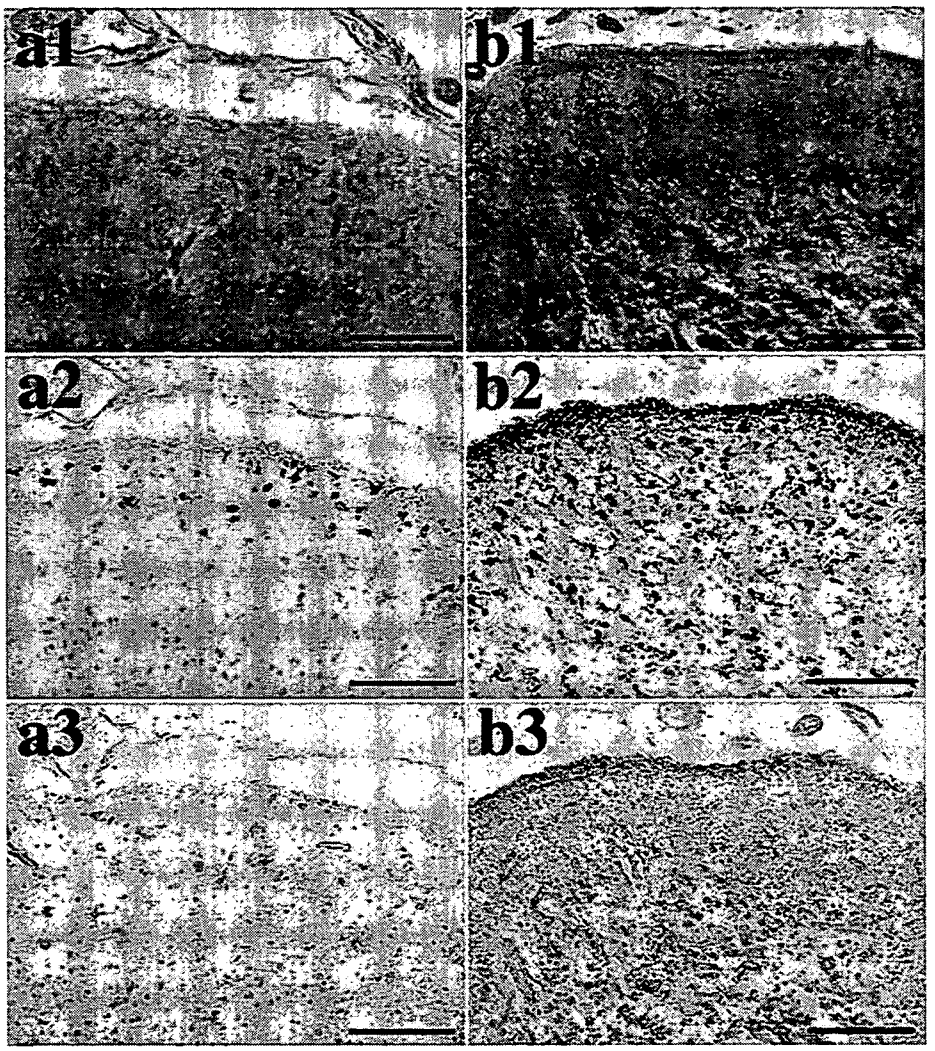

Fig. 7 Two sets of serial sections show the relation between neuropathologic findings, glial fibrillary acidic protein (GFAP) immunoreactivity and aquaporin-4 (AQP-4) immunoreactivity. (a1) In subacute spongiform encephalopathy (SSE), the H&E-stained section shows gliosis and fine vacuole-type spongiform change. (a2) GFAP immunoreactivity is present in the subpial gray matter but less so in the deep cerebral neocortex. (a3) AQP-4 immunoreactivity is widespread in the cerebal neocortex. (b1) In panencephalopathic (PE)-type sporadic Creutzfeldt-Jakob disease (sCJD), the H&E-stained section shows severe gliosis, neuronal loss, tissue rarefaction and status spongiosus. (b2) Widespread GFAP immunoreactivity is present in the subpial gray matter and cerebral neocortex. (b3) Strong AQP-4 immunoreactivity is observed in the cerebal neocortex. The patterns of immunoreactivity of AQP-4 and GFAP differ. Analysis of serial sections showed that spongiform change and gliosis in sCJD appeared at an earlier stage than did strong AQP-4 immunoreactivity. Neuronal loss and tissue rarefaction in sCJD appeared at a later stage than did strong AQP-4 immunoreactivity. Strong AQP-4 immunoreactivity appeared at an earlier stage than did hypertrophic astrocytosis or GFAP-positive astrocytosis. (H&E, hematoxylin and eosin). Bars, 200 m. a, patient 1 (SSE case); b, patient 9 (PE-type sCJD case). a1 and b1, H&E stain; a2 and b2, GFAP immunostaining; a3 and b3, AQP-4 immunostaining.

tions, the development of sCJD pathology, particularly of neocortical lesions, occurs as follows: PrP deposition, spongiform change and gliosis, increased AQP-4 staining, hypertrophic astrocytosis, and neuronal loss and tissue rarefaction. Interestingly, increased AQP-4 staining remained in burnt-out lesions, such as those of status spongiosus.

Determining the relation between clinical data and AQP-4 immunoreactivity was an objective of the present study. We suggest a relation between AQP-4 immunoreactivity and MRI findings, particularly in diffusion-weighted images. AQP-4 immunoreactivity was intensely positive in sCJD cerebral neocortex, which usually shows high signal intensity in diffusion-weighted images in sCJD.[23] Because this high signal intensity may result from variable pathologic factors, including spongiform change, astrocytosis, microglial activation and PrP deposition,[23] evaluation of the relation between MRI findings and AQP-4 immunoreactivity may be difficult. However, we believe that increased AQP-4 expression, particularly in the cerebral neocortex, may be involved in the high signal intensity of diffusion-weighted images in sCJD.

Western blot analysis of protease-resistant PrP showed type 1 PrP in all cases. Because most of our cases were classified as type MM1, with single cases of type MV1 and no cases of type MM2, MV2, VV1 or VV2, assessment of the influence of PRNP polymorphisms at codon 129 and PrP type relative to AQP-4 immunoreactivity in the present study cannot be made. Further studies of PRNP polymorphisms, PrP type and AQP-4 immunoreactivity in sCJD are necessary.

In conclusion, widespread AQP-4 immunoreactivity, particularly in the cerebral neocortex and cerebellar cortex in sCJD, was shown in the present study. Although this may be a secondary change of sCJD pathology associated with intense astrocytosis, it may reflect important aspects of astrocytic pathology associated with sCJD.

