Characterization of matrix metalloproteinase-2 and -9 in cerebrospinal fluid of clinically normal dogs

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Objective—To characterize matrix metalloproteinase (MMP)-2 and -9 in CSF of clinically normal dogs.

Sample Population—Samples of CSF collected from 23 dogs.

Procedure—Dogs were anesthetized, CSF samples were collected, and dogs were then euthanatized. Each CSF sample was evaluated immediately for RBC count, WBC count, and protein and glucose concentrations, and cytologic examination also was performed. Samples were considered normal when protein concentration was < 25 mg/dL and CSF contained < 6 WBCs/μL and < 25 RBCs/μL. Samples were stored at –70°C. Sections of brain tissue were collected and processed for histologic examination. The MMPs were evaluated by use of gelatin zymography and a polyclonal antibody-based sandwich ELISA.

Results—Mean WBC count for CSF samples was < 1 WBC/μL (range, 0 to 3 WBCs/mL). Mean protein concentration was 12 mg/dL (range, 8 to 17 mg/dL). Mean RBC count was 3.65 RBCs/μL (range, 0 to 21 RBCs/μL). All CSF samples generated a clear band on zymography gels that corresponded to the human commercial standard of proenzyme MMP-2. Other major clear bands were not detected on zymography gels. Bands correlating to MMP-9 were not detected in any samples. The ELISA results revealed a mean ± SD proenzyme MMP-2 concentration of 5.61 ± 1.92 ng/mL (range, 3.36 to 10.83 ng/mL).

Conclusions and Clinical Relevance—The proenzyme form of MMP-2 is detectable in CSF of clinically normal dogs, whereas MMP-9 is not detectable. Additional investigation of MMPs in CSF from dogs with various diseases of the nervous system is indicated. (*Am J Vet Res* 2002;63:1359–1362)

Analysis of CSF remains 1 of the most common diagnostic aids used to evaluate diseases of the CNS. With rare exceptions, CSF analysis allows distinction between inflammatory and noninflammatory diseases. However, CSF analysis rarely yields a specific diagnosis.[1] In 1 study,[2] investigators found that the specific cause of inflammation could not be identified on the basis of CSF analysis in a third of dogs with inflammatory or infectious diseases of the CNS. New methods of gaining diagnostic information from CSF are being investigated. For instance, interleukin-8 was evaluated in dogs with steroid- responsive meningitis-arteritis and found to play a role in chemotaxis of leukocytes into the CNS.[3]

A group of enzymes referred to as **matrix metalloproteinases (MMPs)** have been implicated in a number of CNS diseases in humans. Concentrations of these enzymes are altered in the CSF and tissues for many neurologic conditions including viral meningitis, amyotrophic lateral sclerosis, multiple sclerosis, brain tumors, and cerebral ischemia.[4-10] Characteristic patterns of MMPs expression can aid in determining the diagnosis and prognosis of specific diseases in humans. Furthermore, there is ongoing research concerning the role these enzymes play in the pathogenesis of disease and the potential for inhibition of MMP as a means of treatment.

Matrix metalloproteinases have also been identified in dogs. Specifically, canine MMP-2 and -9 have been described.[11-15] Tissues that have been evaluated for MMPs include synovial fluid of dogs, brains of dogs that had clinical signs of Alzheimer's-like disease, myocardium of dogs with cardiomyopathy, and osteosarcomas and mast cell tumors of dogs.[11,13-16] However, to our knowledge, MMP expression has not been evaluated in CSF of dogs.

Zymography is the most common method for use in evaluating samples for MMP. By using substrate-incorporated SDS-PAGE, zymography allows identification of specific MMP on the basis of their enzymatic activity. Zymography can be used to provide a semiquantitative assessment of each enzyme's activity.[17] Zymography has the added benefit of being able to identify the enzyme as well as the proenzyme form in the same assay.[17]

In humans, the **proenzyme form of MMP (proMMP)**-2 is constitutively expressed in the CSF and the brain, whereas MMP-9 is found only during pathologic processes.[18] In the CNS, many types of cells, including neurons, microglia, oligodendrocytes, and astrocytes, produce MMPs.[19] Leukocytes in peripheral blood may also contribute to increases in MMP concentrations in the CNS during disease.

The study reported here was designed to characterize proenzyme and active forms of MMP-2 and -9 in CSF of clinically normal dogs. The study was also designed to provide baseline values for future studies of CSF obtained from dogs with diseases of the CNS.

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Materials and Methods

Animals—Twenty-three clinically normal dogs were used in the study. All dogs had been used in other studies; use in those studies did not affect the nervous system of any of the dogs.

