Quantitative Assessment of HSP70, IL-1β and TNF-α in Spinal Fluid and Spinal Cord Sections of Dogs with Histopathologically Confirmed Degenerative Myelopathy and Control Dogs

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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Abstract

Pro-inflammatory cytokines such as IL-1β and TNF-α appear to play important roles in autoinflammation associated with amyotrophic lateral sclerosis (ALS), but underlying cellular mechanisms related to this process remain to be fully defined. Canine Degenerative myelopathy (DM) is a spontaneous large animal model of familial ALS of man and represents a valuable tool through which to evaluate the role of these pro-inflammatory cytokines in the neurodegenerative process. Here we quantify the expression of IL-1β and TNF-α in conjunction with hsp70 (another potent stimulator of the innate immune response) in cerebrospinal fluid (CSF) and spinal cord sections from control dogs and dogs with histopathologically confirmed DM. Concentrations of hsp70, IL-1β and TNF-α in CSF were determined using high sensitivity ELISA, and protein expression in various regions of the spinal cord was determined by immunohistochemical staining (IHC) and quantified using Aperio Scanscope (Aperio Technologies, Vista, CA). Concentrations of all three proteins were below the limit of assay detection in CSF for both normal (n=10) and DM affected dogs (n=10). Tissue analysis of IHC staining revealed enhanced hsp70 expression in ependymal cells of dogs with DM compared to controls (p=0.003). IL-1β and TNF-α staining was reduced in white matter of DM affected dogs, likely artifactual due to negative staining created by the pathology associated with DM. Based on these findings, hsp70 could serve as an important
biomarker or trigger for sustained inflammation in DM and ALS and further research in this area is warranted.
Dedication

Dedicated to those who came before and those who will come after. May your journey be memorable.
Acknowledgments

I would like to acknowledge my mother Patricia, my father Timothy, and my mentor William Fenner for their support and dedication during this adventure.
Vita

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Major Field: Veterinary Clinical Sciences
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Chapter 1: Introduction

Amyotrophic lateral sclerosis (ALS or Lou Gehrig’s disease) is a chronic, progressive disorder of the central and peripheral nervous systems in people. Clinical signs vary depending on the type of ALS, but may include distal or proximal limb weakness, focal muscle wasting, dysphagia, and/or changes in gait and manual dexterity. As these signs progress, paralysis ensues. Pathologic hallmarks include upper motor neuron (UMN) loss with intraneuronal inclusions and inclusions in lower motor neurons. Some forms of familial ALS (fALS) are associated with mutations in the superoxide dismutase 1 gene (SOD1). While the cause of ALS is currently unknown, various processes such as protein aggregation, oxidative stress and neuroimmune reactions appear to contribute to the motor neuron dysfunction that ultimately leads to paralysis and death in patients with ALS.

The role of inflammation in neurodegenerative diseases such as ALS is an area of active investigation. Recent evidence suggests that inflammatory mediators, such as IL-
IL-1β and TNF-α play a role in the disease progression of ALS. Study of neuroinflammation in rodent ALS models shows distinct regional and temporal roles of activated microglia, by being initially neuroprotective and later transforming into a toxic phenotype. Evidence suggests up-regulation of innate immune responses occur in ALS by multiple mechanisms. These mechanisms lead to microglial activation, and subsequent release of inflammatory mediators such as IL-1β, TNF-α and extracellular heat shock protein 70 (ehsp70). The ultimate result is a cyclical, chronic pro-inflammatory state within the nervous system.

Extracellular hsp70, IL-1β and TNF-α are molecules released into the extracellular space from microglia and astrocytes by a unique secretory pathway enhanced by purinergic receptor signaling. Activation of purinergic receptors in the spinal cord occurs in ALS due to increased extracellular concentrations of ATP, leading to a cascade of inflammatory changes and perpetuation of motor neuron death. In particular, TNF-α and ehsp70 may serve as damage-associated-molecular-patterns (DAMPs) and TLR agonists that stimulate further inflammatory cytokine release and sustain a proinflammatory state.
Canine degenerative myelopathy (DM) is a chronic neurodegenerative disease in adult dogs. DM has many clinical similarities to familial forms of ALS (fALS) in humans. Ultimately, progression of DM leads to paralysis and euthanasia/death. Similar to some forms of fALS, mutations in SOD1 have been associated with canine DM, suggesting that dogs may serve as a valuable naturally occurring disease model of fALS. Although many rodent models of ALS and fALS exist, these models have not served well in translational therapies. Furthermore, the pathology associated with canine DM, mimics the pathology seen in the UMN form of ALS.