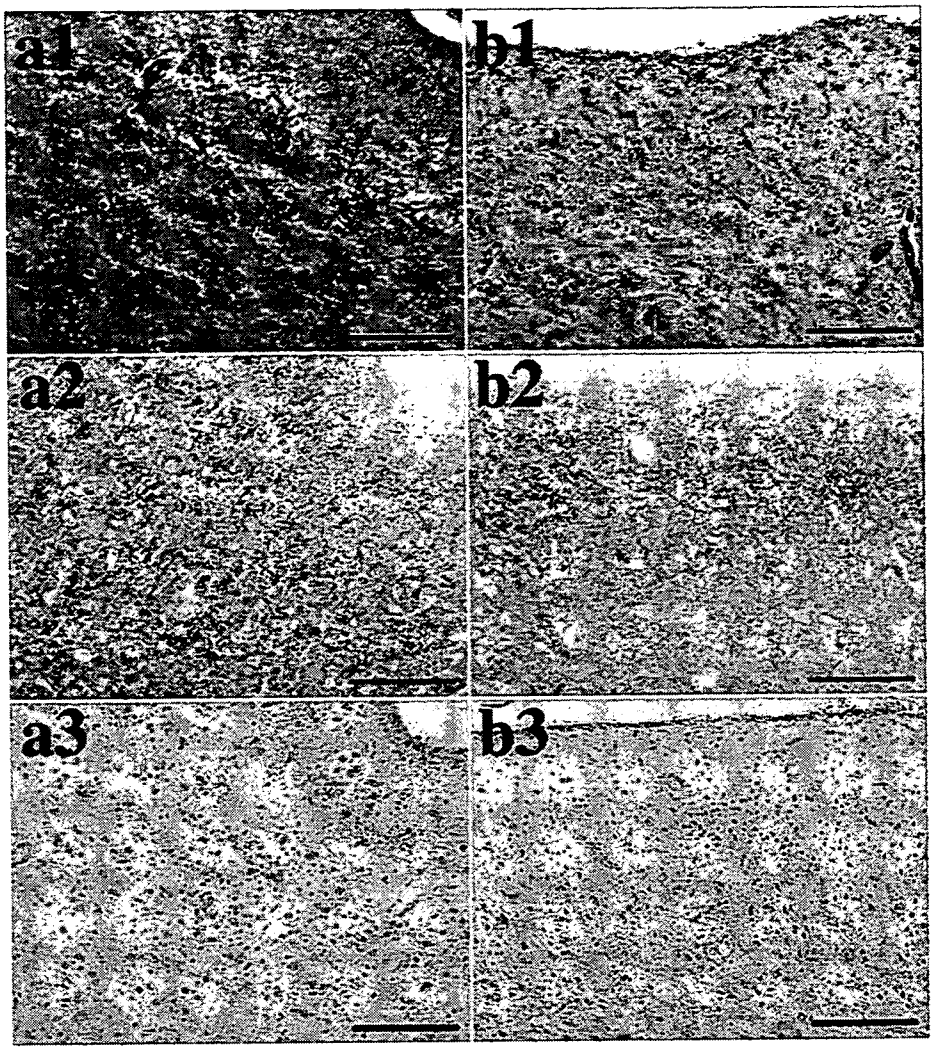

Fig. 8 Two sets of serial sections show the relation between neuropathologic findings, prion protein (PrP) deposition and aquaporin-4 (AQP-4) immunoreactivity. (a1) In subacute spongiform encephalopathy (SSE), the H&E-stained section shows hypertrophic astrocytosis; neuronal loss is not apparent. (a2) Widespread granular PrP immunoreactivity is present in the cerebral neocortex. (a3) Widespread AQP-4 immunoreactivity is present in the cerebral neocortex. (b1) In panencephalopathic (PE)-type sporadic Creutzfeldt-Jakob disease (sCJD), the H&E-stained section shows pronounced hypertrophic astrocytosis, neuronal loss and status spongiosus. (b2) PrP immunoreactivity is relatively decreased in the cerebral neocortex compared to that in SSE. (b3) Strong AQP-4 immunoreactivity is present in the cerebral neocortex. The patterns of PrP deposition and immunoreactivity of AQP-4 differ. PrP deposition appeared at an earlier stage than did spongiform change or gliosis and at an earlier stage than did strong AQP-4 immunoreactivity. Bars, 200 m. a, patient 6 (SSE case); b, patient 8 (PE-type sCJD case). a1 and b1, H&E stain; a2 and b2, PrP immunostaining; a3 and b3, AQP-4 immunostaining.

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Note

Assessment of Prion Inactivation by Combined Use of *Bacillus*-Derived Protease and SDS

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Prions, infectious agents causing transmissible spongiform encephalopathy, retain infectivity even after undergoing routine sterilization processes. We found that MSK103 protease, identified in our previous study, effectively reduces infectivity and the level of misfolded isoform of the prion protein in scrapie-infected brain homogenates in the presence of SDS. The treatment therefore can be applied to the decontamination of thermolabile instruments.

Key words: prion inactivation; protease; scrapie; bioassay; protein misfolding cyclic amplification

Prions are the infectious agents of transmissible spongiform encephalopathies (TSEs), including bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, and Creutzfeldt-Jakob disease (CJD) in humans.[1)] TSE diseases are characterized by accumulation in the central nervous system of PrP^{Sc}, a proteinase K (PK)-resistant misfolded isoform of the cellular prion protein PrP^{C}.[2)] Since prions are highly resistant to conventional sterilization procedures, severe physical and chemical sterilization methods, such as high-pressure sterilization at 134 °C for 18 min or strong alkali treatment, are required to reduce infectivity.[3)]

The development of a secure, simple, and effective disinfection method is required as a preventive measure against prion diseases. We recently identified a *Bacillus* strain that produced protease exhibiting high-degradation activity against scrapie- and BSE-derived PrP^{Sc}.[4)] The isolated protease (MSK103) was found to degrade dried PrP^{Sc} samples more effectively than proteinase K (PK) or a keratinase.[5)] Since this protease can be used under moderate conditions (optimum pH and temperature ranges were 9–10 and 60–70 °C), it can be used to decontaminate fragile, precision, and expensive instruments that are susceptible to PrP^{Sc} contamination.

In the present study, we investigated the efficacy of the MSK103 protease in scrapie prion inactivation. Since protease activity is generally enhanced in the presence of SDS,[6–8)] we examined the additive effects obtained by the combined use of the protease and SDS. The hamster-adapted scrapie strain Sc237 was propagated in the Syrian golden hamster. The brains of hamsters at the terminal stage of the disease, titrating $5 \times 10^{8.5}$ LD_{50} per gram by bioassay, were pooled and homogenized at a concentration of 10% (w/v) in 50 mmol Tris–HCl buffer (pH 9.0). The homogenates were digested with MSK103 protease at a final concentration of 0–8 units (U)/ml at 50 °C for 20 h in the presence of 2% SDS. In a preliminary experiment, PrP^{Sc} samples containing various concentrations of SDS were digested and analyzed by western blot (WB). The addition of 2% SDS to the reaction was most effective for the degradation of PrP^{Sc}. Effective PrP^{Sc} decontamination was observed at a protease concentration of 2–3.8 U/ml against BSE-infected brain homogenate and dried Sc237 PrP^{Sc}, which had firmly attached to a plastic surface in our previous study.[4)] One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glycine per min in a 0.5% solution of human hair-derived keratin powder at 60 °C for 1 h at pH 8.0.

The digested samples were centrifuged at 200,000 *g* for 30 min and a supernatant was carefully removed. No PrP^{Sc} signals were detected in the supernatant by WB analysis. After they were washed three times, the precipitates were resuspended in an initial volume of

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Abbreviations: TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; PrP^{Sc}, misfolded isoform of prion protein; PrP^{C}, cellular prion protein; PK, proteinase K; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; AP, alkaline phosphatase; HRP, horseradish peroxidase; WB, western blot; PMCA, protein misfolding cyclic amplification; PrP^{res}, proteinase-resistant prion protein; ANOVA, analysis of variance

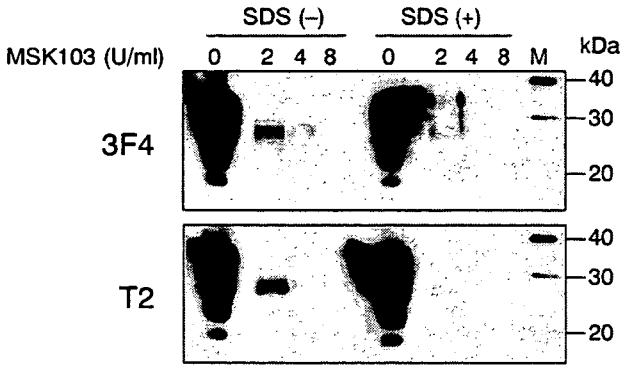

Fig. 1. Western Blot Detection of PrP^{Sc} in Sc237-Infected Hamster Brain Homogenate with the MSK103 Enzyme.

A 10% brain homogenate and 0–16 U/ml of the protease solution were mixed in equal quantities, and the mixture was then incubated at 50 °C for 20 h in the absence or presence of 2% of SDS. The positions of molecular weight standards (Magic Mark XP, Invitrogen) are indicated on the figure (the lane labeled "M").

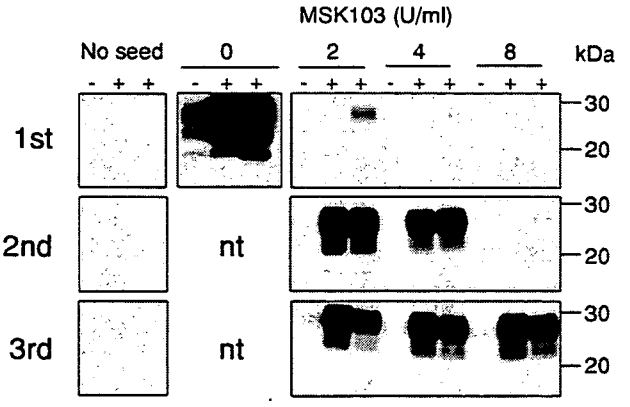

Fig. 2. Protein Misfolding Cyclic Amplification of the Samples Treated with MSK103 Protease.

The samples were analyzed before (−) and after (+, in duplicate) each round of amplification by western blotting following digestion with proteinase K (50 μg/ml). The lanes labeled 'No seed' were the controls, in which PrP^{C} source only was treated in the same manner. No signals were detected in these samples. The positions of the molecular markers corresponding to 30 and 20 kDa, are shown in the figure. nt, not tested.

phosphate-buffered saline (PBS). The samples were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidine fluoride membrane (Millipore, Bedford, MA). After blocking, the membranes were incubated for 1 h with alkaline phosphatase (AP)-conjugated 3F4 antibody (Signet Laboratories, Dedham, MA; 1/10,000) or horseradish peroxidase (HRP)-conjugated T2 antibody (1/10,000).[9] After washing, the blotted membranes were developed with Immobilon western chemiluminescent AP or HRP substrates (Millipore). Chemiluminescence signals were analyzed using a Light Capture system (Atto, Tokyo).

Figure 1 illustrates the results of WB analysis of the digested samples in the absence or presence of SDS. Residual PrP^{Sc} was detected with 3F4 or T2 monoclonal antibodies, which recognize different epitopes of the PrP^{Sc} core fragments. As expected, residual PrP^{Sc} was significantly reduced in the presence of SDS. Although the PrP^{Sc} signal was detected in the sample digested with 2 U/ml of the protease, even in the presence of SDS there were no detectable signals in the samples treated with either 4 or 8 U/ml of the protease. When concentrated to a higher level (16 U/ml), a faint signal of residual PrP^{Sc} was detected by WB analysis, suggesting that protease activity somewhat, decreased probably due to self-digestion of the protease. Hence, the samples treated with a combination of the protease (2–8 U/ml) and SDS were analyzed in further experiments.

In addition to conventional WB analysis, protein misfolding cyclic amplification (PMCA), a highly sensitive method of detecting minute amounts of PrP^{Sc}, was performed as in our previous study.[10] Briefly, the digested sample was diluted 1:10 in 10% uninfected brain homogenate, and one round of the PMCA reaction was performed by applying 40 cycles of sonication followed by incubation at 37 °C for 1 h. Next, a process diluting the PMCA product to 1:10 and its subsequent amplification was repeated twice. With regard to the PMCA products, samples (10 μl) collected before and after each round of amplification were mixed with 10 μl PK solution (100 μg/ml) and incubated at 37 °C for 1 h. The digested materials were analyzed by WB using the 3F4 antibody, as described above.

