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Pyroglutamate-A β 3 and 11 colocalize in amyloid plaques in Alzheimer's disease cerebral cortex with pyroglutamate-A β 11 forming the central core

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Abstract

N-terminal truncated amyloid beta (A β) derivatives, especially the forms having pyroglutamate at the 3 position (A β pE3) or at the 11 position (A β pE11) have become the topic of considerable study. A β pE3 is known to make up a substantial portion of the A β species in senile plaques while A β pE11 has received less attention. We have generated very specific polyclonal antibodies against both species. Each antibody recognizes only the antigen against which it was generated on Western blots and neither recognizes full length A β . Both anti-A β pE3 and anti-A β pE11 stain senile plaques specifically in **Alzheimer's disease** cerebral cortex and colocalize with A β , as shown by confocal microscopy. In a majority of plaques examined, A β pE11 was observed to be the dominant form in the innermost core. These data suggest that A β pE11 may serve as a generating site for senile plaque formation.

Introduction

During the last twenty-five years, much evidence has linked the onset of Alzheimer's disease (AD) to the accumulation of a variety of forms of the amyloid beta (A β) peptide [11]. Full-length A β (amino acid residues 1–40 and 1–42) has been the dominant foci of research, but amino (N) and carboxy-terminally truncated as well as modified, forms of A β also exist. When N-terminal truncation exposes a glutamic acid residue, the amino terminus of A β can become pyrolyzed forming a stable ring [3]. One of these post-translationally modified forms of A β , pyrolyzed A β 3-x (A β pE3), is abundant in brain regions affected in AD [4, 8, 9, 15, 21, 22]. A second form of pyrolyzed A β , A β 11-x (A β pE11) has received less attention, but also colocalizes with A β 1–40/42 containing plaques in AD brain [7, 12]. This presence of A β pE3 and A β pE11 peptides in AD brains is in contrast to full length

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forms of A β that predominate in non-demented elderly control brain tissue [7, 13, 22]. In brain tissue from subjects with Down's syndrome, pathologically similar to that of AD [10], A β pE11 has been identified even before birth [7]. How the various N terminally truncated species of A β , as well as the post-translationally modified derivatives of these species, are generated, and how they contribute to neurodegeneration, are currently the subject of intense research [3].

Studies thus far indicate that generation of A β pE3 is a multi-step process. The first two N-terminal amino acids of A β are sequentially cleaved intracellularly by aminopeptidase A [19]. This cleavage is then followed by pyrolysis of the resulting N-terminal glutamic acid, producing A β pE3 thus rendering it more resistant to further degradation. Cloning of the β -site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE 1) has demonstrated that A β E11 can be generated directly following BACE-1 cleavage of APP [20] followed by γ -secretase cleavage. Additionally, the major proteolytic product of APP, C99, can also produce A β E11 through sequential cleavage by BACE 1 and γ -secretase [6].

Production *in vitro* of A β pE3 and A β pE11 is extremely slow but glutaminyl cyclase (QC) in the brain, predominantly localized in the Golgi apparatus [1], rapidly catalyzes conversion of A β E3 to form A β pE3. QC also catalyzes conversion of A β E11 to A β pE11 [18]. A β pE rapidly adopts a β -sheet conformation and is significantly more toxic and stable than unmodified, full length A β [2, 14, 16]. Recent studies demonstrate increased A β pE3 levels and early accumulation of A β pE3 oligomers in neurons in a transgenic mouse model for AD and in neurons of patients with AD [21]. Passive immunization of the transgenic mice with an antibody that selectively recognizes oligomeric assemblies of A β pE3 not only reduced A β pE3 levels but also normalized behavioral deficits [22]. Moreover, when the transgenic mouse model with abundant A β E3 formation, was crossed with transgenic mice expressing human QC (hQC), the brain tissue from their bigenic progeny showed significant elevation in soluble and insoluble A β pE3 peptides and greater amounts of A β pE3 in plaques. When 6-months old, these bigenic mice also had significant motor and working memory impairment compared to non-hQC transgenic mice. The contribution of endogenous mouse QC (mQC) was examined by then knocking out mQC in the single transgenic AD mouse model. The mQC-KO mice showed significant rescue of wild-type mouse behavioral phenotype [5]. In the same transgenic mouse line, pharmacological inhibition of QC activity produced the same effects as QC KO [17]. The collective data from these strongly support the notion that a A β pE peptide(s) plays a key role in the neuropathology of AD.

To date, there are no studies which have simultaneously examined the deposition and spatial localization of A β pE3 and A β pE11 in brain tissue from AD subjects. To perform these studies, we have generated antibodies that recognize A β pE3 and A β pE11 specifically and used these reagents to elucidate the spatial relationship of A β pE3 and A β pE11 to one another, as well as to A β in AD brain tissue. Here we find that both A β pE3 and 11 are abundant in amyloid plaques and that they colocalize with each other as well as with A β . Additionally, we noted a higher level of A β pE11 in plaque cores when compared with A β pE3 and full length A β species. This observation supports the hypothesis that A β pE11 may be an early aggregating form of A β and thus act as the seed or nidus for senile plaque formation.

