Biological and biochemical characterization of M2B cells: Classical BSE prion is conserved in transgenic mice overexpressing bovine prion protein gene

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ABSTRACT. M2B cells with persistent classical bovine spongiform encephalopathy (C-BSE) have been established previously. In this study, we performed strain characterization of the M2B cell line in bovine PrP\textsuperscript{C} overexpressing mice (Tg 1896). Mice intracranially inoculated with M2B cells and C-BSE survived for 451 ± 7 and 465 ± 31 d post inoculation, respectively. Although biochemical properties, including deglycosylation and conformational stability, differed between M2B cells and C-BSE, inoculation with M2B cell lysate and C-BSE resulted in comparable phenotypes. Comparable vacuolation scores and PrP\textsupersc{Sc} depositions were observed in the brain of Tg 1896 inoculated with both M2B cell lysate and C-BSE. Our results show that biochemical and biological characteristics of M2B cells and C-BSE are classifiable in the same strain.

KEYWORDS. BSE, mouse bioassay, M2B cell, prion, PrP\textsupersc{Sc}, strain characterization

INTRODUCTION

Bovine spongiform encephalopathy (BSE) is one type of transmissible spongiform encephalopathies (TSEs) causing fatal neurodegenerative diseases in animals.\textsuperscript{1} The origin of BSE has not been fully identified; however, meat and bone meals derived from

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Received March 16, 2017; Revised May 4, 2017; Accepted May 10, 2017.
cattle with BSE are regarded as a major route of BSE transmission. Moreover, the agent causing BSE may be identical to that causing variant Creutzfeldt-Jakob disease in humans.2,3

TSEs strains can be defined on the basis of differences in the conformation, glycosylation ratio, protease resistance, and aggregation state of the core of PrPSc.4-9 In addition, mice inoculated with TSEs can serve as strain characterization and provide reliable indicators for incubation periods and vacuolar degenerative patterns, referred to as lesion profiles.10-12 Recently, rare atypical forms of BSE have been reported as active surveillance progressed.13-15 These atypical forms were divided in accordance with Western blot analysis of PrPSc profiles. One of these atypical forms showed lower molecular size (L-BSE) and the other one showed higher molecular size (H-BSE) when compared with the classical type of BSE (C-BSE).14,15 Both, molecular and biochemical properties, including incubation periods, PrPSc profiles, and histopathology, support that L-BSE and H-BSE were differentiated from C-BSE.16 Conceivably, different TSEs strains exhibit different biological and pathological profiles in the central nervous systems.

The mouse bioassay is the gold standard to identify biological properties of TSEs strains. However, there are major disadvantages to this method, such as the long incubation periods required (at least several months even in transgenic mice overexpressing Bovine PRNP) and the number of animals killed.11,17,18 Therefore, considerable effort has been focused on replacing the animal experimental model with an in vitro assay using cell lines susceptible to BSE. Unfortunately, the number of studies investigating cell line with persistent TSE is limited. Rather, mouse-derived models (including a microglial cell line established from PrPSc overexpressing mice, peripheral neuroglial cell line, and murine neuroblastoma cell line) have been intensely investigated.4,19,20

Previously, a cell line with persistent C-BSE (M2B cells) has been established using Madin-Darby Bovine Kidney (MDBK) cells overexpressing bovine cellular prion protein through a lentiviral expression system.21 However, biochemical properties of the M2B cell line, including the glycoform ratio and molecular weight of deglycosylated PrPSc, were different from biochemical properties of C-BSE. To utilize M2B cells in prion studies, it is necessary to confirm that the PrPSc in M2B cells originates from C-BSE.

We hypothesized that PrPSc in M2B was identical to that in BSE. To address this question, we compared histopathological findings in mouse brains inoculated with M2B cell lysates and homogenates of cattle brain with C-BSE. Furthermore, we present biochemical properties of PrPSc of M2B cells by comparing glycoform ratio, deglycosylation, and conformational stability of PrPSc. On the basis of these criteria, we verified that M2B cells had persistent C-BSE, and then it contributes to using M2B cells for therapeutic development, a study on pathogenesis and metabolic interaction.