Figure 2 illustrates the results of amplification of the samples treated with MSK103 protease. In the first round of amplification, weak PK-resistant PrP (PrP^{res}) signals were detected in the sample treated with 4 U/ml of the protease in addition to the sample treated with 2 U/ml of the protease. In the second round of amplification, the signals of both these samples were enhanced, and a weak signal was observed in the sample treated with 8 U/ml of the protease. After three rounds of amplification, PrP^{res} signals were detected in all the digested samples. These results indicate that a trace level of PrP^{Sc}, which was not detected by conventional WB, remained in the samples treated with 4 or 8 U/ml of the protease.

The digested samples were inoculated intracerebrally (20 μl per mouse) into five Tg52NSE transgenic mice[11] that overexpressed hamster PrP^{C} in their nerve systems. The advantage of this bioassay is its ability to confirm the onset of the disease a short period of time after inoculation. The control mice inoculated with the untreated samples developed the disease after an average period of 45 ± 2 d (average ± SD, Fig. 3). The mice that were inoculated with the sample treated with SDS alone died after an average period of 65 ± 4 d. The onset time of the disease in mice inoculated with the sample treated with 2 U/ml of the protease was significantly delayed, but the mice died after an average period of 206 ± 128 d. On the other hand, two out of the five mice that were inoculated with samples treated with either 4 or 8 U/ml of the protease survived for more than

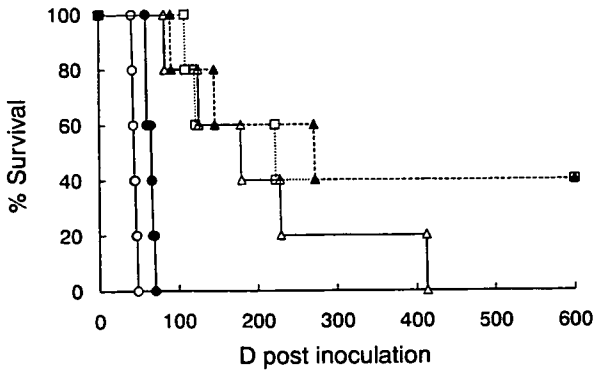

Fig. 3. Survival Curves of Tg52NSE Mice Inoculated with Infected Brain Homogenates Treated with MSK103 Protease.

The control and digested samples were injected intracerebrally into five mice. No treatment (solid line, ○), SDS alone (solid line, ●), 2 U/ml (solid line, △), 4 U/ml (broken line, ▲) and 8 U/ml (dotted line, □).

600 d, although the remaining mice developed the disease after an average period of 170 ± 73 d (4 U/ml) and 151 ± 62 d (8 U/ml). The survival times of the affected mice inoculated with the samples treated with the protease were statistically analyzed by one-way ANOVA. There were no significant differences among the survival times of the three experimental groups.

Although several approaches to prion inactivation by enzymatic degradation have been reported, the infectivity of the degradation products was not examined in some studies.[5,12,13] Where bioassay was performed, only a modest reduction in infectivity (approximately 10^{-3} reduction) present in a low concentration (1%) of infected brain homogenate was achieved following protease digestion.[14] In the present study, we found that combined treatment with MSK103 protease and SDS was effective in reducing the infectivity present in the high concentration (10%) of infected brain homogenate. This led to 40% survival of the inoculated Tg52NSE mice for more than 600 d when the protease treatment was performed under enzyme concentrations of 4 or 8 U/ml at 50 °C for 20 h. The reduction in infectivity was estimated to be less than 10^{-6} on the basis of the incubation time obtained from our previous study.[10] Since a prolonged incubation time (equivalent to a 10^{-3} reduction in infectivity) was also observed in the mice inoculated with the samples treated with SDS alone, the reduction in the amount of infectivity due to the protease was estimated to be less than 10^{-3}.

Since PrP^{Sc} tends to aggregate, the size of the PrP^{Sc} aggregate determines the efficiency of the enzymatic degradation of PrP^{Sc}. If the PrP^{Sc} aggregate is very large, it is possible that core PrP^{Sc} surrounded by many outside PrP^{Sc} remains undigested. This is a serious problem in prion decontamination by enzymatic degradation. The variation in survival times observed in mice inoculated with samples digested with 4 or 8 U/ml of the protease may be due to the fact that core PrP^{Sc} which remained undigested is inhomogeneous in the inoculated samples.

In conclusion, the present study indicates that the combined use of MSK103 and SDS caused effective degradation of PrP^{Sc}, resulting in a significant reduction in infectivity. Efficiency of prion decontamination is possibly improved by repeating enzymatic digestion. However, one should always keep in mind the above-mentioned problem when enzymatic degradation is applied to the decontamination of instruments that are designed for reuse.

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Urinary excretion and blood level of prions in scrapie-infected hamsters

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Prions, infectious agents causing transmissible spongiform encephalopathy (TSE), are composed primarily of the pathogenic form (PrP^{Sc}) of the host-encoded prion protein. Although very low levels of infectivity have been detected in urine from scrapie-infected rodents, no reports of urinary PrP^{Sc} have been substantiated. Studies on the dynamics of urinary PrP^{Sc} during infection are needed to ensure the safety of urine-derived biopharmaceuticals and to assess the possible horizontal transmission of prion diseases. Using the protein misfolding cyclic amplification technique, a time-course study of urinary excretion and blood levels of PrP^{Sc} was performed in Sc237-infected hamsters and a high rate of PrP^{Sc} excretion was found during the terminal stage of the disease. Following oral administration, PrP^{Sc} was present in all buffy coat samples examined; it was also present in most of the plasma samples obtained from hamsters in the symptomatic stage. PrP^{Sc} was excreted in urine for a few days after oral administration; subsequently, urinary PrP^{Sc} was not detected until the terminal disease stage. These results represent the first biochemical detection of PrP^{Sc} in urine from TSE-infected animals.

INTRODUCTION

Prion diseases, commonly referred to as transmissible spongiform encephalopathies (TSEs), are neurodegenerative disorders that have been described in humans as Creutzfeldt–Jakob disease, in sheep and goats as scrapie, and in cattle as bovine spongiform encephalopathy (Prusiner, 1991). A characteristic of TSEs is the accumulation of a protease-resistant, misfolded prion protein (PrP^{Sc}), which is the pathogenic form of the host-encoded prion protein (PrP^{C}) (Caughey *et al.*, 1991; Pan *et al.*, 1993). PrP^{Sc} accumulation occurs in the central nervous system (CNS) of infected subjects through the structural conversion of PrP^{C}. TSE infectivity has also been demonstrated in a variety of peripheral tissues (Prusiner, 1998) and body fluids such as blood (Houston *et al.*, 2000; Taylor *et al.*, 2000; Holada *et al.*, 2002; Hunter *et al.*, 2002; Cervenakova *et al.*, 2003) of infected subjects.

Chronic wasting disease (CWD) is the only prion disease known to affect free-ranging mule and deer (Williams, 2005), and can spread horizontally (Miller *et al.*, 1998, 2000). Therefore, it was suspected that transmission of CWD occurred through contaminated saliva, faeces and urine, and it has been demonstrated that the saliva of infected deer contain the infectious agent (Mathiason *et al.*, 2006). With regard to excrement, a protease-resistant urinary PrP, designated $uPrP^{Sc}$, has been found in the urine of infected animals and humans (Shaked *et al.*, 2001); however, it was reported that the anti-PrP^{Sc} reactivity was primarily due to an immunoglobulin light chain (Serban *et al.*, 2004; Head *et al.*, 2005) or a contaminated bacterial component (Furukawa *et al.*, 2004). There are several reports that demonstrate infectivity in urine during scrapie infection. Urine from scrapie-infected mice with concurrent nephritis was infectious (Seeger *et al.*, 2005). A concentrated sample from pooled urine collected at the terminal stage of the disease exhibited a low level of infectivity in hamsters (Kariv-Inbal *et al.*, 2006). The result obtained in hamsters is important from the viewpoint that the urinary excretion of PrP^{Sc} possibly occurred without severe complications; however, the time course and frequency of appearance of urinary PrP^{Sc} remain to be determined.

As the PrP^{Sc} level in urine is expected to be diminutive when compared with that in the brain tissue, conventional immunoassays might not be applicable to the detection of urinary PrP^{Sc}. Recently, it has become possible to perform *in vitro* amplification of hamster PrP^{Sc} by protein misfolding cyclic amplification (PMCA) (Saborio *et al.*, 2001). By repeated sonication and incubation, PrP^{Sc} converts PrP^{C} to the protease-resistant form (PrP^{res}); this PrP^{res} then becomes the new nucleus, thereby facilitating PrP^{res} formation in the cyclic incubation process. PMCA is a highly effective method for detecting minute amounts of hamster PrP^{Sc} (Castilla *et al.*, 2005a; Saá *et al.*, 2006a) and enables the detection of PrP^{Sc} in the blood of infected hamsters (Castilla *et al.*, 2005b; Saá *et al.*, 2006b).

In the present study, using the PMCA technique, we examined the urinary excretion of PrP^{Sc} in hamsters that were inoculated intracerebrally or orally with the scrapie prion strain Sc237. We also investigated PrP^{Sc} levels in the plasma, buffy coat and urine during the period from latent to terminal stages of the disease and compared PrP^{Sc} dynamics among the above-mentioned samples. This paper is the first demonstration of urinary PrP^{Sc} in any disease model.