Materials and Methods

Peptide synthesis and purification

All A β peptides were synthesized using standard Fluorenylmethyloxycarbonyl chemistry. Synthesized peptides are cleaved off the resin using trifluoroacetic acid (TFA). Crude peptides were purified using preparative reversed-phase high pressure liquid

chromatography (HPLC) using a water and acetonitrile with 0.1 TFA for elution. Peptides were purified to >85% purity as determined by analytical HPLC.

Antibody production

Purified peptides were conjugated to a proprietary mix of carrier proteins (21st Century Biochemicals) and injected into New Zealand white rabbits (Cocalico Farms). A NIH-approved protocol was followed and includes both complete and partial *Freund's* adjuvant with 7 full production bleeds followed by a two-part exsanguination.

Antibody purification

Antibodies were purified using peptides linked to iodoacetyl agarose. Sera showing high antigen reactivity by ELISA against A β pE3–11 or A β pE11–19 respectively, were pooled and extensively immunodepleted with full length and truncated A β peptides. Depleted sera were then purified using either A β pE3–11 or A β pE11–19 and eluted using glycine. Eluates were neutralized and then dialyzed.

Western blotting

SDS-PAGE was carried out using 10–20% or 15% Tris/Glycine gels (Biorad) followed by transfer onto a 0.2 μ m PVDF membrane (Whatman). A slot blot apparatus (Immunetics, Boston, MA) was used to probe duplicate lanes with varying concentrations of antibody. Antibody specificity and sensitivity were detected using enhanced chemiluminescence.

Immunostaining

Double and triple immunofluorescence staining were carried out to assess colocalization by standard fluorescence microscopy or confocal imaging using a Leica SP5 laser scanning confocal microscope. 5 μ m paraffin embedded tissue sections from frontal cortex were obtained through the VA VISN1 Neuropathology Center. Sections were de-paraffinized in xylene and rehydrated through alcohol before being subjected to antigen retrieval in formic acid. Sections were blocked in Super Block buffer (ScyTek) containing 5% normal donkey serum. Primary antibodies [including anti-A β pE3 (21st Century Biochemicals, used at 0.5 μ g/ml) or A β pE11 (21st Century Biochemicals, used at 0.5 μ g/ml) Cy3-tagged anti-A β pE3 (21st Century Biochemicals), Cy5-tagged anti-A β pE11 (21st Century Biochemicals) and anti-A β (Dako, used at 0.5 μ g/ml)] were incubated with tissue sections overnight at 4°C. Where appropriate, sections were then incubated for 1 hour at room temperature with a biotinylated secondary antibody (2 μ g/ml) followed by avidin particles conjugated with either horseradish peroxidase (HRP) (ABC detection system, Vector Labs), Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen, used at 15 μ g/ml). This protocol (beginning with blocking procedures) was repeated for fluorescent secondary antigen detection in sequential staining procedures. Avidin HRP was detected by treating sections for 10 minutes with 3,5-diaminobenzoic acid (DAB) to yield a brown precipitate.

Thioflavin S staining

Rehydrated sections were stained with 1% Thioflavin S solution for 10 minutes. Stained sections were then dehydrated and rinsed in distilled water before mounting with aqueous mounting medium.

Results

To visualize A β pE in brain tissue sections, antibodies were developed against A β pE3 and A β pE11. The sensitivity of these antibodies for protein detection was analyzed by Western blot (Supplementary data. Figure S1). A β pE3–42 (loaded at ~10 ng/lane) was run on a 2D,

10–20% Tris/Tricine gel and immunoblotted with anti-A β pE3 antibody. Figure S1 shows that as little as 5 ng/ml of the antibody can detect A β pE3–42. **Similar results were obtained with the anti-A β pE11 antibody** (Figure S2). Blocking experiments demonstrated specificity of both anti-A β pE3 and anti-A β pE11 antibodies, with complete blocking using the peptide to which the antibody was produced and no blocking with A β 1–40, A β 3–9, or A β 11–19 (Data not shown). The two anti-A β pE antibodies also showed no cross-reactivity with one another (Data not shown).

Previous studies have reported the presence of A β pE species in A β plaques [7, 15]. To assess the colocalization of A β pE3 and A β pE11 with A β plaques using our antibodies, human frontal cortex tissue sections were double immunostained and analyzed by confocal microscopy. Figure 1 is representative of tissue sections labeled with an anti-A β antibody (A,D,G,J,M,P) together with either anti-A β pE3 (B,H,N) or anti-A β pE11 antibodies (E,K,Q). Both anti-A β pE3 and anti-A β pE11 are specifically blocked only by the peptide against which they were produced (K and N respectively), and are not blocked by A β 1–40 (Data not shown) or by a non-target peptide (H and Q). Both A β pE3 and 11 were found within A β containing plaques. The merged images show areas of colocalization (yellow) between A β and A β pE3 (C,I,O) or A β pE11 (F,L,R). Interestingly, A β pE11 was found to be present most heavily in plaque cores, while A β pE3 was observed to colocalize more completely with A β . No staining with either anti-A β pE3 or anti-A β pE11 was seen in the same brain region of 23 year old subject (data not shown).

