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Naturally occurring Antibodies against Synthetic Fragment of Neurotrophin Receptor P75 in Sera of Cognitively Impaired Patients

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Abstract

Aims: Alzheimer's disease is characterized by extracellular inclusions of beta-amyloid peptide. This peptide interacts with neuronal receptors in the human brain such as neurotrophin receptor P75 and neuronal acetylcholine receptor (nAChR) α7-type and pulls them down into plaques. Degraded receptors may introduce new antigenic epitopes to be recognized by specific antibodies in AD patients. The aim of the present work was to estimate the levels of the naturally occurring antibodies against two peptides derived from neuronal receptors –P75 and α7-type nAChR in sera of patients with different cognitive impairment.

Methods: We have used short fragments from the extracellular part of each receptor that participates in the interaction with beta-amyloid peptide, and measured levels of antibodies against them in the serum of different groups of patients (n = 74) by ELISA.

Results and Conclusion: We found antibodies against the fragment of P75 receptor only. These levels tend to be higher in patients with mild cognitive impairment. This study provides findings suggesting the immunologic response to neurotrophin receptor P75 in cognitively impaired subjects.

Keywords: Neuronal receptors; Peptides; Beta-amyloid; Serum antibodies; Alzheimer's disease; Cognitive impairment

Introduction

To date, a lot of effort was made to clarify the pathology of Alzheimer's disease (AD), because of its prevalence in other forms of dementia. The most studied agent in the pathology of this disease has been a beta-amyloid peptide. During AD pathology, several receptors are involved in interaction with toxic beta-amyloid oligomers on the neuronal cell surface which leads to irreversible changes in cells and at the end results in their death [1-3]. We have focused on two neuronal proteins that have been shown to participate in the pathogenesis of AD – neurotrophin receptor P75 and neuronal acetylcholine receptor (nAChR) α7-type [1,4]. Both degraded receptors are found in neuritic plaques in postmortem brain tissue from patients with AD [5,6]. The dying neurons release cell content into extracellular space and released neuronal proteins can be presented to phagocyte/immune system for elimination. Several studies showed differences in specific antibody levels in serum of AD patients and healthy seniors against beta-amyloid and tau protein [7-12].

We propose that presence of natural antibodies against such neuronal receptors, as α7 nAChR and P75, in sera samples of individuals with cognitive impairment can differ from the levels in sera of healthy donors.

In the present research, we have used fragments from extracellular regions of each protein in an ELISA assay to measure and compare levels of naturally occurring antibodies against α7 nAChR and P75 receptors in the groups of cognitively healthy subjects, AD and MCI patient.

Materials and Methods

Participants

All participants signed an informed consent. The research was approved by the Ethics Committee of the University Hospital Kralovske Vinohrady and conducted in accordance with The Declaration of Helsinki.

Serum samples were obtained from 74 participants at the Charles University in Prague, Department of Neurology or Memory Clinic, Czech Republic. Their cognitive functions were evaluated using an updated Czech version of Addenbrooke's Cognitive Examination Revised (ACE-CZ) [13,14]. We were then able to derive MMSE scores from the ACE-CZ. The majority of participants underwent Brain MR or CT imaging and lumbar puncture as part of a routine diagnostic investigation. The biochemical assessment of diagnosis in all participants was done by measuring levels of total tau, phospho-tau$_{181}$ and beta-amyloid$_{42}$ in CSF by commercial Innotest ELISA kits from Fujirebio (Gent,

Belgium) (Table 1). The normal elderly controls were recruited as in-patients from the Department of Neurology. They presented with non-inflammatory conditions, mostly with polyneuropathy and peripheral Bell´s facial palsy, the rest presented with a variety of diseases (e.g., a headache, trigeminal neuralgia and transient unconsciousness). A group of 45 AD patients was categorized to a subgroup of 18 patients with mild cognitive impairment (MCI due to AD; MCI) and a subgroup of 27 patients with mild dementia (Dementia due to AD; AD). They met a diagnosis of AD according to the NIA-AA criteria [15].

Serum samples were collected, centrifuged, and aliquoted in 1 mL polypropylene tubes and stored (on average within 1.5 hours of sampling) at −80°C until analysis. The specimens were thawed just before antibody measurements

Solid-phase peptide synthesis

Peptide fragments with sequence 155-164 aa from P75 receptor and 173-193 aa from the α7-subunit of nAChR were chosen and synthesized as described previously [16,17]. Peptide from P75 receptor was synthesized in a dimeric form bound by ε-aminocaproic acid for better coating on the plates without additional conjugation with a carrier protein. Synthetic peptides were purified by preparative reverse-phase HPLC. The purity of the obtained peptides was estimated as >95%. The data of peptides are summarized in Table 2.

