Short Communication

A β PP Peptide Carboxyl-Terminal to A β Is Neurotoxic

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Extracellular A β -amyloid and intraneuronal paired helical filaments (PHFs) composed of tau protein are the neuropathological hallmark of Alzheimer's disease. A β is a 39- to 43-residue peptide derived by cleavage of a 695- to 770-amino-acid membrane-associated glycoprotein (termed β -protein precursor, β PP). Following the observation that an antiserum to an epitope located between residues 713 and 723 of β PP₇₇₀ (ie, the transmembrane region of the β PP distal to A β) labels PHFs and that a synthetic peptide homologous to residues 713 to 730 of β PP₇₇₀ (β PP713-730) is highly fibrillogenic and interacts with tau *in vitro*, it has been hypothesized that β PP fragments other than A β may feature in the pathogenesis of Alzheimer's disease concurring with neuronal degeneration. To investigate this issue, we have analyzed the effects of the exposure of primary neuronal cultures to the synthetic peptide β PP713-730. Cultures were prepared from rat hippocampus on embryonic day 17 and incubated with the peptide at 2.5 to 30 μ mol/L concentration for 1 to 4 days. Cell viability was compared with that of control cultures exposed to a scrambled sequence of the peptide. A 4-day exposure to 20 μ mol/L β PP713-730 resulted in almost complete neuronal loss, whereas no changes were observed with the scrambled peptide. Degenerating neurons showed DNA fragmentation by agarose gel electrophoresis and apoptotic changes by light and electron microscopy. These findings support the view that β PP sequences other than A β may play a role in nerve cell degeneration in Alzheimer's disease. (*Am J Pathol* 1999, 154:1001-1007)

Alzheimer's disease is a degenerative disorder of the brain that causes a progressive cognitive decline in mid to late adult life. The neuropathological hallmark of the disease is the co-occurrence of extracellular amyloid fibrils and intraneuronal neurofibrillary tangles (NFTs). The major constituent of amyloid fibrils is A β , a 39- to 43-residue peptide^{1,2} derived by proteolytic cleavage of a 695- to 770-amino-acid membrane-associated glycoprotein (β -protein precursor, β PP) that has structural features of an integral transmembrane receptor.³⁻⁶ A β is positioned partly in the extracellular domain and partly in the transmembrane domain, corresponding to residues 672-710/714 of β PP₇₇₀ (Figure 1). NFTs are made up of paired helical filaments (PHFs) composed primarily of microtubule-associated protein tau in an abnormally phosphorylated form (see Ref. 7 for review).

The relationship between amyloid deposition, nerve cell degeneration, and NFT formation is undetermined.^{8,9} A direct link between A β and neuronal degeneration has been suggested by the fact that fibrillar A β is toxic to neurons *in vitro*.¹⁰ However, evidence indicates that A β by itself is not sufficient to cause severe neuronal damage *in vivo*, as A β deposition may occur also in the brain of aged nondemented individuals without significant neuronal loss or dysfunction.¹¹⁻¹⁶

Recently it has been observed that the PHFs are immunoreactive with an antibody to a β PP epitope distal to A β and that a synthetic β PP peptide containing this epitope (β PP713-730) is highly fibrillogenic and binds to tau protein *in vitro*.¹⁷⁻¹⁹ These findings suggest that β PP fragments other than A β might have a role in the process of neuronal degeneration in Alzheimer's disease. To investigate this issue, we analyzed the effects of the peptide β PP713-730 on rat hippocampal neurons *in vitro*.

Supported by the Italian Ministry of Health, Department of Social Services, and by Telethon Italy (grant E360 to G. Giaccone and E482 to G. Marcon).

Accepted for publication December 23, 1998.

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Figure 1. Schematic representation of βPP_{770} with A β (black box) and the synthetic peptide $\beta\text{PP}_{713-730}$ used in this study (dashed bar). A β consists of 28 amino acids in the extracellular domain and 11 to 15 residues within the transmembrane domain. The synthetic peptide $\beta\text{PP}_{713-730}$ corresponds to the 11 last residues of the transmembrane domain and the 7 first residues of the intracytoplasmic domain.