To ascertain that the A β pE3 and A β pE11 containing plaques were composed of amyloid fibers, we localized each pyrolyzed species together with Thioflavin S, a compound that can be detected by fluorescence microscopy when bound to amyloid fibers. Figure 2 shows that both A β pE3 and A β pE11 colocalize with plaques labeled by Thioflavin S staining.

Although A β pE species have been shown individually to be present in amyloid plaques, their colocalization with each other has not been studied. To determine whether or not A β pE3 and A β pE11 colocalize in amyloid plaques, we used confocal microscopy employing purified fluorescently tagged primary antibodies against each derivative. We also employed a mouse monoclonal antibody against A β , which was detected in subsequent steps using a third fluorescent probe. All three primary antibodies were added simultaneously at the same concentration. A representative plaque (Figure 3) demonstrates that both pyrolyzed A β species are present in an A β containing plaque. **In order to determine what percentage of plaques contain each of the three A β species examined, 20 plaques were identified in each of 5 AD cerebral cortex samples using the same triple fluorescent labeling method. All three A β species were observed in each of the plaques examined.** Although all three antigens colocalize in much of the plaque, of particular interest (and in agreement with our previous observation) is the observation that A β pE11 is the dominant form of A β at the core of the plaque.

Discussion

In the present study, we generated two polyclonal antibodies against A β pE species and used them to observe the A β pE profile of human amyloid plaques. Evaluation of these antibodies by Western blot analysis and immunohistochemistry demonstrated that they bind specifically to their targets at low concentrations (Figures S1, S2 and 1).

In AD brain, A β pE3 and 11 were found in abundance in mature senile plaques (Figure 2) where they colocalize with full length A β as well as with each other (Figure 3). In these plaques, we consistently observed A β pE11 rich cores. This was not true of every plaque observed; however, with the use of 5 μ m tissue sections, many of the plaques observed are

not centrally cross sectioned. That being said, A β pE11 core plaques were prominent in the tissue sections observed. Future experiments using thicker tissue sections will strengthen the argument for or against A β pE11 cores. We hypothesize that intracellularly generated A β pE11 aggregates inside neurons, possibly into an oligomeric species as has been shown with A β pE3 [22]. **We hope to determine whether intraneuronal A β pE11 is present at an early stage of AD pathogenesis in future investigations.** We also hypothesize that aggregated A β pE11 enters the **extracellular** space either after cell death or by some other mechanism (e.g., secretion) and serves as seeds for the formation of extracellular amyloid plaques. Further, we predict that the amount of A β pE11 in the CSF and blood may be an early and specific detector of presymptomatic AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A β	Amyloid beta
AD	Alzheimer's disease
N	amino
APP	amyloid precursor protein
BACE 1	beta-site amyloid precursor protein cleaving enzyme 1
QC	glutaminy cyclase
HPLC	high pressure liquid chromatography
KO	knock out
A β pE3	pyrolyzed A β 3-x
A β pE11	pyrolyzed A β 11-x

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Highlights

- Antibodies raised against A β pE3 and A β pE11 recognize their specific antigen in senile plaques
- Both A β pE3 and A β pE11 colocalize with Thioflavin S, a marker of mature amyloid plaques
- A β pE3 and A β pE11 colocalize with each other in amyloid plaques
- A β pE11 species form cores in plaques surrounded by full length and A β pE3

Figure 1.

A β colocalizes with A β pE3 and A β pE11 in the frontal cortex. Confocal microscopy was carried out to assess colocalization of A β pE species with A β plaques. Human brain sections were subjected to immunohistochemistry with antibodies against A β pE3 (green, B,H,N), A β pE11 (green, E,K,Q), and A β (red, A,D,G,J,M,P). Specificity of antibodies to A β pE3 and A β pE11 was confirmed by pre-incubation of each with their target peptide (N or K respectively) or non-target peptide as control (H and Q respectively). Areas of colocalization of A β pE3 and A β (C,I,O) and of A β pE11 and A β (F,L,R) appear yellow in merged panels. White scale bars are 50 μ m in length.

Figure 2.

AβpE3 and AβpE11 are present in senile plaques. Sections from the frontal cortex of AD patients were stained with Thioflavin S dye (green, binds to β-pleated amyloid fibrils found within mature senile plaques). Immunostaining was also performed on the same sections using antibodies against AβpE3 (brown, top) and AβpE11 (brown, bottom). Overlap of AβpE3 and AβpE11 with β-pleated amyloid fibrils was visualized by merging the thioflavin stained and immunostained images.

Figure 3.

A β pE3 colocalizes with A β pE11 in A β plaques. Triple immunostaining was carried out using antibodies against A β pE3 (A), A β pE11 (B) and A β (D). Samples were examined by confocal microscopy for colocalization of A β pE3 and A β pE11 (C) and for colocalization of A β pE3, A β pE11, and A β (E). White scale bar is 50 μ m.