Prion infection impairs the cellular response to oxidative stress

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The molecular mechanism of neurodegeneration in transmissible spongiform encephalopathies remains uncertain. In this study, it was demonstrated that prion-infected hypothalamic neuronal GT1 cells displayed a higher sensitivity to induced oxidative stress over noninfected cells. In addition, the infected cells presented an increased lipid peroxidation and signs of apoptosis associated with a dramatic reduction in the activities of the glutathione-dependent and superoxide dismutase antioxidant systems. This study indicates for the first time that prion infection results in an alteration of the molecular mechanisms promoting cellular resistance to reactive oxygen species. This finding is vital for future therapeutic approaches in transmissible spongiform encephalopathies and the understanding of the function of the prion protein.

The cellular form of the prion protein (PrPc) is a highly conserved cell surface glycoprotein expressed by a broad range of cells and in particular by neuronal cells. In transmissible spongiform encephalopathies (TSEs), this molecule is converted into a conformationally modified protease-resistant isoform called PrPSc (1). The relationship between the PrPSc presence and neurodegeneration is not absolute. In fact, it was shown in vivo that PrPSc deposition in neuronal tissue not expressing PrPC has no pathological consequence (2). Moreover, in both infectious and genetic models of TSEs, neurodegeneration has been detected in the absence of observable protease-resistant PrPSc (3, 4). Recently, it was proposed that a putative transmembrane form of the prion protein (PrP), and doppel, a PrP-related molecule, could be responsible for neurodegeneration in transgenic mice (4, 5), but it is not clear how these two molecules may be implicated in authentic TSEs.

Although advances have been made in understanding prion diseases, the function of PrPC remains elusive. Studies based on structural homology (6) have revealed limited information concerning the function of the protein. However, it was shown that the octapeptide repeat region of the molecule binds Cu (7) and it has been proposed that PrPC may play a role in the oxidative state of the cell through a regulation of the copper transport (8) or through a modification of Cu

Materials and Methods

Reagents and Antibodies. Pefabloc and proteinase K were purchased from Boehringer Mannheim. DMEM was from Life Technologies (Grand Island, NY), and FCS from BioWhittaker. 3-Morpholinosydnonimine (SIN-1) was from Molecular Probes, buthionine sulfoximine (BSO), N-acetylcysteine, and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were from Sigma. Tris hydroxymethyl-aminomethane, thiobarbituric acid, azide, 70% perchloric acid, and potassium cyanide were from Merck, NADPH was from Randox (Crumlin, U.K.) and 1-butanol from Prolabo (Paris). All other reagents were from Sigma.

Rabbit polyclonal antibody P45–66, raised against synthetic peptide encompassing mouse PrP residues 45–66, has been described earlier (11). Scrapie-associated fibril (SAF) 60, SAF 69, and SAF 70 are three mAb produced by J. Grassi and coworkers (Commissariat à l’Energie Atomique-Saclay, Gif sur Yvette, France). They were obtained by using as immunogen SAF that were prepared from infected hamster brains. In enzyme immunometric assays, SAF 60, SAF 69, and SAF 70 were characterized as recognizing the peptide epitope 142–160 of hamster PrP (J. Grassi, personal communication). A mixture consisting of an equal volume of ascites of SAF 60, SAF 69, and SAF 70 antibodies was used to improve PrP detection. Cu/Zn superoxide dismutase (Cu/Zn SOD) polyclonal antibody was from Calbiochem. Secondary antibodies were from Jackson ImmunoResearch.

Cell Culture. Infection of GT1–7 cell lines with Chandler (GT1Chl), 22L (GT122L), and 87V (GT187V) strains is reported elsewhere (10). To generate the two other GT1 cell lines used in this work, GT1Mock and GT1FK, 10% brain homogenate (in PBS containing 5% glucose) of control uninfected C57Bl6 mice and Fukuoka-1-infected mice were used. Briefly, about 1 × 10⁵ GT1–7 cells in a 35-mm culture dish were incubated with 1 ml of the diluted homogenate at 0.2% in serum-free DMEM for 5 h, to which was added 1 ml of DMEM/10% FCS. Once the cells had become confluent, they were split into a 25-cm² culture flask. PrPSc was tested after 5 and 10 passages by immunoblotting as described below. PrPSc was not detected in the control GT1Mock cells, whereas the infection of GT1FK was successful. These ex vivo transmission experiments have been repeated three times with the same outcome.

