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Characterization and functional studies of lipoproteins, lipid transfer proteins, and lecithin:cholesterol acyltransferase in CSF of normal individuals and patients with Alzheimer's disease

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Abstract We investigated the lipoprotein distribution and composition in cerebrospinal fluid (CSF) in a group of patients with Alzheimer's disease (AD) or affected by other types of dementia in comparison to non-demented controls. We found slightly decreased apolipoprotein (apo)E and cholesterol concentrations in CSF of AD patients and moderately increased apoA-I concentrations, while in patients suffering from other types of dementia the apoA-I CSF concentration was increased. ApoA-IV concentrations varied widely in human CSF, but were not associated with any clinical condition. HDL₂-like apoE-containing lipoproteins represent the major lipoprotein fraction. In CSF of normal controls, only a minor HDL₃-like apoA-I-containing lipoprotein fraction was observed; this fraction was more prevalent in AD patients. ApoA-II was recovered mostly in the HDL₃ density range, while apoA-IV was not associated with lipoproteins but appeared in a lipid-free form, co-localizing with LCAT immunoreactivity. Bi-dimensional analysis demonstrated pre- β and α apoA-I-containing particles; apoE and apoA-II were detected only in α -migrating particles. ApoA-IV distributed both to pre- β and γ -migrating particles; the LCAT signal was co-localized in this γ -migrating fraction. Enzymatically active LCAT was present in human CSF as well as PLTP activity and mass; no CETP mass was detected. In CSF from AD patients, LCAT activity was 50% lower than in CSF from normal controls. **CSF lipoproteins induced a significant cholesterol efflux from cultured rat astrocytes, suggesting that they play an active role in maintaining the cholesterol homeostasis in brain cells.**—Demeester, N., G. Castro, C. Desrumaux, C. De Geitere, J. C. Fruchart, P. Santens, E. Mulleners, S. Engelborghs, P. P. De Deyn, J. Vandekerckhove, M. Rosseneu, and C. Labeur. **Characterization and functional studies of lipoproteins, lipid transfer proteins, and lecithin:cholesterol acyltransferase in CSF of normal individuals and patients with Alzheimer's disease.** *J. Lipid Res.* 41: 963–974.

Supplementary key words cerebrospinal fluid • lipoproteins • Alzheimer's disease • cholesterol • efflux

Several observations suggest a close relationship between the lipoprotein metabolism in the brain and the development of Alzheimer's disease (AD) (1, 2). It is clearly established that the inheritance of the apolipoprotein (apo) E4 allele is associated with increased risk of developing AD at a younger age (1, 3). ApoE plays an important role in the cholesterol metabolism, both in the peripheral circulation and in the brain, where it is synthesized by astrocytes and glial cells. Its most important functions are to redistribute lipids needed for neurite growth through receptor-mediated uptake and to interact with the amyloid β peptide (4–7).

In analogy with the metabolism of lipids in the peripheral circulation, cerebrospinal fluid (CSF) lipids are transported by lipoproteins. Although several reports describe the composition of CSF lipoproteins (4, 8–12), their detailed structure and functions are only poorly understood. CSF lipoproteins are needed for delivery of cholesterol during nerve growth, but it is not known whether they are also actively involved in taking up excess cholesterol from nerve cells. This cholesterol could then be removed from the CSF via arachnoid granulations or by redistribution to other cells in the brain compartment (9). Such active lipid redistribution might prove to be functionally important because several recent studies have shown that the neuronal membrane cholesterol content influences processing of the amyloid precursor protein (APP) (13–15). Decreasing the neuronal cell cholesterol content, by com-

Abbreviations: AD, Alzheimer's disease; apo, apolipoprotein; APP, amyloid precursor protein; CETP, cholesterol ester transfer protein; CSF, cerebrospinal fluid; LCAT, lecithin:cholesterol acyl transferase; PLPT, phospholipid transfer protein.

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binning a treatment with lovastatin (HMG-CoA reductase inhibitor) and α -methyl- β -cyclodextrin, reduces amyloid β production. On the contrary, increasing cellular cholesterol concentrations by adding β -VLDL to Cos1 cells causes a decrease of the secretion of the soluble form of APP (sAPP). These observations suggest that CSF lipoproteins may fulfill an important role in maintaining the neuronal cholesterol levels, thereby regulating indirectly the production of amyloidogenic peptides.

Some reports link the development of AD with lipid metabolism in the brain: lipid concentrations, especially that of fatty acid and phospholipids and to a lesser extent cholesterol, which are reduced in the CSF of AD patients (16). Other reports show that a reduction in brain tissue membrane lipids (gangliosides, phospholipids, and cholesterol) is correlated with reduced synaptic function in patients with early onset form (EOAD) Alzheimer's disease, independent of the β amyloid depositions (17).

ApoE and apoA-I are the major CSF apolipoproteins, which are present in high density lipoproteins (HDL), while apoB-containing very low or low density lipoproteins (VLDL or LDL) were never identified in CSF. Early reports by Pitas et al. (4) and later reports by Borghini et al (10) suggest that apoE-enriched HDL is the predominant lipoprotein in CSF. Others report an apoE-enriched very low density HDL₁ fraction that represents a minor subclass (12).

As intact lipoprotein particles cannot cross the blood-brain barrier, the synthesis, remodeling, and redistribution of lipids in the brain must take place in this compartment. It is not known in what form HDL precursors are synthesized in the brain and whether remodeling occurs within the CSF. HDL remodeling in the circulation is largely mediated by the enzyme lecithin:cholesterol acyltransferase (LCAT) and the lipid transfer proteins cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), but in the brain these pathways remain largely obscure.

ApoE secretion by astrocytes, either as a free protein or as a part of lipoproteins, has not been elucidated. It is suggested that the lipoproteins secreted by the astrocytes might resemble (8) the apoA-I pre β -like particles secreted by the liver (18). Pitas et al. (4) reported the existence of discoidal apolipoprotein-lipid complexes in the CSF of dogs. In humans, such pre β particles have not yet been identified, and one of the aims of this study is to identify such precursor particles and the factors that may remodel them. These small particles are likely candidates for participating in the process of "reverse cholesterol transport" as has been suggested for the interstitial fluid lipoproteins. Studies by Ito et al. (19) have demonstrated a unique profile of HDL generation and cholesterol efflux from rat astrocytes, whereby cholesterol-rich (apoE-HDL) and cholesterol-poor (apoA-I-HDL) are generated.

In this study, we carried out a detailed analysis of lipids and lipoproteins in CSF collected from patients affected by AD, by other forms of dementia, or from non-demented controls in order to look for major differences in the lipid and lipoprotein composition among the different diag-

nostic groups. Lipoproteins were isolated by density gradient ultracentrifugation and combined with sensitive and specific apolipoprotein assays enabling the direct lipoprotein analysis on small sample amounts.

In order to understand how the HDL particles in the brain compartment are synthesized and remodeled, we investigated in human CSF the presence of minor lipoprotein subclasses such as pre β particles by bi-dimensional non-denaturing gel electrophoresis. The physiological role of CSF lipoproteins as substrates for lipolytic enzymes such as lecithin:cholesterol acyltransferase (LCAT), and for lipid transfer proteins such as cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) was investigated.

