Matrix Metalloproteinases 2 and 9 in Normal Canine Cerebrospinal Fluid.

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(Abstract)

Cerebrospinal fluid (CSF) analysis is a standard part of a diagnostic evaluation. Commonly evaluated components include the cell count, protein concentration, glucose, and cytology. CSF analysis can be diagnostic in some diseases such as fungal infections and CNS lymphoma. Often, CSF analysis is not specific, but more information can be obtained. Matrix Metalloproteinases (MMPs) are enzymes that have been found in human CSF. They are calcium and zinc dependent endoproteinases with overlapping substrates. They hydrolyze at least one component of tissue extracellular matrix (ECM), such as collagen or elastin. They are important in normal physiologic processes such as angiogenesis, reproduction and wound healing. One class of MMPs, the gelatinases, degrade gelatins and type IV collagen include MMP 2 and MMP 9. MMPs are important in many pathological processes that involve unregulated matrix destruction such as arthritis, neoplasia and CNS diseases. MMP2 is known to be constituitively produced in CSF while MMP 9 is present only in certain pathologic conditions such as multiple sclerosis, neoplasia and inflammatory diseases. We hypothesize that MMP2 is present in normal canine CSF while MMP 9 is absent.

Cerebrospinal fluid samples were taken from 23 normal dogs that were being used for other research purposes. Each CSF sample was evaluated immediately for red blood cells (RBCs), white blood cells (WBCs), protein, and glucose, and then stored at -70°C. Cytological examination was also performed. CSF samples were considered normal if the protein was less than 25 mg/dl, WBCs were less than 6 µl, and RBCs were less than 25 µl. Each dog was euthanized and the brains processed for routine histopathology. MMP analysis was done using gelatin zymography and an enzyme linked immunosorbent assay (ELISA). Bands of enzyme activity were visible following staining due to enzyme degradation of the gelatin. A commercially available polyclonal sandwich ELISA was used to identify the pro form of MMP2.

The mean WBC count for the CSF samples was 0.96 WBC/µl with a range of 0-3 WBC/µl. The mean protein was 12 mg/dl, with a range of 8-17 mg/dl. The mean RBC

count was 3.65 RBC/µl with a range of 0-21 RBC/µl. All normal samples of CSF contained a band of clearing that corresponded to the human commercial standard of proMMP2. No other major bands of clearing were noted on normal samples. The commercial human standards also contained ProMMP2. Other bands were present, but were faint and variable. Using a polyclonal antibody based sandwich ELISA, with samples run in triplicate, the mean pro MMP 2 levels were determined to be 5.61 ng/ml with a range of 3.36 - 10.83 ng/ml.

We conclude that normal CSF values are narrower than what has been previously reported for protein concentration and WBC count. Also, the pro form of MMP 2 is present in normal canine CSF based on results of gelatin zymography and ELISA.

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Chapter 1: Cerebrospinal Fluid Analysis: A review of the literature

Cerebrospinal Fluid (CSF) analysis remains one of the most common diagnostic aids used to evaluate diseases of the central nervous system. While valuable information may be gained from this procedure, results from standard CSF evaluation procedures are often nonspecific. In order to gain a better understanding of the current knowledge of CSF as it relates to veterinary neurology, CSF physiology and methods of analysis will be reviewed. Additional techniques to gain more information from the analysis of CSF have been an area of research in both veterinary medicine and human medicine. Several additional tests have been employed to increase the specificity of CSF analysis with variable rates of success. However, there is a group of enzymes, matrix metalloproteinases (MMPs), that have been evaluated in various diseases of the human CSF. Information about these enzymes may prove to be beneficial. An overview of this topic will also be presented.

Production

About 60-70% of CSF is produced by the choroid plexus in the lateral, third, and fourth ventricles. The remaining portion of CSF originates from blood vessels in the pia–arachnoid and, to some extent, by direct transudation from the brain itself either across the ependymal lining of the ventricles or across the pia covering the surface of the brain. Production of CSF by the choroid plexus arises primarily through two processes, passive diffusion of some components of blood across fenestrated capillaries, and by active secretion by the choroid plexus epithelium and ependymal lining.[1] The production of CSF by the dog has been reported to be relatively constant at 0.05 ml/min.[1,2] Its production is influenced mostly by blood osmotic pressure, and appears to be relatively independent of both blood hydrostatic pressure and ventricular pressure.[3] About 60-70% of CSF is produced in the ventricles (lateral, 3rd and 4th) by the choroid plexus while the remaining volume arises from the subarachnoid space.[1]

Circulation

CSF flow is the result of movement of cilia of the ependymal cells lining the ventricular system as well as the pulsation of blood in the choroid plexus.[3] It circulates through the lateral ventricles into the 3rd ventricle via the interventricular foramina and then into the 4th ventricle by way of the mesencephalic aqueduct. CSF exits the

ventricular system by way of lateral apertures to the subarachnoid space of the brain. It may also go through the central canal of the spinal cord directly from the 4th ventricle.[3]

