NOTE *Pathology*

The Presence of Disease-Associated Prion Protein in Skeletal Muscle of Cattle Infected with Classical Bovine Spongiform Encephalopathy

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ABSTRACT. The aim of this study was to investigate the presence of disease-associated prion protein (PrP^{Sc}) in the skeletal muscle of cattle infected with classical bovine spongiform encephalopathy (C-BSE). The study was carried out systematically in 12 different muscle samples from 43 (3 field and 40 experimental) cases of C-BSE; however, muscle spindles were not available in many of these cases. Therefore, analysis became restricted to a total of 31 muscles in 23 cattle. Even after this restriction, low levels of PrP^{Sc} were detected in the muscle spindles of the masseter, intercostal, triceps brachii, psoas major, quadriceps femoris and semitendinosus muscles from 3 field and 6 experimental clinical-stage cases. The present data indicate that small amounts of PrP^{Sc} are detectable by immunohistochemistry in the skeletal muscles of animals terminally affected with C-BSE.

KEY WORDS: BSE, muscle spindle, prion, skeletal muscle.

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Classical bovine spongiform encephalopathy (C-BSE) in cattle is a fatal neurodegenerative disorder belonging to a group of transmissible spongiform encephalopathies (TSEs). C-BSE was first identified in the United Kingdom in 1986 [18] and subsequently spread to Europe, North America and Japan. The disease is characterized by spongiform changes and the accumulation of a disease-associated abnormal form of prion protein (PrP^{Sc}) in the central nervous system. PrP^{Sc} is commonly accepted as the pathological agent of TSEs and may be a post-translationally modified form of a normal cellular prion protein (PrP^{C}) [14]. The C-BSE agent is transmissible to various mammalian species. For example, the variant form of Creutzfeldt-Jakob disease (CJD) in humans likely resulted from consumption of C-BSE-contaminated foodstuff [19]; therefore, issues regarding C-BSE and variant CJD have increased public health concerns regarding the safety of meat products used for food.

A low level of infectivity was identified in the semitendinosus muscle of a clinically C-BSE-affected field case through a mouse bioassay using highly sensitive transgenic mice overexpressing bovine PrP^{C} [3]. Recently, PrP^{Sc} deposition in skeletal muscles of cattle experimentally infected with atypical BSE was visualized by immunohistochemistry (IHC) [8, 15]. The detailed topological distribution of PrP^{Sc} in the muscular tissues of C-BSE-infected cattle, however, remains unclear [2]. Here, we describe the localization of immunolabeled PrP^{Sc} in the skeletal muscles of cattle with natural and induced C-BSE infections.

All animal handling and experimental protocols were approved by the Institutional Animal Use and Care Committee of both the National Institute of Animal Health and Hokkaido Animal Research Center prior to the experiments being carried out.

This study was carried out in 43 C-BSE-infected cattle including 3 naturally-infected fallen stock cases (BSE/JP17, 21 and 22), 28 orally dosed cattle (6 clinically and 22 preclinically) and 12 intracerebrally administered cattle (9 clinically and 3 preclinically) as previously described in detail [4, 12, 13]. As a control, 4 uninoculated Holstein calves were used, 2 of which were sacrificed at the ages of 27 months and 104 months.

Twelve skeletal muscle samples, including the masseter muscle, pectoral muscle, intercostal muscle, triceps brachii muscle, longissimus thoracis muscle, psoas major muscle, gluteus medius muscle, quadriceps femoris muscle, semitendinosus muscle, diaphragm and tongue (apex and dorsum), were chosen for sampling as these are most frequently consumed by people in Japan. Each muscle sample was collected from each animal at necropsy and cut into 2 pieces, one of which was fixed in 10% buffered formalin solution (pH 7.4) for hematoxylin and eosin (H&E) staining and IHC, while the other was frozen at −80°C for western blotting (WB). IHC for PrP^{Sc} was performed using a mouse monoclonal antibody (mAb) F99/97.6.1 (VMRD, Pullman, WA, U.S.A.) by the 2-step polymer method (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan) or tyramine signal amplification (TSA-biotin system; PerkinElmer, Boston, MA, U.S.A.)

