Short Communication

Accumulation of Mono-Glycosylated Form-Rich, Plaque-Forming PrPSc in the Second Atypical Bovine Spongiform Encephalopathy Case in Japan

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(Received March 23, 2007. Accepted June 1, 2007)

SUMMARY: The recent identification of several atypical cases of bovine spongiform encephalopathy (BSE) has raised the possibility of the existence of distinct strains of BSE agents, arguing against the previous notion that BSE is caused by a single strain. To date, at least two atypical types (L and H) of agent have been reported based on the molecular sizes of the proteinase K-resistant forms of prion protein (PrPSc). These atypical agents were identified first in Japan, Italy, France, and Germany, and later in other European countries. Here, we have identified a case of BSE in a 169-month-old cow (designated as BSE/JP24), in which predominant deposition of the mono-glycosylated form of PrPSc was observed by Western-blot analysis, and plaques of PrPSc were detected in the brain by immunohistochemical analysis. The glycoform ratio of PrPSc was different from that of the typical BSE agent, in which the di-glycosylated form is dominant; instead, the ratio resembled that of type-2 human sporadic Creutzfeldt-Jakob disease and that reported for the L-type BSE. The characteristic glycoform ratio and plaques of PrPSc suggested that the agent in BSE/JP24 was relevant, if not identical, to the agent in bovine amyloidotic spongiform encephalopathy (BASE), an L-type BSE identified in Italy. It was of interest that at the level of the obex, the medulla oblongata was devoid of plaques of PrPSc, and a pathological phenotype similar to that of typical BSE specimens with vacuolations and coarse granular/linear deposition of PrPSc were observed.

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are fatal neurodegenerative disorders that cause severe pathological spongiform changes to the brain, with an accumulation of the disease-causing isoform of prion protein (PrPSc) (1). The experimental results to date support that PrPSc could be the infectious prion agent in TSEs (1,2), although this conclusion remains controversial. PrPSc is a conformer of host-encoded cellular prion protein (PrPC). While PrPC is susceptible to proteolytic digestion by protease K, PrPSc is partially resistant to PrPSc (K) (1). Human prion diseases are known as sporadic, genetic, or infectious disorders, e.g., sporadic Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, kuru, iatrogenic CJD, and variant CJD (vCJD) (1,3). In ruminants, the diseases emerge as scrapie in sheep and goats (1), bovine spongiform encephalopathy (BSE) in cattle (1,4), and chronic wasting disease (CWD) in elk and deer (1).

In human prion diseases and scrapie, a diversity of “prion strains” has been identified, and these strains are associated with the phenotypic variations among diseases in terms of both pathology and biochemistry (1,5). For example, variation is observed in incubation time, the distribution and severity of lesions, and the biochemical properties of PrPSc deposited in the central nervous system of affected hosts. In contrast, experimental transmission of BSE agent into laboratory mice has yielded a uniform lesion profile in the brain, with invariable incubation time, irrespective of the use of different sources of BSE agent (6). The lesion profile and the incubation time in these mice were also basically identical to those of mice inoculated with human vCJD prion (6). In addition, PrPSc derived from BSE cattle and from vCJD patients showed equivalent predominance of the di-glycosylated form of PrPSc over the mono-glycosylated and non-glycosylated forms, according to Western blot (WB) analysis (7). These results strongly suggest that BSE is caused by a single strain of agent, and that the intake of the BSE agent was the most likely cause of human vCJD (1,6,7). However, in recent years, several atypical BSE agents have been reported that exhibit distinct PrPSc migration profiles on WB analysis, as compared to those of the typical BSE agent. Consequently, these atypical cases have raised concerns that more than a single strain is causative of BSE. The atypical BSE agents identified to date are classified into two types, namely, the L- (8-9) and H-types (9-11). These two types were distinguished by the apparent molecular sizes of the non-glycosylated forms of PrPSc according to WB analysis (8-11); in particular, the L-type agent isolated in Italy was distinguished by the formation of amyloid plaques of PrPSc in the central nervous system (the case was designated as bovine amyloidotic spongiform encephalopathy; BASE) (8). Among the cattle flocks in Japan, we previously found an atypical PrPSc in a Holstein steer (referred to as BSE/JP8 (Ibaraki)) (12). The PrPSc in this case showed faster migration of the non-glycosylated form of PrPSc by WB analysis, as was also observed with the L-type agent (12). Here, we report another case identified recently in a Japanese cow.

