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IMMUNODOMINANT EPIOTOPE AND PROPERTIES OF PYROGLUTAMATE-MODIFIED A β -SPECIFIC ANTIBODIES PRODUCED IN RABBITS

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Abstract

N-truncated and N-modified forms of amyloid beta (A β) peptide are found in diffused and dense core plaques in Alzheimer's disease (AD) and Down's syndrome patients as well as transgenic mouse models of AD. Although the pathological significance of these shortened forms A β is not completely understood, previous studies have demonstrated that these peptides are significantly more resistant to degradation, aggregate more rapidly *in vitro* and exhibit similar or, in some cases, increased toxicity in hippocampal neuronal cultures compared to the full-length peptides. In the present study we further investigated the mechanisms of toxicity of one of the most abundant Ntruncated/modified A β peptide bearing amino-terminal pyroglutamate at position 3 (A β N3(pE)). We demonstrated that A β N3(pE) oligomers induce phosphatidyl serine externalization and membrane damage in SH-SY5Y cells. Also, we produced A β N3(pE)-specific polyclonal antibodies in rabbit and identified an immunodominant epitope recognized by anti-A β N3(pE) antibodies. Our results are important for developing new immunotherapeutic compounds specifically targeting A β N3(pE) aggregates since the most commonly used immunogens in the majority of vaccines for AD have been shown to induce antibodies that recognize the N-terminal immunodominant epitope (EFRH) of the full length A β , which is absent in N-amino truncated peptides.

Keywords

N-truncated amyloid beta (A β) peptide; Alzheimer's disease immunotherapy; immunodominant epitope

INTRODUCTION

The accumulation of fibrillar and oligomeric forms of amyloid-beta (A β) peptide in the brain has been hypothesized to play a central role in the neuropathology of Alzheimer's Disease

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(AD) (Masters et al., 1985; Hardy and Selkoe, 2002; Walsh and Selkoe, 2004; Haass and Selkoe, 2007). The main A β variants detected in the human brain are A β 1-40 and A β 1-42, however a significant proportion of AD brain A β consists also of N-terminal truncated/modified species (Mori et al., 1992; Seubert et al., 1992; Saido 1995; Saido 1996; Kuo et al., 1997; Tekirian et al 1998; Russo et al 2001, Miravalle et al., 2005). Although the pathological significance of N-terminally truncated/modified forms of A β is not completely understood, previous studies have demonstrated that these shortened A β forms are significantly more resistant to degradation, aggregate more rapidly *in vitro* and exhibit similar or, in some cases, increased toxicity in hippocampal neuronal cultures compared to the full-length peptides (Pike et al., 1995; Kuo et al, 1997; Tekirian 1999; Russo et al., 2002). Furthermore, N-truncated A β peptides progressively accumulate in the brain of sporadic AD patients, in early onset Familial Alzheimer's disease (FAD) patients and in Down syndrome brain (Naslund et al., 1994; Saido et al., 1995; Tekirian et al., 1998; Huse et al., 2002; Kalback et al., 2002; Piccini et al., 2005; Vanderstichele et al., 2005; Guntert et al., 2006; Liu et al., 2006;). In a recent study, Sergeant and co-workers showed that A β amino truncated peptides aggregate at the earliest stages of AD even before the appearance of clinical symptoms (Sergeant et al., 2003). Moreover, diffuse non-fibrillar preamyloid aggregates contain N-truncated A β , which might play an essential role in neuronal loss and cognitive decline in AD patients (Kumar-Singh et al., 2000). Finally, the presence of intraneuronal pool of N-truncated A β peptides has been shown to correlate with the progression of pathology and neuronal loss in a transgenic mouse model APP/PS1KI, described earlier (Casas et al., 2004, Wirths et al., 2007; Bayer et al., 2008). Thus, the N-terminally truncated/modified A β peptides represent highly desirable and abundant therapeutic targets.

