Study on the Toxic Mechanism of Prion Protein Peptide 106–126 in Neuronal and Non Neuronal Cells

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A synthetic peptide corresponding to the 106–126 amyloidogenic region of the cellular human prion protein (PrP^c) is useful for in vitro study of prion-induced neuronal cell death. The aim of the present work was to examine the implication of the cellular prion protein in the toxicity mechanism induced by PrP 106–126. The effect of PrP 106–126 was investigated both on human neuroblastoma SH-SY5Y cells and on SH-SY5Y overexpressing murine cellular prions (wtPrP). We show by metabolic assay tests and ATP assays that PrP^c expression does not modulate the toxicity of the prion peptide. Moreover, we investigated the effect of this peptide on an established non neuronal model, rabbit kidney epithelial A74 cells that express a doxycycline-inducible murine PrP^c gene. We show for the first time that the prion peptide 106–126 does not exert any toxic effect on this cell line in the presence or absence of doxycycline. Our results show that the PrP 106–126-induced cell alteration is independent of PrP^c expression. Rather, it seems to act via an interaction with lipidic components of the plasma membrane as strengthened by our results showing the differential susceptibility of neuronal and non neuronal cell lines that significantly differ by their membrane fatty acid composition.   © 2006 Wiley-Liss, Inc.

Key words: cellular prion peptide; 106–126 prion peptide; lipid membrane; neurotoxicity

Prion diseases are fatal neurodegenerative disorders affecting the central nervous system (CNS) of humans and animals and characterized by a neuronal vacuolation, astrocytosis, and progressive neuronal degeneration (Gajdusek et al., 1966; Clinton et al., 1993; Fraser, 1993; Jeffrey et al., 2000). These diseases are caused by the intracerebral accumulation of an abnormal isoform of the cellular prion protein (PrP^c), named PrP^{sc} (PrP scrapie) (Caughey and Lansbury, 2003; Collins et al., 2004; Prusiner, 1998). Many lines of evidence suggest that PrP^c acts as a template that promotes the conversion of PrP^c to PrP^{sc} (Caughey and Raymond, 1991). The plasma membrane seems to play a key role in the molecular mechanism implicated in this conversion. Supporting this hypothesis is the subcellular site for the formation of PrP^{sc}; indeed, the conversion occurs after PrP^c reaches the plasma membrane (Taraboulos et al., 1995). Moreover, Baron et al. (2002) have shown, using purified raft membranes, that the conversion of raft-associated GPI-anchored PrP^c to PrP^{sc} requires the insertion of PrP^{sc} into the lipid membrane. However, the mode of interaction of prion proteins with the membranes has not as yet been elucidated: does the interaction occur via a direct insertion of the prion protein into the lipid bilayer or via a putative membrane receptor?

We have shown previously that PrP (106–126), a peptide largely used as a model to study the PrP^{sc} induced neurotoxicity, destabilizes lipid vesicles mimicking the composition of neuronal membranes (liposomes) and induces liposome fusion (Dupiereux et al., 2005a). This destabilization mechanism occurs at low concentrations of the peptide (from 2–10 µM) and via a membrane interaction, as supported by the integrity membrane assay measuring the release of the intracellular lactate dehydrogenase (LDH assay). These results are in agreement with other previous reports. Indeed, several studies have shown that PrP 106–126 forms ion channels in planar lipid bilayers (Arispe et al., 1996; Kawahara et al., 2000) and that it increases the membrane microviscosity of

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neurons and astrocytes (Diomede et al., 1996; Salmona et al., 1997). Nevertheless, the role of the cellular prion protein in the membrane destabilization mechanism induced by this peptide remains as yet non-elucidated. Several works, using knock-out cell lines, show that PrP^c expression is necessary for the cellular toxicity induced by this peptide (Brown et al., 1994; Chabry et al., 2003a; Fioriti et al., 2005b). These results indicate that the membrane destabilization induced by PrP 106–126 could arise from a dual mechanism: a direct peptide insertion in the lipid bilayer or an interaction with PrP^c.

To investigate the role of PrP^c in the neurotoxicity induced by the 106–126 prion peptide we have examined whether an overexpression of murine PrP^c (wtPrP) (Walmsley et al., 2001a) changes the susceptibility of neurons to the peptide. Our results indicate that the toxicity induced by the 106–126 peptide is independent of PrP^c expression level. Moreover, the addition of an anti-prion antibody (SAF 34), able to decrease the basal level of PrP^c, induces, in our experimental conditions, non-significant changes in toxicity, showing that the PrP^c expression is not necessary for the PrP 106–126 toxicity. Additionally, to study the potential relationship between PrP 106–126 and lipidic components of the plasma membrane, we have investigated the effect of the peptide on non-neuronal cells, rabbit kidney epithelial (A74) cells, which express a doxycycline-inducible murine PrP^c gene. We have shown that this cell line is resistant to the PrP 106–126 toxicity confirming that the toxicity of this peptide is independent of PrP^c expression levels. We therefore suggest that this toxicity might be in relation with the lipidic composition of the cell membranes as strengthened by our results after the comparison of the fatty acid composition between neuronal and non-neuronal cells by HPLC fatty acid analysis.

MATERIALS AND METHODS

Chemicals

PrP106–126, derived from amino residues 106–126 of the human prion protein sequence (sequence: Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly) was purchased from Eurogentec SA and scrambled peptide containing the same amino acids in a random order (sequence: Asn-Gly-Ala-Lys-Ala-Leu-Met-Gly-Gly-His-Gly-Ala-Thr-Lys-Val-Met-Val-Gly-Ala-Ala-Ala) was purchased from Bachem. Palmitic acid was purchased from Sigma Aldrich. The secondary antibody rabbit antimouse IgG, A, M/FITC was purchased from Serotec and Streptavidine/FITC from Pharmingen. Rabbit polyclonal antibody P45-66 was supplied by Dr. D. Harris (Washington University, St. Louis, MO) and the secondary antibody HRP-coupled rabbit IgG was from Sigma (St. Louis, MO). The mouse anti-PrP^C SAF34 was kindly provided by Dr. J. Grassi from CEA of Paris. All cell culture supplies were purchased from Life Technologies Inc. and fatty acid supplies were from VWR International and Sigma-Aldrich.