Samples taken from a female Japanese Black (beef cattle) slaughtered in Nagasaki Prefecture (Sasebo Meat Inspection Center) on March 13, 2006, gave a positive signal on the Plateria ELISA kit (BioRad, Hercules, Calif., USA) upon
routine BSE examination performed in abattoirs in Japan (details of this scheme are described in references 12-14). The cow, referred to as BSE/JP24 (Sasebo), was born on February 10, 1992 and was 169-month-old at death. In the abattoir, the cow exhibited clinical symptoms of dysstasia. After detection of a positive signal in the ELISA assay, specimens of medulla oblongata, cerebral cortex, cerebellum, eyeballs, tonsil, adrenal glands, kidney, and lymph nodes were sent to our laboratory (National Institute of Infectious Diseases) for confirmation of the case. Other tissues, including the brain stem, were incinerated in the abattoir and could not be retrieved.

When the PrPSc in the medulla oblongata at the level of the obex was analyzed by WB analysis using an anti-prion protein antibody, 44B1 (14,15), we unexpectedly found that PrPSc in BSE/JP24 gave a predominant signal intensity corresponding to the mono-glycosylated form of PrPSc (Fig. 1A, lane 4). Judging from the signal intensities, the relative amounts of di-, mono-, and non-glycosylated forms were at an approximate ratio of 35:40:25 (Fig. 1C). In contrast, the amount of di-glycosylated PrPSc in typical BSE cases has been found to account for approximately 70% of the total amount of PK-resistant PrPSc (Fig. 1A, lanes 1-3, 5, and 6 and Fig. 1C). The glycoform ratio of PrPSc in BSE/JP24 was reproduced using an anti-prion protein antibody, 6H4 (Fig. 1B); in addition, substantially the same glycoform ratio of PrPSc as that observed in the medulla oblongata was detected in the cerebral cortex, the cerebellum, and the retina. On the other hand, neither atypical nor typical PrPSc was detected in the tonsil, the adrenal glands, the kidney, or the lymph nodes under the analytical conditions used to examine these tissues of BSE/JP24 (data not shown). The distinct glycoform ratio of PrPSc in BSE/JP24 resembled that of BASE, identified in Italy (L-type BSE) (8), and that of type-2 human sporadic CJD (5,8,16). The non-glycosylated form of PrPSc derived from the BASE case (L-type BSE) showed faster migration than that derived from typical BSE cases examined by WB analysis (8); however, the non-glycosylated form of PrPSc from BSE/JP24 exhibited mobility similar to that of typical BSE cases, as far as we examined.

Immunohistochemical (IHC) study using anti-prion protein antibody, T4 (14,17), led to the detection of plaques of PrPSc in some of the brain sections (Fig. 2A and B). Furthermore, histochemical staining with 1-fluoro-2, 5-bis(3-carboxy-}

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**Fig. 1.** Comparison of PrPSc of BSE/JP24 with those of the representative typical BSE cases found in Japan. (A) The homogenates prepared from the medulla oblongata at the level of the obex were digested by proteinase K according to the method previously described (14). The samples were subjected to WB analysis by using NuPAGE Bis-Tris Gels (Invitrogen, Carlsbad, Calif., USA) and PrPSc was detected by using the antibody 44B1 (15), HRP-labeled anti-mouse IgG (GE Healthcare, Uppsala) and ECL-Plus Chemiluminescent reagent (GE Healthcare). The data were captured by LAS-3000mini luminescent image analyzer (FUJIFILM, Tokyo, Japan). The specimens are indicated by the serial codes (BSE/JP) given to the BSE-affected cattle in Japan, together with the ages of the cattle in the parentheses. (B) WB analysis of PrPSc by using the antibody 6H4 (Prionics, Schlieren, Switzerland). (C) The relative amounts of the di-, mono- and non-glycosylated forms of PrPSc in BSE/JP6 (a representative case for typical BSE) and in BSE/JP24 determined in (A), together with BSE/JP8 (the first atypical case in Japan; data adapted from the previous report [12]).

