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Original Article

Infection Route-Independent Accumulation of Splenic Abnormal Prion Protein

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SUMMARY: The accumulation kinetics of the abnormal form of prion protein (PrP^{Sc}) in spleens and brains of scrapie (Obihiro-1)-infected mice at various times after intracerebral (i.c.), intraperitoneal (i.p.), or oral inoculation were studied. PrP^{Sc} was first detected by Western blotting with anti-prion protein antibodies on days 70 and 116 after i.c. (3 μg) in spleens and brains, respectively. Although the amount of cerebral PrP^{Sc} gradually increased to the maximum level on day 152 after i.c. inoculation, splenic PrP^{Sc} established the maximum level on day 116 after i.c. inoculation then registered slight decreases up to day 152 with further incubation. The detectable levels of cerebral PrP^{Sc} by Western blotting were established on day 231 or 259, whereas those of splenic PrP^{Sc} were detected on day 94 or 93, after i.p. and oral infection, respectively. The splenic PrP^{Sc} decreased slightly thereafter. These results indicate that splenic PrP^{Sc} increased before cerebral PrP^{Sc} established a detectable level in a manner independent of the inoculation route.

INTRODUCTION

Prion diseases are neurodegenerative disorders characterized by the accumulation of abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system (CNS) (1). PrP^{Sc} isoform differs from its normal cellular homologue (PrP^{C}) (2) in that it possesses increased beta-sheet conformation (3,4), which is partially protease-resistant and may be associated with amyloid deposition. According to the protein-only hypothesis (1), the essential infectious principle of prion diseases is that PrP^{Sc} is replicated by autocatalytic conversion of PrP^{C} into PrP^{Sc}. The conversion of PrP^{C} into PrP^{Sc} also leads to PrP^{C} deficiency (5). PrP^{C}-deficient cells are susceptible to oxidative stress (6,7) and show abnormal copper metabolism (8). Therefore, both PrP^{Sc} accumulation and PrP^{C} deficiency are thought to be crucial events in the neurodegeneration of prion diseases.

PrP^{Sc}, a reliable indicator of infectivity in most experimental models, is also used as a marker for clinical diagnosis of bovine spongiform encephalopathy (BSE) in ruminants and of other prion diseases such as Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) in humans. Several studies have confirmed that the amount of PrP^{Sc} is proportional to the prion titer (9,10). Preclinical diagnosis is also very important to prevent and arrest the spread of prion diseases, considering the recent outbreak of BSE in European countries. It is even more critical when considering the hazard caused by blood transfusions and transplants from the carriers of prion diseases. Immunostaining of PrP^{Sc} on brain sections and immunoblot analysis of PrP^{Sc} after electrophoresis of brain homogenates are the most reliable and accurate diagnosis methods for prion diseases, as PrP^{Sc} is mainly accumulated in CNS. Although suitable for postmortem diagnosis, these methods are not necessarily appropriate for preclinical diagnosis because brain biopsy samples are impractical to obtain from live animals and humans.

It has been reported that spleens of affected animals serve as a potential source of infection although their infectivity is lower than that of the brain (11-13). Several studies have reported prion infectivity of various organs and tissues of affected animals (14-16), and suggested that spleens acquire infectivity earlier than brains. These facts suggest that spleens are more applicable for preclinical diagnosis of prion diseases than brains. However, it is not clear whether this tendency is inoculation route-dependent or not; parallel studies of PrP^{Sc} accumulation kinetics via various inoculation routes have not been attempted to date. In this study, we studied the route dependencies of kinetics of PrP^{Sc} accumulation in brains and spleens of scrapie (Obihiro-1)-infected mice via intracerebral, intraperitoneal, and oral inoculation.

MATERIALS AND METHODS

Preparation of inoculum: Membrane fractions prepared from the brains of terminally diseased mice with mouse-adapted scrapie (Obihiro-1 strain) were used as an inoculum throughout this study. In brief, the brain (approximately 400 mg) of the affected mouse was homogenized (Physcotron handy micro-homogenizer NS-310E; Niti-On Co., Chiba, Japan) in 4.5 ml of ice-cold PBS before cell debris was removed by centrifugation at 1,500×g for 15 min. From the post-nuclear fraction, membrane proteins were precipitated by centrifugation at 200,000×g at 4°C for 60 min (Optima™ Ultracentrifuge; Beckman Instruments, Fullerton, Calif., USA) using a TL-100 angle rotor. The resulting precipitate

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was carefully resuspended in 1 ml of PBS with a 26-G needle attached to a syringe and stored at –80°C until use.

