Journal of Clinical Laboratory Analysis 17:18–21 (2003)

Detection of Apolipoprotein E Phenotype in Unconcentrated Cerebrospinal Fluid

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We developed a simple method to detect apolipoprotein E (Apo E) polymorphism distribution in approximately 20 μL of unconcentrated cerebrospinal fluid (CSF). A combination of isoelectric focusing in 3 M urea gel and immunoblotting was employed. Apo E phenotypes were identified in CSF samples from 45 patients with probable Alzheimer disease (AD), 15 with multiple sclerosis (MS), and 25 with other neurological diseases (OND). When the data were compared with a set of matched plasma samples, the results were identical. The method is useful for Apo E phenotyping from fresh or frozen unconcentrated CSF, when blood or plasma is not available. J. Clin. Lab. Anal. 17:18–21, 2003.

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Key words: cerebrospinal fluid; apolipoprotein E phenotype; isoelectric focusing; immunoblotting; neurological diseases

INTRODUCTION

Apolipoprotein E (Apo E) is a 34-kd polymorphic protein that is involved in the transportation and redistribution of lipids among various tissues (1). There are three major isoforms of Apo E that have been detected in human plasma: Apo E2, Apo E3, and Apo E4. They are products of three alleles (ϵ2, ϵ3, and ϵ4) at a single gene locus on chromosome 19 (2,3). As a result, six Apo E phenotypes (ϵ2/2, ϵ2/3, ϵ2/4, ϵ3/3, ϵ3/4, and ϵ4/4) are possible. E3 is the most common Apo E isoform, whereas E4 and E2 are less common. Apo E4 differs from Apo E3 due to the presence of arginine instead of cysteine at residue 112, while ApoE2 differs from Apo E3 due to the presence of cysteine rather than arginine at residue 158.

The major apolipoproteins in cerebrospinal fluid (CSF) are E and A-1 (4). ApoE is present in CSF at 3–5% of the plasma concentration (4,5). A number of studies have shown a strong association of Apo E isoforms with certain neurological diseases. The Apo E ϵ4 allele is a significant risk factor for the development of sporadic and familial late-onset Alzheimer disease (AD) (5–7). Apo E4 has also been implicated in development of Creuzfeldt-Jacob disease (8) and Parkinson disease (9). Recent reports also show a link between Apo E ϵ4 allele and the progression of multiple sclerosis (MS) (10,11).

A number of investigators have analyzed Apo E phenotyping in plasma or sera by isoelectric focusing (IEF) followed by immunoblottting (12–15). Recently investigators have examined Apo E isoforms in blood samples by using the polymerase chain reaction (PCR) method (6,7). However, no information has been reported concerning Apo E phenotyping of unconcentrated CSF. We report a simple and accurate method for Apo E phenotyping, in unconcentrated CSF and matched pairs of plasma or serum, using modified IEF and immunoblotting (14). Since both Apo E phenotyping and genotyping techniques have been used to determine ApoE polymorphism, we examined the isoforms of a number of AD and control plasma

Grant sponsor: Pfizer, Inc.; Grant sponsor: New York State, Office of Mental Retardation and Developmental Disabilities.

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Received 3 September 2002; Accepted 10 September 2002
DOI: 10.1002/jcla.10063
Published online in Wiley InterScience (www.interscience.wiley.com).

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samples by phenotyping, and compared them to those found by genotyping.

MATERIALS AND METHODS

Subjects

The study included matched pairs of CSF and plasma or sera samples from 45 patients with probable AD, 15 patients with relapsing-remitting multiple sclerosis (MS), and 25 patients with other neurological diseases (OND). The OND group involved Lyme disease (n = 13), central nervous system lupus (n = 2), polyneuropathy (n = 2), Guillain-Barre syndrome (n = 2), seizure (n = 3), and headache (n = 3). The AD patients ranged in age from 60 to 80 years (mean 71 years), and the OND group from 44 to 63 years (mean 53 years). In addition, we also included plasma and blood from 24 patients with probable AD, and from 31 elderly nondemented controls, and compared the Apo E polymorphism by both phenotyping and genotyping methods.

Apo E Phenotyping

Sample preparation

Fifty μL of CSF were pretreated with 6.25 μL of 5 mM dithiothreitol (DTT), 1% (v/v) Tween-20. Twenty μL each of serum and plasma were treated with 180 μL of the DTT solution. The samples were incubated for 18 hr at 4°C.

