Emergence of a novel bovine spongiform encephalopathy (BSE) prion from an atypical H-type BSE

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The H-type of atypical bovine spongiform encephalopathy (H-BSE) was serially passaged in bovinized transgenic (TgBoPrP) mice. At the fourth passage, most challenged mice showed a typical H-BSE phenotype with incubation periods of 223 ± 7.8 days. However, a different phenotype of BSE prion with shorter incubation periods of 109 ± 4 days emerged in a minor subset of the inoculated mice. The latter showed distinct clinical signs, brain pathology, and abnormal prion protein profiles as compared to H-BSE and other known BSE strains in mice. This novel prion was transmitted intracerebrally to cattle, with incubation periods of 14.8 ± 1.5 months, with phenotypes that differed from those of other bovine prion strains. These data suggest that intraspecies transmission of H-BSE in cattle allows the emergence of a novel BSE strain. Therefore, the continuation of feed ban programs may be necessary to exclude the recycling of H-BSE prions, which appear to arise spontaneously, in livestock. Such measures should help to reduce the risks from both novel and known strains of BSE.
of inoculated mice. When this novel prion was inoculated intracerebrally into cattle, a novel BSE phenotype was confirmed. This study suggests that a novel BSE emerges during intraspecies transmission of H-BSE in cattle.

**Results**

**Serial transmission of H-BSE in TgBoPrP mice.** The results of serial transmission of the H-BSE isolate in TgBoPrP mice are shown in Table 1. All the H-BSE challenged mice developed progressive neurological disease with the incubation periods of 320.1 ± 12.2 days at primary passage. H-BSE-affected animals showed a distinctive clinical sign, namely, constant chewing of the bedding, as reported previously. The incubation periods of the second and third passages were 226.9 ± 4.2 and 215.6 ± 5.0 days, respectively (Table 1). No clear differences were observed in their clinical signs, the banding pattern of PrPSc, and histopathological features from the primary to third passage mice. At the fourth passage, mice from a single experimental group (#3), out of eight experiments, showed shorter incubation periods (108.8 ± 4.0 days) than the other groups. This group was challenged with brain homogenates of a mouse with 221-day incubation period. Group #3 animals showed weight loss, but no constant chewing of the bedding. This short incubation-type of BSE was designated BSE-SW (short incubation with weight loss) strain. BSE-SW was transmitted to TgBoPrP mice with 97.3 ± 3.7 day incubation periods, and their clinical signs were identical to those of mice in the experimental group #3. The other mice in the fourth passage groups showed the symptomatic chewing of the bedding, and their incubation periods were 223.3 ± 7.8 days (Table 1).

**Molecular features of PrPcore of BSE-SW.** Western blot analysis with monoclonal antibody (mAb) 6H4 revealed that the molecular mass of proteinase K (PK) digested PrPSc (PrPcore) of BSE-SW was lower than that of H-BSE, but similar to C-BSE (Fig. 1b). MAb P4, which recognizes the N-terminal region of PrPcore, reacted with H-BSE but not with BSE-SW or C-BSE (Fig. 1a). This revealed a partial similarity between PrPSc of BSE-SW and C-BSE. In contrast, truncated 12-kDa fragments (consistent with PrP 157/163–231) were observed for H-BSE but not with BSE-SW or C-BSE (Fig. 1b). The results of serial transmission of the H-BSE isolate in TgBoPrP mice are shown in Table 1. All the H-BSE challenged mice developed progressive neurological disease with the incubation periods of 320.1 ± 12.2 days at primary passage. H-BSE-affected animals showed a distinctive clinical sign, namely, constant chewing of the bedding, as reported previously. The incubation periods of the second and third passages were 226.9 ± 4.2 and 215.6 ± 5.0 days, respectively (Table 1). No clear differences were observed in their clinical signs, the banding pattern of PrPSc, and histopathological features from the primary to third passage mice. At the fourth passage, mice from a single experimental group (#3), out of eight experiments, showed shorter incubation periods (108.8 ± 4.0 days) than the other groups. This group was challenged with brain homogenates of a mouse with 221-day incubation period. Group #3 animals showed weight loss, but no constant chewing of the bedding. This short incubation-type of BSE was designated BSE-SW (short incubation with weight loss) strain. BSE-SW was transmitted to TgBoPrP mice with 97.3 ± 3.7 day incubation periods, and their clinical signs were identical to those of mice in the experimental group #3. The other mice in the fourth passage groups showed the symptomatic chewing of the bedding, and their incubation periods were 223.3 ± 7.8 days (Table 1).