METHODS

Inoculation of the hamster prion. The hamster-adapted scrapie prion strain Sc237 was propagated in hamsters. The brains of hamsters at the terminal stage of the disease, with a titre of $5 \times 10^{8.5}$ LD_{50} g^{-1} by bioassay (Murayama *et al.*, 2006), were pooled and homogenized at a 10 % concentration (w/v) in PBS. The homogenate was injected intracerebrally (20 µl per hamster) or administered orally (100 µl per hamster) using a gastric tube. All animal experiments were performed in accordance with National Institute of Animal Health guidelines.

Preparation of urine and blood samples. Urine samples were collected at several time points after inoculation. From noon to about 3 p.m., the hamsters were held with both hands for spontaneous urination in a sterilized Petri dish. To separate urinary exudate cells such as leukocytes and epithelial cells of renal tubules from urine, the individual or pooled urine sample (20 µl) was diluted 1 : 10 with PBS and passed through a 0.45 µm membrane filter unit (Ultrafree-MC; Millipore). The flow-through in the collection tube was removed and 40 µl of 2 % Triton X-100, 8 mM EDTA in PBS was added to the membrane. The entire surface of the membrane was rinsed with the detergent solution by pipetting, and the membrane unit was incubated for 30 min at room temperature. After centrifugation, the flow-through was recovered and stored at −80 °C until use.

For blood collection, hamsters were sacrificed at several time points after inoculation and blood (1.5 ml) was collected from the heart and heparinized. Blood samples were centrifuged at 830 *g* for 15 min, and the plasma and buffy coat fractions were recovered. The plasma fraction was centrifuged again at 9170 *g* for 5 min to remove precipitates and the supernatants were stored at −80 °C until further use. Erythrocytes contained in the buffy coat fraction were haemolysed in 0.75 % NH_4Cl, 17 mM Tris/HCl (pH 7.2). The white blood cells and platelets were then washed three times in PBS and the pellets stored at −80 °C until analysis.

PMCA and Western blotting. The PMCA and Western blotting procedures have been described in our previous study (Murayama *et al.*, 2006). Briefly, normal hamster brains were homogenized at a 10 % concentration (w/v) in PBS containing complete protease inhibitors (Roche Diagnostics), 1 % Triton X-100, and 4 mM EDTA. To avoid contamination, the PrP^{C} source was prepared in a laboratory in which infected materials had never been handled. The urine and plasma samples were diluted 1 : 10 and 1 : 100, respectively, in the PrP^{C} source (total 100 µl). Buffy coat samples were prepared by three freeze–thaw cycles and mixed with 100 µl of the PrP^{C} source. Amplification was performed using a fully automatic cross-ultrasonic protein activating apparatus (ELESTEIN 070-GOT; Elekon Science Corp.), which has a capacity to generate high ultrasonic power (700 W). PMCA was performed by 40 cycles of sonication (a pulse oscillation for 3 s was repeated five times at 1 s intervals), followed by incubation at 37 °C for 1 h with gentle agitation. The amplified product obtained after the first round of amplification was diluted 1 : 10 in the PrP^{C} source and a second round of amplification was performed. The process was repeated to obtain the amplified products. Samples were treated with proteinase K (50 µg ml^{-1}), separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). After blocking, the membrane was incubated for 1 h with 1 : 10 000-diluted alkaline phosphatase-conjugated 3F4 monoclonal antibody (mAb; Signet Laboratories). After washing, the blotted membrane was developed using Immobilon Western Chemiluminescent AP substrate (Millipore) according to the manufacturer's instructions. After exposure for 5 min, chemiluminescence signals were analysed using a Light Capture System (ATTO).

Histological analysis. The urinary PMCA product obtained by 160 amplification cycles (see Fig. 1b, lane f) was diluted 1 : 10 with PBS and injected intracerebrally into Tg52NSE mice (20 µl per mouse), which overexpress hamster PrP^{C} in their nervous system (Race *et al.*, 1995). Densitometric analysis of Western blots revealed that the PrP^{res} signal intensity in the urinary PMCA product was approximately one-eighth of that in the 1 % homogenate of Sc237-infected brain. Therefore, the 10 % homogenate of Sc237-infected brain was diluted 1 : 800 and injected into Tg52NSE mice as a control. Tg52NSE mice exposed to the urinary PMCA product and Sc237-infected brain homogenate were sacrificed at 148 and 62 days post-inoculation (p.i.), respectively.

The left hemisphere of the brain was fixed in 10 % buffered formal saline (pH 7.4). Coronal slices of 2 mm thickness were immersed in 98 % formic acid to diminish prion infectivity (Taylor *et al.*, 1997) and embedded in paraffin. Serial sections were mounted on new silane II-coated glass slides (Muto Pure Chemicals Co. Ltd) and routinely stained with haematoxylin and eosin. For the detection of immunoreactive PrP^{Sc}, dewaxed sections were immersed in 98 % formic acid for 5 min and then treated with 3 % hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections were treated by autoclaving for 3 min at 121 °C in a low-ionic-strength buffer [2.1 mM Tris/HCl (pH 7.8), 1.3 mM EDTA, 1.1 mM sodium citrate; Asante *et al.*, 2006]. After washing in distilled water, the sections were placed on an automated immunohistochemical stainer (Autostainer Universal Staining System; DakoCytomation). mAb T1, which was generated in PrP-deficient mice by immunization with recombinant mouse $PrP_{121–231}$ and which recognizes mouse $PrP_{138–144}$, was used as the primary antibody. Anti-mouse universal immunoperoxidase polymer (Histofine Simple Stain MAX-PO (M); Nichirei) was used as the secondary antibody, and 3′-3-diaminobenzidine tetrachloride was used as the chromogen. Astrocytes were immunostained with a mouse anti-human glial fibrillary acid protein antibody (GFAP; DakoCytomation) after heat-mediated antigen retrieval. All sections were counterstained with haematoxylin.

RESULTS

Detection of urinary PrP^{Sc} in Sc237-infected hamsters

In the Western blot analysis, PrP^{Sc} propagation and accumulation in hamster brains became apparent 38 days after intracerebral inoculation (data not shown). The mean incubation time of the inoculated hamsters was 70 ± 2 days (mean $\pm$ SD, $n=58$) and the onset of clinical signs appeared 4–7 days prior to death. Fig. 1(a) illustrates the results of the amplification of the urine samples. No PrP^{res} was amplified in the uninfected control samples and pooled samples obtained during the period 26–46 days p.i. after 280 amplification cycles. In contrast, PrP^{res} was detected

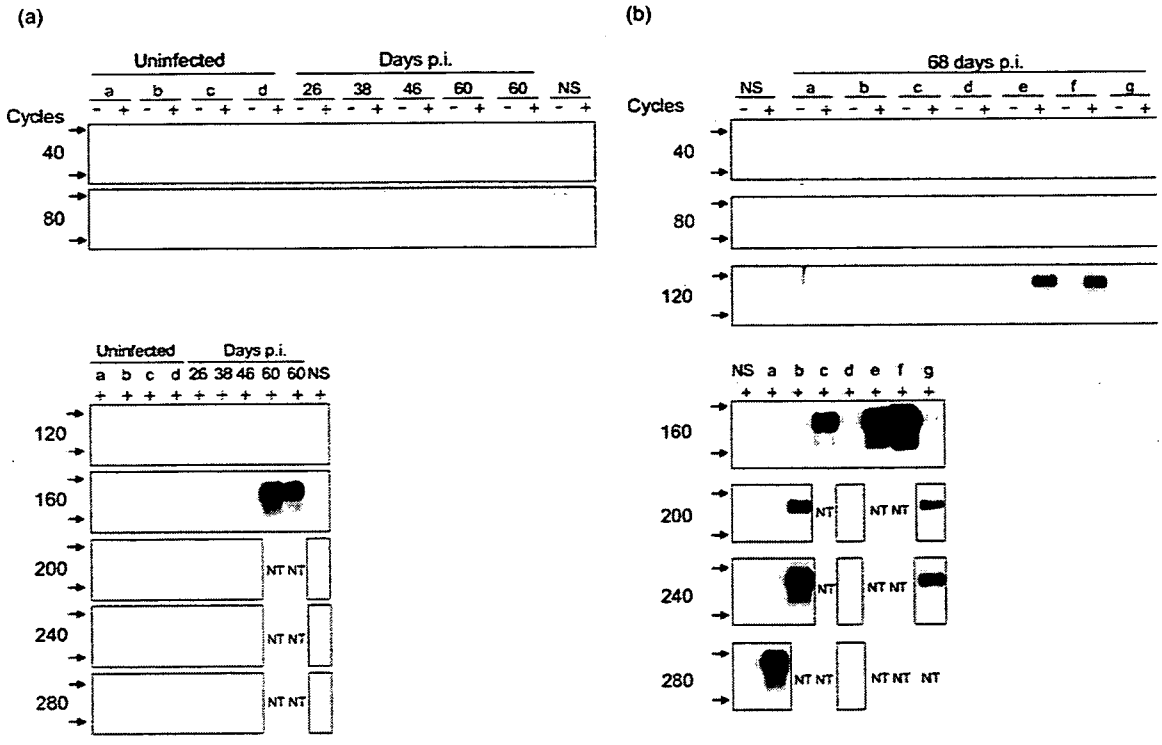

Fig. 1. (a) PMCA results from urine samples obtained from hamsters inoculated intracerebrally with Sc237. The PrP^{C} source was mixed with 0.1 vols of the pooled urine samples prepared as described in Methods, and one round (40 cycles) of the PMCA reaction was performed. This process of 1 : 10 dilution of the PMCA product and subsequent amplification was repeated six times. Samples before (−) and after (+) amplification were analysed by Western blotting following digestion with proteinase K. Lanes: a–d, negative controls in which the urine sample from an uninfected hamster was used for PMCA. No signals were detected in these samples. Arrows indicate the positions of molecular mass markers corresponding to 30 and 20 kDa. (b) PMCA results from urine samples obtained at the terminal stage of disease. Urine samples were prepared from seven individual hamsters (labelled 'a'–'g') and used for sequential PMCA. NS, No seed: negative control reaction in which uninfected brain homogenate (PrP^{C} source only) was treated in the same manner; NT, not tested.