Isoelectric Focusing and Immunoblotting

CleanGel IEF (Amersham Pharmacia Biotech, Piscataway, NJ), a dehydrated polyacrylamide gel, was cut in half and rehydrated in 5.25 mL in a solution containing 3 M urea, 0.12 mL pH 4.5–5.4 Pharmalyte, and 0.24 mL pH 5–8 Pharmalyte (Amersham Pharmacia Biotech, Piscataway, NJ). The gel was placed in a plastic bag on a rocker and rehydrated for 72 hr at 4°C. The rehydrated gel was placed on a precooled flatbed electrophoresis unit, and prefocusing was carried out at 1,000 V, 50 mA, and 25 W for 30 min. The cathode and anode solutions were 1 M sodium hydroxide and 1 M phosphoric acid, respectively. A sample wick was dipped into CSF or plasma, and approximately 15–20 μL were applied on the gel 5 mm from the cathode. Focusing was continued at 1,600 V, 50 mA, and 25 W for 30 min. The sample wicks were removed and focusing was continued at 2,000 V, 25 mA, and 25 W for 2 hr (14). After focusing, the gel was rinsed in Tris-buffered saline (TBS), pH 8.0, and the protein was transferred by simple diffusion as previously described (13).

Briefly, the blot was blocked in 5% nonfat milk in TBS, rinsed with distilled water, and incubated in goat antiserum to human Apo E (1:5,000 dilution) (Incstar Corp., Stillwater, MN) in TBS for 18 hr at 4°C with constant shaking. The blot was washed four times for 5 min in TBS and incubated in rabbit antiserum to goat IgG conjugated to alkaline phosphatase (1:10,000 dilution) (Biosource International, Camarillo, CA) in TBS for 2 hr at room temperature. The blot was developed by incubation at room temperature for 5–10 min in substrate solution: 5-bromo-4-chloro-3-indoxyl phosphate p-toluidine salt (Sigma Chemicals, St. Louis, MO); 5 mg/mL in dimethylformamide and nitro blue tetrazolium chloride (Sigma Chemicals, St. Louis, MO); and 1 mg/mL in 0.1 M Tris-HCl buffer, pH 9.0, 2 M $MgCl_2$. When the bands showed a dark purple color, the enzyme reaction was stopped by rinsing the blot with distilled water.

Apo E Genotyping

Apo E genotypes of blood samples from the AD and control groups were determined using the PCR method as previously described (16). The PCR products were digested with *HhaI* and subjected to polyacrylamide gel electrophoresis. Separated DNA fragments were visualized using ethidium bromide staining.

RESULTS

We examined Apo E polymorphism in a total of 85 matched pairs of CSF and serum samples. Figure 1 shows typical immunoblotting after the IEF patterns of CSF and serum Apo E phenotypes. Apo E4 showed one distinct band toward the cathode. Apo E3 had three distinct bands with different isoelectric points, whereas Apo E2 showed one band toward the anode. The matched CSF and serum samples had identical Apo E phenotypes, but the intensities of the bands differed. The serum samples had denser bands than the CSF samples. The matched pairs of plasma and sera showed identical band patterns (data not shown), indicating that either fluid is suitable for the determination of Apo E allele frequencies. The Apo E polymorphism distribution of the samples is given in Table 1. The higher frequency of Apo E ϵ4 allele reported in the AD group was consistent with previous findings (5). The frequency of Apo E ϵ4 allele in patients with MS and OND was similar to that reported previously (17).

In order to determine whether the Apo E phenotying results were identical to those from Apo E genotyping, we examined ApoE isoforms in plasma samples from 24 AD patients and 31 nondemented controls. There was a 96.3% concordance of results between the two methods (Table 2). The Apo E phenotye and genotype distribu-