**Neuropathological examination.** The degree of brain vacuolation and neuroanatomical distribution patterns of PrPSc in TgBoPrP mice with BSE-SW were different from H-BSE and C-BSE (Fig. 2). The lesion scores of PrPSc staining were 10.8 (5/5) ± 0.3 (7/7) for H-BSE, 1.7 (6/6) ± 0.8 (6/6) for BSE-SW, and 3.0 ± 0.1 (7/7) for C-BSE. These biochemical properties of BSE-SW were maintained in the subsequent passages (data not shown).

| Passage history | Remarks |
|-----------------|---------|
| **First** | **Second** | **Third** | **Fourth** | **Fifth** | **Remarks** |
| 320.1 ± 12.2a (10/10) [318] | 226.9 ± 4.2 (7/7) [230] | 215.6 ± 5.0 (40/40) | | | H-BSE |
| #5 [207] | 220.8 ± 0.3 (7/7) | | | | |
| #1 [209] | 214.0 ± 10.8 (5/5) | | | | |
| #6 [213] | 209.6 ± 0.8 (6/6) | | | | |
| #8 [215] | 223.3 ± 7.8 (4/4) | | | | |
| #2 [216] | 220.0 ± 4.4 (6/6) [234] | 216.7 ± 3.0 (12/12) | | | |
| #7 [216] | 210.3 ± 1.7 (6/6) | | | | |
| #4 [228] | 225.0 ± 0.0 (6/6) | | | | |
| #3 [221] | 108.8 ± 4.0 (20/20) [106] | 97.3 ± 3.7 (10/10) | BSE-SW | | |

Table 1. Transmission of H-BSE in TgBoPrP mice. aAverage ± standard deviation (days). bNumbers of affected/inoculated mouse are shown in parentheses. cIncubation periods of mice used for subsequent passages are shown in brackets. dExperimental group number at fourth passage: each experiment used a different brain sample from affected mice at third passage.
the PrPSc staining pattern in C-BSE was completely different from that in BSE-SW (Fig. 2d,j,g,m). These results indicate that neuropathological properties of BSE-SW in TgBoPrP mice were apparently distinct from those of H-BSE and C-BSE.

Conformational stability studies. MAbs 6H4 and SAF84 were used to conduct conformation stability studies. Western blotting with mAb 6H4 showed that the [GdnHCl]1/2 values (see Methods Section) for PrPSc denaturation in the H-BSE and BSE-SW brains were 3.8 ± 0.4 M and 3.0 ± 0.2 M, respectively (Fig. 3e). This analysis detected PrPcore #1 signal of 17–27 kDa from BSE-SW, and 19–29 kDa from H-BSE (Fig. 3a,c). PrPcore #1 from BSE-SW was more sensitive to GdnHCl treatments than that of H-BSE. A different result was observed with mAb SAF84, which detected both PrPcore #1 and PrPcore #2 (Fig. 3b,d). [GdnHCl]1/2 values of H-BSE and BSE-SW were 3.5 ± 0.3 M and 3.4 ± 0.3 M, respectively (Fig. 3f). These values were not significantly different. Signal intensity of the diglycosylated PrPcore #1 of BSE-SW was weaker than that of H-BSE during 3.0–3.5 M treatment (Fig. 3d). This result was consistent with the mAb 6H4 experiment (Fig. 3c). However, the lower three
bands of PrPcore #2 showed similar signal intensities during 3.5–4.0 M treatment (Fig. 3b,d). This revealed that the conformational stability of PrPcore #1, but not PrPcore #2, is different in PrPSc from H-BSE and BSE-SW.

