Sandwich ELISA for the Measurement of Apo-E4 Levels in Serum and the Estimation of the Allelic Status of Apo-E4 Isoforms

Yoshiaki Uchida,¹ Satoru Ito,^{1*} and Nobuyuki Nukina²

¹Diagnostic Research Laboratories, Fujirebio, Inc., Hachioji, Tokyo, Japan

²Molecular Neuropathology Group, Brain Science Institute, The Institute of Physical and Chemical Research, Saitama, Japan

Apolipoprotein-E (Apo-E) isoforms, especially the Apo-E4 isoform, are considered to be genetic risk factors for familial as well as sporadic Alzheimer's disease (AD). Using monoclonal antibodies specific for Apo-E4 (MAb412-1-7) and Apo-E (LZ18), we established a sandwich ELISA for the measurement of Apo-E4 levels in serum. Total Apo-E also was determined by sandwich ELISA with a second pair of monoclonal antibodies, LZ12 and LZ18. Estimation of a patient's

homozygous or heterozygous status for the Apo-E4 isoform was subsequently calculated as the ratio of Apo-E4/total Apo-E, respectively. Although the antibody for Apo-E used here does not have equivalent reactivity toward Apo-E2, -E3, or -E4, we were able to attain a fairly reliable assay system to estimate the status of the Apo-E4 isoform through a simple formula for the calculation of total Apo-E versus Apo-E4 isoform. *J. Clin. Lab. Anal.* 14:260–264, 2000. © 2000 Wiley-Liss, Inc.

Key words: sandwich ELISA; Apo-E4 isoform; allelic status

Although the issue remains unresolved, numerous studies have been undertaken to attempt to determine the risk factors associated with Alzheimer's disease (AD) (1). The formation of β -amyloid plaque as well as mutations in presenilin-1 and presenilin-2 have been considered to be major risk factors associated with familial AD (2–4). To date, however, little information is available on risk factors associated with sporadic AD. The homozygote of Apo-E ϵ 4 alleles is, however, believed to be a key factor involved in both the sporadic and familial forms of AD (5–8). While genetic analysis is the most common method used to determine the allelic status of Apo-E ϵ , this is a highly technically demanding analysis. Simpler methods for the determination of Apo-E isoform phenotypes would be preferable. Furthermore, a quantitative method for the determination of Apo-E4 levels in serum or plasma could be of benefit for studies into the possible relationship between levels of Apo-E4 and the onset of AD.

We had previously described a monoclonal antibody, MAb412-1-7 (IgG3), capable of specifically recognizing the Apo-E4 isoform in the presence of other isoforms such as Apo-E2 and Apo-E3 (9). In this study, data were presented on an ELISA for the measurement of serum Apo-E4 levels in which wells of plastic plates were coated with serum and Apo-E4 was detected by addition of the monoclonal antibody conjugate. However, this approach proved impractical for routine testing. In the present experiments, a simple sandwich ELISA

system using MAb412-1-7-ALP conjugate and anti-Apo-E (LZ18) on the solid phase was developed to measure serum Apo-E4 levels. In addition, a second sandwich ELISA for total serum Apo-E, using monoclonal antibodies LZ12 for signaling and LZ18 on the solid phase, was developed. Through calculation of the ratio of Apo-E4/total Apo-E, we demonstrate that a reasonable estimate of the phenotypic status of the Apo-E4 isoform of a patient can be made.

MATERIALS AND METHODS

Apolipoproteins E2, E3, and E4, expressed in a baculovirus expression system, were purchased from Biogenesis Ltd. (UK) through Wako Pure Chemicals (Osaka, Japan). Apo-E was obtained from Chemicon International Inc. (Temecula, CA). Lipoprotein fractions were separated by the method of R.J. Havel et al. (10) with minor revision. Rabbit anti-Apo-E was purchased from DAKO Japan (Kyoto, Japan). The conjugation of antibodies with horseradish peroxidase (POD) or alkaline phosphatase (ALP) was carried out using standard protocols.

*Correspondence to: Satoru Ito, Diagnostic Research Laboratories, Fujirebio, Inc., 51 Komiya-cho, Hachioji, Tokyo 192-0031, Japan.
E-mail: sr-itou@fujirebio.co.jp

Received 10 May 1999; Accepted 22 May 2000

The status of Apo-E isoforms in serum was determined by electrophoretic techniques as previously described (11). Serum samples were obtained from patients with AD or from those without neurological symptoms and were stored frozen (-80°C) for over 10 years. Genetic analysis of the isoform alleles was not performed this time.

