NOTE

Pathology

Electrophysiological and histopathological findings of muscular disease suspected as myotonic dystrophy in a Shiba dog

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ABSTRACT. An 8-year-old male Japanese Shiba exhibited muscle wasting and a stiff gait. A low-amplitude myotonic discharge was recorded by needle electromyography (EMG). A histopathological examination on a tru-cut biopsy sample from the muscle revealed myofiber size variations. Internal nuclei and cytoplasmic vacuoles were observed in many fibers. A type 1 fiber predominance and many hybrid type fibers were observed immunohistochemically. On the basis of these EMG and histopathological findings, myotonic dystrophy (DM) was suspected as tentative diagnosis. The cytoplasm around the vacuoles was immunopositive for cytochrome c, tom 20, and SOD-1, suggesting that these vacuoles might occur within mitochondria. Collectively, these results indicate that a mitochondrial abnormality partly play the role on the pathogenesis of present case.

KEY WORDS: dog, immunohistochemistry, mitochondria, muscle, myotonic dystrophy

Myotonic dystrophy (DM) is an autosomal dominant disorder that is characterized by myotonia in association with muscle weakness and wasting. Two forms of DM (DM1 and DM2) that are caused by defects in two different genes have been identified in human cases [2, 5, 10]. In veterinary medicine, equine DM or muscular dystrophy with myotonia has been often reported [6, 9, 11, 15]. There have been several case reports of possible DM in dogs [3, 4, 12, 17, 18], while pathological findings have not been documented in detail. We observed a Shiba dog with a type of DM similar in most respects to human DM. The clinical and histopathological features of our case were described herein, and immunohistochemical features were compared with those of human DM in order to elucidate the pathogenesis.

An 8-year-old male Japanese Shiba exhibited muscle wasting, a stiff gait, and difficulty with walking for a long period of time. During gait, the forelimb and hindlimb muscles could be seen to persist in a state of continuous contraction, which might last after the animal stopped moving. The dog also showed little facial expression and dropped head during gait and rest. The concentration of serum creatine kinase (CK) was markedly elevated (1,791 U/l; reference range: 92–357). Magnetic resonance imaging (MRI) of the head and neck revealed high intensity in the cervical multifidus muscle in T1- and T2-intensified images. MRI examination was not performed in other muscles. Electrophysiological examinations were conducted with a polygraph (Neuropack MEB-9404, Nihon Koden, Tokyo, Japan). Sensory and motor nerve conduction velocities (SNCV and MNCV, respectively) in the tibial and ulnar nerves were within normal ranges. F-wave conduction velocity and F-wave persistence in the tibial and ulnar nerves were also normal in the F-wave examination. A decrementing response was not detected in a repetitive nerve stimulation. Reduced insertion activity and a low-amplitude myotonic discharge were recorded in the cervical multifidus muscle using needle electromyography (EMG) (Fig. 1). The dog died fourteen days after the electrophysiological and MRI examinations. Necropsy was not permitted.

A tru-cut biopsy sample from the cervical multifidus muscle was obtained and fixed in 10% neutral-buffered formalin for a histopathological examination. The tissue was processed routinely and embedded in paraffin wax. Two-micrometer-thick tissue sections were stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS). Immunohistochemistry was performed using the following primary antibodies: anti-slow myosin (clone NOQ7.5.4D; 1:4,000; Sigma-Aldrich, Tokyo, Japan), anti-canine fast myosin (rabbit polyclonal; 1:100; Sigma-Aldrich), anti-nestin (rabbit polyclonal; 1:20; Immuno-Biological Laboratories, Fujioka, Japan), anti-myogenin (clone F5D; 1:50; Dako, Tokyo, Japan), anti-cytochrome c (clone A-8; 1:200; Santa Cruz, Dallas, TX, U.S.A.), anti-tom 20 (rabbit polyclonal; 1:200; Santa Cruz), anti-SOD-1 (rabbit polyclonal; 1:200; Abcam, Cambridge, U.K.),

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Fig. 1. Needle electromyography (EMG) recorded from the cervical multifidus muscle shows myotonic discharges. Amplification by needle insertion (the left most) and discharges (center to right) are low.

