Dynamic Analyses of PrP and PrP^{Sc} in Brain Tissues of Golden Hamsters Infected With Scrapie Strain 263K Revealed Various PrP Forms[1]

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Objective To expatiate dynamic changes in hamsters infected with scrapie strain 263K, to observe the presence and aggravation of various forms of PrP and PrP^{Sc} during incubation period, and to probe primarily the relationship between the onset of clinic manifestations and the presence of different PrP^{Sc} forms. **Methods** Hamster-adapted scrapie strain 263K was intracerebrally inoculated into hamsters. Different forms of PrP and PrP^{Sc} were monitored dynamically by Western blot and immuno-histochemical assays. The presence of scrapie-associated fibril (SAF) was assayed by electron microscopy analysis (EM) and immuno-golden EM. **Results** PrP^{Sc} was initially detected in the brain tissues of the animals in 20 days post-inoculation by immunohistochemistry and 40 days with Western blot. Quantitative evaluations revealed that the amounts of PrP and PrP^{Sc} in brain tissues increased along with the incubation. Several high and low molecular masses of PrP were seen in the brains of the long-life span infected animals. Deglycosylation assays identified that the truncated PrP in the infected brains showed similar glycosylation patterns as the full-length PrP. The presence of short fragments was seemed to relate with the onset of clinical conditions. **Conclusion** These results indicate that infectious agents exist and accumulate in central nerve system prior to the onset of the illness. Various molecular patterns of PrP^{Sc} may indwell in brain tissues during the infection.

Key words: Transmissible spongiform encephalopathies (TSEs); Prion; Scrapie; Glycosylation; Scrapie-associated fibril (SAF)

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are rare degenerative neurological disorders that afflict human beings (Creutzfeldt-Jakob disease, CJD; Gerstmann-Sträussler -Scheinker syndrome, GSS; Kuru; and fatal familial insomnia, FFI), sheep and goat

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(scrapie), cattle (bovine spongiform encephalopathy) and other animals. They may have a sporadic, inherited or transmissible origin[1]. All prion diseases are characterized by the conversion of a normal host-encoded and protease-sensitive glycosylphosphotidylinositol anchored glycoprotein, denoted the cellular prion protein (PrP^{C}) to an abnormal, protease-resistant isoform, designated PrP^{Sc}, existing in highly aggregated and detergent insoluble polymers[2-4]. The relationship between PrP^{Sc} formation and prion replication remains unknown. Caughey and coworkers have demonstrated the ability of PrP^{Sc} to convert PrP^{C} *in vitro* to a form that has proteolytic resistance resembling that of PrP^{Sc}. However, in these studies it was difficult to conduct biological assay of the nascent protease resistant material by measurement of infectivity, because only a small amount of materials was converted to a protease resistant form occurring in the presence of an excess of PrP^{Sc}[5].

Several studies in mice showed that the amount of PrP^{Sc} accumulation had a progressive increase. The accumulation process was monitored in spleen inoculated intraperitoneally with mouse scrapie strain C506M3[6], as well as in brain and spleen inoculated intracerebrally with scrapie agent RML[7,8], preceding the onset of prion disease. PrP^{Sc} accumulations were repeatedly observed in brain and other extra-nerve tissues including spleen and thymus. Moreover, the appearance of PrP^{Sc} accumulation could reach a plateau which might persist until the presence of the symptoms of prion disease[6], correlating well with the development of pathological changes in the tested mice[8,9]. Similar kinetics of prion protein accumulations were also identified both in brain and other organs including lymph nodes, spleen and placenta in scrapie infected sheep[10-12]. However, the detailed dynamic changes presented in the Western blot and immunohistochemistry from the inoculation to the onset of clinical conditions were not expatiated. The references available, particularly in relation to the dynamic profiles of Western blot were absent.

Both PrP^{C} and PrP^{Sc} appear with three electrophoresis-mobilizing patterns, representing diglycosyl, monoglycosyl and aglycosyl forms. In human inherited prion disorders, two different proteinase K (PK) resistant molecules were observed both in naturally occurred patients and in transgenic mice bioassays, a mass of 19 KDa in FFI and 21 KDa in familial CJD and most sporadic CJD[13-15]. The difference in molecular size was shown to be due to different sites of proteolytic cleavage at the NH2 termini of the two human PrP^{Sc} molecules reflecting different tertiary structures[16]. It has been reported that a truncated COOH-terminal fragment of human PrP referred as C1 is present in normal brains and an additional one referred as C2 in CJD cases, with the characteristics as PrP^{C} in C1 and PrP^{Sc} in C2[17]. Different forms of PrP in brain tissues indicate that PrP proteins undergo different routes of proteolytic metabolism according to different PrP molecules and conformations. Although it has been well known that the accumulation of PrP^{Sc} aggravates along with incubation, when different forms of PrP begin to appear in brain tissues during incubation, their properties of glycosylation remain unclear.

