Discussion of the pathophysiology of acute spinal cord injury often refers to primary and secondary events. Primary spinal cord injury refers to the initial mechanical insult to the neuroparenchyma and can be subclassified into compression, contusion, laceration, and distraction. Secondary injury is the biochemical cascade that follows the primary injury and consists of vascular dysregulation, neurogenic shock, oxidative stress, immunologic injury, and excitotoxicity.1,2

Matrix metalloproteinases are zinc-dependent endopeptidases involved in the degradation of the extracellular matrix.1 They have a physiologic role in wound healing, angiogenesis, and embryologic development via their participation in extracellular matrix turnover. They are also involved in neuropathologic processes as diverse as tumor migration, axonal degeneration, lumbar disk herniation, multiple sclerosis, and acute spinal cord injury.4-10 Understanding of the role of MMPs in acute spinal cord injury is rapidly evolving. Recent research with mouse and rat spinal cord injury models suggests that MMP-9, a gelatinase expressed by leukocytes and endothelial cells, is induced within 24 hours of primary spinal cord injury.8,11-13

Expression of MMP-9 has been associated with processes involved in early secondary injury, such as blood-spinal cord barrier permeability and neutrophil migration.8,11 Results obtained with a mouse open spinal cord injury model suggest that inhibition of MMP-9 expression improves locomotor outcome and reduces blood-spinal cord barrier disruption.11,12

Intervertebral disk disease is a frequent problem in dogs, accounts for approximately 2% of all diagnoses of canine disease, and represents the most common cause of acute spinal cord injury in this species.14,15 Intervertebral disk disease occurs most often (66% to 86% of cases) in the thoracolumbar vertebrae in dogs.16,17 Acute thoracolumbar IVDD may lead to sudden functional impairment of the spinal cord, ascending myelomalacia, and, ultimately, shortened duration and quality of life.18,19

Characterizing MMP activity in the CSF and blood of dogs with acute thoracolumbar IVDD is an important step in defining the pathophysiologic features of secondary spinal cord injury attributable to IVDD. It was our hypothesis, based on results from the mouse spinal cord injury studies, that dogs with acute thoracolumbar IVDD would express MMP-9 in the CSF and have increased MMP-9 activity in the blood, compared with controls. It was also anticipated that dogs with MMP-9 activity would have a shorter history of neurologic dysfunction, be more severely affected, and require longer hospitalization stays than injured individuals without MMP-9 activity.

The purpose of the study reported here was to detect MMP-9 in serum and CSF and determine relationships between MMP activity, severity of disease, duration of clinical signs, and duration of hospitalization in dogs with acute IVDD.
Materials and Methods

Animal selection and examination—Client-owned dogs evaluated at Texas A&M University or the University of Missouri-Columbia for acute spinal cord injury resulting from thoracolumbar IVDD were prospectively entered into the study during a 1-year period. Diagnosis of acute spinal cord injury was based on appropriate neuroanatomic localization and a history of neurologic dysfunction or substantial neurologic deterioration (2 or more Frankel grades) of < 7 days’ duration. This timeframe was chosen on the basis of experimental data indicating that MMP-9 induction is most readily detected from 1 to 7 days after trauma.10 Dogs with chronic, progressive neurologic disease or concurrent systemic illness (eg, neoplasia or bacterial infection) were excluded. Thoracolumbar IVDD was defined as disk bulge, protrusion, or extrusion at 1 or more sites from the third thoracic vertebra to the third lumbar vertebra. Disk disease was confirmed in all dogs via necropsy or myelography and surgical exploration. Animal care and use committee approval was obtained for all animals involved in the study.