Finally, cholesterol efflux from rat astrocytes by human CSF lipoproteins was correlated with the CSF (apo)lipoprotein content.

MATERIALS AND METHODS

Patients

All patients underwent a general physical and neurological examination, routine blood screening, neuroimaging consisting of CT scan and/or MRI, electroencephalogram, and extensive neuropsychological examination and lumbar puncture as routinely performed. The control group (CON) had no neurological or psychiatric antecedents and consisted *i)* of subjects with neurological complaints in whom no diagnosis of organic disease involving the central and peripheral nervous system was made after extensive observation and *ii)* of individuals with neurological syndromes involving the peripheral nervous system. Controls underwent lumbar puncture for non-related reasons such as suspected inflammatory disease, polyneuropathy, headaches, unexplained seizures, in which the presence of inflammation was not confirmed. The diagnosis of probable Alzheimer's disease (Dementia Alzheimer Type DAT or AD) was based on the NINCDS/ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders) criteria (20). Patients also fulfilled the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) criteria. The third group (DEM) included a category of samples obtained from dementia patients with an alternative causative diagnosis, such as vascular dementia or other degenerative dementias such as frontotemporal dementia, dementia in Parkinson's disease, and normal pressure hydrocephalus. In the fourth group we included all patients in whom meningitis or encephalitis was diagnosed (MEN). Finally we grouped patients in whom a disruption of the blood brain barrier (BBB) was present. These disruptions were not due to infectious pathology but were caused by active multiple sclerosis or vasculitis. All patients were informed that the remainder of the samples not needed for clinical analysis are used for research purposes. As no procedures were performed that were not clinically indicated, there was no ethical conflict regarding the collection of these samples.

CSF samples

CSF samples were obtained by conventional lumbar puncture in the Department of Neurology, University Gent and in the Memory Clinic, Middelheim Hospital, Antwerp. CSF was collected in polypropylene vials which were immediately frozen at -80°C until analysis. Routine investigation of CSF included cell count, total protein and glucose analysis as well as agar-gel elec-

trophoresis of proteins. All CSF samples contained less than 5 white blood cells/mm³. If needed, the samples were concentrated on ultrafiltration devices.

Materials

Unless otherwise specified, reagents were obtained from Sigma and were of analytical grade. Antibodies against apolipoproteins E, A-I, A-II, and A-IV were raised in rabbits and purified as previously described (21, 22). The anti-PLTP and anti-CETP antibodies were a generous gift from L. Lagrost and C. Desrumaux and were prepared as previously described (23).

Mouse monoclonal antibodies against LCAT were generated by fusing the Sp20 myeloma cell line with spleen cells isolated from a homozygous knock-out mouse previously immunized with purified human recombinant LCAT (24) using standard protocols (S. Roosbeek, F. Peelman, M. Rosseneu, and C. Labeur, unpublished data). Homozygous knock-out mice were a generous gift from E. Rubin. For Western blotting, swine anti-rabbit IgG peroxidase-labeled or rabbit anti-mouse IgG peroxidase-labeled antibodies were used for detection.

Density gradient ultracentrifugation

Lipoproteins in CSF were separated by density gradient ultracentrifugation in a salt-sucrose gradient. The gradient was prepared in 12-ml polyallomer tubes (Beckman) and consisted of 0.5 g sucrose, 5 ml NaCl 4 M, 400 μ l non-concentrated CSF sample, on which 6.2 ml NaCl 0.67 M was layered. Samples were centrifuged for 66 h in a SW41 Ti rotor (Beckman) at 10°C and 38,000 rpm. Fractions (500 μ l) were collected using an auto-densiflow system and stored at 4°C.

Apolipoprotein assays

Apolipoproteins E, A-I, and A-IV were measured by sandwich ELISA using affinity-purified polyclonal antibodies as described previously (21, 22). Briefly, the unlabeled affinity-purified rabbit antibodies were coated onto the ELISA plates (Nunc, Maxisorp). After blocking excess binding sites on the plates with assay buffer containing 0.1% casein (Merck), the diluted samples were incubated on the plates (2 h, 37°C). After washing, bound antigen was detected by incubation with the same affinity-purified polyclonal antibody labeled with peroxidase (2 h, 37°C). Bound peroxidase was visualized by incubation with TMB (tetra-methyl-benzidine, Boehringer Mannheim) as substrate. The reaction was stopped by addition of sulfuric acid (2.5 N) and the intensity was read on a microplate reader at 450 nm (reference filter: 630 nm). The standard curve was fitted using the software provided by the microplate reader (EL-800 Biotek plate reader equipped with KC4 software). The sensitivity of these assays is approximately 10 ng/ml.

Cholesterol and phospholipid measurement

Cholesterol and phospholipid concentrations were measured enzymatically using commercial reagents from Boehringer Mannheim (cholesterol) and Biomérieux (phospholipids). Samples were concentrated 10 times. The amount of free cholesterol in the samples was determined by measurements in the absence and presence of cholesterol esterase.

ApoE and A-IV phenotyping

Both apoE and apoA-IV phenotyping were performed as described by Menzel (25). CSF samples were treated with neuraminidase prior to electrophoresis.

SDS-PAGE and Western blotting, delipidation of CSF samples

The CSF samples were delipidated using ice-cold ethanol-diethylether 3:2 (v/v). One ml CSF was injected into 15 ml ethanol-diethylether mixture at -20°C and centrifuged after 1 h (10 min, 2000 rpm). The pellet was washed twice with 2 ml diethylether, dried, and re-suspended in the electrophoresis sample buffer. On average, one-third of the sample was applied to the SDS-PAGE gel.

SDS-PAGE was performed under reducing or nonreducing conditions (\pm β -mercaptoethanol 0.05%), using standard methodology in a Bio-Rad Mini-Protean system (Bio-Rad). CSF samples were separated by 12.5% or 17% SDS-PAGE. Immunoblotting was performed using the same equipment with a device for wet blotting, using 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer (pH 11) containing 10% methanol onto PVDF membranes (Millipore), using standard procedures. The bound peroxidase was visualized using the CN/DAB substrate kit (Pierce). The blots for CETP were developed using a chemiluminescence substrate (Boehringer).

Bi-dimensional electrophoresis and blotting

CSF samples were concentrated (4°C) at least 30-fold on Microcon concentrators (Millipore, cut off 10 kDa); 20 μ l was applied in the 1.5-cm sample well of a 0.75% agarose gel in 50 mM barbital buffer, pH 8.6, on Gelbond (FMC Bio-Products) at 4°C. Electrophoresis was carried out in the first dimension until an albumin marker stained with bromophenol blue had migrated 7.5 cm. The second dimension electrophoresis was carried out in a 2-15% polyacrylamide gradient gel (15 \times 15 cm). Samples (0.5 \times 7 cm) were cut out from the agarose gels, placed horizontally next to each other on the polyacrylamide gel, and covered with 1% agarose. Electrophoresis was carried out at 4°C in 25 mM tris-(hydroxymethyl) aminomethane (Tris)-glycine buffer (pH 8.3) at 100 V for 19 h. The gels were immunoblotted on nitrocellulose membranes (Sartorius 0.45 μ m) in a 25 mM tris-(hydroxymethyl) aminomethane (Tris)-glycine buffer (pH 8.3), using a semi-dry blotting system (Pharmacia). The membranes were immunoblotted with the apoA-I, anti-A-II, anti-A-IV, anti-E polyclonal antibodies, or with the anti-LCAT antibody as described (18) and developed using chemiluminescence (Amersham, Pharmacia, Biotech).

