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Lipids and Lipoproteins

Apolipoprotein E in Cerebrospinal Fluid: Relation to Phenotype and Plasma Apolipoprotein E Concentrations

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Background: **Apolipoprotein (apo) E may be related to the development of Alzheimer disease, but data on apoE in cerebrospinal fluid (CSF) are limited. The aim of the present study was to measure apoE in CSF and relate its concentrations to apoE phenotype and CSF lipids.**

Methods: **We adapted an assay for CSF apoE sensitivity using an ELISA. It allowed us to measure CSF apoE with sufficient reproducibility and precision.**

Results: **The within- and between-run CVs were <7%, and the detection limit was 0.025 mg/L. No cross-reaction was found for other apolipoproteins. No significant differences related to sex or apoE phenotype were observed in the CSF apoE concentration. The mean CSF apoE concentration was significantly higher in the 0–5 year group (n = 6; 18.47 ± 1.14 mg/L, mean ± SD) than in the >5 year group (n = 34; 8.82 ± 3.31 mg/L). The mean concentrations of total cholesterol (TC) and phospholipid (PL) in CSF were 2.68 ± 2.16 and 6.50 ± 2.84 mg/L (n = 52), respectively. Although no significant differences in TC or PL in the CSF were found with respect to sex or age, the concentrations in subjects with the apoE phenotype E4/E3 were significantly lower than in those with E3/E3 and E3/E2. The concentrations of apoE, TC, and PL in CSF did not correlate with those in plasma. The time-related fluctuations in CSF apoE were independent of those in total protein and IgG. CSF apoE was significantly correlated with TC and PL concentrations in the CSF, but not with the number of cells in the CSF.**

Conclusions: **These findings support the idea that apoE and lipids are unable to cross the blood-brain barrier and that their concentrations in CSF may reflect production in central nervous tissue.**

Apolipoprotein (apo)[4] E, a 35-kDa plasma protein synthesized mainly in the liver, is involved in cholesterol transport and metabolism *(1, 2)*. apoE is also expressed in other tissues, notably the brain, the second most prolific tissue in terms of apoE production *(3, 4)*. Human apoE has three major isoforms, E2(Cys112, Cys158), E3(Cys112, Arg158), and E4(Arg112, Arg158), and these are produced by three independent alleles at a single genetic locus *(5, 6)*. Thus, three homozygous (E2/E2, E3/E3, and E4/E4) and three heterozygous (E2/E3, E3/E4, and E2/E4) phenotypes are represented.

A subject's apoE phenotype is known to affect the plasma total cholesterol (TC) concentration as follows: E2/E2 < E3/E2 < E3/E3 < E4/E3 < E4/E4 (except in type III hyperlipidemia) *(7)*. apoE reportedly exists in the cerebrospinal fluid (CSF) as a component of HDL subfraction 1 *(8, 9)* and transports cholesterol and phospholipid (PL) through the LDL receptors expressed in elements of the nervous system such as neurons and glia *(9–12)*. Over the past 10 or so years, several groups have measured CSF apoE to investigate its clinical relevance to neurological diseases such as multiple sclerosis *(13–15)* and Alzheimer disease (AD) *(16, 17)*. apoE expressed in the brain is also thought to contribute to the growth and repair of the nervous system *(18–20)*. However, information relating to CSF apoE, such as data on the influence of apoE phenotype on apoE and lipid concentrations in the CSF or

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[4] Nonstandard abbreviations: apo, apolipoprotein; TC, total cholesterol; CSF, cerebrospinal fluid; PL, phospholipid; AD, Alzheimer disease; PBS, phosphate-buffered saline; and CNS, central nervous system.

on the relationship between CSF apoE and lipid concentrations, has not been reported in as much detail as that relating to plasma apoE.

The concentration of apoE in the CSF is approximately one-tenth of that in the plasma *(15)*; therefore, the assay method for CSF apoE requires greater sensitivity. We devised an improved assay for apoE by adapting an ELISA method originally developed by Bury et al. *(21)*, which enabled us to use CSF without high multiple predilution. In addition, the TC and PL concentrations in the CSF were also examined, and the composition of the lipoprotein content of the CSF was assessed.

