol/l; triglyceride, 0.08 mmol/l), Tangier disease (E.G., 48 years old, female: total cholesterol, 2.46 mmol/l; triglyceride, 2.71 mmol/l; and J.S., 44 years old, male: total cholesterol, 1.29 mmol/l; triglyceride, 3.95 mmol/l) have been described in previous publications (19, 20).

Fig. 1. Zonal ultracentrifugation analysis of 1.8 ml of PTA/MgCl2-supernatant derived from sera of ten fasting normolipidemic male students. The left plot demonstrates the absorption profiles at 280 nm of supernatants from nonincubated sera (0 hr); the right plot reveals the absorption profiles of corresponding supernatant aliquots after 24 hr incubation of sera at 37°C. All zonal ultracentrifugations were carried out at 250,000 g, 4°C for 18 hr and constant ω × t values.

RESULTS

To study the HDL subfraction distribution in sera of normolipidemic patients and of patients with various forms of dyslipoproteinemia, supernatant fractions of PTA/MgCl2 precipitations were subjected to zonal ultracentrifugation. As shown in Fig. 1, two major fractions were resolved in normolipidemic serum. These fractions were identified by chemical composition and electron microscopy as HDL2 and HDL3. The subfraction distribution of HDL2 and HDL3, as obtained with native serum (not shown), was not affected by the precipitation method employed (HDL2, d 1.15 g/ml NaBr and HDL3, d 1.19 g/ml NaBr, respectively). Furthermore, the chemical and morphologic characteristics of the HDL2 and HDL3 obtained after PTA/MgCl2 precipitation and subsequent zonal centrifugation were indistinguishable from reference preparations obtained from zonal centrifugation fractions of fresh serum. In addition to the two major subfractions, another small peak was detected in the density range of approximately 1.08 g/ml NaBr.
Fig. 3. Zonal ultracentrifugation analysis of fresh (upper) and incubated (lower) serum of a patient with heterozygous familial hypercholesterolemia (G. Sch., heterozygous for FH). After 0 hr and 24 hr incubation at 37°C, serum was treated with PTA/MgCl₂ and the supernatants were subjected to zonal ultracentrifugation.

(Fig. 1, left). During incubation of the serum at 37°C for 24 hr (Fig. 1, right), this fraction increased in concentration simultaneously with the LCAT-mediated interconversion of HDL₃ to HDL₂ (4).

Fig. 2 and Fig. 3 demonstrate the zonal ultracentrifugation pattern of patients with homozygous and heterozygous forms of familial hypercholesterolemia. In the fresh sera of these patients the d 1.08 g/ml NaBr fraction was elevated five to tenfold as compared with normal sera (compare Figs. 2 and 3, top with Fig. 1, left), when the PTA/MgCl₂ supernatants of sera from patients with familial hypercholesterolemia were analyzed by zonal ultracentrifugation before and after in vitro incubation of native sera at 37°C. In addition to the HDL₃/HDL₂ interconversion, a further peak increase in the d 1.08 g/ml NaBr fraction was observed.

Zonal ultracentrifugation analysis of serum from a patient with abetalipoproteinemia (Fig. 4) revealed the near absence of HDL₁ and an abnormal HDL₂/HDL₃ subfraction distribution in fresh serum. However, after in vitro incubation, a substantial peak at d 1.08 g/ml NaBr could be detected. On the other hand, in sera of patients with familial HDL deficiency (Tangier disease), the fraction at d 1.08 g/ml was undetectable both before and after incubation (not shown).

In fresh normal serum the fraction floating at d 1.06–1.09 g/ml NaBr exhibited α₂-mobility in agarose electrophoresis (Fig. 5); electron microscopy of this fraction

Fig. 4. Zonal ultracentrifugation analysis of fresh (upper) and incubated (lower) serum derived from a patient with recessive abetalipoproteinemia (K.M.). After 0 hr and 24 hr incubation at 37°C, serum was directly subjected to zonal ultracentrifugation.
revealed spherical particles with a diameter of 13–19 nm (Fig. 6). Analysis of the fraction after in vitro incubation of normal serum resulted in a slight charge shift in agarose electrophoresis (Fig. 5), but no significant change in size (not shown).

