Immunohistochemical Observation of Canine Degenerative Myelopathy in Two Pembroke Welsh Corgi Dogs

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ABSTRACT. Immunohistochemistry was performed to assess whether oxidative stress and/or denatured proteins play roles in the pathogenesis of canine degenerative myelopathy (DM). Two Pembroke Welsh Corgi (PWC) dogs with a homozygous mutation (c.118G>A) in the canine superoxide dismutase 1 (SOD1) gene were examined. The pathological features of the dogs were consistent with those of previous cases of DM in PWC. In the spinal lesions, diffuse SOD1 expression was observed in the neurons while no inclusion-like aggregates had formed, which disagreed with the findings of a previous study. A unique inducible nitric oxide synthase (iNOS) staining pattern in reactive astrocytes and a significant increase in ubiquitin immunoreactivity in the spinal lesions were also observed. These findings indicate the involvement of oxidative stress and the accumulation of ubiquitinated proteins in the pathogenesis of canine DM, whereas the role of SOD1 remains unclear.

KEY WORDS: canine, degenerative myelopathy, oxidative stress, Pembroke Welsh Corgi.

Canine degenerative myelopathy (DM) is a progressive neurodegenerative disease that affects the spinal cord. Clinically, DM appears in old dogs with progressive ataxia. As dysuria and dyschezia occur in the terminal stages of the disease, euthanasia is usually opted for in the early stages [3, 5, 6, 8, 9, 12–15, 19]. German Shepherd (GS) dogs are the breed most commonly affected by DM [3, 12, 19], although other breeds, such as the Siberian Husky [6], Miniature Poodle [14] and Boxer [15] dogs are also reported to be affected. Recently, DM has been reported in Pembroke Welsh Corgi (PWC) dogs [8, 13].

Several causes of DM have been proposed, including immunologic [5, 19], metabolic [9], and genetic mechanisms [4]. Recently, Awano et al. indicated that a superoxide dismutase 1 (SOD1) mutation (c.118G>A) is associated with canine DM in several breeds including PWC [4, 17]. Considering that SOD1 mutation is known to be a cause of human familial amyotrophic lateral sclerosis (ALS), understanding the pathogenesis of canine DM is important for the development of useful animal models of ALS. The purpose of the present study is to evaluate the neuropathologic, and particularly the immunohistochemical features of DM in two PWC; assess their similarities and differences compared with those of ALS; and elucidate the roles of denatured proteins and oxidative stress in DM.

MATERIALS AND METHODS

The spinal cords of two PWC affected by DM were used. Dog No. 1 was a 12-year-old male PWC, and dog No. 2 was a 14-year-old castrated male PWC. Both dogs showed weakness of the fore and hind limbs and dysuria. Dog No. 1 died suddenly due to an accident, while Dog No. 2 died of respiratory insufficiency. During the necropsy, mild disc herniation (Dog 1: lumbar cord, and Dog 2: thoracic and lumbar cords) was observed. No other significant gross lesion was detected. Histologically, diffuse muscular atrophy considered to be neurogenic was formed in the diaphragm and femur muscles of the dogs. In both dogs, there were no significant histopathological lesions other than the nervous and muscular tissues. A direct gene sequence analysis revealed that both dogs possessed the SOD1: c.118G>A homozygous mutation.

For comparison, the cervical cords of an age-matched Beagle (11-year-old; Dog No. 3), a PWC without nervous signs (6-year-old; Dog No. 4), and a 14-year-old Beagle diagnosed with chronic compressive degeneration due to disc herniation (Dog No. 5) were used. Tissue samples from the spinal cords of the dogs were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. Paraffin sections (4 μm thick) were stained with hematoxylin and eosin (HE) and Luxol fast blue (LFB).

Immunohistochemistry was performed using the Envision polymer method or the labeled streptavidin biotinylated antibody (LSAB) method. Table 1 lists the primary antibodies used. The deparaffinized sections were first autoclaved at 120°C for 10 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval, except for during the detection of glial...
fibrillary acidic protein (GFAP), neurofilament (NF), NF-H&M phosphorylated (pNF-H&M), and ubiquitin. Then, the tissue sections were treated with 3% hydrogen peroxide (H2O2)-methanol at room temperature for 5 min and incubated in 8% skimmed milk-t ris buffered saline (TBS) at 37°C for 40 min to avoid nonspecific reactions. The sections were then incubated at 4°C overnight with one of the primary antibodies. After being washed three time in TBS, the sections were incubated with Envision horseradish peroxidase (HRP) mouse or rabbit polymer (Dako), or HRP-conjugated streptavidin (Dako) for IgG at room temperature for 40 min. Then, the sections  were washed with TBS and visualized with 0.05% 3-3’diaminobenzidine and 0.01% H2O2 in TBS. Counterstaining was performed with Mayer’s hematoxylin.