ELISA assay

The levels of specific naturally occurring antibodies against two neuronal peptides in sera of 3 different groups were measured by ELISA. All samples were measured in duplicate. The assay was optimized to exclude non-specific signal from sample by measuring samples in non-coated, blocked wells with detection by secondary antibody and using negative and positive controls. The assay was carried out at room temperature if not stated otherwise. Blocking buffer (1% BSA in PBS-0.1% Tween 20 (PBS-T) pH 7.2) was used for dilution of all antibodies. Briefly, 0.1 ml/well 20 μg/ml peptide (155-164) from P75 and peptide (173-193) from α7 nAChR in 0.1M sodium bicarbonate buffer pH 9.5 was coated onto the wells of microplate (Gama Group, Ceske Budejovice, Czech Republic) overnight at 4°C. Plates were blocked for 2 hours and washed three times with 0.3 ml/well 0.1% BSA in PBS-T. Subsequently, 0.1 ml/well diluted serum samples (1:20 and 1:40) or rabbit polyclonal antibody against peptide AChR (173-193) [18] as a positive control (1:500) was applied to the wells and incubated for 1 hour. After washing step, 0.1 ml/ well F(ab')2-goat anti-human IgG (Fc specific, highly cross-adsorbed/HRP conjugate, Novex, Life Technologies Carlsbad, CA, USA) at dilution 1:20,000 or Goat anti-rabbit IgG HRP conjugate (Sigma-Aldrich, St. Louis, MO, USA) at dilution 1:10,000, was applied for 30 minutes. It followed by four times washing and the final incubation with TMB substrate for 25 minutes in the dark. The developing color signal was stopped by 0.1 ml/well 1M H_2SO_4 and the absorbance was measured within 30 min after stopping by a Multiskan EX ELISA reader (Thermo Scientific) at wavelength 450 nm and 620 nm as a reference wavelength.

Table 1: Demographic, cognitive and cerebrospinal fluid characteristics of subjects.

	Controls	Mild Cognitive Impairment due to AD	Dementia due to AD	Kruskal-Wallis test
N of subjects	29	18	27	
Female sex (%)	55	50	63	
Age (years)	67 ± 9	74 ± 6*	74 ± 8**	X^2 (2)= 12.51, p = 0.0019
MMSE score[a]	28.9 ± 1.4	25.8 ± 3.2**	19.6 ± 5.3***	X^2 (2)= 36.09, p < 0.001
Total tau[b] (pg/ ml)	200 ± 101	592 ± 501***	701 ± 471***	X^2 (2)= 33.95, p < 0.001
Phospho-tau[b] (pg/ ml)	32 ± 23	64 ± 37***	64 ± 35***	X^2 (2)= 13.14, p < 0.001
Beta-amyloid[b] (pg/ ml)	976 ± 311	586 ± 314**	635 ± 290***	X^2 (2)= 18.99, p < 0.001

Data are presented as the mean ± SD. Statistical significance (Mann-Whitney test) was calculated with respect to controls (* p < 0.05, ** p < 0.01, *** p < 0.001). [a]Mini-Mental Score examination. [b]The concentrations of total tau, phosho-tau and beta-amyloid in cerebrospinal fluid were measured by Innotest® hTau Ag, Innotest® Phospho-tau(181P) and Innotest® β-amyloid$_{(1-42)}$ from company Fujirebio according to manufacturer's instructions.

Table 2: Characteristics of synthetic peptides from P75 neurotrophin receptor and α7 subunit of the nAChR

Peptide abbreviation	Amino acid sequence[a]	Retention time (min)	Molecular mass	
			Calculated, monoisotopic	Found by Mass Spectrometry
P75 (155-164)$_2$Acx[b]	SDEANHVDPC-Acx-SDEANHVDPC-G	10.38	2322.9	2324.8
AChR (173-193)	EWDLVGIPGKRSERFYECCKE	8.06	2543.2	2544.8

[a]Numeration of the peptides is given according to Swiss-Prot database, accession number Q5W554-for human AChR α7-subunit and UniProtKB P08138-for human P75.
[b]Acx – ε-aminocaproic acid.

Statistics

Data were analyzed with GraphPad Prism software Version 5.0 from GraphPad (San Diego, CA, USA) and with software STATISTICA version 9.0 from StatSoft (Tulsa, OK, USA). The global statistical analysis was performed by the Kruskal-Wallis test and for pairwise comparisons we used Mann-Whitney-Wilcoxon test. A p-value <0.05 was considered statistically significant.