Immunohistochemical examination in sheep infected with scrapie agents indicated that the patterns of PrP^{Sc} deposition depended on the strains of the agents and the PrP genotype of the host. Intraneuronal deposition of PrP was the type most closely associated with the development of clinical conditions[18]. The detection of PrP in a murine scrapie model by immunohistochemistry was 20 days earlier than that by Western blot[19]. The positions and patterns of dynamic PrP^{Sc} deposition in brain slices with immunohistochemistry and observations of the possible consistency between Western blot and immunohistochemistry can help us to comprehend the manifestation of clinical symptoms.

In the present study, a hamster-adapted scrapie strain 263K was intracerebrally inoculated into hamsters and the presence of PrP^{Sc} was dynamically monitored. A progressive increase of PrP and PrP^{Sc} in brain tissues was identified. Additionally, the pattern profiles of

the Western blot were changed along with incubation. High molecular weight masses, as well as low molecular weight masses of PrP were repeatedly observed in the brain homogenates in the assays of Western blot, while the latter easily came into view when the clinical symptoms were developed. The low molecular weight masses PrP were confirmed to have similar glycosylation patterns as the full-length PrP. Furthermore, the results of immunohistochemical examination at various post-inoculation times were in accord with those of the detection of PrP^{Sc} by Western blot.

MATERIALS AND METHODS

Bioassay

A total of seventy 14-day old female Chinese golden hamsters were infected intracerebrally with scrapie strain 263K. Prior to inoculation, hamster brain tissues infected with scrapie 263K previously were made into a 10% homogenate with PBS as described elsewhere. Two μL suspension of 10% brain homogenate was injected into hamster's brain mixed with 100 μg/mL ampicillin. All hamsters were fed ad libitum in cages, among which five hamsters were fed as normal control.

Prion Protein Purification

Brain homogenate was prepared and prion protein was purified on the basis of a procedure described previously[20,21], with some modifications. In brief, one gram of brain stem from the inoculated or normal control hamsters was prepared to 10% homogenates in lysis buffer (100 mmol/L NaCl, 10 mmol/L EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mmol/L Tris, pH 7.5). The tissue debris was removed with low centrifugation at 3 000 g for 10 min, and the supernatant was further centrifuged at 20 000 g for 90 min at 4℃. The pellet was re-suspended in 40 μL deionized and distilled water and stored at –80℃ until use.

Western Blot and PrP Quantitative Analysis

Prior to immunoblot analysis, one aliquot of purified PrP^{Sc} was treated with 100 μg/mL proteinase K overnight at 4℃. The protein samples were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes using a Trans-Blot apparatus. After having been blocked with 5% defatted milk in TBST (Tris-buffered saline, pH 7.6, containing 0.5% Tween20) overnight at 4℃, the membranes were incubated with monoclonal antibody 3F4 (Dako) for 2 h at room temperature and the proteins were visualized with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz). Quantitative analyses of the immunoblot images were carried out using computer-assisted software Image Total Tech (Pharmacia). Briefly, the image of immunoblot was scanned with Typhoon (Pharmacia) and digitalized, saved as TIF format. The values of each target blot were evaluated and balanced with that of a genetic engineering expressed HaPrP (recombinant hamster's prion protein)[22], which was used as a control standard.

PrP Deglycosylation

After treatment with PK at 37℃ for 1 h, proteolytic digestions of brain homogenates from normal or infected hamsters were terminated by 5 mmol/L PMSF (phenyl-methylsulfonyl

fluoride). Fifteen μL-aliquot of PK-treated or PK-untreated solutions was boiled for 10 min in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol), and deglycosylated with PNGase F (Biolab, 1 500 U in 1% Nonidet P-40, 50 mmol/L sodium phosphate, pH 7.5) at 37℃ for 12 h. Proteins were precipitated with 4 volumes of cold methanol at –20℃ for 6 h, and centrifuged at 15 000 g for 30 minutes. The pellets were resuspended in sample loading buffer and separated in 15% SDS-PAGE. Western blot assays were done as described above.