For double immunofluorescence staining, phosphate buffered saline (PBS) supplemented with 0.2% Tween 20 was used instead of TBS for the washing steps. The sections were incubated with the first primary antibody and then incubated with the second primary antibody. Then, the sections were incubated with FITC-conjugated anti-mouse IgG (1:100, Invitrogen Life Technologies, Paisley, U.K.) and Alexa 594-conjugated anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA, U.S.A.) at room temperature for 1 hr. After being mounted with Mounting Medium (Vector Laboratories), the specimens were observed and photographed using a DMI3000B microscope (Leica Microsystems, Wetzlar, Germany).

RESULTS

Histologically, axonal degeneration (swelling and fragmentation) or loss, and demyelination of the white matter were observed in the cervical, thoracic, and lumbar spinal cords of both of the dogs affected by DM (Fig. 1a–c). These lesions were most severe in the lower thoracic spinal cord, especially in the dorsolateral funiculi, and were present in almost all funiculi. Moderate to severe astrocytosis characterized by the proliferation of fibrous or gemistocytic astrocytes and in proportion to the grade of demyelination and axonal loss was also observed (Fig. 1d–f). Chromatolysis and a decreased number of large motor neurons were observed in the ventral horns, especially in the thoracic cord. A large amount of lipofuscin was observed in the cytoplasm of neurons. No cytoplasmic inclusion bodies mimicking Bunina bodies were detected on HE sections.

In Dog No. 1 and 2, immunohistochemical analysis revealed the cytoplasmic expression of SOD1 in some reactive astrocytes and neurons with a diffuse pattern mostly involving the gray matter (Fig. 2). Identical results were obtained in the cervical, thoracic, and lumbar cords, regardless of the severity of the lesion. In the spinal cords of the dogs without DM, there was no significant immunoreactivity to SOD1 in the neurons, glial cells, or neuropile. Compared to the control dogs, the spinal cords of Dog No. 1 and 2 displayed a significant increase in ubiquitin immunoreactivity (Fig. 3). Ubiquitin-positive granules were mainly observed as small or large lumps in the neuropiles of the gray and white matter, and few such granules were observed in the cytoplasm of glial cells. No large extracellular ubiquitin-positive granules were seen in the age-matched dogs. No significant positive reaction was observed in Dog No. 4.

In Dog Nos. 1 and 2, immunoreactivity to iNOS was almost completely limited to the gray matter (Fig. 4), regardless of which antibody for iNOS was used. The immunostaining pattern was the same in all examined regions of the spinal cords of the affected dogs. In the gray matter, iNOS immunoreactivity was only observed in the cells that appeared to be reactive astrocytes. No significant immunoreactivity was observed in the spinal cords of the control dogs.

The inflammatory cell population was also examined. Only a few CD3-positive cells were distributed in the spinal lesions of the dogs affected by DM. The number of Iba1-positive microglia was significantly increased throughout the lesion in Dog No. 1, whereas a slight increase was observed in Dog No. 2. There was no significant positive.

<table>
<thead>
<tr>
<th>Antibody1)</th>
<th>Type2)</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>p</td>
<td>1:100</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>GFAP</td>
<td>m</td>
<td>pre-diluted</td>
<td>PROGEN Biotechnik, Heidelberg, Germany</td>
</tr>
<tr>
<td>Iba1</td>
<td>p</td>
<td>1:500</td>
<td>Wako Pure Chemical Industries, Osaka, Japan</td>
</tr>
<tr>
<td>NF</td>
<td>m</td>
<td>pre-diluted</td>
<td>Dako</td>
</tr>
<tr>
<td>pNF-H&amp;M</td>
<td>m</td>
<td>1:1000</td>
<td>Millipore, Billerica, MA, U.S.A.</td>
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<tr>
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<td>p</td>
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<td>Dako</td>
</tr>
<tr>
<td>SOD1</td>
<td>p</td>
<td>1:200</td>
<td>Dako</td>
</tr>
<tr>
<td>SOD1</td>
<td>p</td>
<td>1:2000 (H2O2) 1:200 (IF)</td>
<td>Dako, Cambridge, U.K.</td>
</tr>
<tr>
<td>iNOS</td>
<td>p</td>
<td>1:200 (H2O2) 1:30 (IF)</td>
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</tr>
<tr>
<td>iNOS</td>
<td>p</td>
<td>1:200 (H2O2) 1:50 (IF)</td>
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</tr>
<tr>
<td>CD3</td>
<td>p</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>Dog IgG H&amp;L</td>
<td>p</td>
<td>1:300</td>
<td>American Qualex, San Clemente, CA, U.S.A.</td>
</tr>
<tr>
<td>Dog C3</td>
<td>p</td>
<td>1:50</td>
<td>ICN Biomedicals, Inc., Aurora, OH, U.S.A.</td>
</tr>
</tbody>
</table>

reaction for C3 component or IgG in Dog No. 1 or 2.