**Fig. 2.** Histopathological and IHC analysis of BSE/JP24. (A and B) The plaques of PrPSc in the brain detected by the IHC analysis using the antibody T4 (14,17). (C) Hematoxylin and eosin (HE)-staining of the medulla oblongata at the level of the obex by the method previously described (14). Severe vacuolations in dorsal nucleus of the vagus, nucleus of the solitary tract and nucleus of the spinal tract were observed. (D) IHC analysis of the corresponding area to (C) by using the antibody T4 showed the coarse granular and linear deposition of PrPSc. (E) HE-staining of the retina. (F) IHC analysis of the retina by the antibody T4. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments.
4-hydroxystyryl)benzene, a fluorescent dye used to detect amyloid plaques (Dojindo Laboratories, Kumamoto, Japan) (18,19), gave positive signals that corresponded with the PrP-immunopositive amyloid plaques. The specificity of the dye for staining amyloid plaques was confirmed in a parallel examination of senile plaques in a human brain specimen used as a positive control, which resulted in comparable staining (data not shown). Features of BSE/JP24 such as the predominance of monoclycosylated PrPSc and the deposition of PrP-immunonegative amyloid plaques are similar to those observed in BASE (8). Unfortunately, the brain of BSE/JP24 (except for the medulla oblongata) had disintegrated in the abattoir prior to specimen collection. This unfortunately hampered the accurate assignment of sections to specified regions in the brain. Intact regions including the thalamus, hypothalamus, and hippocampus were not available for examination, and we therefore were unable to determine whether or not the PrPSc plaques occurred in these regions. On the other hand, histopathological examination of the medulla oblongata at the level of the obex revealed severe vacuolation in the dorsal nucleus of the vagus, the nucleus of the solitary tract, and the nucleus of the spinal tract (Fig. 2C). In addition, IHC analysis using the antibody T4 revealed granular and linear deposition of PrPSc, rather than amyloid plaques of PrPSc, in these areas (Fig. 2D). Such histopathological features are similar to those of typical BSE cases (8,10,13), although the granular staining of PrPSc seemed somewhat coarser than that in typical BSE. Intense accumulation of PrPSc was also detected by IHC analysis in the ganglion cell layer, and in the inner and outer plexiform layers of the retina (Fig. 2E and F).

To examine the DNA sequence, 1.2-kbp fragment of DNA including the PrP coding region was amplified by PCR from the genomic DNA of BSE/JP24. The PCR product was then subjected directly to sequence analysis (20). We compared the results with a reference sequence in a public database (accession no. AJ298878) (21), and identified two silent mutations, one at Gln78 (homozygous CAG), and one at Asn192 (homozygous AAT). These mutations have been recognized as common polymorphic variations in cattle breeds in Japan (22), and they appear to have no significant association with BSE susceptibility (Table 1).

From September 2001 to February 2007, 32 cases of BSE were identified in Japan by the national BSE screening and surveillance programs (13). The first atypical PrPSc detected in Japanese cattle was found in an apparently healthy 23-month-old Holstein steer born in October of 2001 (BSE/JP8) (12). Due to the scarce amounts of PrPSc deposited in BSE/JP8, we were unable to accomplish further biochemical characterization of the first atypical PrPSc. An experiment challenging the transmissibility of BSE/JP8 to transgenic mice (TgBoPrP), which expresses bovine PrP (23) and are highly susceptible to BSE agent (24), has been carried out; the results of that study will be published elsewhere (25). The second atypical PrPSc described in the present study was found in an elderly cow (BSE/JP24) born in 1992. BSE/JP8 and BSE/JP24 were kept on the farms located far apart, thus, it is unlikely that the two cases had either an epidemiological or a geographical relationship. In addition, BSE/JP8 and BSE/JP24 exhibit different biochemical features of PrPSc in WB analysis, suggesting no correlation between BSE/JP8 and BSE/JP24 in terms of the biochemistry of prion diseases. Instead, the biochemical features of PrPSc and the pathological phenotypes of BSE/JP24 were similar, if not identical, to those of a case of BASE previously identified in Italy. It was of note that IHC analysis of the BASE case in Italy did not reveal the deposition of PrPSc in the dorsal nucleus of the vagus (8), whereas examination of the medulla oblongata of BSE/JP24 at the level of the obex showed the histopathological and IHC features similar to those observed in typical BSE cases (Fig. 2C and D).