Anti-A β immunotherapy has been shown to disrupt A β aggregates, block aggregation, attenuate toxicity, as well as promote the clearance of the peptide in the central nervous system (CNS). Immunotherapy approaches, both active immunization with A β peptide, or passive transfer of anti-A β antibodies, have shown therapeutic efficacy in several amyloid precursor protein transgenic (APP/Tg) mouse models, which develop AD-like amyloid plaque pathology (Schenk et al., 1999; Bard et al., 2000; Town et al., 2001; Brody et al., 2008), as well as canine and primates models of amyloidosis (Lemere et al., 2004, Head et al., 2008). Interestingly, the majority of the previous studies used mainly A β 1-40 or A β 1-42 as an immunogen for active immunization, which induced antibodies specific for amino-terminal part (EFRH epitope) of A β . However, most of the N-truncated forms of the A β lack this critical B cell epitope. In the present study we have focused on one of the most abundant Ntruncated/modified A β peptide bearing amino-terminal pyroglutamate at position 3 (A β N3(pE)). Previously, this peptide has been found to accumulate in diffuse and mature senile plaques as well as in blood vessels in AD and Down's syndrome brains. (Mori et al, 1992; Saido 1995, Kuo 1997; Harigaya et al., 2000; Russo et al., 2002; Guntert et al., 2006). Also, it has been shown that this peptide is more hydrophobic and has the increased aggregation potential (Pike,1995; He et al, 1999; Schilling et al., 2006). In addition, it has been demonstrated that soluble oligomers of A β N3(pE) impair learning and memory in mice after intracerebroventricular injection and induce a caspase-dependent neuronal apoptosis *in vitro* involving the activation of a cPLA2-arachidonic acid pathway (Youssef et al., 2007).

In the present study we further investigated the mechanisms of toxicity of A β N3(pE) oligomers and demonstrated that they induced phosphatidyl serine externalization and membrane damage in SH-SY5Y and IMR-32 cells. Finally, we produced A β N3(pE)-specific polyclonal antibodies in rabbits, and identified an immunodominant epitope recognized by anti-A β N3(pE) antibodies. We believe our results are potentially important for developing new immunotherapeutic compounds specifically targeting A β N3(pE) aggregates since the commonly used immunogens in the majority of vaccine strategies for AD have been shown to

induce antibodies that recognized the amino-terminal part (EFRH epitope) of the full length A β , which is absent in N-amino truncated peptides.

2. MATERIALS AND METHODS

2.1. Materials

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Synthetic human A β 1-42 and A β 35-25, as well as N-pyroglutamate modified peptides A β N3(pE) and A β N11(pE) were purchased from AnaSpec (San Jose, CA, USA). A non-related peptide (NRP; amino acid sequence: AALSPGSSAYPSATVLA) was synthesized in our laboratory and used as a negative control. A monoclonal anti-A β antibody (4G8) was from Sigma. HRP-conjugated anti-mouse IgG2b and HRP-conjugated goat anti-rabbit IgG were from Zymed (San Francisco, CA, USA). Super Signal West Dura Extended Duration Substrate kit was from Pierce, Rockford, IL, USA. Cell culture media (DMEM/F12, 1:1) were from GIBCO (Grand Island, NY, USA).

2.2. Peptide preparation and WB

A β 1-42, A β N3(pE) and A β N11(pE) were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to allow a conversion to the monomer and, after evaporation of solvent, were stored in aliquots at -20°C. Oligomeric A β 1-42, A β N3(pE) and A β N11(pE) were prepared essentially as described previously (Klein, 2002; Solorzano-Vargas et al., 2008) by incubation of monomers in DMEM/F12 at 4°C or at 37°C for 72 hrs. A β 35-25 and NRP were dissolved in a water at a concentration of 1 mg/ml. Formation of oligomeric A β 1-42, A β N3(pE) and A β N11(pE) species was confirmed by the Western Blot. Briefly, after incubation, peptides were separated by electrophoresis on 4-12% polyacrilamide precast NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) at 100 V for 1 h 45 mins and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) using a semi dry blot system (Bio-Rad) at 25 V for 50 min. Membranes were blocked in PBS/2% non-fat dry milk/0.2% Triton X-100 overnight at 4°C and incubated overnight at 4°C with primary antibodies: 4G8 (1:2000) or rabbit anti-A β N3(pE) polyclonal IgG. After washing with PBS/0.2% Tween, the membranes were incubated with HRP-conjugated anti-mouse IgG2b, 1:2500 or anti-rabbit IgG, respectively, for 2 h at RT. Immunoreactive bands were detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate kit.