Materials and Methods

Cell Cultures

Primary cultures of hippocampal neurons were prepared from fetal rat brains on embryonic day 17. Hippocampal tissue was washed in phosphate-buffered saline (PBS; Dulbecco's without $\text{Ca}^{2+}/\text{Mg}^{2+}$, Biochrom KG-Seromed, Berlin, Germany); the cells were dissociated in Eagle's basal medium Eagle supplemented with 7% NaHCO_3 , 10% fetal calf serum, and 1% L-glutamine (BME-Hanks' salt, Gibco, Paisley, UK) and plated at a density of 3.5×10^5 cells per well in Primaria 15-mm wells (Falcon, Becton Dickinson, Meylan, France), precoated with poly-D-lysine (50 $\mu\text{g}/\text{ml}$; Sigma Chemical Co., St. Louis, MO). The cells were kept at 37°C in a humidified 5% CO_2 atmosphere. Five days after plating, non-neuronal cell division was halted by exposure to 10^{-5} mol/L cytosine arabinoside (Sigma) to prevent overgrowth of glial cells. Additional cultures were prepared in tissue culture chamber slides (Nunc, Naperville, IL) for immunohistochemical investigation.

Peptide Synthesis and Purification

A peptide homologous to residues 713 to 730 of βPP_{770} (ATVIVITLVMKKKQYTS) and a scrambled sequence of this peptide (KVKTTKVTAMIQYSILL) were synthesized by solid-phase chemistry on a 430A synthesizer (Applied Biosystems, Foster City, CA) and purified by reverse-phase high-pressure liquid chromatography (HPLC) as described previously.²⁰ The identity and purity of the peptides were determined by analytical reverse-phase HPLC and amino acid sequencing (Applied Biosystems 477A microsequencer); purity was $>95\%$ for both peptides.

Treatment and Morphological Evaluation of Cultures

The synthetic peptides $\beta\text{PP}_{713-730}$ and scrambled $\beta\text{PP}_{713-730}$ were suspended in sterile distilled water at a concentration of 600 $\mu\text{mol}/\text{L}$. Under these conditions, $\beta\text{PP}_{713-730}$ aggregates into amyloid-like fibrils as previously described,¹⁷ whereas scrambled $\beta\text{PP}_{713-730}$ is soluble. Peptide suspensions were added directly to cul-

ture medium to a final concentration of 2.5, 5, 10, 20, and 30 $\mu\text{mol}/\text{L}$ on day 4 and day 6 after plating. On day 8, cells were analyzed by light and electron microscopy, fluorescence microscopy after staining with the DNA-binding fluorochrome 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI; Boehringer, Mannheim, Germany) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method for *in situ* labeling of fragmented DNA.²¹ For DAPI staining, the cultures were washed with PBS, incubated with aqueous solution of DAPI (1 $\mu\text{g}/\text{ml}$) at room temperature for 5 minutes and then at 37°C for 15 minutes, washed with 100% methanol, rehydrated, and mounted. TUNEL was performed on 4% paraformaldehyde-fixed cultures after microwave treatment (Miele M720 microwave oven, two cycles of 5 minutes at 150 W in 0.01 mol/L citrate buffer, pH 6),²² using the Boehringer kit for *in situ* detection of fragmented DNA (catalog item 1 684 817), according to the manufacturer's instructions. For electron microscopy, cultures were fixed with 2.5% glutaraldehyde in 100 mmol/L phosphate buffer, pH 7.4, at 4°C for 20 minutes, rinsed in Sørensen solution containing 10% saccharose, and post-fixed in 4% OsO_4 at 4°C for 1 hour. After dehydration, the cultures were embedded in Epon (48 hours at 60°C). Ultrathin sections were stained with uranyl acetate and lead citrate and scanned with a ZEISS 109 electron microscope.