Prior to detection of the BSE/JP24 case, it was assumed that the epidemic of BSE in Japan had begun no earlier than

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Table 1. Codon variations in PrP-coding region of BSE/JP24 and several specimens

<table>
<thead>
<tr>
<th>Case (cattle in serial number; month of slaughter)</th>
<th>Age in month</th>
<th>Breed</th>
<th>Codon variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>the second atypical case (BSE/JP24* (Sasebo); Mar. 2006)</td>
<td>169</td>
<td>Japanese Black</td>
<td>CAG/AAT</td>
</tr>
<tr>
<td>the first atypical case (BSE/JP8 (Ibaraki); Sep. 2003)</td>
<td>23</td>
<td>Holstein (steer)</td>
<td>CAG/AAC</td>
</tr>
<tr>
<td>typical BSE (BSE/JP12; Sep. 2004)</td>
<td>62</td>
<td>Holstein</td>
<td>CAG/AAC</td>
</tr>
<tr>
<td>typical BSE (BSE/JP5; Aug. 2002)</td>
<td>80</td>
<td>Holstein</td>
<td>CAG/AAC</td>
</tr>
<tr>
<td>typical BSE (BSE/JP6; Jan. 2003)</td>
<td>83</td>
<td>Holstein</td>
<td>CAA/AAC</td>
</tr>
<tr>
<td>typical BSE (BSE/JP10; Feb. 2004)</td>
<td>95</td>
<td>Holstein</td>
<td>CAG/AAC/T</td>
</tr>
<tr>
<td>typical BSE (BSE/JP13; Sep. 2004)</td>
<td>103</td>
<td>Holstein</td>
<td>CAG/AAC</td>
</tr>
<tr>
<td>healthy (Aug. 2003)</td>
<td>18</td>
<td>Holstein</td>
<td>CAA/AAC</td>
</tr>
<tr>
<td>healthy (Dec. 2004)</td>
<td>26</td>
<td>mixed</td>
<td>CAA/AAC</td>
</tr>
<tr>
<td>healthy (Nov. 2004)</td>
<td>27</td>
<td>Japanese Black</td>
<td>CAG/AAAT</td>
</tr>
<tr>
<td>healthy (Dec. 2004)</td>
<td>32</td>
<td>Japanese Black</td>
<td>CAG/AAC/T</td>
</tr>
<tr>
<td>healthy (Oct. 2004)</td>
<td>71</td>
<td>Holstein</td>
<td>CAG/AAC</td>
</tr>
<tr>
<td>healthy (Jan. 2003)</td>
<td>142</td>
<td>Holstein</td>
<td>CAG/AAC/T</td>
</tr>
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</table>

The DNA fragments encoding PrP were amplified by using Easy-A™ High Fidelity PCR Cloning Enzyme (Stratagene, La Jolla, Calif., USA) and a set of the primers; 5'-TGGCGGCAAGGGTTAGTACAT-3' and 5'-TGGCGGCAAGGGTTAGTACAT-3'. The reaction was started by heating at 94°C for 4 min, then, processed by 25 cycles of PCR at 94°C for 45 s, 60°C for 1 min and 72°C for 1 min. The direct sequencing of the PCR products was carried out by using the above primers and BigDye® Terminators (Applied Biosystems, Foster City, Calif., USA). No codon variation except Gln78 and Asn192 was detected in comparison with the reference sequence (AJ298878).

* The serial numbers of the BSE cases in Japan.
may provide clues to the origin(s) of these atypical BSE-like or human type-2 sporadic CJD in laboratory mice (16,26), that the pooled BSE prion agent(s) propagate as either BSE-10). This observation, taken together with findings showing JP24 case, were all found in cattle born in the early 1990s (8-10). This observation, taken together with findings showing that the pooled BSE prion agent(s) propagate as either BSE-like or human type-2 sporadic CJD in laboratory mice (16,26), may provide clues to the origin(s) of these atypical BSE agents. A transmission study of the PrPSc found in BSE/JP24 is underway, and the partial results of that study are forthcoming (Y. Matsuura et al., in preparation).

ACKNOWLEDGMENTS

We thank to the abattoir and the Meat Inspection Center of Sasebo-city for the collection of the BSE/JP24 specimen. We also thank to the other members of The Expert Committee for BSE Diagnosis, Ministry of Health, Labour and Welfare, Japan for the diagnosis of the specimens. This work was supported by a grant for BSE research from the Ministry of Health, Labour and Welfare of Japan (17270701 to K.H., Y.Y. and T.S.), and a Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (17659024, K.H.).

REFERENCES