Absorption

CSF is absorbed by specialized structures called arachnoid villi, located within intracranial venous sinuses and cerebral veins. Arachnoid villi are tiny processes of the arachnoid membrane that project through the venous sinuses of the dura.[4] A group of arachnoid villi form arachnoid granulations. CSF is absorbed through a one-way valve mechanism of the arachnoid villi. These valves are open when the pressure of CSF is greater than venous pressure.[2] CSF may also be absorbed by lymphatics and veins located around spinal nerves, spinal nerve roots, and cranial nerves I and II.[3]

Barriers

The barriers that separate the systemic circulation from the central nervous system and cerebrospinal fluid have important roles in regulating CSF composition. They are important considerations in health and disease. These barriers are known as the blood-brain-barrier (BBB) and the blood CSF barrier.

The brain is separated from peripheral blood by a barrier referred to as the Blood Brain Barrier (BBB). This barrier is composed of astrocytic foot processes or podocytes, that are located in the perivascular space, a thin basement membrane, and modified vascular endothelial cells with tight junctions.[5] The BBB restricts the size of particles that can enter the CNS.[6]

A second barrier, the blood-CSF barrier, separates peripheral blood from the CSF compartment. This barrier consists of fenestrated capillary endothelial cells and epithelial cells of the choroid plexus or ependyma. Also, in contrast to the BBB it does not have a basement membrane. The blood-CSF barrier is not as exclusive as the BBB as a result of the fenestrated endothelial cells, which allow some ions and small substances such as glucose into the CSF, while preventing the entrance of large molecules such as albumin and other plasma proteins.[4,6] This barrier is found primarily at the circumventricular organs, which include the choroid plexus, median eminence, and the area postrema.[6]

Content

The composition of CSF differs from plasma in that it is an ultrafiltrate. An ultrafiltrate is a liquid, plasma in this case, that that has passed through tiny pores that act as a semipermeable membrane that separate small particles such as ions. The BBB, limits the size of molecules that enter the CSF, as well as restricts the concentration of CSF protein. Together these maintain CSF oncotic pressure below that of plasma.[6] A molecular weight of 65,000 daltons, the size of albumin, is generally the largest protein that will pass through the BBB.[7] Typical total protein concentrations in canine plasma are between 5 and 8 g/dl, while canine CSF protein concentrations are normally less than 25 mg/dl. Albumin is the largest contributor to normal CSF protein concentration and is generally about 10% of serum concentrations. IgG is also present in low concentrations.[1,8] C3, IgM and IgA are normally not detectable.[8,9] Very few components of CSF have been studied in veterinary medicine in comparison to human medicine where the number of CSF constituents studied is tremendous.[10] In human medicine general topics of study include cytological examination, biochemical markers of malignancy, enzymes, amines and amino acids, and proteins.[11]

In comparison to peripheral blood, spinal fluid normally contains fewer cells.[12] The normal cisternal CSF cell count in dogs is less than 5 WBCs/µl, and 0 to 30 RBCs/µl.[7,13] Mononuclear cells are the most common cell types, although neutrophils may be seen in small numbers.[14] Lumbar CSF in dogs has been reported to contain between 1 to 8 WBCs/µl.[14] Generally CSF taken from this space contains fewer WBCs than CSF taken at a more cranial site. Possible explanations given for decreased WBCs in lumbar CSF include a lower rate of WBCs entering the CNS in this region and possibly more WBCs leaving the CNS in this region. Another possibility is that WBCs in this region are lysed more quickly.[15]

CSF also contains less potassium, calcium and glucose, but more chloride, magnesium and sodium than plasma.[12] Normally CSF glucose is 60-80% of blood glucose and changes in blood glucose cause changes in CSF glucose in about 1-3 hours.[12]

Function

CSF maintains homeostasis by providing nutrients as well as providing support and protection to the central nervous system (CNS). In addition, this fluid provides drainage for the CNS in a similar fashion to lymphatics elsewhere in the body. Metabolites, neuropeptides and other materials diffuse into the CSF and are removed, providing a means of waste disposal. CSF is also known to carry neurotransmitters and neuropeptides, which may be altered in diseases such as epilepsy and spinal cord compression.[16-19]

CSF Analysis

Routine analysis of CSF includes noting the appearance of the fluid, in particular, the color and clarity. Quantification of total protein and glucose as well as cell counts for nucleated and non-nucleated cells are also routinely performed. Cytological analysis of cell types also provides useful information. Specific gravity is not routinely determined because it has not been found to be useful information.[20] In addition, antibody titers to infectious agents, microbial cultures, and polymerase chain reaction (PCR) evaluation for specific infectious agents have all been performed on CSF. Finally, CSF concentrations of a variety of enzymes including lactate dehydrogenase and creatine kinase have been described. While each of these enzymes have been correlated to CNS injury, they have not added to the specificity of the test and are not routinely performed. Electrolytes in the CSF are rarely measured.

