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PrP mutants with different numbers of octarepeat sequences are more susceptible to the oxidative stress

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One of the physiological functions of cellular prion protein (PrP^C) is believed to work as a cellular resistance to oxidative stress, in which the octarepeats region within PrP plays an important role. However, the detailed mechanism is less clear. In this study, the expressing plasmids of wild-type PrP (PrP-PG5) and various PrP mutants containing 0 (PrP-PG0), 9 (PrP-PG9) and 12 (PrP-PG12) octarepeats were generated and PrP proteins were expressed both in *E. coli* and in mammalian cells. Protein aggregation and formation of carbonyl groups were clearly seen in the recombinant PrPs expressed from *E. coli* after treatment of H_2O_2. MTT and trypan blue staining assays revealed that the cells expressing the mutated PrPs within octarepeats are less viable than the cells expressing wild-type PrP. Statistically significant high levels of intracellular free radicals and low levels of glutathione peroxidase were observed in the cells transfected with plasmids containing deleted or inserted octarepeats. Remarkably more productions of carbonyl groups were detected in the cells expressing PrPs with deleted and inserted octarepeats after exposing to H_2O_2. Furthermore, cells expressing wild-type PrP showed stronger resistant activity to the challenge of H_2O_2 at certain extent than the mutated PrPs and mock. These data provided the evidences that the octarepeats number within PrP is critical for maintaining its activity of antioxidation. Loss of its protective function against oxidative stress may be one of the possible pathways for the mutated PrPs to involve in the pathogenesis of familial Creutzfeldt-Jacob diseases.

prion protein, oxidative stress, glutathione peroxidase, reactive oxygen species, cell viability

Prion diseases, or transmissible spongiform encephalopathies (TSE), are a group of neurodegenerative diseases that afflict both humans and animals, including bovine spongiform encephalopathy in cattle, scrapie in sheep and goat, and Creutzfeldt-Jakob disease (CJD) in human[1]. The underlying cause of prion disease is the conversion of a host-derived cellular prion protein (PrP^C) to the infectious scrapie prion protein (PrP^{Sc})[2]. Approximately 10%—15% of human prion diseases are associated with the special mutations in the coding region of PrP gene, *PRNP*. Studies have identified that insertions within the octarepeats region are commonly related with familial CJD (fCJD), and so far 1 to 9 extra octarepeats insertions had been described worldwide[3–7]. The mechanism by which a mutated prion protein causes neurodegeneration is not completely understood. It is believed that the CJD-related PrP mutants are inherently unstable, leading to self-association to produce an oligomeric structure[8,9]. Whether prion diseases are, in part, due to the loss of a normal neuroprotective function of PrP^C remains still unsettled.

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The amino terminus of PrP^C contains a series of octarepeats with consensus sequence PHGGGWGQ. This region, which is the conserved region of mammalian PrP[10], has been implicated in binding of divalent metal ions, particularly copper[11]. It is speculated that copper binding endows not only the structure to this region, but also lends stability to the carboxyl terminus[12]. PrP^C has been implicated in copper transport and metabolism[13] and in defense mechanism of the cell against oxidative insult, possibly through a regulation of the Cu/Zn superoxide dismutase (SOD) activity[14].

Oxidative damage to proteins has been implicated in the pathogenesis of many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease[15–17], as well as animal prion diseases. The oxidative stress has been shown to be a pivotal event in the brains of scrapie-infected animals. Both recombinant and brain derived PrP have SOD-like activity, while the SOD activity of the PrP purified from sporadic CJD (sCJD) was markedly reduced to only approximately 15% of the control values. Meanwhile, it shows that the sCJD brains undergo oxidative stress, which is likely to be related to the marked decline of PrP antioxidation activity[18]. PrP has also been implicated in the cellular response to oxidative stress *in vitro*. Cells deficient in PrP are usually less viable in culture compared with cells expressing wild-type PrP and are more susceptible to oxidative agents, such as copper and hydrogen peroxide.

To address the possible differences in anti-oxidation among the PrP proteins with different numbers of octarepeat especially the associated inserted mutations, wild-type and mutated human PrP proteins were expressed from *E. coli* and mammalian cells. We found that the PrP proteins removed octarepeats or inserted with four and seven extra octarepeats were more susceptible to oxidative stress. The cells expressed the mutated PrP proteins were more vulnerable and showed higher level of ROS and the lower level of glutathione peroxidase. It illustrates that the correct numbers of octarepeats is critical for PrP antioxidation capability. Insertions with extra octarepeats result in decline of antioxidation activity of PrP, thereby, possibly contributing to the damage of the cells during the pathogenesis of fCJD.

1 Materials and methods

1.1 Plasmid Construction

The generations of the recombinant prokaryotic protein expressing plasmids pQE30-PrP containing wild-type (WT) human *PRNP* sequence encoding amino acid (aa) 23－231 with five octarepeats[19]. pQE30-PrP-PG9 containing the same length of human *PRNP* sequence with nine octarepeats (PG9) and pQE30-PrP-PG0 containing the same length of human *PRNP* sequence without octarepeats (PG0) were described previously[20]. The human *PRNP* sequence with 12 octarepeats (PG12) was based on the *PRNP* sequence from a Chinese patient with definitely diagnosed familial CJD[21]. Briefly, the *PRNP* sequence with 12 octarepeats was amplified by PCR from brain tissues of the fCJD patient, using forward primer (GGA TCC AAG AAG CGC AAG CCT, with a BamHI site underlined) and reverse primer (AAG CTT TCA GCT CGA TCC TCT GTA ATA, with an Hind Ⅲ site underlined). After verified by sequence analysis, the PCR product was cloned into a prokaryotic expressing vector pQE-30, generating recombinant plasmid pQE30-PrP-PG12.