The cell lines were routinely cultured in DMEM supplemented with 10% heat-inactivated FCS and penicillin-streptomycin and were maintained at 37°C in 5% CO₂ in the biohazard P3 laboratory of our institute.

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Abbreviations: BSO, buthionine sulfoximine; CR, Congo red; GPX, glutathione peroxidase; GR, glutathione reductase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PrP, prion protein; PrPSc, cellular isoform of PrP; PrPSc, scrapie isoform of PrP; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; TSEs, transmissible spongiform encephalopathies; SAF, scrapie-associated fibril; GSH, glutathione; GT1Chl, GT1 cells infected with Chandler; GT122L, GT1 cells infected with 22L; GT187V, GT1 cells infected with 87V.

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Western Blotting. Cells at 90% confluence were washed twice into PBS and lysed for 30 min at 4°C in Triton/deoxycholate lysis buffer [150 mM NaCl/0.05% Triton X-100/0.5% sodium deoxycholate/50 mM Tris-HCl (pH 7.5)], plus protease inhibitors (1 μg/ml pepstatin and leupeptin/2 mM EDTA). After 1 min of centrifugation at 10,000 × g, the supernatant was collected and its total protein concentration measured by using a bicinchoninic acid protein assay (Pierce). Protein concentration was adjusted to its total protein concentration measured by using a bicinchoninic acid protein assay (Pierce). Protein concentration was adjusted with lysis buffer. For detection of PrPSc and Cu/Zn SOD, an equivalent volume of the samples were directly mixed with the same volume of 2X SDS loading buffer.

For detection of PrPSc, equivalent volumes of the samples were digested with 16 μg of proteinase K per mg of total protein at 37°C for 30 min, and the digestion was stopped by incubating with 1 mM Pefabloc for 5 min on ice. The samples were centrifuged at 20,000 × g for 45 min at 4°C, and the pellet resuspended in 30 μl of SDS loading buffer.

Samples were applied onto 12% SDS/PAGE and the proteins were transferred onto a membrane (Immobilon-P, Millipore) in 3-(cyclohexy1amine)-1-propanesulfonic acid buffer containing 10% methanol at 400 mA for 1 h. The membrane was blocked with 5% nonfat dry milk in TBST [0.1% Tween 20/100 mM NaCl/10 mM Tris-HCl (pH 7.8)] for 1 h at room temperature. Nondigested mouse PrPSc was detected by immunoblotting by using a mixture of three mAb, SAF 60, SAF 69, and SAF 70 (mixture of ascitic fluids diluted 1/200 in TBST). The anti-Cu/Zn SOD polyclonal antibody was used at 1/500 in 3% nonfat dry milk in TBST.

DNA Fragmentation Assay. DNA fragmentation was demonstrated in infected cells by the DNA-laddering technique modified from Schatzl et al. (12). Briefly, cells from a 125-cm² flask were washed twice with cold PBS and lysed in 500 μl of hypotonic lysis buffer for 5 min [5 mM Tris-HCl (pH 7.5)/20 mM EDTA/0.5% Triton-X100]. The lysates were centrifuged at 14,000 rpm for 30 min. The supernatants were deproteinized by digestion with 0.3 mg/ml proteinase K for 30 min at 60°C and extracted once in phenol/chloroform and once in chloroform/isoamylalcohol (24:1), and then precipitated in 2.5 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate. After centrifugation at 12,000 rpm at 4°C for 30 min, the pellets were washed in 70% ethanol and resuspended in 25 μl of Tris/EDTA buffer (pH 7.5) and 60 μg/ml RNase A for 1 h at 37°C; 20 μl were supplemented with gel-loading buffer, subjected to electrophoresis on a 1.5% agarose gel, and stained with ethidium bromide.

