Neurotoxicity of prion peptide 106-126 not confirmed

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Abstract Prion-related diseases are accompanied by neurodegeneration, astroglial proliferation and formation of proteinase K-resistant aggregates of the scrapie isoform of the prion protein (PrP^{Sc}). The synthetic PrP fragment 106-126 was reported to be neurotoxic towards cultured rat hippocampal neurons (Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O. and Tagliavini, F. (1993) Nature 362, 543–546) and mouse cortical cells (Brown, D.R., Herms, J. and Kretzschmar, H.A. (1994) Neuroreport 5, 2057–2060). However, we found the viability of these and other neuronal cell types not to be impaired in the presence of PrP106-126 under widely varied sets of conditions. Aged preparations of the peptide as well as synthetic deamidated and isomerized derivatives that correspond to the aging products of the peptide proved also to lack neurotoxicity. Apparently, PrP106-126 cannot serve as a model for the interaction of PrP with neuronal cells.

Key words: Prion peptide PrP106-126; Prion protein; Neurotoxicity; Cell culture; Cortex; Hippocampus

1. Introduction

The cellular prion protein (PrP^{c}) is a glycoprotein of unknown function found at the surface mainly of neurons [1] and glial cells [2]. The normal PrP is converted by an unknown mechanism into its infectious scrapie form (PrP^{Sc}). The PrP fragment 106-126 (PrP106-126) has been reported in numerous publications to elicit several effects of prion pathology, i.e. to be toxic to cultured neurons [3–14] and to support the proliferation of glial cells [15] and microglia [16]. Cultures of cortical neurons derived from $PrP^{0/0}$ mice, i.e. devoid of cellular PrP, have been described to survive the addition of PrP106-126 [4]. PrP106-126 is part of the NH_2-terminal flexible tail (residues 23–126) in the full-length murine PrP with a total of 231 residues [17]. The peptide polymerizes into fibrils [9], assumes a β-sheet structure and forms protease-resistant aggregates of high molecular mass [18]. Here, we describe experiments with neuronal cell cultures in the presence of PrP106-126 under different conditions. The experiments do not confirm the reported neurotoxicity of the PrP fragment.

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Abbreviations: Ara-C, cytosine-arabinoside; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PrP^{Sc}, scrapie isoform of the prion protein; PrP^{c}, cellular isoform of the prion protein; TFA, trifluoroacetic acid

2. Materials and methods

2.1. Peptides

Two batches of PrP106-126 were purchased from Chiron Technologies (Clayton, Vic., Australia) with a purity higher than 95%. The peptide sequence KTNMKHMAGAAAAGAVVGGLG was confirmed by mass spectroscopy, amino acid analysis and sequencing. For aging, the peptide was incubated for at least 30 days at 37°C in 0.1 M sodium phosphate, pH 7.4 [19]. Fibrils of PrP106-126 were prepared by incubating the peptide at a concentration of 500 μM in 0.1 M sodium phosphate, pH 7.4, for 12 h at 25°C. The peptide solution was then subjected to ultracentrifugation at 540 000 × *g* for 3 h at 25°C (Beckmann Optima TLX ultracentrifuge, TLA 100.3 fixed-angle rotor). The resulting pellet was resuspended in H_2O and protein concentrations were determined with the BCA assay from Pierce. PrP106-126 isoforms as they are produced during aging of the peptide [19] were synthesized by replacing L-asparagine in the third position (Asn-108 in PrP) with L-Asp, D-Asp, L-isoAsp or D-isoAsp.

To test whether the reported neurotoxicity of peptide preparations might be due to contaminants from their purification with high performance liquid chromatography (HPLC), peptides were passaged over a reverse-phase HPLC column. They eluted with a final concentration of about 2 μM at approximately 30% acetonitrile/0.1% trifluoroacetic acid (TFA). Peptide-containing fractions of 200 μl each were combined and concentrated 200-fold in a SpeedVac concentrator. The sample was washed twice with water, corresponding to a total dilution factor of eight. The peptide was then dried and resuspended in water to a concentration of 500 μM.

2.2. Animals

Mice (ICR, NMRI and C57Bl/6J) were either from BRL (Füllinsdorf, Switzerland) or from their descendants bred at the Institute for Laboratory Animal Science, University of Zürich. Rats (Wistar and Zur:SD) were from the same two sources.