Collection and initial analysis of CSF samples—Dogs were anesthetized, and CSF samples were collected from the cerebellomedullary cistern of each dog. The CSF was analyzed within 20 minutes after collection. Numbers of RBC and WBC were determined by use of a hemacytometer. Samples were cytocentrifuged and stained with Wright stain, and a differential cell count was then conducted. Protein and glucose concentrations in CSF were determined by use of a colorimetric analysis. After analysis, samples were divided into small aliquots and stored at –70°C until MMP assays were performed. The CSF samples were considered normal when the total WBC count was < 6 WBCs/µL, RBC count was < 25 RBCs/µL, and total protein concentration was < 25 mg/dL.

Collection and processing of brain tissues—Immediately after collection of CSF samples, the dogs were euthanized by administration of an overdose of barbiturate. The brain of each dog was removed and placed in neutral-buffered 10% formalin. Following fixation, each brain was dissected, and the cerebral cortex, hippocampus, midbrain, and cerebellum-brainstem were embedded in paraffin. Tissues were sectioned, stained with H&E, and then evaluated by use of light microscopy to establish that the brains were free of disease.

MMP analysis by use of gelatin zymography—Zymography was performed in mini-gels,[a] using modified procedures described by Rosenberg et al.[20] The resolving gel consisted of 1.74 mL of **high-performance liquid chromatography (HPLC)**-grade water, 45 µL of 10% SDS, 1.5 mL of 30% acrylamide-0.8% bisacrylamide, 0.94 mL of 1.88M Tris, and 0.3 mL of 1.5% gelatin. Gel was polymerized by addition of 22 µL of 10% ammonium peroxodisulfate and 2.2 µL of **N,N,N',N'-tetramethylethylenediamine (TEMED)**. Stacking gel consisted of 500 µL of 30% acrylamide-0.8% bisacrylamide, 500 µL of 1.25M Tris-HCl (pH, 6.8), 50 µL of 10% SDS, and 3.9 mL of HPLC-grade water; the stacking gel was layered on top of the separating gel. The gel was polymerized by addition of 25 µL of 10% ammonium persulfate and 2.5 µL of TEMED. Reservoir buffer consisted of 30 mL of 10X electrode buffer, 3 mL of 10% SDS, and 267 mL of distilled water.

Wells were loaded with samples, each of which consisted of 5 µL of CSF and 5 µL of 2X sample buffer. Sample buffer consisted of 128 µL of 1.25M Tris-HCl (pH, 6.8), 200 µL of 10% SDS, 80 µL of glycerol, and 40 µL of saturated bromphenol blue. Gelatinase zymography standards[b] (0.1 mg/mL) for human MMP-2 and -9 were diluted 1:1,000 with HPLC-grade water and then diluted with sample buffer as described previously for the CSF samples. A tissue homogenate of an osteosarcoma obtained from a dog,[c] which contained canine MMP-2 and -9, also was evaluated by use of gelatin zymography. Gels were electrophoresed at a constant voltage of 200 V for approximately 90 minutes.

Following electrophoresis, gels were rinsed in distilled water and then gently shaken in a solution of 2.5% Triton X-100 for 15 minutes. Gels then were washed, which was followed by shaking in a solution of 2.5% Triton X-100 for 30 minutes. Gels were rinsed and incubated in enzyme buffer that consisted of 50mM Tris-HCl (pH, 7.5), 200mM NaCl, 5mM $CaCl_2$, and 0.02% Brij-35 (30% wt:vol) in 1 L of distilled water. Each gel was incubated in enzyme buffer for 18 to 24 hours at 37°C. Following incubation, gels were rinsed and stained by incubation with 50% methanol-1% acetic acid containing 0.125% Coomassie blue for 1 hour. Gels were destained by incubation with 10% acetic acid for 30 minutes, followed by a second destaining incubation in fresh 10% acetic acid for approximately 2 hours. Identity of the putative enzymes was determined by analysis of the distance that clear bands (indicating areas of hydrolytic enzyme activity) had migrated on the gels, compared with the distance for migration of control samples.