CSF Appearance

Normal CSF is clear and colorless. CSF that is bright red, but clears after centrifugation, indicates iatrogenic or recent hemorrhage.[12] A yellow coloring of the fluid, or xanthochromia, may indicate previous subarachnoid hemorrhage or bilirubin.[12] However, large increases in protein, over 150 mg/dl, may also cause xanthochromia. The presence of oxyhemoglobin may result in a pink or yellow tinge to the fluid.[12] High cell counts, over 500 cells/mm3, may cause the sample to appear cloudy.[12]

CSF Protein

CSF protein can be quantified by various methods. Due to the smaller quantities of protein present in CSF than plasma, more sensitive means for measurement are required.[12] The Pandy and Nonne-Apelt tests are used to identify large molecular

weight proteins such as globulins.[12] The Pandy test utilizes carbolic acid to create turbidity that is indicative of the globulin concentration (over 50 mg/100mL).[14] The Nonne-Apelt test utilizes a solution of saturated ammonium sulfate to identify the presence of increased globulins.[14] Once CSF is placed in a tube with the ammonium sulfate, a white-gray ring forms in the presence of increased globulins.[21] These tests do provide much quantification.[14] More accurate methods of protein determination include the trichloroacetic acid turbidometric method, the Ponceau S red dye binding method, and the Coomassie brilliant blue method.[14] Urinary protein reagent strips can be used for gross identification of protein elevations. Levels of 2+ are considered reliable for protein elevations in the level of (30-100mg/dl), although this method only identifies albumin.[12,22] Storage of CSF samples does not greatly affect protein concentration.[23]

Individual methods for quantifying albumin, IgG, IgM and IgA in normal canine CSF have been described.[9] Rocket immunoelectrophoresis (R-IEP) can be used to measure albumin directly.[8] An alternate method to quantify albumin involves the use of a densitometer scanning technique after electrophoresis of CSF in agarose.[24] The total protein and the percentage of albumin found with electrophoresis is used to determine a specific amount. In addition, this method can also be used to separate alpha, beta and gamma globulins.[7] Immunoglobulins can be quantified in the CSF with radial immunodiffusion and R-IEP.[7,9]

Alterations in CSF Protein

Increases in CSF protein concentrations are common. Unfortunately, elevations in protein are rather nonspecific and can be the result of many different disease processes including degenerative, compressive and inflammatory diseases.[7,25-27] This lack of specificity most likely results from the fact that CSF protein concentration can be affected by several different mechanisms.

There are a number of different mechanisms for increased protein in the CSF. Partial disruptions of the BBB will initially allow albumin to increase within the CSF due to leakage from the serum.[12] However immunoglobulins, which are larger molecules will cross with more severe BBB disruption. Contamination of the CSF with peripheral blood will cause an increase in protein. Another mechanism is intrathecal

immunoglobulin production by leukocytes. Under rare conditions, increased plasma concentration of albumin will be reflected in CSF. Finally occlusion of normal CSF flow will also result in increased CSF protein.[6]

Disruption of CNS barriers can alter CSF protein concentration by at least two different mechanisms. First, inflammation increases the permeability of the BBB by disrupting tight junctions between capillary endothelial cells resulting in a moderate rise in CSF albumin without a corresponding increase in IgG concentration.[6] This occurs because the filtration rate across the BBB is inversely proportional to the molecular weight of the substance; more small particles such as albumin cross the BBB in comparison to larger immunoglobulins.[6] The second type of alteration is a complete disruption of the BBB that may occur in some disease states such as granulomatous meningoencephalitis or viral meningitis that results in accumulation of both albumin and immunoglobulins in the CSF.[6] In dogs, increases in CSF protein concentration may be the result of disruptions in the BBB, with resultant leakage from plasma, or from increased local synthesis of immunoglobulins.[12]

One common finding with many types of CNS disease is albuminocytologic dissociation. This abnormality is an increase in CSF protein with little or no increase in white blood cells.[7] This is a nonspecific finding and results from any disease that causes a non-inflammatory degeneration of nervous tissue or from serum leakage secondary to vascular disruption.[7] It may be present with neoplastic diseases due to tumor compression, or due to other degenerative processes such as hydrocephalus, or as the result of an infarction.

Certainly an increase in protein alone does not provide much information. Determination of the type of protein present may help identify the origin of the protein and a particular disease process. Therefore, in order to determine the source of CSF protein, various formulas have been proposed. These equations are intended to determine the potential cause for increased albumin, or to identify a cause for increased immunoglobulins. These formulas include the albumin quotient, the IgG quotient and the IgG index.