Cell Culture

The human neuroblastoma cell line SH-SY5Y, kindly provided by Professor N.M. Hooper, and the same line stably transfected with the wild-type murine PrP (wtPrP) (Walmsley et al., 2001b) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Inc.), 1% penicillin/streptomycin (Life Technologies Inc.). Cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2. For experiments, cells were maintained in FBS-free DMEM medium containing the neuroblastoma growth supplement N2 (Life Technologies Inc.) and 1% penicillin/streptomycin.

The A74 cell line, initially generated in the laboratory of Drs. Vilette and Laude (INRA, Jouy-en-Josas, France), was established by transfecting rabbit kidney epithelial cells with murine PrP^c. The expression of the murine PrP is doxycycline-inducible via a tetracycline-inducible (tet-on) system (Vilette et al., 2001d). The stable transfectants were selected in the presence of puromycin (10 μg/ml). A74 were cultured in modified Eagle's medium (MEM) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were grown at 37°C in a humidified incubator with 95% air and 5% CO_2. For experiments, cells were maintained in MEM medium containing 2% FBS and 1% penicillin/streptomycin.

Cell Metabolism Assays

SH-SY5Y cells were seeded into 96-well culture plates. Sixteen hours after seeding, the medium was replaced with serum-free DMEM containing the neuroblastoma growth supplement N2 and the cells were treated for 24 hr with different concentrations of PrP 106–126 (10–200 μM). The treatment with the anti-prion antibody SAF 34 was carried out by incubating the cells with an optimized concentration of antibody SAF 34 (1 μg/ml) and after 3 hr, the medium was replaced with fresh medium and cotreated for an additional 24 hr with PrP 106–126 (200 μM) and SAF 34 (1 μg/ml).

A74 cell line in 96-well culture plates were grown overnight in MEM supplemented with 2% FBS. The next day the medium was changed and the cells were treated with doxycycline at 500 ng/ml to induce PrP^c expression. After 24 hr the cells were incubated with PrP 106–126 (50–100 μM) for an additional 24 hr.

To study the effect of lipidic factors, such as palmitic acid, on the toxicity effect of the 106–126 peptide, both the neuronal (SH-SY5Y) and non neuronal cells (A74) were incubated with palmitic acid at a final concentration of 25 μM for 16 hr. Cells were then treated with the palmitic acid (25 μM) and PrP 106–126 (200 μM) for additional 24 hr.

The cell proliferation was measured using the CellTiter 96 AQ$_{ueous}$ Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instruction. The cell toxicity was assessed quantitatively by MTS assay in the presence of phenazine methosulfate (PMS). After addition of 20 μl of the combined MTS/PMS solution in each well, the plates were incubated at 37°C in a humidified atmosphere contain-

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ing 5% CO_2 for 2 hr. The absorbance was measured at 490 nm (EL 312e microplate Bio-Tek Instruments).

All MTS assays were carried out in triplicate. MTS assay is a sensitive indicator of mitochondrial activity.

Cell Toxicity Test

Measurement of ATP levels allows to estimate cellular suffering. Human SH-SY5Y cells were seeded into 96-well culture plates. Sixteen hours after seeding, the medium was replaced with serum-free medium containing the neuroblastoma growth supplement N2 and the cells were treated for 24 hr with different concentrations of PrP 106–126 (10–200 μM).

ATP was quantified using the CellTiter-Glo Lumines-cent Cell Viability Assay (Promega) according to the manufac-turer's instruction. The reagent is a buffered solution contain-ing detergents to break the cell membrane, releasing ATP im-mediately on addition to wells and ATPase inhibitors to stabilize ATP once it is released from cells. After 106–126 peptide treatment, assay plates were removed from the incuba-tor and 100 μl of CellTiter-Glo Assay reagent, equilibrated to room temperature, was added to each well. Plates were shaken for 2 min to mix the contents of the wells. After a 10–15 min incubation at room temperature, luminescence was deter-mined using a Microplate Luminometer MPL2 (Berthold).

PrPc Labeling for Confocal Microscopy

A total of 20,000 SH-SY5Y or wtPrP cells were seeded and left to adhere for 5 hr in 50 μl DMEM 10% FBS and 1% penicillin/streptomycin on uncoated 12-mm circular glass coverslips placed in 24-well culture plates (Greiner). Fresh medium (450 μl) was added. Cells were cultured for 24 hr then fixed during 20 min in 1:1 acetone/methanol at −20°C. The acetone/methanol was discarded and the coverslips were kept at −20°C until use. The cells were then dehydrated at room temperature and rehydrated in phosphate buffered saline (PBS) before labeling. PrP^C was revealed at room temperature as follows: cells were incubated 1 hr with SAF34 10 μg/ml in PBS, washed 3 times with PBS, incubated 30 min with the rabbit anti-mouse IgG, A, M/FITC 1/1,000 in PBS, followed by washing 3 times in PBS. The coverslips were mounted on microscopy glass slides using DAKO fluorescence mounting medium before observation with a Leica TCS SL SP2 confo-cal microscope (Leica, Belgium) equipped with Argon, Ne/ Kr, and He/Ne lasers. One-step scans were carried out. A se-ries of 20 images were acquired and stacked using the Maxi-mum Projection.