Transmission of BSE-SW to cattle. To examine whether the BSE-SW prion could become a threat as a novel prion disease in cattle, three Holstein calves were challenged intracerebrally. All the inoculated animals developed progressive neurological disease. The animals exhibited initial clinical signs of the disease between 11.5 and 12.5 months post-inoculation (mpi), which included disturbance, mild fear or anxiety, mild gait changes, and, occasionally, low head carriage. After one to two months of the initial clinical signs, the animals were leaning towards the floor and rested their heads against the wall, which was followed by ataxia of the hind limbs that progressed to difficulty in getting up without assistance at the clinically terminal stage of the disease. The bodily condition gradually worsened because of a loss of weight during the two to three month clinical duration.

Figure 2. Neuropathological analysis of TgBoPrP mice. Lesion profiles (a), neuroanatomical distribution of PrPSc detected by PET-blot (b–g), and PrPSc staining types assessed by IHC (h–m) in the brains of BSE prion-affected TgBoPrP mice. Vacuolation in the following brain regions was scored, on a scale of 0–4 (mean values): 1, dorsal medulla; 2, cerebellar cortex; 3, superior cortex; 4, hypothalamus; 5, thalamus; 6, hippocampus; 7, septal nuclei; 8, cerebral cortex at the level of the hypothalamus and thalamus; and 9, cerebral cortex at the level of the caudate nuclei. The data are presented as mean ± standard deviation (n = 7). Black circles, H-BSE; Diamonds, BSE-SW; Black triangles, C-BSE. The degree of vacuolation in the brain of BSE-SW was different from H-BSE and C-BSE (a). PET blots with mAb SAF84 corresponding to the brain areas at the level of thalamus (b–d), and the level of medulla and cerebellum (e–g) are shown. IHC was performed with mAb F99/97.6.1. CC, corpus callosum; HB, habenular nucleus; PC, parietal cortex; TC, temporal cortex; H, hippocampus; T, thalamus; HT, hypothalamus; AM, amygdala; DMNV, dorsal motor nucleus of vagus nerve; NC, deep nuclei of the cerebellum. PrPSc deposits and distribution patterns in the BSE-SW brain were distinct from H-BSE and C-BSE.
None of the animals exhibited anorexia, nervousness, or aggression, and responded to visual, acoustic, and tactile stimuli throughout the course of the disease. The cattle were eventually culled at 13.3 mpi, 15 mpi, and 16.2 mpi before astasia, in accordance with the welfare guidelines for animal experiments. The incubation periods of cattle infected with BSE-SW (14.8 ± 1.5 mpi) were shorter than those for H-BSE, C-BSE, and L-BSE (Table 2). Obex samples from these cattle were subjected to routine BSE confirmatory tests.

**Histopathology and PrP\(^{Sc}\) immunohistochemistry of the obex of BSE-SW-affected cattle.**

Mild vacuolation of the extracellular neuropil was observed in the dorsal motor nucleus of the vagus nerve (DMNV), the solitary nucleus, the nucleus of trigeminal nerve spinal tract, and the olivary nucleus in all animals. No intraneuronal vacuolation was seen. Spongy changes were not prominent in the gray matter of medulla oblongata at the obex (Fig. 4a). Immunolabeling of PrP\(^{Sc}\) with mAb F99/97.6.1 resulted in intraneuronal and intraglial patterns throughout the obex (Fig. 4b). Intraneuronal labeling was less common in DMNV and the hypoglossal nucleus compared to other nuclei. Fine and coarse granular PrP\(^{Sc}\) was sparsely distributed throughout the neuropil of reticular formation. No other extracellular types of PrP\(^{Sc}\), such as plaque-like and stellate deposits, were identified in the obex region.

