Characterization of four lipoprotein classes in human cerebrospinal fluid

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Abstract **Lipoprotein metabolism in brain has not yet been fully elucidated, although there are a few reports concerning lipids in the brain and lipoproteins and apolipoproteins in the cerebrospinal fluid (CSF). To establish normal levels of lipoproteins in human CSF, total cholesterol, phospholipids, and fatty acids as well as apolipoprotein E (apoE) and apoA-I levels were determined in CSF samples from 216 individuals. For particle characterization, lipoproteins from human CSF were isolated by affinity chromatography and analyzed for size, lipid and apolipoprotein composition. Two consecutive immunoaffinity columns with antibodies, first against apoE and subsequently against apoA-I, were used to define four distinct lipoprotein classes. The major lipoprotein fraction consisted of particles of 13–20 nm containing apoE and apoA-I as well as apoA-IV, apoD, apoH, and apoJ. In the second particle class (13–18 nm) mainly apoA-I and apoA-II but no apoE was detected. Third, there was a small number of large particles (18–22 nm) containing no apoA-I but apoE associated with apoA-IV, apoD, and apoJ. In the unbound fraction we detected small particles (10–12 nm) with low lipid content containing apoA-IV, apoD, apoH, and apoJ. In summary, we established lipid and apolipoprotein levels in CSF in a large group of individuals and described four distinct lipoprotein classes in human CSF, differing in their apolipoprotein pattern, lipid composition, and size. On the basis of our own data and previous findings from other groups, we propose a classification of CSF lipoproteins.—** Koch, S., N. Donarski, K. Goetze, M. Kreckel, H-J. Stuerenburg, C. Buhmann, and U. Beisiegel. **Characterization of four lipoprotein classes in human cerebrospinal fluid. *J. Lipid Res.* 2001.** 42: **1143–1151.**

Supplementary key words affinity chromatography • apolipoprotein E • apolipoprotein A-I • cholesterol • phospholipids

It is only during the last few years that interest in lipoprotein metabolism in the brain has greatly increased, although the first description of lipoproteins in human cerebrospinal fluid (CSF) by Swahn, Brönnestam, and Dencker (1) was published 40 years ago. This increased interest is due to recent findings on Alzheimer's disease pathology (2, 3) and the description of several lipoprotein receptors in the brain (4, 5) and is reflected by the increasing number of publications dealing with these topics (6–14). However, the structure and function of human CSF lipoproteins (CSF-Lp) has not yet been fully elucidated. To date only a few groups have published on lipoprotein fractionation in CSF (8, 10, 15, 16) by either density gradient or sequential flotation ultracentrifugation, gel filtration, or affinity chromatography [summarized in ref. (17)]. From these and other data there is evidence that apolipoprotein E (apoE) and apoA-I are the key players in transport and delivery of lipids in the brain. ApoE is expressed in the brain mainly by astrocytes (16) and microglia (18) and is secreted by rat astrocytes in discoidal lipoprotein particles (11). In contrast, apoA-I has been described as being synthesized only in the liver and intestine (19) and must cross the blood-brain barrier (BBB) to enter the brain. In accordance with this, apoE and apoA-I have been found in distinct particles but also occur together in "E/A-I particles" (8, 15).

It has been shown that CSF-Lp can both supply neurons with lipids after receptor-mediated endocytosis and promote cholesterol efflux from cholesterol-loaded fibroblasts (14). In addition some other possible functions of CSF-Lp, not related to classic lipoprotein functions, have been described, as the outgrowth of neurites has been shown to be promoted by apoE containing CSF-Lp, a process that is mediated by the LDL receptor-related protein (20). During the regeneration and remyelination of peripheral nerves, lipoproteins containing apoE as well as apoA-I, apoA-IV, and apoD have been suggested to mediate the reutilization of excess lipids, especially cholesterol at the site of the nerve injury (21). In the central nervous system (CNS) only apoE and apoJ have been proposed to be responsible for the recycling of cholesterol after injury (22), during synaptogenesis and the dendritic reorganization of neurons (23). All these studies together with numerous investigations on the role of apoE in Alzheimer's disease, emphasize the importance of this major apolipoprotein in the CNS (24). In contrast, only a few studies have focused on the function of other apolipoproteins that are also syn-

Abbreviations: apo, apolipoprotein; BBB, blood-brain barrier; CSF, cerebrospinal fluid; CSF-Lp, CSF lipoproteins; Q_A, albumin ratio.

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thesized in the brain, namely apoD, apoH (β_2-glycoprotein I), and apoJ (Clusterin) (25–27). It must be noted that these apolipoproteins are not "classic" apolipoproteins either in terms of their structure or their function. For all three proteins physiological functions and pathological associations far beyond lipoprotein metabolism have been reported [see references in refs. (25), (28), and (29)]. The "classic" apolipoproteins, apoA-I, apoA-II, and apoA-IV, which play a role in reverse cholesterol transport and TG metabolism, are not synthesized in the brain but may cross the BBB by transcytosis (30). ApoA-I- and apoA-II-containing lipoproteins could provide an effective removal system for excess cholesterol and other lipid products in the CSF. ApoA-IV has been proposed to act centrally as a satiety signal (31). ApoB, a major apolipoprotein in plasma, has not been detected in the CSF (32).