RESULTS

Survival Times and Clinical Signs in Inoculated Mice

Attack rates and survival times of transgenic bovine mice inoculated with M2B cell lysates (Tg M2B) and homogenates of brains with C-BSE (Tg C-BSE) are shown in Table 1. Both Tg M2B and Tg C-BSE developed the disease during survival times and produced some common clinical signs, such as rough hair coat, sticky eye, and hunched back. Mice reaching the terminal stage of prion disease were killed before showing convulsion, paralysis, and death. Survival times of Tg M2B and Tg C-BSE are shown in Table 1.

| Classification          | Inocula          | Attack Rate | Survival times (dpi) |
|-------------------------|------------------|-------------|----------------------|
| Treatment               | M2B cell lysates | 10/10       | 451 ± 7              |
| Positive control        | C-BSE            | 4/4         | 465 ± 31             |
| Negative control        | C1-2F cell lysates | 0/4         | 480*                 |
|                         | Normal bovine brain | 0/4         | 480*                 |

*Sacrificed
BSE were 451 ± 7 d post inoculation (dpi) and 465 ± 31 dpi respectively. No significant difference between the distributions was observed in an unpaired, 2-tailed t-test (p < 0.05). Both groups showed 100% attack rates. No inoculum specific clinical signs were observed in this assay. Negative controls showed no clinical signs or death.

Pathological Findings in Inoculated Mice

Lesion profiling of Tg M2B and Tg C-BSE was performed using hematoxylin and eosin staining (H&E). When using a semiquantitative method for lesion profiling, vacuolation scores of Tg M2B were comparable to those of Tg C-BSE (Fig. 1). Both groups showed moderate to severe vacuolar degeneration in dorsal medullar nuclei (G1), central thalamus (G5), and septal nuclei (G7). In addition, relatively severe vacuolar degeneration was observed in mesencephalic tegmentum (W2) compared with other white matter lesions (Fig. 2). Less than 10 vacuoles were observed in cerebellar cortex (G2), superior colliculus (G3), hypothalamus (G4), posterior cerebral cortex (G8), anterior cerebral cortex (G9), cerebellar white matter (W1), and pyramidal tract (W3). The patterns of PrPSc depositions of Tg M2B were analogous to those of Tg C-BSE in immunohistochemistry (Fig. 3). Accumulation of PrPSc was observed multifocally in dorsal medullar nuclei, central thalamus and partially in posterior cerebral cortex, hippocampus, and hypothalamus. Both Tg M2B and Tg C-BSE showed some multifocal plaque-like deposition of PrPSc in the central thalamus.

Biochemical Properties of PrPSc

Biochemical properties of PrPSc (molecular weights and relative antibody immunoreactivity) in Tg M2B and Tg C-BSE were compared on the basis of immunoblotting and glycoform ratios. As shown in Fig. 4A, when samples were not treated with proteinase K (PK), both C-BSE and Tg C-BSE showed identical migration patterns. Tg M2B showed comparable patterns with C-BSE and Tg C-BSE; however, M2B cells showed different migration patterns both with and without PK treatment.

To compare PrPSc glycoform ratios in the different preparations, the relative immunoreactivity of diglycosylated, monoglycosylated, and unglycosylated PrPSc was analyzed. The relative immunoreactivity of Tg M2B and Tg C-BSE did not differ significantly at p < 0.05 (Fig. 4B). PrPSc glycoform ratios in both samples were similar to those of C-BSE, although the amount of unglycosylated PrPSc was slightly lower in M2B cells than in the other samples.

To analyze the molecular weight of the PrPSc core, all samples were treated with PNGase F. In Tg C-BSE and Tg M2B molecular weight of the PrPSc core was similar, although there was a slight change in the electrophoretic migration of deglycosylated PrPSc in M2B cells compared with that in C-BSE (Fig. 5). In addition, migration patterns of deglycosylated PrPSc from both Tg C-BSE and Tg M2B were more similar to
migration patterns of C-BSE than migration patterns of M2B cells. All PNGase F treated samples were completely deglycosylated.

In the conformational stability assay, resistance to increasing concentrations of GdnHCl was determined. After PK digestion, GdnHCl\(^{-}\) induced unfolding of PrP\(^{Sc}\) was observed in GdnHCl concentrations of 3.5~4M in all samples (Fig. 6A); the percentage of PrP\(^{Sc}\) decreased as GdnHCl concentration increased. Relative conformational stability was compared on the basis of the GdnHCl concentration at which half of the PrP\(^{Sc}\) was denatured (GdnHCl\(_{1/2}\)). M2B cells and C-BSE showed different denaturation curves and different GdnHCl\(_{1/2}\) values, 1.38 ± 0.04 M and 1.90 ± 0.10 M, respectively. However, Tg M2B and Tg C-BSE showed similar conformational stability, as neither denaturation curves nor GdnHCl\(_{1/2}\) values (2.31 ± 0.06 M and 2.37 ± 0.07 M, respectively) significantly differed at \(p < 0.05\) (Fig. 6B).

