Amyloid Fibrils of Mammalian Prion Protein Are Highly Toxic to Cultured Cells and Primary Neurons*

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A growing body of evidence indicates that small, soluble oligomeric species generated from a variety of proteins and peptides rather than mature amyloid fibrils are inherently highly cytotoxic. Here, we show for the first time that mature amyloid fibrils produced from full-length recombinant mammalian prion protein (rPrP) were highly toxic to cultured cells and primary hippocampal and cerebellar neurons. Fibrils induced apoptotic cell death in a time- and dose-dependent manner. The toxic effect of fibrils was comparable with that exhibited by soluble small β-oligomers generated from the same protein. Fibrils prepared from insulin were not toxic, suggesting that the toxic effect was not solely due to the highly polymeric nature of the fibrillar form. The cell death caused by rPrP fibrils or β-oligomers was substantially reduced when expression of endogenous PrP巡 was down-regulated by small interfering RNAs. In opposition to the β-oligomer and amyloid fibrils of rPrP, the monomeric α-helical form of rPrP stimulated neurite outgrowth and survival of neurons. These studies illustrated that both soluble β-oligomer and amyloid fibrils of the prion protein are intrinsically toxic and confirmed that endogenously expressed PrP巡 is required for mediating the toxicity of abnormally folded external PrP aggregates.

Several neurodegenerative maladies including Alzheimer, Parkinson, Huntington, and prion diseases have been related to the age-dependent accumulation of amyloid deposits in the brain (1, 2). A common feature among these and other “conformational” diseases is the conversion of specific proteins or peptides into polymeric forms that are characterized by cross-β-sheet structures and referred to as amyloid (3). Even though the amyloidogenic proteins have no obvious sequence similarity, they share similar conformational features within the amyloid form (4, 5).

In addition to the amyloid fibrils/deposits, the formation of nonfibrillar soluble oligomers has been observed for a number of proteins associated with conformational diseases including α-synuclein (6, 7), Aβ peptides (8, 9), transthyretin (10), lysozyme (11), and prion protein (12, 13). Soluble oligomers were found either as a prefibrillar intermediate that formed on the kinetic pathway to the mature amyloid fibrils (14–17) or as off-pathway products produced via alternative aggregation mechanisms (13, 18, 19). For the past several years, a substantial body of evidence has accumulated indicating that soluble oligomers are toxic species that are actively involved in the impairment of cellular functions in neurodegenerative diseases. Furthermore, non-fibrillar oligomers have been shown to be intrinsically toxic to cells even when formed from proteins that are not related to any known conformational disease (20, 21). This implies that oligomeric species share a common mechanism of cytotoxicity regardless of the specific protein from which they are generated (22, 23). On the other hand, there is considerable debate as to whether mature amyloid fibrils also exhibit toxicity and are responsible for the onset of neurodegenerative diseases (8, 20, 24–26). In the prevailing opinion, formation of mature fibrils and their co-aggregation into deposits and plaques is considered to be a protective mechanism that evolved in nature to avoid the high intrinsic toxicity of soluble oligomers (20, 24, 25). Determining the relationship between toxicity and the physical state of protein is essential for the identification of the pathogenic species and developing effective therapeutic strategies against neurodegenerative diseases.

To establish a link between toxicity and the physical state of a protein, we tested the effect of full-length mammalian prion protein (rPrP) presented in three conformationally different isoforms to cultured cells and primary neurons. These three isoforms are α-helical monomers, referred to as α-rPrP, β-sheet rich oligomers, referred to as the β-oligomer, and mature amyloid fibrils. To date the studies on toxicity of mammalian prions have been mostly confined to a short synthetic PrP-derived peptide encompassing residues 106–126 or to PrP巡. Although both PrP巡 extracted from scrapie brains and aged solutions of synthetic PrP-(106–126) were reported to induce apoptotic cell death in primary neurons (27–29) and in cultured cells (30, 31), the identity of the cytotoxic species remains uncertain. The difficulties in establishing the origin of cytotoxic effects are largely due to the heterogeneous nature of PrP巡 aggregates, which are known to consist of a mixture of fibrils and non-fibrillar oligomeric species (32) and also due to possible impact of non-protein components that are tightly associated with PrP巡 (33, 34).

Here, we report for the first time that both the soluble β-oligomeric species and mature amyloid fibrils produced from the full-length prion protein are highly toxic to cultured cells and primary neurons. As in the case for PrP巡-induced toxicity, the toxicity of the fibrils and the β-oligomers was dependent on expression of endogenous PrP巡. Several lines of evidence indicate that cell death occurred via an apoptotic mechanism. In opposition to the fibrils and to the β-oligomers, α-rPrP stimulated neurite outgrowth and prolonged neuronal survival. These findings illustrate the remarkable ability of cells to recognize three conformationally different isoforms of the prion protein and provide insight into possible origin of neuron loss during prion infection.

