Coexistence of two forms of disease-associated prion protein in extracerebral tissues of cattle infected with H-type bovine spongiform encephalopathy

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ABSTRACT. H-type bovine spongiform encephalopathy (H-BSE) is an atypical form of BSE in aged cattle. H-BSE is characterized by the presence of two proteinase K-resistant forms of disease-associated prion protein (PrPSc), identified as PrPSc #1 and PrPSc #2, in the brain. To investigate the coexistence of different PrPSc forms in the extracerebral tissues of cattle experimentally infected with H-BSE, immunohistochemical and molecular analyses were performed by using N-terminal-, core-region- and C-terminal-specific anti-prion protein antibodies. Our results demonstrated that two distinct forms of PrPSc coexisted in the various extracerebral tissues.

KEYWORDS: atypical BSE, extracerebral tissue, H-type, peripheral nervous system, prion

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Bovine spongiform encephalopathy (BSE) belongs to a group of prion or transmissible spongiform encephalopathy diseases and is a fatal, progressive degenerative disorder of the central nervous system (CNS) in cattle. The disease is characterized by the development of spongiform changes and accumulation of an abnormal isoform of a prion protein (PrPSc) that is thought to represent a post-translational modification of the normal, host-encoded cellular prion protein (PrP) principally found in the CNS of affected hosts [22]. Currently, BSE is classified into at least three different strains comprising food-borne-related or epidemic BSE, named classical BSE, and putatively sporadic forms of H- or L-type atypical BSEs on the basis of the molecular mass of the proteinase K (PK)-resistant PrPSc [3, 6].

According to western blot (WB) analyses, the molecular characteristics of H-BSE isolates from cattle consist of two forms of PK-resistant PrPSc: PrPSc #1 and PrPSc #2 [4, 11]. Since glycosylated C-terminal PK-resistant PrPSc #2 results from a C-terminal truncation at a position between amino acid residues Ser154 and Glu163 of bovine PrP following PK digestion, detection of PK-resistant PrPSc #1 is possible using either N-terminal-specific antibodies, such as P4 (bovine PrP epitope 101WGQGGSH107), or core-region-specific antibodies, such as F89/160.1.5 (148PLIHFGSD155), 12F10 (154SDYEDRYYRE163) and 6H4 (155DYEDRYYRE163). After deglycosylation, the unglycosylated form of PK-resistant PrPSc #1 was detected as a 19 kDa molecule, which was capable of interactions with various anti-prion protein (PrP) antibodies, while that of PK-resistant PrPSc #2 was detected as a 10–12 kDa fragment recognizable by C-terminal region-specific antibodies, such as SAF84 (175RPVDQY180) or F99/97.6.1 (229YQRE232).

The pathologic and molecular features of experimental H-BSE in cattle have already been described [1, 9, 10, 13, 14, 19]. Previous studies revealed that minimal quantities of PrPSc accumulation could be detected in the extracerebral tissues of experimental H-BSE cattle by immunohistochemistry (IHC) using C-terminal-specific monoclonal antibodies (mAbs) [13, 15]. The different truncated forms of PrPSc depended upon the cell-specific proteolytic cleavage and cell- and tissue-specific PrPSc conformational differences [12]. However, characteristics associated with accumulated PrPSc in the extracerebral tissues of H-BSE-infected cattle remain unclear. The aim of this study was to examine the IHC-staining and molecular properties of PrPSc in cattle experimentally infected with H-BSE.