Monoclonal Antibodies Against Apolipoprotein E

The purified VLDL fraction from plasma (purchased from Cortex through Cosmo Bio, Tokyo, Japan) was used as immunogen, intraperitoneally, with complete Freund's adjuvant, using standard techniques. Harvested spleen cells were fused with myeloma cells (P3U1) using PEG 1500 (Boehringer Mannheim Yamanouchi, Tokyo, Japan) and screened with a recombinant Apo-E3-coated ELISA plate. Monoclonal antibodies LZ12 and LZ18 were subsequently established. The reactivity of these antibodies was confirmed with native Apo-E in lipoprotein fractions, together with a commercially available Apo-E sample, using Western blot analysis as shown in Fig. 1. Monoclonal antibodies were produced in ascites fluid and subsequently purified using protein A-Sepharose 4B column chromatography.

The isotypes of monoclonal antibodies LZ12 and LZ18 were identified as IgG2a using a mouse monoclonal antibody isotyping kit (Amersham, UK).

RESULTS

Determination of Total Serum Apo-E by ELISA

Total serum Apo-E was determined using a combination of monoclonal antibodies LZ18 and LZ12, polyclonal rabbit

anti-Apo-E, and rabbit anti-Apo-E-POD-conjugate, together with 2,2'-azinobis(3-ethylbenzthiazolin-sulfonic acid) (ABTS) as peroxidase (POD) substrate. When the relative efficiencies of LZ12 to LZ18 as the solid-phase capture antibody were compared, it was determined that LZ18 gave far more reproducible data that was consistent with other assay methods, such as immunoturbidity (data not shown). In all subsequent experiments, therefore, LZ18 was employed on the solid phase to capture Apo-E, and an LZ12-ALP-conjugate was used as the signal-generating antibody. Using this ELISA format, the relative reactivity for each of the Apo-E isoforms, using recombinant baculovirus-expressed proteins, was determined, and the results are shown in Fig. 2.

As shown by these data (Fig. 2), the present assay system demonstrated a higher relative reactivity toward Apo-E2, using recombinant Apo-E4 as calibrate. Further, as the reactivity of Apo-E4 was weaker than that of Apo-E3, the current ELISA did not detect each Apo-E isoform with equal sensitivity. Similar results were obtained for the determination of Apo-E levels using an ELISA formatted with polyclonal antibodies. Thus, Apo-E2 was found to have a higher reactivity in general in an ELISA format. Given the observed variations in reactivity, no further quantitative measurements of Apo-E levels were performed. Subsequently, however, we utilized Apo-E4/E4 homozygote serum ($50\times$ dilution) for calibration, rather than recombinant Apo-E4, for the measurement of Apo-E in serum samples. This calibrate proved more convenient and reproducible in the present assay system. The $50\times$ dilution of the Apo-E4/E4 homozygote serum determined to be the most appropriate as compared to immunoturbidity experiments.

Fig. 1. CBB and Western blot data of monoclonal antibodies LZ12 and LZ18 against Apo-E. Lane 1: Molecular size marker (numbers represent kDa). Lane 2: Apo-E (from Chemicon). Lane 3: VLDL. Panels from left to right represent the Coomassie Brilliant Blue (CBB) staining pattern and Western blot visualization of LZ18 and LZ12, respectively.

Fig. 2. Standard curves of Apo-E isoforms in the total Apo-E ELISA system. LZ18 was used for a solid-phase antibody, and LZ12 was used as a conjugate for ELISA. Lines marked with ●, ■, and ▲ depict recombinant Apo-E2, -E3, and -E4, respectively. Recombinant Apo-E4 was used as a standard for calibration.

The present ELISA format resulted in a CV% of 5% ($n = 5$) for E4/E4 homozygote and 4% ($n = 5$) for E4/E2 or E4/E3 heterozygote. Linearity of the ELISA assay for each of the recombinant Apo-E isoforms is shown in Fig. 3a. The concentration of Apo-E in patients' sera was beyond the linear range of the assay, particularly for Apo-E2. Unfortunately, due to a lack of serum sample volume, no further optimization was possible.