Antibodies: Antibody 6H4 (Prionics, Zurich, Switzerland) (17) or P8 (18), used to detect prion protein (PrP) with Western blotting, was diluted at a rate of 1:5,000. Goat anti-mouse IgG-horseradish peroxidase(HRP) (Bio-Rad, Richmond, Calif., USA) and goat anti-rabbit IgG-HRP (Bio-Rad) were used as secondary antibodies for 6H4 and P8 at dilution rates of 1:3,000 and 1:5,000, respectively.

PrP^{Sc} inoculation: PrP^{Sc} was intracerebrally (i.c.), intraperitoneally (i.p.), or orally inoculated into female ICR mice at 4 weeks of age. Inoculation (i.c.) was conducted in the biohazard P3 facilities as follows. PrP^{Sc} inoculum was suspended at a total protein concentration of 3 μg in 30-μl physiological saline. Inocula were injected into the cerebral ventricular system of mice using a microsyringe. For i.p. inoculation, 3 μg of inoculum in 100 μl of physiological saline was injected into the peritoneal cavity of mice using a microsyringe. For oral infection, 3 μg of inoculum in 50 μl of physiological saline was directly introduced into the stomach using a disposal stomach tube. Three mice from each inoculation route were used at the indicated period for examination.

Determination of protein concentrations: Protein concentrations were estimated by the bicinchinonic acid method (Pierce Chemical, Rockford, Ill., USA.) following the manufacturer's instructions.

Proteinase K digestion: To discriminate PrP^{Sc} from PrP^{C}, PrP^{C} in the membrane fraction with a protein concentration at 300 μg/ml was digested with 3 μg/ml proteinase K (PK) at 37°C for 1 h in 100 μl of 50 mM Tris-HCl buffer (pH 8.8) containing 100 mM NaCl and 0.2% *N*-Lauroylsarcosine (Sigma, St. Louis, Mo., USA). In order to terminate the reaction and to precipitate PK-resistant proteins such as PrP^{Sc}, 5 volumes of methanol containing phenylmethylsulfonyl fluoride (PMSF) at 1 mg/ml was added to the reaction mixture. After 60-min incubation at 20°C, the precipitate was collected by centrifugation at 16,000 × g at 4°C for 20 min before analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

SDS-PAGE: Protein samples were dissolved in 20 μl SDS-PAGE sample buffer (70 mM SDS, 10% [w/v] glycerol, 1% 2-mercaptoethanol, 15 mM bromophenol blue, 62.5 mM Tris-HCl, pH 6.8) and incubated at 100°C for 10 min before electrophoresis in 15% polyacrylamide gel (8 cm × 8 cm × 1 mm) containing 0.1% SDS at a constant current of 30 mA for 1 h. After electrophoresis, the proteins in the gel were electrically transferred onto a polyvinylidene difluoride (PVDF) membrane (see below).

Western blotting: Proteins separated by SDS-PAGE were electrically transferred onto PVDF membranes (ProBlot™, Applied Biosystems, Foster City, Calif., USA), which were pretreated with methanol (5 min) and transfer buffer (48 mM Tris, 39 mM glycine, 20% [v/v] methanol and 1.3 mM SDS) for 2 min at a constant voltage of 12 V (ca. 100 mA) for 1 h. After the transfer, the membrane was blocked with defatted milk (Block Ace, Dainippon Pharmaceutical, Osaka, Japan) for 1 h at room temperature (RT) with gentle shaking before incubation with a primary antibody diluted with PBS containing 25% (v/v) Block Ace and 0.05% (v/v) Triton X-100. After 1-h agitation at RT, the membrane was washed thrice with PBS containing 1% (v/v) Tween 20 (PBS-T) for 5 min. The membrane was then incubated with secondary antibody for 1 h before being thoroughly washed with PBS-T. PrP was detected using an ECL™ kit (Amersham Biosciences, Piscataway, N.J., USA) as described in the kit instructions. The electrophoreogram was recorded on RX-U Medical X-ray film (Fuji Photo Film, Kanagawa, Japan). PrP^{Sc} levels were estimated as follows. Each PrP^{Sc} band intensity was measured using an NIH image in Western blotting, and the PrP^{Sc} level of each tissue was estimated as a percentage (compared with the band intensity of cerebral PrP^{Sc} of the terminally affected mouse) as described previously (19).