2.3. Immunization protocol

All experiments with animals were conducted using protocols approved by our Institutional Animal Care Committee. 3-month-old White New Zeland rabbits were immunized subcutaneously (s.c.) at 14 days intervals with 200 μ g of human A β N3(pE) oligomers prepared at 37°C as described above. Freund's complete adjuvant was used for primary injection followed by incomplete Freund's adjuvant for three boost injections. Control rabbits were immunized with adjuvant alone or with a non-related peptide, respectively. Animals were bled on day 0 and 10 days after the third boost injection. The sera were stored at -20°C until use.

2.4. Purification of rabbit IgG

IgG were precipitated from rabbit sera with 1.7 M ammonium sulphate and dialyzed against PBS. Then this solution was applied to columns with Protein G-Sepharose (Zymed) and incubated for 1 h at room temperature. Non-bound proteins were removed by washing with PBS, and IgG was eluted with elution buffer (0.2 M glycine, adjusted to pH 2.8 with HCl). After dialysis against water, antibodies were lyophilized and stored at -20°C.

2.5. ELISA for evaluation of anti- A β N3(pE) antibodies

ELISA analysis was carried out using MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) coated overnight with a synthetic peptides at a concentration of 20 μ g/ml in carbonate buffer (pH 9.6). After washing with phosphate buffer containing 0.2% Tween-20 (PBS-Tween), plates were blocked with PBS/2% non-fat dry milk for 1 h at 37°C. Plates were washed, then rabbit sera diluted in PBS/2% non-fat dry milk/0.2% Triton X-100 were added and after incubation for 1 h at 37°C, plates were washed with PBS/0.2% Tween. Rabbit anti-human A β 1-42 polyclonal antibodies (Zymed) as well as rabbit polyclonal anti-A β N11(pE) antibodies obtained in our laboratory (unpublished data) were used as a positive control to confirm the peptide binding to well. HRPconjugated goat anti-rabbit IgG (Zymed) diluted in PBS/2% non-fat dry milk/0.2% Triton X-100 was added, and plates were incubated for 1 h at 37°C. Plates were washed and 2,2'-azino-bis- (3-ethyl-benzthiazoline-6-sulphonic acid (ABTS) single solution (Zymed) was added. The OD reading at 405 nm was registered using Opsys MR Microplate Reader (DYNEX Technologies, Chantilly, VA, USA). For competition ELISA, sera were preincubated overnight at 4°C with the preparations of serially two-fold diluted synthetic peptides, prior to adding to A β N3(pE)-coated wells. Then the assay was performed as described above.

2.6. Affinity selection of phages binding to anti- A β N3(pE) antibodies

Selection of phages by biopanning was performed as described in our previous studies (Gevorkian et al., 2000; Gevorkian et al., 2004). A Phage Display Peptide Library from New England Biolabs (Beverly, MA, USA) was used. The displayed 7-mer peptides are expressed at the N-terminus of the minor coat protein (cpIII) of M13 phage. MaxiSorp microtiter plates were coated with goat anti-rabbit IgG (Zymed) at a concentration of 5 μ g/ml 1 h at 37°C, washed and blocked with PBS-2% BSA. After washing, polyclonal rabbit anti- A β N3(pE) antibodies diluted 1:200 in PBS-Tween-1% BSA were added and plates were incubated for 1 h at 37°C. Plates were washed, then 10¹¹ plaqueforming units (PFU) from phage library diluted in PBS/BSA 1% were added and plates were incubated overnight at 4°C. Non-specific phages were washed off, and bound phage clones were eluted by triethylamine (0.1 M) and neutralized by Tris-HCl (1M, pH 7.5). Three rounds of biopanning were performed, and 21 individual clones were selected after the third round, amplified and used in ELISA to evaluate their binding to anti-A β N3(pE) antibodies. MaxiSorp microtiter plates were coated with goat anti-rabbit IgG and blocked as described above. Rabbit anti-A β N3(pE) antibodies diluted 1:100 in PBS-Tween-1% BSA were added and plates were incubated for 1 h at 37°C. After washing, 10¹⁰ PFU/ml of each phage clone diluted in PBS-1% BSA were added to plates and incubated overnight at 4°C. After washing, HRP-conjugated anti-M13 monoclonal antibody (Invitrogen) was added and incubated for 1 h at 37°C. After washing, ABTS single solution was added. The OD reading at 405 nm was registered using Opsys MR Microplate Reader (DYNEX Technologies).

Also, single-stranded DNA was prepared from all positive clones and one negative clone as described previously and used for DNA sequencing (Gevorkian et al., 2000). T7 Sequenase version 2.0 Quick Denature Plasmid Sequencing kit (Amersham Pharmacia Biotech, USA) and [α -³⁵S] dATP (Amersham) were used according to the manufacturer's instructions.