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**Figure 3. Conformational stability assay.** The conformation stability of PrP\(^{Sc}\) from BSE-SW and H-BSE. GdnHCl was added at the indicated concentrations and the samples subjected to PK digestion (20 μg/ml). PrP\(^{Sc}\) was detected with mAbs 6H4 and SAF84. Molecular weights are shown on the left (kDa). [GdnHCl]\(^{1/2}\) concentration (M) was calculated based on denaturation curves obtained from densitometric analysis of western blot data. The results are mean ± standard deviation from five experiments. The black and white bars indicate H-BSE and BSE-SW, respectively.
Molecular features of PrPcore of BSE-SW-affected cattle. Western blot analysis detected PrPSc from the obex tissue of the challenged cattle. The molecular features of PrPcore of BSE-SW-affected cattle were distinctly different from C-BSE, L-BSE, and H-BSE (Fig. 5). The molecular mass of PrPcore #1 of BSE-SW, as determined by mAb 6H4, was lower than H-BSE and similar to C-BSE (Fig. 5b). MAb P4 did not detect PrPcore of BSE-SW (Fig. 5a). PrPcore #2 was also observed in BSE-SW (Fig. 5c,d). These results revealed that the biochemical properties of BSE-SW have indeed been transmitted to cattle.

Discussion
Our previous reports have revealed the usefulness of TgBoPrP mice for characterizing BSE prions. In this study, a novel BSE, BSE-SW, was detected using this mouse model. The incubation periods of H-BSE, L-BSE, and C-BSE prions in the TgBoPrP mice were approximately 215 days, 150 days, and 190 days, respectively. The BSE-SW prion showed the shortest incubation period (approximately 90 days) among the known BSE prions. We have previously performed several transmission experiments of sheep scrapie to TgBoPrP mice, but their incubation periods were over 170 days, and we have not observed any prions with ~90-day incubation periods in these mice. In addition, the biochemical and biological properties of PrPSc from BSE-SW were clearly different from C-BSE, L-BSE, H-BSE, and sheep scrapie (data not shown). PrPSc of BSE-SW has some similarity to H-BSE on the account of the presence of truncated 12-kDa fragments (PrPcore #2). Fig. 6 shows the putative PK digestion site of PrPSc from BSE-SW, as assessed by immunoreactivity with mAbs P4, 6H4, and SAF84. These results argue against the possibility that the BSE-SW prion resulted from a contamination of other laboratory prion strains.

It is known that sheep scrapie comprises different prion strains, and some affected sheep harbor these mixed scrapie prion strains. Numerous scrapie strains had emerged in the course of several passage histories. We have also previously isolated distinct scrapie strains from the brain of a scrapie-affected sheep after primary passage in wild type mice. The different scrapie prion strains appeared after primary passage in sheep scrapie cases, which was considered to be due to prion strain selection. For H-BSE prions, French and Polish cases were reported, which transformed into a C-BSE-like phenotype during mouse passages, revealing their potential heterogeneity. However, the BSE-SW prions described herein appear to have emerged by a different manner than our previous scrapie case. The Canadian H-BSE sample that was used in this study was also used to inoculate wild type mice, but
we did not observe the emergence of a C-BSE-like phenotype in those circumstances (unpublished data). The underlying mechanisms of the emergence of new prion strains are important to elucidate prion heterogeneity. This novel BSE strain has never been observed in field BSE cases, but our experiments reveal the potential risk associated with H-BSE.

It has been suggested that different conformations of PrPSc are involved in the prion strain diversity\(^2\), and that rearrangement of PrPSc from a uniform conformation causes the emergence of new host-adapted PrPSc\(^3\). PrPSc of BSE-SW exhibited different conformational stability from H-BSE. It is also known that strain “mutation or transformation” may occur upon intraspecies transmission, where the PrP amino acid sequences of the host and the donor are identical\(^3\). These finding are consistent with the conclusion that PrPSc of BSE-SW has a different conformation than H-BSE.