Concentrations of lipids in CSF from healthy adult donors were first determined in 1959 by Tourtellotte (33), extended in 1971 by Illingworth and Glover (34) and others, and summarized by Davson and Segal (35). The total lipid concentration is 1.25 mg/dl and the levels of the two main lipids, cholesterol and phospholipids, are 0.41 and 0.37 mg/dl, respectively. Fatty acids are not catabolized in the brain, which depends on glucose for energy supply; however, fatty acids as components of phospholipids are of great structural importance in the membranes of neurons. Data on enzymes involved in lipid metabolism, such as phospholipases, esterases, and hydrolases, are given in Davson and Segal (35), as well as in a publication by Demeester et al. (36). Lipoprotein lipase is expressed in the brain and hydrolytic activity has been measured in various regions (37, 38). This enzyme might therefore be important in the development and maintenance of normal neuronal function.

Characterization of CSF-Lp has been impeded by the limited availability of normal human CSF together with the low concentration of apolipoproteins and lipids compared with plasma. Because of these problems no uniform classification and nomenclature for CSF-Lp have yet been proposed. Our aim was to separate different CSF-Lp classes by methods known to minimize changes in particle structure and to characterize their lipid and apolipoprotein composition. On the basis of our own data and previous findings from other groups, we propose a classification of CSF-Lp.

MATERIALS AND METHODS

Patient material

The CSF and plasma samples studied were received as surplus material from normal diagnostic samples from 216 patients who had been admitted to the Neurological Clinic (University Hospital Eppendorf, Hamburg, Germany). The study was approved by the local Ethics Committee at the Ärztekammer Hamburg. The CSF samples were frozen and stored for no longer then 6 weeks before use. Only CSF from patients with intact BBB was used in the pools that were analyzed. None of the patients had a severe neurological disorder. Ten different CSF pools from about 50 different patients were used in the experiments presented here.

Antibodies

Primary and secondary antibodies were obtained from the following companies or colleagues: mouse anti-human apoA-I for Western blotting from Dunn (Asbach, Germany), goat anti-human apoA-I serum for affinity column chromatography from Beckman Coulter (Krefeld, Germany), goat anti-apoA-II from WAK (Bad Homburg, Germany), rabbit anti-apoE from Dako (Hamburg, Germany), and mouse anti-apoJ from Quidel (Surrey, UK). Rabbit anti-apoD antiserum was kindly provided by H. Dieplinger (Innsbruck, Austria) and rabbit anti-apoH antiserum was a generous gift from S. Moestrup (Århus, Denmark). The antibody against apoA-IV was raised in rabbits, using apoA-IV purified from human plasma. Peroxidase-conjugated goat anti-rabbit IgG, peroxidase-conjugated goat anti-mouse IgG, and peroxidase-conjugated donkey anti-goat IgG were obtained from Dianova (Hamburg, Germany).

Lipid and protein quantification

Cholesterol and TG in plasma were determined with enzymatic test kits (Monotest®) from Boehringer Mannheim (Mannheim, Germany). Measurement of fatty acids in CSF were performed with gas chromatography and flame ionization detection as described previously (39) with the following modifications. One hundred microliters of CSF or 300 μl of CSF-Lp was mixed with 2 ml of chloroform–methanol 2:1 (v/v), 100 μl of heptadecanoic acid (200 μg/ml) and 25 μl of 5-α-cholestane (100 μg/ml) were added as internal standards, and 25 μl of butylated hydroxytoluene (0.2 M) was added as antioxidant. The chloroform extract was evaporated under nitrogen, the dried lipids were dissolved in 250 μl of toluene, and fatty acids were derivatized with 500 μl of 0.5 M anhydrous sodium methoxide for 15 min at 50°C. The mixture was neutralized with 1 ml of 2.5% acetic acid and extracted with 250 μl of hexane. The supernatant was evaporated under nitrogen and 100 μl of dimethylformamide was added. Cholesterol was silylated by incubation with *N,O*-bis-(trimethylsilyl)trifluoro acetamide for 30 min at room temperature. After a final evaporation, the pellet was dissolved in 20 μl of toluene, 2 μl of which was chromatographed on a 30-m Hp5MS column using a Hewlett-Packard (Palo Alto, CA) 5890 Series II gas chromatograph.

The other lipids in CSF-Lp were measured enzymatically by fluorometric detection of the reaction products. Cholesterol determination was performed with the Amplex™ Red cholesterol assay kit (Molecular Probes, Leiden, The Netherlands). For phospholipid (PL), TG, and glycerol measurement the following enzymes were used: PL—phospholipase D (100 U/ml; Sigma, St. Louis, MO), choline oxidase (2.8 U/ml; Sigma), and horseradish peroxidase (2 U/ml; Molecular Probes); TG—enzyme tablet from the Boehringer Mannheim kit [lipase, 3 U/ml; glycerokinase (GK), 0.2 U/ml; glycerophosphate oxidase (GPO), 2.5 U/ml; peroxidase, 0.15 U/ml; ATP, 0.5 mM]; glycerol—GK, 0.2 U/ml; and GPO, 2.5 U/ml (Sigma). The enzymes were dissolved in 100 mM Tris, pH 7.4, with 2.5 mM $CaCl_2$, and 300 μM Amplex™ Red (Molecular Probes) was added. These solutions (50 μl each) were then mixed with 50 μl of sample (CSF-Lp, diluted 1:5) and the reaction product was measured after 30 min with a fluorometer (Hewlett-Packard) (excitation/emission, 560/590 nm). Choline measurement was performed as described for PL without phospholipase D.

ApoE concentration was measured by means of a specific sandwich ELISA, using monoclonal antibody EE7 (40) for capturing and the polyclonal anti-apoE antibody from Dako for detection. Bound antibodies were quantified by incubation with an anti-rabbit IgG antibody coupled to horseradish peroxidase. *o*-Phenylenediamine dihydrochloride was used as substrate and the extinction of the reaction product was measured at 490 nm.

ApoA-I was measured with a radioimmunoassay from Mercodia (Uppsala, Sweden). Protein determination of fractionated CSF was performed with Roti®-Nanoquant (Roth, Karlsruhe, Germany).