2.7. Cell culture

Human neuroblastoma SH-SY5Y and IMR-32 cells obtained from the American Type Culture Collection (ATCC, VA, USA) were maintained in DMEM/F12 (1:1) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and penicillin-streptomycin (GIBCO) and differentiated for 8-10 days in the presence of 10 μ M all-trans retinoic acid (SH-SY5Y) or 1mM dibutyl cAMP (IMR-32 cells).

2.7. Annexin V — 7-Amino Actinomycin D (7-AAD) assay

SH-SY5Y cells were seeded in 6-well plates at a density of 10^6 cells/well and exposed to $10\mu\text{M}$ A β N3(pE), A β 1-42 and A β 1-16, diluted in DMEM/F12 without fetal bovine serum for 24 h. After treatment, supernatants were removed and adherent cells were harvested using 0.25% trypsin (Sigma). After dilution in PBS containing 1% fetal bovine serum, the adherent cells were added to detached population floating in supernatants and centrifuged at 600 g for 5 min at room temperature. Cells were resuspended in 500 μl of binding buffer (10 mM HEPES, 140 mM HCl and 2.5 mM CaCl₂) and centrifuged again. Then 5 μl of annexin V-FITC (Invitrogen) diluted in 100 μl of binding buffer were added and cells were incubated for 40 min at room temperature in dark. After washing cells twice with binding buffer, 2.5 μl of 7-AAD (Sigma) were added and cells were incubated for 20 min at room temperature in dark. After washing three times, cells were resuspended in 400 μl of binding buffer and analyzed by flow cytometry. Flow cytometric analysis was performed using a FACScan Flow Cytometer (Becton-Dickinson, NJ, USA). Fluorescence was detected at an excitation wavelength of 488 nm and emission wave-lengths of 525 and 620 nm for the green (FITC) and red (7-AAD) fluorescence, respectively. A total of 10,000 events were acquired for each sample. Unstained cells, annexin V only stained cells and 7AAD only stained cells were run as controls to set gates.

For immunocytochemistry, cells were plated onto round glass coverslips (12 mm diameter) and placed in 24-well-plates (density was 10^5 cells/well). After treatment with peptides as described above, cells were washed with binding buffer and 2 μl of annexin V-FITC and 2 μl of 7-AAD diluted in binding buffer were added to each well. After incubation for 20 min, cells were washed again, fixed for 30 min at room temperature with 4% paraformaldehyde in PBS. After washing with PBS, cells were mounted onto glass slides in ProLong Gold antifade reagent with DAPI (Molecular Probes). Confocal images were collected sequentially at RT on a FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) with a 60x oil immersion objective lens UPLSAPO (NA=1.35). FITC fluorescence was obtained after exciting the samples with a wavelength of 488 nm and reading the fluorescence at 519 nm. For the detection of 7-AAD and DAPI the excitation wavelengths were 543 and 405 and emissions were collected at 619 and 461 nm, respectively. The fluorescence intensity was set optimally for control cells and this exposure was retained for cells treated with peptides in order to determine light intensity. Appropriate threshold was employed to eliminate background signal in the images employing Imaris 5.0.3 software (Bitplane AG, Zurich, Switzerland). Images from controls and treated cells were processed under similar conditions.

2.8. BLAST homology search

A homology between peptide inserts of selected positive clones and known protein sequences was analyzed using BLASTP program (<http://blast.ncbi.nlm.nih.gov>).

2.9. Statistical analysis

Data were analyzed by ANOVA using SPSS statistical software program (Release 9.0).

3. RESULTS

3.1. Characterization of oligomeric A β N3(pE) and A β N11(pE) preparations

Oligomeric A β N3(pE) and A β N11(pE) were prepared from monomers essentially as described previously (Klein, 2002). All preparations were characterized by electrophoresis followed by silver staining (not shown) and immunoblotting. All oligomeric preparations of both A β N3 (pE) and A β N11(pE) contained two prominent bands corresponding to trimers and tetramers as well as a monomers (Fig.1). Higher molecular weight bands corresponding to 12- and 24-

mers were also observed mainly in oligomers preparations incubated at 37°C. All bands were identified by 4G8 antibody known to bind to an internal epitope of A β (aa17-24). It has been reported previously that oligomeric fulllength A β 1-42 peptide also formed mainly trimers and tetramers under conditions used (Chang et al., 2003).