Electron Microscopy Analysis (EM)

Ten μL-aliquot of purified PrP solution from the brain stem described above was absorbed onto 400-mesh carbon-coated cuprum grids for 1 minute. After excess fluid was drained with filter paper, the grids were stained with sodium phosphotungstate, pH 7.5 for about 1 minute. For immuno-golden staining, the grids were absorbed onto 1:100 diluted antibody 3F4 for 8 h and subsequently onto 1:50 diluted golden particles coupled with SPA for 1 h, before the process of negative stain. The presence of SAF was observed with an electron microscope (Philips Tecnai 12) at 80 KV ×38 000-54 000.

Immunohistochemistry (IHC) and HE Staining

Hamster brains inoculated with scrapie strain 263K were fixed in 10% formalin. Tissue blocks were decontaminated in 88% formic acid for at least 1 h prior to embedding in paraffin. After deparaffinization, the slices were treated with the protocol described briefly as follows: microwave irradiation (Galanz, WP1000L30-2) for 30 min, 3% H_2O_2-methanol for 15 min, 0.1% trypsin for 15 min and proteinase K (5 μg/mL) for 3-5 min. The slices were immersed in 1% normal horse serum for 20 min before incubation with the primary antibody (3F4) diluted at 1:400 overnight at 4℃. The incubation of the secondary antibody and visualization of the immunostainings were performed with a commercially supplied VECTASTAIN ABC detection kit (Vector Laboratories, Inc.), according to the manufacturer's instructions. Slices were counterstained slightly with haematoxylin, dehydrated, and mounted in glycerolvinyl alcohol as described elsewhere.

For HE staining, brain sections (5 μm) were stained with haematoxylin and eosin-alcohol after deparaffinization, according to the protocol described elsewhere.

RESULTS

PrP^{Sc} and SAF in Brain Tissues Appeared Earlier Than Onset of Clinical Manifestations

A total of 70 hamsters were inoculated with 263K-scrapie strain, of which 5 hamsters died at the first week after intracerebral challenge, probably due to direct damages to brains. The most definite and commonest clinical sign was ataxia, which appeared roughly in all the infected animals and was defined as the onset of clinical symptoms. Emaciation and shagginess were also observed in most of the animals. From the earliest appearance of clinical symptoms in three hamsters to the latest onset in 15 hamsters, the incubation period varied from 45 days to 72 days (66.7±1.1 days). Except 15 hamsters killed for PrP^{Sc} analysis without detectable symptoms during the incubation period, all the rest showed the symptoms and signs of encephalopathy verified by the appearance of PrP^{Sc} and SAF in brain tissues. The incidence of scrapie infection in inoculated hamsters reached up to 100%.

Five animals were randomly selected on the 20th, 40th, 50th, 60th, 70th and 80th day post-inoculation for the detection of PrP^{Sc} and SAF in brain tissues, as well as the

neuropathological changes. The hamsters killed on the 20th, 40th and 50th day after inoculation were symptom-free, while animals killed on the 60th day had slight ataxia, animals killed on 70th day had severe ataxia, and animals killed on 80th were almost dead (Table 1). Western blot and EM assays failed to identify any PK-resistant protein or SAF in brain homogenates prepared from the first five hamsters on the 20th day post-inoculation (Fig. 1). PrP^{Sc} in the brain tissues was initially detected from five symptom-free hamsters 40 days after inoculation (Fig. 1, Table 1), while SAF was observed in all PrP^{Sc}-positive animals and confirmed by immuno-EM (Fig. 2). The subsequent screenings of the animals in 50, 60, 70, and 80 days post-inoculation showed that all tested hamsters contained PrP^{Sc} and SAF in their central nerve tissues.

In line with the detection of PrP^{Sc} in Western blot and SAF in EM, immunohistochemical and neuropathological assays revealed similar results. PrP^{Sc} deposits were first observed in all five brains collected on the 20th day post-inoculation, which were persisting in all the tested slices prepared from the brain tissues collected subsequently (Figs. 3d-f, Table 1). Compared with the histoblots, the typical spongiform degeneration in brain tissues appeared later, only 1 out of 5 tested brains on the 20th day and 1 out of 4 on the 40th day were shown to be positive. Extensive spongiform changes were observed in the animals in 50, 60, and 70 days post-inoculation (Table 1), mainly in layers Ⅲ, Ⅳ and Ⅴ of cerebral cortex (Figs. 3a-c). These results suggested that the scrapie infectious agents, and even neuropathological changes, appeared in brain earlier than the onset of illness.

