Biochemical Assay for AD7C-NTP in Urine as an Alzheimer's Disease Marker

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A reliable and specific immunoassay has been developed to detect and measure AD7C-NTP, a biochemical marker for Alzheimer's disease, in urine. The urine samples are first processed by centrifugation and ultrafiltration to fractionate and concentrate AD7C-NTP. The urinary AD7C-NTP has the same molecular weight as AD7C-NTP in brain and cerebrospinal fluid by size exclusion chromatography. It has also retained the binding properties to the monoclonal and polyclonal antibodies developed against recombinantly produced AD7C-NTP. This assay is an enzyme linked sandwich immunoassay (ELISA) using 96 well microtiter plates. The plate surface is coated

with a monoclonal antibody (N3I4) which has a high affinity and specificity for AD7C-NTP, capturing it effectively from the samples. The detection was achieved using a polyclonal antibody (ADRI). The utility of the assay has been demonstrated using urine specimens from Alzheimer's disease (AD) patients and non-Alzheimer's controls. Urinary AD7C-NTP in the AD group (2.5 ng/mL, n=66) was significantly higher than the non-AD group (0.8 ng/mL, n=134). Using 1.5 ng/mL as cut off, in this patient population, specificity and sensitivity of urinary AD7C-NTP were comparable to CSF AD7C-NTP. *J. Clin. Lab. Anal.* 12:285–288, 1998. © 1998 Wiley-Liss, Inc.

Key words: NTP; Alzheimer's diagnostic test; Alzheimer's marker; urinary AD7C test

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a chronic deteriorating course of impaired intellectual function and memory loss. The definitive diagnosis of AD is made by pathologic examination of postmortem brain tissue (1). Antemortem diagnosis, however, is by exclusion (2), as there are currently no reliable antemortem biochemical tests available. Apolipoprotein E₄ (ApoE₄) allele is merely a risk factor consideration (3). Amyloid β -protein level in CSF has been reported to decrease in AD, and Tau protein level (4–11) in CSF has been reported by several groups to increase in AD patients. However, a significant overlap between AD and non-AD levels limits the usefulness of both amyloid β -protein and Tau. A CSF-based Alzheimer's test based on the combination of amyloid β -protein and Tau has been suggested, but it still suffers from excessive overlap of AD and non-AD and has no diagnostic value when both levels are either high or low (8). AD7C-NTP in CSF has been reported to be helpful in diagnosing AD (12–14).

AD7C-NTP is present in the long axonal processes which emerge from the nerve cell body and is found in large amounts associated with the neurofibrillary tangles of Alzheimer's disease (12,13). There is evidence that AD7C-NTP is involved in neuronal repair and regeneration in the brain. AD7C-NTP gene expression is also elevated in Alzheimer's brain tissue

(in situ hybridization). The gene encoding AD7C-NTP has been cloned and sequenced, and the protein has been produced by recombinant techniques (14). This protein has been used to generate both monoclonal and polyclonal antibodies to Neural Thread Protein (NTP). An immunoassay for measuring AD7C-NTP in CSF has been previously described (15). There is a significant need for a sensitive clinical biochemical marker for AD that can be easily accessed by noninvasive means. Neural thread protein (AD7C-NTP) has been established as a biomarker for AD and is selectively increased in AD brain and AD cerebrospinal fluid (CSF). We investigated the levels of AD7C-NTP in urine samples from patients with AD as compared to non-AD controls.

MATERIALS AND METHODS

Materials

Multistix® 10 SG reagent strip for urinalysis was purchased from Bayer™. Acetate membrane filter units (0.2 μ) were obtained from Nalgene™. The 10 kD MW cutoff concentrator used was the Amicon® Centricon-10. Sodium chloride, potas-

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Received 4 February 1998; Accepted 3 March 1998

sium chloride, Tween-20, casein, and Trizma base were purchased from Sigma™ Chemicals. Coomassie protein assay dye reagent was purchased from Bio-Rad™. Bovine gamma globulin standard, bovine serum albumin standard, o-phenylenediamine dihydrochloride (OPD) tablets, stable hydrogen peroxide (H₂O₂), goat antirabbit IgG HRP, and purified Tween-20 are from Pierce™ Chemicals. Sodium hydroxide (2N), hydrochloric acid (6N), high and low binding 96 well ELISA plates, and sulfuric acid (2.5N) were purchased from Fisher™ Scientific.