after 160 cycles in two different pools of urine collected from five hamsters at 60 days p.i. To examine the incidence of urinary PrP^{Sc} excretion at the terminal stage of the disease, urine was collected from seven individual hamsters at 68 days p.i. and amplified (Fig. 1b). PrP^{res} signals were detected in two samples (Fig. 1b, hamsters 'e' and 'f') after 120 amplification cycles. Based on previous data (Murayama *et al.*, 2006), we estimated that the level of PrP^{Sc} present in these samples corresponded to that present in a 10^{-9}-diluted infected brain homogenate; such a dilution is well below the level detectable by any other method. PrP^{res} signals were detected in one sample (Fig. 1b, hamster 'c') after 160 cycles, in two samples (Fig. 1b, hamsters 'b' and 'g') after 200 cycles, and in one sample (Fig. 1b, hamster 'a') after 280 cycles of amplification. In contrast, no signals were detected in hamster 'd' after 280 amplification cycles.

We also examined the detection limit of our amplification system (Fig. 2) and confirmed that PrP^{Sc} present in an infected brain homogenate diluted $1:10^{10}$ could be detected after 160 cycles, and that PrP^{res} signals were enhanced in a similar manner during the further amplification process in the duplicated samples. However, in the more extreme dilution range, the number of amplification cycles required to amplify PrP^{Sc} to a detectable level varied in the duplicated samples. After 240 amplification cycles, both samples diluted $1:10^{12}$ became positive for PrP^{res}; however, the PrP^{res} signal remained very weak in one of the samples that was diluted $1:10^{14}$, even after 280 cycles. As PrP^{Sc} tends to aggregate, the weak detection of PrP^{res} in the reaction may have been due to the near-absence of PrP^{Sc} seed, which would have been almost completely diluted out. Therefore, it was assumed that PMCA with 280 cycles could reproducibly detect PrP^{Sc} present in the samples at a level equivalent to that present in an infected brain homogenate diluted $1:10^{12}$. Taken together, the PMCA analysis results indicated that PrP^{Sc} was excreted in the urine in a higher rate (86 %, six out of seven hamsters in Fig. 1b) at the terminal stage of the disease, although PrP^{Sc} concentrations differed considerably among the urine samples.

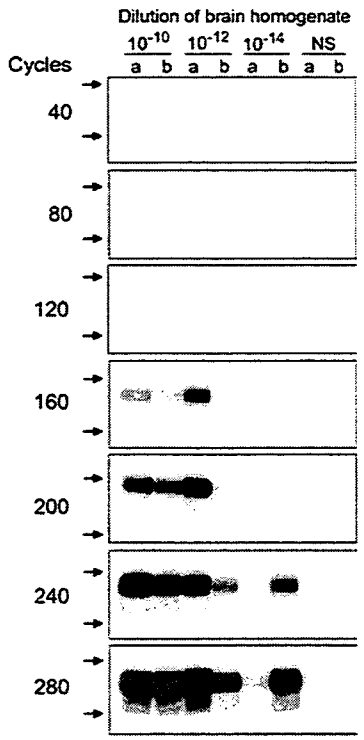

Fig. 2. Sensitivity of detection of hamster scrapie Sc237 using sequential PMCA. The PrP^{Sc} seed was diluted from 10^{-10} to 10^{-14} and used as the PrP^{C} source. Lanes a and b represent the results of duplicate samples. Arrows indicate 30 and 20 kDa molecular mass markers. NS, No seed.

Histological analysis of brain tissue infected with urine-derived PMCA product

Neuropathologically, vacuolar degeneration was detected in the thalamus, mesencephalon and medulla oblongata of Tg52NSE mice inoculated with Sc237 and the PMCA product (Fig. 3). This vacuolation was present mainly in the neuropil but was rare in the neurons. No vacuolation was observed in control mice brains. Granular-type PrP^{Sc} accumulation principally associated with the presence of vacuolation was observed in the neuropil and neurons of the thalamus, mesencephalon and medulla oblongata stained with mAb T1. Swollen or hypertrophic astrocytes proliferated in the same lesion that showed PrP^{Sc} accumulation. No significant difference was observed in vacuolar change, PrP^{Sc} accumulation and astrogliosis between Tg52NSE mice inoculated with Sc237 and those inoculated with the PMCA product.

PrP^{Sc} levels in the blood

As it has been demonstrated that PrP^{Sc} binds to a blood component (Fischer *et al.*, 2000), we examined PrP^{Sc} levels in both the plasma and buffy coat samples collected during the period of detection of urinary PrP^{Sc}. Fig. 4(a) shows the results of the amplification of buffy coats obtained at 60 and 68 days p.i. All samples became positive for PrP^{res} signals after 160 (60 days p.i.) or 120 (68 days p.i.) amplification cycles; therefore, we confirmed that PrP^{Sc} could be detected in the buffy coat samples by PMCA, as reported previously (Castilla *et al.*, 2005b). With regard to

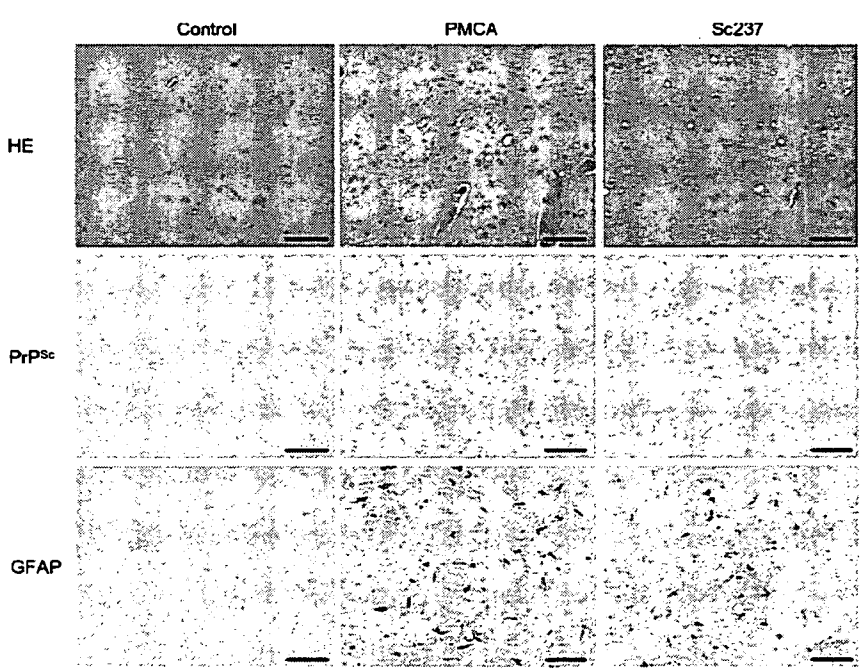

Fig. 3. Pathology of Tg52NSE mice exposed to urine-derived PMCA product from hamster 'f' in Fig. 1(b) (PMCA) and Sc237-infected brain homogenate (Sc237). Thalamus sections of mice that succumbed to scrapie after intracerebral inoculation with the PMCA product or the infected brain homogenate showed similar levels of vacuolation, PrP^{Sc} accumulation (labelled with mAb T1) and astrogliosis (labelled with anti-GFAP antibody). Bars, 50 μm.