TABLE 1

Presences of Clinical Sign, PrP^{Sc} and Neuropathological Change in the Hamsters Inoculated With Scrapie 263K

Tested Number	Post-inoculation (day)	Clinical Sign (ataxia)[a]	Western Blot (PrP^{Sc})	Immunohisto-chemistry (PrP^{Sc})	HE Stain (spongiform)
5	20	0/5	0/5	5/5	1/5
5	40	0/5	5/5	4/4	1/4
5	50	0/5	5/5	5/5	4/5
5	60	5/5	5/5	5/5	4/4
5	70	5/5	5/5	5/5	2/2
5	80	5/5	5/5	ND[b]	ND[b]

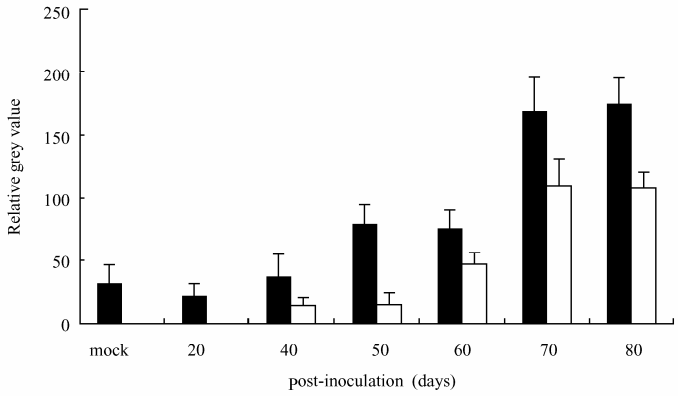
Note. [a]using detectable ataxia as the clinical sign; [b]ND represents not done.

Amounts of Total PrP and PrP^{Sc} Increase Along With Disease Progression

In order to address the possible quantitative changes of PrP in brain during progression, the brain tissues collected at different time-points were comparatively visualized with Western blot assays. All the tested samples from brain stem were equilibrated for weight-balance before PK treatment. Using commercially supplied software Image Total Tech, the developed protein bands of both total PrP and PrP^{Sc} were quantitatively analyzed. The amount of PrP in normal animals in the whole incubation period was not changed significantly (data not shown). Compared with the 20-day post-inoculation animals' results, the average PrP amount of 40-, 50-, 60-, 70-, and 80-day post-inoculation hamsters showed 1.7-, 3.61-, 3.45-, 7.67-, and 7.94-fold increased respectively (Fig. 1). However, analysis of PrP amount of normal hamsters used as control at the same time-point failed to reveal the similar increasing (data not shown). These results suggested that the total amount of brain PrP increased during disease progression, probably due to the accumulation of abnormal

isoforms.

In line with the increase of total PrP amount, the amount of brain PrP^{Sc} also revealed an increasing trend. Fig. 1 shows that although PrP^{Sc} was first visible in animals in 40-days post-inoculation, the amount of brain PrP^{Sc} at this time-point was only 14.34% of that of the animals with severe ataxia in 80 days post-inoculation. More PrP^{Sc} was detected in the brain tissues of the hamsters sacrificed subsequently (Fig. 1). Compared with the average amount of PrP^{Sc} found in the 80-day hamsters, the average amount of PrP^{Sc} of 50-, 60-, and 70-day animals was 14.37%, 44.19%, and 102.8%, respectively (Fig. 1).



X-fold PrP increasing compared with that of 20 days	ND	1.00	1.70	3.61	3.45	7.67	7.94
X% of PrP^{Sc} compared with that of 80 days	–	–	14.34	14.37	44.19	102.08	100.00

FIG. 1. Both PrP and PrP^{Sc} in the brain tissues of the infected hamsters increase along with incubation time. Quantitative analysis of PrP and PrP^{Sc} in Western blot was performed using computer-assistant software described in Methods. All the tested samples from brain stem were equilibrated for weight-balance before further processing. The data were expressed as $\bar{x}\pm s$. Relative grey value was calculated from five tested hamsters. Mock represents the hamsters inoculated with PBS intracerebrally. Samples treated with or without PK were denoted as white bars and black bars, respectively. The average values of X-fold increase of PrP and X% of PrP^{Sc} are shown in lower part.