LCAT activity measurements

Acyltransferase activity of LCAT in CSF was measured on discoidal complexes consisting of ether-DPPC, cholesterol/apoA-I and containing 1,2-bis-(1-pyrenebutanoyl)-*sn*-glycero-3-phosphocholine as a substrate, prepared by cholate dialysis (26). As reference material we used the supernatant of a stable transfected human LCAT BHK cell line, which on average contains \pm 1.5 μ g LCAT/ml (24). The acyltransferase activity of the WT LCAT and mutants was determined by HPLC by measuring the cholesteryl-1-pyrenebutyrate formed by hydrolysis of 1,2-bis-(1-pyrenebutanoyl)-*sn*-glycero-3-phosphocholine and the transfer of the 1-pyrenebutyric acid to the β -hydroxyl of cholesterol by LCAT (27). The assay mixture contained approximately 2 μ M of 1,2-bis-(1-pyrenebutanoyl)-*sn*-glycero-3-phosphocholine, 4 μ M free cholesterol (FC) incorporated in the discoidal complexes, 4 mM 2-mercaptoethanol, and 400 μ l cell culture medium or CSF. A 10-mM Tris-HCl buffer (pH 8.0), 0.15 M NaCl, 3 mM EDTA, 1 mM Na₃N was added to give a final volume of 500 μ l; the mixture was incubated for 2 h at 37°C. The reaction was stopped by adding 4 ml of hexane-isopropanol 3:2 (v/v) containing cholesteryl-1-pyrenehexanoate as internal standard (60 nM). The cholesteryl-1-pyrenebutyrate formed during the enzymatic reaction was quantified by isocratic HPLC (Waters 600E) on a reversed-phase

ODS C18 column (Merck Licrocart 2504), eluted with acetonitrile–isopropanol 90:10 (v/v), and detected at 240 nm.

Determination of PLTP and CETP mass and activity

PLTP and CETP mass were determined by a competitive ELISA, as described (23, 28). PLTP activity was determined by calculating the rate of increase in pyrene-monomer fluorescence intensity that occurs upon transfer of the pyrene-labeled phospholipids (Molecular Probes) from quenched phosphatidylcholine vesicles to HDL acceptor particles, as previously described (29). CETP mass was measured according to the method described (28) and CETP activity was measured using the Wak Chemie (GmbH, Germany) fluorescence activity kit using 200 μ l CSF liquid and a 3-h incubation time in order to increase the sensitivity of the assay.

Determination of the cholesteryl esters in CSF

The distribution of the CSF cholesteryl esters was determined by HPLC analysis on a RP C18 column run by isocratic separation in acetonitrile–isopropanol 1:1 and detection at 210 nm. Samples (min. 500 μ l) were delipidated by extraction with hexane–isopropanol 3:2 (v/v) in the presence of an internal standard (cholesterylheptadecanoate, Sigma). The amount and the composition of the cholesteryl esters in the samples were calculated by comparison with a standard mixture (30) as described.

Astrocyte cultures and cholesterol efflux assays

Astrocyte cultures. Primary cultures of mixed astrocytes were made from newborn rat cerebral cortex. After removing the meninges, the brain tissues were gently forced through a nylon sieve. Astrocytes were plated on 6-well dishes (Nunclon) at a density of 120,000 cells/ml in 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone Laboratories), 2 mM glutamine, and 50 μ g/ml gentamicin (31). After 2 weeks in culture, astrocytes were labeled by a 48-h incubation with [3 H]cholesterol (NEN, DuPont de Nemours, 2 μ Ci/well); then the cells were rinsed and incubated for 24 h in DMEM containing 0.5% bovine serum albumin. After this incubation, cells were washed with phosphate-buffered saline (PBS) and incubated at 37°C with CSF diluted in DMEM for 7 h. At the end of this incubation the medium was removed, the cell monolayer was washed three times with PBS and harvested in 1 ml 0.1 M NaOH. Radioactivity was measured in both medium and cells. Cholesterol efflux, expressed as percentage, was calculated as the amount of label recovered in the medium divided by the total label in each well. Each sample was tested in triplicate.

CSF samples. CSF samples for the efflux experiments were concentrated approximately 15 to 20 \times on Centricon 10 concentrators (Millipore). The concentration of apoA-I and apoE was measured in each sample as mentioned above; the samples were rediluted in incubation medium to a final apolipoprotein concentration of \pm 10 μ g/ml. In separate wells, purified apoA-I or apoE were incubated with the cells at a concentration of 5 μ g/ml.

RESULTS

Study population

The composition of the study group is given in **Table 1**, where the number of cases and the apoE phenotyping results are given for each diagnostic group. The selection criteria and patient definitions are described in Materials and Methods. Patients in whom clinical observations suggested a defect in the blood–brain barrier were grouped separately. Although the number of Alzheimer patients is

TABLE 1. Classification and apolipoprotein E phenotypes in each diagnostic group

Diagnosis	Number of patients	ApoE4 (at least 1 E4 allele)
CON	55	10/50
AD	17	12/17
DEM	11	2/8
MEN	10	ND
DEF BBB	11	ND

Abbreviations: CON, non-demented controls; AD, patients with Alzheimer's disease; DEM, patients suffering from other forms of dementia; MEN, patients affected by meningitis; DEF BBB, patients with a defective blood–brain barrier; ND, not determined.

relatively small, the high frequency of the E4 allele in this group compared to the controls is obvious.

Lipids and apolipoprotein concentrations in human CSF

The CSF apolipoprotein and cholesterol concentrations are listed in **Table 2**. ApoA-I levels in CSF from AD patients are slightly, but not significantly, higher than in controls or in patients affected with other forms of dementia.

ApoA-IV concentrations varied strongly in all groups, ranging from 0.06 up to 1.76 μ g/ml. Due to the non-Gaussian distribution of these values, median apoA-IV concentrations and concentration ranges are given in **Table 2**. In patients with blood–brain barrier defects, apoA-IV median values were significantly elevated in comparison with other diagnostic groups. In general, all parameters were slightly elevated in the patients with a defective blood–brain barrier, although this increase was only statistically significant for apoA-IV and cholesterol values.

ApoE values were slightly but not significantly decreased in AD patients, but significantly increased in patients affected by other types of dementia.

Cholesterol levels were decreased in AD in comparison with controls, while patients suffering from meningitis had significantly elevated cholesterol levels. Correlation coefficients (r) between apoA-I, apoE, and cholesterol values were, respectively, 0.72 and 0.66, confirming that the majority of the cholesterol in the CSF is transported on lipoprotein particles carrying apoA-I or apoE. ApoA-IV levels correlated less strongly with cholesterol levels ($r = 0.48$), while no significant correlations were observed between apoE and apoA-I or apoA-IV levels.

Western blotting for apolipoproteins, LCAT, PLTP, and CETP

The presence of apolipoproteins A-I, A-II, E, and A-IV and of LCAT, CETP, and PLTP in CSF samples was tested by immunoblotting, as depicted in **Figs. 1–3**.