The Albumin Quotient (AQ)

The Albumin Quotient (AQ) is used to assess the integrity of the BBB and is based on the fact that albumin originates from serum.[24] The ratio of CNS albumin to serum albumin is increased due to serum leakage across the BBB. The AQ = (CSF albumin ÷ serum albumin) x 100. Increases in the AQ greater than 2.35 indicate BBB disruption.[7,12] Inflammatory diseases may result in a wide range of AQ values, anywhere from a normal value up to a dramatic increase. Degenerative conditions typically result in moderate increases in the AQ. Neoplastic diseases may cause marked increases in the AQ because of relative degrees of BBB disruption with each disease.[7]

Beta and gamma globulins may originate in serum or by intrathecal production, while alpha globulins are from serum only.[7] The AQ can be used to aid in the interpretation of CSF alpha, beta, and gamma globulin fractions in that an increase in this ratio suggests BBB disruption.[26] In a study of 52 dogs, electrophoresis was used to determine the AQ and alpha, beta and gamma globulin portions of CSF protein. The electrophoresis findings allowed diseases to be grouped into categories that agreed with the actual diagnosis in 73% of the cases; samples with low to normal CSF protein values showed the least amount of differentiation by this method.[26] In 15% of the cases the electrophoretic pattern was suggestive of CNS disease.[26] It was determined that increases in albumin concentration were the most consistent sign of CNS disease.

There are many variables found in the analysis of CSF protein in cases of CNS neoplasia. With this type of disease the AQ may be increased without a concurrent increase in gamma globulins because of partial disruption of the BBB.[7] Elevations in CSF protein may occur secondary to neoplasia, without subsequent changes in cellularity due to tissue necrosis, fenestrations in tumor capillaries, or leakage of protein from the serum.[7,24] In addition, immunoglobulins may be produced near the site of the neoplasm.[7,26] One study found that in dogs with neoplasia the AQ, albumin values, alpha globulin and beta globulins were elevated, indicative a serum transudation.[24]

IgG Quotient and IgG Index

CSF IgG normally originates from lymphoid tissue outside the CNS. It can reflect disruption of the BBB. However with inflammatory disease IgG can be

synthesized intrathecally by activated lymphocytes that have entered the CNS.[6] The IgG quotient is a formula that compares serum IgG with CSF IgG. When used with the AQ it can help distinguish between BBB disruption and intrathecal immunoglobulin production. The IgG index is calculated by the formula (CSF IgG concentration/serum IgG concentration)/ AQ.[28] A value over 1.00 suggests production of IgG in the CNS and is most likely the result of an inflammatory disease.[7] Examples of inflammatory diseases that may have an increased IgG Index include granulomatous meningoencephalitis, Toxoplasmosis, and chronic canine distemper viral encephalitis.[7] Unfortunately the accuracy of these formulas have questioned.[26]

Using rocket immunoelectrophoresis or agarose electrophoresis and the formulas discussed previously, four possibilities for classification of CSF protein exist: 1)unaltered CSF, 2)BBB disturbance, 3)local IgG synthesis, 4)BBB disturbance + local IgG synthesis.[24,28] These techniques of protein classification are reported to be sensitive but not specific. Therefore attempts have been made to categorize diseases based certain patterns that emerge after electrophoretic analysis of CSF. In dogs with canine distemper, gamma globulin concentrations were elevated while in dogs with granulomatous meningoencephalitis, the beta and gamma globulin values were elevated.[24]

Cell Counts

The most common technique for determining total CSF cell counts is by counting cells on a hemocytometer. It is generally accepted that cell counts should be obtained within about 30 minutes of collection due to cellular deterioration in the low oncotic environment of CSF.[13] Some have suggested that serum may be added to CSF to preserve nucleated cells for a few hours.[7,21,29] A recent study found that the addition of 11% autologous serum to CSF samples stored at 4° C allowed samples to be evaluated for at least 48 hours without significant loses in cell morphology.[30]

An increase in the amount of white cells in CSF is called a pleocytosis.[14] This change frequently occurs due to migration of peripheral white blood cells across the blood brain barrier in response to an inflammatory stimulus.[23] The more disruption of BBB present, the larger the increase in white blood cell count.[7] Several diseases have

characteristic cytological changes. As a rule, viral diseases tend to cause a lymphocytic pleocytosis, while neutrophils are more common with pyogenic bacterial infections. Unfortunately, mixed cell responses are typical of a number of different infectious agents as well as non-infectious inflammatory diseases such as granulomatous meningoencephalitis and type I intervertebral disc disease.

