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Canine degenerative myelopathy: Biochemical characterization of superoxide dismutase 1 in the first naturally occurring non-human amyotrophic lateral sclerosis model

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ABSTRACT

Mutations in canine superoxide dismutase 1 (SOD1) have recently been shown to cause canine degenerative myelopathy, a disabling neurodegenerative disorder affecting specific breeds of dogs characterized by progressive motor neuron loss and paralysis until death, or more common, euthanasia. This discovery makes canine degenerative myelopathy the first and only naturally occurring non-human model of amyotrophic lateral sclerosis (ALS), closely paralleling the clinical, pathological, and genetic presentation of its human counterpart, SOD1-mediated familial ALS. To further understand the biochemical role that canine SOD1 plays in this disease and how it may be similar to human SOD1, we characterized the only two SOD1 mutations described in affected dogs to date, E40K and T18S. We show that a detergent-insoluble species of mutant SOD1 is present in spinal cords of affected dogs that increases with disease progression. Our *in vitro* results indicate that both canine SOD1 mutants form enzymatically active dimers, arguing against a loss of function in affected homozygous animals. Further studies show that these mutants, like most human SOD1 mutants, have an increased propensity to form aggregates in cell culture, with 10–20% of cells possessing visible aggregates. Creation of the E40K mutation in human SOD1 recapitulates the normal enzymatic activity but not the aggregation propensity seen with the canine mutant. Our findings lend strong biochemical support to the toxic role of SOD1 in canine degenerative myelopathy and establish close parallels for the role mutant SOD1 plays in both canine and human disorders.

Introduction

For two decades, data from human studies and transgenic animal models have shown that mutations in superoxide dismutase 1 (SOD1) cause a form of dominantly-inherited amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disorder resulting in motor neuron loss, paralysis, and death 3–5 years after symptom onset (Kiernan et al., 2011; Rosen et al., 1993; Turner and Talbot, 2008). To date, over 160 mutations have been identified in SOD1, which normally functions as a Cu,Zn-metalloenzyme that converts superoxide anions to molecular oxygen and H_2O_2 (for a list of mutations, see http://alsod.iop.kcl.ac.uk/). Although SOD1 mutations occur throughout the protein and result in a range of disease durations, they are united in their reduced stability and increased ability to aggregate. In fact, SOD1 aggregates in motor neurons are the histopathological hallmark of SOD1 ALS, a property that extends to animal models, cell culture overexpression systems, and experiments with recombinant protein. As SOD1-mediated ALS is a dominantly-inherited disease, this toxic gain of function becomes important in the setting of the remaining functional copy of wild-type (WT) SOD1, yet the mechanism responsible for how this toxic gain of function contributes to disease is unknown.

Until recently, with the exception of the artificial G86R and G93R mutations created in mouse and zebrafish SOD1, respectively, humans were the only organisms in which ALS occurred, and the only organisms where mutations in SOD1 responsible for causing this disease have been described (Ramesh et al., 2010; Ripps et al., 1995). That changed with GWAS data linking a mutation in canine SOD1 (cSOD1) to canine degenerative myelopathy (DM), a progressive neurodegenerative disorder in dogs with striking similarities to the clinical progression of human ALS (Awano et al., 2009). Specifically, canine DM affects multiple dog breeds toward the latter half of their lifespan and is characterized by an often asymmetric onset of paraparesis and ataxia in the hind limbs that progresses to paraplegia and muscle atrophy within one year from onset of signs. Although elective euthanasia occurs for a majority of these dogs by this stage, those that have been allowed to

Abbreviations: ALS, amyotrophic lateral sclerosis; DM, degenerative myelopathy; GWAS, genome-wide association study; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SOD1, superoxide dismutase 1; TFE, trifluoroethanol.