Gel filtration, immunoaffinity, and heparin chromatography

Plasma (100 μl) or 200 μl of a 10-fold concentrated CSF pool (Amicon Miniplus concentrator; Amicon, Danvers, MA) was separated on a Superdex 200 column (Amersham, Freiburg, Germany) in 100 mM NaCl, 10 mM Tris-HCl, pH 8.0. Fractions of 500 μl were collected and apoE, cholesterol, and PL were measured.

CSF-Lp were isolated by consecutive affinity chromatography as shown in Fig. 3. Affinity columns with monospecific antibodies directed against either apoA-I or apoE (2 mg of antibody per ml of gel) were prepared as described (41), using CNBr-activated protein G-Sepharose (Sigma) for goat anti-apoA-I serum and protein A-Sepharose CL-4B (Amersham) for affinity-purified rabbit anti-apoE. First, lipoproteins containing apoE were isolated from CSF by incubating 10 ml of CSF with the anti-apoE affinity column for 2 h at room temperature. Second, the eluted E-particles and the unbound material were fractionated on an anti-apoA-I column in the same way. Elution of bound particles was performed after extensive washing with PBS, pH 7.3, followed by a short wash with PBS, pH 6.6, with 100 mM glycine, pH 2.5, into 1 M Tris, pH 8.5. Fractions containing eluted proteins (as determined by measuring the optical density at 280 nm) were pooled and stored at 4°C for further analysis.

Heparin chromatography was performed as previously described (42). Briefly, 5–10 ml of CSF pool containing 25 mM $MnCl_2$ was applied to a 1-ml heparin column (Amersham) and washed, and bound material was eluted in two subsequent steps, using first a manganese-free buffer and then a high salt buffer to separate lipoproteins with low and high affinity for heparin. The unbound material and the two eluates were analyzed by Western blot for apolipoprotein content.

SDS-PAGE and Western blot analysis

For immunoblot analysis of apolipoproteins in CSF-Lp fractions, equal protein amounts of CSF-LpE, CSF-LpEA, and CSF-LpA and 6- to 17-fold more protein of the unbound fraction (sCSF-Lp) were precipitated and separated under reducing conditions on a 15% SDS-polyacrylamide gel according to Neville (43). After electrophoresis, Western blotting was performed with the indicated antibodies with the following dilutions: anti-apoA-I, 1:1,000; anti-apoA-II, 1:250; anti-apoA-IV, 1:1,000; anti-apoD, 1:1,000; anti-apoE, 1:5,000; anti-apoH, 1:500; anti-apoJ, 1:500. The immunoblots were developed with secondary antibodies linked to peroxidase (1:5,000 for goat anti-rabbit IgG and goat anti-mouse IgG and 1:2,500 for donkey anti-goat IgG), using the ECL system (Amersham).

Electron microscopy

For visualization of the lipoproteins, CSF-Lp fractions after affinity chromatography were diluted 1:100 with 10 mM Tris, 100 mM NaCl, pH 8.0, and negatively stained with 2% sodium phosphotungstate, pH 7.5 (44), on Formvar-coated grids. The lipoproteins were examined with a CEM 902 A (Zeiss, Oberkochen, Germany) electron microscope and AnalySIS® software (SIS, Münster, Germany).

RESULTS

Lipid and apolipoprotein analysis of human CSF

To establish normal values for lipids and the major apolipoproteins in human CSF we analyzed CSF samples from 216 patients who had been admitted to the Neurological Clinic. All patients had a normal BBB function in CSF diagnosis, determined by an albumin ratio (CSF/blood, Q_A) of $<8 \times 10^{-3}$. **Table 1** shows the lipid and apolipoprotein values for CSF samples compared with plasma values. The plasma cholesterol and TG levels in the patients (105 males, 111 females; mean age, 49 years) were normal, as were the apoE and apoA-I levels in plasma. The CSF cholesterol level of 0.6 mg/dl was comparable to values in the literature (0.2–0.8 mg/dl) (16, 15, 34, 45, 46). Also, the apoE and apoA-I levels in CSF (0.3 and 0.37 mg/dl, respectively) corresponded well with published data, which indicate 0.3–0.6 mg/dl for apoE (16, 47–49) and 0.45 mg/dl for apoA-I (50). Total fatty acids were measured by gas chromatography and the value obtained was 2.3 mg/dl, comparable to the normal value of 2 mg/dl in the literature (51). Total fatty acids in plasma were not measured in this group of subjects and we therefore used in Table 1 values obtained in a parallel study of 142 patients who were also submitted to the Neurological Clinic of our hospital. To determine PL and TG in CSF sensitive fluoro-

TABLE 1. Lipids and apolipoproteins in human plasma and CSF

	n	CSF	Plasma	% in CSF
Total cholesterol	216	0.6 ± 0.2 mg/dl 15.5 μM	219 ± 58 mg/dl 5.7 mM	0.27
Phospholipids	39/27	0.55 ± 0.3 mg/dl 7.1 μM	231± 60 mg/dl 2.9 mM	0.25
Total fatty acids	204	2.3 ± 0.7 mg/dl 85 μM	422 ± 244 mg/dl[a] 14.9 mM	0.54
ApoE	216	0.3 ± 0.2 mg/dl 0.08 μM	6.8 ± 4.6 mg/dl 1.8 μM	4.41
ApoA-I	46	0.37 ± 0.08 mg/dl 0.13 μM	141 ± 48 mg/dl 50 μM	0.26

Plasma and CSF samples from 216 patients were obtained from the Neurological Clinic and analyzed for various biochemical parameters. The main lipids (total cholesterol, phospholipids, and total fatty acids) and the major apolipoproteins are listed in terms of mass and molar concentration. In addition, the percentage in CSF from plasma levels is indicated. The percentage for albumin is 0.5%.