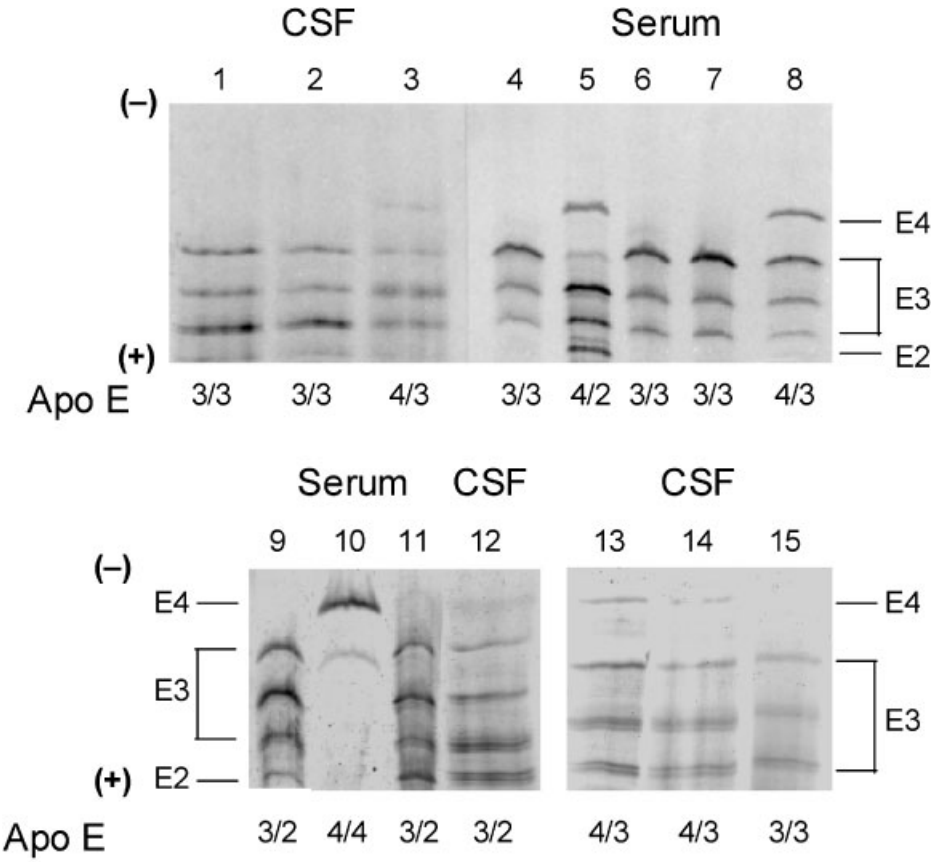

Fig. 1. Top: Apo E immunoblots showing five of the six possible phenotypes. **Lanes 1–3:** MS CSF. **Lanes 4 and 5:** ApoE standards. **Lanes 6–8:** MS sera. Bottom, **Lanes 9 and 10:** ApoE standards. **Lanes 11 and 12:** OND serum and CSF. **Lanes 13–15:** AD CSF.

TABLE 1. ApoE phenotypes in AD, MS and OND CSF

Group	ApoE polymorphism distribution					
	E4/E4	E4/E3	E4/E2	E3/E3	E3/E2	E2/E2
AD (n = 45)	8	22	1	13	1	0
MS (n = 15)	0	4	0	8	3	0
OND (n = 25)	1	4	0	18	2	0

TABLE 2. ApoE phenotype and genotype in AD and control plasma

Group	ApoE polymorphism distribution					
	E4/E4	E4/E3	E4/E2	E3/E3	E3/E2	E2/E2
AD (n = 45)						
Phenotype	6	11	1	4	1	1
Genotype	6	11	1	4	1	1
Controls (n = 31)						
Phenotype	0	7	0	19	5	0
Genotype	1	5	0	20	5	0

tion was identical in the AD group and in 29 of 31 individuals in the elderly nondemented control group. Two samples that showed the E4/E3 isoform by phenotyping showed E4/E4 and E3/E3 by the genotyping technique.

DISCUSSION

Apo E phenotyping was originally carried out using IEF of ultracentrifugally isolated very-low-density lipoprotein (18,19). However, several investigators have recently reported the use of simple and rapid IEF following immunoblotting for the detection of ApoE phenotyping (14,15). Although the latter method was found to be useful for Apo E phenotyping of plasma and synovial fluid (20), the Apo E phenotype had not heretofore been identified in unconcentrated CSF. In a large number of CSF samples, we showed that the method is useful for Apo E phenotyping in patients with neurological diseases.

The differences in CSF and serum Apo E band intensities (Fig. 1) reflect higher concentrations of Apo E in serum than in CSF (4,5). The accuracy of our method was confirmed by comparing the genotype of blood samples identified by using the PCR method.

The concordance rate of 96.3% in this study was in accordance with other studies (14,21). There was complete agreement between the two methods in AD patients. The reason for the discrepancy seen in the Apo E isoforms in two nondemented control samples is not known. However, it may be due to other Apo E variants, as reported previously (22).

In the present study we examined Apo E phenotypes of a small number of MS CSF and sera samples. Our findings of Apo E isoform distribution between the MS and OND groups are consistent with published reports (17). It has been reported that Apo E ϵ4 allele is a high risk factor for the progression of disability in MS (10,11). However, other studies could not confirm those findings (23,24). Further studies with a greater number of samples from patients with primary progressive MS are essential to clarify these controversial findings.

Brain banks at medical centers often collect only CSF, since it is not common practice to collect matching blood. In such situations our method is very useful for determining Apo E phenotypes, since only a small amount of fresh or frozen unconcentrated CSF is required. In summary, we have developed a simple, inexpensive, and reliable method for ApoE phenotyping in unconcentrated CSF. The advantages of our method are: 1) small volumes; 2) no need for ultracentrifugation to isolate lipoproteins, or pretreatment with neuraminidase; and 3) commercially available rehydrating dry polyacrylamide gel.