**DISCUSSION**

M2B cells with persistent C-BSE have been established previously\(^{21}\) and the M2B cell strain has been characterized in conventional VM/Dk mouse (unpublished). However, whether M2B cells and C-BSE induce the same neurodegenerative disease in cattle remained to be elucidated. Transgenic mouse models are useful tools to indirectly characterize the effect of M2B cells in cattle. Although the measurement of survival times is expensive and time-consuming, they are an important indicator for the disease: Survival times depend on PrP\(^{Sc}\) deposition lesions and neurodegenerative disease progress, which are caused by PrP\(^{Sc}\) inoculation.\(^{11}\) The survival times of Tg M2B mice and Tg C-BSE were 451 ± 7 dpi and 465 ± 31 dpi, respectively (Table 1). No significant difference was observed when comparing survival times after M2B cell lysate and C-BSE inoculation. However, Tg M2B showed shorter survival times of about 2 weeks. Different doses
of different inocula might influence survival times. Therefore, we used semi-quantitative titration by enzyme-linked immunosorbent assay (ELISA) to reduce the difference between inocula. Furthermore, in primary passage, the same strain of PrP<sup>Sc</sup> may result in different survival times.<sup>22</sup>

Tg M2B and Tg C-BSE showed similar lesion profiles, consistent with previous reports on lesion profiles of Tg mice treated with C-BSE in the UK (APHA, unpublished data). In our study, both groups of inoculated mice showed higher scores for dorsal medullar nuclei (G1), central thalamus (G5), septal nuclei (G7), and mesencephalic tegmentum (W2) lesions than for other lesions (Fig. 3). Depositions of PrP<sup>Sc</sup> detected by immunohistochemistry were consistent with lesion profiles. Taken together, these results indicate that the accumulation of PrP<sup>Sc</sup> caused vacuolar degeneration. In addition, both Tg M2B and Tg C-BSE showed comparable multifocal plaque-like depositions in the central thalamus (G5). Strain specific pathological properties of C-BSE were reproduced in M2B cells. However, different biochemical features were observed between C-BSE and M2B cells in biochemical analysis. First, immunoreactive bands of M2B cells were conserved after PK digestion, while immunoreactive bands of C-BSE were diminished after PK treatment. This possibly resulted from disturbed PK digestion due to some endogenous extracts in the M2B cell condition. This phenomenon has also been observed in mouse neuroblastoma cells with persistent scrapie.<sup>4</sup> In addition, other endogenous enzymes could have affected M2B cells before PK digestion. PK acts on the PK cleavage site (β-cleavage site) and after digestion of the C-terminal, the PK-resistant core of PrP<sup>Sc</sup> remains.<sup>23</sup> However, the other cleavage site (α-cleavage site) resides closer the C-terminal than the β-cleavage site. This digestion causes shorter residues of PrP<sup>Sc</sup>. Several enzymes have generally been identified including ADAM family,

FIGURE 3. Immunohistochemistry of Tg M2B and Tg C-BSE using S1 antibody. PrP<sup>Sc</sup> deposition in the central thalamus in thalamic sections of Tg M2B (A) and Tg C-BSE (C), small window: Plaque like depositions of PrP<sup>Sc</sup>. PrP<sup>Sc</sup> deposition in the dorsal medullar nuclei in medulla section of Tg M2B (B) and Tg C-BSE (D).
plasmin and plasminogen. These enzymes might be activated during cell culture or the cell lysis procedure.

Secondly, PrP$^C$ appears in 3 different glycoforms, un-, mono- and diglycosylated form. Glycosylation happens in the endoplasmic reticulum by the attachment of glycan to the N-linked glycosylation site. The 3 different glycoforms showed tissue-specific biochemical properties. Because the M2B cell line originated from the MDBK cell line (derived from kidney epithelium), it makes sense that the structure of prion proteins differs between brain tissue and M2B cells. Conclusively, these results were compatible with previous reports that persistently infected cell lines preserved properties of PrPSc in serial passages.

Previous studies have shown that migration patterns of the PrPSc core fragment after digestion with PK represent strain specific properties (strain characterization). However, recent studies have shown that biochemical properties such as glycoform profiles and molecular size of deglycosylated PrPSc were not conserved in persistently infected cells. Furthermore, murine GT1 cells persistently infected with different Creutzfeldt-Jakob disease agents showed identical PrPSc migration patterns. Taken together, the conventional strain characterization method has certain limitations in persistently infected cells. Thus, a mouse bioassay was essential for strain characterization of M2B cells. Future studies should include in vitro bioassays of prion strain characterization to overcome limitation of mouse bioassays such as being time-consuming and posing ethical and financial problems.