[a] This value is derived from another group of comparable patients (n = 142) and compares there to a CSF value of 2.1 mg/dl.

metric measurements were performed. The mean value for PL was 0.55 mg/dl, corresponding to values between 0.2 and 0.65 mg/dl in the literature (35, 45, 46). The glycerol values, determined fluorometrically before and after hydrolysis by TG lipase, indicated that there were only trace amounts of TG in human CSF (data not shown).

Separation of CSF-Lp by gel filtration

Because of the high and similar densities of CSF-Lp, gel filtration (fast protein liquid chromatography, FPLC) rather than density gradient ultracentrifugation was used for separation of the various lipoprotein classes. The Superdex 200 column provides an optimal resolution in the molecular mass range of 10–600 kDa and is suitable to separate particles in the size range of HDL. For standardization plasma lipoproteins were separated on the column and the positions of eluting VLDL, LDL, and HDL were assessed by cholesterol and phospholipid measurement as shown in **Fig. 1A**. The cholesterol peak in fractions 17 and 18 represents VLDL and LDL and in fractions 22–26 HDL. The detection of apoE in fraction 17 indicates the position of VLDL and in fractions 21–23 HDL_E is detectable, which is distinct from the cholesterol/phospholipid peak in fractions 24 and 25 representing HDL_2 and HDL_3. In Fig. 1B the separation of a typical CSF pool by FPLC is shown. Detection of CSF lipoproteins after gel filtration was performed by measuring cholesterol, PL, and apoE in the fractions. Both lipids were found mainly in fractions 18–23, corresponding to a size range between those of LDL and HDL. ApoE eluted in a broad peak together with the major lipid peak. Other apolipoproteins (apoA-I, apoA-II, apoA-IV, and apoD) were analyzed in all fractions by Western blotting (data not shown). ApoA-I, apoA-II, and some apoA-IV and apoD were found to coelute with apoE; however, apoA-IV and apoD were also detected in fractions 27–30 together with some fatty acids (data not shown).

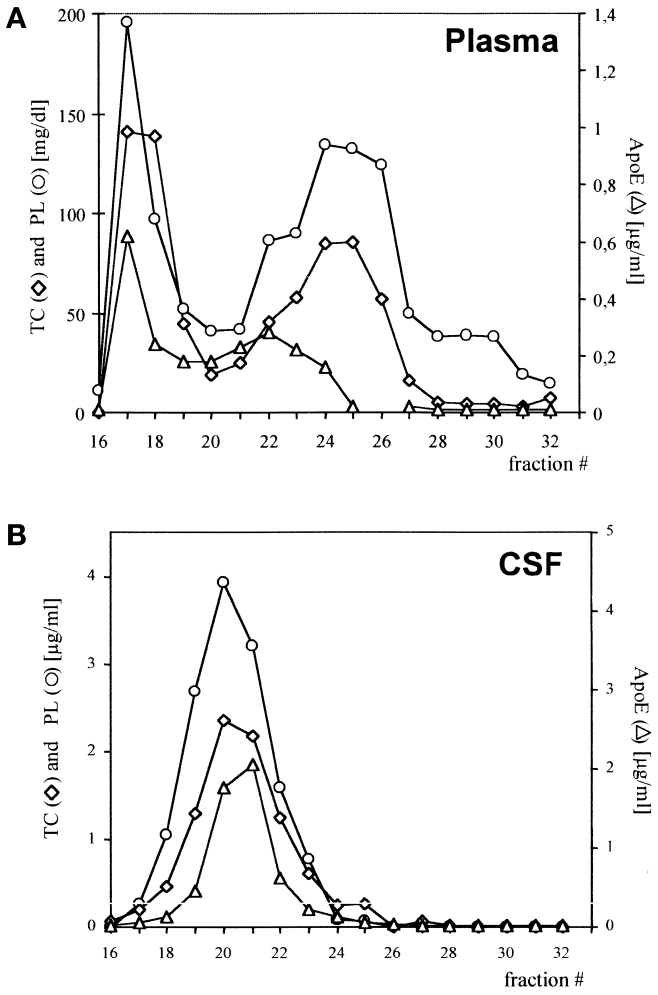

Fig. 1. Gel filtration of plasma and CSF. One hundred microliters of normal human plasma (A) and 200 µl of 10× concentrated normal human CSF (B) were separated on a Superdex 200 column. ApoE (open triangles), total cholesterol (TC; open diamonds), and PL (open circles) were measured in each fraction. The peaks are (A) VLDL in fraction 17, LDL in fraction 18, HDL_E in fractions 22 and 23, and HDL_2 and HDL_3 in fractions 24–26. Albumin (68 kDa) can be detected in fraction 29 (not shown). CSF-Lp (B) eluted in a broad peak between plasma LDL and HDL. Most of the cholesterol, PL, and all apoE can be found in these fractions (18–23). No TG were detectable in the CSF profile; glycerol eluted in fraction 42 (not shown). One representative FPLC profile is shown out of 14 separate FPLC runs using 10 CSF pools.