Materials and Methods

PROTEINS AND ANTIBODIES

Purified apos (apoAI, apoAII, apoB, apoCII, apoCIII, and apoE), anti-apoE polyclonal antibody (rabbit), anti-apoE monoclonal antibody, and horseradish peroxidase-conjugated anti-mouse IgG (goat) were purchased from Chemicon International Inc., Dako, Progen Biotechnik, and MBL Co., respectively.

SUBJECTS

One hundred sixteen CSF samples with no contamination by erythrocytes were obtained from subjects 0–82 years of age (mean ± SD, 45.1 ± 22.3 years). In 40 of these subjects, serum samples were obtained in pairs with the CSF samples on the same day. These samples were stored at −80 °C before use. All subjects gave their informed consent before participation. One hundred ten of these CSF samples were obtained from subjects >5 years of age; these were used to compare apoE concentrations between groups with (n = 76) or without (n = 34) neurological disease. TC and PL were measured in the 52 of the 116 CSF samples that were of sufficient volume.

ELISA PROCEDURE

Sample preparation. All samples were diluted with an equal volume of phosphate-buffered saline (PBS), pH 7.4, containing 1.0 g/L Tween 20 (PBS-Tween 20).

Calibrators. Calibrators were prepared by dilution of reference serum (purchased from Daiichi Pure Chemicals Co. Ltd.) to 0, 0.05, 0.10, 0.50, 5.0, and 25.0 mg/L using PBS-Tween 20.

Assay procedure. Polystyrene microtiter plates (Nunc TM) were coated with anti-apoE polyclonal antibody (rabbit; Dako) in 0.1 mol/L Na_2CO_3, pH 9.6 (1.2 mg protein/L) and incubated at 4 °C overnight. Plates were washed five times with PBS-Tween 20 after each of the subsequent incubation steps. Unoccupied sites were blocked with 10 g/L skim milk in PBS-Tween 20 for 2 h at room temperature. The calibrators and samples were then added at 100 μL/well and incubated for 2 h at 25 °C. Anti-apoE monoclonal antibody in PBS (0.25 mg protein/L) was added at 100 μL/well and incubated for 1 h at 37 °C. Peroxidase-conjugated anti-mouse IgG (goat; MBL), diluted 3000-fold with PBS, was then added at 100 μL/well and incubated for 1 h at 37 °C. After the final washing, the color reaction was developed with 100 μL/well of 5 g/L *t*-methylbenzidine dihydrochloride and hydrogen peroxide, followed by 100 μL/well of 0.4 mol/L sulfuric acid to stop the reaction. The absorbance at 450 nm was measured by a Behring ELISA processor II (Behringwerke). A calibration curve was generated using a semilogarithmic scale, and the apoE concentration in the CSF was calculated from the curve. Each assay was carried out in triplicate.

ISOELECTRIC FOCUSING

Sample preparation. CSF (15 μL) was incubated with 5 μL of neuraminidase (20 kU/L; Nakarai Chemical) in 0.1 mol/L citric acid buffer containing 10 g/L Tween 20 for 12 h at 37 °C, and then treated with 5 μL of 75 mmol/L dithiothreitol (Wako Pure Chemicals) in 10 g/L Tween 20 for 1 h at room temperature.

Electrophoresis and immunoblotting. Immunoblotting was carried out as described previously *(22)*. Briefly, 20 μL of the prepared sample was electrophoresed on a 4.8% polyacrylamide gel containing 8 mol/L urea and 20 mL/L ampholine (pH 4~6) using 3.3 mmol/L phosphoric acid as the anode buffer and 20 mmol/L NaOH as the cathode buffer. Electrophoresis was performed overnight at 4 °C under constant voltage (200 V). The separated proteins were electrophoretically transferred onto nitrocellulose membranes, which were then incubated with 50 mmol/L Tris-HCl, pH 8.0, containing 20 g/L skim milk (blocking buffer) for 30 min at room temperature. The membranes were then incubated with anti-apoE polyclonal antibody in blocking buffer for 1 h at room temperature. After washing, the membranes were incubated with peroxidase conjugated anti-rabbit IgG for 1 h at room temperature. The bands representing the various apoE isoforms were developed using 3,3′-diaminobenzidine tetrahydrochloride (Dojin Chemical Co.) and hydrogen peroxide (Wako).