Fig. 2. Cervical multifidus muscle; Shiba dog. Size variations in myofibers, the loss of myofibers, fat infiltration, and internal nuclei. Small angular fibers are occasionally observed (arrows). H&E. Bar, 100 μ m.

Fig. 3. Cervical multifidus muscle; Shiba dog. Severely atrophied myofibers with pyknotic nuclear clumps (arrows). H&E. Bar, 30 μ m.

anti-LC3 (rabbit polyclonal; 1:3,000; Cell Signaling Technology, Danvers, MA, U.S.A.), anti-ubiquitin (clone 10C2-2; 1:100; Abcam), anti-p62 (rabbit polyclonal; 1:1,000; MBL, Nagoya, Japan), anti-NBR 1 (rabbit polyclonal; 1:200; Proteintech, Rosemont, IL, U.S.A.), and anti-active caspase 3 (rabbit polyclonal; 1:200; Promega, Tokyo, Japan). Normal control specimens of canine multifidus muscles were selected from dogs without clinical signs of myopathy.

Microscopically, the cervical multifidus muscle showed a loss of myofibers, fat infiltration, myofiber size variations (atrophy and hypertrophy), and internal nuclei in many fibers (Fig. 2). Pyknotic nuclear clumps were often observed mainly in severely atrophied myofibers (Fig. 3). Vacuolization of the cytoplasm was also detected in many fibers (Fig. 4). Although small angular fibers were occasionally observed, there was no group atrophy. In the longitudinal section, internal nuclei were arranged in chains. PAS staining did not show sarcoplasmic masses and ringed fibers. The biopsy muscle sample did not contain any intramuscular peripheral nerve tissues and any histological changes were not observed in the control biopsies.

Immunohistochemistry for slow and fast myosins revealed a type 1 fiber predominance, i.e. almost all fibers in the section were immunopositive for slow myosin (Fig. 5a). Many hybrid type fibers that were immunopositive for slow and fast myosins were also observed (Fig. 5b–c). Internal nuclei were observed in all type 1, type 2 and hybrid type fibers. Severely atrophied myofibers with pyknotic nuclear clumps were immunopositive for fast myosin (Fig. 5c), but immunonegative for slow myosin and nestin. The cytoplasm around the vacuoles was immunopositive for cytochrome c (Fig. 6a), tom 20 (Fig. 6b) and SOD-1 (Fig. 6c), and also immunopositive for nestin (data not shown). All myonuclei were immunonegative for myogenin. No immunoreactivity to LC3 was found in myofibers. Ubiquitin-positive structures were observed in a few fibers and were also immunopositive for p62 and NBR 1.

Fig. 4. Cervical multifidus muscle; Shiba dog. Myofibers with cytoplasmic vacuolization. H&E. Bar, 30 μ m.

Fig. 5. Cervical multifidus muscle; Shiba dog. Most myofibers are immunopositive for slow myosin, indicating a type 1 fiber predominance. Immunohistochemistry for slow myosin. Bar, 100 μ m. (a). Most myofibers are hybrid type fibers immunopositive for slow (b) and fast (c) myosins. However, severely atrophied myofibers with pyknotic nuclear clumps are only immunopositive for fast myosin (arrows). Immunohistochemistry for slow (b) and fast (c) myosin. Bar, 100 μ m.

The cytoplasm around the vacuoles was immunonegative for ubiquitin, but immunopositive for p62 and NBR 1 (data not shown). No immunoreactivity to active caspase 3 was found.

Myotonia is often more apparent in DM1 compared with DM2 and facial and neck weakness are common in DM1 but rare

Fig. 6. Cervical multifidus muscle; Shiba dog. The cytoplasm around the vacuoles of affected myofibers are immunopositive for cytochrome c (a), tom 20 (b) and SOD-1 (c). Immunohistochemistry for cytochrome c (a), tom 20 (b) and SOD-1 (c). Bar, 15 μ m.

