Transmissible spongiform encephalopathies (TSE) are neurological disorders having common pathological signs like vacuolization of the neuropil, astrocytosis, and loss of neurons (2). TSE are characterized by the accumulation of large aggregates of the prion protein, PrP-res, in both human and animal brains. The abnormal PrP-res isoform has a high content of β-sheet secondary structure, forms amyloid fibrils, and is partially resistant to proteolysis (3–5). The precursor of the pathological isoform PrP-res is a normal host glycoprotein (termed PrP-sen) widely expressed in the central nervous system and in peripheral tissues. Unlike PrP-res, PrP-sen is a protease-sensitive protein, soluble in mild detergents, and has mainly α-helix structures (6–8). Interestingly, the development of spongiform pathologies requires the presence of both isoforms PrP-res and PrP-sen (9), the level of PrP-sen expression being an essential factor in the pathology (10). In experimental transmigrations with transgenic mice, it was found that increasing the number of PrP gene copies led to a decreased incubation time of the disease (11, 12).

The in vivo mechanisms leading to the synaptic loss and neuronal death are not elucidated, but the possible involvement of apoptotic processes has been postulated and is consistent with the lack of inflammation stigmata in TSE-affected brains. The neural tissue damages could be due exclusively to PrP-res deposits, and/or to disruption of PrP-sen physiological functions, and/or to cytotoxic effects induced by PrP fragments or PrP-res aggregates. PrP-res and a synthetic peptide fragment (P106–126) have been shown to be cytotoxic in vitro likely via the programmed cell death pathway (1, 13). Like PrP-res, the P106–126 peptide is partially resistant to proteinase K, presents a high β-sheet enriched structure, and forms amyloid fibrils in vitro (14). Hope et al. (15) have shown that the expression of PrP-sen is necessary for P106–126 to exert its cytotoxic effects.

Whether the apoptotic process could account for in vivo neurodegeneration taking place during the development of TSE remains unclear. The induction of apoptosis has been suggested in scrapie-infected mouse brain (16), as well as in Creutzfeldt-Jakob disease (17) and bovine spongiform encephalopathy (18).

In the present report, we examined whether the PrP fragment P106–126 can induce apoptotic-mediated cell death in vivo. To address this question, we directly administered P106–126 into rat eyes taking advantage of the fact that the retina is an integral part of the central nervous system. Apoptotic process induced by P106–126 was demonstrated by means of the terminal deoxynucleotidyl transferase dUTP-end labeling (TUNEL) staining and of the DNA fragmentation technique. Neuronal death was confirmed by measurement of the electrical activity of the retina.

**EXPERIMENTAL PROCEDURES**

**Peptides**—The human sequence of the prion protein fragment (P106–126; KTNNHMAGAAAGAVVGLG) was purchased from Bachem. The prion fragment used as a control peptide (P98–103; GQGGGTHNQWKNPKSK) was synthesized by Eurogentec. Lyophilized peptides were dissolved in deionized water at a concentration of 5 mM, distributed into 20-μl aliquots, and stored at −20 °C. Peptides were diluted twice with 2× PBS minus Ca2+ and immediately used or aged by incubation at room temperature for 3 days.

**Intravitreal Injection**—Adult male albino Wistar rats (35–55 days old) were anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital followed by a topical application of 0.4% oxybuprocaine hydrochloride. The injections of 2 μl of peptides or vehicle (phosphate-buffered solution) were done unilaterally with a 30-gauge needle introduced into the posterior chamber on the upper pole of the eye directed toward the center of the vitreous. The injections were performed slowly to allow a better diffusion of the peptide and avoid any ocular hypertension. For negative controls, we both considered the uninjected contralateral eyes and eyes injected in the same conditions with 2 μl of vehicle (PBS). At least four animals were used in each group of experimental conditions.

**In Situ Labeling by the TUNEL Method**—Rats were euthanatized with an overdose of sodium pentobarbital 3 days post-intravitreal injections. The eyes were enucleated, and a puncture was made at the limbus to permit the infusion of the fixative solution. The eyes were immediately fixed in ice-cold 4% paraformaldehyde in PBS for 4 h then cryoprotected overnight in PBS containing 20% sucrose and embedded in Tissue-Tek® (Sakura). Frozen sections (10 μm) were cut on a cryostat (Leica), and slides were heated at 50 °C for 60 min then stored at −20 °C until use. The in situ cell death detection was performed fol-
lowing the manufacturer's recommendations (Roche Molecular Biochemicals) then revealed using a 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories) and processed for detailed examination by light microscopy. Morphological and histological observations of retinas were done by staining the sections with 1% cresyl violet.