Currently, there are no studies that have investigated the roles of inflammatory mediators such as ehsp70, IL-1β and TNF-α in DM-affected dogs. However, each of these molecules has been implicated as a potential marker or mediator of disease in study of ALS. Investigation of the role each of these inflammatory mediators in canine DM may provide better understanding of the pathology underlying DM, as well as provide evidence for DM as a disease model of the neuroinflammatory component of ALS.

Chapter 2: Literature Review

2.1 Canine Degenerative Myelopathy
Canine DM has been recognized as a clinical entity since the early 1970’s. Originally described in the German Shepherd Dog (GSD), the disease is now recognized to have a clinical spectrum and affect many other breeds including the Pembroke Welsh Corgi (PWC), Boxer, Siberian Husky, Miniature Poodle, Chesapeake Bay Retriever, Rhodesian Ridgeback, as well as mixed breed dogs. Prevalence is estimated to be 0.19%. Clinical diagnosis is based on appropriate signalment, history and neurologic examination findings, and exclusion of other causes of myelopathy.

The onset of clinical signs begins in adulthood, typically with a mean age of 9 years in the larger dog breeds and 11 years in the Pembroke Welsh Corgi. No predilection for sex has been identified. Common neurologic examination findings early in the disease include upper motor neuron (UMN) spastic paresis with general proprioceptive ataxia in the pelvic limbs. It is not uncommon for these findings to be asymmetric. A clinical hallmark that differentiates this disease from compressive myelopathy is a lack of spinal hyperpathia.

A four-point classification system has been developed to document the stage of disease in dogs with DM (Figure 1). The UMN paraparesis invariably ensues to paraplegia. If allowed to progress further, lower motor neuron signs develop. This
results in hypotonia, hypo- or areflexia, and generalized muscle atrophy suggestive of lower motor neuron dysfunction of the pelvic limbs\textsuperscript{21,24,25,31}. The thoracic limbs become affected in later stages of the disease. Cranial nerve involvement has been reported very late in the disease, typically manifesting as dysphagia and dysphonia\textsuperscript{16,24,25}. Urinary and fecal incontinence also occur during the disease course\textsuperscript{21,24,27,31}. 
Dogs typically become non-ambulatory within 12 months. Average time from disease onset to euthanasia in large breed dogs is 6 months\textsuperscript{15}. Owners often elect euthanasia when dogs become unable to ambulate without assistance. In contrast to what is typically seen in large breed dogs, the mean disease duration in one study focusing on PWCs was 19 months\textsuperscript{24}. Many of these patients had signs of thoracic limb involvement.
at the time of euthanasia. The advanced disease progression in this breed has been attributed to the small size of the PWC making at-home care easier\textsuperscript{24,25}.

A tentative clinical diagnosis of DM is based on the exclusion of other causes of a chronic, progressive UMN myelopathy such as intervertebral disc herniation, spinal or spinal cord neoplasia, or myelitis. The results of clinicopathologic testing are typically within the normal reference ranges. CSF analysis and finding on magnetic resonance (MR) imaging or computed tomography (CT) are typically unremarkable\textsuperscript{15}. Occasionally, analysis of lumbar CSF reveals albuminocytologic dissociation, although this is considered a non-specific finding of CNS pathology. MR imaging reveals a normal thoracolumbar spinal cord, excluding compressive or neoplastic etiologies.

Definitive diagnosis of DM is made by histopathologic examination of the thoracolumbar spinal cord\textsuperscript{15}. Gross necropsy findings are typically unremarkable. Light microscopy reveals segmental degeneration of the axon and myelin replaced by astrogliosis, but without the presence of inflammatory infiltrates or spheroids\textsuperscript{21-23,26,29}. Lesional distribution is most predominant in the dorsal portion of the lateral funiculus and dorsal funiculus\textsuperscript{21-23,26} (Figure 2).
Figure 2: Histopathology of the thoracic spinal cord. Comparison of Luxol fast blue with a periodic acid-Schiff counterstain (LFB/PAS) staining (A–C) and immunohistochemistry detecting glial fibrillary acidic protein (D–F) from a normal unaffected 14.5-year-old Pembroke Welsh Corgi (PWC) (A, D), a 10-year-old PWC with clinical signs of DM for 6 months and mild paraparesis and pelvic limb ataxia (B, E), and a 14-year-old PWC with clinical signs of DM for 48 months and flaccid tetraplegia (C, F). Myelin loss in the white matter is depicted by loss of blue color with LFB (B, C). Note the severity of pallor in the white matter and increased areas of astrogliosis in the PWC with longer disease duration\(^\text{15}\). Figure courtesy of Dr. Joan Coates and reproduced with permission.