Animal experiments were approved by the Institutional Animal Care and Use Committee at the National Institute of Animal Health (approval ID: 07-9). Experimental transmission of H-BSE to cattle has previously been described (case No. 1, code 7749; case No. 2, code 9458; and case No. 3, code 0728) [19]. Two calves inoculated with 10% normal brain homogenates prepared from healthy cattle served as controls and were sacrificed at the age of 35 months. The tissue samples examined in this study were as follows: brains, spinal cords (C8 or L6), spinal nerves (C8 or L6), dorsal root ganglia (C8 or L6), cauda equina, trigeminal ganglion, retinas, optic nerves and adrenal glands. Histopathologic analysis was performed on hematoxylin and eosin (HE)-stained tissue sections. After epitope retrieval [17], the deposition of PrPSc was determined by IHC using four mAbs, namely P4 (0.2 µg/ml; R-Biopharm, Darmstadt, Germany), F89/160.1.5 (0.1 µg/ml; SPI-bio, Montigny Le Bretonneux, France), 12F10 (0.1 µg/ml; SPI-bio) and F99/97.6.1 (0.1 µg/ml; VMRD, Pullman, WA, U.S.A.), followed by administration of the TSA-biotin system (PerkinElmer, Boston, MA, U.S.A.) according to previously described protocols [16]. For WB analysis, tissue samples were dissolved in sodium
dodecyl sulfate polyacrylamide gel electrophoresis sample buffer and assayed using standard WB procedures. PK-resistant PrPSc signals were detected with mAbs P4 (0.2 µg/ml), 6H4 (0.2 µg/ml; Prionics, Schlieren, Switzerland) and SAF84 (0.2 µg/ml; SPI-bio), followed by incubation with a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Rockford, IL, U.S.A.) according to a previously described method [18].

In accordance with IHC results described previously [7, 18], intracellular (intraneuronal and intraglial types) and extracellular (particulate, granular, stellate and plaque-like types) PrPSc deposits were immunolabeled with mAb F99/97.6.1. However, immunolabeling intensity reflecting the detectable PrPSc in the brain and extracerebral tissues varied with the antibodies tested (Figs. 1 and 2). In general, intracellular PrPSc deposits in the Schwann cells of the cauda equina or spinal nerves, the ganglion cells and satellite cells of ganglia, the ganglion cells of the retina, and the glial cells of the optic nerve that were immunolabeled with mAb F99/97.6.1 exhibited stronger signals, while those labeled with mAb 12F10 exhibited weak to moderate signals, respectively (Fig. 2). Immunolabeling intensity and patterns of PrPSc with mAb F89/160.1 were similar to those observed with mAb 12F10 (data not shown). Additionally, granular deposits of PrPSc were detected at the periphery of axons, referred to as adaxonal PrPSc deposits, with mAb F99/97.6.1 [15], but these were not detectable using other
antibodies (data not shown). In the adrenal gland, immuno-
labeled PrPSc was located between chromaffin cells, which
participate in intercellular processes involving sympathetic
neurons (Fig. 2). In the retina, granular PrPSc accumulation
was pronounced in the ganglion cell layer, as well as the
inner and outer plexiform layers, using both C-terminal-
and core-region-specific antibodies (Fig. 2). Interestingly,
faint intraglial staining was seen with mAb P4 in the optic
nerve, but mAb P4 did not yield any immunoreactions in
other tissues, such as the trigeminal and dorsal root ganglia,
cauda equina and spinal nerves (Fig. 2). By conventional
WB analysis, all antibodies used in this study resulted in
signals detected in both the brain and all extracerebral tis-
sues (Fig. 3). However, the signal intensity with mAb P4 in
the peripheral nervous tissues, i.e., the trigeminal ganglion
and cauda equina, was apparently weaker than that observed
with mAb 6H4. Moreover, the multiple banding patterns of
PrPSc #1 and PrPSc #2 with an additional 10–12 kDa band
were detected in these tissues with mAb SAF84 (Fig. 3).
Additionally, a smaller 7 kDa fragment was identified in the
cauda equina and optic nerve using mAb P4 (arrowhead in
Fig. 3).

Infectivity of the cauda equina from case 1 was assessed
by intracerebral injection into bovine PrP-overexpressing
(TgBoPrP) mice as described previously [18]. Although
the incubation period of TgBoPrP mice inoculated with the
cauda equina from case 1 was 354.9 ± 84.5 days (n=8) and
appeared to be longer than that for mice inoculated with the
brain tissue from the original cattle (315.8 ± 11.6 days, n=10)
[18], no significant difference was observed in the mean in-
cubation periods analyzed using Instat3 software (GraphPad
Software; La Jolla, CA, U.S.A.), indicating that the cauda
equina exhibited a high level of infectivity. Additionally,
the unique clinical signs, the vacuolar lesion scores in HE-
stained sections, the neuroanatomic distribution patterns of
immunolabeled PrPSc and the molecular features of PrPSc
in the brains of TgBoPrP mice inoculated with the cauda
equina were identical to those observed in TgBoPrP mice
inoculated with the brain homogenate from H-BSE-infected
cattle, which constituted the original cattle inoculum (data
not shown) [18].