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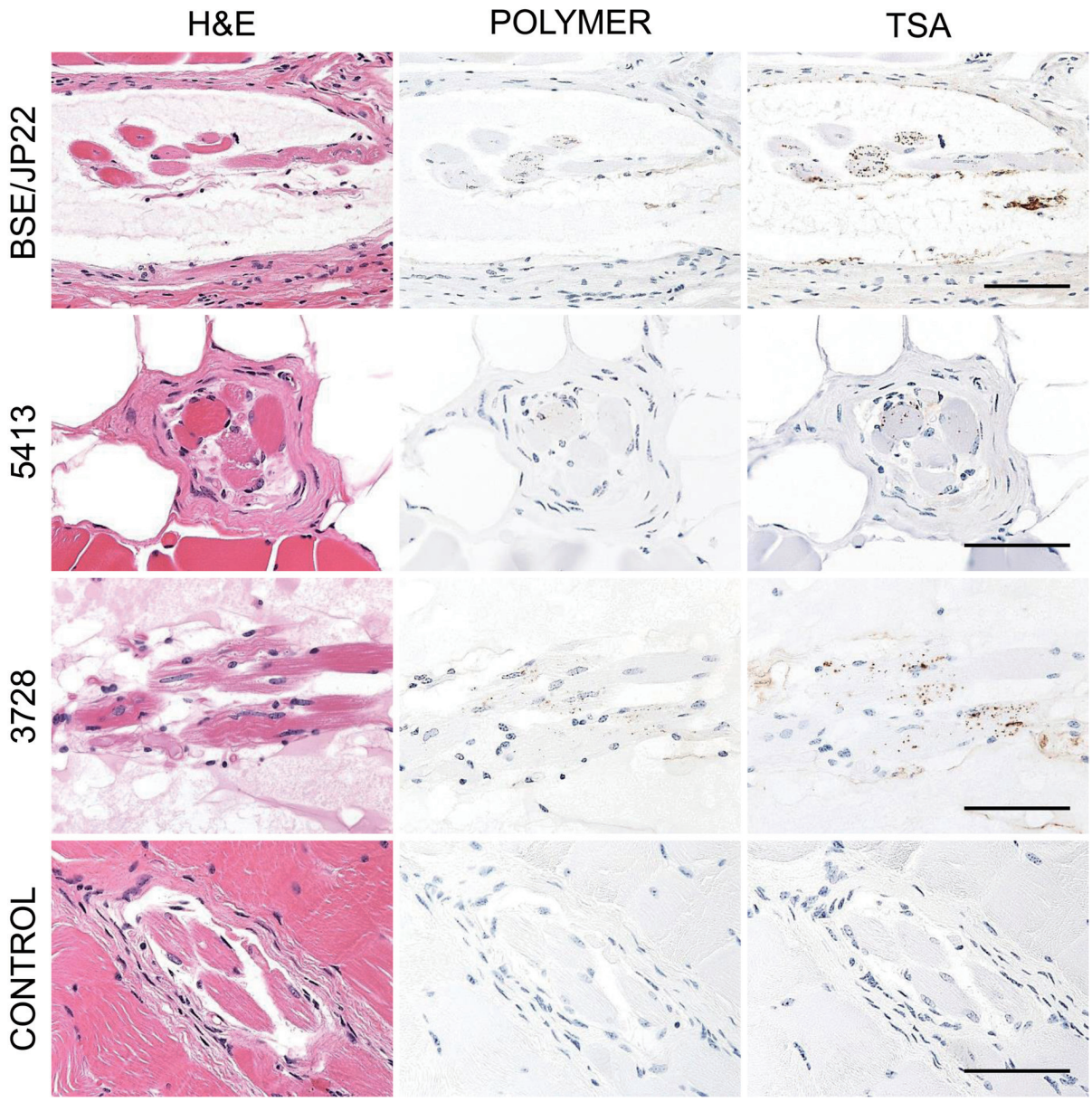

Fig. 1. Sections of skeletal muscles from cattle infected with classical BSE. Detection and localization of PrP^{Sc} in muscle spindles of the triceps from a field case (BSE/JP22), quadriceps from an orally-infected case (ID#5413), psoas major from an intracerebrally infected cattle (ID#3728) and pectoralis (control). Granular PrP^{Sc} immunolabeling (brown) was observed in intrafusal myofibers. Depicted are H&E staining (left column) and PrP^{Sc} staining by IHC using the conventional 2-step polymer (center column) or the TSA biotin system (right column). Bar=50 μm.