Furthermore, our new strain was successfully transmitted to cattle. Standard diagnostic testing for BSE confirmed the presence of spongiform changes associated with PrPSc accumulation in the obex, and the challenged cattle fulfilled BSE criteria (Figs 4 and 5). The disease phenotype and features of PrPSc, different from the known types of BSE, indicated that this prion could cause a novel type of atypical BSE. The shorter incubation periods in cattle were consistent with the relative incubation periods in TgBoPrP mice, and indicate high virulence of this

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**Figure 5. Western blot analysis of PrPcore from BSE-SW-affected cattle.** PrPcore from the affected cattle were detected using mAbs: P4 (a), 6H4 (b), and SAF84 (c). Lane 1, H-BSE; Lane 2, BSE-SW; Lane 3, C-BSE; Lane 4, L-BSE. All the samples were digested with 40 μg/ml PK at 37 °C for 1 h. Digested aliquots were treated with PNGaseF, and were probed using mAb SAF84 (d). Equivalents of 0.5 mg brain tissues were loaded. Molecular markers are shown on the left (kDa).

**Figure 6. Schematic representation of PrPcore of BSE prions.** Putative PK cleavage sites and PrPcore are shown, as estimated by immunoreactivity with mAbs P4, 6H4, and SAF84. PrPcore #1 is an unglycosylated PrPcore with a molecular weight of 17–19 kDa. PrPcore #2 is an unglycosylated PrPcore with a molecular weight of 12 kDa.
novel prion. Further analysis of diseased cattle is necessary to clarify its characteristics. Such studies could help to elucidate the mechanisms of conformational change in PrPSc, which lead to the propagation of new prion strains.

The ban on meat-and-bone meal in livestock feed has contributed to the decline in C-BSE occurrences6. Recently, easing of the BSE-related regulations and control measures has been discussed. The origin of atypical BSE remains unknown, but it has been proposed to be spontaneous or sporadic19. H-BSE has been reported to transform into a C-BSE-like phenotype during animal passages18–20. Furthermore, we have shown here that the sequential transmission of H-BSE in TgBoPrP mice, i.e., to a homologous bovine PrP context, generated a novel type of BSE. Considering these observations, a continuous feed ban program may be necessary even after C-BSE is eradicated. Prohibiting the recycling of spontaneously occurring H-BSE prions in cattle should help to prevent both re-emerging and emerging types of BSEs.

**Methods**

**Ethics statement.** Procedures involving animals have been approved by the Animal Care and Use Committee at the National Institute of Animal Health (approval ID: 11-008, 13-005). Animal experiments were performed in accordance with the Guidelines for Animal Transmissible Spongiform Encephalopathy Experiments of the Ministry of Agriculture, Forestry, and Fisheries of Japan.

**Transgenic mice.** TgBoPrP mice overexpressing the bovine PrP gene (encoding BoPrP) in a mouse PrP deficient background were used. These mice harbored the cattle PrP gene containing six copies of the octarepeat sequence (EMBL, X55882), and produced approximately eight times more BoPrP per gram of protein than found in the cattle brain96.

**BSE material.** Brain samples of Canadian H-BSE cattle, courteously provided by Dr. S. Czub, were used in this study31. Mouse-passaged L-BSE and C-BSE prions were also used. These prions were routinely maintained by serial passaging into TgBoPrP mice, as described previously21,22. C-BSE and L-BSE prions were adapted to TgBoPrP mice by serial passaging, and their incubation periods were approximately 190 days and 150 days, respectively. Brain samples from C-BSE, L-BSE, and H-BSE-affected cattle were also used10,32,33.

**Mouse transmission study.** Brain tissues from BSE-affected animals were homogenized in nine volumes of phosphate buffered saline (PBS) using a multi-bead shocker (Yasui Kikai) and centrifuged at 1,000×g for 5 min at room temperature (RT). Three-week-old female TgBoPrP mice were inoculated intracerebrally with 20 μl supernatant. Following inoculation, clinical status of the mice was monitored daily to assess the onset of neurological signs. The brains of diseased mice were removed and stored at -80°C for biochemical analysis or fixed for histopathological examination (i.e., BSE confirmatory tests).

**Cattle transmission study.** Three female 3–4-month-old Holstein calves were challenged intracerebrally with 1 ml of 10% brain homogenate of BSE-SW-affected TgBoPrP mice, as described previously10. Animals were euthanized before axia deterioration. The brains of diseased cattle were removed and stored at -80°C for western blotting analysis or fixed for pathological examination (i.e., BSE confirmatory tests).