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5 The abbreviations used are: rPrP, recombinant full-length prion protein; PrP巡, cellular isoform of the prion protein; PrP巡, disease-associated isoform of the prion protein; PLL, polylysine; siRNA, small interfering RNA.
Materials and Methods

Protein Purification and Refolding—Recombinant mouse full-length PrP encompassing residues 23–230 was expressed, purified, and refolded into the α-helical form (α-rPrP) as described previously (35). The purified α-rPrP was confirmed by SDS-PAGE and electrospray mass spectrometry to be a single species with an intact disulfide bond (purity >99%). The conformation and monomeric state of α-rPrP was confirmed by circular dichroism and by size-exclusion chromatography, respectively (13). Conversion of α-rPrP to the β-oligomeric form and to the amyloid fibrils was performed as previously described using partially denatured conditions (13). To form amyloid fibrils from insulin, an aqueous solution of bovine insulin (Sigma) was prepared at a concentration of 1 mM, the pH was then adjusted to 2.0 with HCl, and the solution was incubated overnight at 65 °C as described previously (36). The β-oligomers and the amyloid fibrils of α-rPrP and insulin were dialyzed in 10 mM sodium acetate buffer, pH 6.0, and kept in stock at concentrations equivalent to 50 μM. rPrP isoforms and insulin fibrils were administered directly to the cultured cells by diluting the stock solutions to final concentrations of 0.1–1 μM as indicated.

Cell Cultures—Human SH-SY5Y neuroblastoma cells (ATCC), mouse N2a neuroblastoma cells (ATCC), mouse fibroblasts NIH-3T3 cells (ATCC), and human NT2 (nondifferentiated) cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultured cells were plated at a density of ~8000 cells/cm² in 8-well Pemason Lab-Tek chamber slides (Nagel NUNC Inc.) coated with poly-I-lysine (PLL, 10 μg/ml) and incubated at 37 °C in humidified 5% CO₂. Because toxicity experiments lasted for up to 4 days, we blocked the cell proliferation by reducing the fetal bovine serum percentage in the culture medium from 10 to 1% when rPrP isoforms were administered to the culture medium.

Primary Neuronal Cells—Hipocampi were obtained from E18 Wistar rats as described by Maar et al. (37) with modifications (38). After dissection, the hippocampi were cleared from blood vessels and meninges, mechanically chopped, and trypsinized. The neurons were washed in the presence of DNase and trypsin inhibitor (Sigma), and the cellular debris was pelleted by centrifugation. Cells were plated at a density ~40,000 cells/cm² in 8-well Lab-Tek Pemanox chamber slides either directly or after PLL-coating in Neurobasal medium supplemented with 2% B27 (Invitrogen), 2 mM Glutamax, 100 units/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, and 4% w/v bovine serum albumin. Cultures were incubated at 37 °C in humidified 5% CO₂. Cerebella neurons were isolated from 5-day pups and cultivated using the same procedure. rPrP isoforms were administered at final concentrations of 0.1–1 μM as indicated either 1 h or 2 days after plating neuronal cells, and the cells were incubated for an additional period of time before fixation with 4% paraformaldehyde in phosphate-buffered saline and 0.01 mM sucrose for 20 min. Neurons were identified with primary rabbit anti-GAP43 antibodies (1:700), and secondary antibodies were labeled with Alexa 488 (1:1000). During the final washing stages the cells were counterstained with Hoechst-33258 to visualize their nuclei.

To analyze the neurite outgrowth, images of at least 300 neurons for each group were obtained with a 20×/0.50 NA objective using a cooled 12-bit CoolSnap HQ CCD camera (Photometrics). From these images neurite outgrowth was estimated using the software program Process Length (Protein Laboratory, Copenhagen, Denmark) as described by Ronn et al. (39).

Cytotoxicity Assay and Apoptotic Cell Death—The toxicity of rPrP isoforms was measured on living cells using staining with Hoechst-33342 (Molecular Probes). Hoechst-33342 permeates non-fixed cells and, therefore, has been commonly used for distinguishing dying cells from living cells based on the intensity of nuclei staining. Hoechst-33258 has been used for fixed cells. Apoptotic cells were identified by characteristic bright blue fluorescence of nuclei that appears due to condensed or fragmented chromatin. Digital images were captured from 10 random fields for each sample (~500 cells total) using an inverted microscope. Cell death rate was determined by counting bright cells including cells with fragmented or condensed nuclei and expressed as a percentage of total number of cells. Statistical analysis was made for 3 or 4 independent experiments for each cell type.