CSF characteristics may change depending on the stage of the disease. This has been demonstrated that dogs with Steroid Responsive Meningitis, initially have a dramatic neutrophilic pleocytosis, but then progress to a monocytic pleocytosis over the ensuing months.[31] CSF may also be reevaluated to monitor response to therapy.[14,23] This has been advocated in diseases such as steroid responsive meningitis and cryptococcal meningitis.[31,32] In human medicine repeated analysis of CSF is useful because cell counts and cell differentials can change during the course of a disease for instance a chronic bacterial meningitis will in latter stages become neutrophilic. Also repeated analysis of CSF is used in monitoring response to therapy in cases of infectious meningitis (tuberculous meningitis and syphilis).[33]

Iatrogenic hemorrhage is a major concern in the analysis of CSF. While it is assumed that peripheral blood contamination will increase both red and white blood cell numbers as well as protein concentrations, this has not be carefully evaluated in veterinary patients. Based on earlier assumptions, formulas have been derived to correct for hemorrhage. For example, for each 500 RBCs/µl present as a result of blood contamination, the WBC numbers in CSF increase by approximately 1 WBC/µl and protein by 1mg/dl.[15,23] Another method evaluates the patient's blood at time of CSF collection, the plasma WBC-RBC ratio x RBC concentration in CSF = expected number of WBCs from contamination. Expected contamination WBC count is used to determine significance of the measured WBC count in CSF. Adjusted WBC count (observed minus expected contamination), or the observed-expected WBC ratio can be measured.[23] It has been stated that the Observed-expected WBC ratio more is more accurate than the adjusted in determining true WBC elevation.[23] Other studies have found that blood contamination had little effect on white cells found in the CSF, but the effect on protein concentration is not clear.[34,35]

Cytological Evaluation

Cytological evaluation of CSF in all cases, is indicated even when the nucleated cell count is within normal limits.[36] In some instances, (i.e. lymphosarcoma and fungal encephalitis) cytological evaluation can provide a definitive diagnosis.[27] Due to a normally small amount of cells in the CSF, some means of concentrating cells is necessary. A number of techniques have been described, including direct smears, sedimentation, and filtration, and are described in detail in the appendix. The technique thought to provide optimum preservation of cell morphology is cytocentrifugation.

While some authors have described specific patterns of cytological changes in various categories of CNS disease, most have reported a tremendous amount of overlap between and within disease categories.[27] For example, meningiomas are typically thought to result in an increase in CSF protein without a concomitant increase in white blood cells (albuminocytologic dissociation). However, one study found that the CSF of dogs with confirmed meningiomas had a mean WBC count of 200 cells/µl with greater than 50% neutrophils.[37] Similarly a neutrophilic pleocytosis has also is been reported with other forms of neoplasia including oligodendrogiliomas and astrocytomas.[15] Therefore, it is impossible to discriminate between inflammatory and neoplastic diseases on the basis of CSF alone.[7] Furthermore, in some infectious diseases (i.e. canine distemper infections) CSF can be conspicuously normal. Therefore cytological evaluation has limited utility.

Glucose

Glucose in measured routinely in CSF and has been reported to be decreased in the presence of bacterial infections.[13] The change in glucose due to bacterial infections is not well proven in dogs.[23] Furthermore, decreases in CSF glucose may also result from red blood cell metabolism when there has been significant contamination and a delay in processing of CSF.[12]

CSF Titers

Antibody titers to numerous infectious diseases including viral, fungal, protozoal, and rickettsial agents, may be determined from CSF. Toxoplasmosis, Cryptococcosis, Lyme Borreliosis have been evaluated by CSF titers.[23,38,39] In some instances, (i.e. Toxoplasmosis and Cryptococcosis) CSF titers may be more reliable that serum titers for

active infections.[23] CSF titers may be more reliable in nonbacterial than bacterial causes of meningitis.[23] The most common titer clinically evaluated is for canine distemper virus (CDV) antibody. When performed alone, intrathecal antibody production can be difficult to differentiate from BBB breakdown. Therefore, either an AQ should be evaluated or titers for diseases that are not likely to result in intrathecal antibody production, such as parvovirus, can be compared to CDV titers to aid in this differentiation.[40] To further aid in the diagnosis of distemper CDV viral antigens may also be detected in CSF cells with immunofluourescent antibodies.[41] In addition, antimyelin basic protein antibody may be present in the CSF in some cases of Chronic CDV encephalomyelitis, where chronic demyelination may be related to an immune response.[42]

Enzymes

Enzymes including creatine kinase (CK) and lactate dehydrogenase (LDH) have been evaluated in the CSF.[7,12,43] Increased enzyme activity in the CSF may be the result of diffusion from plasma across a disrupted BBB, leakage into the CSF from diseased CNS tissues, or discharge from inflammatory cells, neoplastic cells, or microorganisms.[12] A specific brain isoenzyme of CK has been identified although it has not proven to be useful.[7,12] Lactate dehydrogenase levels have been evaluated in an attempt to identify disruptions in the BBB.[43] The level of LDH in the serum of healthy dogs was about ten times the amount in CSF in one study and the authors speculated that alterations in this ratio might help to identify BBB disruption. Unfortunately most enzyme assays lack specificity for disease identification.[15]