The eukaryotic expressing plasmid pcDNA-PrP-PG5 containing full-length WT human *PRNP* segment encoding aa 1-253 was generated as previously descrybed[22]. Plasmid pcDNA-PrP-PG0 removed octarepeats segment was generated by a splint PCR technique using plasmid pcDNA-PrP-PG5 as backbone. Briefly, a 171-bp long *PRNP* sequence encoding the peptide flanking the octarepeats region from aa 1 to 51 and from aa 91 to 96 was amplified from plasmid pcDNA-PrP-PG5 by PCR, using forward primer PrP1 (GGA TCC AGT GCG AAC CTT GGC TGC TG, with an BamHI site underlined) and a splint reverse primer (G ATC GTG GCC ACC TCC *TGG GTA GCG GTT GCC TCC*, in which the sequence encoding aa 48－51 was illustrated in capital and the sequence encoding aa 91-96 was shown in italic). Using the 171-bp PCR product as the forward primer and primer PrP762 (TTC TCA T CAT CCC ACT ATC AGG, with an EcoRI site underlined) as the backward primer, the full length *PRNP* gene that removed octarepeats region was constructed by PCR technique. The PCR product was cloned into an eukaryotic expressing vector pcDNA3.1, generating recombinant plasmid pcDNA-PrP-PG0.

Plasmid pcDNA-PrP-PG9 was generated by overlap PCR technique using pQE30-PrP-PG9[20] as backbone. Firstly, a 734-bp long human *PRNP* sequnece encoding aa 23-231 with 9 octarepeats was amplified from the plasmid pQE30-PrP-PG9, using forward primer PrP63 (CTG CAA GAA GCG CCC GAA GCC TG) and re-

verse primer PrP701 (AGG ACC ATG CTC GAT CCT CTC TGG TAA TAG), and a 153-bp long *PRNP* segment encoding aa 1-51 was amplified from plasmid pcDNA-PrP-PG5 using forward primer PrP1 and reverse primer PrP153 (AGG TGG GTA GCG GTT GCC TCC AGG G). An 800-bp long *PRNP* fragment (N-terminal) encoding aa 1—231 with 9 octarepeats was generated in a reaction containing above two products by overlap PCR method. Subsequently, a 411-long *PRNP* fragment (C-terminal) encoding aa 116—253 was amplified from plasmid pcDNA-PrP using forward primer PrP348 (AGC AGC TGG GGC AGT GGT GGG G) and reverse primer PrP762 (GC GGC CGG TCA TCC CAC CCA CTA TCA GG, with an NotI site underlined). The full length *PRNP* gene (aa 1—253) with 9 octarepeats was generated in the reaction containing N- and C-terminal products by overlap PCR method. After verified with sequence assay, the full length *PRNP* gene (aa 1—253) with 9 octarepeats was cloned into pcDNA3.1, constructing plasmid pcDNA-PrP-PG9. *PRNP* sequence with 12 octarepeats was amplified by PCR from the brain tissues of the fCJD patient [21], using forward primer PrP1 and reverse primer PrP762. The PCR product was cloned into pcDNA3.1, generating recombinant plasmid pcDNA-PrP-PG12.

1.2 Protein Preparation

Purification and refolding process were carried out with a chromatograph of nickel-ion-charged Sepharose. Briefly, *E. coli* M15 fleshly transformed with plasmids pQE-rPrP-PG0, pQE-rPrP-PG5, pQE-rPrP-PG9 and pQE-rPrP-PG12 were incubated in LB medium at 37℃ till the A_{600} reached 1.0, and induced with 100 mmol/L IPTG for 5 h. Cells were harvested by short centrifugation, lysed and sonicated according the protocol described previously[23]. Protein purification was performed with a chromatograph of nickel-NTA agarose (Pharmacia Biotech). The purified proteins were dialyzed into PBS and stored at −80℃.

1.3 Western blot

Various purified PrP proteins were separated by 12% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After blocking with 5% deffated milk in PBST (phosphate buffered saline, pH 7.6, containing 0.05% Tween-20) overnight at 4℃, the membranes were incubated with 1:4000 PrP specific monoclonal antibody (mAb) 3F4 (Dako) for 2 h at room temperature, and then incubated with 1:2000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz). The protein bands were visualized by ECL kit (PE Applied Biosystems, Foster City, USA).

1.4 Cell culture and transient transfection

Human neuroblastoma cell line SH-SY5Y and human cervical carcinoma cell line HeLa which did not express endogenous prion protein were maintained in DMEM (Gibco BRL, USA). Cells were plated into 6-, 24- or 96-well plates (Falcon, Japan) one day before transfection. Different amounts of various recombinant PrP plasmids, as well as vector pcDNA3.1, were transfected into the monolayer cells with Lipofectamine 2000 transfection reagent (Invitrogen, USA). 48 hours after transfection, the cells were harvested in PBS, pelleted by centrifugation and suspended in the lysis buffer (10 mmol/L Tris-HCl, pH 7.8, 0.5% sodiumdeodycholate, 0.5% Nonidet P-40, 100 mmol/L NaCl, 10 mmol/L EDTA), supplemented with complete protease inhibitor mixture. The protein content of each lysate was determined using BCA (Beyotime Biotechnology, China) assay kit.