Clinical information recorded included age, breed, duration of neurologic signs, and administration of glucocorticoids by the referring veterinarian. Dogs in the study group received complete neurologic assessments and physical examinations. Pelvic limb motor function was determined by walking the affected dog with tail support and evaluating its movement. Superficial nociception was assessed by applying hemostats to the interdigital skin of the pelvic limb and observing heart rate, respiratory rate, and the dog’s reaction. Deep nociception was determined by cross-clamping the metatarsals with hemostats (using either the nose or handles) and monitoring similar variables. A modified numerical Frankel spinal cord injury scale was used to grade all dogs at initial evaluation as having paraplegia with no deep nociception (grade 0), paraplegia with no superficial nociception (grade 1), paraplegia with nociception (grade 2), nonambulatory paraparesis (grade 3), ambulatory paraparesis and ataxia (grade 4), or spinal hyperesthesia only (grade 5).

Treatment of affected dogs—Blood was collected for clinical biochemical tests of serum and heparinized plasma. Portions of these samples were divided into aliquots and frozen within 30 minutes of collection at –20°C for 2 to 12 hours; samples were then moved to a –80°C freezer. All dogs received general anesthesia according to standard veterinary protocols. Cerebrospinal fluid was collected from the cisterna magna or the subarachnoid space. The resulting CSF was examined cytologically, and aliquots were excluded from the study because of the possibility of contamination on detection of MMPs. Serum and plasma samples were separated by centrifugation and stored at –80°C.

Control dogs—Eight apparently healthy military working dogs were used as noninjured controls. Age and breed were recorded. All control dogs underwent complete neurologic and physical examinations carried out by 1 investigator (JRC). Results were within reference ranges. Serum and CSF were collected and stored in a similar manner to study dogs. Electromyography and motor nerve conduction velocity studies were also performed to exclude subclinical neurovascular and lumbosacral disease. Results of CSF analysis and electrophysiologic testing had to be within reference ranges to meet study inclusion criteria. Permission for procedures on control dogs was granted by the United States Army, San Antonio, Tex.

Laboratory methods—With the exception of initial, clinically indicated CSF analyses, all laboratory analyses on all samples were carried out at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University. Samples were shipped frozen on dry ice to the laboratory by overnight carrier and stored at –80°C until analysis. Samples were batched to reduce intra-assay variability.

Serum or plasma was diluted 1:50 with PBS solution. Total protein concentration was determined in the diluted serum or plasma samples and undiluted CSF by use of a protein assay kit as per manufacturer’s instructions. Bovine serum albumin was used to establish a standard curve.

Proteins in the diluted serum or plasma and undiluted CSF were separated by electrophoresis on precast 10% polyacrylamide, 1% gelatin zymography gels as described by the manufacturer. All gels were loaded with human MMP-2 and MMP-9 standards. The proteins on the gels were renatured by washing in the buffer according to the manufacturer’s directions. Gels were soaked in developing buffer for 30 minutes. Developing buffer was changed and the gels were incubated for 24 hours at 37°C to enable the gelatinases to cleave gelatin. Gels were stained with Coomassie blue for 45 minutes. Gels were destained with a solution of 40% ethanol and 10% acetic acid for 1 hour with constant agitation.

After destaining, gels were evaluated by use of transillumination and digital capture with a digital gel documentation system. Black and white points of the capture device were adjusted to avoid saturation of any pixels in the gel image. Band detection was performed on the resulting digital images with gel analysis software. Band detection threshold was set at a minimum 5% change in density relative to the maximum density in each lane. Expected gel migration distances in each lane were normalized across gels by reference to the human pro–MMP-9, pro–MMP-2, and active MMP-2 standards loaded on each gel.

The occurrence of pro–MMP-9, active MMP-9, pro–MMP-2, and active MMP-2 was recorded in each serum and CSF sample. To assess the possibility that blood contamination could result in additional MMP activity in the CSF, particularly MMP-9, the effect of various degrees of blood contamination on detection of MMPs was assessed. Canine blood was diluted in PBS solution to 100,000 RBCs/µL and a doubling dilution series was generated to 625 RBCs/µL. Samples were analyzed via zymography as described.