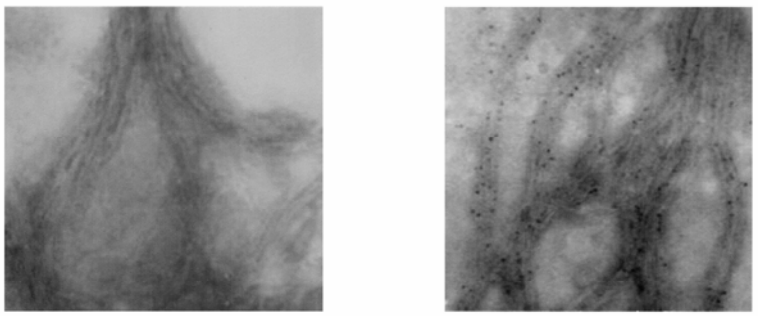


FIG. 2. Observation of scrapie-associated fibrils from brains of hamsters inoculated with scrapie strain 263K 40 days after infection. No detectable clinical sign was found. (a): Ordinary negative-stain electron microscopy. (b): Immuno-electron microscopy. Magnification ×38 000.

PrP^{Sc} deposits were first observed in brain tissues of the animals in 20 days after inoculation, in which a few punctate deposits could be seen without typical spongiform (Fig.

3d). Much larger granules, as well as morula, were repeatedly identified in the hamsters in 50- and 60-day post-inoculation (Fig. 3e). More extensive distributions of PrP^{Sc} with severe spongiform degeneration were found in the animals in 70-day post-inoculation with distinct symptoms, which involved almost the whole cerebral cortex (Fig. 3f). Interestingly, the amount of the PrP^{Sc} deposits located at one pole of vacuoles, which looked like a ring of pearls[23]. In line with the spongiform degeneration, PrP^{Sc} deposits lay mainly in layers Ⅲ, Ⅳ and Ⅴ of cerebrum, enlarging along with incubation (Figs. 3d to 3f).

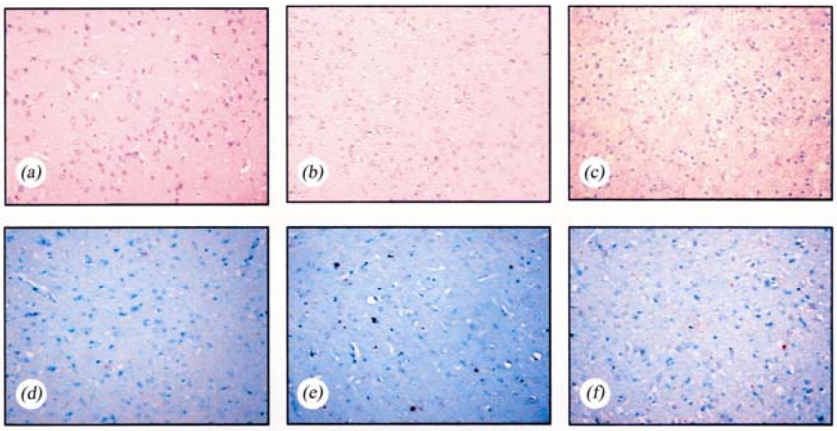


FIG. 3. PrP^{Sc} deposits and spongiform degenerations detected prior to onset of illness aggravate along with the incubation time. (a) to (c): HE stain of cerebrum, (a): 20 days after infection, (b): 50 days after inoculation, (c): 70 days after inoculation. (d) to (f): Immunohistochemistry using PrP specific monoclonal antibody 3F4 as the primary antibody, (d) to (f): Cerebrum from the animals 20, 50, and 70 days after inoculation, respectively. Magnification ×400.

High and Low Molecular Masses of PrP Appeared in Brain Tissues of Long Life-span Animals

Except for the typical PrP bands mobilized around 27 to 35 KDa, several high molecular masses (HMM) could also be recognized by PrP specific monoclonal antibody 3F4, which mobilized at the position of 50 to 140 KDa (Fig. 4a). The appearance and augment of these masses were clearly related to the incubation time (Fig. 4b). Treatment of PK for 1 h at 37℃ removed all signals at the same mobilizing position, even in the preparations with higher concentrated large-molecular PrP from the animals 80-day post-infection (Fig. 4a).