CSF Culture

Various types of culture and organism identification have been employed for viral, bacterial and fungal infections. Both aerobic and anaerobic bacterial cultures of CSF may be done when an infectious organism is suspected. Fungal culture of CSF can be used to isolate Cryptococcus and viral isolation has also been used to aid in cases of viral meningoencephalitis.[38,44-46] However, positive culture results are uncommon in veterinary medicine even with cytological evidence of inflammation.[23,25] A lack of positive cultures may be the result of problems with sample handling, culturing media, or

a low number of organisms in the CSF.[23] Indications for anaerobic cultures include the presence of a mass lesion or the presence of multiple organisms in the CSF.[23]

Identification of antigens

Identification of antigens is the most specific means of infectious disease diagnosis.[40] This is possible in some diseases such as cryptococcal meningitis or in canine distemper virus (CDV) through latex agglutination or fluorescent antibody testing respectively.[32,41] However, for many diseases antigen detection has not been possible. Polymerase chain reaction (PCR) can be used to overcome the limitations of small amounts of DNA. This process is used increases an infectious agent's DNA or RNA, which can then be measured by other processes. This is helpful in cases where the organism cannot be cultured.[40] In veterinary medicine PCR has been used to amplify DNA from Sarcocystis neurona, the causative agent of Equine Protozoal Myeloencephalitis, in the CSF of horses.[47] However since the Sarcocystis organism rarely occurs in the CSF, the test is usually negative, and is therefore not clinically useful.[48] It has also been used to identify Toxoplasma gondii from the CSF of dogs and cats, and CDV in dogs.[49,50]

CSF Collection

CSF is usually collected from the cerebellomedullary cistern or from the lumbar spaces L5-L6 or L6-L7 in dogs. The technique for CSF collection has been reviewed elsewhere.[29] The site of collection varies with the suspected site of the lesion. Due to CSF flow, samples should be collected caudal to the site of the suspected lesion.[51] This means that for suspected spinal cord diseases, CSF from a lumbar collection should be analyzed. However, lumbar samples tend to contain more iatrogenic hemorrhage, are smaller in sample size, and may be technically more difficult to obtain.[15,51]

Contraindications

CSF collection is a relatively safe procedure, however there are some risks with the procedure. It is contraindicated if the animal cannot undergo general anesthesia. Increased intracranial pressure is also a contraindication for CSF collection due to the risk of brain herniation. Therefore, advanced imaging of the brain such as CT or MRI is suggested before collecting CSF.[29] Herniation results from a sudden change in the

pressure that occurs when CSF is removed from the cerebellomedullary cistern or the lumbar CSF space.[29]

Summary

With this review of CSF analysis as it pertains to veterinary medicine, it should become apparent that CSF analysis provides some diagnostic information. It generally allows categorization of the disease into inflammatory and non-inflammatory categories. However CSF rarely provides a specific diagnosis.[6] In one study, it was found that in 1/3 of dogs with an inflammatory or infectious disease it was difficult to impossible to identify the specific cause of the inflammation.[25] New methods of gaining diagnostic information from CSF are under investigation. For instance interleukin-8 activity has been evaluated in dogs with steroid responsive meningitis-arteritis and found to be elevated.[52] Nonetheless much more information can be gained from CSF analysis.

In human medicine a group of enzymes, matrix metalloproteinases (MMPs), have been shown to correlate with certain diseases of the CNS. CSF and tissue concentrations of these enzymes are altered in many neurologic diseases including viral meningitis, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), brain tumors, and cerebral ischemia.[53-58] MMP expression may show characteristic patterns, which can aid in both diagnosis and prognosis of specific diseases in humans. Further more, there is extensive research involving the role these enzymes play in the pathogenesis of disease and the potential for MMP inhibition as a form of therapy.

Chapter 2: Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a large group of zinc dependent endoproteinases that have the ability to degrade components of the extracellular matrix (ECM).[59] This group of enzymes is part of a larger class of endoproteinases that all hydrolyze the internal bonds of various proteins.[60] MMPs first received attention in 1962 for their role in the dissolution of tadpole tails.[61] Since that time, research in this field has grown exponentially. At least 17 different enzymes have been described that play an important role in a number of normal physiologic processes as well as pathological events.[62]

In addition to having the ability to degrade the ECM, MMPs have a number of other common features. All are secreted in an inactive or latent form. MMPs can be activated by organomercurial compounds, and inhibited by tissue inhibitors of metalloproteinases (TIMPs). Furthermore, they all have regions of homologous DNA.[63] Finally, these enzymes are calcium-dependent, which is important in inhibition.[64]

MMP terminology is confusing as multiple names have been used to describe both the group of enzymes as well as individual MMPs. Similar substrate specificity has resulted in MMPs being named by a particular substance they degrade, even though they may be capable of degrading other substances.[63] MMPs can be divided into four groups based on substrate specificity, these include collagenases, stromelysins, gelatinases, and membrane-type MMPs.