RESULTS

PrP^{Sc} can be detected and distinguished from PrP^{C} by Western blotting after treatment with PK (Fig. 1). As a primary antibody for cerebral PrP, 6H4 (Fig. 1; lanes 1-4), an EU committee recommended anti-PrP mouse monoclonal antibody, was used. For splenic PrP, P8 (Fig. 1; lanes 5-8), a newly produced anti-PrP rabbit polyclonal antibody, was used to avoid possible cross-reaction of secondary antibody with endogenous mouse immunoglobulins, which are abundant in spleens. We have confirmed that P8 could detect PrP with a high specificity, as high as that of 6H4. Without digestion, cerebral PrP was manifested as three separate bands (ca. 34, 31, 28 kDa) by Western blotting with 6H4 (Fig. 1; lanes 1 and 3) or P8 (Fig. 1; lanes 5 and 7). The bands corresponded to PrP as di-, mono-, and non-glycosylation, respectively. Without PK treatment, PrP^{Sc} was indistinguishable from PrP^{C}. Upon digestion with PK, the bands from uninfected mice disappeared (Fig. 1; lanes 2 and 6), whereas three bands in infected mice remained detectable, albeit they were shifted approximately 4 kDa lower (ca. 30, 27, 24 kDa) due to partial degradation of PrP^{Sc} (Fig. 1; lanes 4 and 8). Thus, PrP^{C} was completely digested by PK, whereas PrP^{Sc} was only partially degraded by PK. It should be noted that P8 was as sensitive as 6H4 in detecting PrP^{Sc}. Because PrP^{Sc} was selectively detected, the above method appears to provide the most reliable and accurate diagnosis for prion diseases. At the indicated times after inoculation, PrP^{Sc} was detected in membrane fractions of the brains and spleens.

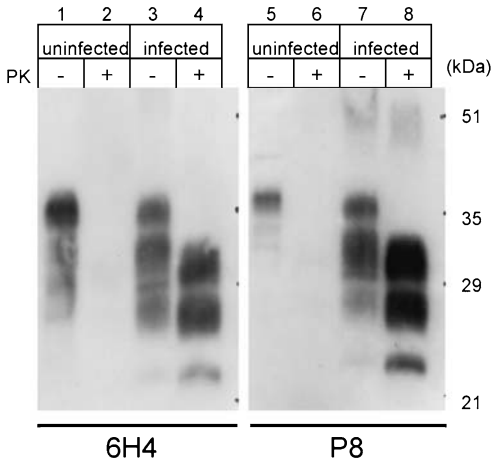

Fig. 1. Detection of PrP^{Sc} in brain of terminally diseased mice. PrP from brain membrane fractions (30 μg protein per lane) of non-infected mice (lanes 1, 2, 5, 6) and terminally diseased mice intracerebrally inoculated with Obihiro-1 prions (lanes 3, 4, 7, 8) were detected by Western blotting with anti-PrP 6H4 (lanes 1-4) and P8 (lanes 5-8). Each fraction was treated (+) (lanes 2, 4, 6, 8) or non-treated (–) with proteinase K (PK) (lanes 1, 3, 5, 7).

By measuring PrP^{Sc} band intensity from Western blot images, the relative PrP^{Sc} levels were determined. Various dosages (3 μg, 100 ng, 3 ng, 100 pg) of inoculum showed a decrease of incubation time in a dose-dependent manner until PrP^{Sc} in brains and spleens established respectively detectable levels (data not shown). After 3-μg i.c. inoculation, cerebral PrP^{Sc} was detected in all 3 mice after 116-day incubation (Fig. 2A). Splenic PrP^{Sc} was detected earlier than cerebral PrP^{Sc}. PrP^{Sc} was detected clearly in spleens of all 3 mice after 70-day incubation (Fig. 2A). Note that splenic PrP^{Sc} gradually decreased after the initial increase (Fig. 2A). Inoculation (i.p.) with 3-μg inoculum showed that PrP^{Sc} accumulation was first detected in spleen of 1 out of 3 mice after 94-day incubation, whereas PrP^{Sc} was detected in brain of 2 out of 3 mice after 231-day incubation (Fig. 2B). Although the order of incubation time between spleens and brains for PrP^{Sc} to establish detectable levels coincided well that of i.c. inoculation, a marked difference in incubation time for detection of cerebral PrP^{Sc} was observed. In addition, oral administration with 3-μg inoculum showed that