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Western Blotting

A74 cells (seeding density 5×10^6) were grown in T75 cm^2 flasks. The next day the cells were treated for an additional 24 hr with different concentrations of doxycycline (10–500 ng/ml). After 24 hr, the cells were washed twice with cold PBS, calcium- and magnesium-free, and lysed for 30 min at 4°C in Triton-deoxycholate lysis buffer (1× buffer is 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxy-cholate, and 50 mM Tris–HCl, pH 7.5) containing protease inhibitors. After 1 min of centrifugation at 10,000g, the super-natant was collected, and its protein concentration was meas-

ured by the BCA assay (Pierce). The equivalent of 20 μg of total protein in SDS loading buffer was subjected to 12% SDS-PAGE electrophoresis followed by electroblotting on polyvinylidene difluoride in Tris-glycine buffer containing 20% methanol. The membrane was blocked with 5% non-fat dry milk in TBST (0.1% Tween 20, 100 mM NaCl, 10 mM Tris-HCL, pH 7.8) for 1 hr at room temperature, and PrP was detected by immunoblotting with P45-66 antibody. After adding the second antibody (horseradish peroxidase-coupled rabbit IgG), immunoreactive proteins were detected with the ECL Western blot system.

Flow Cytometry

Human SH-SY5Y cells seeded in 6-well plates (1.6 × 10^6 cells/well) were incubated for 24 hr in the presence or absence of SAF34 at 1 μg/ml. The cells were then rinsed in PBS and collected after incubation at 37°C for 10 min with 500 μl of cell dissociation buffer (enzyme free; Invitrogen). Samples of 100 μl containing 1×10^6 SH-SY5Y cells were incubated with the biotinylated antibody SAF34 (10 μg/ml) for 30 min at 4°C in PBS, washed and then incubated for an additional 30 min with a fluorescein isothiocyanate-conjugated streptavidin (diluted 1/1,000). The control cells were not in-cubated with the antibody (biotinylated SAF34). After rinsing, the resuspended cells were immediately analyzed in a FACS-can (Becton-Dickinson, Sunnyvale, CA).

Fatty Acid Analysis

Phospholipid extraction was carried out on SH-SY5Y and A74 cells. The cells were placed in 0.1 ml of a mixture of butylated hydroxy toluene (BHT)/CH_2Cl_2 (0.0204 mg of BHT in 1 ml of CH_2Cl_2) and 4 g of Na_2SO_4 and crushed with a cooled mortar. After 1 min, Celite 545 (3 g) was added and the cells were further crushed for 1 min. The mixture was transferred in a chromatography column filed with glass wool and 2 g of a mixture of $CaHPO_4.2H_2O$ and Celite 545 in a proportion of 1:9. The mortar was washed with 15 ml of the mixture CH_2Cl_2/CH_3OH (90:10) and transferred onto the column. About 50 ml of the mixture CH_2Cl_2/CH_3OH was added and the eluent was collected and diluted in 1 ml of ethyl acetate and the phospholipid fatty acids were separated with a SPE method (solid phase extraction). The trans-esterifi-cation procedure of the carboxylic group with methanol in hot acid medium produced the fatty acid methyl esters (FAME). The FAME were separated by gas chromatography (GC), Agi-lent 6890, on a capillary column with helium as the carrier gas. The analysis was realized with a flame ionization detector. The chem station software, on the GC Agilent 6890, measured the height of the different peaks and calculated the concentrations of the different FAME.

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RESULTS

Toxicity of PrP 106–126 Is Independent of the Level of Cellular PrP Expression

To study the relationship between the toxicity of PrP 106–126 and the expression levels of the cellular prion protein, we compared the effect of the peptide on SH-SY5Y neuroblastoma cells, expressing a basal level of
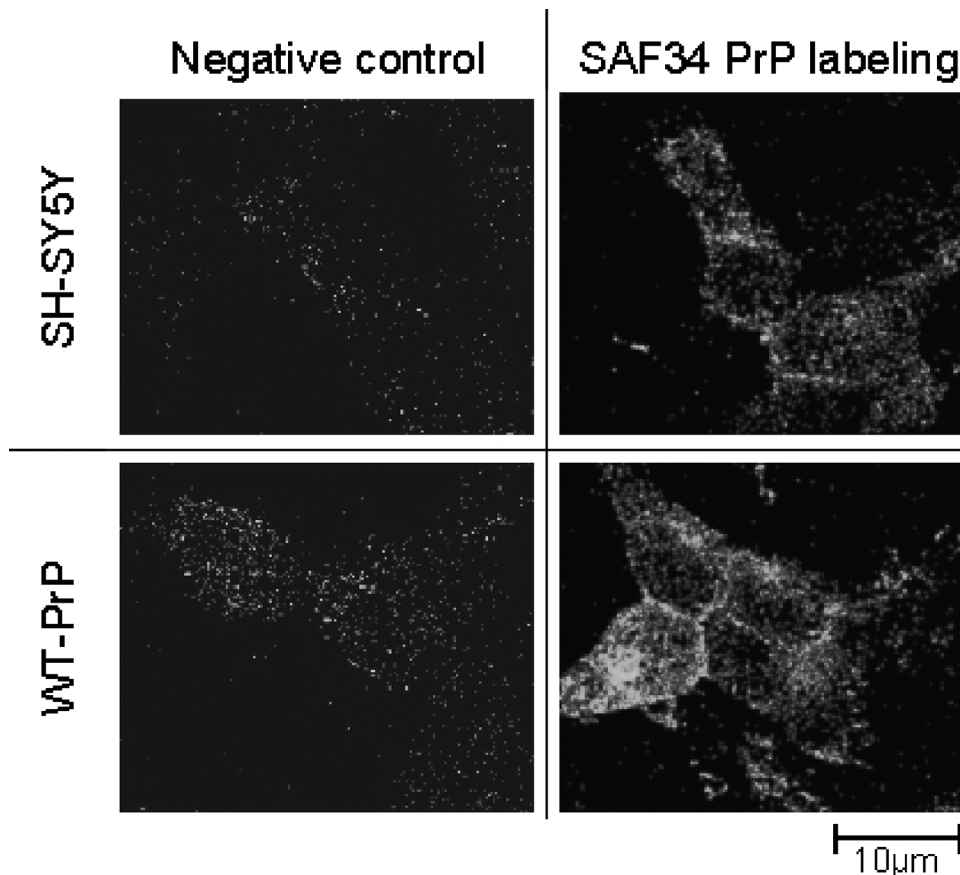
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Fig. 1. Immunofluorescence detection of the prion protein on SH-SY5Y and wtPrP cells. PrP^c was detected using SAF 34 antibody (10 μg/ml) followed by a FITC-conjugated secondary antibody. Cells were viewed by a Leica laser scanning confocal microscope. A more pronounced staining was observed on wtPrP cells indicating a high expression of PrP. Figure can be viewed in color online via www.interscience.wiley.com.

