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Evaluation of a technique to identify acetylcholinesterase C-terminal peptides in human serum samples

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ABSTRACT

A novel theory for neurodegeneration is that non-cholinergic functions of acetylcholinesterase (AChE) are responsible for the progressive death of global neurons. The C-terminal region of AChE has been shown to be responsible for non-cholinergic actions of AChE by binding to an allosteric site on the alpha 7-nicotinic acetylcholine receptor, thereby causing calcium influx; the resultant signal has trophic effects in immature neurons, but toxic effects in mature neurons. Although there is strong *in vitro* and *in vivo* evidence for the involvement of this C-terminal region of AChE in neurodegeneration, a cleaved C-terminal peptide has not yet been identified in human brains. This preliminary study aimed to identify the cleaved AChE C-terminal peptide in serum from human Alzheimer's disease patients using immunoaffinity purification. A number of antibodies were tested for sensitivity and specificity towards peptide sequences from the C-terminus. Although the antibodies were able to identify peptide *in vitro*, peptide was not detected using immunoaffinity purification of human serum, possibly due to insufficient detection limits of the antibody. Therefore more sensitive techniques are required to identify cleaved AChE C-terminal peptides in human samples. None the less, C-terminal AChE peptide might act as a signalling molecule in an as yet unexplored system.

1. Introduction

The neurodegeneration characterising diseases such as Alzheimer's (AD), Parkinson's (PD) and motor neuron disease (MND), could be an aberrant form of normal development, triggered at the wrong time and in the wrong context, i.e. in response to an insult, stroke or accumulation of toxins. The neurons that are selectively vulnerable in all three of these diseases – basal forebrain, substantia nigra and motor neurons, respectively – are part of a wider yet distinct neuronal population called global neurons, comprising a continuous hub extending from brainstem to basal forebrain [38]. The term global neurons encompasses cholinergic and monoaminergic neurons which form a global network, generating widespread patterns of activity and operating as a unified system. Global neurons are neurochemically distinct from other 'serial' neurons which generally relay sensory information about the external and internal environment [12]. Global neurons appear to be capable of transforming incoming sensory data into memories [38]; as part of this function, global neurons retain plasticity into maturity [4,15] and are therefore sensitive to trophic factors which enhance calcium entry into the neurons [38] in order to stimulate intracellular signalling pathways. However, calcium tolerance declines significantly in maturity, therefore calcium influx stimulated by trophic factors may cause toxicity and neuronal cell death. The more cells die, the more their death will provide the trigger for the process to repeat as global neurons attempt to replace damaged cells; this would instigate a vicious cycle of neurodegeneration. The not infrequent co-pathology of these diseases suggests a common feature that might be pivotal to neurodegeneration [17]; we suggest that if large areas of these global neurons are damaged, then more than one neurodegenerative disease state will occur [17]. A possible causative factor behind this mechanism of neurodegeneration is suggested by a common feature of global neurons; regardless of the mode of synaptic transmission used, all express AChE even though the substrate acetylcholine is not always present within the various nuclei [35]. Our group have been among the first to show that AChE has novel, non-classical actions including the enhancement of outgrowth of neurons [10,18,19,21,22]. Moreover, AChE is now well-established generally as a signalling molecule that has trophic activity in a wide variety of tissues and situations [20,23–26,36]. The Greenfield group identified the salient region of the AChE molecule, a C-terminal domain peptide [10], which could mediate such non-classical actions and also demonstrated toxic effects including cell death due to increased calcium influx [5,9,13].

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; PD, Parkinson's disease; MND, motor neuron disease; ACh, acetylcholine; α7-nAChR, alpha 7-nicotinic acetylcholine receptor.

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More recently, they obtained direct evidence that the respective target for this peptide is an allosteric site on the alpha 7-nicotinic acetylcholine receptor (α7-nAChR), interaction with which causes calcium influx and up-regulation of receptor expression [6,39].