Cell Viability Assay. Cell viability was assessed by using a modified MTT assay from Hansen et al. (13). MTT is a sensitive first indicator of mitochondrial damage induced by oxidative stressors (14). Briefly, around 10,000 GT1 cells per well were plated in 96-well microtiter plates with 100 μl of medium. The next day, the medium was changed and the cells were challenged for 24 h with the indicated concentration of BSO or SIN-1. The medium was then changed and the cells were incubated for an additional 24 h without the drugs. For the MTT assay, 10 μl of MTT (5 mg/ml stock in PBS) was added to each well for 1 h at 37°C; 100 μl of 50% DMSO/5% SDS solubilization solution was then added, and adsorption readings were performed at 570 nm with reference at 630 nm.

Lipid Peroxidation. The lipid peroxidation was evaluated by using an assay based on fluorescence of a malondialdehyde thiobarbituric acid adduct measured after extraction with 1-butanol (15). Subconfluent cells were trypsinized in 75-cm² flasks, washed three times by 10 ml of isotonic, trace element-free 400 mM Tris-HCl buffer (pH 7.3), and then lysed in hypotonic 20 mM Tris-HCl buffer by five freeze-defrost cycles; 750 μl of a mixture of thiobarbituric acid at 8 g/7% perchloric acid (2/1) were added to 100 μl of sample. After agitation, the mixture was placed in a 95°C water bath for 60 min and then cooled in an ice bath. The fluorescent compound was extracted by mixing with 1-butanol for 2 min. After centrifugation, the fluorescence in the 1-butanol phase was determined with an Amino-Bowman fluorimeter with excitation at 532 nm and emission at 553 nm. A blank was run for each sample. The calibration curve was created with a stock solution of 1,1,3,3-tetraethoxypropane prepared in alcohol. The results were expressed as μmol malondialdehyde/g protein.

SOD Activity. For SOD activity, the method from Oberley et al. (16) was first used. After two washes in PBS, the cells were sonicated in 0.015 M sodium phosphate buffer (pH 7.8) and centrifuged at 4,000 rpm for 4 min. The supernatant was then tested for total SOD activity. To determine the Mn SOD activity, the supernatant was incubated with 4 mM potassium cyanide for 20 min before testing to inhibit the Cu/Zn SOD activity. One unit of SOD is given as that level allowing for a 50% inhibition of formazan production. The specific activity was determined as units/mg protein.

As a control, an alternative method was used as follows. Subconfluent cells in 75 cm² flasks, were washed three times and collected in 10 ml of isotonic, trace element-free 400 mM Tris-HCl (pH 7.3), and then lysed in hypotonic 20 mM Tris-HCl buffer by five freeze-defrost cycles. After 10 min of centrifugation at 4,000 rpm and 4°C, the lysate was assayed for metalloenzyme activities and soluble protein content. Total SOD, Mn SOD, and Cu/Zn SOD were determined by using the pyrogallol assay following the procedure described by Marklund and Marklund (17), based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD, spectrophotometrically read at 420 nm. Briefly, 50 μl of the sample was added with 1,870 μl of 50 mM Tris/1 mM diethylenetriaminepentaacetic acid/20% cacodylic acid buffer (pH 8.3) and with 80 μl of 10 mM pyrogallol to induce an absorbance change of 0.02 in the absence of SOD. The amount of SOD inhibiting the reaction rate by 50% in the given assay conditions was defined as one SOD unit. The specific Cu/Zn SOD inhibition by KCN (60 μl of 54 mM KCN added to 300 μl of lysate) allows the Mn SOD determination in the same condition. Each sample was assayed twice, and results were expressed as SOD units and normalized to the cell protein content.

Analysis of Glutathione (GSH)-Dependent Antioxidant System. For the determination of total GSH levels, after washing twice in PBS, confluent 25-cm² cells were scraped into 1 ml of PBS, centrifuged at 7,000 rpm for 5 min, the pellet was resuspended in 100 μl of cold H2O2, and vortexed for 5 min; 5 μl was then removed for protein determination (bicinchoninic acid) and 95 μl of cold 10% trichloroacetic acid (wt/vol) was added. After 10 min at 4°C, the solution was spun at 3,000 rpm for 15 min and the supernatant was then assayed for total GSH content according to the method of Akerboom and Sies (18).