Viability of Cultures

Viability of cultured cells was assessed by crystal violet staining on day 8 after plating. Cultures were incubated with 0.5% crystal violet in water/methanol (4:1) for 4 minutes, washed with water, and dried. The mean optical density and the percent area covered by cultured cells with respect to the whole well area were determined using the image analyzer IBAS 2.0 (Kontron-Zeiss). These two parameters allowed us to calculate the weighted optical density (mean optical density $\times$ percent area), which has been demonstrated to be proportional to the number of living cells.²³

DNA Analysis

To evaluate the DNA fragmentation, the hippocampal cells from 15-mm wells were washed with PBS, pH 7.4, and, after addition of cold lysis buffer (10 mmol/L Tris/HCl, 20 mmol/L EDTA, 0.5% Triton X-100, pH 8.0), collected, transferred to microtubes, and kept on ice for 15 minutes. After centrifugation at $13,000 \times g$ for 20 minutes, the supernatant (containing low molecular weight DNA) was incubated with RNase A (100 $\mu\text{g}/\text{ml}$ at 37°C for 1 hour); the pellet (containing high molecular weight DNA) was incubated with proteinase K (100 $\mu\text{g}/\text{ml}$ at 48°C overnight). DNA was extracted with phenol/chloroform solution and precipitated with NaCl (0.5 mol/L final concentration) and ethanol (3 vol) at -70°C overnight. It was then resuspended in 10 mmol/L Tris/HCl, 1 mmol/L EDTA, pH 7.4, and electrophoresed through 1.1% agarose gel.

Figure 2. Primary cultures of hippocampal neurons exposed for 4 days to vehicle solution (**a** and **c**) or to the peptide β PP713–730 at a concentration of 20 μ mol/L (**b**, **d**, and **e**). **a** and **b**: Phase-contrast photomicrographs showing remarkable neuronal degeneration in culture treated with the β PP peptide (**b**) as compared with control culture (**a**). **c** to **e**: TAU1 immunocytochemistry showing a dense network of strongly decorated axons in control culture (**c**) and almost complete loss of axonal processes in culture exposed to the β PP peptide (**d**), the residual immunostaining being largely confined to a few cell bodies (**e**). Magnification, $\times 210$ (**a**), $\times 420$ (**b**), $\times 155$ (**c** and **e**), and $\times 100$ (**d**).

DNA bands were visualized by staining with ethidium bromide.

For a quantitative evaluation of DNA fragmentation, the cells were lysed with lysis buffer as described above and centrifuged at $13,000 \times g$ for 20 minutes. Both the supernatant and the pellet were treated with trichloroacetic acid (12.5% final concentration) at 4°C overnight. After centrifugation at $13,000 \times g$ for 5 minutes, the DNA in the pellet was quantified by the diphenylamine reaction²⁴ with the spectrophotometrical analysis at 600 nm with an automated microplate reader (Titertek Multiskan, ICN, Costa Mesa, CA).

Immunocytochemistry

Cultures were washed with PBS, pH 7.2, fixed for 30 minutes in 4% paraformaldehyde in PBS, pH 7.2, washed with 50 mmol/L Tris buffer, pH 7.6, and permeabilized with 0.3% Triton X-100 in Tris buffer for 5 minutes. Cultures were immunostained with the monoclonal antibody TAU1 (1:100; Boehringer), which detects a nonphosphorylated epitope of tau including Ser199-Ser202, as well as with an anti-MAP2 monoclonal antibody (clone AP20, Boehringer) revealed by an avidin-biotin system with biotinylated anti-mouse serum raised in sheep (1:100;

Amersham, Little Chalfont, UK) followed by streptavidin-horseradish peroxidase (1:100; Amersham), using 3-3'-diaminobenzidine as chromogen.

Results

The normal hippocampal cultures initially consisted of undifferentiated round cells. After 2 to 3 days, the cells began to generate processes and to acquire neuronal morphology. Axonal processes elongated and organized in bundles that formed a loose network (Figure 2a). The axonal processes were strongly immunoreactive with the monoclonal antibody TAU1 (Figure 2c), whereas cell bodies and dendrites were labeled by anti-MAP2 antibody. These morphological and immunohistochemical features are typical of neuronal cultures of fetal rat hippocampus.²⁵

No difference from this pattern was observed in cell cultures treated with the scrambled β PP713-730 peptide or the vehicle solution. Conversely, the neuronal cultures exposed to 20 μ mol/L β PP713-730 underwent severe degeneration. The first changes were detected 2 days after peptide treatment and consisted in loss of phase brightness of cell bodies followed by shrinkage. After a 4-day treatment, the few surviving cells displayed a collapse of the dendritic arbor and an almost complete loss of axonal processes (Figure 2, b and d), the scanty residual Tau1 immunostaining being confined to some degenerating neuronal cell bodies (Figure 2e). No other specific derangement of the cytoskeleton and in particular no abnormal cytoplasmic inclusions were observed in the β PP713-730-treated neurons.