Apo-E4 Isoform-Specific ELISA

In this assay format, monoclonal antibody LZ18 was used to coat a 96-well tray, and monoclonal antibody MAb412-1-7, conjugated with ALP, was used as a secondary antibody and signal generator. The combination of these two monoclonal antibodies provided a reasonable signal for the determination of Apo-E4. As described above, the Apo-E4/E4 homozygote serum was employed as a standard in further experiments. The current assay format (MabLZ18/Mab412-1-7) resulted in a CV% of 9% ($n = 5$) for the determination of the E4/E4 homozygote and 13% ($n = 5$) for both the E4/E3 and E4/E2 heterozygotes. Both linearity and specificity for Apo-E4 were acceptable, as shown in Fig. 3b.

Initially, sera from patients whose Apo-E4 allelic status already had been determined, using isoelectric focusing/immunoblotting with anti-Apo-E, were used to confirm the relationship between the ratio of Apo-E4/Apo-E deduced from the present ELISA method and data derived from electrophoretic methods. The results are summarized in Table 1.

The allelic status deduced from the ratio of Apo-E4/Apo-E using the present method was in reasonable agreement with that obtained by the more classical electrophoretic techniques. Although we could have used OD values to indicate the amount of Apo-E, we preferred to use the weight form description taken for other apolipoprotein parameters.

As further confirmation of the descriptive value of the present ELISA format, the assay was applied to blinded serum samples, and the results are shown in Fig. 4. The phenotype of each serum sample, determined using the classical electrophoretic methodology, also is shown in Fig. 4.

DISCUSSION

Many reports have described risk factors associated with familial Alzheimer's disease, including β -amyloid plaque formation and mutations of presenilins (2–4). However, sporadic cases of Alzheimer's disease mostly have been excluded from these discussions. However, some studies have suggested that the Apo-E $\epsilon 4$ allelic composition of an individual may be a risk factor in familial as well as sporadic AD. As such, it is believed that there is a higher probability of risk for AD in Apo-E $\epsilon 4$ allele homozygous individuals compared to Apo-E $\epsilon 4$ allele heterozygous individuals (5). Further, it has been suggested that there is a significant inverse relationship between anti-cholinergic drug responses and the allelic state of Apo-E ϵ genes (12). However, this study provided only genotype information and lacked the phenotypic data needed to further evaluate this issue. To the authors' knowledge, there

Fig. 3. Serial dilution curve of Apo-E isoforms in the total Apo-E ELISA system and in the Apo-E4-specific ELISA system. (A) Behavior of the serial dilution curves of the Apo-E isoforms was shown in the total assay system. (B) Behavior of the serial dilution curves of the Apo-E isoforms was shown

in the Apo-E4-specific ELISA system. The assay was carried out using Apo-E4/E4 homozygote serum as a calibration standard. Diluted samples were applied for this ELISA system. Lines marked with $\bullet$, $\blacksquare$, and $\blacktriangle$ depict recombinant Apo-E2, -E3, and -E4, respectively.

7. Saunders AM, Strittmatter WJ, Schmechel D, et al. Association of apolipoprotein E allele ϵ 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 1993;43:1467–1472.
8. Mayeux R, Saunders AM, Shea S, et al. Utility of the apolipoprotein E genotype in the diagnosis of Alzheimer's disease. *New Engl J Med* 1998;338:506–511.
9. Nukina N, Uchida K, Sakamoto T, Ide K, Kanazawa I. Monoclonal antibody against the polymorphic site distinguishes apolipoprotein E4 from other isoforms. *Biochem Biophys Res Commun* 1995;216:467–472.
10. Harvel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955;34:1345–1353.
11. Kataoka S, Paidi M, Howard BV. Simplified isoelectric focusing/immunoblotting determination of apoprotein E phenotype. *Clin Chem* 1994;40:11–13.
12. Poirier J, Delisle M-C, Quirion R, et al. Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. *Proc Natl Acad Sci USA* 1995;92:12260–12264.
13. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD. Apolipoprotein E: high-avidity binding to β -amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 1993;90:1977–1981.
14. Corder EH, Robertson K, Lannfelt L, et al. HIV-infected subjects with the E4 allele for Apo-E have excess dementia and peripheral neuropathy. *Nat Med* 1998;4:1182–1184.