3.2. Production of anti-A β N3(pE) antibodies and their binding to synthetic A β peptides

To produce anti-A β N3(pE) antibodies, rabbits were immunized with peptide preparation mixed with adjuvant. Animals were bled 10 days after the third boost injection and the production of specific antibodies was tested by ELISA. Anti-A β N3(pE) antibodies were found to bind specifically to A β N3(pE) peptide while no binding to A β 1-42 or A β N11(pE) was observed (Fig.2). No binding of preimmune serum or anti-NRP serum to A β N3(pE) was detected. Also, after pre-incubation of anti-A β N3(pE) antibodies with A β 1-42 and A β N11(pE) there were no inhibition of binding of anti-A β N3(pE) antibodies to A β N3(pE) (Fig.3).

Anti-A β N3(pE) antibodies were tested in Western Blot and found to be specific for oligomeric A β N3(pE), while no binding to A β N11(pE) was observed (Fig.1).

3.3. Affinity selection of phages binding to anti- A β N3(pE) antibodies

To identify immunodominant region of A β N3(pE) peptide, the library of random heptapeptides displayed as a fusion to the minor coat protein of M13 phage was screened with rabbit anti-A β N3(pE) antibodies. Three rounds of biopanning were performed and 21 clones were randomly selected from the eluate after the third round. Binding of these clones to anti-A β N3(pE) antibodies was tested in ELISA. DNA sequences of heptapeptides coding inserts of 6 positive and 1 negative phage clones were determined and the deduced amino acid sequences are shown in Table 1. Peptide inserts of the three positive clones had the same sequence QFRHDWY, and two other positive clones (C11 and C12) had a homologous sequence QFRT(V)D(P)D(Y)P. Thus, after third round of biopanning five positive clones bearing inserts with peptide sequences homologous to A β N3(pE) were selected. One positive clone (C8) had a peptide sequence WPVGGEH with no homology with A β N3(pE). BLAST search revealed a homology of this sequence with a hexapeptide present in the growth factor receptor-bound protein 7 (GRB7), an adaptor molecule that mediates signal transduction from multiple cell surface receptors to various downstream signaling pathways.

Interestingly, neither anti-full length A β 1-42 antibodies nor anti- A β N11(pE) antibodies bound phage clones with a consensus sequence QFRH(T,V)D(P)W(D,Y)Y(P).

3.4. A β N3(pE) induces phosphatidyl serine (PS) externalization and increases the membrane permeability in SH-SY5Y and IMR-32 cells

To assess the cell death mechanisms induced by A β N3(pE), we used annexin V and 7-AAD dual staining approach to differentiate between early apoptotic, necrotic/late apoptotic and live cells. It is known that in cells undergoing apoptosis, the membrane PS translocates from the inner face of the plasma membrane to the cell surface and can bind to annexin V (Vermes et al., 1995). 7AAD is a fluorescent, cell-impermeable DNA-binding agent and is used to assess the cell membrane integrity. Both cell lines (SH-SY5Y and IMR-32) used in this study were characterized extensively in previous reports and shown to have biochemical properties of human neurons *in vivo* (Sherer et al., 2001) and, after differentiation, to have also all the characteristics of adult neurons in the brain and to be sensitive to degeneration evoked by A β (Lambert et al., 1994). That is why we differentiated cells for 7 to 10 days before conducting all experiments.

After treatment with A β N3(pE) for 24 h, differentiated SH-SY5Y cells were stained as described in Materials and Methods section and analyzed by flow cytometry. Four populations

of cells were detected (Fig. 4). Live cells, which were not affected by the oligomeric A β N3 (pE), are located in the lower left quadrant. The lower right quadrant represents early apoptotic (annexin V-positive and 7AAD-negative) cells while the upper right quadrant represents late apoptotic/necrotic cells (annexin V-positive and 7-AAD positive). A small number of dead cells were observed in the upper left quadrant (annexin V-negative and 7-AAD-positive). In summary, exposure to 10 μ M A β N3(pE) induced a significant ($p < 0.05$) decrease in the number of viable cells (29.3% and 27.5%) compared with control untreated cells and control cells treated with A β 1-16 peptide, respectively (Fig. 5). Also, exposure to A β N3(pE) induced a 3-fold increase in the number of early apoptotic cells (annexin V positive and 7-AAD negative cells) compared with control cells. A three-fold increase in the number of annexin V-positive and 7-AAD-positive (necrotic/late apoptotic) cells compared with control untreated cells or A β 1-16 peptide treated cells was observed too.