human PrP, with the same SH-SY5Y cells overexpressing a murine prion protein (wtPrP). The level of PrP expression was analyzed by confocal microscopy using the SAF 34 antibody. As shown in Figure 1, a more intense immunostaining was observed on wtPrP cells, whereas a weak staining was detected on SH-SY5Y cells. These results confirm that the wtPrP cells overexpress PrP in comparison with the SH-SY5Y cells that express a basal level of PrP.

Neuronal injuries induced by the 106–126 peptide were monitored by measuring the reduction of the mitochondrial activity using the MTS assay and the ATP assay monitoring cell alteration. MTS is converted to a formazan product by dehydrogenase enzymes, which become inactive as the cell suffers. Measurement of this formazan product is an indicator of cell metabolism and viability. As shown in Figure 2A, PrP 106–126 treatment (10–200 μM) induces a concentration-dependent toxicity for both SH-SY5Y and wtPrP cells. No significant difference in the toxicity induced by the peptide was observed between the two cell lines; indeed, PrP 106–126 caused a 50% decrease at 200 μM in SH-SY5Y and wtPrP (45.9 ± 3.6% and 55.8 ± 11.2%, respectively). In contrast, 200 μM of scrambled peptide had no effect (99.5 ± 0.96%) (Fig. 2B). These data indicate that the toxicity induced by the peptide 106–126 is independent of the level of PrP expression.

By using the ATP assay, the effect of PrP 106–126 on the intracellular content of ATP was measured in relation with the level of PrP expression (Fig. 3). A 24 hr treatment with different concentrations of the peptide (10–200 μM) induced a dose-dependent decrease in the ATP level without any difference between the two tested cell lines (SH-SY5Y and WtPrP). These data are in agreement with those obtained with the MTS assay confirming that the toxicity induced by PrP 106–126 is independent of the level of PrP^c expression.

To elucidate whether the neurotoxic effect of PrP 106–126 observed on the neuroblastoma cell lines is mediated by the PrP basal level expression, we blocked PrP^c surface expression with an anti-PrP antibody. We used SAF34, known to interact with PrP^c and prevent the interaction between PrP^c and PrP^{sc} (Perrier et al., 2004; Feraudet et al., 2005a).

To investigate the effect of the SAF34 antibody on PrP^c expression, we analyzed by flow cytometry the level of PrP^c on SH-SY5Y neuroblastoma cells incubated for 24 hr in the presence or absence of the SAF34 antibody. As shown in Figure 4a, in the absence of SAF34 treatment, the neuroblastoma cells SH-SY5Y present a positive immunostaining indicating that they express a basal level of PrP^c as shown by confocal microscopy (Fig. 1). Conversely, when the cells were pre-incubated with the SAF34 antibody for 24 hr (Fig. 4b) no staining was
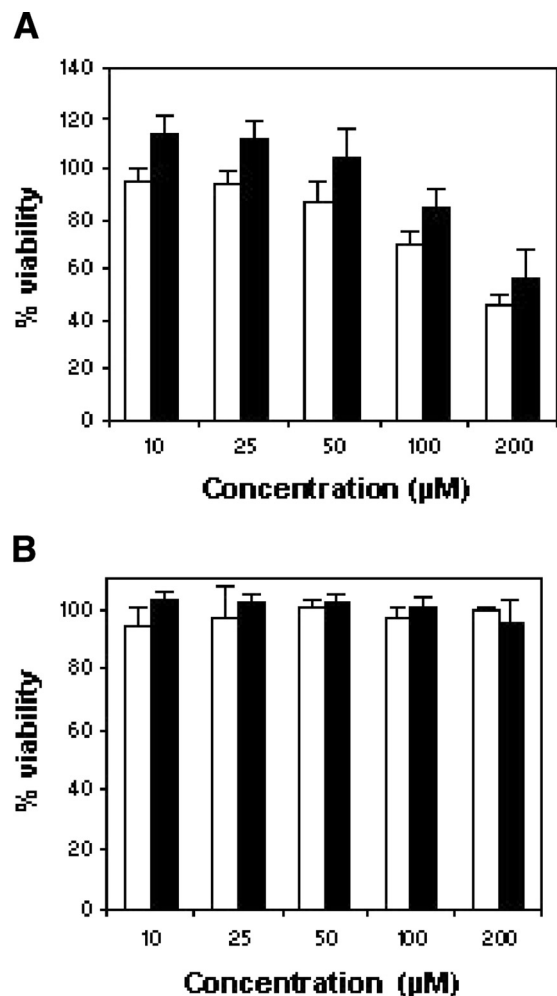
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Fig. 2. PrP 106–126 neurotoxicity in SH-SY5Y and wtPrP cells. PrP 106–126 (**A**) or scrambled (control) PrP 106–126 (**B**) was added at concentrations of 10–200 μM. Cell metabolism (viability) was measured 24 hr later using the MTS assay. PrP 106–126, but not scrambled peptide, altered the metabolic activity of SH-SY5Y (white bars) and wtPrP (black bers) cells in a dose-dependent manner. No significant differences were observed in relation with the level of PrP^c expression. Each value represents the mean percentage ± SD from triplicate experiments repeated three times (nine observations).

observed, suggesting that PrP^c was either cleared or masked by the interaction with SAF34.