Buffers and Solutions

Coating buffer: tris buffered saline (TBS) pH 7.5

Blocking buffer: 0.5% casein in TBS pH 7.5

Antibody and Standards buffer: 0.1% casein in TBS pH 7.0 with 0.005% purified Tween-20

Sample buffer: 0.2% casein in TBS pH 7.0 with 0.01% purified Tween-20

Wash solution: TBS pH 7.0 with 0.05% Tween-20

Color substrate: OPD tablets in deionized water with H₂O₂

Stop solution: 2.5N sulfuric acid

Equipment

Fisher Scientific™ Model 225 Centrifuge™ Centrifuge

Baxter™ Model 2503 Labofuge A

Marathon™ 13 K/M microcentrifuge

Bio-Rad™ Model 550 Microplate Reader

Boekel™ Model #133000 Dry Incubator

Nunc™-Immunowash 12 Wash Block Plate Washer

Mettler™ AE163 Analytical Balance

Antibodies to AD7C-NTP

Monoclonal antibodies (MAbs) were produced against recombinant AD7C-NTP as described by de la Monte et al (13). The MAbs were purified on protein G columns (Pharmacia™). Polyclonal antibodies were also raised against recombinant AD7C-NTP. Rabbits were immunized with purified recombinant AD7C-NTP and boosted according to a monthly schedule. Bleeds were screened using direct ELISA by coating plate wells with AD7C-NTP in the coating buffer. After desired antibody titers were achieved, the rabbit antisera was purified by protein A/G affinity chromatography (Pierce™). All antibody solutions were centrifuged for 3 min at 10,000 rpm immediately before use.

Coating the Plates

Microtiter plate wells were coated with MAb using 0.25 µg antibody per well in the coating buffer and incubated for 60 min at 37°C. The wells were then washed with the wash solution and blocked with the blocking buffer for 60 min at 37°C.

Patient Description

The clinical diagnosis of probable AD was rendered according to the criteria established by the National Institute of

Neurological and Communication Disorders and Stroke/ Alzheimer's Disease and Related Disorders Association or DSM-IV. The non-AD patients were simply identified as per their current chart or were "normal" volunteers. The subjects are living and therefore there has been no possibility of postmortem confirmation of the diagnosis. Patients with known renal disease or urinary abnormalities were excluded from the study. The majority of patients are male which merely reflects the availability of acceptable samples for this study. The normal and AD groups, however, were age-matched. The participants provided urinary samples after giving written informed consent. For the patients with AD, informed consent was also obtained from relatives. The studies were separately approved by each of the Institutional Review Boards of the participating institutions. All clinical data used in the studies were compiled without knowledge of the assay results and similarly assay results were generated without knowledge of the clinical data.

Sample Collection and Preparation

Twenty-four-hour samples were collected to minimize concentration fluctuation due to fluid and renal factors. Fifty mL aliquots of urine were centrifuged for 15 min at 3,000 rpm. The supernatant was then passed through a 0.2 µ acetate filter under vacuum. From the filtrate, 2.0 mL was pipetted into the 10 kD MW cutoff concentrator, centrifuged at 5,000 rpm for one hr, and washed twice with 1 mL of TBS pH 7.0 in the concentrator at 5,000 rpm for 30 min. The entire retained material was quantitatively transferred to small tubes, and the weight was brought up to 500 mg (500 µL) with TBS pH 7.0.

Urinalysis

Prior to sample preparation a complete urinalysis was carried out by standard and routinely accepted methods by a clinical laboratory.

Sample Selection

Exclusion and inclusion criteria for the urine samples were based on visual observation and standard urinalysis data. Cloudy and highly colored samples were excluded. The clear samples were sent for routine urinalysis. Samples with any of the following abnormalities were rejected: (a) protein (24 hr) less than 40 mg or more than 200 mg; (b) presence of any significant numbers of white blood cells, red blood cells, or bacteria; (c) crystalluria; (d) abnormal specific gravity; (e) abnormal pH; (f) presence of glucose, ketones, nitrites, bilirubin, or urobilinogen.

Protein Assay

The total protein content of the AD7C-NTP stock solution was determined by using the Coomassie protein assay per instructions.

AD7C-NTP Assay

The assay was performed according to a set of detailed CLIA approved Standard Operating Procedures (available from the authors). The plate format and other details have been previously described (15). The standard and sample well volume was 200 μ L, with the exception of 300 μ L in all wells for the blocking step. The processed urine samples were diluted with equal volume of the sample buffer. Blanks were the same as standards except for the absence of AD7C-NTP.

Samples, standards, and blank wells on the coated and blocked plate were filled with 200 μ L of the appropriate solutions. The plate was covered and incubated overnight at 4°C. The plate was washed 5 times with the wash solution. The appropriate, diluted ADRI was placed into all wells. The plate was covered and incubated for 60 min at 37°C. The plate was washed 5 times with the wash solution. The goat antirabbit IgG HRP was diluted according to the product insert and added to all wells. The plate was covered and incubated at 37°C for 60 min. The plate was washed 5 times and 200 μ L of the OPD solution, prepared as per product insert, was added to each well. The plate was covered and incubated at 37°C for 60 min. Finally, the substrate reaction was stopped by adding 100 μ L of 2.5 N sulfuric acid. The plates were read at 490nm in the microplate reader and absorbance values were analyzed.

