

1-1-2001

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PİRİM, İBRAHİM (2001) "Sandwich-Type Enzyme Immunoassay for Amyloid A4 Protein in Cerebrospinal Fluid From Patients with Head Trauma," *Turkish Journal of Medical Sciences*: Vol. 31: No. 1, Article 7. Available at: <https://journals.tubitak.gov.tr/medical/vol31/iss1/7>

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Sandwich-Type Enzyme Immunoassay for Amyloid A4 Protein in Cerebrospinal Fluid From Patients with Head Trauma

Received: December 14, 1999

Abstract : The aim of this study was to design a simple method for detecting the amyloid A4 protein in the cerebrospinal fluid (CSF) of patients who had severe brain trauma caused by traffic or other accidents and to evaluate its diagnostic and prognostic value. For this purpose, a sandwich type ELISA assay was used. A4, a 4kDa protein of 39-43 amino acids, is a metabolic product of a membrane-spanning, 695-770 aa, precursor molecule, the β -amyloid precursor protein (β -APP). The polyclonal rabbit antibody against synthetic A4 protein (1-40 residues) was used as an immobilized antibody. A mouse monoclonal antibody against synthetic A4 protein (1-28 residues) was used as a different immunoglobulin to attach the A4. Enzyme-labelled anti-immunoglobulin (peroxidase rabbit anti-mouse antibody, PRAM) was used for reaction with mouse immunoglobulin. The assay was

highly specific for A4, demonstrating no cross reactivity between polyclonal anti A4 and monoclonal A4 antibodies (<0.7% cross reactivity). The lowest detectable value in the assay was 100ng/ml (1ng/well). Our results showed that, in CSF samples of 30 patients with head trauma caused by traffic or other accidents and 14 controls drawn from children and adults who presented with acute headache or meningeal irritation and were tested by ELISA for A4 protein, only the CSF samples of patients with head trauma displayed elevated A4 reactivity. This assay thus permits the detection of abnormal fragments of β -APP (A4) within the CSF. It means that severe head trauma may cause abnormal production of A4 which leads to amyloid deposition and, in turn, neuronal degeneration.

Key Words: Beta-Amyloid protein (A4), Head Trauma, ELISA

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Introduction

A4, a 4kDa protein of 39-43 amino acids, is a metabolic product of a membrane-spanning, 695-770 amino acid, precursor molecule, the β -amyloid precursor protein (β -APP). Amyloid precursor protein 695-770 aa (β -APP), is a transmembrane protein which is encoded by a gene on chromosome 21 and is constitutively secreted by both brain and non-brain cells into extracellular fluids throughout life (1,2). Intensive investigations have focused on the metabolic mechanisms generating A4 (3,4). APP can be metabolically processed by at least two pathways. The α -secretase pathway cleaves β -APP in the central section of the A4 domain (5,6) and thereby precludes amyloidogenesis, whereas the β -secretase pathway generates the free N-terminus of intact A4 (7). It is now widely accepted that the deposition of the A4 within the brain parenchyma and in cerebrospinal fluid blood vessels

is an early and necessary feature of Alzheimer's disease, which is the most common cause of age-related mental failure (8-10). The relationship between head trauma and the immediate production of A4, in turn diffusing into CSF, is not clear, since A4 production and accumulation in the brain take years. It is, however, likely that severe head trauma leads to the production of A4, which passes into CSF.

The aim of this work was to measure the abnormal fragment of amyloid precursor protein A4 within the CSF in the case of head trauma using a sandwich-ELISA method. Here, we report A4 appearance in the CSF samples of patients who had severe head trauma.

The aim of this study was to design a simple method for detecting the A4 protein within CSF samples from patients who had brain trauma, and to evaluate its diagnostic and prognostic value. For this purpose, a sandwich type ELISA assay was used.

Materials and Methods

Patients and control:

All patients (21 male 43±21; 9 female, 41±22) who had head trauma were investigated for intracranial hemorrhage. The controls (9 male, 26±22; 5 female, 33±24) were drawn from children and adults who presented with acute headache or meningeal irritation and who had a lumbar puncture performed for investigation of suspected meningitis.

Specimen Collection and Analysis

CSF was obtained by lumbar puncture under sterile conditions. Blood-stained CSF samples were centrifuged promptly. The CSFs were stored at 4°C for no longer than 4 days.

Specimen collection (including sample volume) was dictated solely by clinical considerations and no additional specimens were collected for the purposes of this study.