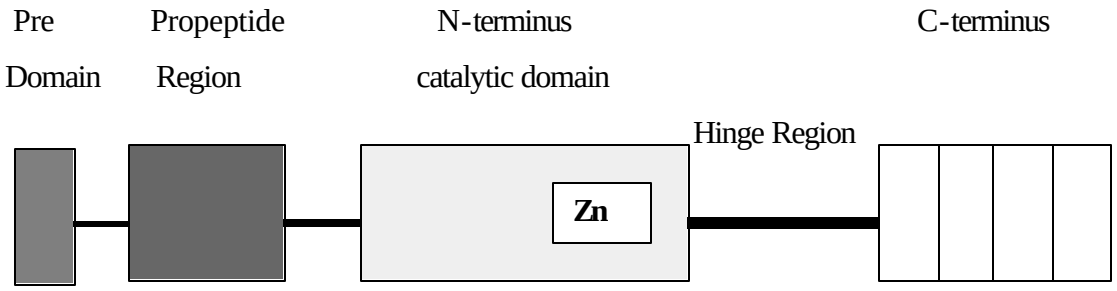
MMP Structure

Structural similarities and differences between MMPs allows further distinction and classification. Members of each MMP family have conserved domains that distinguish them as a group.[65] Many share the same propeptide and N-terminus catalytic domains.[66] They are grouped by fibronectin-like repeats, transmembrane domains and C-terminus hemopexin-like domains into collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMP).[66]

Each MMP contains a pre-domain that is a leader sequence of the enzyme.[67] (Figure 2-1) The leader sequence labels the enzyme for secretion, but is absent in the enzyme's next form, the latent enzyme.[65,68] The next region is the pro-domain, which

is removed at the time of enzyme activation. This portion of the domain, the N-terminal domain, is highly conserved and is responsible for enzyme latency.[67,68] The catalytic domain contains a conserved sequence that is the zinc binding site.[65,67,68] The hemopexin domain is a heme-binding peptide at the carboxyl-terminal end.[67] It is present in all MMPs but matrilysin, and is responsible for substrate specificity.[65,68] The gelatinases have a collagen-binding domain known as the fibronectin domain.[67]

Figure 2-1: Domain Structures of MMPs



MMP 2

MMP-2, also known as Gelatinase A, may have the widest physiologic distribution.[69] The latent form, which is inactive, is also referred to as the proenzyme. ProMMP-2 has a molecular weight of 72 kDa while the active form is 62 kDa.[63] Based on in vitro assays of multiple substrates MMP-2 degrades denatured collagen (gelatin), type IV (basement membrane) collagen, type V collagen, and elastin.[63,70] Tissue inhibitor of metalloproteinase 2 (TIMP-2) binds with MMP-2 to inhibit this enzyme's activity.[66,71] There has been some variability in the molecular weights reported for these enzymes. Active MMP 2 has also been reported to be 64 kDa.[72]

MMP 9

MMP-9 is also known as Gelatinase B. In the latent form Pro-MMP9 may be 92 or 100 kDa, and there have been 130 kDa and 250 kDa variations of MMP 9 reported;[72] the active form is 84 kDa.[63] This enzyme is capable of degrading denatured collagen (gelatin), type IV (basement membrane) collagen, type V collagen, and elastin.[63] Because large amounts are present in neutrophils, it has also been called neutrophil elastase.[69] Plasmin is known to be an activator of MMP 9.[62] TIMP-1 inhibits the activity of MMP 9.[66,71]

Cells that express MMPs

Leukocytes, endothelial cells, trophoblasts, and tumor cells all express MMP 2.[69] MMPs may be secreted into the ECM or expressed in a plasma membrane bound form.[62] Cells that secrete MMPs do not store them for later use.[69] Secretion may be stimulated by multiple factors including growth factors, oncogenes, and cytokines.[67,73]

Regulation

Since MMPs are active in so many physiological processes, their activity must be tightly controlled. There are three different mechanisms by which MMP function can be controlled; regulation of gene expression and translation, secretion of the enzymes in an inactive form that require activation, and inhibition by tissue inhibitors of MMPs. (TIMPs).[74,75] Many MMPs are not expressed constituitively and must therefore be expressed when needed.[76] There are multiple compounds that mediate MMP expression. Growth factors such as Epidermal growth factor, and cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1 are known to induce transcription.[70,77] Prostaglandin E2 and other eicosanoids transforming growth factor beta have an inhibitory influence on transcription.[62,77] Production of MMPs can be stimulated by TNFalpha, lipopolysaccharide, immediate early genes, c-fos and c-jun, IL-1beta and others.[78] Free radicals can also activate MMPs.[78]

Another mechanism of regulation is the secretion of MMPs in latent, or inactive forms. Most are then activated by removal of the amino-terminal propeptide.[76] The process of activation results in a decrease in molecular weight of the enzyme due the loss of the propeptide. The proMMP form is present where a zinc atom in the catalytic

domain is bound to cysteine in the pro-peptide region. When the bond is broken and the catalytic site is exposed, the enzyme becomes intermediately active. This can then lead to a process, known as the cysteine switch, where the enzyme autocatalyzes and cleaves the propeptide to become fully activated.[62,79] Proteinases, such as plasmin, trypsin, or kallikrein, activate MMPs in a stepwise manner.[62,77] In addition, pathways that result in activation work synergistically and lead to activation of other MMPs.[66]