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progress show ascension of the disease to include the forelimbs, ultimately resulting in flaccid tetraplegia and dysphagia. The entire course of the disease can last as long as 3 years (Coates and Wininger, 2010). Histopathology of spinal cords from affected dogs show a similar pattern of myelin and axonal loss replaced with astrogliosis seen in human ALS, and immunohistochemistry reveals the same SOD1 aggregates in motor neurons that are present in humans and rodent models with human SOD1 (hSOD1) mutations (Awano et al., 2009; Bruijn, 1998).

Dog models of human disease offer specific advantages over traditional rodent models. For example, many human diseases occur naturally in dogs that would otherwise need to be artificially induced in rodent models. Furthermore, the limited genetic diversity of dog breeds, faster aging, shared environment with humans, and availability of advanced medical care facilitate expedited clinical trials for promising pharmacological agents targeting human disease. One example is that many canine models of human cancers, which develop sporadically in dogs, have been found to share several genetic similarities with human cancers and have been successfully used in clinical trials (Rankin et al., 2012; Rowell et al., 2011). Therefore, with these advantages in mind, further characterization of the canine model of ALS is warranted. Here, we report the first biochemical characterization of the only two cSOD1 mutations known to be associated with canine DM—the E40K mutation originally described in a number of breeds and the T18S mutation from a case report detailing a Bernese Mountain dog with canine DM (Awano et al., 2009; Wininger et al., 2011). We show that spinal cords of DM dogs contain detergent-insoluble mutant SOD1 that correlates with disease severity and that both mutants are enzymatically active dimers that possess an increased aggregation propensity *in vitro*. Our results elucidate important similarities between human and canine SOD1 with respect to enzymatic function and aggregation propensity, adding a biochemical dimension to the clinical and histopathological similarities between canine DM and SOD1-mediated human ALS.

Materials and methods

Detergent extraction of SOD1 aggregates

Frozen thoracic spinal cord sections were obtained from age-matched control dogs and dogs with increasing severity of canine DM. Detergent extraction of SOD1 aggregates was preformed as previously described (Prudencio et al., 2010). Briefly, 100 mg of frozen tissue was thawed on ice and homogenized *via* hand blender in 10 w/v TEN buffer (10 mM Tris, pH 8, 1 mM EDTA, and 100 mM NaCl) with protease inhibitors (Sigma). The homogenate was then mixed with an equal volume of 2× Extraction Buffer (10 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl, 1% NP-40, and protease inhibitors), mixed *via* sonication, and spun cold at 100,000 ×*g* for 10 min in a Beckman Ultracentrifuge. The supernatant, representing the detergent soluble fraction S1, was transferred to a new tube. The pellet was washed twice by resuspension in 1× extraction buffer *via* sonication and spun at 100,000 ×*g* for 10 min, cold. After the final wash, the supernatant was discarded and the pellet, designated as P2, resuspended *via* sonication in Buffer 3 (10 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl, 0.5% NP-40, 0.25% SDS, 0.5% Na-deoxycholate, and protease inhibitors). Protein concentration for the S1 and P2 fractions was determined by BCA assay (Pierce). The experiment was repeated once.