OTHER ASSAYS

Serum apoE. The serum apoE concentration was determined by turbidimetric immunoassay using a commercially available kit (ApoE Auto N DAIICHI; Daiichi) and a Hitachi 7170 automated analyzer.

Total protein in CSF. The total protein concentration in CSF was determined by the Pyrogallol red method (Wako) using a Hitachi 7170 automated analyzer.

TC and PL in CSF. The TC and PL concentrations in CSF were determined, respectively, by the cholesterol-oxidase method (Kyowa Medex Co. Ltd.) and the choline-oxidase method (Wako) using a Hitachi 7170 automated analyzer. The assay conditions were modified by increasing the

ratio between sample volume and reagent volume to 15-fold higher than the ratio used for serum.

IgG in CSF. IgG in CSF was determined by laser nephelometric immunoassay using a Behring Nephelometer-Analyzer (Behringwerke).

STATISTICAL METHODS

The CSF apoE value for each phenotype was expressed as mean and SD, as calculated using Microsoft Excel Ver. 5.0® (Microsoft). apoE values obtained by this method in diluted patient sera and by turbidimetric immunoassay were compared by linear regression analysis. The relationships among CSF apoE, serum apoE, CSF lipid, and CSF total protein were determined from the correlation coefficient obtained by linear regression analysis. The statistical difference between any two groups was assessed by the Student or Welche *t* test, $P < 0.05$ being considered significant.

Results

ASSAY CHARACTERISTICS

Calibration curve. A typical calibration curve for apoE obtained using the new assay is shown in Fig. 1. The absorbance of the zero calibrator (PBS-Tween 20), as measured on five different plates as nonspecific binding, was 9.4 ± 1.4 ($\times 10^{-3}$, mean ± SD). The minimum detection limit, calculated from the mean absorbance of the zero calibrator plus 2 SD (based on 10 replicate measurements of PBS-Tween 20), was 0.025 mg/L. The upper limit of the assay, as determined by a series of dilutions, was 25 mg/L.

Precision. The within-run reproducibility was determined by making 20 replicate measurements of three diluted serum samples on the same plate (CV, 2.4–7.0%). The between-run reproducibility was determined by making triplicate measurements of two diluted serum samples on each of 10 consecutive days (CV, 5.7–9.0%; Table 1).

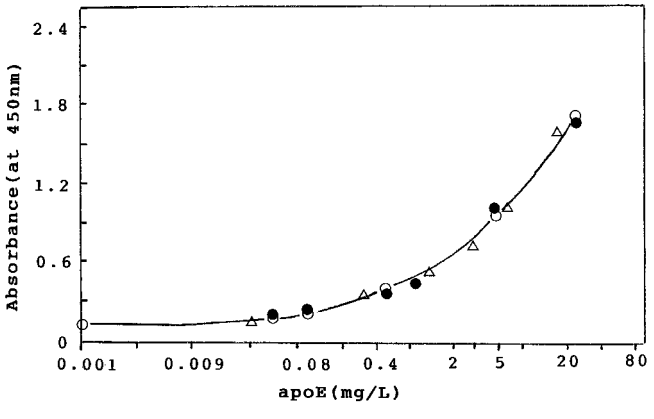

Fig. 1. Typical calibration curve for apoE in CSF.
○, reference serum; ●, purified apoE; △, patient CSF.

Table 1. Within- and between-run reproducibility.

	Sample[a]	Mean[b] (SD), mg/L	CV, %
Within-run	L	0.10 (3.50×10^{-3})	3.5
(n = 20)	M	2.05 (4.92×10^{-2})	2.4
	H	25.25 (1.769)	7.0
Between-run	M	2.01 (0.115)	5.7
(n = 10)	H	23.83 (2.145)	9.0

[a] Samples were prepared using pooled serum diluted with PBS-Tween 20 and were stored at −80 °C prior to use. *L*, *M*, and *H* indicate low, middle, and high concentrations of apoE, respectively.
[b] Mean of triplicate measurements.