Statistical analysis—The frequency of detection of MMP activities in CSF and serum of dogs with IVDD was compared with that of the noninjured control group via contingency table analysis (Fisher exact test). Odds ratios and confidence intervals of the odds ratios for detection of MMP-2 and MMP-9 in serum and CSF versus IVDD versus control dogs were calculated. The frequency of detection of MMP-9 activity in CSF of affected dogs was analyzed relative to the neurologic severity index with the χ² test for trend. Median duration of hospitalization was compared between IVDD dogs with pro–MMP-2 activity and the dogs with pro–MMP-9 activity by use of the Mann-Whitney U test. All statistical analyses were performed with a commercially available software package. For all comparisons, P < 0.05 was considered significant.
Results

Thirty-five dogs met the criteria for inclusion. The majority (n = 23) of the dogs were miniature or standard Dachshunds. Also represented were mixed-breed dogs (n = 4); Pekingeses (3); and 1 each of Bassett Hound, Bichon Frise, Cocker Spaniel, Welsh Corgi, and Shih Tzu. Mean ± SD age of the study population was 5.5 ± 2.4 years (2 dogs had unknown ages and were excluded from this calculation). There were 14 female dogs and 21 male dogs. Nineteen of 35 dogs received glucocorticoids prior to admission (prednisolone, 9; dexamethasone, 7; and methylprednisolone, 3).

The majority of dogs (27/35) were nonambulatory at the time of admission; 13 of 35 dogs were paraplegic, and 2 of 35 dogs lacked deep nociception. Regarding spinal cord injury scale, 2 dogs had grade 0, 4 had grade 1, 7 had grade 2, 16 had grade 3, 5 had grade 4, and 1 had grade 5. Duration of clinical signs was variable, with 13 animals evaluated to 0 hours after onset, 5 evaluated 24 to 48 hours after onset, 7 evaluated 49 to 72 hours after onset, and 10 evaluated from 73 hours to 7 days after onset.

Cerebrospinal fluid was collected in all patients, and serum was reserved for MMP analysis in 31 of 35. Myelography and laminectomy were performed on 34 of 35 dogs; 1 dog was euthanized and necropsied, and the diagnosis of thoracolumbar IVDD was confirmed. One dog died during surgery.

Cerebrospinal fluid analysis of the affected dogs revealed median RBC count of 6 cells/µL (range, 0 to 2,317 cells/µL; reference range, 0 to 5 cells/µL), mean WBC count of 2.3 cells/µL (range, 0 to 11 cells/µL; reference range, 0 to 5 cells/µL), and mean protein concentration of 18.5 mg/dL (range, 10 to 42 mg/dL; reference range, <25 mg/dL). One dog had high CSF protein and WBC count, 4 dogs had CSF pleocytosis and serum was reserved for MMP analysis in 31 of 35.

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severity score, or administration of glucocorticoids and presence of various metalloproteinasises or gelatinase isoforms in the serum or CSF. There was no significant association between serum and CSF MMP-9 activity.

Discussion

Matrix metalloproteinase-9 activity was detected in the CSF of 6 of 35 dogs with acute spinal cord injury resulting from IVDD. Typically, CSF samples expressing MMP-9 were collected early in the course of disease. Dogs with detectable MMP-9 activity in the CSF often had severe dysfunction (paraplegia); however, this may reflect a greater tendency for referral of severely affected IVDD dogs to our institution. Control dogs and clinically normal dogs evaluated in another study rarely had detectable MMP-9 in the CSF as detected via zymography.2 Matrix metalloproteinase-9 activity that was identified in 1 control dog may have been attributable to sample contamination or unidentified neurologic disease. All affected dogs and controls had detectable MMP-2 in the CSF; consistent with published data.