Similar to certain forms of ALS (UMN-onset), DM dogs ultimately progress to develop low motor neuron (LMN) signs. Pathologic hallmarks of ALS include muscle and axonal degeneration/loss, astrogliosis, intracellular protein-aggregate formation and neuronal cell body death (UMNs and LMNs). Changes in the peripheral nervous system in ALS are suggestive of a distal axonopathy\(^\text{18}\). These changes are similar to those seen with canine DM, including axonal degeneration, secondary demyelination, and thinly myelinated fibers, although motor neuron loss is not a typical finding in DM\(^\text{16}\).
Canine DM has recently been associated with mutations in the gene encoding for the protein SOD1. The SOD1 protein is a ubiquitous protein in the central nervous system (CNS) that functions primarily as a free radical scavenger. Mutations in SOD1 occur in some patients with fALS. To date more than 140 different mutations having been identified. Emerging evidence from studies of motor neurons, microglia and astrocytes supports that SOD1 mutations underlie the initiation and progression of ALS.

With the discovery of SOD1 mutations in the mid-1990s, transgenic rodent models over-expressing SOD1 were developed. The study of these laboratory models have documented numerous detrimental effects of mutations in SOD1, including oxidative cellular damage, neuroinflammation, protein misfolding and aggregation, and mitochondrial dysfunction and endoplasmic reticulum stress. The precise mechanisms by which these activities are initiated remain to be elucidated.

Studies in transgenic SOD1 mouse models have shown that SOD1 mutations result in a toxic gain-of-function, rather than a loss of function. Two hypotheses have been suggested to explain the mechanism by which this gain of function results in disease. The first postulates the mutant enzyme has a “gain of function” that results in
toxic catalytic properties. The second is a “gain of interaction” hypothesis that assumes the misfolding of the mutated protein becomes toxic.39

2.2 Chronic inflammation in fALS

There is a growing body of evidence to support that motor neuron death in patients with fALS is the result of non-neuronal cell mediated chronic inflammation4,5,7,9,40. Activated microglia and astrocytes maintain a proinflammatory environment by up-regulation of purinergic (P2X) receptors, most notably the P2X7 receptor (P2Xr)4,5,9. The P2X7r is an ionotropic ligand-gated cation channel that when stimulated by ATP increases intracellular calcium resulting in up-regulation and release of inflammatory mediators such as hsp70, IL-1β and TNF-α, among others41,42 (Figure 3). The P2X7r has the highest affinity for extracellular ATP of all the P2X receptors and inflammation of the spinal cord results in increased levels of extracellular ATP5,43-46. P2X7r stimulation results in severe morphologic changes in microglia, enhance the activity of TNF-α, and results in toxic effects on neurons expressing the mutant SOD19.

In both fALS patients and in transgenic SOD1 rodents, there is increased immunoreactivity for P2X7r in spinal cord microglia47,48. SOD1 mutant microglia induce
IL-1β-mediated inflammation and accelerate the pathogenesis of ALS in transgenic mice\textsuperscript{49}. Glial cells, particularly astrocytes with mutant SOD1 also have aberrant ATP signaling, resulting in an ATP-dependent neurotoxic phenotype\textsuperscript{5}. On the whole, up-regulation of the P2X\textsubscript{7} receptor in transgenic SOD1 mice has been linked to chronic inflammation and subsequent motor neuron death\textsuperscript{5,9}.

Hsp70, IL-1β, and TNF- α are molecules released into the extracellular space by microglia and injured astrocytes via a unique secretory pathway that is enhanced by P2X\textsubscript{7}r signaling\textsuperscript{10-13} (Figure 3). Extracellular hsp70, IL-1β, and TNF- α represent important contributors to a pro-inflammatory state and may contribute significantly to the pathology of ALS. IL-1β appears to be associated with amyloid-like misfolding in addition to stimulating inflammation, potentially by metabotropic glutamate receptors\textsuperscript{50,49}. TNF- α potentiates the effects of neurotoxic glutamate via nuclear factor-kappaB. Extracellular hsp70 serves to stimulate toll-like receptors by acting as a DAMP, ultimately stimulating pro-inflammatory cytokine release\textsuperscript{51,52}. 
2.3 Major Inducible 70 kDa Heat Shock Protein (hsp70)

Heat shock proteins (HSPs) are a group of functionally related proteins induced in various states of stress or injury. These proteins exist in both intracellular and extracellular forms. As one of many roles, these proteins serve as molecular chaperones tasked with the prevention of protein misfolding and aggregation, in a complex
intracellular environment\textsuperscript{53}; however when released into the extracellular space, many of these proteins appear to be potent stimulators of innate and adaptive immune responses\textsuperscript{54}.