Apoptosis was monitored by measuring DNA fragmentation with Hoechst-33342, caspase-3 activation, the movement of phosphatidylserine from the internal to the external surface of the cell membrane (annexin assay), and membrane blebbing (calcein assay). The caspase-3 activity was estimated by a caspase 3-R110 assay (Molecular Probes) using D2-R110 as a substrate according to the manufacturer’s procedure. D2-R110 is cell-permeable and was used on living cells (40). Phosphatidylserine on the external surface and propidium iodide uptake were visualized simultaneously using the annexin V-Alexa 488 apoptosis detection kit (Vibrant apoptosis assay kit 2, Molecular Probes) according to the manufacturer’s recommendations. This double staining discriminates the early stages of apoptosis from necrosis. Membrane blebbing was visualized by staining non-fixed cells with calcein AM (41) (live/dead viability/cytotoxicity kit, Molecular Probes) according to the manufacturer’s procedure.

Caspase-3 Activity—SH-SY5Y cells were cultivated in 60-mm Petri dishes for 24 h before administration of rPrP (1 μM) and then incubated for additional 20 h. Non adherent and adherent cells were collected and solubilized in SDS sample buffer and analyzed by SDS-PAGE followed by Western blot with rabbit anti-caspase-3 antibody (cleaved caspase-3 (Asp-175) antibody, Cell Signaling, Beverly, MA) that recognizes active caspase-3 subunits.

Small Interfering RNAs (siRNA) Transfection—SH-SY5Y cells were transfected with siRNA specific for human PrP (siGENOME SMARTpool siRNA, Dharmacon, Dallas, TX) or with non-targeting siRNA pool (Dharmacon). One day before the transfection cells were plated at a density of ~60,000 cells/well in 24-well culture plates or in 4-well Lab-Tek Pemanox chamber slides in 400 ml of growth medium without antibiotics. The siRNAs were transfected at final concentrations of 50 or 150 nM mixed with 1.2 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Cells were cultured for 3 days, and PrP gene knockdown was confirmed by fluorescence immunostaining using 3F4 antibody (1:1000) followed by secondary goat anti-mouse antibodies labeled with Alexa 488 and by Western blot using anti-PrP AG4 antibodies (1:1000). For fluorescence, immunostaining cells were treated with 0.2% saponin. siGLORISC-Free siRNA (Dharmacon) was used as negative control and as transfection marker. Efficiency of transfection for SH-SY5Y cells was ~80%.

Fluorescence Microscopy—Microscopy was carried out on an inverted microscope (Nikon Eclipse TE2000-U) with an illumination system X-Cite 120 (EXFO Photonics Solutions Inc.) connected through fiber optics using a 1.3 aperture Plan Fluor 100× NA and 20×/0.50 NA objectives. Digital images were acquired using a cooled 12-bit CoolSnap HQ CCD camera (Photometrics).

Statistical Analysis—Data on cell death were analyzed by one-way analysis of variance for repeated measurements supplemented with Newman-Keuls post-comparison test. Statistical comparison of results was made in the GraphPad Prism software package, Version 4 (San Diego, CA). Significance was expressed as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
**Toxic Prion Protein Fibrils**

![Image](https://example.com/image1)

**FIGURE 1. Immunofluorescent imaging of the β-oligomers and amyloid fibrils.** a, immunostaining of β-oligomers incubated in the cell culture medium in the absence of cells (panel 1) and in the presence of SH-SYSY cultured cells (panel 2). b, immunostaining of amyloid fibrils incubated in 10 mM sodium acetate buffer, pH 5.0 (panel 1), in cell culture medium in the absence of cells (panel 2), and in cell culture medium in the presence of SH-SYSY-cultured cells (panel 3). Both the β-oligomer and the amyloid fibrils were stained using Fab P (1:1,000) (green); cell nuclei were stained with Hoechst-33258 (blue). Scale bars ~ 10 μm.