The glutathione peroxidase (GPX) activity was assayed by the method of Gunzler et al. (19). GPX was measured in a coupled reaction with glutathione reductase (GR). Tert-butyl hydroperoxide was the substrate. Briefly, 25 μl of the sample was added with 990 μl of 50 mM Tris/1 mM Na2EDTA/4 mM azide buffer (pH 7.6) (azide was included in the assay mixture to inhibit interference of catalase) and with 20 μl of 0.15 M GSH/20 μl of 200 units/ml GR/20 μl of 8.4 mM NADPH in order and left 1 min for mixture equilibrium. Tert-butyl hydroperoxide (20 μl) was then added and the decrease in absorbance was monitored for 200 s. The difference in absorbance per min was used to calculate the enzyme activity and results were expressed as GPX units/g protein.
In this study, Congo red (CR)-treated GT1 Chl cells (GT1Chl-CR) would represent a suitable model to study prion infection. Although the total amount of full-length PrPC was not modified when compared with GT1–7 (Table 1), indicating that the oxidative state modified in infected cells. Interestingly, GT1 cells were successfully infected and producing PrPSc (Fig. 1C). GT1Mock, GT1 87V, and GT1 FK, with only the latter being successfully infected and producing PrPSc (Fig. 1B).

In a first attempt to evaluate the variation in cellular redox state between infected and control cells, the lipid peroxidation was measured by determining the formation of malondialdehyde (25). Cellular damages were significantly higher in GT1Chl cells when compared with GT1–7 (Table 1), indicating that the oxidative state was modified in infected cells. Interestingly, GT1 cells have been previously used to investigate neuronal cell death, Bcl-2 function, and reactive oxygen species (ROS)-induced stress (14, 26). These cells have been shown to display high sensitivity to the toxicity from BSO, a y-glutamylcysteine synthetase inhibitor that results in the depletion of GSH in the cytosol and mitochondria of cells (14). To test the consequences of prion replication, infected and control GT1 cells were treated with BSO (100 μM or 1,000 μM) for 24 h and their viability assessed after 48 h by a modified tetrazolium salt (MTT) assay, an assay of mitochondrial activity proportional to cell viability (13). Each of GT1Chl, GT122L, and GT1FK displayed a concentration-dependent sensitivity to BSO that was significantly higher than that of the noninfected and the control cell lines (GT1Chl-CR, GT1Mock, and GT187V) (Fig. 2A). These results were confirmed by using as additional cell survival test, a Trypan blue exclusion assay (data not shown), and it is noteworthy that our results have been reproduced in several independently cultured cell lines. A visual illustration of BSO-induced neuronal death in infected cells was obtained in phase contrast microscopy (Fig. 2B). Because BSO sensitivity differed between the infected and noninfected cells, it was important to measure GSH levels before and after the treatment (18). GT1–7, GT122L, and GT1Chl all had similar GSH content and exposure to increasing concentrations of BSO had an equivalent effect on the depletion of GSH between the cell types (Fig. 2C). This indicated that differences among cell lines were not related to various levels of available GSH. Importantly, N-acetylcysteine, a known precursor of GSH and a ROS scavenger, allowed for protection against BSO-induced toxicity in our model (Fig. 2D). Taken together, our results suggest that prion infection increases sensitivity of neuronal cells to oxidative stress and ROS are an important death factor in TSEs.