1.5 Detection of PrP proteins expressed in cells transfected with PrP expressing plasmids by Immunofluorescent staining

Cells were plated onto microscope slides which were placed into 24-well plates one day before transfection. 48 hours after transfection, the cells on microscope slides were fixed by a solution of phosphate-buffered formaldehyde 15 min at room temperature. After washed with PBS for three times, the slides were incubated with PrP mAb 3F4 (diluted 1:500 with PBS) at 37℃ for 2 h, and then, incubated with FITC-labeled anti-mouse IgG (Santa Cruz, diluted 1:100 with PBS and 0.01% Evans blue) at 37℃ for 1 h. The slides were washed, dried and monitored under a fluorescent microscope.

1.6 Detection of carbonyl groups of the recombinant PrP proteins expressed in E. coli and the cells transfected with PrP expressing plasmids

To measure the activities of oxidative damage on the purified PrP proteins, 2 μg each PrP-PG5, PrP-PG0, PrP-PG9 and PrP-PG12 were incubated in 135 mmol/L NaCl, 1.3 mmol/L KCl, 3.2 mmol/L Na_2HPO_4, 0.5 mmol/L KH_2PO_4, pH 7.4 (PBS) with different concentration of H_2O_2 at room temperature for 30 min. Protein oxidation was measured by OxyBlot Protein Oxidation

Detection Kit (Intergen, Purchase, NY, USA), according to the manufacturer's instruction. Briefly, H_2O_2-treated proteins were derivatized to 2, 4-dinitrophenyl (DNP) by reaction with 2,4-dinitrophenyl hydrazine (DNPH) at room temperature for 30 min before loading onto SDS-PAGE gel. The proteins were electro-transferred, probed with 1:150 a rabbit antiserum (Chemico, USA) against DNP-modified carbonyl groups for 1 h at room temperature and subsequently incubated with 1:300 horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. The bands were visualized by ECL kit (PE Applied Biosystems, Foster City, USA).

To measure the carbonyl groups in the cells expressing various PrPs, cells were transfected with 2 μg PrP expressing plasmids in 6-well plates. Twenty-four hours posttransfection, cells were exposed to the DMEM containing 100 μmol/L H_2O_2 for another 24 h. Cells were harvested and lysed according to the protocol described above. After determining the protein concentration by BCA kit, 20 μg each preparation was employed into the measurement procedure of OxyBlot Protein Oxidation Detection Kit as described before.

1.7 Measurement of intracellular oxidative activity and glutathione peroxidase activity

Determination of the level of intracellular reactive oxygen species was carried out with Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechonolgy, Jiangsu, China). Briefly, the cells were treated with dihydrodichlorofluorescein diacetate (DCF-DA) delivered in serum-free medium at 37℃ for 30 min and harvested in PBS. The fluorescence of each well was then measured on Flow CytoMeter at the condition of 480 nm excitation and 530 nm emission wavelengths.

The glutathione peroxidase activity was measured with Cellular Glutathione Peroxidase Assay Kit (Beyotime Institute of Biotechonolgy, Jiangsu, China). Each lysate was mixed in the solution containing 50 mmol/L $Na_2HPO_4/NaHP_2O_4$, pH 7.0, 1 mmol/L EDTA, 1 mmol/ L NaN_3, 0.2 mmol/L NADPH, 1 mmol/L glutathione and 1 U/mL glutathione reductase in 0.1 mL volumn at 25℃ for 5 min. The reaction was initiated by the addition of 1.5 mmol/L cumene hydroperoxide and the absorbance measured for 3 min at 340 nm. All samples were corrected with the total protein content.

1.8 Trypan blue and MTT assays

Cells were cultured in 24-well tissue culture plates and transiently transfected with each PrP expressing plasmids. Forty-eight hours after transfection, cells were harvested by centrifugation at 1000 r/min for 10 min and stained with 0.4% DNA-binding dye trypan blue (Sigma) for 30 min. The numbers of trypan blue-positive stained cells each well were counted under microscope for quantification of death cells. To evaluate the cell viability, cells were plated as normal in 96-well trays. Cells were exposed to 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) in culture medium for 4 h. After lysed, the formazan product dissolved in dimethylsulfoxide (Me_2SO). Absorbance was measured at 540 nm in a spectrophotometer.

1.9 Statistic analysis

Statistical analysis was performed using the Kruskal-Wallis nonparametric one-way analysis of variance. Probabilities of less than 0.05 were considered to be statistically significant.

2 Results

2.1 PrP mutants with inserted octarepeat sequences expressed in E. coli were more likely affected by oxidation

Equal amounts (2 μg) of rPrP-PG0, rPrP-PG5, rPrP-PG9 and rPrP-PG12 were incubated with different concentrations of H_2O_2 ranging from 100 μmol/L to 500 μmol/L at room temperature for 30 min and analyzed by SDS-PAGE and Western blots. It showed that in the presences of 100 to 500 μmol/L H_2O_2 the electrophoretic positions of rPrP-PG0 and rPrP-PG5 remained almost unchanged (Figure 1(a)), whereas the molecular masses of rPrP-PG9 and rPrP-PG12 became significant larger, moved at positions of M_r 35 and 40 kD (Figure 1(a)), respectively. Furthermore, to investigate the possible difference in the generation of carbonyl groups among the recombinant PrP proteins containing difference octarepeats, equal amounts (2 μg) of rPrP-PG0, rPrP-PG5, rPrP-PG9 and rPrP-PG12 were also incubated with different concentrations of H_2O_2 ranging from 100 to 500 μmol/L at room temperature for 30 min, and then subjected to DNPH modification. Immunoblotted with anti-DNP antibody demonstrated clear signals of carbonyl group in the preparations of rPrP-PG9 and rPrP-PG12 after treated with H_2O_2 (Figure 1(b)). In contrast, no positive signal was detectable in the reactions of rPrP-PG0 and rPrP-PG5 at the expected position (Figure 1(b)). These