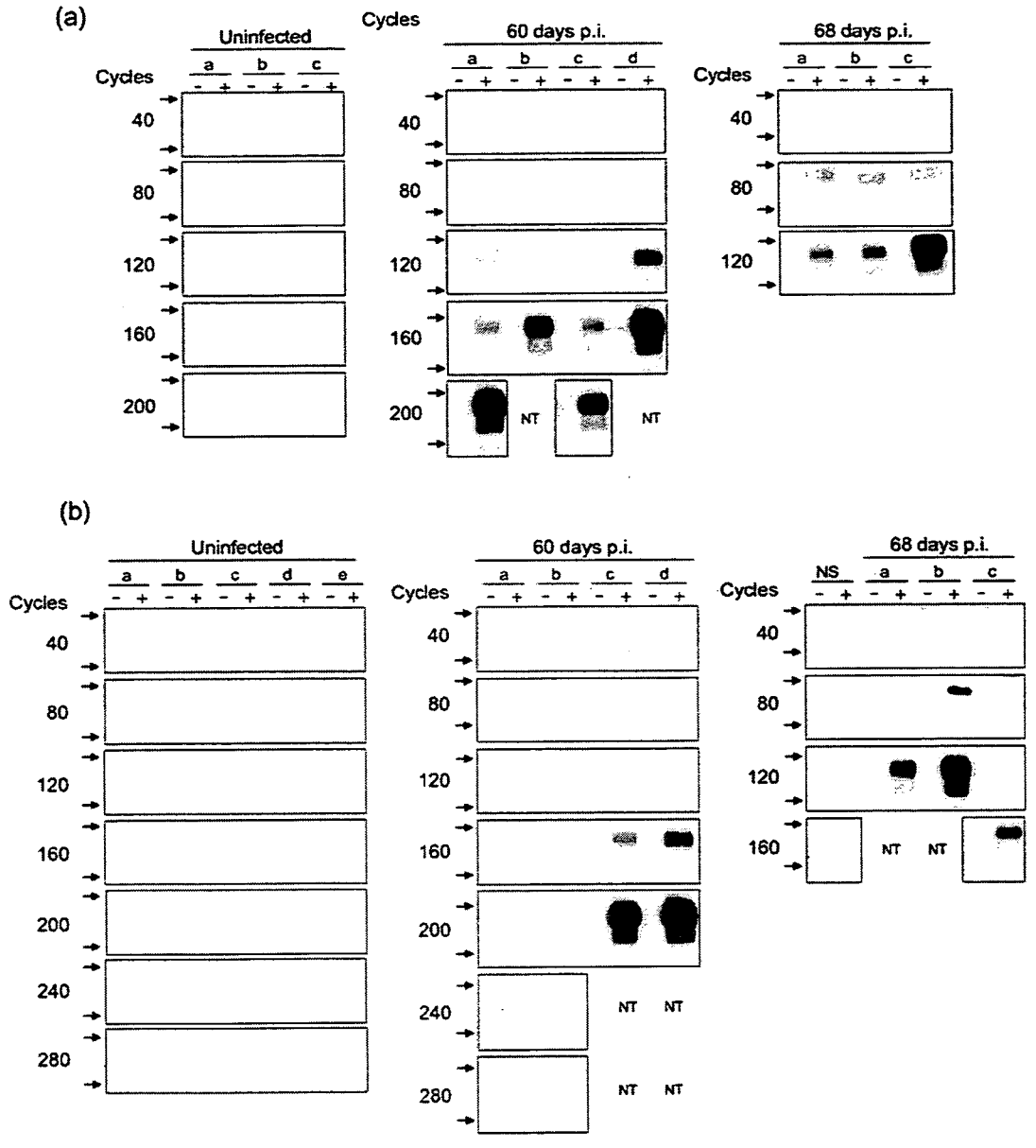

Fig. 4. PMCA of buffy coat (a) and plasma (b) samples obtained at 60 and 68 days p.i. from individual hamsters inoculated intracerebrally with Sc237. The PrP^{C} source was mixed with the buffy coat sample prepared as described in Methods or with a 1 : 100 dilution of the plasma sample. Sequential PMCA was performed on three to five hamsters (labelled 'a'–'e'). Arrows indicate 30 and 20 kDa molecular mass markers. NS, no seed; NT, not tested.

the plasma samples, PrP^{res} signals were detected after 160 amplification cycles in two samples ('c' and 'd') collected at 60 days p.i., whereas no signal was detected, even after 280 cycles, in other samples (Fig. 4b). In the three samples collected at 68 days p.i., 80–160 amplification cycles were required to amplify PrP^{res} to a detectable level. These results indicated that PrP^{Sc} was also present in the plasma at symptomatic and terminal stages of the disease. However, the detection sensitivity of PrP^{Sc} in plasma was low compared with that in buffy coats.

PrP^{Sc} levels in urine and blood after oral administration

The mean survival time of orally administered hamsters was 171 ± 15 days (mean $\pm$ SD, $n=18$). Fig. 5 illustrates the results of the amplification of urine samples collected between 4 and 155 days p.i. PrP^{res} was detected after 200–240 amplification cycles in two of four samples collected at 4 days p.i., and an additional sample became positive for PrP^{res} after 280 cycles. In contrast, PrP^{res} signals could not

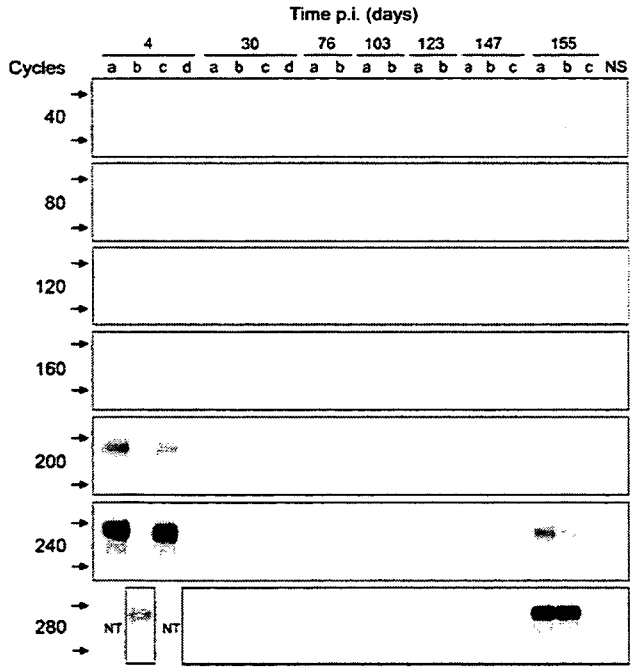

Fig. 5. PMCA results from urine samples obtained from individual hamsters after oral administration. Urine was collected from two to four hamsters (labelled 'a'–'d') at each time point. Arrows indicate 30 and 20 kDa molecular mass markers. NS, no seed; NT, not tested.

be detected in urine obtained between 30 and 147 days p.i. At 155 days after administration, urinary PrP^{Sc} was detected in two of three samples after 240–280 amplification cycles. This observation was in agreement with the results that urinary PrP^{Sc} was detected in the symptomatic and terminal stage of disease after intracerebral inoculation.

Fig. 6 (a) and (b) illustrate the results of amplification of plasma and buffy coats, respectively, obtained from animals sacrificed during the period 30–166 days p.i. PrP^{Sc} was not detected in plasma collected at the asymptomatic stage (<75 days p.i.) (Fig. 6a). At 103 days p.i., the propagation and accumulation of PrP^{Sc} became apparent in brains (Fig. 6c); at this stage, PrP^{res} signals were detected in the plasma after 160–200 amplification cycles (Fig. 6a). However, no PrP^{res} signal could be detected in one plasma sample (sample b) collected at 103 days, even after 280 amplification cycles. No remarkable PrP^{Sc} accumulation was observed in the brain of the plasma donor (Fig. 6c, 103 days p.i., sample b) when compared with the other donor (Fig. 6c, 103 days p.i., sample a). With regard to the buffy coats, PrP^{res} signals were detected after 120–160 cycles in all hamsters examined during the period 75–166 days p.i (Fig. 6b). We could not examine the buffy coat samples at 30 days p.i. because of a technical failure and could not verify the previous observation that PrP^{Sc} disappeared in the buffy coat fraction during the period of asymptomatic stage of the disease (Saá *et al.*, 2006b).

DISCUSSION

In the present study, using PMCA, we have revealed for the first time the presence of PrP^{Sc} in the urine of hamsters infected intracerebrally or orally with scrapie, without any dialysis and concentration procedures. A characteristic property of the urinary PrP^{Sc} was its detectability only at the terminal stage of the disease, except for a few days immediately after oral administration (Fig. 7). We also examined PrP^{Sc} levels in blood and clarified that PrP^{Sc} could be detected in plasma as well as in buffy coat fractions. There was an obvious time lag between PrP^{Sc} excretion in the urine and the appearance of PrP^{Sc} in the blood, and urinary PrP^{Sc} did not increase in proportion to PrP^{Sc} accumulation in the CNS. Furthermore, in some cases, urinary PrP^{Sc} could not be detected, even during the terminal stage of the disease, using the highly sensitive PMCA technique. Hence, urinary PrP^{Sc} appears to be an unsuitable marker for the early diagnosis of prion infection.

With regard to urinary PrP^{Sc} amplification, 120 or more cycles were required to amplify PrP^{Sc} to a detectable level, indicating that the amount of PrP^{Sc} present may be insufficient to induce infection. Although an extremely small amount of PrP^{Sc} is excreted in the urine, PrP^{Sc} can survive in the natural environment for a long time. In fact, prions remain infectious after incubation for several years in soil (Brown & Gajdusek, 1991), and recent research has demonstrated that PrP^{Sc} adsorbs firmly to soil components (Leita *et al.*, 2006) and that the soil minerals–PrP^{Sc} complex remains infectious (Johnson *et al.*, 2006). Therefore, it is conceivable that PrP^{Sc} is captured and absorbed effectively on soil and is then concentrated. Furthermore, it has been indicated that soil ingestion behaviours have been observed frequently in wild animals including mule deer (Arthur & Alldredge, 1979). A recent study indicated that excrement-borne transmission was not proved in an experimental CWD infection (Mathiason *et al.*, 2006); however, the possibility exists that some prion diseases may spread via contaminated soil in nature.