Accuracy. The accuracy of this assay was confirmed by analytical recovery studies using purified human apoE. The recovery rates from samples to which 0.50 and 1.45 mg/L of apoE had been added were 84.0% and 89.0%, respectively.

Lot-to-lot variability. To assess the lot-to-lot variability, we analyzed 32 CSF samples twice using two different plates coated with anti-apoE polyclonal antibody on different occasions. The mean apoE values were 10.9 ± 5.6 and 10.3 ± 5.4 mg/L (mean ± SD), respectively. The linear regression equation was: $y = 0.960x - 0.133$; $r = 0.998$; $S_{y|x} = 0.3285$.

Specificity. Assay specificity was estimated by measuring the cross-reactivity with other apos. The absorbance values measured for apoAI, apoAII, and apoB at a maximum concentration of 500 mg/L, and for apoCII and apoCIII at a maximum concentration of 100 mg/L in place of apoE were roughly similar to that obtained for the zero calibrator (data not shown).

Intermethod comparison. We examined the correlation between the apoE values obtained using the present assay (y) and those obtained using a turbidimetric immunoassay (x) using diluted and undiluted serum samples, respectively. The mean values for apoE obtained using the present assay and the turbidimetric immunoassay were 51.6 ± 20.2 and 53.4 ± 23.7 mg/L (mean ± SD), respectively. The linear regression equation was: $y = 0.768x + 10.521$; $r = 0.903$.

CLINICAL OBSERVATIONS

apoE concentration. The age-related distribution of CSF apoE for the 41 of 116 subjects who had no neurological disease is shown in Fig. 2. The concentration in the 0–5 year age group (n = 6; age, 3.1 ± 2.0 years) was significantly higher (mean concentration, 18.47 ± 1.14 mg/L) than that in the group >5 years of age (n = 34; 8.82 ± 3.31 mg/L). No significant difference in CSF apoE concentration was observed between males and females or among the three major apoE phenotypes; however, a significant difference was observed between the controls and those with a neurological disease (Table 2) *(23)*.

Table 2. apoE concentrations in CSF, in mg/L.

	Neurological disease			Control[a]		
	n	Mean	SD	n	Mean	SD
Total	76	10.85[b]	6.37	34	8.82	3.31
Male	43	11.26	7.13	18	9.26	3.44
Female	33	10.25	5.70	16	8.37	3.21
apoE3/E2	10	11.75	4.33	2	6.30	2.83
apoE3/E3	51	9.88	6.17	25	9.27	3.61
apoE4/E3	14	11.99	7.29	6	7.80	2.49
apoE5/E3[c]	1	20.90		1	9.40	

[a] Control subjects were patients >5 years of age without neurological disease.
[b] $P < 0.05$ vs control.
[c] From Tajima et al. (23).

Lipid concentration in CSF. The within- and between-run CVs for the TC and PL assays using diluted serum (mean TC, 1.56 mg/L; mean PL, 1.99 mg/L) were <6.3% and 9.7%, respectively. The minimum detection limits for TC and PL, calculated from the mean values for the zero calibrator plus 2 SD (based on 10 replicate measurements of PBS), were 0.10 and 0.08 mg/L, respectively. The mean concentrations (with SD) of TC and PL are shown in Table 3. Significantly higher concentrations of both lipids were found in phenotype E3/E2 (or E3/E3) than in E4/E3. However, age, sex, and the presence of neurological disease had no effect on these concentrations (data not shown).