Two forms of globular AChE are predominantly present in the brain; a soluble monomeric form (G1) and a tethered tetrameric form (G4) which is bound to cell membranes [16]. In Alzheimer's disease, levels of G4 expression decrease, whereas those of G1 increase [3,33,34]. G1 has also been identified in developmental situations, e.g. human umbilical vein endothelial cells [29] and fetal bovine serum [31]. Interestingly, analysis of G1 isolated from fetal bovine serum has shown that the C-terminal region is not present [31], perhaps because it has been cleaved to function as an independent signalling molecule in these scenarios of degeneration and development. This preliminary study aimed to identify cleaved AChE C-terminal peptide in human serum samples from Alzheimer's disease patients, compared with age-matched controls, using immunoaffinity purification.

2. Materials and methods

2.1. ELISA

Anti-AChE antibodies were tested for specificity and sensitivity towards AChE C-terminal peptides using conventional enzyme-linked immunosorbent assay (ELISA) [14]. Peptides were obtained from Genosphere (Paris, France), truncated erythrocytic AChE not coding for the C-terminal region (T548) was a kind gift from Palmer Taylor (Department of Pharmacology, University of California, San Diego, CA, USA), and full length synaptic AChE and beta amyloid peptide amino acids 25–35 (AβP) were obtained from Sigma–Aldrich (Dorset, UK). The 96-well plates were coated (in duplicate) with 0.5 μg antigen at 100 μl/well diluted in TBS. The plates were incubated overnight at 4 °C, washed with Tris-buffered saline (TBS) 0.1% Tween-20 (TBST) and blocked with 2% chicken ovalbumin for 1 h at 4 °C. Primary antibodies were diluted in TBST and incubated at 4 °C for 1 h. The wells were washed thoroughly with TBST, and incubated for 1 h at 4 °C with alkaline phosphatase conjugated secondary antibody (Sigma, Dorset, UK) diluted in TBS. Plates were washed with TBST and colour was developed by adding 100 μl of the substrate, p-nitrophenyl phosphate (Sigma, Dorset, UK) and incubating the plates at RT for 30 min; absorbance at 405 nm was recorded. All primary antibodies were purchased from Santa Cruz Biotechnology (CA, USA); sc80614 (4E11), sc6431 (N-19), sc60430 (C-16), sc11409 (H-134), sc-58481 (190-01) and sc6432 (E-19).

2.2. AChE C-terminal peptide interactions with albumin

T30 peptide (250 ng) diluted in 200 μl PBS was incubated at 37 °C for 1 h, either alone or with 10 mg/ml bovine serum albumin (BSA) and then separated using centrifugal molecular weight cut-off (MWCO) columns (Millipore, Watford, UK). Columns were centrifuged at 4 °C for 15–60 min depending on the MWCO of the column until all liquid had passed through, taking care not to totally dry out the column membrane. Protein concentration of filtrate and retentate was measured in triplicate using DC Protein Assay (Bio-Rad, Germany), absorbance recorded at 590 nm and the percentage of loaded protein recovered in each fraction calculated. ELISA, as described in Section 2.1, was used to measure AChE C-terminal T30 peptide in filtrate and retentate.

2.3. Immunoaffinity purification of AChE C-terminal peptides from human serum

The anti-AChE C-terminal specific antibody (4E11, SantaCruz, CA, USA) was used to carry out immunoaffinity purification (IP) of native serum samples from AD patients and age-matched controls. Serum samples were kindly donated by Professor Nigel Hooper, Leeds University. AD patients were recruited through memory clinics in Leeds and Dewsbury (West Yorkshire, England). Control subjects were recruited through the Leeds Family Health Services Authority, day hospitals and elderly medicine outpatient clinics within the Leeds area. All participants were of European Caucasian extraction and gave written informed consent (assent from relatives of the AD patients was given where appropriate) according to a protocol approved by the Leeds Teaching Hospitals NHS Trust Research Ethics Committee. 100 μg 4E11 diluted in PBS was bound per 100 μl of protein A/G agarose bead resin (Pierce, Northumberland, UK) and cross-linked using DSS (Pierce, Northumberland, UK). Spin columns were used to carry out the IP (Pierce, Northumberland, UK). 500 μl serum samples were pre-cleared using 4% beaded agarose to reduce background interactions and the flow through incubated with 50 μl antibody-bound beads over night at 4 °C with gentle rocking. After thoroughly washing off un-bound proteins with PBS, acid elution of bound peptides was carried out using 50 μl 100 mM glycine–HCl pH 2.8 per elution. Eluted samples were separated using 16% tricine SDS-PAGE [32]. In-gel rapid silver staining of protein bands was used [30] as well as immunodetection of AChE C-terminal peptides following electrotransfer of proteins onto nitrocellulose [32].