Figure 1 shows results obtained for apoA-I and apoA-IV blotting. A band at 28 kDa corresponding to the molecular mass of apoA-I (**Fig 1, lane 1**) and fainter but distinct bands at the top of the gel were detected, suggesting that despite the treatment of the samples with SDS, part of the apolipoproteins remained associated with lipoproteins with a size too large to enter the gel. After sample delipi-

TABLE 2. Mean (\pm SD) and median concentrations of apolipoprotein and cholesterol concentrations ($\mu\text{g}/\text{ml}$) in the CSF of patients classified in the different diagnostic groups

Diagnosis	ApoA-I	ApoA-IV ^a	ApoA-IV Median	ApoA-IV Range	ApoE	Cholesterol ^a
CON	0.96 \pm 0.47	0.36 \pm 0.21	0.39	0.11–0.67	2.89 \pm 0.91	4.40 \pm 1.31
AD	1.53 \pm 0.60	0.36 \pm 0.18	0.31	0.08–1.31	2.40 \pm 0.97	3.50 \pm 1.12
DEM	1.02 \pm 0.61	0.36 \pm 0.22	0.28	0.06–1.35	3.20 \pm 1.44 ^b	4.71 \pm 2.20
MEN	0.94 \pm 0.61	0.45 \pm 0.31	0.29	0.09–1.76	3.00 \pm 1.08	6.13 \pm 4.30 ^b
DEF BBB	1.08 \pm 0.53	0.59 \pm 0.36 ^b	0.48 ^b	0.08–1.32	3.10 \pm 1.20	7.21 \pm 3.73 ^c

Abbreviations: see Table 1.

^a Concentrations determined in a subset of the population (n = 8).

^b $P < 0.1$, Student's *t*-test.

^c $P < 0.01$, Student's *t*-test.

dation (Fig. 1, lane 2), all apoA-I immunoreactivity was restricted to one band with a molecular mass of 28 kDa. All consecutive analyses were carried out on delipidated samples. ApoA-IV reactivity (Fig. 1, lane 3) is associated with one band corresponding to the molecular mass of apoA-IV.

The apoA-II and apoE immunoblots were more complex, as summarized in Fig. 2, where the delipidated samples of a E3/E3 homozygous individual were analyzed in the absence or presence of reducing agents (Fig. 2). Non-reduced samples displayed several bands for apoE at, respectively, 83, 69 (weak), 42, and 34 kDa (Fig. 2A, lane 1). Because apoE3 contains free cysteine residues and is capable of binding to apoA-II, the observed bands could correspond to E3–E3, E3–A-II homo- and heterodimers and an apoE monomer band (34 kDa). Upon reduction (Fig. 2A, lane 2), all bands turned into one sharp band at the position of apoE3 monomer (34 kDa), except for a diffuse band at 69 kDa, which could not be explained. In non-reduced samples (Fig. 2B, lane 1), reactivity for apoA-II showed distinct bands at 50 (apoE–A-II heterodimer), and 17 kDa (apoA-II homodimer); upon reduction of the samples, the 50 kDa band disappeared, confirming the presence of apoA-II–apoE heterodimers. No apoB reactivity could be demonstrated in all CSF tested (data not shown).

LCAT and PLTP immunoreactivity were demonstrated in human CSF (Fig. 3, lanes 1 and 2, respectively). However, we could not demonstrate the presence of CETP in

human CSF by immunoblotting, even after detection with the sensitive luminescence reagent (sensitivity 25–50 ng).

Isolation of CSF lipoproteins by density gradient ultracentrifugation

Lipoproteins were isolated from CSF by density gradient ultracentrifugation. In the respective density fractions, lipoproteins were identified by measuring apoE, apoA-I, and apoA-IV. The density gradient profiles of the CSF from three controls (Fig. 4A) are compared to those of three samples collected in AD patients (Fig. 4B). In all controls (apoE3/E3) the major lipoprotein fraction consists of an apoE-containing HDL fraction; in sample a (Fig. 4A) this fraction was heterogeneous with maximal densities at 1.08 and 1.12 g/ml. In the two other control subjects (Fig. 4A, b and c) the apoE-rich HDL was more homogeneous with one maximum at 1.10 g/ml. In these three control subjects analyzed, apoA-I is associated with smaller particles of higher density (1.15 g/ml) and of low concentration.

The density profiles of the CSF lipoproteins in AD pa-

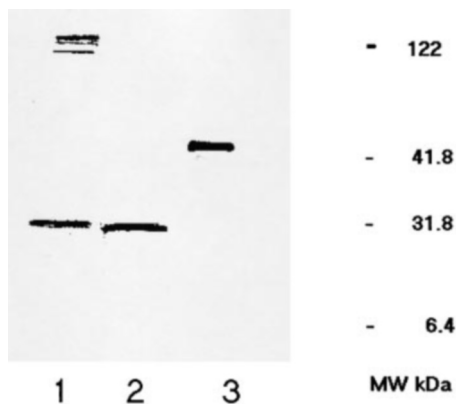


Fig. 1. Western blot analysis of human CSF with anti-apoA-I antibody. Lane 1, sample before and lane 2, after delipidation. In lane 3, the immunoblotting for apoA-IV (delipidated sample).

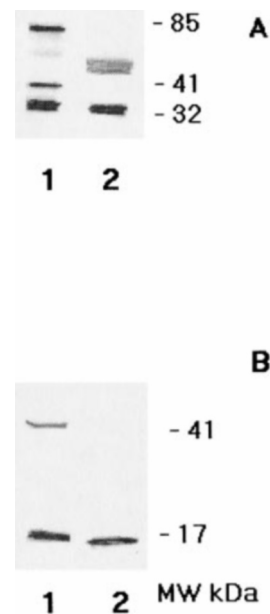


Fig. 2. Western blot analysis of delipidated human CSF with (A) anti-apoE and (B) anti-apoA-II, respectively without (lane 1) or with (lane 2) reducing agent.

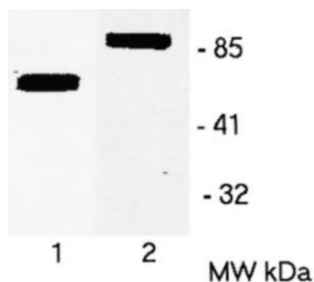


Fig. 3. Western blot analysis of delipidated human CSF with anti-LCAT (lane 1) and anti-PLTP antibody in non-reducing conditions (lane 2).

tients (Fig. 4B) are different from controls. The major lipoprotein fraction also consists of an apoE-rich HDL₂-like particle with maximal density around 1.10 g/ml. In the two E3/E4 heterozygotes (Fig. 4B, b and c), the apoE peak was slightly heterogeneous with a clear maximum between 1.10 and 1.12 g/ml. A clear apoA-I-enriched fraction with a maximal density at 1.13–1.14 g/ml was detected in all AD samples. The apoE/apoA-I concentration ratio in the CSF of controls or AD patients is, respectively, 2.2 and 0.94, reflecting decreased apoE and increased apoA-I values in AD patients. The same observation could be made for the ratio of the sum of apoE/sum of apoA-I in the density gradient fractions, which amounts to 11.1 in non-demented controls compared to 3.3 in AD patients.