Hsp70 is highly conserved across mammalian species, and occurs in both a constitutively expressed form as well as a highly stress-inducible isoform\textsuperscript{55}. The inducible form of hsp70 occurs with a variety of pathological states within the CNS, including neurodegenerative disease, trauma, epilepsy, autoimmunity, and ischemia\textsuperscript{53,56-58}.

\textit{2.3.1 Intracellular hsp70}

In healthy mammals, intracellular hsp70 (ihsp70) is found in low or undetectable levels\textsuperscript{59}. Intracellular hsp70 is involved in protein translation, translocation across membranes, assembly and disassembly of macromolecular complexes or aggregates, and apoptosis\textsuperscript{60}. Intracellular hsp70 also plays a role with non-native polypeptides utilizing ATP for binding and hydrolysis\textsuperscript{61,62}. This role can be divided into three activities: prevention of aggregation, promoting folding to the native state, and solubilization and refolding of aggregated proteins\textsuperscript{60}.

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In response to stress or injury, ihsp70 plays a neuroprotective role. In experimental models, ihsp70 is induced within neural tissue (cerebrum, cerebellum, brainstem) within 1.5 hours following hyperthermia and these levels persist for as long as 24 hours\textsuperscript{63,64}. Spinal cord ischemia also induces hsp70 expression as early as 12 hours after reperfusion. This expression typically peaks within 24 hours following the insult\textsuperscript{65,66}.

Experimental ischemic brain injury models using permanent occlusion of the middle cerebral artery in both transgenic mice over-expressing hsp70 and wild type mice have demonstrated that over-expression of intracellular hsp70 confers neuronal protection\textsuperscript{67,68}. Similar results are seen after exposure to kainic acid in transgenic mice that over-express ihsp70 and in astrocyte cultures from transgenic mice exposed to hydrogen peroxide\textsuperscript{69}.

2.3.2 Extracellular hsp70

The role of ehsp70 in neuroinflammation is an active area of investigation. In contrast to that of ihsp70, when released into the extracellular space, ehsp70 appears to serve as an immunoregulatory protein by acting as a DAMP to
stimulate toll-like receptors\textsuperscript{70,71}, activate neutrophils, microglia, dendritic cells, T and B lymphocytes, and NK cells, and stimulating release of IL-1\textbeta and TNF-\textalpha\textsuperscript{52,71-78}. In models of CNS infection, this may represent an adaptive response that serves to eliminate pathogens; however, in the context of neurodegenerative disease it may represent a maladaptive response that potentiates disease.

Increases in serum ehsp70 concentrations occur in various systemic inflammatory conditions such as Behçet’s disease and rheumatoid arthritis. Although the hsp70 protective system is ubiquitous, some types of neurons do not express the hsp70 protein\textsuperscript{79}. However, many types of cells in the CNS can release ehsp70 in a biologically active form that can be taken up by neighboring cells, allowing ehsp70 to serve an important role in cell to cell signaling\textsuperscript{79-81}. While the mechanism by which this occurs is unclear, ehsp70 can be internalized in the cytoplasm and nucleus of varying cell types\textsuperscript{82-88}.

Sustained ehsp70 release is described in the inflammatory sequel to ischemic-reperfusion injury in spinal cord of dogs and people\textsuperscript{54,89}. Increase in ehsp70 concentrations occurs in CSF of dogs with an immune-mediated disorder steroid responsive meningitis-arteritis\textsuperscript{58}, and an hsp70 response also occurs in spinal cord of dogs after acute injury\textsuperscript{90}.
Through an exosomal release process, astrocytes release hsp70 into the extracellular environment\(^9\). Extracellular hsp70, via interactions with cell surface receptors, stimulates release of proinflammatory cytokines and chemokines, and activate dendritic cells\(^7,8,9,2-9,6\). Thus, hsp70 can continually perpetuate the cycle of neuroinflammation.

2.3.3 Roles of IL-1β in inflammation and neurodegenerative disease

IL-1β is a protein encoded by the IL-1β gene and is a member of the interleukin 1 cytokine family. IL-1β is produced by activated macrophages as a proprotein that must be activated by caspase 1. An important mediator of the inflammatory response, IL-1β is involved in cell proliferation, differentiation, and apoptosis.

IL-1β appears to play a role in the progression of many neurodegenerative diseases, including ALS\(^9,7,9,8\). Furthermore, mutant SOD1 proteins have been shown to activate caspase 1 and IL-1β in microglia\(^4,9,9,7\). Inhibition of IL-1β activation of IL-1β slows progression of ALS and reduce ischemic and excitotoxic neuronal damage in rodent models\(^9,8,9,9\). The role of IL-1β in the
pathogenesis of canine DM has not been studied to date, but given its importance in the progression of ALS, it warrants further investigation.