The microplate reader's data analysis program calculates the mean of the blank values, the background corrected means ($\pm$ SD), and CVs for standards and samples. The program also plots the standard curve, constructs a linear curve fit equation, and calculates the AD7C-NTP concentration for all samples.

Size Exclusion Chromatography

The molecular mass of AD7C-NTP was determined in several samples by high performance liquid chromatography (HPLC) (size exclusion column: Supelco™ TSK, G2000SW, flow rate 1 mL/min). In brief, 100 μ L processed urine was passed through the column along with molecular weight standards and 50 fractions of 0.5 mL were collected. Each fraction was analyzed for AD7C-NTP immunoreactivity by ELSIA using the MAb (N3I4) AD7C-NTP capture antibody linked to the solid phase support followed by detection with the appropriate ADRI prepared against the recombinant protein. The molecular weight has been confirmed by capillary electrophoresis (BioRad™ cartridge 1423050) based on size exclusion of the fractions that contained AD7C-NTP immunoreactivity.

RESULTS

Urinary AD7C-NTP as Compared to CSF and Brain AD7C-NTP

The HPLC fractions from a representative AD sample and 3 normal age-matched control urine samples are plotted

against AD7C-NTP concentration for the fractions in Figure 1. There is a consistent peak for the $\approx$ 41 kD AD7C-NTP fraction #20 in AD urine. This peak is similar to the peak found in AD CSF (14), and it is not present in non-AD control samples. The capillary electrophoresis studies have demonstrated the N3I4 immunoreactive proteins to be 39–41 kD in size. Therefore, the AD7C-NTP immunoreactive proteins detected in urine have the same molecular mass as the proteins detected in human CSF and cerebral tissue (14).

Recovery of AD7C-NTP

Recovery of the AD7C-NTP from urine was determined by adding a known volume of CSF with a known concentration of AD7C-NTP to the urine prior to sample processing. The recovery of added AD7C-NTP was determined to be from 90 to 101%.

Assay Parameters

The assay is essentially the same as the sandwich enzyme immunoassay for measuring AD7C-NTP as an AD marker in CSF (15). The MAb (N3I4) was used for capture and ADRI for detection. The assay is linear in the 0 to 2 ng range ($r^2 > 0.99$) and its sensitivity is from 30 to 50 pg. The assay reproducibility is indicated by intra-plate, inter-plate, and inter-operator CVs of 10% or lower (15).

Sample Handling and Storage

AD7C-NTP levels of fresh urine (processed and assayed without freezing) were not significantly different from AD7C-NTP levels in the same sample but frozen at dif-

Fig. 1. AD7C-NTP concentrations for HPLC fractions from AD and control urine samples. AD urine contains a peak for the 41kD AD7C-NTP in fraction #20 which is not present in controls.

ferent stages of processing. Moreover, unprocessed and processed urine samples were stored frozen (-20°C or -80°C) for at least 3 months without any significant change in their AD7C-NTP levels.

Clinical Utility

The utility of AD7C-NTP as an Alzheimer's disease marker in CSF has been previously documented (14). It has been found that 89% of patients with early AD have AD7C-NTP levels of above 2 ng/mL of CSF and 89% non-AD cases have AD7C levels of less than 2 ng/mL of CSF. In a subsequent study it was observed that only 6% of non-AD demented cases had AD7C-NTP levels of more than 2 ng/mL (16). A large clinical study for urinary AD7C-NTP is underway. The results for 79 cases are reported here as a demonstration of urinary AD7C-NTP having the potential as a peripheral AD marker and hence to be useful in aiding in the diagnosis of AD.

The mean value of urinary AD7C-NTP concentration in the AD group was 2.5 ng/mL ($n=66$), significantly elevated (t -test $P < .001$) compared to the mean value of 0.8 ng/mL ($n=134$) for non-AD controls. Using a cutoff of 1.5 ng/mL, the specificity of urinary AD7C-NTP in this study was 91%. Based on the accuracy of clinical diagnosis, which is expected to be in the 75%–90% range, the urinary AD7C-NTP sensitivity is in the 80% to 85% range, which is comparable to those reported for CSF AD7C-NTP (14, 16).

DISCUSSION

The method described in this article can reliably measure AD7C-NTP levels in urine specimens. AD7C-NTP is a proven Alzheimer's disease marker in brain as well as in CSF (14,16). Hence, its measurement in urine provides a unique opportunity of a peripheral marker for Alzheimer's disease. This represents a significant step towards a reliable and readily available laboratory test that can aid physicians in the early and more accurate diagnosis of AD, with significant time and cost saving implications.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the valuable technical assistance of Anita Mattero, Christopher Donaldson, and

Kilian Songwe. We thank Drs. Jack Wands and Suzanne de la Monte of Massachusetts General Hospital, Boston, for their invaluable suggestions and ideas.

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