Additional small bands that mobilized below the aglycosyl PrP^{C} were observed in Western blot assays (Fig. 4a). These 19-23 KDa bands were first identified in the brain homogenates prepared from the symptom-free animals in 50 days after inoculation and became more abundant in that of the diseased animals in 60, 70, and 80 days after inoculation (Fig. 4b). Compared with the preparations treated with PK, the smallest band (about 19 KDa) moved at the same position as the aglycosyl PrP^{Sc}. Quantitative analyses showed that the presence and augment of the low molecular masses (LMM) were similar to those of the large molecular weight masses of PrP (Fig. 4b).

To find out whether the appearance and gradual augment of the HMM and LMM were normal phenomena during growth, the brain homogenates prepared from the same age normal hamsters used as mock were comparatively studied. Western blot assays failed to find LMM, even in the older hamsters (Fig. 4b), indicating that the presence of low

molecular PrP might be disease-related even disease-specific. Only very tiny signals of HMM in normal hamsters were observed (Fig. 4b). However, compared with the increased curve of HMM in the infected animals, the increased degree of HMM in uninfected hamsters was very restricted.

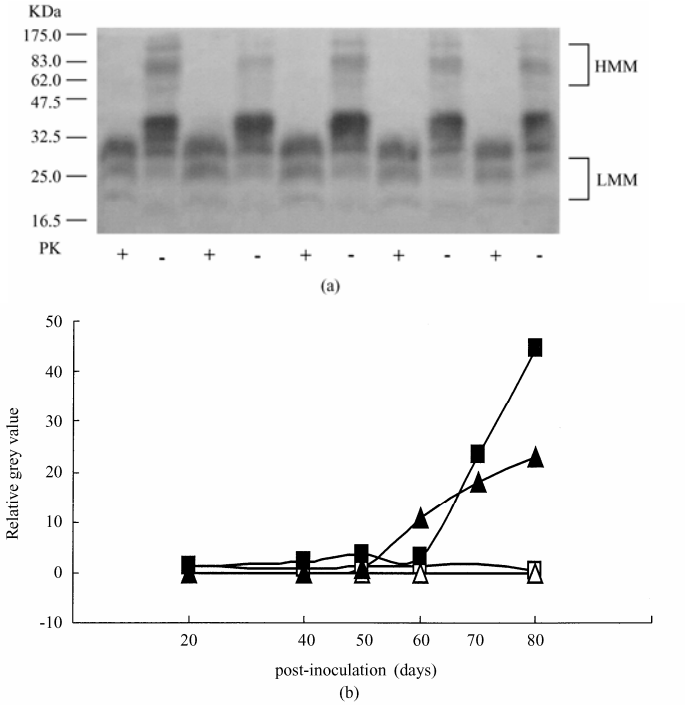


FIG. 4. Accumulation of high molecular masses (HMM) and low molecular masses (LMM) of PrP proteins during pathogenesis. (a): The HMM and LMM of PrP were marked on the chart. (b): Quantitative analysis of the HMM and LMM of PrP was carried out with a computer software described in method. ■: HMM in the inoculated hamsters, □: HMM in the normal hamsters, ▲: LMM in the inoculated hamsters,△: LMM in the normal hamsters.

LMM of PrP in Infected Animal Brains May Possess Similar Glycosylation Patterns to Those of Full-length PrP

To address whether LMM presented in the infected hamsters' brains was glycosylated as the full-length PrP^{C} and PrP^{Sc}, the brain homogenates were subsequently incubated with PNGase F, after treatment with or without PK. Interestingly, after deglycosylation two bands were recognized by antibody 3F4 in the preparation treated without PK, which mobilized at the same positions as the aglycosyl PrP^{C} (band A) and the PK-digested aglycosyl PrP^{Sc} (band B), respectively (Fig. 5a, lane 2). However, in the preparation previously treated with PK, band A no longer existed, whereas band B seemed not to be affected by PK digestion (nominated as band B*) and even became much thicker (Fig. 5a, lane 4). Quantitative evaluation of bands A, B and B* showed that the amount of band B* was equal roughly to that of bands A and B (data not shown). Analyses of brain homogenates of normal control hamsters after having been treated with PNGase F showed only band A at the aglycosal position (Fig. 5b). However, no molecular mass similar to band B was observed. Furthermore, all bands disappeared after treatment with PK (Fig. 5b). These results

suggested that LMM of PrP might be specific to the disease, and possessed the similar glycosylation patterns to those of the full-length PrP. The hydrolyzed site of truncated PrP in cells seemed to be also similar to that of PK *in vitro*.