ROS toxicity has been implicated in several other neurological disorders. Such toxicity could result from an imbalance between antioxidant enzymes which normally have an appropriate relationship to each other (27). Among the multileveled interdependent antioxidant systems, which aerobic cells have evolved to protect against oxidant injury, is the family of SODs, which represent a good culture model avoiding clonal differences (21). These cell lines were not recently developed in the laboratory (10). These cell lines were not cytokines and mitochondria (14). To test the consequences of prion replication, infected and control GT1 cells were treated with BSO (100 μM or 1,000 μM) for 24 h and their viability assessed after 48 h by a modified tetrazolium salt (MTT) assay, an assay of mitochondrial activity proportional to cell viability (13). Each of GT1Chl, GT122L, and GT1FK displayed a concentration-dependent sensitivity to BSO that was significantly higher than that of the noninfected and the control cell lines (GT1Chl-CR, GT1Mock, and GT187V) (Fig. 2A). These results were confirmed by using as additional cell survival test, a Trypan blue exclusion assay (data not shown), and it is noteworthy that our results have been reproduced in several independently cultured cell lines. A visual illustration of BSO-induced neuronal death in infected cells was obtained in phase contrast microscopy (Fig. 2B). Because BSO sensitivity differed between the infected and noninfected cells, it was important to measure GSH levels before and after the treatment (18). GT1–7, GT122L, and GT1Chl all had similar GSH content and exposure to increasing concentrations of BSO had an equivalent effect on the depletion of GSH between the cell types (Fig. 2C). This indicated that differences among cell lines were not related to various levels of available GSH. Importantly, N-acetylcysteine, a known precursor of GSH and a ROS scavenger, allowed for protection against BSO-induced toxicity in our model (Fig. 2D). Taken together, our results suggest that prion infection increases sensitivity of neuronal cells to oxidative stress and ROS are an important death factor in TSEs.

ROS toxicity has been implicated in several other neurological disorders. Such toxicity could result from an imbalance between antioxidant enzymes which normally have an appropriate relationship to each other (27). Among the multileveled interdependent antioxidant systems, which aerobic cells have evolved to protect against oxidant injury, is the family of SODs, which catalyzes the conversion of the superoxide ion (O2−) into hydrogen peroxide (H2O2). It has been suggested that PrPSc itself may play a role in the response of the cell to oxidative stress possibly through its involvement in copper metabolism and/or in SOD activity. Indeed, it was shown in various experimental models that cells derived from PrPnull mice were more sensitive to oxidative stress, copper toxicity, and had a reduced SOD activity when compared with control cells (7, 28, 29). Finally, modulation of SOD activity by PrP has been reported to result from an

Table 1. Determination of lipid peroxidation and SODs, GPX, GR activities

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GT1–7</th>
<th>GT1Chl</th>
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<tbody>
<tr>
<td>MDA</td>
<td>69.3 ± 13.9</td>
<td>113.3 ± 8.0**</td>
</tr>
<tr>
<td>Total SOD</td>
<td>15.2 ± 0.4</td>
<td>9.9 ± 1.6*</td>
</tr>
<tr>
<td>Mn SOD</td>
<td>6.4 ± 0.6</td>
<td>4.1 ± 0.04*</td>
</tr>
<tr>
<td>GPX</td>
<td>40.4 ± 2.6</td>
<td>20.1 ± 4.6**</td>
</tr>
<tr>
<td>GR</td>
<td>368.4 ± 97.1</td>
<td>174.1 ± 57.7*</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD for three independent determinations. MDA is in nmol/g of protein; SOD activities are in unit/mg of protein; and GPX and GR activities are in unit/g of protein. Significantly different from GT1–7 value (*, P < 0.05; **, P < 0.01, Student’s t test).
alteration in the incorporation of copper in this enzyme (7). However, these latter data were not confirmed recently (30) and analyses in affected brains have not permitted a verification of these data (31). To investigate further whether increased sensitivity to stress of infected cells may be linked to an alteration in their enzymatic defenses, SOD activities present in the cultures were measured by using the xanthine and Nitro Blue Tetrazolium technique (16). A significant reduction in both Cu\textsubscript{Y}Zn and Mn\textsubscript{SOD} activities was observed in both GT1\textsubscript{22L} and GT1\textsubscript{Chl} when compared with GT1–7 and GT1\textsubscript{Chl–CR} control cells (Fig. 3A). The latter results were also reproduced by using an alternative method of SOD detection, that of autooxidation of pyrogallol (25) (Table 1). Interestingly, Western blot detection of Cu\textsubscript{Y}Zn SOD in GT1 lines indicated that the total level of the protein was significantly reduced in infected cells (Fig. 3B). This may either reflect a down-regulation of expression of the enzyme or a degradation because of fragmentation under conditions of higher oxidative stress (32). Either way, the consequences of lowered SOD activity is an increased O\textsubscript{2}\textsuperscript{−} that combined with nitric oxide could produce peroxynitrite the toxicity of which is well recognized (33). Interestingly, SIN-1, which generates nitric oxide (14) induced in GT1 cells a rapid and reproducible neuronal cell death (Fig. 3C) that was higher in infected cells. This confirmed the higher sensitivity of infected cells to oxidative stress and suggests that peroxynitrite may play an important role in the neuropathology of TSEs.