A semiquantitative evaluation of cell viability by crystal violet staining demonstrated that the neurotoxic effect of β PP713-730 was dose dependent. The neuronal loss was 56%, 62%, and 75% at concentrations of 5 μ mol/L, 10 μ mol/L, and 20 μ mol/L of β PP713-730, respectively. By contrast, the viability of cultures treated with the scrambled peptide did not differ significantly from that of cultures exposed to vehicle solution (Figure 3).

To investigate whether a mechanism of programmed cell death was involved in the neurotoxicity induced by β PP713-730, control cultures and cultures treated for 4 days with 20 μ mol/L β PP713-730 were stained with the DNA-binding fluorochrome DAPI or labeled with TUNEL. Microscopic examination showed that a large number of neurons exposed to β PP713-730 exhibited pyknotic or fragmented nuclei that were marked by TUNEL (Figure 4, a and c), whereas only rare apoptotic cells were present in cultures treated with scrambled β PP713-730 or vehicle solution (Figure 4, b and d). At the electron microscopic level, the altered neurons displayed changes corresponding to different stages of the apoptotic process. Some cells showed aggregation of chromatin into large compact granular masses lying on the nuclear membrane (Figure 4e); in others, the nucleus had condensed and broken up into fragments that formed intracytoplasmic apoptotic bodies.

Agarose gel electrophoretic analysis of DNA extracted from cultured cells was carried out to evaluate the frag-

Figure 3. Quantitative evaluation of the neurotoxic effect of β PP713-730 as a function of peptide concentration. After a 4-day exposure of cultures to β PP713-730 (5, 10, and 20 μ mol/L) or to a scrambled sequence of β PP713-730 (20 μ mol/L), nerve cell viability was assessed by crystal violet staining followed by densitometry. Viable neurons are expressed as a percentage of the number of neurons in vehicle-treated cultures. The data are the mean $\pm$ SE of 12 to 20 determinations.

mented DNA (Figure 4f) that was then quantified by the diphenylamine method. In cultures treated with 20 μ mol/L β PP713-730 for 4 days, the fragmented DNA was $12.5 \pm 3.4\%$ (mean $\pm$ SD; $n = 13$) of total DNA, whereas in control cultures the fragmented DNA was $6.1 \pm 3.1\%$ (mean $\pm$ SD; $n = 19$) of total DNA. This difference was statistically significant ($P < 0.001$, Student's *t*-test).

Discussion

The hypothesis of a neurotoxic effect of A β originated from the observation that in Alzheimer brains A β amyloid deposits are closely associated with abnormal neurites and was supported by the finding that A β induces nerve cell death *in vitro*.¹⁰ A possible role of β PP peptides other than A β has not been extensively investigated to date, although immunohistochemical analyses indicated that β PP and/or fragments thereof accumulate in dystrophic neurites of senile plaques.^{26,27} Our study shows that a synthetic peptide homologous to residues 713 to 730 of β PP₇₇₀, corresponding to the β PP region located carboxyl-terminally to the A β sequence, is neurotoxic and suggests that β PP cleavage products other than A β may feature in the pathogenesis of Alzheimer's disease.

Several lines of evidence support this view, including the early studies by Yankner and co-workers²⁸ that showed that PC12 cells transfected with the 105 carboxyl-terminal amino acids of β PP (β PP-C105) degenerated when induced to differentiate into neuronal cells by nerve growth factor. Subsequently, this neurotoxic effect was entirely ascribed to A β , based on the results obtained with relevant synthetic peptides.¹⁰ However, the neurotoxicity of β PP-C105 to rat cortical neuronal cultures was stronger than that observed with any A β fragment.²⁹ Therefore, the possibility should be considered that