Affinity chromatography of CSF-Lp

Gel filtration of CSF does not separate distinct lipoprotein classes. To determine whether apoE and apoA-I, the main apolipoproteins in the CSF, are found on the same or separate particles we therefore used two immunoaffinity chromatography columns. First, apoE-containing particles were isolated and the nonbinding material, devoid of apoE, was applied to an anti-apoA-I column. The resulting three CSF-Lp fractions, that is, E-particles (apoE-containing lipoproteins), CSF-LpA (apoA-I-containing lipoproteins), and sCSF-Lp (small CSF-Lp), were analyzed by Western blotting for the distribution of apoE and apoA-I (**Fig. 2A**). As expected, apoE could be detected only in the E-particles. ApoA-I was found in two fractions, both in association with apoE and on separate particles (CSF-LpA). Neither apoE nor apoA-I was detected in the unbound material (sCSF-Lp). We used electron microscopy (EM) to visualize lipoproteins in these three fractions (Fig. 2B–D). Relatively homogeneous populations of intact particles were observed in all lipoprotein fractions after affinity chromatography. The size range determined by EM was 14–22 nm, which corresponds to the FPLC elution profile, where we find these CSF-Lp between HDL (10–13 nm) and LDL (22–29 nm). These findings show the existence of spherical lipoprotein in CSF and verify the existence of small particles also in the unbound material, the sCSF-Lp.

As shown in **Fig. 3** we further analyzed the apoE-containing lipoproteins on an anti apoA-I column to separate apoE- and apoA-I-containing particles from those that do not contain apoA-I. We thereby obtained CSF-LpE and CSF-LpEA. Western blot analysis revealed the apolipoprotein pattern of the four distinct lipoprotein classes as shown in **Fig. 4**. All four lipoprotein classes contained comparable amounts of apoJ. CSF-LpE contained apoA-IV and apoD

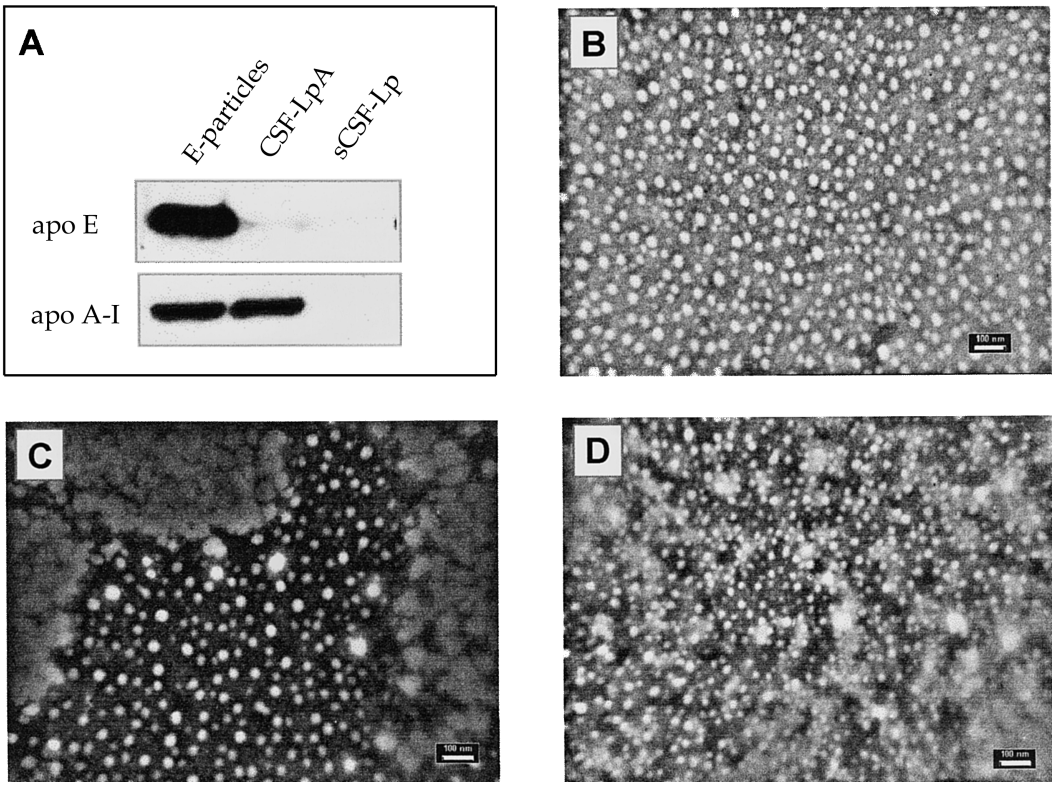

Fig. 2. Analysis of three CSF-Lp classes by Western blot and EM. A: ApoE-containing E-particles were isolated by using an anti-apoE affinity column. The unbound material was further fractionated into apoA-I-containing particles (CSF-LpA) and a fraction devoid of apoE and apoA-I (sCSF-Lp). Sixteen micrograms of apoE-containing particles or CSF-LpA and 100 μg of unbound material (sCSF-Lp) were separated by SDS-PAGE and apoE and apoA-I were detected by Western blotting. B–D: The three lipoprotein fractions were negatively stained and viewed by EM. B: E-particles. C: CSF-LpA. D: sCSF-Lp. Magnification bars: 100 nm.

in addition to apoE. CSF-LpEA differed from CSF-LpE by the additional presence of apoA-I and apoH. CSF-LpA, by definition, did not contain apoE, but apoA-II and apoD as well as a small amount of apoA-IV were present. The major apolipoprotein in the smallest apoE- and apoA-I-free lipoprotein fraction (sCSF-Lp) was apoD; in addition sCSF-Lp contained apoA-IV, apoH, and apoJ.