[11]. The biochemical properties of PK-resistant PrP^{Sc} were analyzed by WB using mAb T2 as described in detail elsewhere [10].

PrP^{Sc} was only detected in muscle spindles, while no other structures of the muscle samples showed reactivity by IHC. Specifically, PrP^{Sc} immunolabeling was not detected in myofibrils, intramuscular nerve fascicles or in most ganglia of tongue muscular tissues for all muscle samples examined. The PrP^{Sc} immunolabeling intensity was weak using the conventional 2-step polymer method, and the TSA method gave the best results (Fig. 1). The morphological appearance of PrP^{Sc} presented as granular or dot-like deposits. Although the study was carried out systematically in 12 skeletal muscles including tongues and diaphragm of 43 C-BSE-infected cattle, muscle spindles were not available in many of these samples. Therefore, the study was restricted to analysis on H&E-stained sections of 23 animals (Table 1). The total number of muscle sampled owing to usable spindles was

Table 1. Immunohistochemical detection of PrP^{Sc} in the skeletal muscle of 23 natural and experimental BSE cases

Animal ID	*Inoculation*	*MPI*	*Disease status*	*Muscular tissue*							
				MM	*PM*	*IM*	*TBM*	*LM*	*PMM*	*QFM*	*SM*
BSE/JP17	Field	54*	undefined	+							
BSE/JP21	Field	69*	undefined				+				+
BSE/JP22	Field	64*	undefined				+				+
0886	IC	18	preclinical			–					
3728	IC	21	clinical	+					+	+	
4612	IC	24	clinical				+				
5536	PO	18	preclinical		–						
1936	PO	20	preclinical						–		
1945	PO	20	preclinical			–					
1952	PO	20	preclinical		–						
6416	PO	24	preclinical		–			–			
8104	PO	24	preclinical	–							
9787	PO	30	preclinical	–							
4008	PO	30	preclinical	–							
7342	PO	30	preclinical					–			
2072	PO	30	preclinical		–						
5529	PO	36	preclinical		–						
5468	PO	42	clinical	+							
5598	PO	46	preclinical			–					
5550	PO	58	clinical	–							+
5420	PO	60	preclinical	–							
5413	PO	66	clinical	+		+	+				
5444	PO	84	clinical			+					

MPI, months post-inoculation; IC, intracerebral; PO, per os; MM, masseter muscle; PM, pectoral muscle; IM, intercostal muscle; TBM, triceps brachii muscle; LM, longissimus muscle; PMM, psoas major muscle; GMM, gluteus medius muscle; QFM, quadriceps femoris muscle; SM, semitendinosus muscle. –, negative in muscle bundles by IHC; +, positive in muscle bundles by IHC. Blank cells mean that no muscle spindles have been detected in the muscle samples used in this study. Asterisks in MPI denote the age that the animal died.

31. No muscle spindles were detected in the gluteus medius muscle, diaphragm or tongue. Even after this restriction, the global frequency of immunolabeled PrP^{Sc} detection was 9 (3 natural and 6 experimental) of 23 cases. Positive immunolabeling was detected in 16 of the 31 muscle samples containing spindles, including the masseter muscle (4/9; number of positive samples/number of detected samples), intercostal muscle (2/5), triceps brachii muscle (4/4), psoas major muscle (1/2), quadriceps femoris muscle (1/1) and semitendinosus muscle (3/3). In experimentally challenged animals, PrP^{Sc} detection was associated with clinical symptoms, but not with preclinical status. No positive spindles were observed in the 14 preclinical and 4 control animals. By WB analysis, a very weak signal for PrP^{Sc} was detected in 2 different muscle samples from naturally (masseter muscle from BSE/JP17) and experimentally (intercostal muscle from ID#5413) C-BSE-infected animals (Fig. 2). However, a detectable PrP^{Sc} signal was obtained from only 1 of 3 tissue pieces; adjacent locations were used for PrP^{Sc} immunohistochemistry within the same tissue in each case.

The results of this study indicate that low amounts of PrP^{Sc} are deposited in the muscle spindles of C-BSE-infected cattle. To the best of our knowledge, this is the first, or at least the most comprehensive, report on the localization of PrP^{Sc} by IHC in skeletal muscles of C-BSE affected cattle. Localization of PrP^{Sc} in the muscle spindles of skeletal muscles in the study was consistent with that of atypical BSE in cattle [8] and natural scrapie in sheep [1]. In addition, immunolabeled PrP^{Sc} localized at the terminal nerve endings of myofibrils has been reported in hamsters with scrapie and [16] in rodent and primate models with C-BSE and CJD, respectively [6, 9, 17].