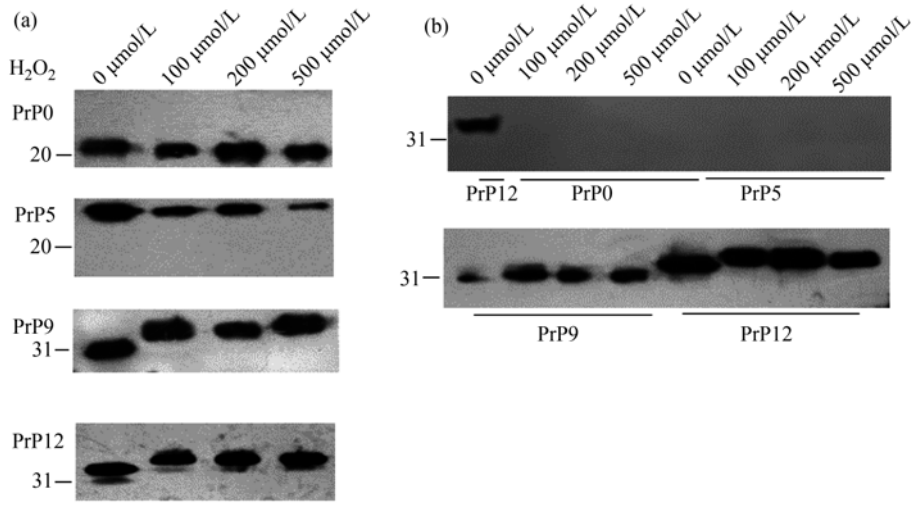

Figure 1 PrP mutants with inserted octarepeat sequences were more likely affected by oxidation A. Western blot analysis of aggregations of recombinant PrP-PG12 and PrP-PG9 after treatment of H_2O_2. 2 μg of PrP-PG0, PrP-PG5, PrP-PG9 and PrP-PG12 were incubated with 0, 100, 200 and 500 μmol/L of H_2O_2 at room temperature for 30 min. PrP-specific mAb 3F4 was used as the primary antibody. B. Recombinant PrP-PG9 and PrP-PG12 are more susceptible to oxidative damage. 2 μg of PrP-PG0, PrP-PG5, PrP-PG9 and PrP-PG12 were incubated with 0, 100, 200 and 500 μmol/L of H_2O_2 at room temperature for 30 min. The carbonyl groups in PrP samples were derivatized to DNP by reaction with DNPH and visualized in Western blot with anti-DNP antibody.

results suggested that the PrP mutants with inserted octarepeats, rPrP-PG9 and rPrP-PG12, were more likely affected by oxidation.

2.2 Cells expressing the mutated PrPs within octarepeats are less viable than the cells expressing wild type PrP

To address the difference of wild-type and mutated PrP proteins in the response activity to oxidative stress, various PrP expressing recombinant plasmids were transfected into the cultured SH-SY5Y and HeLa cells. Western blot of the cellular lysates transiently transfected with the PrP expressing plasmids with mAb 3F4 identified three predominant positive bands at the expected positions in each preparation, representing di-, mono- and un-glycosylated PrP proteins (Figure 2(a)). Immunoflurescent assays revealed the four different PrP proteins were expressed in the individually transfected cells, mostly on the surface of the cells (Figure 2(b)).

To see the influence of expressions of various PrP proteins on the growth abilities of the cultured cells, the transfected cells were measured with Trypan blue and MTT assays. The results of Trypan blue revealed that 48

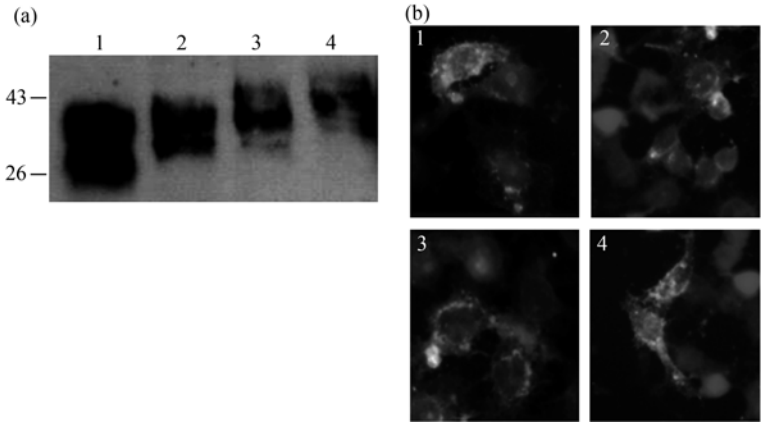

Figure 2 Expressions of the wild-type PrP and PrP mutants in the cultured cells. Cells were harvested 48 h after transfection. (a) Western blot analysis with mAb 3F4. Lane 1: PrP-PG0; lane 2: PrP-PG5; lane 3: PrP-PG9; lane 4: PrP-PG12. (b) Indirect fluorescent assays with mAb 3F4. Panel 1: PrP-PG0; panel 2: PrP-PG5; panel 3: PrP-PG9; panel 4: PrP-PG12.