3. Results and discussion

3.1. Selecting an antibody for identifying AChE C-terminal peptide

A number of commercially available AChE antibodies were tested for specificity and sensitivity towards a number of different AChE C-terminal peptides (Fig. 1a). All antibodies detected full length AChE whereas those specific to the N-terminus of AChE (N-19 and E-19) could not detect AChE C-terminal peptides T40, T30 and T15 (Fig. 1b). Antibodies specific to the C-terminus (C-16, 190-01 and 4E11) detected all three AChE C-terminal peptides as well as the full length AChE, but not truncated AChE (T548) which does not express the C-terminal region as expected (Fig. 1b). H134 raised against full length AChE was able to detect AChE, T548 and T15. AβP is also associated with neurodegeneration and homology between the β-amyloid precursor protein and the AChE C-terminus was used by this group to identify the 40 amino acid region of AChE potentially involved in neurodegeneration [17]. For this reason, it was important that the antibodies used did not cross-react with AβP which may also be present in serum from patients with neurodegeneration. The antibodies tested did not react with the biologically active 25–35 AβP fragment. The antibody 4E11 was chosen to carry out further studies to identify the AChE C-terminal peptide in human serum samples. The sensitivity (Fig. 1c) and affinity (Fig. 1d) of 4E11 was tested and shown to detect levels of T30 peptide down to 100 nM using ELISA.

3.2. Interactions of peptide with albumin

Albumin is a highly abundant serum protein which can associate with small peptides, preventing their removal during kidney filtration of the blood and extending their half-life [11,27]. The interaction between the biologically active AChE T30 peptide and albumin was investigated due to the fact that human serum samples were to be analysed for the presence of peptide; an important factor contributing to whether or not peptide would indeed be present in these serum samples if the half-life of the peptide is increased due to albumin interactions, therefore preventing immediate excretion into the urine by the kidneys. MWCO columns were used to investigate the interactions between BSA and T30 peptide

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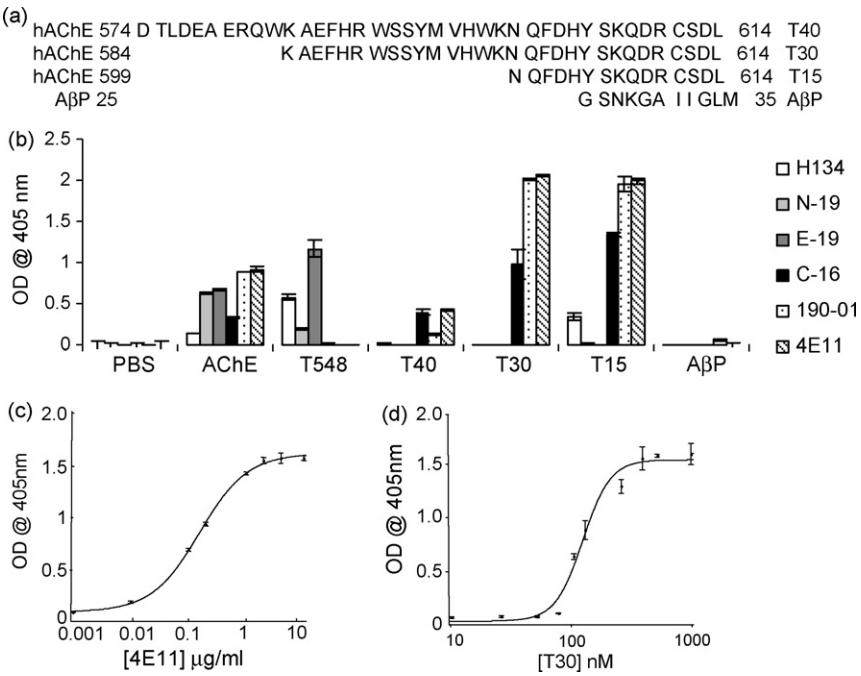