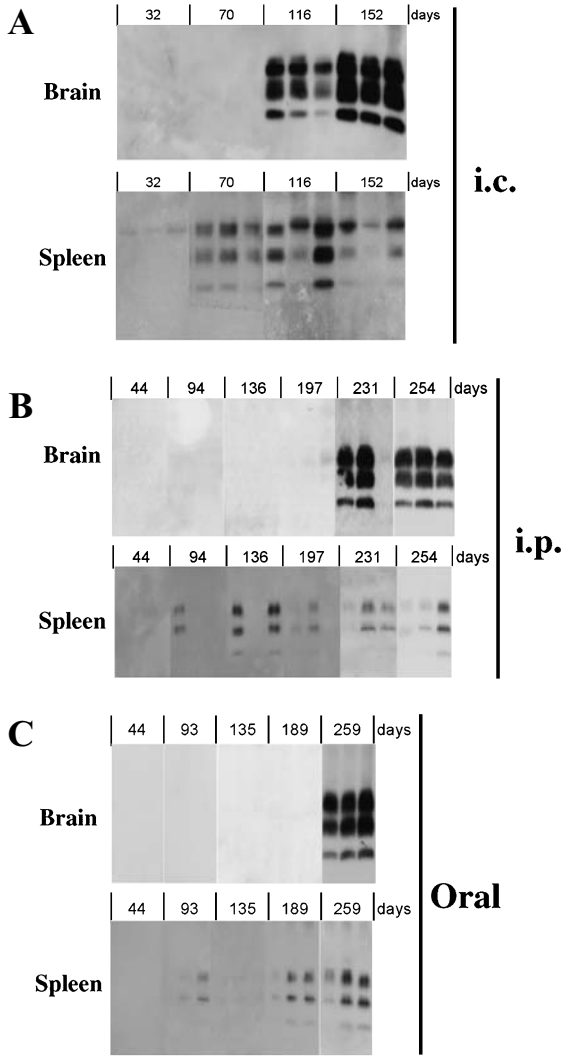

Fig. 2. Kinetics of PrP^{Sc} accumulation in brains and spleens of mice treated with intracerebral (i.c.), intraperitoneal (i.p.) and oral inoculation of Obihiro-1 prions. PrP^{Sc} in brains or spleens of mice treated with (A) i.c., (B) i.p. or (C) oral inoculation of 3-μg inoculum at the indicated times were analyzed by Western blotting with 6H4 (in brain) or P8 (in spleen).

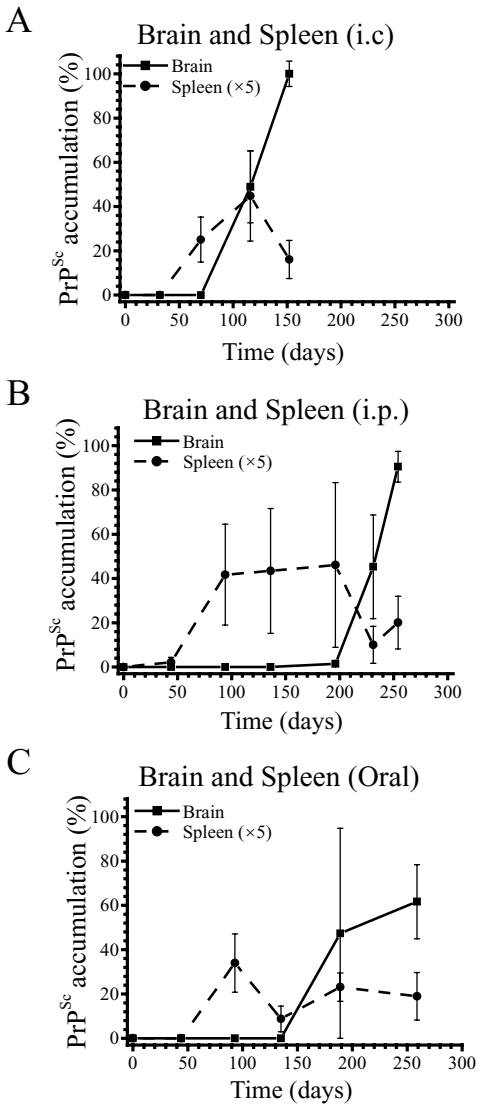

Fig. 3. Kinetics of PrP^{Sc} accumulation in brains and spleens of mice infected with Obihiro-1 prions via various inoculation routes. The relative concentrations of PrP^{Sc} in brains (solid line and circles) and spleens (dashed line and squares) of mice infected with Obihiro-1 prions (3-μg inoculum) via (A) intracerebral (i.c.), (B) intraperitoneal (i.p.), and (C) oral inoculations were estimated by densitometric measurements of the PrP^{Sc} signal at different time points. The value of PrP^{Sc} accumulation in brains of mice that had succumbed to terminal prion disease (day 152, i.c. inoculation) was taken as 100%. For convenience, amounts of splenic PrP^{Sc} are denoted as quintuples (× 5). Error bars indicate standard deviations (SD).