h after transfection, the viabilities of both SH-SY5Y and HeLa cells expressed PrP-PG5 were comparable with that transfected with plasmid pcDNA3.1, even slightly higher (Figure 3(a)), while those of the cells expressing PrP-PG0, PrP-PG9 and PrP-PG12 displayed remarkably reduced viabilities, showing statistic differences compared with PrP-PG5 that of (P<0.05, Figure 3(a)). MTT analysis revealed the similar results as Trypan blue (Figure 3(b)). The mean OD values of SH-SY5Y and HeLa cells expressing PrP-PG5 were 1.41 and 1.34, while that of the cells expressing PrP-PG9 and PrP-PG12 were 1.244 and 1.123 in SH-SY5Y and 1.18 and 1.11 in HeLa, respectively, revealing statistical differences in SH-SY5Y (P=0.022 and P=0.045) in HeLa (P=0.037 and P=0.005). Meanwhile, the mean OD values of SH-SY5Y and HeLa cells expressing PrP-PG0 were 1.03 and 1.09, showing statistical differences compared with that of PrP-PG5 in HeLa and SH-SY5Y (P=0.029 and P=0.022).

2.3 Cells expressing the PrP mutants showed high levels of intracellular free radicals and low levels of glutathione peroxidase

To address the possible influence of the expressions of the PrP mutants with extra-octarepeats on intracellular oxidation process, free radicals generation in the cells receiving various PrP expressing plasmids were measured by staining with fluorescent dye DCF-DA. The fluorescence of each well was then measured on Flow CytoMeter at the condition of 480 nm excitation and 530 nm emission wavelengths (Figure 4(a)). Compared with the preparation transfected with pcDNA3.1, the ROS level in the cells expressing PrP-PG5 was obviously lower (Figure 4(b)). Meanwhile, the cells expressing inserted extra-octarepeats PrP-PG9 and PrP-PG12 induced significantly higher ROS levels (Figure 4(b)). The average ROS levels in the groups of PrP-PG9 and PrP-PG12 were 1.65- and 1.78-fold increased in HeLa cells and 1.519- and 1.433-fold increased in SH-SY5Y cells, revealing statistical differences in HeLa (P=0.0175 and P=0.0033) and in SH-SY5Y (P=0.0137 and P = 0.0435). Interestingly, in the preparation transfected with plasmid expressing PrP-PG0, the average ROS levels were also significantly higher than that of PrP-PG5 (P = 0.0012 in HeLa and P=0.021 in SH-SY5Y, Figure 4(a)).

Furthermore, cellular glutathione peroxidase activities in the cells expressing wild type PrP and mutant PrPs were comparably measured. In line with the results of intracellular ROS levels, higher level of glutathione peroxidase activity was observed in the cells expressing wild-type PrP-PG5, while markedly lower activities were identified in the cells expressing mutated PrP-PG9 and PrP-PG12, as well as PrP-PG0 (Figure 4(c)). Compared with the preparation of PrP-PG5, the average glutathione peroxidase activities in the preparations of PrP-PG9, PrP-PG12 and PrP-PG0 decreased to 74.0, 61.5 and 67.3% in HeLa cells and 72.9, 67.8 and 73.2% in SH-SY5Y cells, with statistical difference. These results suggest that expressions of PrP mutants in cultured cells may weaken the cellular protective capacity against oxidation.

2.4 Cells expressing the PrP mutants produced more carbonyl groups after treatment of H_2O_2

To test the possible difference in the production of carbonyl groups in the cells expressing various PrPs, cells were exposed to various concentrated H_2O_2 for 24 h and the total intracellular carbonyl groups were measured by

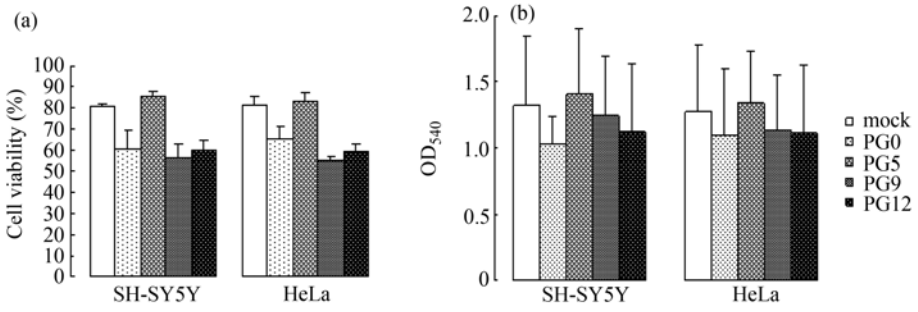

Figure 3 Evaluations of the viabilities of the cells expressing various PrPs. Cells were analyzed 48 h after transfection. (a) Trypan blue staining. The average cell viabilities (%) were illustrated at the left of panels. (b) MTT. The average OD values were indicated at the left of panels. SH-SY5Y and HeLa cells were indicated at the bottom. The average data of each preparation was calculated based on three independent experiments and represented as mean ± S.D.