Generation of SOD1 constructs

Total RNA was extracted with TRIzol (Invitrogen) from frozen brain tissue obtained from control and DM-affected dogs according to the manufacturer's protocol. Total RNA was then reverse transcribed using oligo dT primers to generate cDNA (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen). Wild-type (WT) and E40K cSOD1 were amplified *via* PCR using primers with engineered restriction endonuclease sites at the 5′ ends. To subclone cSOD1 cDNA into pGEX4T-1 (GE Healthcare) using EcoRI and NotI, the forward primer was 5′-agtcgaattcATGGAGATGAAGGCCGTGTGC-3′ and the reverse primer was 5′-atcggcggccgcTTATTGGGCGATCCCAATG ACA-3′. To subclone into pEYFP-C1 (Clontech) using EcoRI and BamHI, the forward primer was 5′-agtcgaattcaATGGAGATGAAGGC CGTGTGC-3′ and the reverse primer was 5′-atcgggatccTTATTGG GCGATCCCAATGACA-3′. The T18S mutant was obtained by site-directed mutagenesis of the wild-type SOD1 using the primers 5′-GTGGAGGGC**T**CCATCCACTTCGTGCAGAAG-3′ and 5′-CTTCTGCAC GAAGTGGATGG**A**GCCCTCCAC-3′. Positive clones were verified by sequencing and subcloned into pGEX4T-1 and pEYFP-C1 using primers previously described. The human E40K mutant was generated *via* site-directed mutagenesis of wild-type hSOD1 using the primers 5′-GCATTAAAGGACTGACT**A**AAGGCCTGCATGGATTC-3′ and 5′-GAAT CCATGCAGGCCTT**T**AGTCAGTCCTTTAATGC-3′. The human E40G mutant was similarly generated using the primers 5′-GCATTAAAGG ACTGACTG**G**AGGCCTGCATGGATTC-3′ and 5′-GAATCCATGCAGGCCT **C**CAGTCAGTCCTTTAATGC-3′. Positive clones were verified by sequencing and subcloned into pEYFP-C1 with the primers 5′-agtcgaattcaGCC ACGAAGGCCGTGT-3′ and 5′-atcgggatccTTATTGGGCGATCCCAATTACA CC-3′ and the restriction endonucleases EcoRI and BamHI.

Untagged versions of the above plasmids for mammalian expression were created by subcloning human and canine SOD1 constructs into pCI-NEO (Promega) with a forward primer containing an EcoRI site and a Kozak sequence and a reverse primer containing a NotI site. For cSOD1, the primers were 5′-agtcgaattcgccaccATGGAGAT GAAGGCCGTGTGC-3′ and 5′-atcggcggccgcTTATTGGGCGATCCCAATG ACA-3′. For hSOD1, the primers were 5′-agtcgaattcgccaccATGGCG ACGAAGGCCGTG-3′ and 5′-atcggcggccgcTTATTGGGCGATCCCAATTAC ACC-3′. Positive clones were verified by sequencing.

Cell culture, transfection, and microscopy

NSC34 cells (CELLutions Biosystems, Inc., Burlington, ON) were grown in DMEM containing 10% fetal bovine serum with 10 U/mL penicillin and 10 μg/mL streptomycin. Cells were split into 12-well plates at 5×10^5 cells/well 18 h prior to transfection with media lacking antibiotics. Cells were transiently transfected with 900 ng of midi-prep DNA (Promega) and 6 μL Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty four hours later, cells were split into 24-well plates at varying densities for microscopy, with a small aliquot taken for protein analysis. Forty eight hours after transfection, cells were washed once with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min at room temperature, followed by three PBS washes. Cells were counterstained with DAPI in PBS containing 0.1% Triton X-100 for 10 min, followed by three PBS washes. Images were captured on a Nikon Eclipse TE300 inverted microscope using Metamorph software (Molecular Devices). Observers were blinded during cell counts, and at least 120 cells were counted for each condition in three separate experiments.

Recombinant SOD1 production

Rosetta 2 *E. coli* (Novagen) containing GST-tagged cSOD1 WT, E40K, and T18S and hSOD1 WT, G85R, or E40K in pGEX4T-1 were grown in LB media with 100 μg/mL ampicillin and 25 μg/mL chloramphenicol at 37 °C overnight, then diluted 1:10 in pre-heated LB with antibiotics and grown for 2 h at 37 °C. Protein production was induced for a further 4 h after the addition of 1.0 mM IPTG. Cells were pelleted by centrifugation at 3500 ×*g* for 25 min, washed with 1× PBS, centrifuged again, and frozen until needed for purification.

Cell pellets were thawed on ice and resuspended in lysis buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 5 mM DTT, pH 8.0). Cells were incubated with 4 mg/mL lysozyme (Sigma)