Relationship between CSF apoE and other components. As shown in Fig. 3, the apoE, TC, and PL concentrations in the CSF showed no correlation with those in the serum (n = 40; apoE, $r = 0.100$; TC, $r = 0.221$; PL, $r = 0.161$). However, the lipid concentrations in the CSF correlated significantly with the CSF apoE (n = 40; vs TC, $r = 0.604$, $P < 0.001$; vs PL, $r = 0.702$, $P < 0.001$; Fig. 4). The relationships between apoE and other major components (total protein, IgG, and number of cells in the CSF) were investigated by linear regression analysis. CSF apoE showed extremely weak correlations with total protein (n = 112; $r = 0.288$; $P < 0.01$) and IgG (n = 95; $r = 0.273$; $P < 0.01$), but no correlation with the number of cells (n = 95; $r = 0.028$). The time-related fluctuations in apoE, total protein, and IgG concentrations in the CSF of three different patients with neurological disease are shown in Fig. 5 . The changes in these values seemed to be quite independent of each other in all three cases. In each case, apoE varied more than total protein.

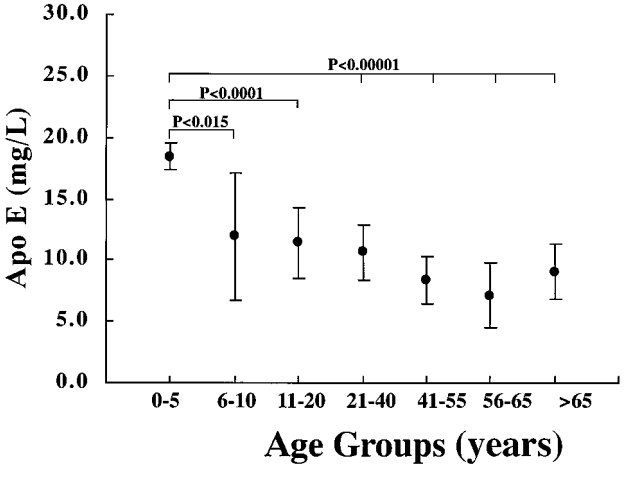

Fig. 2. CSF apoE concentrations in different age groups.

Number of subjects in each age group: six in the *0–5 group*; five in the *6–10 group*; three in the *11–20 group*; six in the *21–40 group*; seven in the *41–55 group*; seven in the *56–65 group*; and six in the *>65 group*. No patients with neurological disease were included. The values are expressed as mean ± SD (*bars*), and *P* values were calculated by comparison with the 0–5 group by Student *t*-test.

Discussion

CSF apoE plays an important role in lipid redistribution for the repair of degenerative myelin and in the regulation of cholesterol homeostasis through the LDL receptor *(9–12)*. This means that information about CSF apoE is essential to our understanding of both the metabolism of lipids in the brain and the pathology of central nervous system (CNS) disease. For routine detection of a slight change in CSF apoE concentrations, it is necessary to establish a simple assay that has sufficient accuracy and

Table 3. Effect of apoE phenotype on lipid concentrations in CSF.[a]

		apoE phenotype		
	Total	E3/E2	E3/E3	E4/E3
n	52	6	38	8
Age (±SD), years	42.0 (23.7)	43.7 (31.3)	44.1 (21.3)	41.0 (28.9)
TC, mg/L				
mean	2.71	2.98	2.78	1.34[b]
SD	2.176	2.391	1.295	0.723[c]
PL, mg/L				
mean	6.54	7.88	6.66	4.99[d]
SD	2.850	2.604	2.969	1.840[c]

[a] Patients: 20 without neurological disease (2 E3/E2, 15 E3/E3, 3 E4/E3) and 32 with neurological disease (4 E3/E2, 23 E3/E3, 5 E4/E3).
[b–d] Student *t* test: [b] $P = 0.004$ vs E3/E3; [c] $P = 0.029$ vs E3/E2; [d] $P = 0.031$ vs E3/E2.