Double immunofluorescence staining was performed using tissue samples from Dog 1 (Fig. 5). SOD1 did not colocalize with NF or pNF-H&M, while some GFAP-positive astrocytes in the gray matter showed SOD1 immunoreactivity in their cytoplasm. Ubiquitin did not colocalize with NF or pNF-H&M, and some GFAP-positive astrocytes in the gray matter showed ubiquitin immunoreactivity in their cytoplasm.

Fig. 1. a–c: Lesions of the cervical (a), thoracic (b), and lumbar (c) spinal cords of Dog No. 1. LFB-HE. Severe myelin loss is observed. The lesion is most severe in the thoracic cord. d–f: Cervical spinal cord, dorsal funiculus. Degeneration (d; LFB-HE), axonal loss (e; immunostained for neurofilament), and astrocytosis (f; immunostained for GFAP).

Fig. 2. a–c: SOD1 expression in the neurons in the ventral horns of the cervical lesions. Dog No. 1 (a), Dog No. 2 (b), and control Dog No. 5 (c). Insert: Higher magnification.

Fig. 3. a–c: Ubiquitin-positive structures in the gray matter of the cervical cords of Dogs No. 1 (a), 2 (b), and 5 (c).

Fig. 4. a–c: Immunostaining of the cervical lesions for iNOS. Dog Nos. 1 (a), 2 (b), and 5 (c). iNOS-positive reactive astrocytes are observed in the gray matter of the dogs with DM. Insert: Higher magnification.
with NF or pNF-H&M. In contrast, iNOS was localized in GFAP-positive cells within the gray matter. No colocalization of iNOS and NF or pNF H&M was observed.

**DISCUSSION**

The pathological changes observed in the spinal cords of the two dogs affected by DM in the present study (demyelination, axonal loss or degeneration, and astrocytosis) are consistent with those outlined in previous reports describing canine DM [4, 8, 13, 15], and the distribution of the spinal lesions was almost identical to that of the previous reports on DM in PWC [8, 13].

In human ALS, three types of cytoplasmic inclusion bodies are observed: 1) Bunina bodies, 2) ubiquitin-positive skein-like inclusions (SLI), and 3) round hyaline inclusions (RHI), most of which are positive for both SOD1 and ubiquitin [7, 10]. A recent report [4] on canine DM revealed that affected dogs with an SOD1 A/A homozygous mutation consistently display a high frequency of well-defined SOD1-positive cytoplasmic inclusion bodies in their spinal neurons. However, in the present DM dogs, no cytoplasmic inclusion bodies were detected and the SOD1 immunoreactivity in their neurons was not well defined; instead, it was diffuse and granular [4]. These results indicate that the SOD1-protein is not sufficiently aggregated to form inclusion bodies, at least in the present dogs. Since SOD1 expression was only seen in the neurons and astrocytes of the DM dogs and not in the controls, SOD1 might play a role in the progression of canine DM, as is supposed to occur in familial ALS patients and SOD1 transgenic mice [10,18]. However, the change of expression of the molecules might

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**Fig 5.** Results of double immunofluorescent staining. The gray matter of the cervical lesion, Dog No. 1. a–c: SOD1 (a; red), neurofilament (b; green) and merged (c). d–e: SOD1 (d; red), GFAP (e; green) and merged (f). SOD1 colocalized with GFAP, but not neurofilament. g–i: iNOS (g; red) colocalized with GFAP (h; green) in astrocytes (i; merged).
not be the exclusive factor for the pathologic events on canine DM.

On the other hand, abnormal accumulations of ubiquitin were observed in neurofilament-negative structures in the lesions of the present DM cases, but not in the control dogs. This indicates that unknown ubiquitinated proteins might play important roles in the pathogenesis of canine DM, as is found in ALS.

There have been several reports concerning the involvement of oxidative stress due to the overproduction of NO in human ALS [2, 18] and other neurodegenerative diseases [2]. In the present canine DM cases, significant iNOS expression was detected in the gray matter without significant infiltration by CD3-positive T cells. This indicates that the iNOS expression was not induced by a T cell-mediated pathway. This unique iNOS expression pattern is similar to that reported in ALS patients [16, 18] and SOD1-transgenic mice [1, 11]. The absence of inflammatory T-cells from the present cases may indicate that abnormal glial iNOS expression in the spinal gray matter is a primary factor in the pathogenesis of canine DM. In fact, no iNOS expression was detected in the control dogs.

In conclusion, the present study revealed the involvement of oxidative stress and ubiquitinated proteins in the spinal lesions of DM-affected PWC. Since no SOD1-positive “inclusion bodies” were found within the motor neurons of the affected PWC, the role of altered SOD1 expression in the pathogenesis of canine DM, and its similarity to ALS remain unclear.

REFERENCES