REFERENCES

1. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 1988;240:622–630.
2. Rall Jr SC, Weisgraber KH, Mahley RW. Human apolipoprotein E. The complete amino acid sequence. J Biol Chem 1982;257:4171–4178.
3. Paik YK, Chang DJ, Reardon CA, Davies GE, Mahley RW, Taylor JM. Nucleotide sequence and structure of the human apolipoprotein E gene. Proc Natl Acad Sci U S A 1984;82: 3445–3449.
4. Roheim PS, Carey M, Forte T. Apolipoproteins in human cerebrospinal fluid. Proc Natl Acad Sci U S A 1979;76:4646–4649.
5. Lehtimaki T, Pirttila T, Mehta PD, Wisniewski HM, Frey H, Nikkari T. Apolipoprotein E (apoE) polymorphism and its influence on ApoE concentrations in the cerebrospinal fluid in Finnish patients with Alzheimer's disease. Hum Genet 1995;95: 39–42.
6. Mayeux R, Saunders AM, Shea S, et al. Utility of the apolipoprotein E genotype in the diagnosis of Alzheimer's disease. N Engl J Med 1998;338:505–511.
7. Saunders AM, Strittmatter WJ, Schmechel D, et al. Association of apolipoprotein E allele ϵ4 with late-onset familial and sporadic Alzheimer's disease. Neurology 1993;43:1467–1472.
8. Zerr I, Helmhold M, Poser S, Armstrong VW, Weber T. Apolipoprotein E phenotype frequency and cerebrospinal fluid concentration are not associated with Creutzfeld-Jakob disease. Arch Neurol 1986;53:1233–1238.
9. Tang G, Xie H, Xu L, Hao Y, Lin D, Ren D. Genetic study of Apolipoprotein gene, alpha-1-chymotrypsin gene in sporadic Parkinson disease. Am J Med Genet 2002;114:446–449.
10. Chapman J, Vinokurov S, Achiron A. APOE genotype is a major predictor of long-term progression of disability in MS. Neurology 2001;56:312–316.
11. Evangelou N, Jackson M, Beeson D. Association of the APOE ϵ4 allele with disease activity in multiple sclerosis. J Neurol Neurosurg Psychiatry 1999;67:203–205.
12. Hackler R, Schoffer JR, Motzny S, et al. Rapid determination of apolipoprotein E phenotypes from whole plasma by automated isoelectric focusing using PhasSystem™ and immunofixation. J Lipid Res 1994;35:153–158.
13. Kamboh MI, Ferell RE, Kottke B. Genetic studies of human apolipoproteins. V. A novel rapid procedure to screen apolipoprotein E polymorphism. J Lipid Res 1988;29: 1535–1543.
14. Kataoka S, Paidi M, Howard BV. Simiplified isoelectric focusing/ immunoblotting determination of apolipoprotein E phenotype. Clin Chem 1994;40:11–13.
15. Hill JS, Pritchard PH. Improved phenotyping of apolipoprotein E: application to population frequency distribution. Clin Chem 1990;36:1871–1874.
16. Hixson JE, Vernier DT. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. J Lipid Res 1990;31:545–548.
17. Oliveri RL, Cittadella R, Sibilia G. APOE and risk of cognitive impairment in multiple sclerosis. Acta Neuro Scand 1999;100: 290–295.
18. Utermann G, Hees M, Steinmetz A. Polymorphism of apo E and occurrence of dysbetalipoproteinemia in man. Nature 1977;269:604–607.
19. Zannis VI. Genetic polymorphism in human apolipoprotein E. Methods Enzymol 1986;128:823–851.
20. Adiloglu AK, Gurakur-Osborne A, Prete PE. Standardized determination of apolipoprotein E phenotypes by simplified isoelectric focusing/immunoblotting in plasma and synovial fluid. Clin Chim Acta 1998;273:99–102.
21. Dupuy A, Badiou S, Ritchie K. Discrepancies between apolipoprotein E phenotyping and genotyping in the elderly. Clin Chem 2001;39:405–413.
22. Wardell MR, Brennan SO, Janus ED, Fraser R, Carell RW. Apolipoprotein E2-Christchurch (136 Arg Ser). New variant of human apolipoprotein E in a patient with type III hyperlipoproteinemia. J Clin Invest 1987; 80:483–490.
23. Ferri C, Sciacca FL, Veglia F. APOE ϵ2-4 and -491 polymorphisms are not associated with MS. Neurology 1999;53:888–889.
24. Weatherby SJM. No association between the APOE ϵ4 allele and outcome and susceptibility in primary progressive multiple sclerosis. J Neurol Neurosurg Psychiatry 2000;68:532.