MMP analysis by use of an ELISA—To quantify the amount of proMMP-2 in samples, a commercially available ELISA[d] was used in accordance with the manufacturer's instructions, except for described modifications. The kit contained polyclonal antibodies against human proMMP-2. The kit was used because of the homology between canine and human MMPs.[12] Samples (25 µL) of CSF obtained from the dogs were diluted with 75 µL of sample diluent, and each sample was assayed in triplicate. A dose-response curve was generated by use of human standards provided in the immunoassay kit. Negative-control samples consisted of sample diluent without CSF and calibrator diluent without the proprietary standard. Mean ± SD values were calculated for each type of negative-control sample, and mean ± 3 SD of the absorbance of the negative-control sample with the highest background was used as the cutoff to determine a positive reaction. Samples with an absorbance of ≥ 0.10 at 450 nm were considered positive. Recombinant canine MMP-2[e] was also evaluated as a positive-control sample to ensure that the antibodies recognized canine MMP. Absorbances of various concentrations of the human standard were used to generate an equation, using linear regression analysis. Absorbances of samples were then compared to this equation to estimate concentrations of canine MMP-2. Final concentrations were corrected for the dilution factor of the sample.

Statistical analysis—Data from the CSF analysis and ELISA were used for correlation analysis. Concentrations of MMP-2 were correlated with WBC counts and protein concentrations.

Results

Histologic examination and initial analysis of CSF—All dogs were considered clinically normal on the basis of results of histologic examination. Mean ± SD total WBC count was 1 ± 1 WBC/µL of CSF (range, 0 to 3 WBCs/µL). Mean RBC count was 4 ± 5 RBCs/µL of CSF (range, 0 to 21 RBCs/µL). Mean total protein concentration was 12 ± 2.15 mg/dL of CSF (range, 8 to 17 mg/dL). Cytologic distribution varied for the CSF samples. However, predominantly mononuclear cells (lymphocytes or monocytes) were detected.

MMP analysis by use of gelatin zymography—All CSF samples had a distinct clear band that corresponded in size (68 kd) to the commercial human standard proMMP-2 **(Fig 1)**. A sample from an osteosarcoma of a dog that had high amounts of MMP-2 and -9 activity[15] was evaluated to confirm that migratory patterns were similar between canine and human MMPs **(Fig 2)**. Other bands were detected; however, these were extremely faint and varied in location when compared with the commercial standard. A faint and somewhat variable band was evident at the region that corresponded to the standard activated form of MMP-2 (62 kd). We did not detect clear bands that corresponded to commercial proMMP 9 (92 kd). Furthermore, we did not identify any other specific bands.

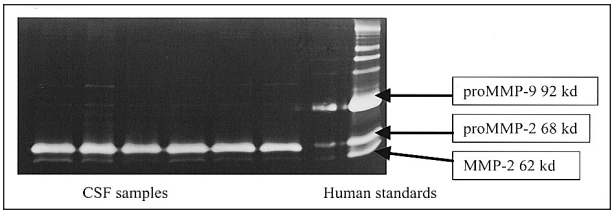

Figure 1—Results of gelatin zymography for analysis of matrix metalloproteinase (MMP) in CSF samples obtained from anesthetized clinically normal dogs (6 left lanes) and commercial human standards (right lane). ProMMP = Proenzyme form of MMP.

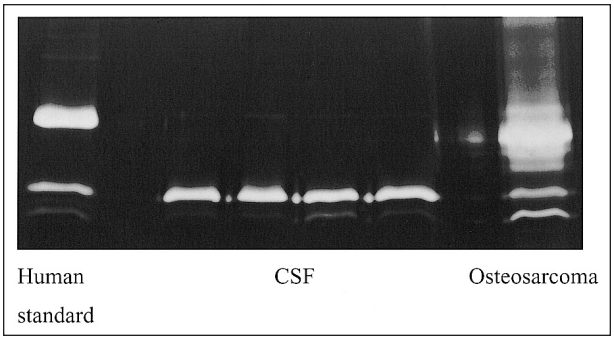

Figure 2—Results of gelatin zymography for analysis of MMP in commercial human standards (left lane), CSF samples obtained from clinically normal dogs (4 middle lanes), and tissue homogenate of an osteosarcoma obtained from a dog (right lane). The osteosarcoma was known to have high amounts of MMP activity.

MMP analysis by use of an ELISA—All CSF samples had positive results for proMMP-2 when assayed by use of the ELISA that had a polyclonal antibody to human MMP-2. A recombinant canine MMP-2 product was used as a positive-control sample. Mean concentration of proMMP-2 was 5.61 ± 1.92 ng/mL (range, 3.36 to 10.83 ng/mL). Linear regression analysis indicated that there was not a significant correlation between the proMMP-2 concentration and WBC count (correlation coefficient, 0.0017) or the proMMP-2 concentration and protein concentration (correlation coefficient, 0.39).

Discussion

Proenzyme MMP-2 was detectable in the CSF of clinically normal dogs. The active form of MMP-2 also was detected in some samples but at extremely low amounts. Amounts of MMP-9 were less than the detection limit of the gelatin zymography assay. When WBC counts and protein concentrations of CSF were used to assess correlations with MMP-2 concentrations, we did not detect an association. However, the relative homogenous character of the CSF samples may have made it difficult to detect any correlations.