PrP^{C} is primarily expressed in neural tissues, but is also distributed in non-neural organs and tissues, such as the spleen, lymph node, heart, skeletal muscle, kidney, uterus, adrenal gland, intestine and mammary gland [7]. In muscle tissues, PrP^{C} is located primarily at the neuromuscular junction [5]. Somatic motor neurons known as efferent nerve fibers arise from the ventral horn of the spinal cord and innervate skeletal muscle tissues at the neuromuscular junction via alpha motor neurons or intrafusal muscle fibers of the muscle spindles via gamma motor neurons. In addition, type Ia afferent sensory fibers connect to the muscle spindles. However, there are no precedents for a primary role for sensory neural pathways in the pathogenesis of BSE [10]. PrP^{Sc} accumulation in the peripheral nervous tissues may be attributed to a high degree of neurotropism in C-BSE [3, 10]. Our results indicate the centrifugal spread of the infectious agent from central nervous tissues through the somatic motor and/or sensory pathways to the muscle spindles of various muscle tissues during the clinical stage of the disease in both naturally occurring and experimentally-induced C-BSE

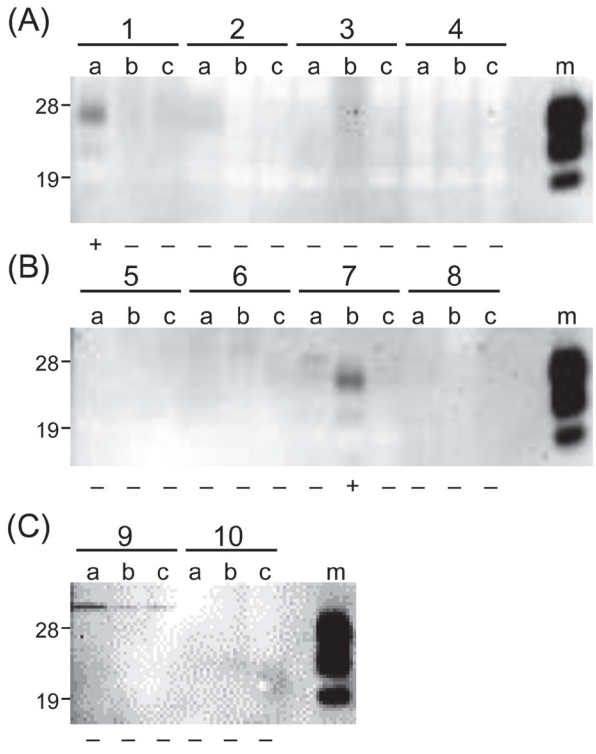

Fig. 2. Western blot analysis of proteinase K-digested PrP^{Sc} in skeletal muscle samples of cattle infected with natural and experimental C-BSE. Detection of PrP^{Sc} in the skeletal muscle samples from cattle (lane 1–3, BSE/JP17; lane 4, BSE/JP22) with natural BSE (A), cattle (ID#5413) with experimental C-BSE through an intracerebral route (B) and cattle (ID#5466) with experimental C-BSE through an oral route (C). The muscle tissues tested are indicated above the lanes: 1, masseter muscle; 2, semitendinosus muscle; 3, triceps brachii muscle; 4, semitendinosus muscle; 5, masseter muscle; 6, triceps brachii muscle; 7, intercostal muscle; 8, quadriceps femoris muscle; 9, triceps brachii muscle; and 10, intercostal muscle. Lanes a, b and c represent the results of triplicate samples. The equivalent of 100 mg of tissue was loaded in each lane. Western blots were probed with a monoclonal antibody T2 to detect PrP^{Sc}. +, positive; −, negative. Lane m, mouse scrapie-infected brain was used as the positive control (1.6 μg brain eq.). Molecular markers are shown on the left (in kDa).

animals.

Although we did not attempt a mouse bioassay to demonstrate infectivity in skeletal muscles, small amounts of PrP^{Sc} were detected in the skeletal muscles of C-BSE cattle in the clinical stage of the disease. The study suggests that it is important to investigate the presence of PrP^{Sc} in muscle tissues of C-BSE-affected cattle using immunohistochemical analysis.

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