2.3.4 Roles of TNF-α in inflammation and neurodegenerative disease

TNF-α is a proinflammatory cytokine that belongs to the tumor necrosis factor superfamily. TNF-α is mainly secreted by macrophages, but is also is produced by lymphoid cells, mast cells, and neuronal tissue, among others. It is involved in an array of biological processes including cell proliferation and differentiation, apoptosis, and coagulation, and has been implicated in various autoimmune diseases, insulin resistance, and cancer.

Tissue TNF-α levels are elevated in ALS. Also, TNF-α enhances susceptibility to slow excitotoxic injury in motor neurons. This type of injury is believed to contribute to progression in ALS. Down-regulation of TNF-α is associated with longer survival in murine models of ALS. As such, the role of TNF-α in the pathogenesis of canine DM warrants further investigation.
3.1 Objective:

Our objective was to examine whether hsp70, IL-1β and TNF-α responses occur in DM-affected dogs. We hypothesized expression of hsp70, IL-1β and TNF-α will be increased in CSF and spinal cord tissue of dogs with DM. Our first aim was to quantify hsp70, IL-1β and TNF-α in cerebrospinal fluid (CSF) of dogs with clinical and histopathologically confirmed DM, and to compare those results to those of neurologically normal age-matched dogs. Our second aim was to evaluate for the presence of tissue hsp70, IL-1β and TNF-α responses within the spinal cord of DM-affected dogs, and to again compare those results to tissue from clinically and histologically normal, age-matched controls.

3.2 Materials and Methods:

All samples were collected with owner consent and under approved animal care and use protocols in accordance with the investigators' institutions.

3.2.1 Cerebrospinal fluid samples

CSF samples were collected from the cerebellomedullary cistern of DM-affected (n=10), and clinically normal, age-matched controls (n=10). Samples were processed routinely and the cell-free supernatant was collected and stored at
-80°C until hsp70, IL-1β, and TNF-α analysis was performed. A diagnosis of DM was ultimately confirmed by histopathology in all affected dogs, and all DM-affected dogs were homozygous for the SOD1 mutation. All control dogs were homozygous negative for the SOD1 mutation and were free of clinical and histopathologic signs of neurologic disease.

3.1.2 Spinal cord tissue samples

Sections of midthoracic spinal cord were collected from DM-affected dogs (n=4) presented to the Veterinary Medical Diagnostic Laboratory at University of Missouri College of Veterinary Medicine and from neurologically normal, age-matched control dogs euthanized for other reasons (n=5). Spinal cord tissue samples were processed for routine histologic evaluation. Tissues were fixed in 10% neutral buffered formalin and paraffin-embedded. Histopathologic diagnosis of DM was based on previously published criteria. All DM-affected dogs were homozygous for the E40K SOD1 mutation and all dogs were either grade 3 or 4, equating to late or end-stage disease. All control dogs were homozygous negative for the SOD1 mutation and were free of clinical and histopathologic signs of neurologic disease.
3.3 Sample analysis

3.3.1 Hsp70 ELISA

The concentration of ehsp70 in each CSF sample was determined using an hsp70 high sensitivity ELISA (Assay Designs, Ann Arbor, MI; sensitivity 90 pg/ml). This assay has been previously validated in our laboratory for detection of hsp70 in canine CSF. Each sample was diluted 1:5 in phosphate buffered saline and analyzed in duplicate. Samples below the limit of detection of the assay were reported as “zero”.

3.3.2 IL-1β ELISA

The concentration of IL-1β in each CSF sample was determined using an IL-1β high sensitivity ELISA (canine specific, USCN Life Sciences, sensitivity 7.3 pg/ml). Each sample was diluted 1:4 in phosphate buffered saline and analyzed in duplicate. Samples below the limit of detection of the assay were reported as “zero”.

3.3.3 TNF-α ELISA
The concentration of TNF-α in each CSF sample was determined using a TNF-α high sensitivity ELISA (canine specific, R&D Systems, sensitivity 4.2 pg/ml). Each sample was diluted 1:4 in phosphate buffered saline and analyzed in duplicate. Samples below the limit of detection of the assay were reported as “zero”.