To study the effect of the antibody treatment on the toxicity induced by the prion peptide 106–126, we have carried out toxicity assays on cells treated for 24 hr with the antibody. The MTS assay (Fig. 5) showed that the measured values remained equivalent in the presence of the SAF antibody suggesting that the toxicity of the peptide is independent of the cellular prion protein.

We have shown previously that the PrP 106–126 induced a destabilization of lipidic liposomal vesicles indicating that the toxicity effect of this peptide could occur via a membrane interaction (Dupiereux et al., 2005b); this in turn suggests that lipidic components

could play a key role in the toxicity of PrP 106–126 peptide.

Rabbit Kidney Epithelial Cells Are Resistant to Prion Peptide 106–126 Toxicity

To further investigate the relationship between the basal expression of PrP^c and the neurotoxicity induced by the peptide 106–126 we used a cell model (rabbit kidney epithelial cells) that expresses low levels of PrP^c but in which murine PrP^c can be selectively induced by doxycycline (Vilette et al., 2001c).

Treatment with doxycycline (10–1,000 ng/ml) induced the expression of PrP^c, as shown by Western blotting (Fig. 6). Conversely, in the absence of doxycycline no endogenous PrP^c was detected in these cells (Vilette et al., 2001b).

In these cells, we have shown that the absence or the presence of doxycycline (absence or presence of PrP^C expression), does not change the PrP 106–126 toxicity (Fig. 7). The incompatibility between the human sequence of PrP 106–126 and the murine PrP can be discarded; indeed, we have shown by MTS assay, that the same peptide is neurotoxic for murine neuroblastoma cells (N2a) expressing a murine PrP in the same concentration range of PrP 106–126 inducing 50% cell death at 200 μM (data not shown). These data indicate that the PrP expression is not required for PrP106–126 toxicity.

The difference in the susceptibility of these two tested cell lines (neuronal and non neuronal), toward the PrP 106–126 peptide, might be related to the composition of the lipidic cell membrane. To study the putative implication of the lipids, the fatty acid composition of the cellular membrane of SH-SY5Y and A74 was compared (Table I). Significant differences were observed between these two cell lines and in particular in the composition of their unsaturated fatty acids. Indeed, the content in unsaturated fatty acids in the neuroblastoma cells was significantly higher than in the rabbit kidney epithelial cells. The most unsaturated fatty acids in the neuronal cells were palmitoleic acid (16:1), vaccenic acid (18:1), arachidonic acid (20:4), docosapentaenoic acid (22:5), and docosahexaenoic acid (22:6). These data suggest that the susceptibility to the prion peptide 106–126 could be related to the fatty acid composition of the cellular membranes. Our observation reinforces the hypothesis of a direct interaction of the prion 106–126 peptide with the lipidic membrane.

Enhanced Toxicity of PrP 106–126 on the Neuroblastoma Cell Line in the Presence of Palmitic Acid

Based on fatty acid analysis we observed different saturated and non saturated fatty acid composition between the neuronal and non neuronal cell lines used in this study. Indeed, amongst others, palmitic acid, vaccenic acid and docosahexaenoic acid were present in low amounts in the PrP 106–126 resistant non neuronal
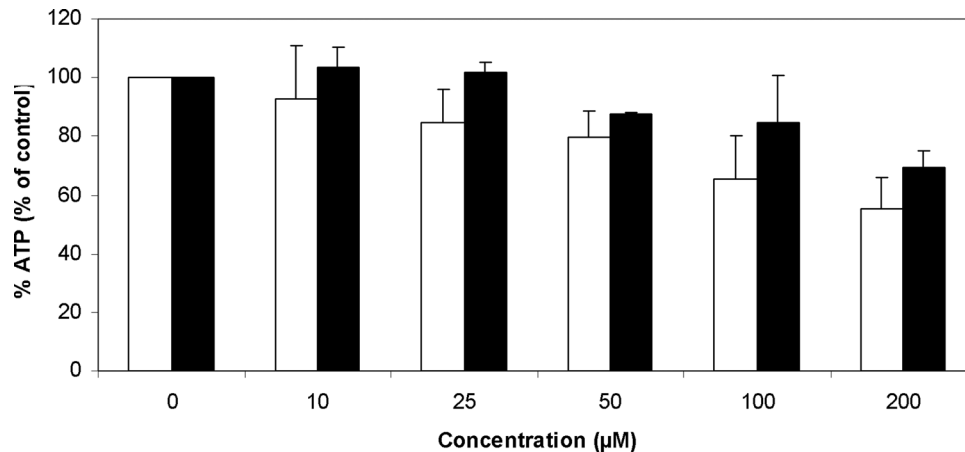
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Fig. 3. Effect of PrP 106–126 on intracellular ATP levels of cells. SH-SY5Y (white bars) or wtPrP (black bars) were treated for 24 hr with different concentrations of PrP 106–126 (10–200 μM). Data are expressed as a percentage of control (non-PrP 106–126 treated cells) and represent the mean percentage ± SD from triplicate experiments repeated three times (nine observations).
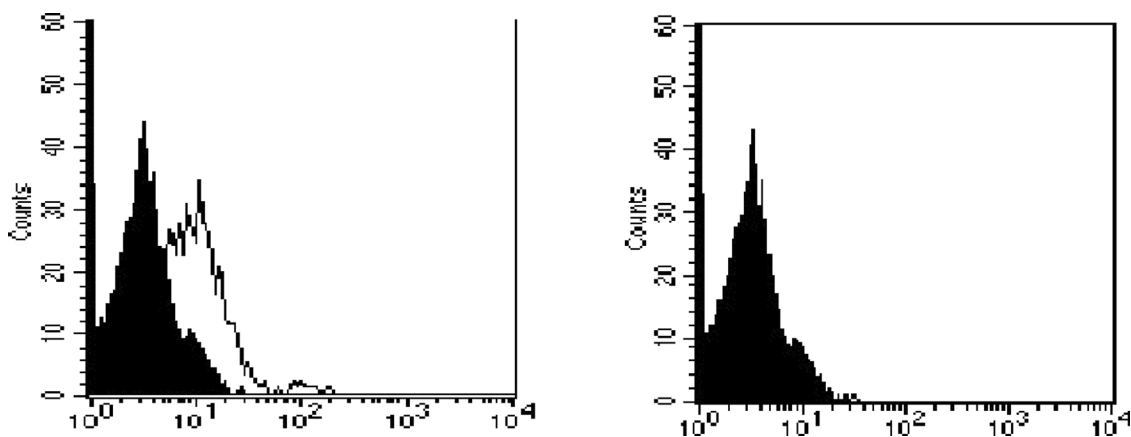