Where is the urinary PrP^{Sc} derived from? PrP^{res} was amplified successfully from urinary material trapped on a filter membrane. The urinary material probably included leukocytes and epithelial cells from the renal tubules. In preliminary experiments, PrP^{res} could not be amplified when the flow-through fraction was concentrated and used as a PMCA seed (data not shown); this suggests that PrP^{Sc} was primarily present in the insoluble and solid fraction in the urine samples. Kidney dysfunction or bacterial urinary tract infection might occur following disease progression, and lymphocyturia might arise at the terminal stage of the disease. However, the above elucidation does not provide a reasonable explanation for amplification of PrP^{res} in urine

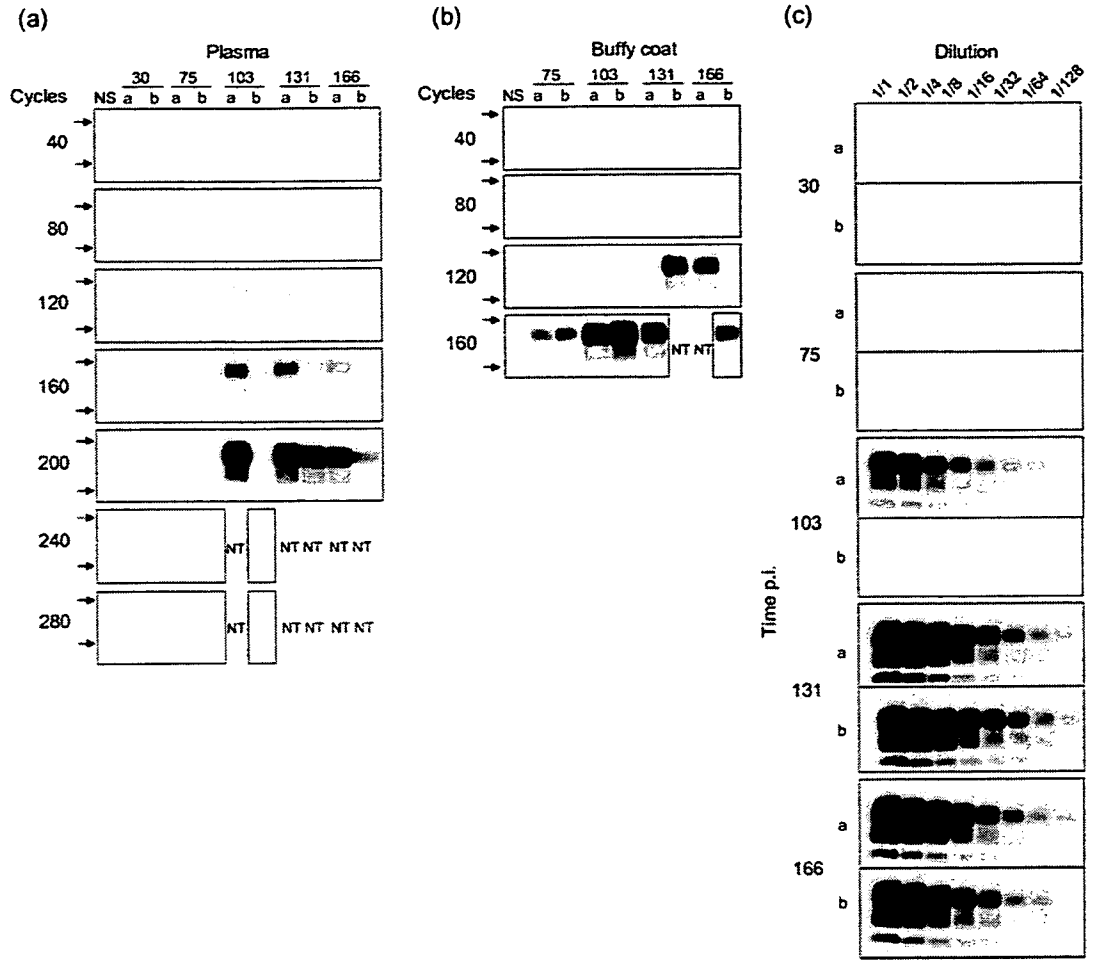

Fig. 6. (a, b) PMCA results from plasma (a) and buffy coat (b) samples obtained at 30–166 days p.i. from hamsters inoculated orally with Sc237. Two hamsters (labelled 'a' and 'b') were sacrificed at each time point, and blood and brain samples were prepared. (c) PrP^{Sc} accumulation in the brains of hamsters after oral administration of Sc237. Serial twofold dilutions of 10% brain homogenate were prepared and the results analysed by Western blotting. NS, no seed; NT, not tested.

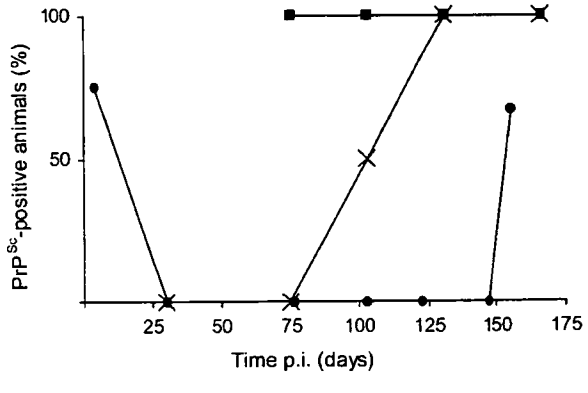

Fig. 7. Time course for the appearance of PrP^{Sc} in urine (●), plasma (×) and buffy coat (■) of orally infected hamsters.

samples collected at 4 days after peroral inoculation. It has been demonstrated that PrP^{Sc} can be detected on migrating intestinal dendritic cells (DCs) immediately after oral administration (Huang *et al.*, 2002), and that infected DCs can transmit the PrP^{Sc} prion to the CNS in recombination activating gene (RAG) knockout mice (Aucouturier *et al.*, 2001). These observations indicated that DCs could transport PrP^{Sc} from the intestinal tract to other tissues and body fluids without any propagation of the prion in the peripheral tissues. DCs also exist in the kidney, and it has been demonstrated that renal DCs form a contiguous network that continuously surveys the tubulointerstitium (Soos *et al.*, 2006). Renal DCs would capture PrP^{Sc} from the tubular lumen. Therefore, migrating DC-mediated transport may also be involved in urinary excretion of PrP^{Sc}, particularly in the early stage of infection.

The results of PrP^{res} amplification in the plasma samples after oral administration provide a possible explanation for the origin of plasma PrP^{Sc}. PrP^{res} could not be amplified from the plasma samples collected at 30 and 75 days p.i. and from one sample at 103 days p.i.; PrP^{Sc} accumulation could not be detected in the brains of these hamsters (Fig. 6). In contrast, PrP^{res} could be amplified from all plasma samples that were obtained when PrP^{Sc} propagation was apparent in the brains. These observations imply that PrP^{Sc} might leak into the plasma from the infected brain, probably due to destruction of the blood–brain barrier. The source of PrP^{Sc} in the buffy coats during the asymptomatic stage appeared to be the spleen and other lymphoid organs, as suggested previously (Saá *et al.*, 2006b). A more detailed analysis needs to be performed to confirm whether plasma PrP^{Sc} can serve as an indicator for the propagation of PrP^{Sc} in the CNS.

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Note

Assessment of Prion Inactivation by Fenton Reaction Using Protein Misfolding Cyclic Amplification and Bioassay

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An abnormal isoform of the prion protein, associated with transmissible spongiform encephalopathies, retains infectivity even after undergoing routine sterilization processes. We found that a formulation of iron ions combined with hydrogen peroxide effectively reduced infectivity and the level of abnormal isoforms of the prion protein in scrapie-infected brain homogenates. Therefore, the Fenton reaction has potential for prion decontamination.

Key words: prion inactivation; Fenton reaction; scrapie; bioassay; protein misfolding cyclic amplification

Prions, infectious agents causing transmissible spongiform encephalopathy (TSE), primarily comprise the proteinase K (PK)-resistant pathogenic isoform (PrP^{Sc}) of the host-encoded prion protein (PrP^{C}).[1)] Prion diseases include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep and goats.[2)] PrP^{Sc} accumulates in the central nervous system of the infected subjects *via* structural conversion of PrP^{C}.[3,4)] PrP^{Sc} deposition or TSE infectivity has also been found in a variety of peripheral tissues[1)] and body fluids, such as the blood[5)] of scrapie-infected subjects. Since PrP^{Sc} has also been detected in the peripheral tissues of CJD patients,[6)] there is a serious risk of iatrogenic transmission through human-derived products, medical equipment, and instruments such as endoscopes.[7)]

PrP^{Sc} retains infectivity even after undergoing routine sterilization processes, and complete inactivation necessitates very harsh treatment such as complete incineration, strong alkali treatment, or high-temperature autoclaving.[8)] Hence, the livestock and healthcare industries desire the development of practical techniques for PrP^{Sc} inactivation. Hence, novel sterilization methods involving chemical[9,10)] or enzymatic[11,12)] treatment have been devised to inactivate prions effectively.

In the present study, we investigated the efficacy of the Fenton reaction in scrapie prion inactivation. The Fenton reaction consists of the reduction of Fe (III) by an electron donor and the generation of a hydroxyl radical *via* the reduction of hydrogen peroxide (H_2O_2) by the reduced metal.[13–15)] Iron is an essential redox transition element that is capable of inducing ion-mediated damage in proteins. Here, we present evidence justifying interest in an Fe (II)/H_2O_2 formulation for use in prion inactivation.