3.3.4 Immunohistochemistry (IHC)

Sectioned mid-thoracic spinal cord from DM-affected (n=4) and control (n=5) dogs underwent IHC staining for hsp70, IL-1β and TNF-α. Antibody sources, specificities, and dilutions are included in table 1. After deparaffinization, sections were treated with 3% hydrogen peroxide for 10 min. Species-specific biotin conjugated second step antibodies were added at a working dilution of 1:50–1:100 (Table 1). Peroxidase labeling was visualized with 3,3-diaminobenzidine. Sections were counterstained with hematoxylin and eosin. Appropriate isotype controls were used to eliminate nonspecific staining as a reason for IHC positivity.
Table 1: List of antibodies used, including name, specificity, source, and dilution

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal</td>
<td>IL-1β</td>
<td>Abbiotec, San Diego, CA</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit polyclonal</td>
<td>TNF-α</td>
<td>Abbiotec, San Diego, CA</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse polyclonal</td>
<td>hsp70/hsp72</td>
<td>Enzo Life Sciences, Farmingdale, NY</td>
<td>1:50</td>
</tr>
</tbody>
</table>

3.3.5 Evaluation of IHC images

Digital quantitative analysis of IHC staining was performed. Individual slides were digitized using the Aperio ScanScope (Aperio Technologies, Vista, CA, USA) and digital images were analyzed using Aperio ImageScope software. Regions of interest (ROI) were chosen for examination: ependymal lining of the central canal, spinal cord ventral grey matter, spinal cord dorsal grey matter, spinal cord ventral white matter, and spinal cord dorsal white matter. White matter was identified by heavily myelinated axons and the absence of neurons. Dorsal and ventral tracts were identified by previously published criteria\textsuperscript{108}. Grey matter was consistently identified by the presence of neurons and absence of heavily myelinated axons. Dorsal and ventral sections were identified by drawing a line in the dorsal plane through the central canal. For each slide examined, regions were
manually outlined by a single investigator (MCL), using 20× magnification, on the ImageScope software. A pre-programmed algorithm for analysis of IHC staining consisted of the following: hue value of 0.1 (consistent with recognition of brown pixels), hue width of 0.5 and color saturation of 0.04. These parameters allowed for consistent identification of brown pixels (positive immunoperoxidase signal) and consistent exclusion of pixels containing other colors. Positive pixel counts were performed for three representative areas within each ROI, and values were reported as “positivity” (calculated as the number of positive pixels identified by the algorithm in a defined field, divided by the number of total pixels in the field). Possible values for positivity ranged from 0-1.0. The three values obtained for each ROI were averaged to produce a single mean positivity from each ROI.

3.3.6 Statistical analysis

CSF concentrations of hsp70, IL-1β and TNF-α, and mean positivity of IHC staining for ROIs from the group of DM affected dogs were compared to that of normal controls using a student’s t-test. P< 0.05 was considered statistically significant. All values are presented as mean ± standard error of the mean (SEM).

Chapter 4: Results
4.1 Cerebrospinal fluid analysis:
Concentrations of all three proteins were below the limit of assay detection in CSF for both normal (n=10) and DM affected dogs (n=10).

4.2 IHC analysis:

4.2a Hsp70

Mean positivity for all five ROIs in control and DM affected dogs and are presented in Figure 4. IHC staining for hsp70 was higher in ependymal cells lining the spinal cord central canal for DM-affected dogs compared to controls Figure 5 (p=0.003). IHC staining for hsp70 was lower in ventral white matter for DM-affected dogs compared to controls (p=0.029).
**Figure 4:** Histogram representing mean positivity for hsp70 in control and DM affected dogs. *Denotes statistical significance.
Figure 5: IHC for hsp70 in the ependyma of the thoracic spinal cord of DM-affected dogs (5a) and controls (5b) 4X magnification, inlay 20X magnification. Scale bar is 50 microns.

4.2b TNF-α
Mean positivity for all five ROIs in both control and DM-affected dogs is presented in Figure 7. IHC staining for TNF-α was lower for ependymal cells, dorsal white matter and ventral white matter (Figure 8) in DM-affected dogs compared to controls (p=0.012, p=0.006, and p=0.001, respectively).

Figure 6: Histogram representing mean positivity for TNF-α in control and DM affected dogs. *Denotes statistical significance.
Figure 7: IHC for TNF-α in the ventral white matter of the thoracic spinal cord in control dogs (7a) and those with DM (7b) 4X magnification, inlay 20X magnification. Scale bar is 50 microns.

4.2c IL-1β
Mean positivity for all five ROIs in both control and DM-affected dogs is presented in Figure 9. IHC staining for IL-1β was lower for ependymal cells, ventral grey matter (Figure 10) and ventral white matter in DM-affected dogs compared to controls (p=0.004, p=0.039, and p=0.049, respectively).