Fig. 1. Testing AChE antibodies using ELISA. Specificity of the following AChE antibodies were tested (b); H134 against full length AChE, N-19 and E-19 against the N-terminus, and C-16, 190-01 and 4E11 against the C-terminus of AChE. The antibodies were tested against 0.5 µg of full length human AChE (AChE), truncated AChE amino acids 1–548 (T548) and AChE C-terminal peptides (T40, T30, T15 and T14) (a). Amyloid beta peptide (AβP) and PBS were used as a negative control. (c) Sensitivity of 4E11 was tested against the T30 peptide (250 nM) and varying concentrations of T30 (d) tested with 2 µg/ml 4E11. Graphs show standard error bars from two separate experiments carried out in triplicate.

as these columns allow rapid separation of proteins depending on the MW, therefore separating un-bound T30 (3.8 kDa) from BSA (69.3 kDa). Alone, approximately 100% of BSA loaded was retained by the 3 and 50 kDa column, with half the amount retained by the 100 kDa column (Fig. 2a). Alone, T30 was only retained by the 3 kDa column suggesting that the peptide does not form oligomers of large enough molecular weight to be retained by 50 or 100 kDa MWCO columns under these conditions (Fig. 2b). Analysis of BSA/T30 incubated samples showed high levels of T30 peptide in retained fractions from the 3, 50 and 100 kDa columns, above the MW of the peptide (Fig. 2a) and in the same fractions which showed high levels of BSA (Fig. 2b). This suggests that T30 peptide interacts with albumin causing the retention of the peptide at MW significantly higher than the 3.8 kDa of the peptide since alone, the peptide was not retained in these fractions. Interaction of the peptide with albumin would increase the half-life of the peptide in the blood stream and therefore increase the chances of detection.

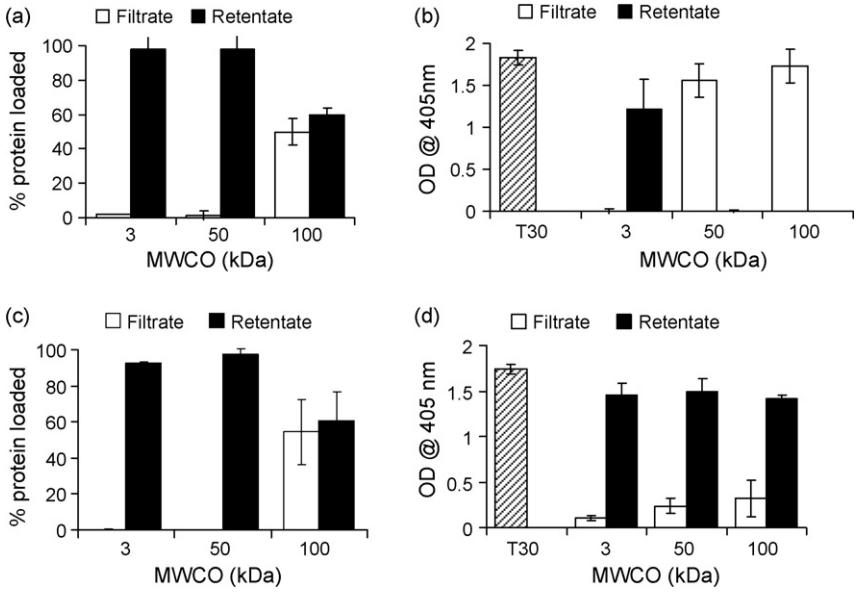

Fig. 2. AChE C-terminal peptide interactions with albumin. 200 µl of 1.25 µg/ml T30 AChE C-terminal peptide and 10 mg/ml BSA were diluted in PBS and centrifuged through 3, 50 and 100 kDa MWCO columns either alone (a and b) or after incubation together for 1 h at 37 °C (c and d). DC protein assay was used to measure total protein in filtrate and retentate and the percentage of total protein loaded recovered in each fraction shown (a and c). ELISA using 4E11 antibody was carried out on filtrate and retentate to detect T30; 250 ng T30 was used as a positive control (b and d). Graphs show standard error bars from two separate experiments carried out in triplicate.