**RESULTS**

Preparation of Conformationally Distinct Isoforms of rPrP—Elucidating the potential toxicity of conformationally distinct isoforms requires preparation of relatively homogeneous species of each isoform. All three isoforms of rPrP (α-rPrP, β-oligomer, and amyloid fibrils) were prepared according to the experimental procedures that we have previously described (13). To prepare homogeneous samples of the β-oligomer and fibrils, we took advantage of the fact that these two isoforms can be produced selectively under different solvent conditions and that the β-oligomer is not on the kinetic pathway to amyloid fibrils (13, 18, 42). Differences in the physical properties of rPrP isoforms were confirmed using several biophysical techniques including circular dichroism and Fourier transform infrared spectroscopy (to confirm secondary structure), size-exclusion chromatography (to confirm the oligomerization state), thioflavin T assay, electron microscopy, and epifluorescence microscopy (to confirm fibrillar structures) (13, 35). In the preparation of α-rPrP no fibrils were found; however, ~5% of total rPrP was the β-oligomer species as determined by the size-exclusion chromatography (data not shown). The preparation of β-oligomer contained less than 10% of the monomeric α-rPrP species as was quantified by size-exclusion chromatography (data not shown). However, samples of the β-oligomers were free of amyloid fibrils as judged from electron microscopy, thioflavin T binding assay (data not shown), and epifluorescence microscopy (Fig. 1a). The preparations of amyloid fibrils contained less than 1% of the monomeric α-rPrP as determined by the size-exclusion chromatography (data not shown) but were free of any detectable amount of the β-oligomeric species as judged from electron microscopy (data not shown) and epifluorescence microscopy (Fig. 1b). Most importantly, the preparations of the β-oligomers were free of amyloid fibrils, and vice versa.

Amyloid Fibrils and the β-Oligomers but Not α-rPrP Are Toxic to Cultured Cells and Primary Neurons—The toxicities of all three rPrP isoforms were assessed using four cultured cell lines and two primary neuron cultures. rPrP isoforms were added to the cultured cells at concentrations equivalent to 0.5 μM and incubated for 48 h, and the percent cell death was measured using the Hoechst-33342 assay (Fig. 2). Dying cells stain very strongly in the Hoechst assay and show bright fluorescence, whereas nuclei of healthy cells display only dim staining (Fig. 2e). Amyloid fibrils were found to be toxic to all cultured cell lines tested: 3T3, NT2, N2a, and SH-SYSY (Fig. 2, a–d). The percent cell death observed in cells treated with the β-oligomer was lower than that found in cells treated with fibrils. α-rPrP showed a very minor toxic effect, which was comparable with that observed in control cells. This minor toxic effect could be due to the presence of minor amounts of the β-oligomer in the preparation of the α-monomeric species. Cell lines showed the same rank order with respect to their susceptibilities to toxicity induced by the amyloid fibrils and the β-oligomer (Fig. 2, a–d).

To test whether the toxic effect of amyloid fibrils is limited to cultured cells or not, we used primary hippocampal and cerebellar neurons. Primary neuronal cultures are a more appropriate model to study toxicity because they are terminally differentiated. Two protocols for plating neuronal cells were applied: cells were plated on PLL either for 1 h (i) or for 2 days (ii) before administration of rPrP isoforms. The second format allowed cells to grow and produce neurites before incubating with rPrP. We found that both the β-oligomer and amyloid fibrils were highly toxic to hippocampal neurons (Fig. 3, a and h) and cerebellar neurons (Fig. 3c). High toxicity of both abnormal isoforms was observed regardless of the format for plating cells. Specifically, the β-oligomer and fibrils induced death in up to ~90 and ~80% of the population of neuronal cells, respectively, that were precultivated for 1 h before the addition of rPrP (Fig. 3a). Precultivation of cells for 2 days reduced the size of the cell population susceptible to the toxic effects of the β-oligomer and amyloid fibrils to ~60 and 50%, respectively (Fig. 3b). Toxicity was dose-

![Image](https://example.com/image2)

**FIGURE 2. Cytotoxic effect of the β-oligomer and amyloid fibrils assayed on cultured cells.** Percentage of cell death in SH-SYSY neuroblastoma cells (a), N2a neuroblastoma cells (b), NT2 cells (c), and 3T3 cells (d) upon incubation of cells with α-rPrP (A), β-oligomer (B), and amyloid fibrils (F) as measured using Hoechst-33342 assay. Cntr, control. Cells were cultured for 1 day before administration of rPrP isoforms (0.5 μM) and then incubated for 48 h after administration of rPrP isoforms. Each data set represents a mean value of four experiments; >500 cells were counted for each data point in each experiment. e, Hoechst-staining of N2a cells treated with α-rPrP (left panel), β-oligomer (middle panel), or amyloid fibrils (right panel). Mean values ± S.E. (n = 4) are shown, p < 0.05 (*) and p < 0.001 (***); compared with untreated control, ***, p < 0.01 compared β-oligomer versus fibrils.
Toxic Prion Protein Fibrils