Figure 4. Apoptosis of hippocampal neurons treated with β PP713-730. **a** and **b**: Photomicrographs of hippocampal cultures stained with the TUNEL method for *in situ* labeling of fragmented DNA (**a** and **b**) and with the DNA-binding fluorochrome DAPI (**c** and **d**) after a 4-day exposure to 20 μ mol/L β PP713-730 (**a** and **c**) or to vehicle solution (**b** and **d**). After β PP713-730 treatment, several nuclei are strongly labeled by TUNEL (**a**) and show a typical pyknotic morphology (**c**). At variance, in control culture, nuclei labeled by TUNEL (**b**) or showing condensation and fragmentation after DAPI (**d**) are very rare. Magnification, $\times 150$ (**a** and **b**) and $\times 420$ (**c** and **d**). **e**: Electron micrograph of a neuron from hippocampal culture treated for 4 days with 10 μ mol/L β PP713-730, showing aggregation of chromatin into large compact granular masses lying on the nuclear membrane. Magnification, $\times 14,000$. **f**: Agarose gel electrophoresis of the supernatant (lanes 1 and 3) and the pellet (lanes 2 and 4) of DNA preparations from hippocampal neurons exposed for 4 days to 20 μ mol/L β PP713-730 (lanes 1 and 2) or to vehicle solution (lanes 3 and 4). Fragmented DNA, which separates in the supernatant as a ladder, is more abundant in cultures treated with β PP713-730 (lane 1) than in control conditions (lane 3). Conversely, high molecular weight DNA, which separates in the pellet, is reduced in β PP713-730-treated cultures (lane 2) as compared with controls (lane 4).

$A\beta$ and β PP sequences carboxyl-terminal to $A\beta$ act synergistically in determining the neurotoxic effect of β PP-C105.

It is known that the processing of β PP generates several carboxyl-terminal peptides including residues 713 to 730, although the biochemical characterization, cellular trafficking, and topology of such fragments have not been completely established. By the endosomal-lysosomal pathway, β PP is cleaved by β -secretase, yielding a 12-kd product containing $A\beta$ at its amino terminus. Additional enzymatic attack by γ -secretase generates $A\beta$ and releases a complementary carboxyl-terminal fragment (usually indicated as p7) whose amino terminus corresponds to β PP713–730.^{30,31} It is conceivable that p7 and possibly larger fragments containing the sequence 713 to 730 have physicochemical properties and biological activities analogous to those of the β PP713–730 peptide. This scenario would parallel with the finding that peptides homologous to portions of $A\beta$ (eg, residues 25 to 35) retain the fibrillogenic and neurotoxic properties of the entire amyloid subunit.¹⁰ It is noteworthy in this regard that β PP peptides corresponding to the 100 carboxyl-terminal residues accumulate consistently in neurons expressing any of the five known mutations of β PP associated with familial Alzheimer's disease³² and that these fragments share the ability with $A\beta$ and β PP713–730 to assemble into amyloid-like fibrils when expressed in COS cells.³³

Our experiments also indicate that the dose-dependent neuronal death induced by the prolonged exposure of rat hippocampal neurons to β PP713–730 peptide occurs by an apoptotic mechanism. This may be relevant to the process of neurodegeneration in Alzheimer's disease, considering that 1) cells that express β PP mutants and accumulate carboxyl-terminal β PP fragments undergo apoptosis³⁴ and 2) extensive DNA fragmentation has been demonstrated in Alzheimer brains.³⁵

Additional studies are needed to clarify how the β PP713–730 peptide might affect neurons. On the other hand, despite extensive research in the past years, the mechanisms of neurotoxicity of $A\beta$ are not fully understood. Free radicals, increased sensitivity to excitotoxicity, disruption of Ca^{2+} homeostasis, and interference with ubiquitin-dependent protein degradation have been considered (see Ref. 9 for review). The analogy between the changes evoked in cultured neurons by $A\beta$ and β PP713–730 peptides raises the possibility of similar mechanisms of action. Alternatively, as it has been suggested that the tau-binding properties of the β PP region distal to $A\beta$ may reflect a physiological tau-mediated interaction between full-length β PP and the cytoskeleton,^{18,19} the mechanism of neuronal damage induced by β PP713–730 may involve a disruption of this binding.

In conclusion, our findings provide evidence for a neurotoxic activity of the synthetic peptide homologous to region 713 to 730 of β PP770, which lies outside the extensively studied $A\beta$ region and may represent a novel molecular landmark for efforts to elucidate the relationship between the complex cascade of events triggered by β PP metabolites and neurodegeneration in Alzheimer's disease.

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