The general composition of the four CSF-Lp classes is shown in **Table 2**. Total cholesterol and PL were measured as well as the protein content. The sCSF-Lp fraction contained all soluble CSF proteins, because these small lipoproteins cannot easily be separated from them. In the three other lipoproteins we find 53–66% protein and 20–32% PL, which is in the same range as found for plasma HDL. Because only trace amounts of TG were detected in whole CSF, we did not determine TG in the isolated lipoproteins, and free fatty acids are not expected to be transported in lipoproteins but should be bound to albumin. CSF-LpE contained relatively more cholesterol than both CSF-LpEA and CSF-LpA. The presence of cholesterol and PL in sCSF-Lp confirmed the EM data and showed that the apolipoproteins detected in this fraction were indeed bound to lipoproteins. Further analysis of the sCSF-Lp is required to show whether the various apolipoproteins form distinct particles or whether they join on the same particles.

We wanted to know whether the lipid and apolipoprotein composition in CSF is determined by the apoE isoforms and analyzed the data according to the apoE phenotypes. The group contained 131 patients (62%) with apoE 3/3, 39 patients (18%) with apoE 3/4, and 24 patients (11%) with apoE 2/2 (the rest were apoE 2/4 and apoE 4/4). We found no difference in composition in any of the determined parameters; even the apoE mass was comparable with 0.3, 0.32, and 0.25 mg/dl, respectively.

To confirm the integrity and size of the particles in the four CSF-Lp fractions after affinity chromatography, gel filtration was performed followed by Western blot detection of one of the major apolipoproteins in the fractions (**Fig. 5**). CSF-LpE and CSF-LpEA were visualized with the anti-apoE antibody. CSF-LpE eluted with a peak in fractions 20 and 21 in contrast to CSF-LpEA, which were found in a broad peak in fractions 20–25, thereby overlapping in size with both CSF-LpE and CSF-LpA, which were mainly found in fractions 22 and 23. FPLC analysis of sCSF-Lp revealed that apoA-IV (46 kDa) eluted in fractions 28–30 and apoD (29 kDa) eluted in fraction 28. The elution of albumin has been shown to be in fraction 29. The presence of apoA-IV in fractions 29 and 30 indicates that there is a reasonable amount of free apoA-IV in CSF. The presence of apoE and apoA-I in fractions 17–19 can be explained by larger particles as also detected in the EM pic-

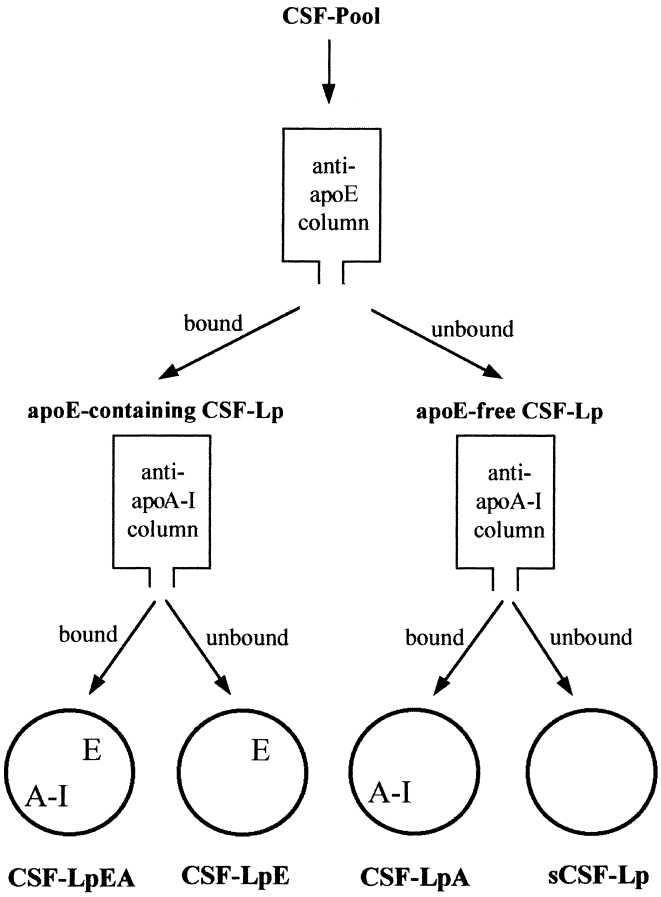

Fig. 3. Scheme of fractionation of CSF-Lp by affinity chromatography. Pools of normal human CSF were subjected to anti-apoE affinity chromatography. The unbound apoE-free CSF-Lp and the eluted particles (apoE-containing CSF-Lp) were subsequently applied to anti-apoA-I affinity columns. The resulting four samples, two eluates and two unbound fractions, were named CSF-LpEA, CSF-LpE, CSF-LpA, and sCSF-Lp according to their main apolipoproteins as determined by Western blotting (Fig. 4) or to their small size (sCSF-Lp) as determined by gel filtration (Fig. 5). The experiments were performed 14 times with 10 different pools (each consisting of 10 different samples) of CSF.