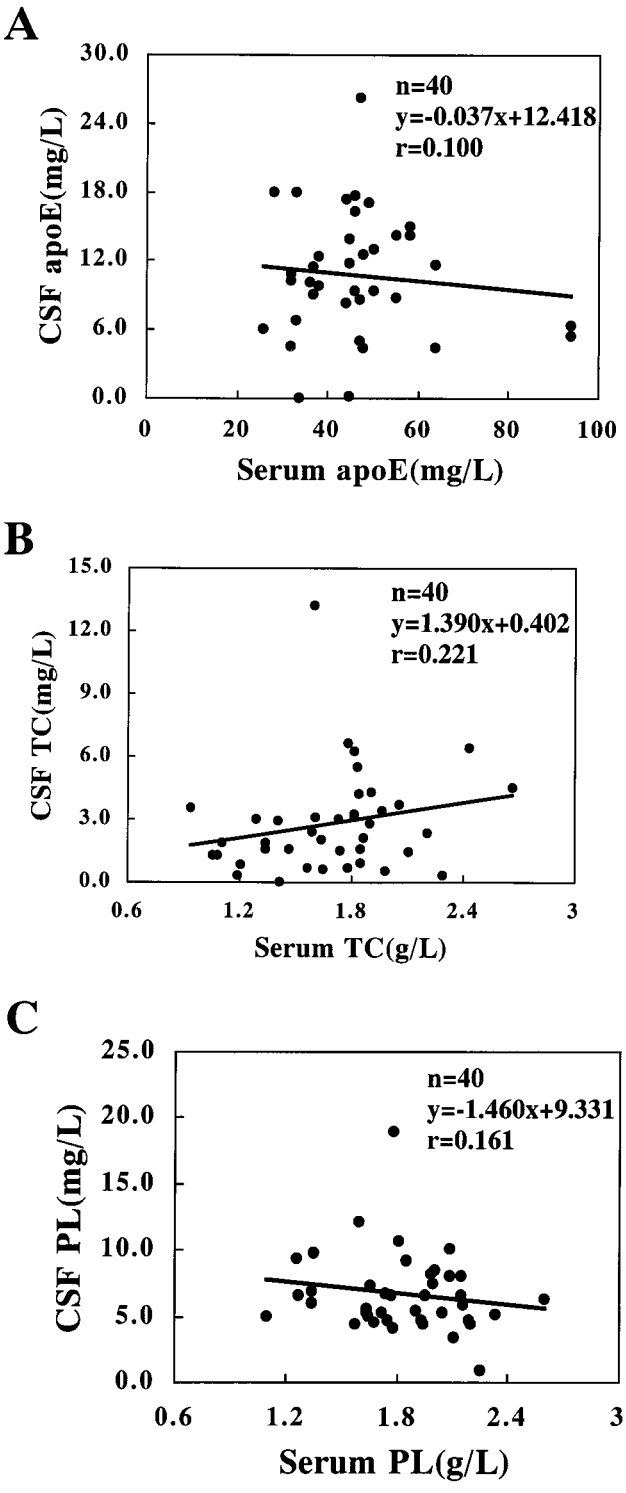

Fig. 3. Comparison between serum and CSF concentrations for apoE (*A*), TC (*B*), and PL (*C*).

CSF and serum samples were obtained on the same day from each subject. There were 40 subjects in all.