Reagents

Synthetic A4 peptide fragment (1-40) of β -APP, polyclonal anti-A4(1-40) antibody and peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (PRAM) were obtained from Sigma (A1075, A8326 and A9044 respectively). Monoclonal anti A4-antibody (1-28) was kindly provided by Dr. M. Landon, Nottingham Univ., Biochem. Dept., England.

Assay

In preparation for the assay, 96-well Maxisorp plates (Cat. no.469949; Nunc Roskilde, Denmark) were passively coated at 37°C for 24 h with polyclonal anti-A4 antibody (200 μ l/well). After five washes with washing buffer (distilled water containing 0.5ml/l Tween 20), 100 μ l of different concentrations of synthetic A4 (1-40 peptide), (concentration range 10, 30, 50, 70, 90, 100 ng/well) and CSF of the patients (neat) were added to the wells. Then, 100 μ l mouse monoclonal anti A4 antibody (1:1000 dilution) was added to each well. We then added 100 μ l of peroxidase-labelled anti-mouse immunoglobulin antibody (dilution of 1:500) to each well. After 4h incubation and 10 washes with 300 μ l of washing buffer per well, to each well we added 200 μ l of substrate solution (0.1%w/w ABTS (2,2azino-bis, 3-ethylbenzthiazoline-6-sulphonic acid), 0.003%v/v H₂O₂ (100 vol) in citrate/phosphate buffer pH4). After incubation for 15 min, the reaction was terminated by 0.1 M citric acid. The absorbance of the plates was read

at 405 nm with a spectrophotometer. The colour change was proportional to the amount of antigen in the test solution.

Results

Plates passively coated with polyclonal anti-A4 antibody were used for the CSF samples of the patient and control groups. In addition, in order to draw a standard curve, different synthetically produced A4 peptide (A1075 Sigma) concentrations were prepared (50 μ g/ml, 10 μ g/ml, 1 μ g/ml, 0.1 μ g/ml, 0.01 μ g/ml and 0.001 μ g/ml) and constant monoclonal anti-A4 peptide antibody was used (1:1000 dilution). The interassay reproducibility (expressed in μ g/ml) was examined with A4 standard samples of the dilution curve. The interassay variation was monitored by running the assay three times. The detectable value of the assay was 1ng/well for synthetic peptide. The lower limit of the assay always present problems in ELISA. In our study, we used a lower limit of 1.5 times the background value.

The background was defined in two ways: either from a well containing all reagents except the sample, or from a well containing a known negative sample. The graph for absorbance versus the amount of A4 peptide was drawn (Figure 1). The A4 peptide concentrations in the CSF of the samples were determined with a standard A4 peptide concentration curve. The control and patient groups were sorted according to age groups (Table 1). The graph for the patient and control groups versus A4 peptide concentration was drawn (Figure 2).

When patients with head trauma were compared to the control group, increased A4 concentrations were determined in patients with head trauma. Older patients with trauma showed higher concentrations than both younger patients with trauma and the control group.

Discussion

In this sandwich-type enzyme immunoassay for β -A4 protein, antibody is labelled with classical peroxidase (Sigma, PRAM). The peroxidase-labelled Fab fragment of antibody is directed against monoclonal antibody which is against the 1-28 residues of the β -A4 fragment and the immobilised polyclonal antibody coated onto the surface of the wells of 96-well plates is directed against the 1-40 residues of β -A4. This sandwich-type enzyme

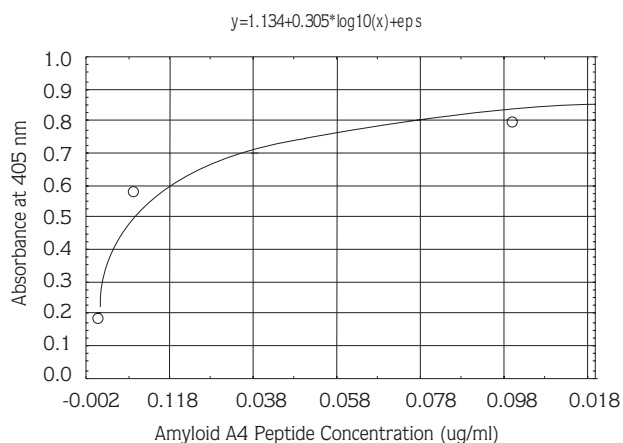


Figure 1. Passively polyclonal anti-A4 antibody-coated ELISA plates were used for different concentrations of synthetic A4 peptide (1-40 amino acid residues of APP). Monoclonal anti-A4 antibody was then used. The sandwich-ELISA complex was identified by enzyme-labelled anti-mouse immunoglobulins.