**FIGURE 3. Cytotoxic effect of the β-oligomer and amyloid fibrils assayed on primary neurons.** Percentage of cell death was measured after incubation of primary neuronal cells with α-rPrP (A), β-oligomer (B), and amyloid fibrils (F). Cntr, control; a, hippocampal neurons were plated for 1 h on PLL before administration of rPrP isoforms followed by cultivation for an additional 24 h (black bars), 2 days (light gray bars), or 4 days (dark gray bars) in the presence of rPrP. b, hippocampal neurons were cultured for 2 days on PLL, then rPrP isoforms were added at the following final concentration: 0.1 μM (black bars), 0.5 μM (light gray bars), and 1 μM (dark gray bars); cells were incubated for an additional 4 days, c, cerebela neuronal cells were cultured for 2 days on PLL, rPrP isoforms were added to the final concentration of 0.5 μM, and cells were incubated for an additional 4 days. In panels a, b, and c, each data set represents a mean value of three experiments; n = 300 cells were counted for each data point. Cell death was measured using a Hoechst-33258 assay. Mean values ± S.E. (n = 3) are shown, t, p < 0.05 compared β-oligomer versus fibrils. d, Hoechst-33258 staining of hippocampal neurons illustrates cell death. Cells were cultured on PLL for 2 days before administration of rPrPs and incubated for an additional 4 days with α-rPrP (0.5 μM, left panel), or fibrils (right panel); e, hippocampal neurons were cultured on PLL for 1 h before administration of α-rPrPs, incubated with rPrP isoforms (0.5 μM) for an additional 24 h, and stained with Hoechst-33258 (blue) and GAP43 (green). Total neurite length per cell (in μm) is shown for each group.

% Cell Death

![Graph](image)

**FIGURE 4. Effect of siRNAs on PrPC expression and toxicity of the β-oligomer and amyloid fibrils tested in SH-SY5Y cells.** Dose-response of siRNAs on PrPC expression analyzed by fluorescence immunostaining (a) and by Western blot of total cell extracts (b) is shown. Cells were transfected with PrP-specific siRNAs (50 or 150 nM, as indicated) and incubated for 72 h. For immunostaining, cell were fixed and stained with mouse anti-β-PrP antibody; Western blot was stained with anti-PrP mouse AG4 (1:1000). After stripping, the same membrane was incubated with mouse anti-actin antibody (1:5000, Sigma) used as a control for protein loading. c, Hoechst-33342 staining of cells treated with fibrils (panel 1), cells transfected with PrP-specific siRNAs (150 nM) and treated with fibrils (panel 2) or with the β-oligomer (panel 3), and cells transfected with non-target siRNAs (150 nM) and treated with fibrils (panel 4). d, quantification of cell death in SH-SY5Y cells transfected with PrP-specific siRNAs (150 nM) (gray bars) or with non-target siRNA (150 nM) (black bars). Approximately 800 cells were analyzed for each group in each individual experiment. Data are shown as the mean ± S.E. of three independent experiments. p < 0.001 (***), or p < 0.001 (+ + +) compared cells transfected with PrP siRNAs versus non-targeting siRNAs. rPrP isoforms (0.5 μM) were added 24 h after transfection of cells with siRNAs, and cells were incubated for additional 48 h before staining with Hoechst-33342. Cntr, control; α-α-rPrP; β, β-oligomer; F, amyloid fibrils.

% Cell Death

![Graph](image)

**TOC**

**Toxic Prion Protein Fibrils**

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Amyloid Fibrils and the β-Oligomer Induce Apoptosis—Cell death can be divided into two main types, programmed (apoptotic) and necrotic cell death. During apoptosis the cells display membrane blebbing, flipping of phosphatidylserine in the plasma membrane, nuclear fragmentation, and activation of a family of cysteine proteases, referred to as caspases. To test whether the cells treated with abnormal isoforms of rPrP undergo apoptosis, we employed several aforementioned assays.

In the early stage of apoptosis the plasma membrane still maintains its integrity; however, the cells lose the plasma membrane asymmetry and expose phosphatidylserine on the cell outer surface. Phosphatidylserine can be visualized by fluorescently labeled annexin V, a phospholipid binding protein that has a high affinity for phosphatidylserine. We found only cells that were either treated with the β-oligomer or with amyloid fibrils, but not with α-rPrP, showed positive staining in the annexin V assay (Fig. 5a, third row). Activation of caspases is regarded as another early sign of apoptosis. Consistent with the result of phosphatidylserine assay, we observed that the number of cells that showed positive staining for caspase-3 increased upon incubation either with the β-oligomer or with amyloid fibrils but not with α-rPrP (Fig. 5a, second row). Activation of caspase-3 in cells treated either with the β-oligomer or with fibrils was also detected by Western blot (Fig. 5b).