In summary, the present study revealed biochemical and histopathological similarities of PrPSc obtained from M2B cells and C-BSE in transgenic mouse. The present data demonstrates the suitability of M2B cells as a tool to investigate prion diseases. However, it remains unclear why M2B cells, which showed different biochemical properties from C-BSE, induced the same pathological changes, and eventually recovered original properties. Further investigation of this phenomenon of a structural shift of PrPSc in cell cultures is necessary.

**MATERIAL AND METHODS**

**Inocula Preparation**

An M2B cell line with persistent C-BSE has been established in a previous study. MDBK cell line (C1–2F) overexpressing bovine PRNP gene at an advance stage of persistent infection, was prepared as a negative control. M2B cells were cultured in a biosafety level III laboratory. Permission has been obtained from the Korea Centers for Disease Control and Prevention. The cells were grown in completed medium

![FIGURE 4. Western blot analysis and relative glycoform ratio of PrPSc. (A) C-BSE and mouse brain homogenates infected with C-BSE and M2B cells show different patterns before and after treatment with proteinase K. In contrast, M2B cells maintained the same pattern before and after digestion with proteinase K. (B) Relative proportion of PrPSc detected as diglycosylated (Di), monoglycosylated (Mono), unglycosylated (Un) PrPSc. Error bars, SEM.](image)
Dublecco’s modified Eagle’s medium/F12 supplemented with 10% fetal bovine serum, 100 μg/ml antibiotics (penicillin and streptomycin), non-essential amino acid, L-glutamine.

Inocula were prepared from the M2B cell lysates at 35th passage in saline (3 × 10⁷ cells/ml). As a positive control, 10% (w/v) cattle brain homogenates with C-BSE (APHA, UK) were prepared in normal saline. MDBK C1–2F cell lysates (3 × 10⁷ cells/ml) and 10% (w/v) normal cattle brain homogenates in saline were prepared as negative controls. Cell lysates were homogenized by freezing and thawing 3 times.

Mouse Inoculation and Sampling

Transgenic Tg 1896 mice overexpressing bovine PRNP gene were obtained from the Animal and Plant Health Agency (APHA), and were kept under specific-pathogen-free (SPF) conditions during the experiments. All procedures involving mice were approved by Animal Ethics Committee (AEC), Animal and Plant Quarantine Agency (APQA) under the Animal Protection Act 1991 (No. APHA-AEC-2016–449). Before injection, the genetic background of all transgenic mice was assessed by polymerase chain reaction using bovine PRNP primers. Eight-week-old mice were anaesthetized with isofluorane/O2 and inoculated intracranially with 20 μl of the each inoculum. Ten mice were inoculated with M2B cell lysates. For positive and negative controls, 4 mice were inoculated respectively.

Inoculated mice were housed in isolators placed in the animal care facility of the biosafety level III laboratory. Health status and clinical signs were observed daily. Clinical signs included rough hair coat, sticky eyes, emaciation, hunched back, limb paresis, convulsion, and depression. When a mouse showed more than 3 of these symptoms over one week, euthanasia and necropsy were performed. One half of each brain was fixed in 10% neutral buffered formalin for histopathology and the other half was homogenized at 10% (w/v) concentration in normal saline for biochemical assays. Survival times were defined as the number of days from inoculation to the terminal stage of the disease. Statistical analysis was performed using the GraphPad-Prism software.

Western Blotting

Brain homogenates prepared in saline were mixed with 15 μl of PrPSc lysis buffer, composed of PBS containing 0.5% TritonX-100, 5 mM EDTA, 150 mM NaCl, 0.05% Digitonin (Sigma-Aldrich) and complete mini protease inhibitors (Roche Applied Science). The samples were mixed with 5 μl collagenase (20 mg/ml) and 8 μl of DNase I (10 mg/ml) and incubated for 1 h at 37°C. To digest PrPC, 8 μl of PK (1 mg/ml) was added and incubated at 37°C for 1 h. The digested samples were mixed with 8 μl of pefabloc and centrifuged at 20,000 g for 15 min. After discarding the supernatant, 20 μl of 4% sodium dodecyl sulfate (SDS) was added. Samples were boiled at 100°C for 5 min, and 400 μl of pre-cooled methanol were added. Samples precipitated overnight at −20°C. After precipitation, dried pellets were collected after 10 min of centrifugation at 20,000 g. Dried pellets were boiled at 100°C for 5 min in 20 μl of SDS sample buffer and separated by electrophoresis at 100 V for 90 min in NuPAGE 12% Bis-Tris gel. Proteins were transferred onto PVDF membranes (Immonilon P, Merk Millipore) in transfer buffer at 400 mA for 90 min. Membranes were blocked with 0.02% I-Block (Tropix) in tris-
buffered saline (TBS) for 30 min and rabbit anti-PrP polyclonal antibody S1 was applied (produced by the QIA). Immunoreactive bands were developed with CDP STAR (Applied Biosystems) and analyzed using an LAS 4000 chemiluminescence system (Fuji). Prior to the analysis, PrPSc quantities were equalized by serial loading volumes. Glycoform profiles (i.e., ratio of diglycosylated, monoglycosylated and unglycosylated PrPSc) were analyzed by ImageQuant TL 1D gel analysis (Fuji).