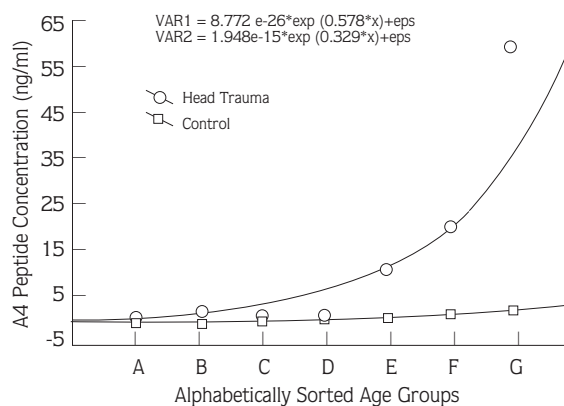


Figure 2. Polyclonal anti-A4 antibody coated ELISA plates were used for CSF samples of head trauma and control groups. Monoclonal anti-A4 antibody was then added. The sandwich-ELISA complex was identified by enzyme-labelled anti-mouse immunoglobulins. The graph was drawn according to the concentration values in Table 1.

Table 1. Patients and control group sorted according to age. Each group is listed alphabetically. Arithmetic means and SD of A4 absorbance values of CSF are given for each group. Concentration of A4 within CSF was calculated from the standard curve (Fig.1).

AGE GROUPS		Head Trauma, CSF A4 Absorbance and Corresponding Concentration (ng/ml)		Control, CSF A4 Absorbance and Corresponding Concentration (ng/ml)	
0-9	A	(n:3) 0.290±0.02	1.8±0.06 ng/ml	(n:4) 0.133±0.02	*
10-19	B	(n:3) 0.373±0.15	2.8±0.09 ng/ml	(n:3) 0.115±0.01	*
20-29	C	(n:4) 0.354±0.17	2.3±0.1 ng/ml	(n:1) 0.199	*
30-39	D	(n:3) 0.369±0.14	2.6±0.27 ng/ml	(n:1) 0.219	*
40-49	E	(n:3) 0.544±0.19	11.8±2.08 ng/ml	(n:2) 0.270±0.1	*
50-59	F	(n:7) 0.625±0.18	21±3.67 ng/ml	(n:1) 0.279	2.1 ng/ml
60-69	G	(n:7) 0.777±0.34	60.1±8.97 ng/ml	(n:2) 0.332±0.13	2.2±0.04 ng/ml

* Values were below or at the cut-off ELISA absorbance range

immunoassay is quite sensitive, detecting 1 ng β -A4 per well. The method is also specific, the cross-reactivity found with PRAM and immobilised polyclonal antibody being either undetectable or very small. The intra- and interassay reproducibility tests carried out with standard samples of A4 peptide gave results in the range of values which we obtained.

This study of 14 control subjects of both sexes between the ages of 7 and 62 allowed us to show a very low level of absorbance at 405 nm which was not significant for ELISA detection. However, the CSF

samples of the head-trauma patients exhibited increasing concentrations of A4 together with age, time of LP (lumbar puncture) and severity of trauma. The earliest-performed LP in the patients was 5 h after the accident occurred. The relationship between traumatic brain injury and the precocious development of neurodegenerative cascades, including diffuse deposits of β -A4 proteins in the injured brain and the leaking of it in CSF, will enable the design of a diagnostic test for brain-cell degeneration. At present, no diagnostic technique for Alzheimer's disease, motor neurone disease or similar

neurodegenerative disease exists for patients except at postmortem. Neuronal cell death is a very important factor in all these diseases and in severe head trauma, involving the deposition of β -A4 protein. It has been postulated that on the death and subsequent lysis of these cells, the previous constituents of the cells, including the β -A4 protein, will leak out into the CSF and serum. Thus, if it were possible to detect the β -A4 protein in a sample of CSF taken from a living patient suspected of having a neurodegenerative disease or severe neuronal damage, an accurate diagnosis could be reached while the patient was still alive.

It has been shown that A4 peptide can be detected immunocytochemically at sites of axonal injury in the brain. It was recently found to be a useful marker for

injured axons in patients who survived for only 3h after head trauma (11). β -A4 immunocytochemistry may be useful in the detection of traumatic axonal injury in its early stages (12).

Animal experiments have revealed similar results. In one study, rats were subjected to brain injury. At 1h, 2h, 48h, 1 week or 2 weeks after injury, the animals were killed and their brains were analysed immunohistochemically. β -A4 immunoreactivity increased as early as 1h after injury and persisted for at least 2 weeks (13).

In conclusion, by using the Sandwich-type ELISA method described in this study, it is possible to measure the A4 level within CSF, and it may indicate the extent of neuronal degeneration caused by trauma.

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