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# ACCUMULATION AND AGGREGATE FORMATION OF MUTANT SUPEROXIDE DISMUTASE 1 IN CANINE DEGENERATIVE MYELOPATHY

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**Abstract—Canine degenerative myelopathy (DM) is an adult-onset progressive neurodegenerative disorder that has recently been linked to mutations in the superoxide dismutase 1 (SOD1) gene. We generated a polyclonal antibody against canine SOD1 to further characterize the mutant SOD1 protein and its involvement in DM pathogenesis. This antibody (SYN3554) was highly specific to canine SOD1 and had the ability to reveal distinct cytoplasmic aggregates in cultured cells expressing canine mutant SOD1 and also in the spinal neurons of symptomatic homozygotes. A similar staining pattern was observed in asymptomatic homozygotes. SOD1 aggregates were not detected in the spinal neurons of heterozygotes; the accumulation of SOD1 was also detected in the reactive astrocytes of homozygotes and heterozygotes to a similar extent. Our results support the hypothesis that the cytoplasmic accumulation and aggregate formation of the mutant SOD1 protein, especially in astrocytes, are closely associated with the pathogenesis of DM. Therefore, this disease is regarded as a spontaneous large-animal model of SOD1-mediated amyotrophic lateral sclerosis in humans.**


**Key words: aggregates, canine, degenerative myelopathy, superoxide dismutase 1, Pembroke Welsh Corgis.**

## INTRODUCTION

Canine degenerative myelopathy (DM) is an adult-onset progressive neurodegenerative disorder that affects the spinal cord. Although the incidence of DM is high in the German Shepherd Dogs (Averill, 1973; Griffiths and Duncan, 1975; Braund and Vandevelde, 1978), it has also been reported in multiple breeds (Bichsel et al., 1983; Matthews and de Lahunta, 1985; Coates et al., 2007; Awano et al., 2009). The early signs of DM are pelvic general proprioceptive (GP) ataxia and upper motor neuron (UMN) paraparesis (Averill, 1973; Matthews and de Lahunta, 1985; Kathmann et al., 2006; Coates et al., 2007). Clinical signs progress to lower motor neuron (LMN) paraplegia, with many large dog breeds being elected for euthanasia at or prior to this point (Averill, 1973; Griffiths and Duncan, 1975; Braund and Vandevelde, 1978). Therefore, the later clinical course of this disease has not been documented in detail. In our experience, many affected dogs are able to live with extensive care until respiratory failure develops, which is commonly approximately 3 years or later after the disease onset.

The histopathological hallmark of DM is axonal loss and demyelination of the spinal cord, which predominantly occurs in the caudal thoracic area. These changes have been detected in all funiculi and involve the somatic sensory, GP sensory, and motor tracts; however, the dorsolateral part of the lateral funiculus was previously suggested to be the most severely affected (March et al., 2009). The pathology of the gray matter remains largely unknown. However, neuronal cell body degeneration and loss were recently observed in the spinal cord of DM cases (Ogawa et al., 2014).

Although many previous studies have attempted to elucidate the pathophysiological mechanisms underlying DM, its precise mechanisms remain largely unknown. DM-affected dogs were recently found to carry a mutation in the superoxide dismutase 1 (SOD1) gene. The resequencing of SOD1 from normal and DM-affected dogs revealed a c.118G > A missense mutation in exon 2 that predicted an E40K substitution (Awano et al., 2009). A homozygous c.52A > T missense mutation was more recently identified in the SOD1 gene in a Bernese Mountain Dog, which predicted a T18S substitution (Wininger et al., 2011). DM has since been

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*Abbreviations:* ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DAPI, 4′,6-diamidino-2-phenylindole; DM, degenerative myelopathy; GP, general proprioceptive; GST, glutathione S-transferase; HEK, human embryonic kidney; PVDF, polyvinylidene difluoride; PWC, Pembroke Welsh Corgis; SDS, sodium dodecyl sulfate; SOD1, superoxide dismutase 1; UMN, upper motor neuron.

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proposed to represent a spontaneous animal model of a SOD1-mediated human neurodegenerative disorder, namely amyotrophic lateral sclerosis (ALS) (Boillee et al., 2006). A recent study characterizing the biochemical properties of canine mutant SOD1 further indicated a strong link between SOD1-mediated diseases in humans and dogs (Crisp et al., 2013).

We generated a polyclonal antibody that specifically recognizes canine SOD1 in order to further characterize the canine mutant SOD1 protein (E40K) and its involvement in DM pathogenesis. This antibody was especially useful in revealing SOD1 aggregates in cultured cells as well as in the spinal neurons of affected dogs. We demonstrated that the canine mutant SOD1 protein had the propensity to form distinct cytoplasmic aggregates in cultured cells and in the spinal neurons of symptomatic dogs homozygous for the SOD1:c.118A allele. The staining pattern of spinal neurons was similar in homozygotes without clinical signs, which suggested that pathological changes had occurred in these dogs, but were in a subclinical or preclinical state. The spinal neurons of dogs heterozygous for SOD1:c.118 were free of SOD1 aggregates, but had moderate immunoreactivity in the gray matter. Furthermore, double immunofluorescence staining revealed that astrocytes around spinal neurons accumulated SOD1 in dogs carrying A/A or A/G.