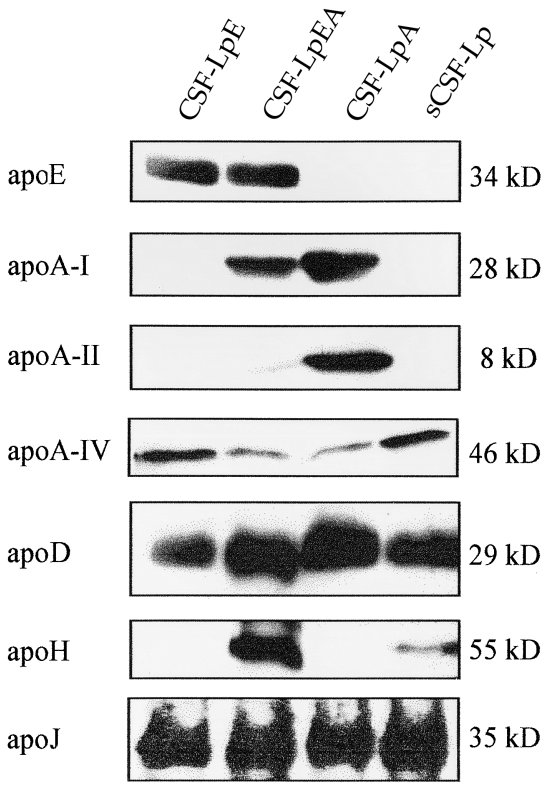

Fig. 4. Analysis of the four CSF-Lp fractions by Western blot. After fractionation of CSF-Lp by affinity chromatography (Fig. 3), the apolipoprotein patterns of CSF-LpEA, CSF-LpE, CSF-LpA, and sCSF-Lp were determined by Western blotting. The precipitated protein was subjected to 15% SDS-PAGE under reducing conditions. Western blotting was performed after electrotransfer to nitrocellulose. The nitrocellulose was cut horizontally for detection of the different-sized apolipoproteins with specific antibodies. In every fraction a certain set of apolipoproteins could be detected and apoE and apoA-I were distributed as expected from the affinity fractionation.

ture in Fig. 2B. These large lipoproteins could be artifacts caused by particle fusion.

A summary of the general characterization of the four isolated CSF-Lp classes is given in **Table 3**. In addition to size, density, and apolipoprotein pattern we have used heparin-Sepharose to analyze the heparin-binding capacity of the lipoproteins, as described for plasma lipoproteins (42). As expected, the apoE-containing CSF-Lp were able to bind to heparin whereas CSF-LpA and sCSF-Lp did not.

DISCUSSION

Lipoproteins in human CSF have not yet been studied in a large population and the standard values for CSF

TABLE 2. Lipids in the four CSF-Lp classes

	CSF-LpE		CSF-LpEA		CSF-LpA		sCSF-Lp
	μg/ml	%	*μg/ml*	%	*μg/ml*	%	*μg/ml*
Cholesterol	0.37 ± 0.5	17	0.8 ± 0.7	14	0.8 ± 0.7	15	0.48 ± 0.52
PL	0.6 ± 0.26	27	1.1 ± 0.7	20	1.7 ± 0.6	32	4.2 ± 5.7
Protein	1.27 ± 1.13	56	3.7 ± 4.2	66	2.9 ± 2.8	53	—[a]

Values given in this table are expressed as micrograms in 1 ml of the original CSF pool. The data represent the mean from five different preparations.

[a] This fraction contains all soluble proteins in addition to the apolipoproteins and therefore no protein value can be given for the lipoprotein composition.

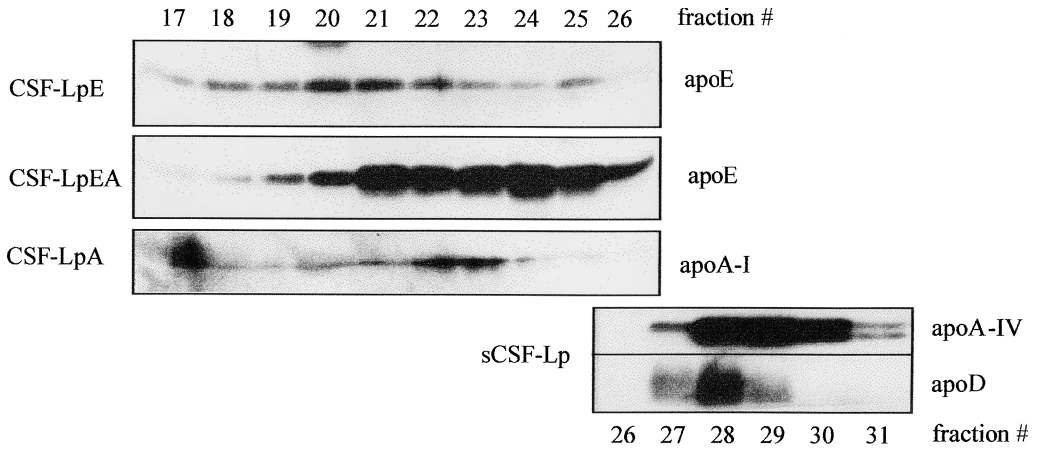

Fig. 5. Analysis of the four CSF-Lp classes by gel filtration. The integrity and size of the four CSF-Lp populations were analyzed by gel filtration on a Superdex 200 column. Two hundred microliters of each lipoprotein class was applied on the column, and fractions 17–31 were precipitated by TCA and analyzed by Western blotting, using the indicated antibodies. CSF-LpE, as shown by the detection of apoE, were found mainly in fractions 18–22, indicating a size equivalent to that of small LDL (Fig. 1). CSF-LpEA particles, here also detected with anti-apoE antibodies, showed a broader distribution ranging in size from LDL to small HDL (fractions 19–26). CSF-LpA are smaller than CSF-LpE; the apoA-I was found mainly in fractions 22 and 23. ApoA-IV and apoD, which mark the lipoproteins devoid of apoE or apoA-I, were both found mainly in fraction 28. ApoA-IV in fractions 29 and 30 represents most free apolipoprotein.

lipid and apolipoprotein levels are based on rather small sample sizes. This might be because CSF protein and lipid levels are extremely low, which makes a comprehensive analysis difficult. We analyzed total cholesterol, PL, total fatty acids, and the major apolipoproteins in plasma and CSF of a large group of patients with normal BBB function and no severe neurological disorder. The data obtained here confirm the published data, which had been obtained from postmortem samples (45) or in smaller (35) or diseased (Alzheimer's disease) populations (46). In the CSF of patients with intact BBB the percentage of apoA-I (0.26%) and cholesterol (0.3%) in CSF relative to plasma is similar to the percentage of albumin (0.5%) and fatty acids (0.54%). In contrast, there is 4.4% apoE in CSF, which underscores the fact that apoE is synthesized in the brain. This is consistent with reports in the literature, where independent apoE metabolism in brain has been demonstrated in patients after liver transplantation (52).