In the same assay, exposure to A β 1-42 induced an increase (although less than in the case of A β N3(pE)) in the number of early apoptotic cells compared with control untreated cells and cells treated with A β 1-16 (Fig.4).

The same mechanisms of toxicity were observed by immunocytochemical analysis of cholinergic IMR-32 cells treated with oligomeric A β N3(pE). After 24 h of incubation with 10 μ M A β N3(pE) cells were stained with annexin V-FITC and 7-AAD. Cells treated with oligomeric A β N3(pE) showed both translocation of PS to the outer side of the plasma membrane (green color) and DNA staining with 7-AAD (red color) (Fig.6). At the same time, cells treated with a control peptide (A β 1-16) were annexin V/7AAD negative.

DISCUSSION

Soluble A β aggregates present in the brain of sporadic AD patients and in Down syndrome were significantly different and more toxic compared with A β present in normal brain, and this was correlated with the predominance of the N-truncated species over full length A β 1-42 (Kalback et al., 2002; Piccini et al., 2005; Vanderstichele et al., 2005). Importantly, A β N3(pE) is the most abundant one in AD brain and is absent in normal aging, while other N-truncated species were detected in both AD and aged control brains (Schilling et al., 2008). Moreover, the resistance toward proteolytic degradation by aminopeptidases decreases the rate of A β N3 (pE) clearance and enhances its accumulation. In addition, pyroglutamate-containing A β species have been shown to be potential seeding species of aggregate formation *in vivo* and may play an important role during the initiation of the disease (Schilling et al., 2006). Importantly, it has been demonstrated that intracerebroventricular injection of a low dose of soluble A β N3(pE) oligomers impaired memory in male C57BL/6 mice suggesting that this peptide might cause impairments of synaptic integrity and plasticity (Youssef et al., 2007). Previously, it has been reported that in APP^{SL}-PS1KI transgenic mice, emerging of A β N3(pE) peptide is detected at an age when the first signs of hippocampal neuronal loss (Casas et al., 2004). Finally, it has been demonstrated recently that inhibition of glutaminy cyclase, an enzyme responsible for A β N3(pE) formation, reduced plaque load in two different transgenic mouse models of AD accompanied by alleviated plaque-associated inflammation and a significant memory improvement (Schilling et al., 2008). Thus collectively these results provide support for a role for A β N3(pE) in A β aggregate formation. We believe our results are important in the development of immunogens capable of inducing antibodies specifically targeting A β N3(pE), which may inhibit the formation, as well as enhance clearance of the more pathological forms of A β peptide in the CNS.

In the current study we demonstrated that the immunodominant region of one of the most abundant N-truncated/modified A β peptide, bearing aminoterminal pyroglutamate, A β N3(pE), is located at its amino terminus. Interestingly, although the epitope recognized by anti-A β N3

(pE) antibodies contains FRH motif, anti-A β N3(pE) serum did not bind to phage clone bearing EFRH-containing peptide selected in our previous studies (Gevorkian et al., 2004). Also, rabbit anti-A β N3(pE) antibodies are highly specific against this modified peptide and do not recognize A β N11(pE) nor the full length A β 1-42 peptides. These results emphasize the need to search for immunogens capable to target N-truncated/modified species, since previously reported immunotherapy studies are based on EFRH epitope.

The molecular mechanisms of neuronal death induced by A β N3(pE) oligomers were addressed in a few studies (Piccini et al., 2005; Youssef et al., 2007). It has been shown that A β N3(pE) oligomers altered the membrane structure and permeability of liposomes (Piccini et al., 2005). Also, it has been reported that A β N3(pE) oligomers induced a redox-sensitive neuronal apoptosis involving caspase activation and an arachidonic acid-dependent proinflammatory pathway (Youssef et al., 2007). In the present study we demonstrated in two human neuroblastoma cell lines - dopaminergic SH-SY5Y cells and cholinergic IMR-32 cells - that oligomeric A β N3(pE) induced PS externalization and loss of membrane integrity measured by annexin V/7AAD staining. Our results indicate that both apoptosis and necrosis can occur in these two cell lines after exposure to 10 μ M oligomeric A β N3(pE).