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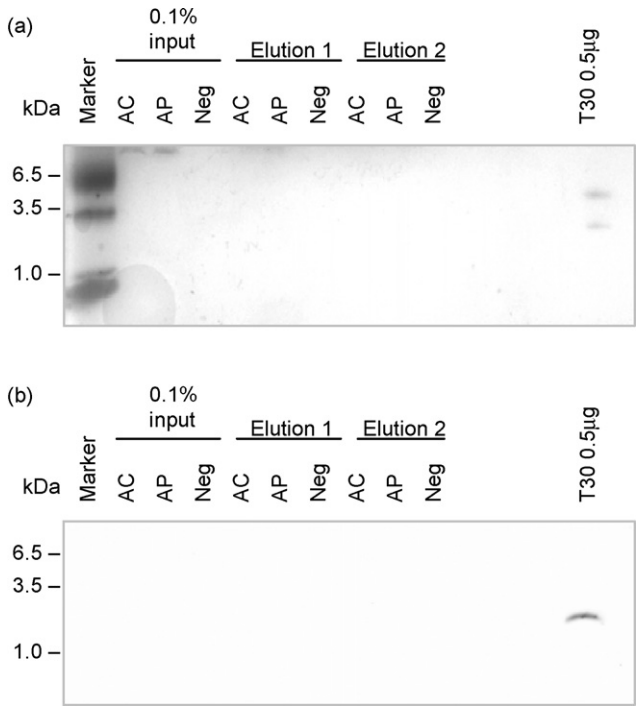

Fig. 3. Immunoaffinity purification of human serum samples. IP of control (AC) and patient (AP) serum samples was performed using 4E11. PBS was used as a negative control (Neg). Acid elution was performed twice and elutes separated by tricine SDS-PAGE and analysed by (a) silverstain and (b) western blotting, probing with 4E11. 0.5 μg T30 was used as a positive loading control.

3.3. Identification of peptide in human samples using immunoaffinity purification

IP of native human serum from AD patients (AP) and age-matched controls (AC) was performed using the 4E11 antibody against the AChE C-terminal. Eluted material was separated using tricine SDS-PAGE and analysed by silver stain in-gel protein detection (Fig. 3a) and by western blotting immunodetection (Fig. 3b). These experiments were carried out for six further samples of patient and control serum and similar results gained as shown in Fig. 3. No bands correlating to the weight of T30 were identified using silver staining and no bands were detected by western blotting using AChE C-terminal specific antibodies; however, 500 ng of T30 (25 mg/l) was loaded as a positive control in both cases and was only weakly detected. Low abundance peptides, which we expect the AChE C-terminus peptide to be, are generally below the ng/l concentration [2], therefore this technique does not appear to be suitable for these detection limits using the current method. A considerably more sensitive antibody would be required in order to pursue this technique as well as concentration of serum samples, possibly by deproteination and reduction of volume using vacuum drying.

4. Conclusions

The IP method used in this study does not have adequate sensitivity to detect AChE C-terminal peptide in serum. A number of changes to the method could be carried out in order to increase the probability of detecting AChE C-terminal peptides which are discussed below. The use of a custom-made antibody would be advantageous as this would not only produce antibody on a large-scale, but would also enable the production of a more sensitive antibody to a specific AChE C-terminal region. However, initial attempts to produce a custom antibody by this group have proved difficult due to the highly conserved nature of the AChE C-terminus between humans and animals commonly used for this purpose. Deproteination of serum, for example by using acetonitrile or trichloroacetic acid to precipitate larger proteins, would allow the concentration of samples so that more could be loaded onto the column without overloading. Freeze-vacuum drying of sample and reconstituting in smaller volumes would also further concentration of peptide. The use of liquid chromatography followed by mass spectrometry (LC/MS) to analyse deproteinated serum samples would be another technique to consider as this would allow the analysis of numerous peptides present in the serum. This would have the advantage that different versions of the AChE C-terminal sequence could be detected; since the exact region of the AChE C-terminus involved is unknown, separation of different peptides by liquid chromatography would allow subsequent analysis to ascertain the activity and potential involvement in neurodegeneration. Incorporating such refinements into the method would be a worthwhile undertaking as the non-cholinergic properties of AChE C-terminal peptides present an interesting potential for a previously unexplored signalling mechanism.

Conflict of interest

None.

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