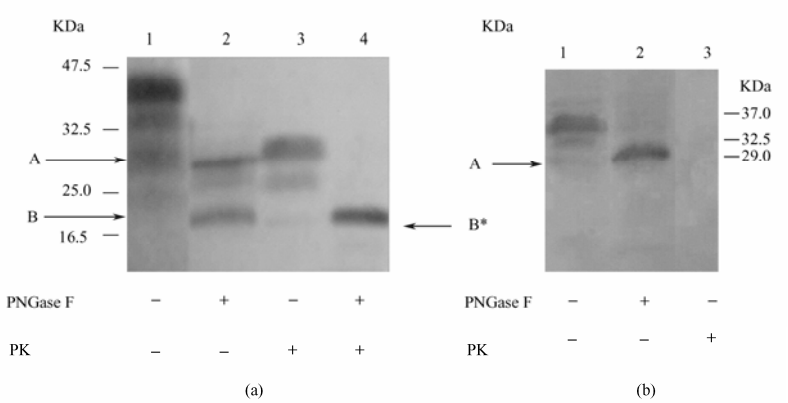


FIG. 5. Similar glycosylation patterns as the full-length PrP. in shorter fragments of PrP of infected animal brains. (a): The glycosylation patterns of PrP in the infected animal brains. Molecular mass markers are indicated to the left. (b): The glycosylation patterns of PrP in the normal brains. Molecular mass markers are shown to the right. Large and small fragments of PrP generated by PNGase F were named as bands A and B, respectively. The fragment of PrP^{Sc} generated by PNGase F and PK is indicated as band B*. PK represents Proteinase K.

DISCUSSION

When prions were transmitted from one animal species to another, a significant species barrier exists in interspecies transmissions. In other words, inefficiency in these processes was manifested by a lack of clinical symptoms or prolonged incubation periods in the recipient species[24,25]. For instance, once scrapie was transmitted to mice, the incubation periods were longer than subsequent mice to mice passages. However, following serial passages, the incubation time became shorter, and stable pathological properties were acquired[26]. In our study, scrapie strain 263K was the second passage from hamsters to hamsters in our laboratory. The incubation period in the first passage was 81-110 days[27], while in this experiment it became much shorter (45-72 days). A kinetic numerical model proposed that generally a strain may be stabilized after three passages[28].

The pathogenesis of TSEs is extremely long, usually for years and even for decades. During the incubation period the TSEs agents may replicate and accumulate in central nerve tissues till the disease occurs. The presence of PK-resistant PrP and its polymerized form SAF in brain tissues is a diagnostic marker for TSEs. Several studies confirmed that PrP^{Sc} and SAF might appear in brain tissues before the onset of clinical manifestations[7, 8,11]. In this work, both PrP^{Sc} in Western blot and SAF in EM were detectable in the symptom-free animals in 40 days post-inoculation. PrP^{Sc} in IHC was even much earlier, which was roughly 30 days earlier than the onset of the disease. In our other tests, PrP^{Sc} and SAF emerged about in 150 days and 200 days before disease onset, when the mice-adapted scrapie strain 139A[29] (incubation time 385-405 days) and strain ME7[30](incubation time 455-550 days) were inoculated into hamsters. Earlier detectable infectious agents in brain tissues within the prolonged incubation period could serve as a useful diagnostic signal.