The hamster-adapted scrapie prion strain Sc237 was propagated in hamsters. The brains of hamsters at the terminal stage of the disease, titrating $5 \times 10^{8.5}$ LD_{50} per gram by bioassay,[16)] were pooled together and homogenized at a concentration of 20% (w/v) in phosphate-buffered saline (PBS). The homogenate was mixed with an equal volume of 0.2% (w/v) $FeCl_2$-PBS solution in the presence of 5% (v/v) H_2O_2. The samples were heated to 50 °C and kept for 22 h. The Fenton reaction was performed on the basis of experimental results obtained in our previous study.[15)] A high concentration of H_2O_2 was used in the present study, but H_2O_2 itself does not disinfect prions.[17)] After treatment, the samples

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Abbreviations: TSE, transmissible spongiform encephalopathy; PK, proteinase K; PrP^{Sc}, pathogenic isoform of prion protein; PrP^{C}, host-encoded prion protein; CJD, Creutzfeldt-Jakob disease; BSE, bovine spongiform encephalopathy; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; WB, western blot; PMCA, protein misfolding cyclic amplification; PrP^{res}, protease-resistant prion protein

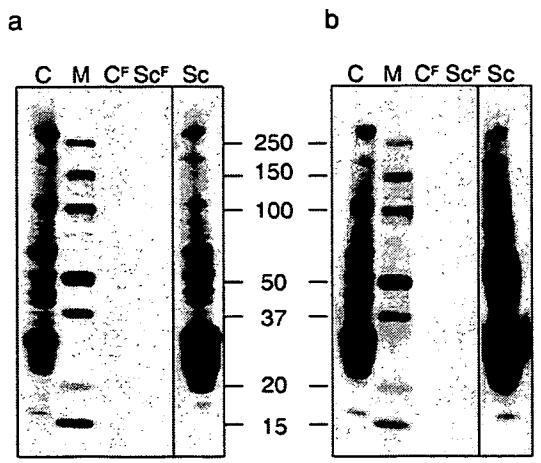

Fig. 1. Western Blotting Analysis of the Prion Protein in Hamster Brain Homogenate Following the Fenton Reaction Using 3F4 (a) or SAF32 (b) Antibodies.

Uninfected brain homogenate before (C) and after (C^F) the Fenton reaction; infected homogenate before (Sc) and after (Sc^F) the Fenton reaction. The positions of molecular weight standards (15–250 kDa) are also shown in the figure (the lanes labeled M).

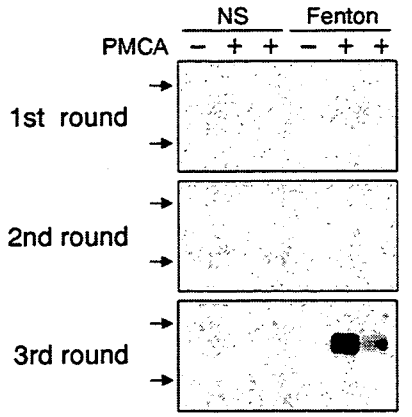

Fig. 2. Protein Misfolding Cyclic Amplification of Samples Treated by the Fenton Reaction.

The samples were analyzed by western blotting before (–) and after (+, in duplicate) each round of amplification following digestion with proteinase K. The lanes labeled NS denote the controls, in which the uninfected brain homogenate (PrP^C source only) was treated in an identical manner. No signals were detected in these samples. The arrows indicate the positions of the molecular weight markers corresponding to 30 and 20 kDa respectively.

were washed three times by ultracentrifugation to remove the H_2O_2, and the pellets were resuspended in an initial volume of PBS.

The treated samples were separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated 3F4 (Signet Laboratories, Dedham, MA) or SAF32 (Cayman Chemical, Ann Arbor, MI) antibodies, which recognize different epitopes of the prion protein. After it was washed, the blotted membrane was developed by the ECL+Plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, England), according to the manufacturer's instructions. Chemiluminescence signals were analyzed by the Light Capture System (Atto, Tokyo).

Figure 1 illustrates the results of western blot (WB) analysis performed on the samples treated by the Fenton reaction. Following the Fenton reaction, no detectable PrP signals were observed in the uninfected or infected samples. In addition to conventional WB analysis, protein misfolding cyclic amplification (PMCA), a highly sensitive method of detecting minute amounts of PrP^{Sc},[18,19] was performed to detect residual PrP^{Sc}. The PMCA procedure followed was based on that reported in our previous study.[16] Briefly, the treated sample was diluted 1:10 in 10% uninfected brain homogenate, and one round of the PMCA reaction was performed by applying 40 cycles of sonication, followed by incubation at 37 °C for 1 h. Next, the process diluting the PMCA product to 1:10 and its subsequent amplification was repeated two times. With regard to the PMCA products, samples (10 µl) collected before and after each round of amplification were mixed with 10 µl PK solution (100 µg/ml) and incubated at 37 °C for 1 h. The digested materials were analyzed by WB using the 3F4 antibody, as described above.

Figure 2 illustrates the results of amplification of the samples treated by the Fenton reaction. In the first and second rounds of amplification, no signals from the protease-resistant prion protein (PrP^{res}) were detected in the samples. After three rounds of amplification, PrP^{res} signals were detected in both the duplicated samples. These results suggest that only a trace amount of PrP^{Sc} remained in the sample treated by the Fenton reaction.

The sample treated by the Fenton reaction was inoculated intracerebrally (20 µl per mouse) into 3–5 Tg52NSE transgenic mice that overexpressed hamster PrP^C.[20] The control mice inoculated with the untreated samples developed the disease after an average period of 45 ± 2 d (average ± SD, n = 5). The mice that were inoculated with the sample subjected only to heat treatment died after an average period of 46 ± 0 d (n = 3). The disease onset time significantly decreased in the mice inoculated with the sample treated by the Fenton reaction, but the mice died after an average period of 163 ± 28 d (n = 3). Based on the incubation time obtained in a previous study,[16] the reduction in infectivity was estimated to be approximately 10^{-6}.

According to the Fenton reaction, Fe (II) generates a hydroxyl radical *via* the breakdown of H_2O_2. This radical is a highly reactive oxygen species that causes direct damage to nucleic acids and proteins, a property that has been already used in the inactivation of conventional pathogens.[21] A copper-H_2O_2 formulation has been proved an effective method for prion decontamination.[10] We also confirmed that the most effective oxidative deamination by H_2O_2 was obtained in the presence of Cu^{2+},[15] but we adopted the iron-H_2O_2

formulation for prion decontamination considering all possible risks relating to chemical toxicity against a living body, corrosive impact against medical apparatus, and environmental pollution. The present study indicated that iron ions cause effective oxidative damage to PrP^{Sc} in the presence of high concentrations of H_2O_2, resulting eventually in an effective reduction in infectivity and a significant decrease in PrP^{Sc} levels. Hence, the Fenton reaction using iron ions can be applied in decontamination of fragile, precision, or expensive instruments that are susceptible to PrP^{Sc} contamination.

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Prion inactivation by the Maillard reaction

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Abstract

Since variant Creutzfeldt-Jakob disease (vCJD) has been suspected to be attributable to the infectious agents associated with bovine spongiform encephalopathy (BSE), it is important to prevent the transmission of pathogenic forms of prion protein (PrP^{Sc}) through contaminated feeding materials such as meat and bone meal (MBM). Here, we demonstrate that the Maillard reaction employing a formulation of glucose in combination with sodium hydrogen carbonates effectively reduced the infectivity (approximately 5.9-log reduction) of a scrapie-infected hamster brain homogenate. In addition to a bioassay, a protein misfolding cyclic amplification (PMCA) technique, in which PrP^{Sc} can be amplified *in vitro*, was used as a rapid test for assessing PrP^{Sc} inactivation. The PMCA analysis also indicated that the PrP^{Sc} level in the infected material significantly decreased following the Maillard reaction. Therefore, the Maillard reaction can be employed for the decontamination of large amounts of byproducts such as MBM.

Keywords: Prion inactivation; Meat and bone meal; Maillard reaction; Bioassay; Protein misfolding cyclic amplification

Prion diseases or transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders that have been described in humans as Creutzfeldt-Jakob disease (CJD), in sheep and goats as scrapie, and in cattle as bovine spongiform encephalopathy (BSE) [1]. A characteristic of TSE is the accumulation of a protease-resistant, misfolded prion protein (PrP^{Sc}), which is a pathogenic variant of the host-encoded prion protein (PrP^{C}) [2,3]. Since PrP^{Sc} retains its infectivity after undergoing routine sterilizing processes, harsh physical or chemical sterilization procedures are required for its inactivation [4,5].

BSE is an emerging disease that first appeared in the United Kingdom after 1986 [6]. It appears that the cause of the BSE outbreak was feeding PrP^{Sc}-contaminated meat and bone meal (MBM) acquired from rendered carcasses of BSE- or scrapie-infected ruminants to healthy cattle [7,8]. It is thought that the recycling of BSE-infected bovine tissues augmented the concentration of PrP^{Sc} in commercial MBM, thus causing the subsequent BSE epidemics [9]. Thus, in order to ensure that byproducts such as MBM can be safely used in the future, the development of a technology that can be used for treating large amounts of byproducts at low costs is required.

In the present study, we report a novel method for prion inactivation by chemical modification of proteins based on the principle of the Maillard reaction [10–13]. The Maillard reaction is a very complicated reaction between reducible carbohydrates and reactive amino acid residues of proteins such as lysine, arginine, and tryptophan [14–16]. The reaction products consist of a large variety, for example, brown protein polymers called "melanoidins." Since the Maillard reaction irreversibly modifies the proteins, the structural changes in PrP^{Sc} induced by the Maillard reaction are expected to be effective for reducing the infectivity. We therefore investigated the efficacy of the Maillard reaction in scrapie prion inactivation by a bioassay that examined

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