**Dose-Response Effect of PrP Peptide P106–126**

The concentration was determined by measuring the absorbance at 260 nm. Samples of 10 µg/ml of protein were diluted (corresponding to 0.05, 0.5, and 5 nmol) and precipitated in a solution of 0.3 M sodium acetate in ethanol overnight at -20 °C. The DNA concentration was determined by measuring the absorbance at 260 nm. Samples of 10 µg of DNA were electrophoresed through 1.2% agarose gel containing 1 µg/ml of ethidium bromide. DNA bands were visualized by a UV light transilluminator and photographed.

**Electroretinograms (ERGs)**—Dark-adapted ERGs were performed as described previously (19). Full-field ERG responses were obtained overnight with dark-adapted rats prepared under dim red light before recording. The pupils of anesthetized rats were dilated with a drop of 0.5% tropicamide. A silver chloride ring-recording electrode was placed on the cornea, and the reference electrode, with a silver-silver chloride tip, was connected to the ear. Light stimulus (10 µw) was provided from a stroboscopic flash (Grass Instruments Inc.) placed 0.25 m in front of the rat. The ERGs were recorded using the visual electroretinogram test system (LRC Technologies, Inc.) and then stored and analyzed. Amplitude of the a-wave was measured from the trough to the peak of the b-wave. The averaged responses represent the mean of five white flashes delivered four min apart. Electroretinograms were recorded before treatment and then at different times of recovery (1, 2, 3, and 7 days).

**Statistical Analysis**—The amplitude values of a- and b-waves obtained with each animal were normalized to baseline amplitudes at each point of the study and expressed as percentage of baseline value. Data were expressed as mean ± S.E. of values obtained with 4 rats per group. Results from the ERG recordings were not distributed normally and had unequal variance. Therefore, the Mann Whitney U test was used to compare the peptide and the vehicle-treated group of rats. Differences were considered statistically significant when P values were less than 0.02.

**RESULTS**

**In Vivo Induction of Retina Cells Apoptosis by Peptides**—The effect of freshly prepared or 3-day-aged P106–126 and soluble P89–103 PrP peptides on the retina cell death was examined after intravitreal injection. Fig. 1 shows representative photomicrographs of treated retina sections processed through the TUNEL labeling assay (Fig. 1, B–E) and the histology of PBS-treated retina stained with cresyl violet (Fig. 1A). After intravitreal injection of aged P106–126 (5 nmol), TUNEL-positive cells were observed in the inner and outer nuclear layers, as well as in the ganglion cells layer (Fig. 1D). When freshly prepared P106–126 was intravitreally injected, only occasional TUNEL-positive cells were observed (Fig. 1C) suggesting that the cytotoxic effect of the peptide was linked to its physicochemical state. Intact retinas (data not shown) and retinas treated with PBS alone (Fig. 1B) or soluble PrP peptide P89–103 (Fig. 1E) showed only few TUNEL-positive cells. No differences in labeling patterns were observed between the lower and the upper poles of the retina where injections were done.

**Dose-Response Effect of PrP Peptide P106–126 on TUNEL Labeling**—To assess whether the cytotoxic effect was dose-dependent, rat eyes were treated for 72 h with PBS alone or containing 0.05, 0.5, or 5 nmol of aged P106–126. Corresponding retina sections were processed through the TUNEL-labeling assay and revealed with the DAB substrate. The nuclei of TUNEL-positive cells are black. Magnification, × 10. Bar, 50 µm. The cell layer abbreviations are as follows: G, ganglion cell; IP, internal plexiform; INL, inner nuclear; EP, external plexiform; ONL, outer nuclear; IS, inner segments of rods; OS, outer segments of rods and cones.

**Fig. 1. Effect of PrP peptides P106–126 and P89–103 on retina cell death.** Representative microphotographs showing an un injected rat retina stained by 1% cresyl violet (A) and peptide-treated retina sections revealed by the TUNEL-labeling method (B–E) are shown. Rats were intravitreally injected with 2 µl of PBS alone (B) or containing 5 nmol of freshly prepared (C) and 3-day-aged PrP peptides P106–126 (D) and P89–103 (E), respectively. Rat eyes were enucleated 72 h after injection, fixed, and cut on a cryostat. Retinal sections (10 µm) were processed through the TUNEL-labeling protocol and revealed with the DAB substrate kit. The nuclei of TUNEL-positive cells are black. Magnification, × 10. Bar, 50 µm. The cell layer abbreviations are as follows: G, ganglion cell; IP, internal plexiform; INL, inner nuclear; EP, external plexiform; ONL, outer nuclear; IS, inner segments of rods; OS, outer segments of rods and cones.
nuclear layer. The retinal DNA was extracted and electrophoresed by the PrP peptide P106–126 on retina cells. The b-wave amplitudes were normalized and expressed as the percentage of vehicle alone and of aged peptide P106–126 (5 nmol), respectively. The a- and b-wave amplitudes were expressed as the change from control. Each group represents four animals injected unilaterally; ten sections were cut per eye. The relative intensity of DNA was determined in a 10-fold magnification photograph of retina sections and quantified with the NIH image software. The data represent the means ± S.E. of two independent experiments expressed in arbitrary units. G, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