In this study, Western blot analysis revealed that there was a dynamic accumulation of

PrP^{Sc} in brains of hamsters infected with 263K-scrapie strain during the incubation period. Moreover, it seemed that the more severe the disease was the more PrP^{Sc} could be detected. This process was concordant with nucleated polymerization hypothesis on a model of prion propagation, suggesting that the inocula from brain homogenate of the hamster infected with scrapie strain 263K might likely seed the formation of PrP^{Sc}, and the amount of PrP^{Sc} which needed to be accumulated to a certain degree caused the onset of scrapie disease. It has been proposed that the prion protein lie in equilibrium between a dominant native state, PrP^{C}, and a stable minor oligomeric structure, PrP^{Sc} produced by self-assemble in an ordered manner. Once a stable "seed" structure was formed for some reasons, it could thereby recruit PrP^{C} to form more aggregation of PrP^{Sc}, thus leading to an autocatalytic irreversible cascade[31,32]. On the other hand, slowly escalating PrP^{Sc} in central nerve tissues along with disease progression could predict that infectious factors to a certain extent might be a threshold for disease onset, although they might vary dramatically among the TSEs strains. A symptom-linked accumulation of PrP^{Sc} might indicate that a dose-dependant phenomenon could control the clinical features to a certain extent. How the accumulated PrP^{Sc} causes the disease remains unclear. Nevertheless, several possible mechanisms have been proposed, including the direct neurotoxic effects on brain cells due to the aggregation of PrP^{Sc} in which prion protein encompassing residues 106-126 might affect the normal cellular metabolism[33], and the defect of PrP^{C} which has been proposed to have a function as an antioxidant molecule might result in an increased oxidative stress in neurons[34,35] and the disturbance of normal cellular levels of PrP^{C} during infection process might promot apoptosis[36,37]. The earlier accumulation process of PrP^{Sc} before the appearance of clinical symptoms in animal bioassays would help us to have a better understanding of TSEs pathogenesis.

The amount of large-molecular bands (HMM) in Western blot assays increased with prolonged incubation time. Polymer forms of PrP might be present during disease progression. Although the large-molecular PrP seemed to be sensitive to protease, it was still hard to exclude the possibility of PrP^{Sc} in the polymers. It lacks direct evidence to describe either the chemical bonds between PrP molecules, or the potential domain of PrP itself involved in molecule interaction during the PrP aggregation. Virtuelly all the reactions digested with PK revealed low-molecular PK-resistant bands ranging usually from 19-27 KDa in Western blot tests. One might hypothesize that proteinase K would have destroyed the PrP polymer, even PrP^{Sc} polymer, leading to release of the monomer of PrP^{Sc}.

The presence of short fragments of PrP (LMM) was correlated well with the progression of illness, indicating that it might be used as an additional diagnostic marker. The evidences of deglycosylation we proposed in this study highly indicated that two molecular patterns of PrP^{Sc}, the full-length and the truncated PrP^{Sc} were present in the brain tissues of the animals infected with scrapie, both sharing the similar glycosylation patterns. The presence of these truncated PrP^{Sc} glycoforms implied that they underwent normal glycosylation secreting process. The aberrant production of PrP^{Sc} might have two possible explanations. One explanation is that PrP^{Sc} was produced during transferring process of PrP^{Sc} to the cell surface or during endocytosed recycle, and then it produced and retained a series of truncated forms within endosomes, possibly due to a more slow degradation of PrP^{Sc} than that of PrP^{C}[38]. Recently, an experiment indicated that impairing the plasma membrane recycling of PrP^{C} and stimulating the retrograde transport of the molecules towards to the endoplasmic reticulum increased the formation of PrP^{Sc} conformation in prion-infected neuroblastoma N2a cells[39]. The other explanation is that the nascent PrP^{Sc} vice versa destroyed or interfered with the normal metabolism of PrP^{C}, damaged the transport process of PrP^{C} to the cell membrane or the endocytosed recycle. As a result, in some acid endocytic

compartments a few of truncated PrP^{C} forms were produced, and then these molecules converted corresponding PrP^{Sc} forms. A recent study indicated that the PrP^{C} with deleted N-terminus prolonged the half-life time significantly, delayed the transit from the secreting compartment to the cell membrane[40]. Acid condition could facilitate the formation of PrP^{Sc}-like isoforms, and moreover, trace PrP^{Sc} could largely enhance the production of such molecules[41]. The truncated aglycosyl PrP^{Sc} formed *in vivo* and the aglycosyl PrP^{Sc} generated by PK treatment *in vitro* could mobilize at the same position in SDS-PAGE, indicating a similar proteolytic region for PrP^{Sc}. Although the explicit hydrolytic site(s) of the truncated PrP^{Sc} *in vivo* remain to be verified, it might somehow explain various N-terminal PrP fragments in the TSE infected brains[16], if the endogenetic proteolytic site(s) of the truncated PrP^{Sc} is slightly different from that of PK digestion.

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