Another early characteristic sign of apoptosis is blebbing of plasma membrane that can be visualized using a calcein assay (41). Because of the ubiquitous intracellular distribution of esterase that converts non-pH-sensitive cell-permeant calcein into highly fluorescent calcein, non-apoptotic cells are characterized by a smooth plasma membrane as observed in the calcein assay. In opposition to this, we found intense membrane blebbing in cells treated with amyloid fibrils and, to a lesser extent, in cells incubated with the β-oligomer but not with α-rPrP (Fig. 5a, first row).

Differentiating apoptosis and necrosis morphologically is often based upon nuclear changes, where apoptotic cells display nuclear condensation and fragmentation. These peculiar changes can be visualized using Hoechst staining. Again, we found that nuclear fragmentation was typical for cultured cells treated with the β-oligomer or amyloid fibrils but not with α-rPrP (Fig. 5a, fourth and fifth rows).

In addition to apoptosis we were also interested in determining the extent to which a subpopulation of cells may die in parallel via necrosis. The propidium iodide assay is used routinely to detect necrotic cells. The intact membrane of living cells excludes propidium iodide, whereas necrotic cells are quickly stained by short incubations with this dye due to extensive membrane damage. We found that among the cells pretreated with the β-oligomer or with amyloid fibrils, only very minor fractions were positive in the propidium iodide assay (Fig. 5a, third row).

To test whether rPrP-induced apoptotic death is specific to the SH-SY5Y cell line or not, we used the aforementioned assays with three other cultured cells and primary neuronal cultures. All three cultured cell lines (N2a, 3T3, and NT2) showed extensive apoptosis but not necrosis upon treatment with either β-oligomer or amyloid fibrils but not with α-rPrP as judged by membrane blebbing, flipping of phosphatidylserine, nuclear fragmentation, caspase activation, and propidium iodide assays (data not shown). Similarly, hippocampal neuronal cells showed extensive nuclear fragmentation upon incubation with β-oligomer or amyloid fibrils but not with α-rPrP (Fig. 6b). Taken together our data suggest that both the β-oligomer and the amyloid fibrils induced apoptosis in cultured cells and primary neuronal cells. Only a very minor fraction of necrotic cells was found.

rPrP Amyloid Fibrils but Not β-Oligomers Induced Cell Aggregation—While studying the toxic effects of abnormal rPrP isoforms, we noticed remarkable differences in the way cells responded to the addition of the β-oligomers and amyloid fibrils (Fig. 7). Amyloid fibrils, but not β-oligomers or α-rPrP, caused spatial redistribution and severe aggregation of cultured cells on a surface. Cellular redistribution and aggregation occurred over time, whereas cells remained partially attached to the plastic or PLL (cells were plated for 24 h before the administration of fibrils). The first signs of aggregation were noticed upon incubation of cells with fibrils for ~7–8 h (Fig. 7, first row). Over time, cell aggregation developed further, showing
formation of small cell clumps at 24 h (Fig. 7, second row) and much larger clumps after 2–4 days. This phenomenon was observed for N2a, NT2, and SH-SYSY cells and to much lesser extent for 3T3 cells. Notably, aggregated cells still remained at least partially attached to the plastic. Some level of aggregation was also observed upon prolonged incubation of cells with the β-oligomers (for more than 4 days); however, the extent of aggregation was much less than that in cells incubated with the fibrils.

Endogenously expressed PrP<sup>C</sup> was previously shown to be involved in cell adhesion, which leads to intercellular aggregation of N2a neuroblastoma cells (45). Intercellular adhesion seems to be mediated via interaction of the N-terminal region of PrP<sup>C</sup> expressed on the cell surface with some other surface molecules (45). Because the N-terminal region is exposed in the amyloid fibrils and because fibrils are highly polymeric by nature, it is reasonable to speculate that the physical interaction of fibrils with the cell surface accounts for fibril-induced aggregation of cultured cells. Therefore, we were interested in knowing whether cellular aggregates were formed in close proximity to the amyloid fibrils. It is important to note that in cell media, pH 7.4, rPrP fibrils always aggregated in clumps of variable sizes (Fig. 1b). To detect fibrils in cell culture media, we used several assays including staining with thioflavin T and immunostaining with Fab P. In the first 30 min after the addition of fibrils to the media, we used several assays including staining with thioflavin T and immunostaining with Fab P. In the first 30 min after the addition of fibrils to the media, we used several assays including staining with thioflavin T and immunostaining with Fab P. This was followed by redistribution of nearby cells and their coaggregation with fibrillar clumps ~7–8 h after administrating fibrils to cell media (Fig. 8a).