We tried to identify the positions of apoA-IV and apoA-II in the lipoprotein profiles obtained by density gradient ultracentrifugation. ApoA-IV was recovered in the density fraction >1.21 g/ml, suggesting that apoA-IV might be released from lipoproteins during centrifugation. However, a parallel analysis of samples separated by gel filtration (data not shown) demonstrated that a small apoA-IV-containing fraction appeared at higher elution volumes, suggesting that apoA-IV in CSF is not associated with lipoproteins of HDL size and density. In the gel-filtrated samples, the lipoproteins identified corresponded to those observed by density gradient ultracentrifugation, a large less dense apoE containing fraction and a denser, smaller, and less pronounced apoA-I-containing fraction, where the apoA-II immunoreactivity also co-localizes. LCAT and apoA-IV immunoreactivity were detected in the fractions of lower molecular mass, i.e., in the lipoprotein-free fractions.

Bi-dimensional electrophoresis and blotting

The presence of small pre- β -like particles in CSF was investigated by bi-dimensional electrophoresis, as described in Materials and Methods. Gels were immunoblotted for apoA-I, apoE, apoA-II, apoA-IV, and LCAT and results are summarized in Fig. 5. Lipoproteins are identified according to α , pre- β , or γ -mobility. ApoA-I immunoreactivity was distributed over a broad range of α -migrating particles, while apoE immunoreactivity was associated with a more homogeneous subclass of α -migrating particles, confirming to a large extent our results obtained by density gradient centrifugation. Pre- β migrating apoA-I-containing particles were identified in some samples, but not in all.

ApoA-IV reactivity was recovered only in pre- β and γ -migrating particles and not in α -migrating particles. Interestingly, LCAT immunoreactivity co-localized in part with apoA-IV reactivity, suggesting that in CSF LCAT is bound to small γ -like particles. Analysis of different CSF samples demonstrated that the LCAT immunoreactivity varied significantly among these samples. LCAT distribution in a fresh plasma sample (data not shown) was identified in the same γ -like particles as in the CSF; moreover, a faint reactivity was identified in smaller α -migrating particles, while no reactivity was associated with large α -migrating particles.

Quantification of cholesteryl esters in human CSF

The cholesteryl ester and free cholesterol content, together with the cholesteryl esters distribution, was analyzed in a limited number of CSF samples. The results summarized in Table 3 show that approximately 70% of cholesterol in the CSF is esterified, as compared to 75% cholesteryl esters in human plasma. A comparison of the cholesteryl esters distribution in CSF and plasma shows that relatively more cholesteryl oleate (18:1), more cholesteryl palmitate (16:0), and cholesteryl linoleate (18:2) are found in CSF as compared to plasma.

Activity measurements for LCAT, PLTP, and CETP

LCAT activity, PLTP and CETP activity, and mass were measured in a subset of CSF samples obtained from seven non-demented patients and one AD patient. The results summarized in Table 4, show that the mean LCAT activity in these samples was 0.010 ± 0.002 nmol CE/ml sample per h, which corresponds to about 2.5% of the activity of serum LCAT. These preliminary results suggested that LCAT activities in CSF samples from the one AD patient tested were low. Therefore a new set of well-characterized fresh CSF samples was collected (10 AD cases and 10 controls) and the LCAT activity in these samples was compared. The average LCAT activity in the 10 AD patients measured was significantly lower, 0.004 ± 0.02 nmol CE/ml sample per h ($P < 0.1$, Student's *t*-test) than in the control samples. As previous reports suggested impaired cholesterol esterification in some neurological disorders (32, 33), these data might corroborate these findings.

The PLTP mass and the activities measured in human CSF correspond to approximately 1% of the serum values (23). In this subset of samples, cholesterol and phospholipid concentrations are comparable and the correlation between these parameters is $r = 0.9$. Moreover we found a significant correlation between PLTP mass and activity ($r = 0.6$), and between LCAT and PLTP activity and cholesterol concentrations (both correlations $r = 0.6$).

In the same set of fresh samples we could not demonstrate any significant CETP activity or mass as measured by the fluorescence activity assay and ELISA (sensitivity 25 ng/ml).

Cholesterol efflux from astrocytes

The results of the cholesterol efflux experiments are given in Fig. 6. Primary rat astrocyte cultures were prepared, labeled with cholesterol, and subjected to a 7-h incubation with human CSF from various sources. All samples

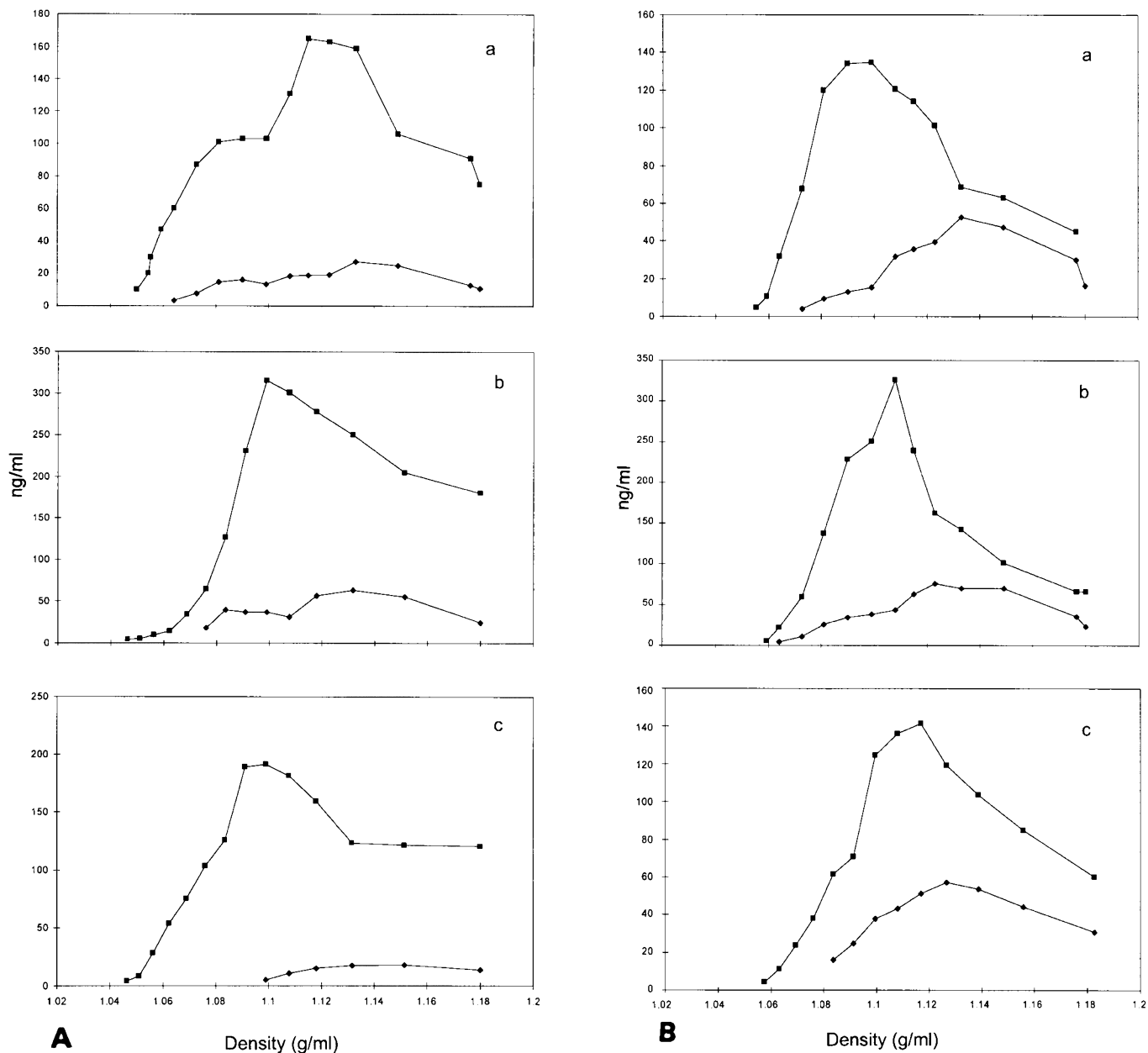


Fig. 4. Gradient density ultracentrifugation profiles of CSF samples from (A) three non demented controls and (B) three AD patients. Apoe (■) and apoA-I (◆) (ng/ml) measured in each fraction as described.