**Figure 8**: Histogram representing mean positivity for IL-1β in control and DM affected dogs. *Denotes statistical significance.
Figure 9: IHC for IL-1beta in the ventral grey matter of control dogs (9a) and DM affected dogs (9b) 4X magnification, inlay 20X magnification. Scale bar is 50 microns.
Chapter 5: Discussion

5.1 Extracellular concentrations of hsp70, IL-1β and TNF-α

Chronic neuroinflammation appears to play a critical role in many human neurodegenerative diseases, with hsp70, IL-1β and TNF-α all serving as potentially prominent players. What is not fully understood is how these molecules establish and maintain the neuroinflammatory response in chronic disease and whether these proteins represent part of a disseminated global extracellular response (i.e. propagated by CSF), or a response that is of primary importance in the extracellular microenvironment of the spinal cord. Our results demonstrate CSF levels in DM-affected dogs are below the limits of detection for the assays used in this study.

The lack of protein detection in CSF of DM-affected dogs may indicate one of the following:

5.1a: Hsp70, IL-1β and TNF-α may not be present in CSF of DM-affected dogs.
While IL-1β and TNF-α have been shown to play important roles in disease progression of ALS, they may not play a role in DM. CNS pathology in DM, while in many ways similar to ALS, shows some key differences. For example, while motor neuron loss is a hallmark finding in ALS, this has not been recognized as a histopathologic finding in DM-affected dogs. DM, in contrast, is predominantly an axonopathy. Just as differences in histopathology exist, so might differences in inflammatory mediators of disease.

Hsp70 is elevated in many patients with acute CNS injuries\textsuperscript{54,58,89,90}. In these patients, the type of injury sustained results in a breakdown of the blood-CSF barrier (BCB) from the acute injury. This can result in “contamination” of CSF with hsp70, IL-1β and TNF-α as a direct result of cell membrane rupture and exposure to blood and extracellular fluids. In DM-affected dogs, the integrity of the BCB has not been determined. A response may be found in the extracellular microenvironment of the spinal cord, but may not be reflected within the CSF.
Chronic inflammation associated with neurodegenerative disease likely has temporal changes in inflammatory mediator release. Initially, the release of hsp70, IL-1$\beta$ and TNF- $\alpha$ may be more robust from acutely injured neurons. In a rodent model of acute spinal cord injury, CSF levels of extracellular hsp70 peaked within 3 days$^{54}$. However as the course of the disease progresses, further injury may lead to a decline in the release of these proteins. The patients included in this study were grade 3 and grade 4 (non-ambulatory, flaccid tetraplegia with brainstem signs) when samples were collected. This is considered to be the end stage manifestation of the disease. Detectable levels of extracellular hsp70, IL-1$\beta$ and TNF- $\alpha$ may therefore occur earlier in the course of the disease. Such cellular responses could be depleted in the late stages of disease.

5.1b: Hsp70, IL-1$\beta$ and TNF- $\alpha$ may be present below the limits of detection for the assays chosen.

Each assay is sensitive to levels at or below 90 pg/ml (range 4.2-90 pg/ml). It is not know what quantity of protein must be present to incite an inflammatory response. It remains possible that very low levels of protein may be enough to incite or maintain chronic neuroinflammation.
5.1c: Hsp70, IL-1β and TNF-α may not be in a detectable form

Another possible explanation for failure of protein detection may involve the form in which the proteins exist in CSF. For example, exosomally bound hsp70. Exosomes are vesicles that function to promote intercellular communication and serve as reservoirs of hsp70 among other molecules\textsuperscript{109}. Neurons have been shown to produce and release exosomes into the extracellular space\textsuperscript{110}. The role of exosomes in DM is currently unknown. However, when contained within an exosome hsp70 may not be detectable by ELISA and therefore the assays chosen might fail to detect bound hsp70. Future studies should be directed at evaluating whether detection of hsp70 by ELISA is influenced by containment within exosomes.

5.2 IHC
Immunohistochemical staining revealed enhanced hsp70 expression in ependymal cells lining the spinal cord central canal for dogs affected with DM when compared to controls. This finding is indicative of a pro-inflammatory phenotype and is consistent with a pro-inflammatory response seen in several other models of spinal cord disease \(^{54,58,89,90}\). Specifically, enhanced hsp70 expression by ependymal cells of the central canal has been previously linked to enhanced extracellular release of hsp70 into the CSF in the acute stages of the neuroinflammatory response \(^{54}\).

Increased ihsp70 within ependymal cells may serve as a marker of disease. Increases in intracellular hsp70 have been documented in response to ischemia, hyperthermia, and oxidative injury \(^{63-66,69}\). Chronic neuroinflammation leads to oxidative injury that is further perpetuated in ALS patients \(^{111}\). Therefore the increased intracellular hsp70 may simply be a response to oxidative/cellular stress, which may be exaggerated in dogs with DM.