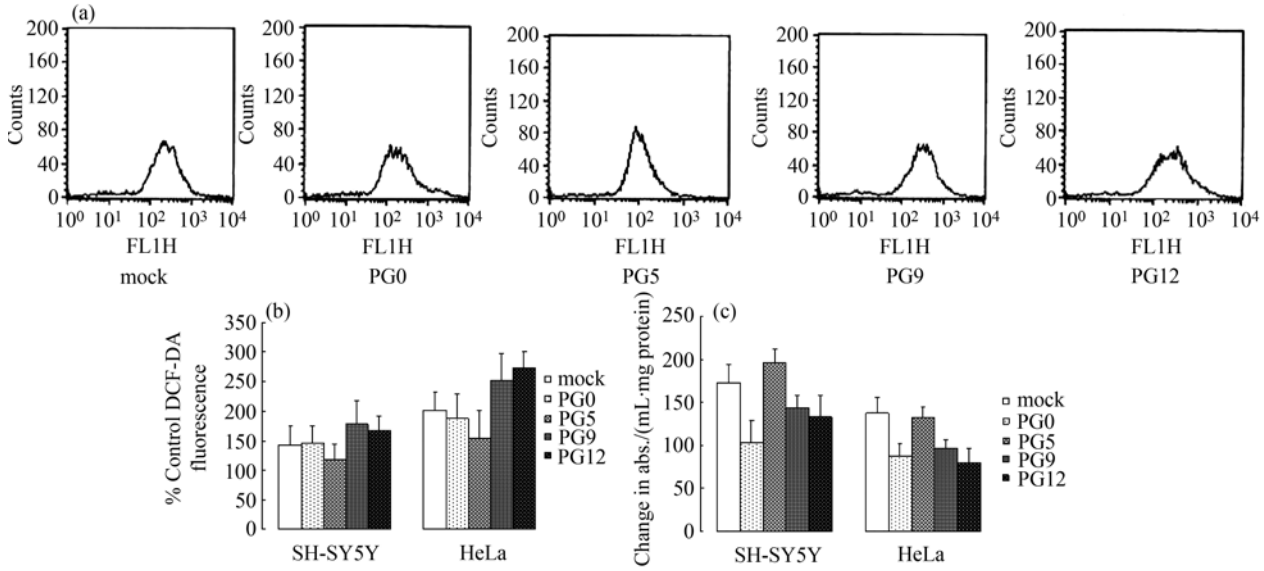

Figure 4 Evaluations of the levels of intracellular free radicals and glutathione peroxidase of the cells expressing various PrPs. Cells were analyzed 48 h after transfection. (a) Measurements of the cells stained with fluorescent dye DCF-DA on Flow CytoMeter at the condition of 480 nm excitation and 530 nm emission wavelengths. (b) The average ROS values. The ROS values were indicated at the left of the panels. (c) The values of glutathione peroxidase. The average OD values were shown at the left of the panels as change in abs./(mL·mg protein). SH-SY5Y and HeLa cells were indicated at the bottom. The average data of each preparation were calculated based on three independent experiments and represented as mean ± S.D.

Western blot. No significant signal of carbonyl groups was detected in the cells without treatment of H_2O_2 at 48 h posttransfection (data not shown). However, clear carbonyl groups-specific reactive bands were observed in all preparations treated with 100 μmol/L H_2O_2 and actin as control (Figure 5). Compared with the preparation of PrP-PG5, more carbonyl groups were detected in that of PrP-PG0, PrP-PG9 and PrP-PG12 (Figure 5(a)). Analysis of the intensities of the specific-signals revealed that expressions of PrP-PG0, PrP-PG9 and PrP-PG12 in the cultured cells induced statistically more carbonyl groups than that of PrP-PG5 (P<0.05, Figure 5(b)).

2.5 Cells expressing the PrP mutants were more sensitive to the challenge of H_2O_2

To see the possible antioxidation capacity by the of expression of exogenous PrP in cultured cells, SH-SY5Y cells transfected with wild-type and mutated PrP expres-

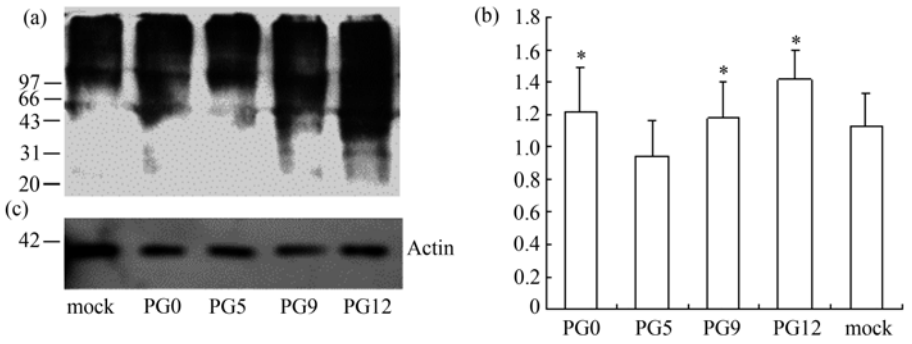

Figure 5 Cells expressing PrP mutants with different numbers of octapeptide repeats produced more carbonyl groups after challenged with H_2O_2. (a) Western blots with carbonyl groups specific antibody. Cells transfected with various PrP expressing plasmids were maintained in the medium containing 100 μmol/L H_2O_2 for 24 h and cell lysates were separated in 12 % SDS-PAGE. Mock represented as the cells transfected with the vector pcDNA3.1. Various PrPs, including PrP-PG0, PrP-PG5, PrP-PG9 and PrP-PG 12 were shown at the bottom. (b) Analyses of the relative gray value of each sample. The relative gray value of the individual preparation was the ratio of gray value of the carbonyl group specific signal of each reaction in Western blot to that of the cells without transfection. The average data of each preparation were calculated based on three independent experiments and represented as mean ± S.D. "*" represented statistic difference (P<0.05) between the groups of wild-type PrP (PG5) and other individual preparation. (c) Actin-specific Western blots of each preparation.