2.3. Cell cultures

Cortex and hippocampus were dissected as described [20] and cell cultures were prepared according to the published protocols from the laboratories of Forloni and Kretzschmar [3,4]. Cells were obtained at embryonic (E) day 17 and in certain cases at day E16 or E18. The tissue was treated for 5 min at 37°C with 2.5 mg/ml trypsin (Life Technologies) and 200 μg/ml DNAse (Boehringer Mannheim), centrifuged, washed five times with 3 ml of serum-free Dulbecco's minimal essential medium (DMEM, Life Technologies) containing 5 mg albumax/ml (Life Technologies) and then mechanically dissociated in serum-free medium containing 5 mg albumax/ml. Per well of a 96 well microtiter plate, 40 000 cells in 40 μl were placed corresponding to a cell density of 5.2×10^5 cells/cm^2. Either a solution of peptide in water or water as a control was added to a final volume of 50 μl. The medium was exchanged (40 μl) every second day. In 24 well plates, cells were cultured in a total volume of 400 μl at a cell density of 1.8×10^5 cells/cm^2, corresponding to 320 000 cells per well, the medium being exchanged (350 μl) every second day. All wells were coated with 50 μg/ml poly-D-lysine (Sigma).

To suppress residual trypsin activity, 5 mg albumax/ml was added throughout the culture period unless indicated otherwise. Cells were incubated at 37°C in a 5% CO_2 atmosphere. Under serum-free conditions, DMEM was supplemented with the defined protein mix B27 (Life Technologies) [21,22], without adding cytosine-arabinoside (Ara-C) or antibiotics. The final concentrations of ingredients of N2-supplemented (Life Technologies) medium were: insulin (5 μg/ml), human transferrin (100 μg/ml), progesterone (6.3 ng/ml), putrescine (16.11 μg/ml) and selenium (5.2 ng/ml). In certain experiments, 10% heat-inac-

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tivated fetal calf serum (FCS) was used instead of the defined protein mix B27. Serum batches from different companies were tested (Amimed, Hyclone, Boehringer Mannheim, Biospa and Sigma).

Staining for living and dead cells was performed with the LIVE/DEAD Viability/Cytotoxicity kit L-3224 according to the recommendations of the supplier (Molecular Probes, Eugene, OR, USA). Fluorescent cell stains were calcein AM for living cells and ethidium homodimer-1 for dead cells. The preparation was examined with a fluorescence microscope (Leitz DMR, Leica).

2.4. Assessing cell viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [23,24]: a sterile-filtered stock solution of 5 mg MTT (cell culture grade, Sigma) per ml of phosphate-buffered saline was added directly to the wells to give a final concentration of 0.5 mg/ml. The cells were incubated with MTT for 3 h at 37°C. In the case of 24 well plates, 300 μl of the supernatant was subsequently carefully removed and 300 μl acidic isopropanol (0.04 N HCl in isopropanol) was added to each well. In microtiter plates, 35 μl was removed and 100 μl acidic isopropanol was added. The plates were then placed onto a lab shaker continuously shaking at 200 rpm for 10 min to dissolve the formazan crystals. Plates were read on a microtiter plate reader (Dynatech MR7000) at 550 nm versus the reference wavelength of 750 nm. At least every second day and just before starting the MTT assay, cells were visually inspected with a Nikon microscope using phase contrast optics.

3. Results and discussion

3.1. PrP106-126 does not impair cell viability

PrP106-126, when added to cultures of mouse cortical neurons in different concentrations up to 200 μM, did not show any toxic effect (Fig. 1). This finding is in contrast to previous reports that described a cytotoxic effect of the peptide at a concentration of only 20 μM under comparable conditions with hippocampal cultures from rats [3] and cortical cultures from mice [4]. PrP106-126 proved to be non-neurotoxic also towards hippocampal and cortical neurons from Wistar rats, outbred NMRI mice and inbred mice C57BL/6J (Table 1). Toxicity could depend on accompanying cells, e.g. astrocytes or microglia which have been reported to support toxicity of PrP106-126 [5] and to mediate a proliferative effect of PrP106-126 on astrocytes [16]. Microglial cells start to be found in the brain around day E14 [25], whereas GFAP-positive glial cells proliferate and differentiate later [26]. To guarantee that non-neuronal cell types were present, neurons were cultured from different developmental stages, i.e. at days E16, E17 and E18. None of these cultures was affected by PrP106-126 (not shown).