## EXPERIMENTAL PROCEDURES

### Plasmid construction

A mammalian expression plasmid for FLAG-tagged canine SOD1 at the C-terminus (WT-SOD1-FLAG or E40K-SOD1-FLAG) was generated as described previously (Urushitani et al., 2002) with minor modifications. Canine mutant SOD1 was cloned by a reverse transcription polymerase chain reaction (RT-PCR) from poly-A RNA, which was extracted from the peripheral blood of a normal dog or DM-affected dog known to have an E40K genotype. After reverse transcription using reverse transcriptase (Life Technologies, Carlsbad, CA, USA), cDNA for canine SOD1 was amplified by conventional PCR to be subcloned into a pcDNA3 vector (Life Technologies) at EcoRI/XhoI sites. The primer pair used was 5′-GCCGAATTCATGGAGATGAAGGCCGTGT-3′ and 5′-GGCCTCGAGTCACTTGTCGTCATCGTCTTTGT AGTCTTGGGCGATCCCAATGACA-3′. In the bacterial expression plasmids for the purification of canine recombinant E40K SOD1 proteins, the above-mentioned cDNA was amplified by PCR with the primer pairs; 5′-ATGGA GATGAAGGCCGTGTG-3′ and 5′-TTGGGCGATCCCAA TGACAC-3′. The PCR products were subcloned into the pGEX4T-1 vector containing a glutathione S-transferase (GST) tag (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

### Generation of an anti-canine SOD1 polyclonal antibody

The pGEX4T-1-E40K canine SOD1 vector was introduced into *Escherichia coli* (*E. coli*) BL21 and the protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). Crude protein was extracted from *E. coli* by extensive sonication. GST-tagged E40K-SOD1 was separated from the crude protein using affinity chromatography. GST was digested with GST-specific protease and the recombinant E40K-SOD1 protein was purified. A five-week-old BALB/c mouse was immunized with 500 μg of the recombinant E40K-SOD1 protein emulsified with an equal volume of Freund's complete adjuvant. An intraperitoneal injection was performed once a week for 3 weeks with the recombinant E40K-SOD1 protein emulsified with Freund's complete adjuvant and with 500 μg of the recombinant E40K-SOD1 protein only in the subsequent week. Serum was collected from the vaccinated mouse a week after the last immunization. The antiserum was titered against the recombinant E40K-SOD1 protein using an enzyme-linked immunosorbent assay. We hereafter referred to the antiserum as SYN3554.

### Cell culture and transfection

All cell culture studies used human embryonic kidney (HEK) 293A cells (Life Technologies). HEK293A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mg penicillin, and 100 μg/ml streptomycin in a 6-well plate for the Western blot analysis or in 8-well chamber slides for immunocytochemistry. Using Lipofectamine™ 2000 (Life Technologies), 3 μg of vector DNA was transfected per well for the Western blot analysis, and 0.4 μg of vector DNA was transfected per well for immunocytochemistry. Cells were fixed or harvested 48 h after transfection.

### Western blot analysis

Cells were collected by scuffing each well with a cell scraper, washed in PBS, and incubated in TNG-T (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 10% glycerol, and 1% Triton X-100) with protease inhibitor cocktail (complete mini, Roche, Basel, Switzerland) for 30 min at 0 °C. After the addition of 4% sodium dodecyl sulfate (SDS), the mixture was sonicated and boiled at 95 °C for 5 min. Cell lysates were separated onto 15% SDS–PAGE gels (Supersep Ace, Wako, Osaka, Japan) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Membrane, Millipore, Bedford, MA, USA). Membranes were incubated overnight at 4 °C in 1% skim milk with 0.1% Tween 20-TBS followed by immunoblotting with SYN3554 (1:2000), a rabbit polyclonal anti-SOD1 antibody (1:1000) (SOD-100, Assay Designs Stressgen, Ann. Arbor, MI, USA), or a rabbit polyclonal anti-FLAG antibody (1: 1000) (Abnova, Taipei, Taiwan) for 45 min at room temperature. The secondary antibodies used were horseradish peroxidase (HRP)-tagged goat anti-mouse IgG (1:5000) for SYN3554 or HRP-tagged goat anti-rabbit IgG (1:10,000) (all from Jackson ImmunoResearch, Pennsylvania, USA) for the anti-SOD1 antibody and anti-FLAG antibody. Membranes