Fig. 4. Flow cytometry detection of PrPc expression in the absence (**A**) or in the presence (**B**) of SAF 34. SH-SY5Y cells were incubated for 24 hr with the anti-prion antibody SAF 34 and PrP^c was detected with a biotinylated antibody SAF34 (10 μg/ml) for 30 min at 4°C (white histogram). The control cells were not incubated with the antibody biotinylated SAF34 (black histogram).

cell line. In the present work we were mainly interested on the potential implication of palmitic acid, a representative cell membrane fatty acid, in this resistance.

To evaluate whether palmitic acid triggered PrP 106–126 cytotoxicity, we determined the synergistic toxic effect of palmitic acid and prion peptide on the neuronal (SH-SY5Y) and non neuronal (A74) cell lines. Cell viability as measured by MTS assay is shown in Figure 8. We compared the viability of the palmitic acid pre-treated cells after a 24 hr exposure to 200 μM PrP 106–126. As expected, PrP 106–126 led to a 50% survival for the neuronal cell line (SH-SY5Y) whereas no toxicity was observed for the rabbit kidney epithelial cell line (A74). In contrast, SH-SY5Y pre-treated with palmitic acid showed significant susceptibility to the toxic effect of prion peptide with a cell death increase of 10%, whereas palmitic acid alone did not affect cell viability. Surprisingly, the non neuronal A74 cell line pre-treated with palmitic acid remained resistant to the PrP 106–126 toxicity.

DISCUSSION

PrP 106–126 peptide is a useful model for the in vitro study of prion-induced cell suffering. Indeed, this peptide exhibits some of the pathogenic and physico-chemical properties of PrP^{sc}. It is able to form protease-resistant fibrils, induce neuronal toxicity, and has a relatively high β-sheet content (Tagliavini et al., 2001). However the mechanism by which this peptide induces alterations in neuronal cells is not well understood. Does this occur by direct membrane interactions or via membrane receptors?

We have studied the relationship between the expression of PrP^c, the lipidic membrane composition, and the toxicity induced by the prion peptide 106–126.

It is widely accepted that PrP^c is necessary for the toxicity of PrP^{sc} (Bueler et al., 1993; Brandner et al., 1996). This dependence on PrP^c and toxicity of PrP 106–126 has also been indicated (Brown et al., 1994; Chabry et al., 2003b; Fioriti et al., 2005d). Nevertheless,

Fig. 5. Effect of SAF 34 on the toxicity induced by PrP106–126 in SH-SY5Y and wtPrP cells. The viability was measured in untransfected SH-SY5Y cells (white bars) or wtPrP-expressing cells (black bars) both exposed to PrP 106–126 at 200 µM for 24 hr after a pre-incubation of 3 hr in the presence or absence of SAF 34. Each value represents the mean percentage ± SD from triplicate experiments repeated three times (nine observations).
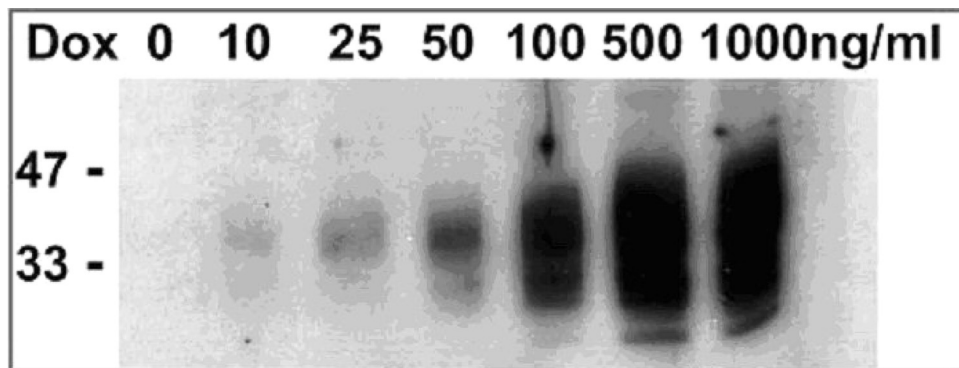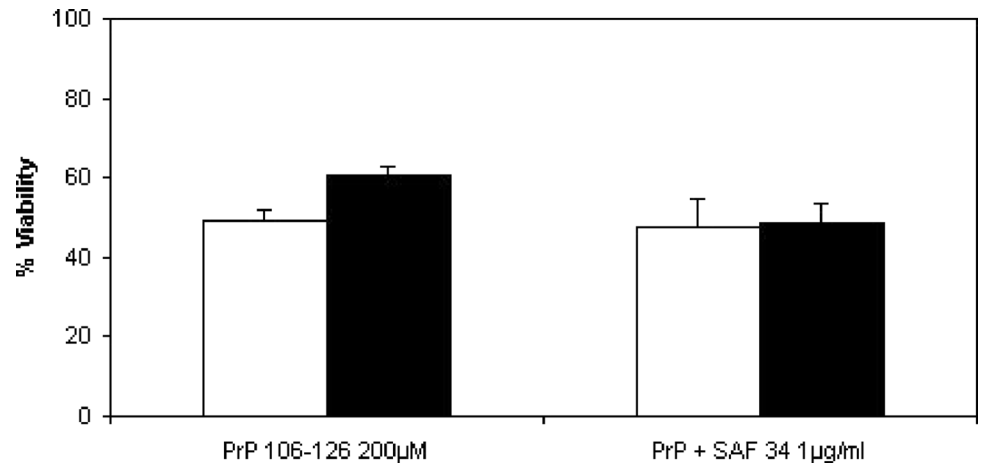