Interestingly, the peptide insert of one of the positive clones selected in this study had a homology with a hexapeptide WPVGGD present in the growth factor receptor-bound protein 7 (GRB7), an adaptor molecule that mediates signal transduction from multiple cell surface receptors to various downstream signaling pathways (Daly, 1998; Shen and Guan, 2004). It has been reported previously that A β may alter the molecular composition of focal adhesions that would have multiple downstream effects such as rapid tyrosine phosphorylation of neuronal proteins and induction of an unscheduled cell cycle leading to death, among others (Zhang et al., 1994; Berg et al., 2002; Lambert et al., 1998; Williamson et al., 2002). Importantly, both proteins, A β and GRB7, were demonstrated to participate in the integrin signaling pathway by activating the focal adhesion proteins (Grace and Busciglio, 2002; Shen and Guan, 2004; Frasca et al., 2008). The sequence homology described in our study may explain, in part, these observations. However, further studies will have to be conducted.

In conclusion, by designing new immunogens (anti-A β N3(pE) epitope) capable of inducing antibodies against N-amino-truncated/modified A β peptides, one may specifically target the most pathological species of the A β peptide. This should significantly enhance the efficacy of immunotherapy in the CNS of AD patients, because only approximately 0.1% of the antibody in the blood gains entry into the brain.

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Fig.1.

Characterization of aggregated A β N3(pE) and A β N11(pE) peptides by PAGE and Western blot analysis. Oligomers were prepared and analyzed as described in Materials and methods section. (A) A monoclonal anti-A β antibody (4G8) binding to a central part of A β 1-42 was used to detect oligomers of A β N3(pE) and A β N11(pE) incubated at 4°C and at 37°C. (B) Rabbit antihuman A β N3(pE) polyclonal IgG were used to detect oligomers of A β N3(pE) incubated at 4°C and at 37°C. Rabbit preimmune serum was used as a negative control. Migration of the molecular mass standards is indicated by arrowheads.

Fig.2.

Rabbit anti-AβN3(pE) IgG are specifically binding in ELISA to AβN3(pE) peptide, while recognition of AβN11(pE) and Aβ 1-42 is negligible. AβN3(pE), AβN11(pE) and Aβ 1-42 were prepared as described in Materials and methods and used for covering microtiter plates. Optical densities (OD) were registered at 405. Data are means of three independent experiments $\pm$ SD.

Fig.3.

Rabbit anti-AβN3(pE) antibodies are highly specific against AβN3(pE) peptide as confirmed by competition ELISA. Sera were preincubated overnight at 4°C with the preparations of serially two-fold diluted synthetic AβN3(pE), AβN11(pE) and Aβ 1-42, prior to adding to AβN3(pE)-coated wells.

Fig.4.

A β 3N(pE) peptide toxicity in differentiated dopaminergic SH-SY5Y cell cultures.

Representative dot-plots of annexin V-FITC and 7-AAD staining of untreated cells (A), cells treated with A β 1-16 (B), cells treated with A β 1-42 (C), A β 3N(pE) (D) and cells treated with ionomycin (E).

Fig.5.

Quantification by flow cytometry of AβN3(pE)-induced cell death. Data shown are means $\pm$ SD of 3 independent experiments. Means denoted with different letters are statistically different ($P < 0.05$).

Fig.6.

A β N3(pE) induces phosphatidyl serine (PS) externalization and increases the membrane permeability as observed by immunocytochemical analysis. Cells were treated with A β N3(pE) as described above and stained with annexin V-FITC and 7-AAD (upper panel). A substantial incorporation of annexin V and 7-AAD is observed. Incubation of cells under same conditions with a negative control peptide (A β 1-16) (lower panel) did not induce toxicity. We observed similar immunostaining patterns in four independent experiments.

Table 1Peptide sequences and reactivity of selected phage clones with rabbit anti-A β N3(pE) serum

Phage clone	Sequence	OD 405nm
C5	T P S Y V S K	0.03±0.001
C8	W P V G G E H	0.8±0.008
C3, C10, C18	Q F R H D W Y	1.42±0.04
C11	Q F R H D D P	1.39±0.046
C12	Q F R V P Y P	0.78±0.06
C17	Q F R S D S T	0.82±0.142
C3.15	S Y E F R H H	0.03±0.005