Aggregation of nearby cells into larger groups continued over time (Fig. 8b). Remarkably, cells tend to surround fibrillar clumps, forming co-aggregates together with fibrils. Noteworthy, most of the apoptotic cells were found later on within these clumps of aggregated cells and fibrils (Fig. 5, see staining with annexin; Fig. 8c, panel 1). In contrast, cells treated with the β-oligomers did not aggregate (Fig. 8a) and displayed apoptotic cells scattered throughout the cell population.

To test whether fibril-induced aggregation is mediated by endogenously expressed PrP<sup>C</sup>, we transfected SH-SYSY cells with anti-PrP siRNAs. The aggregation level was substantially reduced in the cells, where the expression of PrP<sup>C</sup> was down-regulated by siRNAs (Fig. 8c, panels 1 and 3). Remarkably, cells tend to surround fibrillar clumps, forming co-aggregates together with fibrils. Noteworthy, most of the apoptotic cells were found later on within these clumps of aggregated cells and fibrils (Fig. 5, see staining with annexin; Fig. 8c, panel 1). In contrast, cells treated with the β-oligomers did not aggregate (Fig. 8a) and displayed apoptotic cells scattered throughout the cell population.
incubated with the β-oligomer (Fig. 9). Cells treated with α-rPrP did not show any aggregation and, to our surprise, developed long neurites. Furthermore, when incubated with α-rPrP, cells survived longer than those in control samples (data not shown).

Taken together, our results indicate that both amyloid fibrils and the β-oligomer induced cell apoptosis, but the β-oligomer did not cause cell aggregation. α-rPrP did not display any toxic effect and, even more, stimulated neurites outgrowth under stress conditions that were not optimal for cultivating neuronal cells. Because all three isoforms (α-rPrP, β-oligomers, and fibrils) were prepared from the same stock solution of rPrP, the lack of toxicity in preparations of α-rPrP ruled out the possibility that toxicity was caused by minor impurities co-purified with rPrP.

**DISCUSSION**

This report is the first direct demonstration that mature amyloid fibrils generated from the full-length rPrP can be highly toxic to cultured cell lines and to primary neurons. Cytotoxicity of the fibrillar form was time-dependent, dose-dependent, and comparable with that displayed by small β-oligomeric particles. As in the case for PrPSc-induced toxicity, the toxicity of the fibrils and the β-oligomers was found to be dependent on expression of endogenous PrPSc.

There is considerable debate as to whether soluble non-fibrillar oligomers or mature amyloid fibrils are the pathogenic species responsible for the onset of neurodegenerative diseases (8, 20, 24–26). In the case of some neurodegenerative disorders such as Alzheimer disease, the literature contains conflicting data as to whether soluble oligomers of fibrils are the toxic species. In the last few years the pathologic role of non-fibrillar oligomers in neurodegenerative disorders has emerged. Substantial evidence has accumulated suggesting that soluble oligomeric species generated from a variety of proteins and peptides rather than mature amyloid fibrils are inherently highly cytotoxic (20). Impairment of cellular functions and high toxicity of soluble oligomers were reported for a number of polypeptides including α-synuclein (6, 7), Aβ peptides (8, 9), transthyretin (10), and lysozyme (11) as well as for proteins that are not associated with any conformational disorders (20, 21). These findings lead to a dominating perception that formation of amyloid fibrils/plaques might be a protective process by which cells sequester more dangerous oligomers to avoid their highly toxic effects. The presented findings provide new opportunities for reexamining our current view and for identifying cytotoxic protein states.

The soluble β-oligomers produced from different proteins and peptides seem to exhibit a common mechanism for pathogenesis and toxicity regardless of the amino acid sequence (22). These soluble oligomers were shown to induce an increase in membrane conductance and permeability followed by rapid elevation of intracellular Ca2+ (22, 23). The mechanism of toxicity exhibited by soluble oligomers seems to be unified regardless of specific proteins involved, which is not surprising considering that β-oligomeric species produced from different proteins also shared a generic substructure (46). It would be difficult to explain, however, the substantial variations in pathogenesis and neuropathology observed in different classes and even within the same class of neurodegenerative maladies if one ignores the possibility that fibrillar forms may also possess toxic effects and result in impairment of cellular functions. Sporadic Creutzfeldt-Jakob disease is known to exhibit extreme phenotypic heterogeneity with respect to neuropathological features including substantial variations in lesion profile and PrPSc deposition, clinical symptoms, and age at onset (47, 48).