Fig. 6. Doxycycline-dependent induction of PrP^c expression in A74 cells. Dox was added at different concentrations (0, 10, 25, 50, 100, 500, and 1,000 ng/ml) to medium for 24 hr, and PrP^c expression was determined in A74 cells by Western blot. The equivalent of 20 µg of protein were subjected to SDS-PAGE and PrP^c was detected with antibody P45-66 raised against the N terminus of the protein. PrP^c expression reaches a maximum at 500 ng/ml of dox; after this concentration we reach a plateau. Molecular mass markers are indicated on the left in kDa.
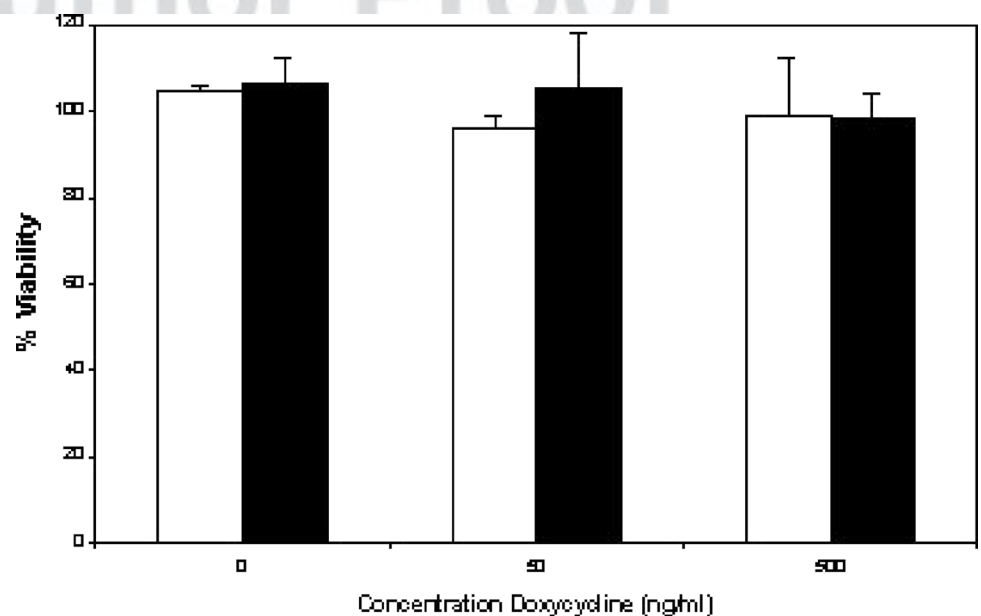

Fig. 7. Effect of PrP 106–126 on a rabbit kidney epithelial cells (A74) in relation with the expression level of PrP^c. A74 cells were seeded at 5,000 cells/well and stimulated with doxycycline (50 and 500 ng/ml) 24 hr before PrP 106–126 exposure at different concentrations: 50 µM (white bars); 100 µM (black bars). Viability was measured by MTS assay. Each value represents the mean percentage ± SD from triplicate experiments repeated three times (nine observations).

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the mechanism of toxicity remains unclear and contradictory data about the relation between PrP^c expression and the toxicity induced by PrP^{sc} or PrP 106–126 have been reported. Several works have shown that the prion peptide 106–126 is toxic to cultured neurons and that neurons derived from Prnp$^{-/-}$ mice are resistant to this peptide (Brown et al., 1994; Chabry et al., 2003c). Fioriti et al. (2005a) using cerebellar granule neurons derived from wild-type mice and Tg mice overexpressing mouse PrP, did not detect any difference in the toxicity of PrP 106–126 on both cell lines. Conversely, they did not observe any toxicity of the peptide on the knock-out cell line. These data suggest that the expression of PrP^c is necessary for the toxicity induced by the peptide but that the level of expression does not modulate this neurotoxicity. The authors propose to explain the toxicity of PrP 106–126 by the physiologic loss of PrP^c. Furthermore, Gu et al. (2001) have shown that in a human neuronal cell line resistant to the PrP 106–126 toxicity, the prion peptide binds at the cell surface and that some of the resistant neuronal cells internalize the peptide that accumulate in intracellular compartments. They concluded that the resistance to the toxicity induced by PrP 106–126 seemed to be related to an aberrant binding of the peptide with the membranes. Currently, it is known that such an aberrant binding with the cellular membrane can be induced by an alteration in the membrane lipidic composition.

In the present study, we have analyzed the toxicity of the peptide on a neuroblastoma cell line expressing different levels of PrP^c. Our results show that the toxicity of PrP 106–126 is independent of the expression level of the cellular prion protein, as shown by Fioriti and collaborators (Fioriti et al., 2005c). To study the implication of the endogenous levels of cellular prion protein in the toxicity induced by the peptide, we have used an original strategy based on the ability of an antiprion antibody (SAF34) to inhibit PrP^{sc} replication and to decrease the levels of total PrP^c and PrP^{sc} in an infected cell model (Feraudet et al., 2005b). We have shown by MTS assay that an incubation of SH-SY5Y with SAF34 did not reduce the toxic effect of the peptide 106–126 indicating that endogenous levels of the cellular prion protein is not directly implicated in the toxicity of the peptide. Because we cannot exclude the possibility that residual PrP^c is left in our cells after the antibody treatment, we propose that a sub-physiological level or an appropriated lipid composition could be required for peptide toxicity.