The data reported here will serve as a starting point for future studies of MMPs in dogs. In addition, a consistent method of performing discontinuous zymography by use of dilutions of CSF obtained from dogs was developed by modifying the method described by Rosenberg et al.[20] This method produced distinct bands that can be compared with commercially available standards or known samples. There did not appear to be a substantial difference between the migration of MMP-2 and -9 proteins from human standards, samples of an osteosarcoma obtained from a dog, or CSF obtained from clinically normal dogs. This is not surprising, because these enzymes are highly conserved among species.[12]

Multiple faint clear bands were evident in CSF obtained from clinically normal dogs and samples of an osteosarcoma obtained from another dog. These bands were not observed for the commercial human standards. These were large molecules with a mass > 88 kd. These unknown bands could have been dimers of MMP-2 or -9. Furthermore, it has been reported[5,21] that bands not corresponding to known MMP standards could be other gelatinases or complexes of MMPs and tissue inhibitors of MMPs. Various methods have been used to verify that clear bands are truly indicative of gelatinase activity. One technique is to incubate samples with an enzyme buffer that contains calcium-chelating agents such as EDTA or phenanthrolene, which inhibit enzyme activity.[22] This was not done in the study reported here. However, an ELISA was used to confirm that substantial amounts of proMMP-2 were detectable in the samples.

An ELISA for MMP-9 was not used in the study. Because gelatin zymography is considered a more sensitive technique than an ELISA,[4,23] and MMP-9 was not detected in the zymograms, the ELISA for MMP-9 in CSF of clinically normal dogs was not considered helpful. Such an ELISA may be developed for use in future studies that have zymograms with positive results for MMP-9.

Zymography appears to be useful for the evaluation of CSF, because it requires only small amounts of CSF (5 to 10 µL/sample). This is helpful, because only limited amounts of CSF are acquired from some clinically affected dogs. Furthermore, MMPs appear to be relatively stable, and samples can be stored at –70°C for long periods without loss of MMP activity. Many of the samples used in this study were stored for > 1 year.

Zymography has been used as a semiquantitative assay for evaluating MMPs by use of densitometers and arbitrary units of activity.[7] This was not done in the study reported here, because zymography is only a measure of enzyme activity and cannot determine the total amount of enzyme. It would also not be of use for comparing the quantity of 2 enzymes, because each MMP has differing amounts of activity and, therefore, differing capabilities to create clear bands on zymograms. For example, MMP-9 has 25 times more activity against gelatin, compared with the activity of MMP-2 against gelatin.[24]

The concentration of canine proMMP-2 was determined by use of an ELISA. This provided an estimate of the amount of enzyme and also verified that the bands identified by use of zymography were MMP-2. This commercially available assay consistently identified proMMP-2 in the CSF samples obtained from the clinically normal dogs. The range of values reported here (3.36 to 10.83 ng/mL) was slightly greater than that reported in humans (0.51 ng/mL [ie, detection limit of the assay] to 1.95 ng/mL).[4,23]

The ELISA for MMP-2 is useful, because samples can be analyzed within 4 to 5 hours, whereas zymography requires 2 to 3 days. A potential disadvantage of this ELISA is the volume of CSF required for each well

(25 µL). This volume was required to obtain an optical density that would be within the values for the curve generated by use of standards supplied with the kit. This is only a concern when the volume of CSF is limited for a particular animal.

Samples were not centrifuged to remove all WBCs, which can produce MMPs. However, this was not considered a problem, because the samples were relatively acellular (mean, 1 WBC/µL of CSF). Alterations in MMP activity may have resulted if there was variation in the time between collection of the samples and freezing of the samples at –70°C. However, it has been reported[5] that CSF samples can be refrigerated for up to 7 days without a significant change in MMP activity. Because freeze-thaw cycles can potentially decrease MMP activity,[9] every attempt was made to prevent multiple freeze-thaw cycles.

[a]Biorad Miniprotean II system, Bio-Rad Laboratories, Hercules, Calif.
[b]Gelatinase Zymography Standards, Chemicon International Inc, Temecula, Calif.
[c]Canine osteosarcoma tissue homogenate, provided by Dr. Susan Lana, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colo.
[d]Bindazyme ProMMP-2 enzyme immunoassay kit, The Binding Site, Birmingham, England.
[e]Recombinant canine MMP-2, provided by Dr. Barbara Kitchell, Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, Urbana, Ill.

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