were adjusted to a final apolipoprotein concentration in the incubation medium of $\pm 10 \mu\text{g/ml}$ (sum of apoA-I and apoE concentrations, see Fig. 6B). The efflux induced by CSF from three AD patients, three patients with other types of dementia (DEM), and three control samples are compared (Fig. 6A). In separate wells the efflux induced by $5 \mu\text{g/ml}$ purified apoA-I or apoE was determined. The data summarized in Fig. 6A show that all CSF samples induced a significant cholesterol efflux to the medium, which was significantly lower ($P < 0.05$, Student's *t*-test) for the CSF samples collected in the patients suffering from other types of dementia as compared to the control group. Of note, the efflux induced by $5 \mu\text{g/ml}$ apoA-I is slightly lower than for $5 \mu\text{g/ml}$ apoE, while the total percentage of efflux induced by the CSF lipoproteins (in-

cubated at concentrations of $10 \mu\text{g/ml}$) is comparable to the efflux induced by lipid-free apolipoproteins. The data given in Fig. 6B demonstrate that the amount of apolipoprotein measured in the CSF is directly and strongly correlated ($r = 0.95$) with the percentage of cholesterol efflux induced. The percentage efflux correlated less strongly with the CSF apoE concentration ($r = 0.82$) than with the CSF apoA-I concentrations ($r = 0.93$).

DISCUSSION

This study on the heterogeneity and function of lipoproteins and lipoprotein-associated enzymes in human cerebrospinal fluids revealed some interesting new observations.

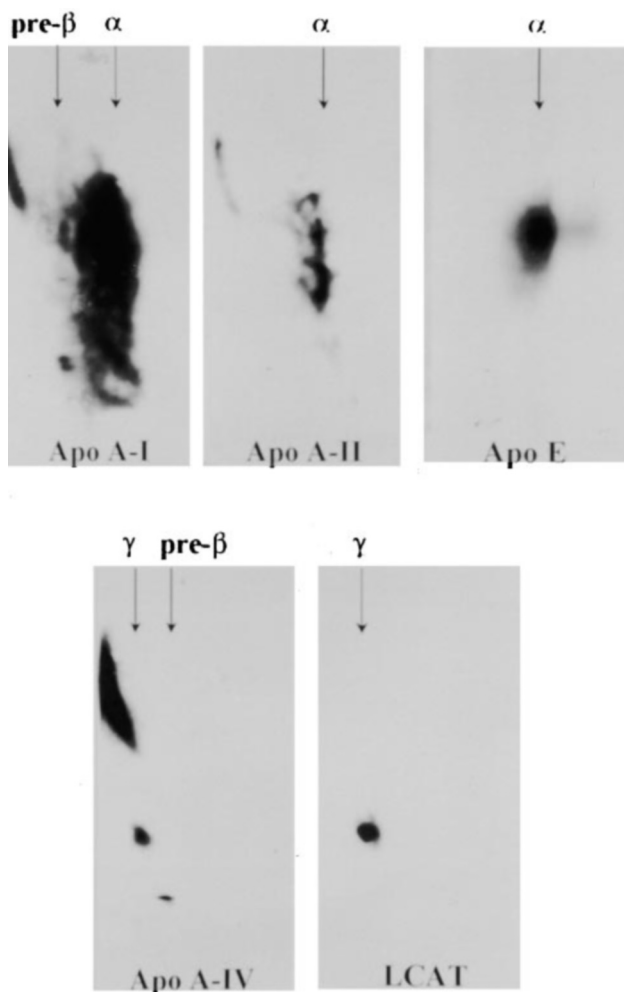


Fig. 5. Bi-dimensional electrophoresis and immunoblotting for apoA-I, apoA-II, apoE, apoA-IV, or LCAT of a human CSF sample. The relative positions of the pre β , γ , and α positions are indicated by arrows.

ApoE4 allele frequency in the study population

In spite of the small size of the patient and control groups, the apoE phenotype distribution confirmed the increased apoE4 allele frequency in the group of Alzheimer patients, compared to our non-demented controls, and to patients diagnosed with other types of dementia, as previously reported (34–36). As Csaszar et al. (37) had suggested that an A-IV 2 phenotype might constitute another susceptibility factor for the development of AD, the apoA-IV phenotypes were determined in a subset of our study population (data not shown). However, all patients and controls expressed the same apoA-IV 1 phenotype; therefore the small size of our study population was not informative in this respect.

Apolipoproteins, lipid transfer proteins, and LCAT identified by Western blotting

The presence of apoE, apoA-I, apoA-II, and apoA-IV in human CSF was initially confirmed by immunoblotting.

ApoE appears both as homo- and heterodimers with apoA-II, while apoA-II dimers were also identified, con-

TABLE 3. Percent (mean \pm SD) of cholesteryl esters and distribution of cholesteryl esters in the CSF obtained from normal controls ($n = 9$), patients with Alzheimer's disease ($n = 2$), and patients affected with other types of dementia ($n = 3$)

Diagnosis	% Distribution of Cholesteryl Esters				
	% CE ^a	16:0	18:1	18:2	20:4
CON	70 \pm 15	29	36	31	4
AD	71 \pm 3	29	41	27	3
DEM	60 \pm 6	14	40	46	1
Plasma	75	13	22	58	6

^a Percent of total cholesterol esterified. 16:0, cholesteryl palmitate; 18:1, cholesteryl oleate; 18:2, cholesteryl linoleate; 20:4, cholesteryl arachidonate.

firming the findings of Montine et al. (38). The presence of apoA-II in the brain was previously questioned by others. Montine et al. (38) also describe hetero- (apoA-II:E) and homodimers in apoE3 carriers, while heterodimers were not observed in E4 homozygotes, lacking free cysteines. ApoA-II in the brain is plasma derived, while apoE is synthesized locally; heterodimerization must therefore occur in the brain compartment. It is not known whether apoE heterodimerization affects its functions in the brain.

Enzymes and transfer proteins involved in HDL remodeling were identified in the CSF by immunoblotting; although strong signals were observed for LCAT and PLTP in human CSF, no CETP signal could be found in human CSF (either from controls or demented patients) even after sensitive detection with a chemiluminescent substrate or by ELISA. These results are in contrast with the findings of Albers et al. (39) who identified significant amounts of CETP in human CSF. In the sera of the same patients, normal CETP levels were measured that were not significantly different between patients and controls ($2.5 \pm 1.2 \mu\text{g/ml}$ for normal controls and $2.6 \pm 1.0 \mu\text{g/ml}$ for AD patients, $P = 0.2$).