5.2a: Hsp70 as a mediator of disease at the tissue level
Injured cells can release hsp70 into the extracellular spinal cord microenvironment. Extracellular hsp70 interacts with cell surface receptors such as TLRs to stimulate innate immunity via signaling pathways such as NF-κB\textsuperscript{112,113}. NF-κB then stimulates various neuroinflammatory cascades including induction of IL-1β. To that effect, extracellular hsp70 may then serve to perpetuate neuroinflammation.
Neural stem cell niches have been recognized in the brain and spinal cord that serve as a source of pluripotent neural stem cells\textsuperscript{114}. Ependymal cells possess latent neural stem cell properties and recent work has identified a unique population of cells within the subependymal area of the spinal cord that express markers of both ependymal cells and neural precursors\textsuperscript{115}. However, the ependymal cells lining the central canal have limited ability to produce progenitor cells\textsuperscript{115}. The role of this unique population of cells remains to be defined, but their presence has been correlated with higher numbers of proliferating ependymal cells\textsuperscript{115}. This may support their role in neural regeneration. Neural stem cells are activated in response to injury and possess regenerative capabilities\textsuperscript{116}. These capabilities include trophic and immune modulatory effects\textsuperscript{117}. However, these cells are sensitive to injury. Release of hsp70 from ependymal cells into the subependymal spinal cord parenchyma may incite inflammatory responses that may inhibit pluripotent neural stem cells from producing precursor cells needed for repair.

5.2b: Hsp70 as a mediator of disease throughout the CNS
Hsp70 may not only act within the parenchyma of the spinal cord, but may have the potential to act on distant sites. Recent evidence suggests hsp70 may be released from cells via extracellular vesicles such as exosomes and microvesicles (EMVs)\textsuperscript{118}. Hsp70 has been documented within the subventricular zone of the lateral ventricles in mouse brain tissue, as well as in neuronal bodies and the dentate gyrus of the hippocampus\textsuperscript{119}. EMV bound hsp70 may therefore be released into the CSF.

Dissemination of EMVs throughout the CSF may be an important mechanism for propagation of neuroinflammation. In this capacity hsp70 may be transported throughout the CNS. DM is initially characterized as a T3 to L3 myelopathy. As the disease progresses, sites both cranially and caudally become involved. Exosomally bound hsp70 may play a role in the spread of disease pathology and perpetuation of neuroinflammation that ultimately may lead to progressive neurodegeneration in DM

5.2c: Decreased IL-1β and TNF-α expression in DM-affected dogs
An unexpected finding in this study was decreased IHC staining for IL-1β and TNF-α in white matter of DM-affected dogs. This was likely due to the increased negative staining (i.e. “white space” on the slide) associated with axonal degeneration in dogs with DM. By comparison, control dogs have preservation of normal spinal cord architecture and less white space. This likely caused an artifactual decrease in IHC positivity in the white matter of DM-affected dogs.

Decreased IL-1β and TNF-α expression was noted in ependymal cells of DM-affected dogs compared to control. Temporal changes in the course of DM may result in changes in the neuroinflammatory milieu over the course of the disease. As the course of the disease progresses, a decline in the release of these molecules or a decrease in their production may occur. The DM-affected dogs included in this study were almost exclusively at the end stage of the disease, immediately preceding death. Further studies of dogs at different disease stages and at different levels of the CNS are needed to clarify these temporal changes. A similar phenomenon may also explain the decrease in IL-1β staining noted in the ventral gray matter of DM affected dogs. This may represent degradation of stores of IL-1β in microvesicles or exosomes that release their contents post-mortem. Again, for the DM dogs, these stores may be depleted and therefore no release occurs. Further investigation into the normal staining patterns of ventral grey matter of control dogs is warranted.
Chapter 6: Conclusions

While concentrations of hsp70, IL-1β and TNF-α were below the limits of assay detection in CSF from DM-affected dogs and controls, enhanced expression of hsp70 was identified in the ependymal cells lining the central canal of the spinal cord of DM-affected dogs. This staining pattern is consistent with a pro-inflammatory phenotype and resembles that seen in other spinal cord disease models, for which a profound extracellular release of hsp70 is noted to occur\(^ {54}\). The significance of this enhanced hsp70 expression in DM warrants further investigation. Future investigations should be directed at the mSOD1/hsp70 interaction with regard to the precise role extracellular and intracellular hsp70 play in chronic neuroinflammation.
References:


(27 degrees C), NMDA receptor activation and potassium evoked depolarization on the induction. *Neurochem Int* 2004;44:53-64.