PrP^{Sc} accumulation could be detected in spleens of 2 out of 3 mice after 93-day incubation, whereas PrP^{Sc} was detected in brain of all 3 mice after 259-day incubation (Fig. 2C). PrP^{Sc} accumulation kinetics was monitored by densitometric analysis via inoculation routes at the same dosage (i.e., 3 μg protein of inoculum); viz., i.c., i.p. and oral inoculations (Fig. 3). After 94-day incubation, splenic PrP^{Sc} could be detected via all inoculation routes. However, in the case of cerebral PrP^{Sc}, a longer incubation time was needed for PrP^{Sc} to establish a detectable level with i.p. or oral tha with i.c. inoculation (Fig. 3). Interestingly, cerebral PrP^{Sc} gradually increased in a time-dependent manner, whereas splenic PrP^{Sc} indicated an increase followed by decreases via all inoculation routes (Fig. 3).

DISCUSSION

In this study, we investigated accumulations of splenic and cerebral PrP^{Sc} and elucidated the accumulation kinetics of PrP^{Sc} in brains of scrapie-infected mice during the development of prion disease, using Western blotting. This study design is important in the context of diagnostic and preventive medicine, where highly sensitive detection methods are employed to prevent the spread of prion diseases.

PrP^{Sc} accumulated in brains established detectable levels within 116 days, and the levels on day 152 after i.c. inoculation (3 μg) of PrP^{Sc} were comparable to those accumulated in brains of terminally affected mice. Furthermore, PrP^{Sc} accumulated in spleens of infected mice (<20% of brain sample) was detected by Western blotting on day 70 after i.c. inoculation (data not shown). In contrast to the accumulation of cerebral PrP^{Sc}, not only did the splenic PrP^{Sc} levels of infected mice not increase by further incubation of up to day 152, but showed decreases. Spleens revealed some shrinkage at the terminal stage of infection. However, this splenic-PrP^{Sc} decrease is not directly due to the shrinkage,because the PrP^{Sc}-decrease was observed much earlier than the splenic shrinkage. Upon i.p. or oral administration of 3-μg inoculum, PrP^{Sc} was also detected in spleens before PrP^{Sc} established detectable levels in brains. Cerebral PrP^{Sc} did not establish detectable levels until days 231 and 259, whereas splenic PrP^{Sc} levels were detected as early as days 94 and 93 after i.p. and oral infections, respectively. Taken together, our results show that PrP^{Sc} was detected much earlier in spleens than in brains, regardless of the inoculation route.

Immunostaining for PrP^{Sc} in mouse spleens indicates that PrP^{Sc} accumulated in follicular dendritic cells (FDCs) (20), suggesting that prion replication occurs in FDCs. The accumulation of PrP^{Sc} in brains appears to be preceded by that in spleens and FDCs (14,21). Interestingly, present findings indicate that inoculation routes did not influence the incubation periods with regard to establishing a detectable level of splenic PrP^{Sc}, although a great difference (>80 days; see Fig. 3) was observed in the case of cerebral PrP^{Sc}. Among the various infection routes, oral administration may be the most likely natural invasion route of pathogens in animals and humans. Oral inoculations required a longer incubation time for PrP^{Sc} to be detected in the brain. Furthermore, although cerebral PrP^{Sc} indicated gradual increases to establish the peak level, splenic PrP^{Sc} manifested an increase followed by slight decreases via all inoculation routes tested in this study. These results indicate that splenic PrP^{Sc} established a detectable level, independent of the inoculation route, before cerebral PrP^{Sc} did. The slight decreases of splenic PrP^{Sc} might be due to activation or targeting of splenic macrophages. Splenic macrophage is a natural host defender against scrapie agent invasion (22). Activation or targeting of these cells may thus represent a therapeutic pathway to explore in cases involving a natural route of prion infection. Activation of splenic macrophage with CpG motifs has been discussed as a therapy to delay prion disease on the basis of promising results in mouse models of scrapie (19). Above all, detection of splenic PrP^{Sc} may provide a more timely diagnosis, although the method may not be the most convenient in terms of practicality.

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