Matrix metalloproteinases are involved in both secondary spinal cord injury and repair mechanisms. Rat and mouse spinal cord injury models reveal MMP-9 in neuroparenchymal endothelium, macrophages, and neutrophils within 24 hours of trauma.8,11-13 Blood-spinal cord barrier disruption has also been identified and likely results from extracellular matrix degradation secondary to MMP-9 expression.5 Spinal-injured mice lacking MMP-9 expression because of pharmacologic inhibition have reduced neutrophil migration and blood-spinal cord barrier permeability. These mice also have more rapid locomotor recovery than those with MMP-9 activity.8 By 7 days after injury, MMP-9 expression is relatively dampened and MMP-2 activity (which is present constitutively) is upregulated.13 This increase in MMP-2 expression may be needed for neovascularization, glial scar formation, and remyelination.8,10,13 Although injured canine spinal cords have not been examined to determine the particular cell populations involved in the generation of MMP-9, results in all species studied thus far have supported a clear expression pattern and role for MMP-9 in secondary spinal cord injury. Given that MMP-9 is not normally expressed in canine CSF and is not typically present in spinal cord homogenates of healthy animals of other species (i.e., it cannot be released as a product of normal cells but must be induced), its presence in the CSF of dogs with acute IVDD suggests an active role in spinal cord trauma.

The findings in the study reported here agree well with experimental spinal cord injury model data.8,11-13 Several investigators have found that MMP-9 expression peaks at 24 hours after injury in rat and mouse spinal cord homogenates.8,11-13 In the study reported here, dogs with IVDD often expressed MMP-9 in the CSF when neurologic dysfunction had been present for < 24 hours. Our data also concur with a graded spinal cord injury study in mice that revealed more MMP-9 induction in mice with severe spinal cord injury, compared with mice with mild or moderate trauma. Dogs that were paraplegic because of IVDD more frequently expressed MMP-9 than those that had paraparesis and ataxia or no neurologic deficits. We were unable, however, to detect a relationship between modified Frankel grade and MMP activity in CSF.

This study did not reveal that lack of MMP-9 expression in the CSF is a positive prognostic indicator, although spinal cord injury models have established that rodents with lower MMP-9 activity have more rapid recovery.8,13 The relatively small number of dogs evaluated in our study and methods for measurement of outcome may have led to the inability to correlate MMP-9 expression to slower recovery. Many dogs with IVDD did not return to the participating institutions for reassessment, which limited the ability to obtain postoperative neurologic scores at various time intervals. Duration of hospitalization was examined with reference to MMP-9 activity, and although dogs that expressed MMP-9 did have longer hospitalization (median, 8 days vs 6 days), this difference did not reach significance.

Duration of hospitalization may not be an accurate indicator of the degree of neurologic dysfunction. Some clients wish to house relatively healthy dogs in the hospital for convenience or physiotherapy, whereas others desire to take extremely dysfunctional dogs home.

Serum MMP activity is apparently predominantly derived from leukocytes (principally neutrophils) and may reflect inflammation or degeneration in multiple systems.25 Although results are not as specific to the CNS as to tissue or CSF, serum offers the advantage of being readily obtainable. Dogs with IVDD had MMP-9 activity in the serum more frequently than did controls. Dogs with mammary adenocarcinoma have high serum MMP-9 expression.25 Leukocyte chemotaxis and inflammation play a central role in both diseases. The activity of MMP-9 in the serum thus may indicate leukocyte margination.24,25 It is likely that the prognostic value of testing for serum MMP-9 activity will be low in spinal cord trauma, given the lack of specificity of this assay and the observation that nearly all dogs with IVDD express MMP-9 activity in the serum.

Further studies of CSF and serum MMP expression in dogs with IVDD are indicated to better assess the prognostic value of MMP-9 in the CSF and to evaluate the potential benefit of MMP-9 inhibitors. The wide administration of glucocorticoids, which act as weak nonspecific MMP inhibitors, to dogs with acute thoracolumbar IVDD in this study may have dampened the expression of both MMP-2 and MMP-9.26 Examination of a population of dogs with acute spinal cord injury that have not received glucocorticoids may facilitate the detection of MMP-9 in a greater number of individuals.
References