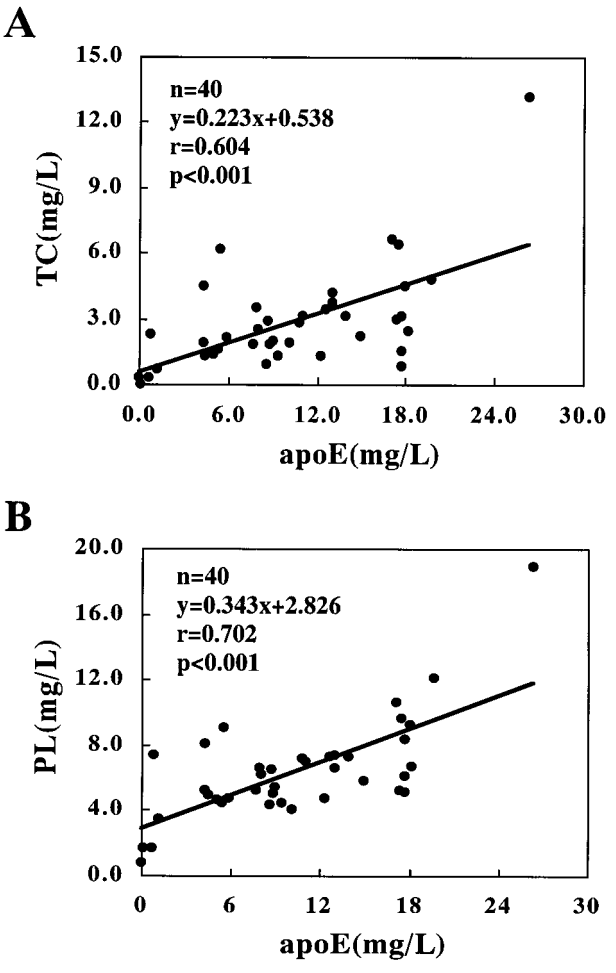

Fig. 4. Comparison between apoE concentration and TC (*A*) and PL (*B*) concentrations in CSF.

precision. Our improved assay has adequate sensitivity and a minimum detection limit similar to that of the assay for serum apoE developed by Bury et al. *(21)*. The present assay was not inferior to the method of Bury et al. in terms of precision, accuracy, or specificity, and it can be applied to CSF samples without the need for high multiple dilution, which sometimes causes measurement errors.

The reference interval calculated from the CSF apoE concentrations of the subjects without neurological disease was similar to previous data presented by Carlsson et al. *(15)*. Although the CSF apoE concentrations were not sex-related, they were significantly higher for children <5 years of age than for subjects >5 years of age. Neuronal growth, such as the extension of neurons, is remarkably active at an early age. Previous studies have also demonstrated that apoE synthesized within the CNS plays an important role in the growth, repair, and maintenance of neuronal tissues *(9–12)*. This could account for the relatively high concentration of CSF apoE seen at an early age. Indeed, in newborn infants CSF apoE concentrations would be expected to be even higher than the concentrations reported here.

The TC and PL concentrations in serum generally are known to increase with age and to be distinctly different between males and females *(24)*. However, we did not

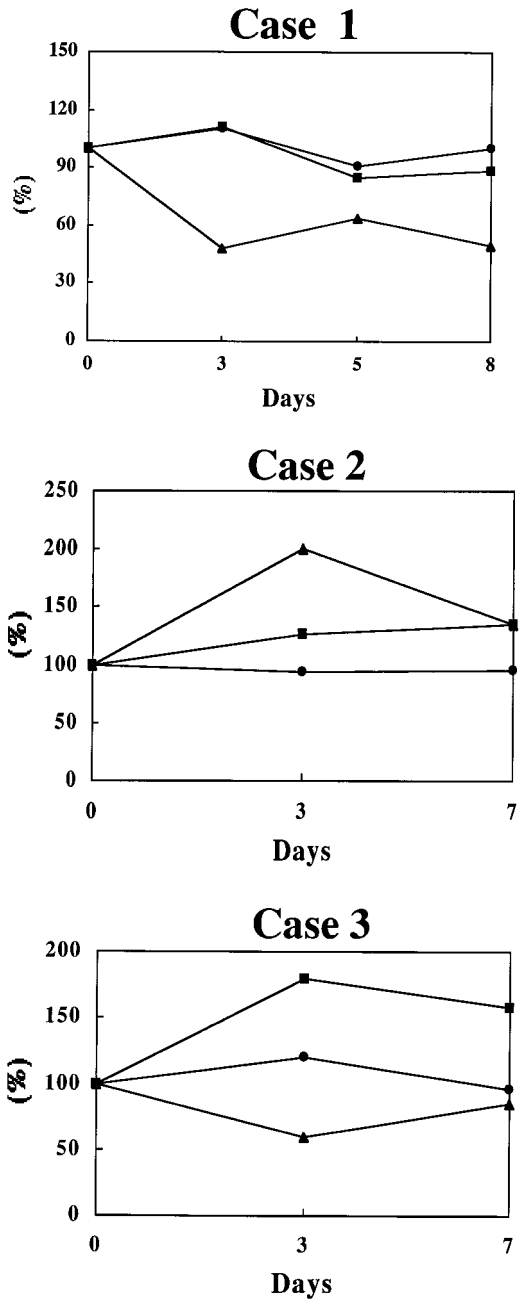

Fig. 5. Time-related changes in apoE, total protein, and IgG concentrations in CSF in three subjects.