Several publications have described different classes of lipoproteins in CSF (8, 10, 11, 15, 16). However, the methods used were limited in the degree of separation achieved, in light of the similar physicochemical properties of the particles. By using density gradients we obtained similar results, for example, incomplete separation (data not shown). Taking note of published data, we next used gel filtration for separation (11). The size separation demonstrated the overlapping size range for the CSF-Lp (10–24 nm) (Fig. 1).

We first used affinity chromatography to isolate apoE-containing CSF-Lp and CSF-LpA as well as lipoproteins containing neither apoE nor apoA-I. We checked the integrity of these lipoproteins by EM and were able to confirm that the isolated lipoprotein fractions indeed contained spherical particles with a size range between those of plasma HDL and LDL.

For a further fractionation of apoE-containing lipoproteins these particles were applied to an apoA-I affinity column. In this way we isolated CSF-LpE and CSF-LpEA in addition to the above-described CSF-LpA and sCSF-Lp. CSF-LpE is the particle most resembling the large apoE-containing CSF-Lp described by Guyton et al. (8). CSF-LpA was found to be smaller than CSF-LpE, which is in agreement with Pitas et al. (16). The major additional apolipoproteins in this lipoprotein class were apoA-II and apoD and therefore this particle most resembled a plasma HDL_3. The unbound material from the apoA-I column contained, as expected, no apoE and no apoA-I, but apoA-IV, apoD, apoJ, and apoH. In electron micrographs we were able to demonstrate that these apolipoproteins seem to form small spherical lipoproteins and that they elute from the FPLC in a size range slightly larger than that of albumin. To date we have not been able to analyze whether these apolipoproteins are all on one small lipoprotein or whether we are seeing a mixture of small particles, each consisting of one or two apolipoproteins with some lipids. This set of data corresponds well to the data from Pitas et al. (16), who also used a sequence of affinity columns (in the reverse order) and described apoE- and apoA-I-containing particles. Also, Borghini et al. (15) described

TABLE 3. General characterization of the four CSF-Lp classes

	CSF-LpE	CSF-LpEA	CSF-LpA	sCSF-Lp
Size (nm)	18–22	13–20	13–18	10–12
Apolipoproteins	E, A-IV, D	E, A-I, A-IV, D, H	A-I, A-II A-IV, D	AI-V, D, H, J
Lipid:protein ratio	0.76:1	0.51:1	0.86:1	—
Heparin binding	++	+	—	—

CSF was fractionated as described in Fig. 3. Sizes were determined by EM and represent the range from five preparations. Apolipoprotein composition was analyzed by Western blot (Fig. 4). Heparin binding was determined on a heparin affinity column (42) and the relative affinity (+ or ++) was determined by the salt concentration at which the lipoproteins eluted.

CSF-LpE and CSF-LpA, which eluted after gel filtration with similar size. Both groups did not further separate the fractions and therefore would not have detected the CSF-LpEA as described here. Koudinov et al. (10) reported on four lipoprotein classes with different densities corresponding in general to the particle classes described here. However, the lipoproteins described by Koudinov et al. had less distinct apolipoprotein compositions, most probably because of their overlap in density.

So far there are few data available on the composition of CSF lipoproteins and we are the first, to our knowledge, to determine the lipid and protein composition in the four distinct lipoprotein classes in CSF. However, in comparing the relative amounts of cholesterol and the four classes of CSF-Lp, CSF-LpE has more cholesterol than the other lipoproteins. This is in agreement with data presented by Pitas et al. (16), who showed more cholesterol in "CSF-E" than in "CSF-A-I."

Several groups have analyzed the CSF apolipoprotein pattern with respect to apoE isoforms (12, 14, 46). An analysis of lipoprotein composition was performed by Rebeck et al. (14) and they found no isoform-specific differences in the composition of the total lipoprotein fraction. We analyzed the CSF lipid and apolipoprotein levels in 216 patients according to the apoE phenotype and also found no differences.

With this report we describe four distinct lipoprotein classes in human CSF with specific apolipoprotein and lipid composition and differences in size. CSF-LpA particles are believed to be derived from small HDL-like plasma particles and to enter the CNS parenchyma via the BBB. They might mediate cholesterol efflux from neuronal cells and transport it back to the liver via the CSF. There are, however, no data yet to confirm such a role for the HDL-like CSF-Lp in "reverse cholesterol transport." In contrast, apoE particles are synthesized as discoidal precursors from astrocytes (11) and it is hypothesized that they are converted to spherical particles by uptake of cellular cholesterol within the parenchyma of the CNS before reaching the CSF (CSF-LpE). We propose that CSF-LpEA might be formed by interaction between these two particles either in the CNS parenchyma or in the CSF. ApoA-IV, apoD, apoH, and apoJ seem to be lipoprotein associated in the CSF. While apoD, apoH, and apoJ are synthesized within the brain and, in the case of apoD and apoJ secreted as lipoproteins (11, 27), apoA-IV and apoH might enter the brain as "lipid-poor" particles or as free apolipoproteins. To date, only limited data are available on the differential functions of lipoproteins in brain, and where studies have been performed they have used either artificial lipoproteins or plasma lipoproteins that do not occur in the brain. With the isolation of four distinct lipoprotein classes from human CSF we now have the tools to study cerebral lipid metabolism in cell culture and learn more about the interaction of these lipoproteins with their receptors as well as their potential function in the brain.

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