3.2. Variation of cell culture conditions

To exclude the possibility that an unknown toxic component is attenuated by bovine serum albumin or particularly effective antioxidative components of the B27 medium supplement used by us, control cultures were kept in the minimum medium supplement N2. Since the neurotoxic effect was originally shown in media containing 10% FCS [3], we tested several batches of FCS (see Section 2). Again, all these conditions were tested without finding any evidence for PrP106-126 being toxic (Table 1). Due to its higher protein content, neurons generally show higher viability in B27-supplemented than in N2-supplemented culture medium. Nevertheless, B27-supplemented medium proved adequate to detect the neurotoxicity of the Alzheimer amyloid β-protein 1-42 [27], a finding that was reproduced in our laboratory. PrP106-126 was also found to be non-neurotoxic in low-density cultures using mouse hippocampal neurons at a density of 100 000 cells per ml (or per 8.5 cm^2), followed by staining for living and dead cells (not shown). In the studies reporting PrP106-126 to be toxic, Ara-C was used to inhibit the proliferation of dividing cells [3,4]. However, Ara-C also inhibits the proliferation of microglial cells [28], calling into question the statement that microglia support PrP106-126 toxicity [5]. We did not observe any toxicity of the peptide in control cultures to which Ara-C as well as the antibiotics penicillin and streptomycin were added (not shown).

3.3. Neurotoxicity is not elicited by aggregation of PrP106-126

Conceivably, a prerequisite for neurotoxicity to become apparent could be the formation of aggregates or fibrils of

Table 1
Viability of cultured neuronal cells: synopsis of conditions tested

Experiment	PrP106-126, 80 μM	Species	Strain	Tissue	Medium	MTT ratio peptide/H_2O ± S.D.	*n*
1	Authentic	Rat	Wistar	**Hippocampus**	B27, albumax	0.98 ± 0.04	4
2	Authentic	Rat	Wistar	**Cortex**	B27, albumax	1.01 ± 0.03	4
3	Authentic	Mouse	C57BL/6J	Cortex	**B27**	1.09 ± 0.04	4
4	Authentic	Mouse	C57BL/6J	Cortex	**B27, albumax**	1.02 ± 0.04	4
5	Authentic	Mouse	NMRI	Cortex	**FCS**	0.96 ± 0.11	4
6	Authentic	Mouse	NMRI	Cortex	**N2**	0.99	1
7	Authentic	Mouse	NMRI	Cortex	**B27**	1.07 ± 0.18	2
8	**Authentic**	Mouse	NMRI	Hippocampus	B27, albumax	1.13 ± 0.07	3
9	**Aged in media**	Mouse	NMRI	Hippocampus	B27, albumax	1.07 ± 0.16	3
10	**Authentic, HPLC**	Mouse	NMRI	Hippocampus	B27, albumax	0.47 ± 0.05	2
11	**Aged in phosphate, HPLC**	Mouse	NMRI	Hippocampus	B27, albumax	0.50 ± 0.02	2
12	**HPLC buffer**	Mouse	NMRI	Hippocampus	B27, albumax	0.49	1

Bold print indicates the particular condition tested. PrP106-126 was used at 80 μM throughout these experiments. 'Authentic' stands for a stock solution of the peptide freshly prepared in water, sterile-filtered and frozen for storage. 'MTT ratio' gives the absorption ratio of cultures containing PrP106-126 to control cultures with H_2O added. The absorption in a MTT assay corresponds to the number of viable cells. Experiments 1 and 2 compare hippocampal with cortical neurons. Experiments 3 and 4 address the question if albumax as a medium supplement prevents neurotoxicity of PrP106-126. Experiments 5–7 test for a difference under the specified media conditions. (5) 10% FCS was used instead of medium supplements. The MTT ratio corresponds to the average value from five different batches of serum. (6) The minimal medium supplement N2 was used which has a lower antioxidative capacity than B27 used in (7). Experiments 8–12 serve to demonstrate that contaminants of HPLC buffer such as acetonitrile or TFA might be toxic. Experiments 8 and 9 are the controls for experiments 10–12: (8) authentic peptide, (9) PrP106-126 aged in cell culture medium without supplement for 2 weeks at 37°C, (10) PrP106-126 eluted from a reverse-phase HPLC column with acetonitrile/TFA, (11) aged PrP106-126 after HPLC with acetonitrile/TFA, (12) HPLC buffer, i.e. 30% acetonitrile/0.1% TFA after concentration by SpeedVac. For details of the experiments, see Section 2.