In addition, on further examining the interdependent oxidative enzymes, although GSH levels were equivalent between cells, it was observed that both the GPX and GR activities were significantly reduced in our infected cells (Table 1) (25). We recently confirmed by using N2a cell lines infected with the Chandler prion strain (10) that SOD as well as GPX activities were also affected by prion replication in these cells. Values for control vs. infected N2a cells, calculated as described in Table 1, were for SOD: 24.8 ± 3.7 and 10.8 ± 1.9 (P < 0.01, Student’s t test) and for GPX: 454.6 ± 16.3 and 299.1 ± 33.8 (P < 0.01, Student’s t test), respectively. Previous results obtained with infected hamsters revealed an increase of the activities of these two enzymes in the brain (34). This discrepancy may be related to the fact that in the latter study, the enzymatic activities included those of glial and microglial cells that are known to be activated in TSEs. In our case, the lowering of GSH enzymes could be explained by a cellular response to a lowered SOD activity (27). However, because the infected cells displayed a higher sensitivity to SIN-1, it is possible that the lowered activities may be caused by an increased protein nitration (35). The latter could also explain the reduced Mn SOD activity (36). Interestingly, it was reported that PrP\textsuperscript{cara} cells possessed a higher sensitivity to H\textsubscript{2}O\textsubscript{2}, which was attributed to a lowered GR
 altering the cellular redox state (37). Our observation that both GPX and GR were lowered during prion infection suggests, therefore, that prion diseases may evolve from a disturbance of PrPC function by PrPSc.

The results presented here demonstrate for the first time that prion-infected cells are more susceptible to imposed oxidative stress caused by disturbance of oxidative defense and suggest that ROS play an important role in TSEs. Three main hypotheses can now be proposed to explain neurodegeneration in prion diseases (Fig. 4): (i) It is possible that the pathological conversion of PrPC to PrPSc may lead to an aberrant copper metabolism altering the cellular redox state and the activities of important oxidative enzymes. However, knowing the recent results (30) obtained in transgenic animals, this may be unlikely and studies on the uptake of Cu by infected cells could test this hypothesis; (ii) The conversion of PrPC to PrPSc may lead to a general increase in ROS levels that would weaken cellular defenses and finally trigger cell death. Recent data showed that PrP itself could have a SOD-like activity (38), conversion into PrPSc could result in loss of activity leading to increased ROS or toxic gain of function as in amyotrophic lateral sclerosis (39); and (iii) on the other hand, modification of the PrP function may result in a modulation of oxidative defenses possibly involving protein regulation or (metal-regulated) transcription factors. This idea is supported by the fact that GPX, GR, Cu/Zn SOD, and Mn SOD partially share common signaling and biochemical pathways. In this case, PrP would function as a specific stress sensor leading to resistance of cells to oxidative stress through a yet unknown mechanism. This exciting idea is reminiscent of "the γ receptor hypothesis" formulated by others (40) and we are now looking for a possible signaling pathway in which PrP could be involved.

In conclusion, it is becoming more and more evident that although the etiological factors of neurodegenerative diseases may differ, the neurodegeneration process present in such disorders share some common mechanisms. The implication of ROS and oxidative stress in TSEs allows now the possibility of new therapeutic approaches to substitute and balance antioxidant deficiencies in the disease state.

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