It is quite possible that, in contrast to the generic mechanism of toxicity proposed for soluble β-oligomers, fibril toxicity may have multiple mechanisms. In fact, studies presented here demonstrate that the fibrillar form of rPrP was highly toxic to several cultured cell lines and primary neurons, whereas fibrils of insulin were not. In our consideration of toxic effects imposed by abnormally folded states of proteins, we should acknowledge the fact that fibrils exhibit much greater conformational diversity than that found in soluble oligomeric particles. Substantial conformational variations were described for fibrils produced from different proteins as well as for fibrils generated within the same amino acid sequence (49, 50) including fibrils generated from rPrP (51). Different strains of transmissible spongiform encephalopathy that display substantial differences in neuropathological profile and in the age of onset of prion disease were linked to conformational variations within PrPSc (52–54). Two conformationally different “strains” of amyloid fibrils produced in vitro from Aβ1–40 peptide were shown to have significantly different toxicities in neuronal cells (55). It is quite likely that conformational variation in fibrils determine in large part the range and extent of cellular impairments. The toxic effect of amyloid fibrils demonstrated in the current studies was consistent with the earlier observation that spongiform degeneration is colocalized with sites of PrPSc accumulation in the brain (56). PrPSc purified from scrapie brains was shown to be neurotoxic to cultured neurons in vitro (30, 57). Because PrPSc is heterogeneous by nature and relatively impure, synthetic PrP-derived peptides were exploited in numerous studies in an attempt to identify the toxic conformational. Specifically, a short PrP-derived peptide encompassing residues 106–126 was used to model prion toxicity in vitro (31, 58, 59). An aged solution of PrP-(106–126) was previously found to induce apoptosis in primary hippocampal (27) and cerebella neurons (29) and in mouse retinae in vivo (60). Because
PrP-(106 –126) peptide has never been found in vivo, the relevance of these findings is limited to the extent to which this short peptide mimics the conformation of PrPSc.

In our studies the cytoxicity of the fibrils and the β-oligomers was shown on four cultured cell lines and in hippocampal and cerebellar primary neuronal cells. These data argue that the effect of the β-oligomer and fibrils was not limited to a specific cell line but was rather general. Noteworthy, cells seem to have an intrinsic ability to recognize conformationally different isoforms generated from the same protein. Although the β-oligomer and the amyloid fibrils were both toxic, cells responded in a different manner to the exogenous administration of these two forms. Amyloid fibrils caused cell aggregation that was followed by apoptosis observed primarily in the subpopulation of cells that formed aggregates. In contrast, cells treated with the β-oligomer developed apoptosis before showing any signs of aggregation. Notably, cell aggregation seemed to be a cooperative process that was initiated by the physical attachment of fibrils to the cell surface but continued to develop further over a period of several days when no free fibrils were found in cultured media. The amyloid fibrils but not the β-oligomers were also found to induce aggregation of primary neurons. When endogenous expression of PrP was down-regulated using siRNAs, cell aggregation was substantially reduced. Therefore, cell aggregation cannot simply be due to the physical interaction of fibrils with the cellular membrane but, rather, requires endogenous PrP. The two different patterns of cellular response could be linked to the significant differences in size and structure between the two abnormal rPrP isoforms. Our previous studies established that the β-oligomer and amyloid fibrils produced from rPrP have a different substructure and are formed via distinct kinetic pathways (13, 18, 42).

Several lines of experimental evidence indicate that PrPSc is required for mediating the neurotoxicity of PrPSc. Mice devoid of endogenous PrPSc showed no signs of neuropathology when inoculated with PrPSc (61). Grafting of neuronal tissues expressing PrPSc into the brain of PrP−/− mice revealed that the graft but not the surrounding tissue aggregation was substantially reduced. Therefore, cell aggregation can respond in a different manner to the exogenous administration of α-PrPSc-stimulated neurite outgrowth were consistent with the neuroprotective function of PrPSc. Previous studies have also showed that PrP is involved in neurite outgrowth and neuronal survival via different signal transduction pathways (73, 74). Even though the mechanism of α-PrPSc-induced stimulation of neurite outgrowth is beyond the scope of the current studies, this finding emphasized the striking differences between the effects of correctly folded and misfolded states of rPrP as tested in neuronal cells.

Our studies demonstrate for the first time that fibrils prepared from the full-length rPrP are as toxic to cells as soluble oligomers. The extent to which toxicity of the amyloid form is a general or unique characteristic of PrP fibrils needs to be elucidated in future studies. Nevertheless, these findings illustrate the remarkable ability of cells to recognize three conformationally different isoforms of the prion protein and provide insight into the possible origin of neuron loss during prion infection.

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