Concentration of apolipoproteins and lipids in human CSF

Apolipoprotein and cholesterol measurements in CSF from patients and controls show that apoE is the most abundant apolipoprotein in the CSF; the apoE and cholesterol levels reported here agree with those reported by other groups (16, 40–45). Interestingly, apoE CSF con-

TABLE 4. Mean (\pm SD) PLTP and LCAT concentrations, activities, and cholesterol (CHOL) and phospholipid (PL) concentrations measured in human CSF ($n = 8$)

	Concentration	Activity ^a
	$\mu\text{g/ml}$	nmol/ml/h
PLTP	0.04 \pm 0.02	3.30 \pm 2.21
LCAT	0.10 \pm 0.05 ^b	0.01 \pm 0.002
CHOL	3.13 \pm 1.33	
PL	3.54 \pm 1.33	

^a PLTP activity = nmol PL/ml/h and LCAT activity = nmol cholesteryl ester/ml/h.

^b LCAT mass was estimated by comparing the activity of the samples with that of the reference LCAT preparation.

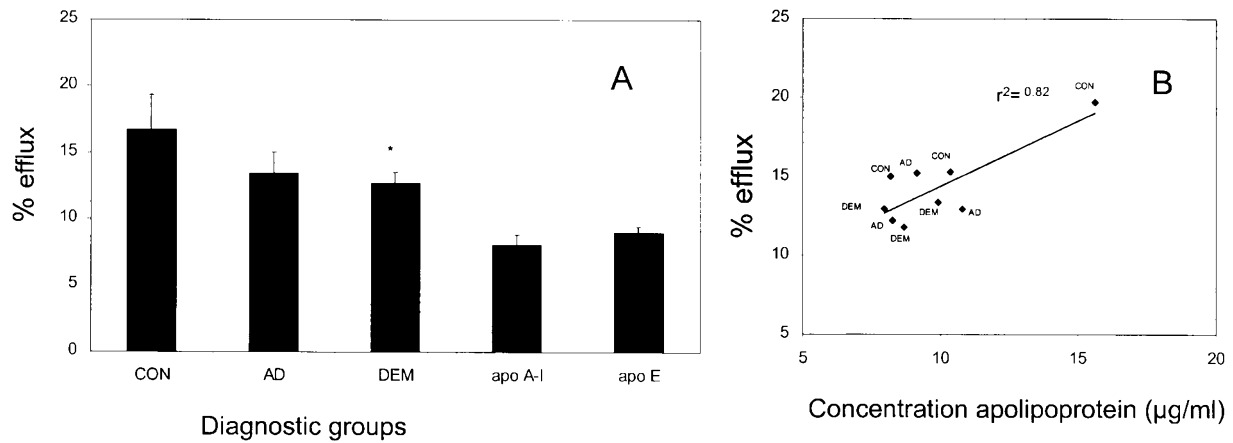


Fig. 6. Cholesterol efflux from rat astrocytes by human CSF lipoproteins or purified apolipoproteins. A: % efflux induced by CSF collected in patients from different diagnostic groups (3 samples/group, measured in triplicate) compared with purified apoA-I or apoE incubated at a concentration of 5 $\mu\text{g/ml}$ in the medium, where *: significantly different from controls $P < 0.05$, Student's t -test. B: Correlation between % efflux and concentration of apolipoproteins ($\mu\text{g/ml}$) present in the human CSF.

centrations were slightly lower (but not statistically significant) in AD patients versus controls; however the CSF samples obtained from patients suffering from other types of dementia showed significantly increased apoE concentrations in their CSF. While most authors describe reduced apoE concentrations in CSF from AD patients (40–45), others failed to observe these differences.

Cholesterol levels measured in our AD patients were decreased compared to controls and other diagnostic groups, in agreement with the report by Mulder et al. (16). Defects in the blood–brain barrier filtration seem to increase CSF cholesterol concentrations significantly.

ApoA-I levels in CSF amount up to one-third of the apoE concentrations, suggesting that there is little or no synthesis of apoA-I in the brain. The mechanism through which apoA-I crosses the blood–brain barrier is not known. De Vries et al. (46) suggested that apoA-I might be transported by cerebral endothelial cells. In our population, however, the “leaky” blood–brain barriers allow a significant influx of apoA-IV-containing particles, while apoA-I levels are only moderately increased. Others suggest that apoA-I synthesis is present in the brain, and reported mRNA expression both for apoE and apoA-I in the brain (47), which is corroborated by in situ hybridization and direct immunostaining of neurons (48). These authors observed apoA-I immunostaining in some senile plaques, confirming the in vitro findings that the amyloid β can associate with apoA-I (47). Our apolipoprotein measurements agree with the reports by Harr et al. (48) who estimated by Western blotting that the amount of apoE in CSF is 3-fold higher than that of apoA-I. However, the increase in apoA-I levels in our AD patients, compared to controls, was not observed in the previous study. Montine et al. (38) suggested that the apoE/apoA-I ratio is identical in patients and controls, while we observed a decreased apoE/apoA-I ratio (due to lower apoE and higher apoA-I values) in our AD patients compared to controls. As will be discussed later, these findings were confirmed by careful analysis of the

apolipoprotein and lipoprotein distribution in the CSF of controls and AD patients.

Mean apo-IV concentrations in human CSF samples were comparable to the apoA-I and apoE levels, although there was a large variation in the population tested. This is in contrast with the mean concentration and range observed for plasma apoA-IV ($12.8 \pm 5.7 \text{ mg/dl}$) (49, 50). Both Harr et al. (48) and Koudinov et al. (51) reported the presence of apoA-IV in CSF by immunoblotting, which is associated with HDL particles. The specific role of apoA-IV in the brain remains obscure. As described above, the apoA-IV 2 phenotype has been associated with increased susceptibility for the development of AD in patients, while significant differences between carefully age-matched controls and AD patients were not confirmed by others (52). On the other hand, apoA-IV 2 phenotype is considered as a longevity factor. ApoA-IV has been detected in rat astrocytes (53), but it is still unclear whether apoA-IV is synthesized locally or whether it is derived from plasma. After fat feeding, plasma apoA-IV is associated with both chylomicrons and HDL lipoproteins, while it is also present in a lipoprotein-free form in the circulation. The exact function of apoA-IV in lipid and lipoprotein metabolism has not yet been elucidated. ApoA-IV was proposed to be involved in the reverse cholesterol transport in the circulation as it constitutes one of the best LCAT activators, and as apoA-IV lipid complexes induce significant efflux from cholesterol-loaded cells (54). Animal studies show increased apoA-IV serum and brain concentrations induced by fat feeding. Direct injections of apoA-IV into the third ventricle inhibited food uptake, suggesting that apoA-IV acts centrally as a neuromodulator to inhibit gastric secretion (55). The variation in apoA-IV levels in human CSF could not be related to individual nutritional status, as no systematic record of food uptake was available. However, the large variation observed for the apoA-IV concentrations in human CSF are puzzling and still unaccounted for.