sion plasmids were challenged with different amounts of H_2O_2 for 6 h and cell viability was evaluated by MTT assays. It showed that the number of living cells reduced along with the increases of the amount of H_2O_2 in all preparations (Figure 6). Compared with the preparations of PrP-PG0, PrP-PG9, PrP-PG12 and mock, more live cells were observed in that of PrP-PG5. To address the difference in reduction extend of live cells among various PrP preparations after challenge with H_2O_2, the relative cell viability of each sample was calculated by dividing the OD value of exposing at various concentration of H_2O_2 with that of the same preparation without treatment of H_2O_2. Statistical analysis demonstrated the difference (P<0.05) in the relative cell viabilities between PrP-PG5 preparation and every other preparations

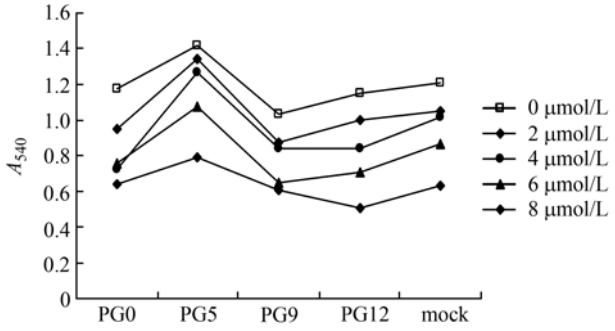

Figure 6 Cells expressing wild-type PrP possessed anitoxidation activity against H_2O_2 at certain extent. MTT assays. Cells transfected with various plasmids were exposed to the medium containing 2 to 8 μmol/L H_2O_2 for 6 h. Panel 1: PrP-PG0; panel 2: PrP-PG5; panel 3: PrP-PG9; panel 4: PrP-PG12; panel 5: pcDNA3.1. The average OD value of each reaction was calculated with three independent tests.

exposing to 2 μmol/L H_2O_2 (Table 1). Statistic difference (P<0.05) were also detected between PrP-PG5 and every other PrP mutants, including PrP-PG0, PrP-PG9 and PrP-PG12 when exposing to 4 or 6 μmol/L H_2O_2, but no difference compared with the mock (Table 1). When the final concentration of H_2O_2 increased to 8 μmol/L, the relative cell viabilities between PG5 and every other reaction showed no difference. It implies that expression of wild-type PrP benefits the host cells against the challenge of H_2O_2 at certain extent.

2.6 Cells expressing the PrP mutants possessed more free radicals and less glutathione peroxidase after challenged with H_2O_2

To see the possible correlation between cell death and oxidative stress after challenged with 6 μmol/L H_2O_2, levels of intracellular free radicals and glutathione peroxidase of the cells expressing various PrPs were measured. Obvious high intracellular free radicals and low glutathione peroxidase were see in all preparations after treatment of 6 μmol/L H_2O_2, compared without challenge of H_2O_2. As expected, stronger increase of intracellular free radicals and reduction of glutathione peroxidase activity were identified in the cells expressing PrP-PG0, PrP-PG9 and PrP-PG12 (Tables 2 and 3), showing statistic difference compared with the cells expressing PrP-PG5 (P<0.05).

3 Discussion

Oxidative damage to proteins has been implicated in the pathogenesis of many neurodegenerative disorders, such as Alzheimer's disease, Parkinson disease, as well as prion disease. Previous studies propose that PrP^C impli cates in the cellular response to oxidative stress and functions as SOD-like activity. Cells deficient in PrP^C

Table 1 Reduction of cell viability in the cells expressing various PrPs after challenge with different amounts of H_2O_2

H_2O_2 (μmol/L)	PG5 ($\bar{x}$ ±SD%)	PG0 ($\bar{x}$ ±SD%)	PG9 ($\bar{x}$ ±SD%)	PG12 ($\bar{x}$ ±SD%)	Mock ($\bar{x}$ ±SD%)
0	100	100	100	100	100
2	94.44±2.85	74.62±10.38*	79.80±9.98*	83.51±2.33*	83.72±4.19*
4	88.70±5.92	62.46±5.14*	72.83±8.00*	69.01.±10.20*	81.33±9.39
6	75.49±2.89	60.34±3.62*	62.07±5.44*	60.48±6.56*	72.14±4.77
8	56.11±4.03	55.69±7.17	59.55±3.62	45.02±16.47	55.52±17.70

x% of each preparation was the percentage of cell viability after exposing to H_2O_2 compared with the individual sample without challenge of H_2O_2. The average data of each preparation was calculated based on three independent experiments and each experiment contained three wells per reaction. *, Statistic difference (P<0.05) comparing with the cell viability of the preparation of PG5 exposing to the same amount of H_2O_2.