In Vivo Cytotoxicity of Peptide P106–126

Effects of PrP peptide P106–126 on the retinal DNA laddering pattern. The retinal DNA was extracted and electrophoresed on a 1% agarose gel as described under "Experimental Procedures." Each lane was loaded with 10 µg of retinal DNA extracted from four retinas treated with PBS (control) or aged peptide P106–126 for 24, 48, and 72 h. DNA standard ladders are indicated on the left side of the gel. bp, base pair.

For the last decade, many reports have shown that PrP has been extensively studied in vitro (30, 31). The b-sheet structure adopted by P106–126 and its aggregation state are crucial (1, 15). Our results are consistent with these previous reports, because intravitreous injections of freshly diluted P106–126 failed to cause cell death and modification of electrical activity of the retina. The observed effects of the peptide are monitored by DNA fragmentation and electroretinogram recording analysis and can be evidenced as soon as 24 h after intravitreal injection. Moreover, P106–126 causes long-term damages on the physiological activity of retina, because no recovery of the deleterious effects can be observed 7 days after peptide inoculation.

The molecular mechanisms involved in the P106–126 toxicity are not completely understood. The relationship between toxicity and physicochemical properties of amyloid peptides has been extensively studied in vitro (30, 31). The b-sheet structure adopted by P106–126 and its aggregation state are crucial (1, 15). Our results are consistent with these previous reports, because intravitreal injections of freshly diluted P106–126 failed to cause cell death and modification of electrical activity of the retina. Moreover, Jen et al. (32) have shown recently that the amyloid peptides Aβ1–40 and Aβ1–42 involved in Alzheimer's disease also induced photoreceptor cells apoptosis after intravitreal injections. Here again, cell death was observed only with aged (aggregated) peptides, whereas freshly prepared solutions were devoid of cytotoxicity. As a positive control to our own experiments, the apoptotic response induced by aged solutions of Aβ1–40 have been reproduced in the present work (data not shown). There exists no sequence homology between Aβ1–40 and P106–126 peptides. However, both peptides share the same propensity to form b-sheet structures and sedimentable aggregates. Taken together these data strongly suggest that the specific mode of the aggregation of P106–126, rather than its amino acid sequence, is responsible for the observed in vivo cell death.

Apart from programmed-cell death, other molecular mechanisms of P106–126 toxicity have been proposed but are still controversial (33). The possible insertion of the peptide into the cell membrane could lead to channel formation, the subsequent disruption of ion homeostasis being responsible for the cell death (34, 35). Our results strongly suggest that at least part of the neuronal loss observed in TSE diseases is because of the toxic effects of PrP-res and/or its degradation products. In contrast with Alzheimer's disease in which the overproduction of the amyloid peptides Aβ1–40 and Aβ1–42 is well documented, the catabolism of the PrP molecules remains unclear. An amino-terminally truncated fragment of PrP insoluble in detergents and resistant to protease has been purified from brains of
Creutzfeldt-Jakob disease affected patients (36). Caughey et al. (37) have also shown that a PrP-res isofom bracketed at residue 90 is formed in scrapie-infected mouse neuroblastoma cells. Furthermore, fragments of PrP-sen have been identified in extracts of normal brain, and the precise cleavage site has been mapped to His-111 or Met-112 (36). Interestingly, the cleavage occurring in physiological conditions disrupts the neurotoxic region of PrP-sen comprising amino acid residues 106–126. Thus, pathological conditions could promote the appearance of PrP toxic fragments that are never formed in physiological situation.

In conclusion, our study provides the first in vivo evidence of the involvement of an apoptotic process in the cytotoxic mechanisms of the prion protein fragment P106–126 suggesting that similar mechanisms also occur in TSE. The retina model used in the present work is a powerful tool to further identify the molecular mechanisms leading to the neuronal loss that could be a general characteristic of several neurodegenerative diseases. Finally, the retina model should serve for screening drugs able to limit cell damage and prevent neuronal loss.

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