**Extraction of PrPSc from BSE-affected TgBoPrP mice and cattle.** Brain tissues were homogenized in PBS (20% homogenate, w/v) using a multi-bead shocker. The brain homogenate (125 μl) was mixed with an equal volume of buffer containing 4% (w/v) Zwittergent 3–14 (Calbiochem), 1% (w/v) Sarkosyl, 100 mM NaCl, and 50 mM Tris–HCl (pH 7.6), and incubated with 6.25 μl of 40 mg/ml collagenase solution. The samples (50 μg/ml for mice brain tissues, and 40 μg/ml for cattle brain tissues) were then subjected to PK (Roche Diagnostic) digestion at 37°C for 1 h to detect PK-resistant PrPSc fragments (PrPcore). PK digestion was terminated with 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc; Roche Diagnostic). The samples were mixed with equal volumes of 2-butanol:methanol mixture (5:1) and centrifuged at 20,000×g for 10 min. The pellets were resuspended in gel-loading buffer containing 2% (w/v) SDS, and were then boiled for 10 min before western blotting. After PK treatment, some samples were deglycosylated with N-glycosidase F (PNGaseF; New England Biolabs), following the manufacturer's instruction.

**Antibodies.** The following monoclonal antibodies (mAbs) against PrP were used in this study: P4 (R-Biopharm), 6H4 (Prionics), SAF84 (SPI-bio), and F99/97.6.1 (VMRD). MAb P4 recognizes amino acid residues 101–107 of bovine PrP sequence34. MAbs 6H4 and SAF84 recognize amino acid residues 156–16335 and 175–18036, respectively, of the bovine PrP sequence. F99/97.6.1 recognizes C-terminus of PrP, amino acid residues 229–23210.

**Western blot analysis.** Samples were separated by SDS-PAGE and blotted electrically onto a PVDF membrane (Millipore). The blotted membrane was incubated with mAbs P4, 6H4, and SAF84 at RT for 1 h. MAb binding was detected by horseradish peroxidase-conjugated anti-mouse IgG. Signals were developed with a chemiluminescent substrate (SuperSignal; Pierce Biotechnology).

**Neuropathology, immunohistochemistry, and PET-blot analysis.** Histopathological analysis of TgBoPrP mice and cattle was performed according to a previously described method10,16,21. Briefly, the brains were fixed in 10% buffered formalin solution (pH 7.4) containing 10% methanol. The formalin-fixed brains were immersed in 98% formic acid, and embedded in paraffin wax. Sections (4 μm thick) were cut and stained with hematoxylin and eosin (HE). The lesion profile was determined by scoring the vacuolar changes in nine standard grey matter areas, as previously described37. For PrPSc immunohistochemistry (IHC), sections were
incubated with mAbs SAF84 or F99/97.61, followed by incubation with anti-mouse universal immunoperoxy-
dase polymer (Histofine Simple Stain MAX-PO (M), Nichirei) as the secondary antibody, and visualized using 3,3′-diaminobenzidine tetrachloride as the chromogen. Finally, the sections were counterstained with hemato-
des polymer (Histofine Simple Stain MAX-PO (M), Nichirei) as the secondary antibody, and visualized using 3,3′-diaminobenzidine tetrachloride as the chromogen. Finally, the sections were counterstained with hemato-
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Conformational stability assay. Conformation stability assay was performed according to a previously described method with minor modification35. Briefly, 50 μl of 10% brain homogenate were added to an equal volume of guanidine hydrochloride (GdnHCl), concentration range 0-8 M. Mixed samples were incubated at 37 °C for 1h. The samples were diluted by the addition of 850 μl Tris buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton-X, and 0.5% deoxycholate. Following this, 50 μl GdnHCl were added to each sample to obtain 0.4 M final concentration. Next, the samples were digested with 20 μg/ml PK at 37 °C for 1h. PrPSc concentration and western blot analysis were carried out as described above. Conformational stability was examined using mAbs H64 and SAF84. Denaturation curves were obtained by densitometric analysis using Fluorochem soft-

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**Author Contributions**

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**Additional Information**

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