The *x-axis* and *y-axis* indicate, respectively, the ordinal number of the assay and the concentration relative to that in the first assay. ▲, apoE; ●, total protein; ■, IgG.

observe these tendencies in the CSF. Our data indicate that the lipid concentrations in the CSF are not affected by the physiological status of the individual and that they are regulated at a relatively constant concentration in each individual. Interestingly, significant differences were recognized in both TC and PL concentrations among the three major apoE phenotypes. Serum TC concentration, except in the case of type III hyperlipidemia, is known to vary in the rank order E2/E2 < E3/E2 < E3/E3 < E4/E3 < E4/E4 *(7)*. In contrast, the CSF TC concentration was higher in E3/E2 (or E3/E3) than in E4/E3.

apoE4 is known to be a risk factor for AD, because the frequency with which the apoE-4 allele occurs in AD patients is significantly higher than in control subjects *(25–28)*. Recently, the isoform-specific binding of apoE to β-amyloid peptides, which form a major component of senile plaque and neurofibrillary tangles *(24, 29)*, has been studied *(30–32)* to elucidate the mechanism underlying the participation of apoE in AD and the differences in the effects of the various apoE isoforms on the development of AD. However, the conclusions reached in these studies are not entirely consistent with each other. It is believed that β-amyloid peptides bind to the domain near the carboxy terminus of apoE *(33)*, which is also the region with the highest potential for lipid binding *(1)*. This indicates that competition may exist between β-amyloid peptides and lipids for apoE. Our finding of a significant difference in CSF lipid concentrations between the apoE phenotypes E4/E3 and E3/E2 (or E3/E3), could be the key to the mechanism underlying the participation of apoE in the development of AD.

The mean (TC + PL)/apoE ratio in the CSF was 1.17. This supports the idea that the main lipoprotein in the CSF may consist of lipids and apoE in a ratio of ~1:1. This result agrees with previous reports that apoE exists in CSF in the form of HDL1 *(8, 9)*. Recently, apoJ and traces of apoAI and AII have been identified in CSF, in addition to apoE *(9, 34, 35)*. LaDu et al. *(34)* suggested that the CSF lipoprotein-containing apoE exists in large particles, that apoAI and apoAII are localized in smaller particles, and that apoJ is evenly distributed among all particle sizes. In addition, Guyton et al. *(36)* identified a novel large apoE-containing lipoprotein in CSF with a density of 1.006–1.060 kg/L in addition to HDL1, which has a density of 1.063–1.21 kg/L. In the present study, a significant correlation was observed between lipids and apoE, but the scatter around the regression lines was quite large. Taken together, these findings indicate that CSF lipoproteins may be microheterogeneous in size and composition and that the various particle sizes may be in a different ratio in each individual. A child <5 years of age and a subject with apoE phenotype E4/E3 would be expected to be extreme cases and would help to verify these ideas.

The apoE, TC, and PL concentrations in CSF did not correlate with those in serum. As indicated previously *(15)*, such findings suggest that the apoE and lipids in the plasma are unable to cross the blood-brain barrier. Thus, the concentrations would reflect the local production within the CNS. The lack of strong correlations between apoE and total protein, IgG, and the number of cells in the CSF also indicates an independent regulation of CSF apoE.

Rifai et al. *(13)* suggested that the "apoE Index" (calculated from the concentration of apoE and albumin in CSF and serum) is useful in discriminating between

remission and exacerbation in multiple sclerosis patients. Blennow et al. *(16)* and Lehtimäki et al. *(17)* showed a significant reduction in CSF apoE concentrations in AD patients compared with controls. These results indicate why we need a simple assay for CSF apoE of the kind described here, i.e., because of the importance of CSF apoE as an independent parameter in CNS disease.

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