The present study demonstrated that two different forms
of PrPSc, PrPSc #1 and PrPSc #2, could propagate both in the
CNS and in extracerebral tissues analyzed by WB. However,
IHC was unable to discriminate between PrPSc #1 and PrPSc
#2 using different antibodies. The strain-specific molecular
features of H-BSE characterized by the presence of an addi-
tional 10–12 kDa fragment detected with mAb SAF84 were
maintained in the extracerebral tissues tested in this study
(Fig. 3). Peripheral PrPSc accumulation is commonly thought
to result from centrifugal spreading by nerve pathways dur-
during the clinical stage of the disease [2]. The presence of higher
PrPSc levels in the optic nerve relative to other peripheral
nervous tissues according to IHC and WB analyses could be
attributed to the optic nerve being involved with the CNS
rather than the peripheral nervous system. Detection of two
PrPSc forms in the sympathetic terminal nerve endings of the
adrenal medulla or Schwann cells of the peripheral nerves
may be attributed to a high degree of peripheral neurotro-
pism in H-BSE [13–15, 18]. Low PrPSc levels were detected
by IHC in the muscle spindles of skeletal muscles of cattle
terminally affected with H-BSE [13]. In general, PrPSc levels
in the peripheral tissues were believed to be much lower than
those found in the brain of animals at the terminal stage of
the disease.

Immunolabeling intensities of PrPSc in extracerebral tis-
sues were different from those observed using other antibod-
ies. The C-terminal mAb F99/97.6.1 immunolabeled a wide
variety of different PrPSc stains and always returned the best
results [19]. Moreover, immunoreactivity of mAb P4 for

![Fig. 3. Proteinase K-resistant PrPSc profiles of H-BSE prions analyzed with different mAbs (P4, 6H4 and SAF84). The arrowhead indicates a ~7 kDa fragment detected with mAb P4. The arrow indicates an additional 10–12 kDa band detected with mAb SAF84. Each number in parenthesis indicates tissue equivalent (mg) loading. Molecular markers are shown to the left (kDa).](image-url)
PrPSc was apparently weaker than that of the core-specific PrP antibodies 12F10 or 6H4 according to both IHC and WB results (Figs. 1–3). Minimal levels of PrPSc were detected with mAb P4 in the optic nerve, but not in other tissues (Fig. 2). The differences in immunoreactivity observed from the N-terminal and core-specific PrP antibodies may be explained by variations in antibody affinities and immunodetection procedures, including epitope retrieval and reagents [7–12]. Since antigen-epitope retrieval might involve specific amino acids in specific locations, the chemical-based and/or autoclaving-antigen unmasking techniques used in this study could be insufficient at denaturing the PrPSc protein, which would hinder immunodetection with mAb P4 by IHC [8]. Therefore, we might exclude the possibility that mAb P4 would have a weaker affinity for PrPSc according to IHC relative to other antibodies used in this study [7].

A C-terminal 10–12 kDa fragment detected with C-terminal-specific antibodies in H-BSE may be the result of enzymatic truncation and digestion occurring at a site distant from residue 163 of bovine PrP [3, 4]. Similarly, the presence of a 10–12 kDa low molecular-weight PK-resistant C-terminal fragment in the brain was reported in various forms of Creutzfeldt-Jakob disease (CJD) in humans [5, 23, 25]. Interestingly, the C-terminal fragment was associated with non-plaque-type dura CJD [23]. In H-BSE, an additional ~7 kDa fragment detected with mAb P4, but not with mAb SAF84, by WB analysis was distinct from the 10–12 kDa fragment. This ~7 kDa fragment might be derived from the cleaved N-terminal product from the full-length PrPSc, PrPSc#1, resulting from PK digestion between amino acids of aggregated PrP Sc in the extracerebral tissues of H-BSE-fragments might be strongly associated with the deposition of PrP-amyloid plaques in the brain [4, 19] and the presence of aggregated PrPSc in the extracerebral tissues of H-BSE-infected animals [15].

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