Deglycosylation of PrPSc

To remove N-linked glycosylation, PNGase F (New England BioLabs) was used. 5 μl of brain homogenate were mixed with 15 μl of PrPSc lysis buffer and 4 μl of PK (1 mg/ml) and incubated at 37°C for 1 h. The incubated samples were centrifuged at 20,000 g for 1 h and 8 μl of 30% sarkosyl was added. After discarding the supernatant, samples were dissolved in 10 μl of glycoprotein denaturation buffer and heated at 100°C for 10 min. After cooling heated samples, 2 μl of 10% NP40, 2 μl of 10 × GlycoBuffer, 2 μl of PNGase and 4 μl of distilled water were added. The samples were incubated overnight at 37°C. Subsequently, samples were incubated with 20 μl of SDS sample buffer at 100°C for 5 min. Samples were separated and blotted following a described previously western blotting method.

Conformational Stability

Five microliters of a brain homogenate were mixed with 15 μl of PrPSc lysis buffer and 1 μl of PK (10 mg/ml) and incubated at 37°C for 1 h. Guanidine hydrochloride solution (GdnHCl) at concentrations ranging from 0 to 4 M (final concentration) were added and incubated at 20°C for 1 h while shaking gently. Subsequently, samples were centrifuged at 20,000 g for 1 h and 8 μl of 30% sarkosyl were added. After discarding the supernatant, samples were boiled at 100°C for 5 min, 400 μl of precooled methanol were added, and samples were allowed to precipitate overnight at −20°C. After precipitation, dried pellets were collected.

FIGURE 6. Conformational stability of PrPSc. (A) Western blotting of the samples. The amount of PrPSc was inversely proportional to the concentration of GdnHCl in all samples. (B) Fraction of PrPSc as a function of GdnHCl. PrPSc from M2B cells showed 1.38 ± 0.04 M at GdnHCl1/2 and PrPSc from C-BSE showed 1.90 ± 0.10 M at GdnHCl1/2. Conformational stability of PrPSc was comparable in Tg M2B and Tg C-BSE brain homogenates. GdnHCl1/2 values were 2.31 ± 0.06 M and 2.37 ± 0.07 M, respectively. Error bars, SEM.
by centrifugation at 20,000 g for 10 min. Dried pellets were boiled at 100°C for 5 min in 20 μl SDS sample buffer. Samples were separated and blotted according to described previously western blotting methods. Statistical analysis was performed using the GraphPad-Prism software.

**Histopathology**

Mouse brains were fixed in 10% neutral buffered formalin and immersed in 88% formic acid for 1 h to inactivate PrPSc. After tissue processing and paraffin embedding, serial 5 μm thick sections were stained with H&E. The pattern of vacuolar changes was scored in 12 areas, namely the dorsal medullar nuclei (G1), cerebellar cortex (G2), superior colliculus (G3), hypothalamus (G4), central thalamus (G5), hippocampus (G6), septal nuclei (G7), posterior cerebral cortex (G8), anterior cerebral cortex (G9), cerebellar white matter (W1), mesencephalic tegmentum (W2), and pyramidal tract (W3). The vacuolation score was established based on the pattern, size, and density of the vacuoles using standard criteria (grade 0 for no and grade 5 for maximum vacuolation).11

**Immunohistochemistry**

Tissue sections were dewaxed, rehydrated with routine pretreatments including 30-min hydrated autoclaving at 121°C in 500 ml of antigen-retrieval solution (DAKO). A polyclonal rabbit anti-PrP antibody (S1), biotinylated anti-rabbit secondary antibody, alkaline phosphatase-streptavidin conjugate, and 3-amino-9ethylcarbazole substrate were used for immunolabeling. Immunostaining was performed using a semi-automated immunostaining machine (BenchMark, Roche).

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

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