Heparin-Sepharose affinity chromatography was performed to further identify the nature of the lipoprotein floating at d 1.085 g/ml NaBr and here designated HDL₁. As shown in Fig. 7, the d 1.08–1.09 g/ml NaBr fraction bound to the column and was eluted with increasing concentrations of NaCl at 0.15 M. Two minor fractions were also resolved and eluted at 0.07 M and 0.3 M NaCl. The nature of these minor fractions was not further investigated.

The apolipoprotein composition of the d 1.08–1.09 g/ml NaBr fraction, analyzed by SDS gel electrophoresis and isoelectric focusing, revealed apoA-I, apoA-II, and apoE as the major apolipoproteins in fresh (Fig. 8, Table 1) and incubated sera (not shown). The fraction isolated from fresh serum also contained C-apolipoproteins, whereas in the fraction derived from incubated serum C-peptides were barely detectable.

Quantitative analysis of lipids by HPTLC of the d 1.08–1.09 g/ml NaBr fraction revealed cholesterol, mainly cholesteryl esters, as the predominant lipid moiety; di- and triglycerides were also present (Table 1). The phospholipid concentration was low with a relative phosphatidylcholine/sphingomyelin molar ratio of about 4.1:1. Lipid analysis of the d 1.08–1.09 g/ml NaBr fraction after in vitro incubation of fresh serum revealed an increased concentration of cholesteryl esters, mainly cholesteryl linoleate, and a decreased concentration of free cholesterol and triglycerides (Table 1).

To investigate the metabolic origin of the HDL₁ subfraction, HDL-E-containing particles were isolated from the HDL₃ fraction of fresh serum (which was obtained by ultracentrifugation) by means of heparin-affinity chromatography. After ¹²⁵I-labeling, the ¹²⁵I-HDL-E was again added to fresh normal serum and subjected to zonal ultracentrifugation. In fresh serum, the major peak of radioactivity was detected in the HDL₃-fraction.
**DISCUSSION**

The present studies indicate that a lipoprotein with the compositional (apoE as major apoprotein), electrophoretic (α2-mobility), and morphologic (13–19 nm diameter) characteristics of HDL1 can be isolated from the PTA/MgCl₂ supernatants of fresh normolipidemic sera by zonal ultracentrifugation in the density range of 1.08–1.09 g/ml NaBr. This ultracentrifugal fraction, in contrast to the major fractions HDL₂ and HDL₃, binds to a heparin-Sepharose affinity column and can be eluted by increasing concentrations of NaCl. Thus, this fraction has characteristics similar to the lipoprotein HDL₁₅ previously characterized by Mahley (21) as a major lipoprotein in cholesterol-fed animals. This lipoprotein increases in concentration upon incubation of serum at 37°C. It is present in high concentrations in fresh sera of patients with familial hypercholesterolemia and could not be detected in native or incubated sera of patients with Tangier disease. However, it was found to be present in the incubated serum of a patient with abetalipoproteinemia.

We have previously demonstrated that LCAT participates in the interconversion of HDL₃ to HDL₂ (4). In those studies, only whole sera were analyzed by zonal centrifugation and the d 1.08–1.09 g/ml NaBr fraction could not be resolved due to the overlap of apoB-containing lipoproteins in the ultracentrifugal flotation profile. The HDL₁ subfraction, as shown in the present studies, could only be separated by zonal ultracentrifugation after prior precipitation of apoB-containing lipoproteins.

The term HDL₁ has been used in the past by different authors for the apoprotein E-containing HDL (21–23). As shown by Mahley and collaborators, HDL₁ increases...
concentrations in the sera of hypercholesterolemia patients. The precise metabolic origin of HDL_{1} in human plasma remains unknown. However, the fact that HDL_{1} was undetectable in fresh and incubated Tangier serum, but present in incubated serum of a patient with abetalipoproteinemia, suggests a metabolic relationship to the major HDL subfractions normally present in serum. Furthermore, the involvement of LCAT not only in the interconversion of HDL_{3} to HDL_{2} (4), but also in the formation of HDL_{1}, is supported by our data. Whether HDL_{1} is generated from either HDL_{3} or HDL_{2}, or alternatively serves as a substrate for LCAT or an acceptor for newly formed cholesteryl esters, remains to be clarified. Elucidation of the potential role of HDL_{1} as a lipoprotein involved in the clearance of cholesterol from peripheral cells and/or delivery of cholesterol to the liver also awaits further studies.

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