Table 2 Changes of intracellular ROS in the cells expressing various PrPs after challenge with 6 μmol/L H_2O_2

H_2O_2	PG5 ($\bar{x}$ ±SD%)	PG0 ($\bar{x}$ ±SD%)	PG9 ($\bar{x}$ ±SD%)	PG12 ($\bar{x}$ ±SD%)	Mock ($\bar{x}$ ±SD%)
0 mmol/L	100	100	100	100	100
6 mmol/L	207.144±21.58	250.86±1.45*	265.39±30.51*	245.19±11.17*	224.67±39.75

x% of each preparation was the percentage of ROS after exposing to H_2O_2 compared with the individual sample without challenge of H_2O_2. The average data of each preparation was calculated based on three independent experiments and each experiment contained three wells per reaction. *, Statistic difference (P<0.05) comparing with the cell viability of the preparation of PG5 exposing to the same amount of H_2O_2.

Table 3 Changes of intracellular glutathione peroxidase in the cells expressing various PrPs after challenge with 6 μmol/L H_2O_2

H_2O_2	PG5 ($\bar{x}$ ±SD%)	PG0 ($\bar{x}$ ±SD%)	PG9 ($\bar{x}$ ±SD%)	PG12 ($\bar{x}$ ±SD%)	Mock ($\bar{x}$ ±SD%)
0 mmol/L	100	100	100	100	100
6 mmol/L	87.69±3.97	66.67±9.27*	73.45±3.88*	72.21±2.83*	84.36±8.57

x% of each preparation was the percentage of glutathione peroxidase after exposing to H_2O_2 compared with the individual sample without challenge of H_2O_2. The average data of each preparation was calculated based on three independent experiments and each experiment contained three wells per reaction. *, Statistic difference (P<0.05) comparing with the cell viability of the preparation of PG5 exposing to the same amount of H_2O_2.

are less viable in culture compared with cells expressing wild-type PrP, and are more susceptible to oxidative damage and toxicity caused by agents such as copper and hydrogen peroxide[24−26]. In this study, we propose the evidences the fCJD-associated PrP proteins are more sensitive to the challenges of oxidation, both in the levels of recombinant proteins and cultured cells.

About 10 to 15% human TSE are related with a series of mutations in *PRNP* gene, among them inserts of various numbers of extra octarepeats are repeatedly reported. The octarepeats of PrP seems to be the functional region, showing several activities, e.g. binding with metal ion Cu^{2+}, interacting with numerous neuron proteins, functioning as SOD-like activity. Interestingly, the exact numbers of octarepeats within PrP peptide has been confirmed to be critical for its biological characteristics. Alterations of the numbers of octarepeats within PrP will change its biological features, even lead to a fatal familial disease[27]. Our data of prokaryotic expressed PrP proteins to oxidation agent and eukaryotic expressed PrP proteins on the growth of the cultured cells illustrated again the consequence and significance of the accurate numbers of octarepeats within PrP.

We observe for the first time by the Western blot that rPrP-PG9 and rPrP-PG12 are more likely affected by oxidation agent H_2O_2, leading to aggregating to higher molecular mass. Meanwhile, marked increase of carbonyl groups have been observed in the H_2O_2-treated rPrP-PG9 and rPrP-PG12. This is consistent with the previous observations that PrP-PG8 and PrP-PG10 are more prone to oxidative damages [28]. Requenan et al has reported that histidine residue within PrP peptide is the main target of the Cu-mediated oxidation [29]. There are total nine histidine residues in PrP peptide, five locating in the region of octarepeats. Increase of numbers of histidine residues through octarepeats insertion will probably sensitize the susceptibility to oxidation.

PrP^C has also been implicating in the cellular response to oxidative stress, that the cells deficient PrP^C are less viable in culture compared with the cells expressing wild-type PrP and are more susceptible to oxidative damage and toxicity caused by agents such as copper and hydrogen peroxide. The octarepeats can bind Cu^{2+}, possibly making PrP have SOD-like activity. Consistent with the influences on cell growth after transfected with various PrPs, remarkable decreases of the activities of cellular glutathione peroxidase have been observed in the cells expressing PrP octarepeats mutants. It has been believed that the reduced form of glutathione is critical for detoxification of cellular H_2O_2, prevention of lipid and protein peroxidation and the transport, detoxification and sequestration of copper. An increased glutathione peroxidase activity has been already described in the cell expressing wild-type PrP. Lower cellular glutathione peroxidase activity may decrease the cellular capacity of reduction of H_2O_2, which in presence of redox active metals could lead to cell damage due to the formation of toxic hydroxyl radicals [30].

Oxidative stress is caused by an imbalance in the level of ROS that are the products of normal cellular metabolism. Brain tissue has a remarkably high respiration rate, constituting only about 2% of the adult body mass, but consuming about 20% of its resting oxygen of the whole body, independent of the state of mental activity, therefore, brain tissue is much more sensitive to ROS [31]. Links among oxidative stress, ROS, and neurological disorders have been reported in a serial of neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, amylotrophic lateral sclerosis, and even aging itself. Oxidation seems to play also roles in the pathogenesis of TSE, in which oxidation of PrP molecule at its octarepeats region may be an important factor. This study and previous studies [32] repeatedly confirmed that expressions of PrPs with extra insertion of octarepeats in cells lead to raising the cellular ROS. Higher ROS levels in the cells possessing fCJD-associated PrP indicate a relative vulnerable status, in which the cells are more likely to be injured. Alterations of the numbers of octarepeats in PrP peptide may not only result in loss of the cellular protective activity of PrP, but also turn the protein itself to an oxidative event.

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