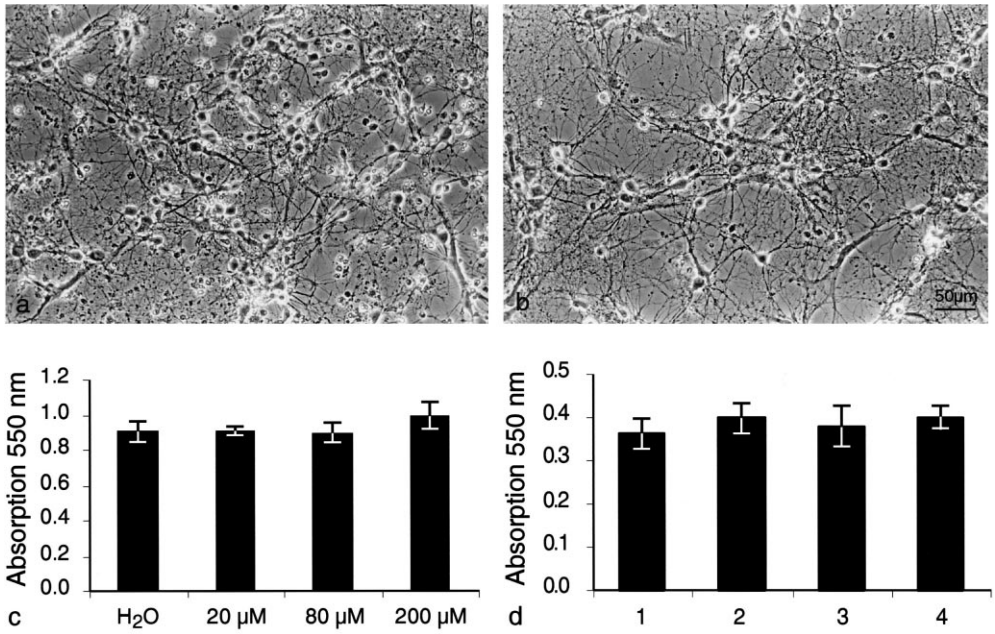

Fig. 1. Viability of cultured cortical mouse neurons after exposure to PrP106-126. The cells were obtained on day E17. (a) Control culture without peptide. (b) Culture in the presence of 200 μM PrP106-126. The bar represents 50 μm. (c) MTT assay with neurons cultivated for 10 days in the absence and in the presence of 20, 80 and 200 μM peptide. (d) MTT assay of cells cultured for 8 days in the presence of 80 μM aggregated and non-aggregated peptide: (1) control culture without peptide, (2) culture medium containing non-filtered PrP106-126, (3) sterile-filtered PrP106-126 or (4) fibrillar PrP106-126 (see Section 2). Error bars show the S.D.s of 3–4 experiments.

PrP106-126. New stock solutions of PrP106-126 were routinely filtered to maintain them sterile and endotoxin-free for direct use in cell cultures without antibiotics. Removal of 'seeds' by filtering the peptide preparation through a 0.2 μm membrane might abolish toxicity. A sterile-filtered sample of PrP106-126 was tested together with non-filtered material, a preparation of fibrils of PrP106-126 (see Section 2) and a control without peptide. Visual inspection by phase contrast microscopy did not reveal any toxic effects under any condition. The results were verified by MTT viability tests with cell cultures after 8 days (Fig. 1). No significant difference in cell viability between control and addition of sterile-filtered PrP106-126, non-filtered or fibrillar peptide was observed. Floating, translucent material thought to represent peptide aggregates was observed 1 day after the peptide was added in all wells of 24 well plates containing 80 μM PrP106-126.

3.4. None of the isoforms of PrP106-126 exerts a toxic effect on neurons

Asn-108 of PrP106-126 becomes spontaneously deamidated to aspartic acid and isoaspartic acid with a half-time of 12 days [19]. Consequently, an aged solution of the peptide is to be expected to be contaminated with these isoforms, which might be responsible for the putative neuronal toxicity observed in other laboratories. The situation could be the same as for Alzheimer amyloid β-peptide 1-42, the toxicity of which has been shown to depend on aged peptide material [29] and the content of β-sheet structure which is adopted by incubation in water for several days [30]. For PrP106-126, a report claiming an increased efficacy upon aging in inducing cation permeability of artificial bilayer membranes suggested a similar process [31].

Aged PrP106-126 (see Section 2), peptide aged in culture medium for 14 days, the corresponding separate isoforms of PrP106-126 (containing L-Asp, L-isoAsp, D-Asp, D-isoAsp instead of Asn-108) and an equimolar mixture of the isoforms (20 μM each) were tested for toxicity. We found no toxic effect of all these peptides (for the results with aged PrP106-126, see Table 1; the data with the isoforms are not shown).

3.5. Components of HPLC buffer are toxic to neurons

In our hands, the only conditions where we found neuronal toxicity of a preparation of PrP106-126 was after its passage over an HPLC column under certain conditions. When the peptide was subjected to reverse-phase chromatography at a low concentration (2 μM) and was concentrated to a final concentration of 500 μM (see Section 2), the peptide preparation became toxic. The toxic effect was comparable to that of HPLC buffer in fractions from runs without peptide subjected to the same concentration procedure (Table 1). In summary, the toxic effect of PrP106-126 preparations seems to be due to an unidentified contaminant, e.g. of microbial origin or resulting from the synthesis or the HPLC purification, rather than due to the peptide itself.

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