Our finding is consistent with other reports indicating that the toxicity of PrP^{sc} and PrP 106–126 is independent of the expression of PrP^c. Indeed, it has been shown that PrP^{sc} and PrP 106–126 may cause an upregulation of the MAP kinases signalling pathway inducing neurotoxicity in Prnp$^{-/-}$ cells (Gavin et al., 2005; Marella et al., 2005). Furthermore, McHattie et al. (1999) have studied the cellular trafficking of the prion peptide 106–126 and have observed that cultured cells are capa

TABLE I. Comparative Study of Fatty Acid Composition of the Cellular Membrane of SH-SY5Y and A74 Cells

| Phospholipidics fatty acids	Neuronal cells (SH-SY5Y) μmol/kg	Non neuronal cells (A74) μmol/kg
Myristic acid	1,129	923
Nentadecyclic acid	144	674
Nalmitic acid	10,247	5,765
Nalmitoleic acid	1,276	701
Stearic acid	3,596	2,460
T-vaccenic acid	187	68
Oleic acid	8,928	8,814
C-vaccenic acid	3,165	195
Linolenic acid	572	520
Gamma linolenic acid	25	22
Alpha linolenic acid	49	32
Gadoleic acid	209	329
Dihomo-g-linolenic acid	124	177
Arachidonic acid	1,974	1,390
Eicosapentaenoic acid	96	612
Docosapentaenoic acid	999	98
Docosahexaenoic acid	2,420	1,280
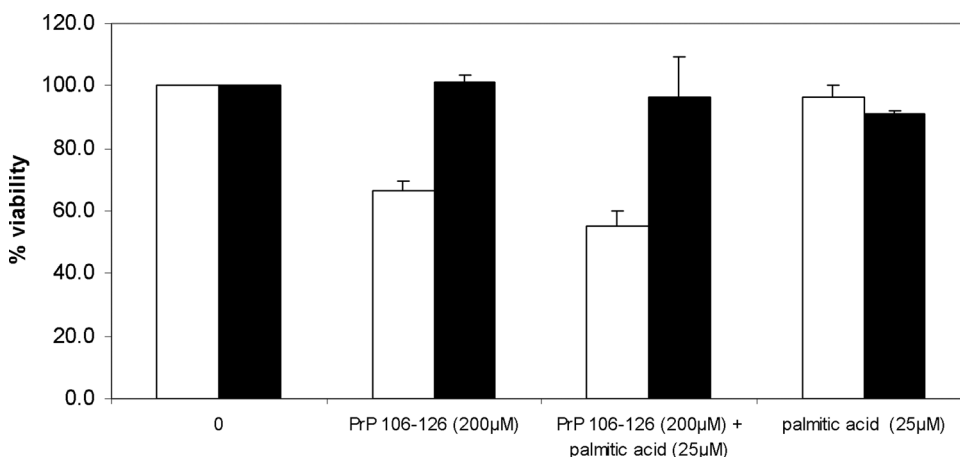
Fig. 8. Effect of palmitic acid on the toxicity induced by PrP 106–126 in SH-SY5Y and A74 cells. The viability was measured in SH-SY5Y cells (white bars) and A74 cells (black bars) both exposed to PrP 106–126 at 200 μM for 24 hr after a pre-incubation of 16 hr in the presence or absence of palmitic acid (25 μM). Each value represents the mean percentage ± SD from triplicate experiments repeated three times (nine observations).

ble of sequestering the peptide independent of PrP^c expression.

These results suggest that the toxic mechanism of this peptide is independent of a direct interaction with PrP^c and indicate that a direct insertion of PrP 106–126 into the cell membrane, particularly in cholesterol–rich lipid domains, named rafts, could induce disturbances. We have shown recently that the 106–126 peptide de-stabilizes lipid vesicles mimicking the composition of neuronal membranes and that it induces liposome fusion. This destabilization occurred via a membrane interaction as was shown by the integrity membrane assay (Dupier-eux et al., 2005c).

Using a non-neuronal cell line in which the expression of PrP^c could be selectively induced by doxy-cycline (Vilette et al., 2001a), no toxicity was observed in the presence and in the absence of PrP^c. These data are in agreement with the results obtained on the neuro-blastoma cells and suggest that the toxicity induced by PrP 106–126 could be dependent on the composition of the cellular membrane as was shown by the fatty acids analysis. We have observed that the effect of toxicity induced by the PrP 106–126 prion peptide was more pronounced in the presence of palmitic acid whereas, no effect was observed for the non-neuronal cell line A74. The mechanism as to how palmitic acid could increase the prion peptide toxicity was not investigated in the present study and needs to be further explored; these results must be thoroughly studied by testing different other lipid factors but at the current state of the work, we can suggest that the palmitic acid enhances binding or internalization of the peptide into the lipid mem-brane. It has been reported that fatty acids with longer acyl chains (C12–18) cause a reduction in the fluidity of the cellular membranes affecting cell membrane func-tion (Johnson et al., 2003) and inducing apoptosis. Our results seem to indicate that the cellular membrane fatty acid composition could be an important modulator factor in the neurodegeneration observed in prion pa-thology. This is probably by altering the physicochemi-cal properties of cell membranes favoring the membrane interaction of pathologic prion protein with the cell membranes.

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In conclusion, we reinforce the hypothesis that the toxicity induced by the prion peptide 106–126 may not be due only to direct interaction with PrP^c and that the key event in this toxicity could be a direct interaction with the lipidic components of cell membranes. A desta-bilization of membrane lipids could interfere with PrP^c function and may facilitate access of the peptide to intra-cellular targets.

Our current results reinforce previous observations (Chen et al., 1995; Jeffrey et al., 2004b) proposing that, during prion diseases, the novo-transconformed PrP^{sc} could be cleaved in toxic prion protein fragments re-sponsible of neuronal dysfunction and death. Even more, these peptides may diffuse and intoxicate adjacent PrP-negative cells; this hypothesis might explain neuronal death in transgenic mice knock-out for the Prnp gene

except in astrocytes after PrP^{sc} infection (Jeffrey et al., 2004a).

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