Results

Table 1 shows basic characteristics of the 74 study participants. The participants in the MCI and AD groups were older than healthy subjects. However, the analysis of age covariance did not show any effect on the analysis. Statistical analysis revealed significant drops in MMSE score in all groups with cognitive impairment when compared to controls. The CSF biomarkers showed significant differences between groups of MCI and AD patients when compared to healthy subjects.

We measured levels of naturally occurring antibodies against an extracellular non-structural loop of α7 nAChR and P75 receptors. Characteristics of peptides used in this study are indicated in Table 2. We were able to detect naturally occurring antibodies against one fragment only, particularly against P75 peptide. The AChR peptide showed very weak signal if any in all sera of subjects (Figure 1A). For P75 peptide, the presence of antibodies was shown in all the groups. We observed only a tendency for an elevation of antibodies levels against P75 fragment in the MCI group in comparison to control group (Figure 1B), but without statistical significance (Mann-Whitney test, $p = 0.07$). We did not observe any correlation between antibody levels and concentrations of CSF biomarkers.

Discussion

Previously, we have shown that immunization of mice either with synthetic fragment (155-164) from neurotrophin receptor P75 or with peptide (173-193) from α7-subunit of the nAChR prevented the loss of the memory and led to a significant decrease in the level of brain beta-amyloid observed in the animals with experimentally induced form of AD [16,17]. Moreover, it was shown that antibodies specific to synthetic fragment (173-193) from α7 nAChR protected neuronal cells against beta-amyloid toxicity in tests in vitro [18]. Thus, our previous results have demonstrated that antibodies specific to these particular sequences of each protein can play a functionally important role in the AD pathology. Therefore, the presence of naturally occurring antibodies against these significant parts of these receptors could be a hallmark of irreversible processes leading to the development of the disorder.

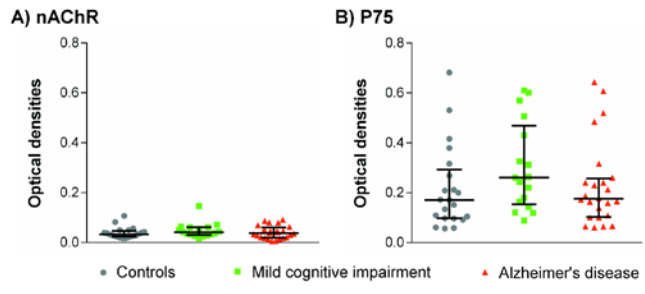

Figure 1: Serum antibodies levels against the fragment of the α7-subunit of nAChR (173-193 aa)
(A) and fragment with sequence (155-164 aa) derived from neurotrophin receptor p75.
(B) in control group of cognitively normal subjects, patients with mild cognitive impairment and patients with dementia due to Alzheimer's disease. Lines represent median values with error bars showing 25th -75th percentiles.

According to our current data from ELISA measurements of sera samples, there were naturally occurring antibodies only against the fragment (155-164) from neurotrophin receptor P75 (Figure 1). The levels of antibodies against the peptide (173-193) from nAChR turned out to be under detection limit in all investigated groups. The density of nAChR α7-type is relatively low and moreover is decreasing during AD [19] and that could explain an absence of antibodies against it in all groups of individuals. On the contrary, the P75 receptors are more abundant in the brain but also found in other tissues such as perivascular cells, dental pulp cells, lymphoidal follicular dendritic cells, basal epithelium of oral mucosa and hair follicles, prostate basal cells, myoepithelial cells and hepatocyte cells [20-24]. However, the expression level of P75 remains stable or down-regulated in adult age and is increased only during some forms of cancer, injury or neurodegeneration that is characteristic of AD pathology [25-27]. This is in agreement with detected basal levels of natural antibodies against P75 in cognitively normal individuals. Moreover, we observed elevated levels of antibodies against the P75 peptide in sera samples of MCI patients, which can reflect an initial pathological increase of P75 receptor levels in these patients. However, this condition could not be sustainable possibly leading to the drop of antibodies levels with the progression of neurodegeneration. The MCI group is, however, small. Therefore, these preliminary results should be confirmed by analysis of a larger cohort of MCI patients. Nevertheless, considering a difference between the two investigated fragments, the peptide (155-164) from neurotrophin receptor P75 appears promising for detection of naturally occurring antibodies in human sera and observing early changes in the course of memory impairment due to neurodegeneration.

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Conflict of Interest

The authors have declared no conflict of interest.

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