Isolation of lipoproteins from human CSF by density gradient ultracentrifugation and identification of pre- β migrating particles by bi-dimensional electrophoresis and blotting

Direct analysis of the CSF lipoproteins obtained in nondemented controls and AD patients showed significant differences. Our lipoprotein profiles confirm the observation that apoE is mainly associated with larger HDL₂-like particles of lower density, while ApoA-I and apoA-II are mainly associated with smaller HDL₃-like particles of higher density, but are also found in the HDL₂ density range. These data are confirmatory of the apoE distribution obtained by immunoblotting reported in other studies (48, 51). However, we observed differences in the amounts of apoA-I-containing lipoproteins in CSF between AD patients compared to controls. The specific function of this distinct apoA-I-enriched lipoprotein fraction remains to be investigated.

In contrast to plasma, apoA-IV was not associated with lipoproteins but rather appeared as a lipid-free protein. While apoA-I is associated with both small and larger α -migrating particles, apoA-II and apoE are restricted to particles with a more homogeneous size, as observed in the lipoprotein profiles. However in some patients, especially those who suffered from a cerebrovascular accident, we identified a pre- β migrating apoA-I fraction, while such precursor type particles were absent from others, maybe due to a very rapid turnover or limited stability of these particles. The bi-dimensional immunoblots for apoA-IV and LCAT confirm the gel filtration results, showing that apoA-IV and LCAT in CSF belong to lipid-poor γ -migrating lipoprotein particles. ApoA-IV is not associated with mature HDL particles in CSF, but is identified only in pre- β and γ -migrating particles.

One can speculate on the role and function of these different lipoproteins in CSF and especially whether they are actively involved in the regulation of cholesterol redistribution among the cells of the brain. Pitas et al. (4) have estimated that there is a flux of cholesterol from the brain into the cerebrospinal fluid of 1–2 mg/day. Others have reported that the conversion of cholesterol into 24(S)-hydroxycholesterol significantly increases the transfer of this oxysterol through the blood–brain barrier and estimated that 4–6 mg/day of cholesterol are cleared from the brain by this mechanism (56). The lipoprotein-mediated clearance of cholesterol is thus likely to be less important. Brain cholesterol is efficiently protected from any exchange with circulating lipoproteins by the blood–brain barrier. Recent reports suggest that the majority of brain cholesterol is synthesized in situ and that synthesis and remodeling of lipoproteins probably takes place in this compartment.

Lipid transfer proteins and LCAT in human CSF

Several plasma proteins contribute to HDL interconversion, including both lipoprotein and hepatic lipases, LCAT, CETP, and PLTP.

In this paper we demonstrated that significant amounts of enzymatically active LCAT and PLTP are present in the CSF. LCAT gene expression was detected in the brain by

Warden et al. (57) and the physiological role of LCAT *in vivo* was illustrated in patients with LCAT deficiency who accumulated cholesterol in peripheral tissues and developed premature atherosclerosis and central nervous system impairments. Secretion of LCAT by brain neuroglial cell lines (58) was demonstrated; moreover, these authors show that apoA-I can activate the brain LCAT. The activity and LCAT mass reported here are comparable to those observed by Albers, Marcovina, and Christenson (33). Careful analysis of a second set of samples demonstrates that LCAT activity in CSF from AD patients is significantly lower than in normal controls. Others have reported that cholesterol esterification is impaired (32, 33) in AD patients and in patients with Down's syndrome; these authors reported a 20% decrease in LCAT activity where we report a 50% decrease in activity. ApoA-I and apoA-IV are the best activators of LCAT in plasma (24). In the present study we observed that apoA-IV and LCAT co-migrate on the same HDL-precursor type in the CSF, thus suggesting that apoA-IV might be a physiologically important LCAT activator in the brain. However, apoA-I or apoE probably can activate LCAT in the brain as well. In CSF the amyloid β peptide was found associated with the HDL particles, and shown to inhibit LCAT in a dose-dependent way (49), which might explain the lower LCAT activity in CSF from AD patients.


The presence of CETP activity in CSF was reported by Albers et al. (39) who reported an activity around 12% of that found in plasma, suggesting a local synthesis of this protein in the brain. However, we were unable to demonstrate any significant CETP activity or mass in human CSF from normal controls and AD patients. In plasma, CETP catalyzes the hetero-exchange of cholesteryl esters and triglycerides between HDL and apoB-containing lipoproteins and, in conjunction with hepatic lipase, the CETP is involved in HDL remodeling (59, 60). As the human CSF does not contain any apoB or triglyceride-rich particles, the absence of this transfer protein is not surprising. CETP mRNA was detected in several tissues in humans, including spleen, liver, small intestine, adrenal glands, and adipose tissue, but not in the brain (60, 61).

The presence of PLTP in the brain had not yet been demonstrated. PLTP plays an important role in phospholipid transfer among lipoproteins, as it transfers phospholipid from VLDL to LDL and HDL, and it is also involved in HDL interconversion. High levels of PLTP mRNA were detected in mouse brain (60) whereas the PLTP mRNA signal in human brain is low. In human CSF we detected PLTP mass, with an average activity and mass 1% of that of plasma.

Cholesterol efflux from rat astrocytes induced by human CSF lipoproteins

Our cholesterol efflux studies on primary rat astrocytes revealed that CSF lipoproteins are capable of inducing a significant cholesterol efflux, suggesting that CSF lipoproteins are actively involved in the redistribution of lipids in the brain. Studies by Ito et al. (19) have shown that exogenously added apoA-I can induce a cholesterol efflux to a lipid-poor particle, where endogenously synthesized apoE produces

cholesterol-rich HDL. Our data show that after a 7-h incubation, the cholesterol efflux induced by purified apoA-I or apoE is comparable to that induced by a comparable amount of apolipoproteins presented as CSF lipoproteins. We show that the apolipoprotein concentration in the CSF is highly correlated with efflux, and that this correlation is stronger with the apoA-I than with the apoE concentration. These data corroborate the findings of Ito et al. (19), that apoA-I and apoE have different functions in the process of cholesterol efflux. Moreover, the identification of pre- β -like apoA-I particles, but not of pre- β -like apoE-containing particles in human CSF, suggests that apoA-I particles in the brain may have specific functions. In these experiments we did not correlate efflux with apoA-IV concentrations in the samples, which means that we cannot rule out the possible effects of apoA-IV-mediated efflux. The finding that AD patients display a more pronounced apoA-I-enriched fraction in their lipoprotein profile suggests that this particular subclass may have important functions in the lipoprotein-mediated lipid transport in the brain.

In summary, we describe the complex lipoprotein and apolipoprotein distribution in human CSF. The most abundant apoE-containing lipoproteins are of larger size and lower density, while smaller apoA-I-containing particles of higher density are present at low concentrations in nondemented patients, as compared to AD patients. We demonstrated, moreover, the presence of apoA-I and apoA-IV-containing pre- β -migrating particles in CSF. Significant levels of LCAT and PLTP mass and activity were identified in CSF, suggesting that in the brain lipoproteins are actively involved in the cholesterol metabolism. Interestingly, the LCAT activity in CSF from AD patients is markedly reduced. Moreover, the lipoproteins present in human CSF are capable of inducing a significant cholesterol efflux from rat astrocytes, demonstrating that human CSF lipoproteins are actively involved in the cholesterol redistribution in the brain. 

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