in DM2 [5, 10]. In the present Shiba case, little facial expression and dropped head were observed, similar to human DM1. As reported previously in horses and dogs [1, 8], a stiff or impaired gait was observed in the present Shiba case. Myotonic discharges are observed by EMG in all patients with DM1 and 90–100% of patients with DM2, and serum CK levels may be normal to moderately elevated in human cases [5]. The most common pathological features in human DM1 and DM2 are myofiber size variations and multiple internal nuclei. The lesion is progressive with a loss of myofibers and increasing amounts of fat and fibrous tissues, as reported in other muscular dystrophies [2]. Sarcoplasmic masses and ring fibers, which can be seen in particular PAS and oxidative enzyme stains, are typical features of human DM1, but they are non-specific and can occur in other myopathies [2]. In dogs and horses with DM, myotonic discharges were observed by EMG [3, 4, 6, 9, 11, 12, 15, 17, 18]. Myofiber size variations, myofiber splitting, multiple internal nuclei, occasional necrosis, fat infiltration, and fibrosis were histopathologically observed in the dogs and horses. Sarcoplasmic masses and/or ring fibers were also reported in the horses, while not in the dogs [3, 4, 6, 9, 11, 15, 17, 18]. In the present Shiba case, the pathological changes were not identical with those seen in humans and horses, but the main difference was the absence of myofiber splitting, sarcoplasmic masses and ring fibers. Although only a small muscle sample was assessed in our study, DM was suspected as tentative diagnosis on the basis of histopathological findings together with clinical signs including the results of EMG. The amplitude of the myotonic discharge was low, and was considered to be associated with the loss of myofibers and fat infiltration.

Although the presence of small angular fibers in the muscular lesion suggested denervation, there was no group atrophy, fiber type grouping or peripheral nerve abnormalities in human DM [2]. However, fiber type grouping was observed in some equine cases and a canine case [3, 6, 9, 11]. Nerve conduction is generally normal in human patients as well as in the previous cases of DM in dogs and horses [4, 5, 11, 12, 18]. Small angular fibers were observed in the present Shiba case, which is consistent with the previous human and canine cases; however, neither group atrophy nor peripheral nerve abnormalities were detected. The results of the immunohistochemical analysis revealed the absence of fiber type grouping. Collectively, these results indicate that denervation did not occur in the present Shiba case, as reported previously in the human cases; however, a histopathological evaluation of peripheral nerve tissues was not performed.

A type 1 fiber predominance accompanied by a variable degree of hybrid type fibers are common features in human DM1 and DM2, which has been only documented in the case of DM in a horse [2, 6]. The average proportion of type 1 fibers is 70% in the normal dog multifidus muscle [13], but was almost 100% in the present Shiba case. Severely atrophied myofibers with pyknotic nuclear clumps are only observed in the late stages of DM1 and early stages of DM2 in humans and atrophied fibers are immunopositive for slow and fast myosins in DM1, but for fast and fetal myosin in DM2 [2]. Severely atrophied myofibers in the present Shiba case were immunopositive for fast myosin only, similar to human DM2.

In the present Shiba case, regenerative changes such as satellite cell or myoblast proliferation and centrally located nuclei possessing prominent nucleoli were not observed. We previously reported that regenerating myofibers with centrally located nuclei in dogs are immunopositive for nestin and myogenin [16]. However, myofibers with internal nuclei in the present Shiba case were immunonegative for nestin and myogenin, indicating that these fibers were not regenerating, as has been inferred in human DM cases [2]. Although regenerating fibers in dogs are partly the hybrid type, which is immunopositive both for slow and fast myosins [16], these fibers in the present Shiba case were immunonegative for nestin and myogenin. The reason why many hybrid type fibers existed in the present Shiba case as well as in human cases currently remains unclear.

Mitochondrial abnormalities have been reported in some human DM patients and also in a mouse model of DM [7, 14]. In the present Shiba case, the cytoplasm around the vacuoles was immunopositive for mitochondrial markers such as cytochrome c, tom 20, and SOD-1. These results suggest that mitochondrial vacuolization might occur in the affected myofibers. The cytoplasm around the vacuoles was immunopositive for nestin, indicating that the accumulation of nestin is possibly associated with mitochondrial degeneration. Furthermore, there was no evidence of the abnormalities associated with autophagic and/or apoptotic pathways in the present Shiba case because no immunoreactivities to the related molecules such as LC3 and active caspase 3 were detected in myofibers. Further studies including the identification of causative genetic alterations are needed in order to elucidate

the pathogenesis of canine DM in more detail.

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