The third control mechanism is inhibition by tissue inhibitors of metalloproteinases. TIMPs regulate MMPs once they have been activated through a process of 1:1 noncovalent complexing.[66] However, TIMPs can also bind with proMMPs to regulate MMP activity.[66] TIMPs bind to MMPs and greatly reduce their proteolytic activity.[53] Alpha macroglobulins found in serum have a large molecular weight that can inhibit MMPs by stopping them from entering tissue spaces.[69]

Physiologic Processes

The ECM is composed of proteoglycans and proteins that exist in a highly organized fashion and play a role in structural integrity. By participating in processes such as cell proliferation, differentiation, and adhesion and migration of cells, the ECM is important in physiologic events such as development of the organism, remodeling of tissues, inflammation and invasion of tumors.[76] The ECM is important in maintaining the structure of the CNS in addition to its role in transport of ions, cell migration and delivery of growth factors.[54] In the CNS, connective and vascular tissues contain most of the collagen, fibronectin, and laminin.[80] The basal lamina is composed primarily of laminin and type IV collagen and is an important structure because of its role as a barrier in the CNS.[81]

Normal physiologic processes that involve MMPs include angiogenesis, reproduction (embryo development and tissue involution)[82] and wound healing.[62,74,83] Angiogenesis is aided by MMP mediated destruction of the ECM, which allows capillary growth and expansion. The process of wound healing is similar in that MMP activity allows new cells to expand into the ECM. Inflammatory processes induce MMP expression secondary to cytokine stimulation. The MMPs then degrade the ECM and allow leukocyte invasion into the surrounding tissues.

In the brain, cerebellar granule cells and astrocytes synthesize MMPs during development. Glial cells and neurons produce MMP during neovalscularization and growth of neurites.[76] Cells in the CNS that produce gelatinases include astrocytes and microglial cells.[80,84] T cells and macrophages produce both gelatinases, which are involved with breakdown of the subendothelial basement membrane and their subsequent migration out of the vessels.[84] MMPs 2 and 9 are found mostly in microglia and astrocytes in normal white matter.[80]

Pathology

MMP activity is normally tightly controlled. Loss of regulation results in pathology.[66] The basis for the role MMPs play in disease has two components. When there is more enzyme than inhibitor, destruction of tissues or invasion of tissues by other cells occurs. When there is more inhibitor than enzyme, fibrosis occurs. Furthermore, a lack of MMP production has been incriminated in fibrotic diseases such as fibrotic lung disease, glomerulonephritis, and hepatic cirrhosis.[85]

The knowledge of the role these enzymes play in various diseases is rapidly expanding. Diseases for which excessive MMP activity has been implicated in the pathophysiology include cardiovascular disease such as atherosclerosis, cancer, rheumatoid arthritis, glomerulonephritis, periodontal disease, fibrotic lung disease, and tissue ulceration.[62,74,83,86] Gelatinase activity is increased in neoplastic cells with a higher metastatic potential.[87] Through its ability to degrade type IV collagen, MMP 9 is important in the destruction of the basal lamina, which allows for tumor cell invasion and metastasis.[86]

Canine MMPs

MMPs have been described in dogs. Specifically, canine MMP2 and MMP9 have been identified in a variety of tissues.[88-92] These MMPs have been qualitatively measured by sodium dodecylsulfate polyacrylimide gel electrophoresis (SDS-PAGE) with degradation of a substrate, referred to as zymography. ELISA and western blot techniques, using human anti-metalloproteinase antibodies, have also been used. Coughlan and others evaluated canine synovial fluid taken from dogs with rheumatoid arthritis for the presence of MMP2 and MMP 9. With the use of zymography it was found that there was gelatinase activity at 62 kDa (MMP2) and at 240 and 88 kDa

(MMP9) bands.[88] MMP 9 levels were elevated in arthritic joints in comparison to normal joints.[88] This study found that canine MMP9 is probably 88 kDa based on electrophoretic migration.[88]

In another study, MMP 2, a 62 kDa band measured by zymography, cross reacted to polyclonal antiserum against human MMP-2 and was not elevated in normal synovial fluid.[89] In this same study a 15 N-terminal residue of 62 kDa canine protein band showed 87% homology to the human proMMP2 sequence.[89]

MMP activity has been found in various other canine pathological conditions. MMP 9 has been characterized in the brains of dogs that displayed Alzheimer's disease type clinical signs. This was done by using a combination of zymography and ELISA that used human monoclonal antibodies against MMP 9.[91] In dogs with cardiomyopathy, pro MMP9 (90 kDa) was elevated in myocardial tissue from Doberman pinschers when compared to normal samples, suggesting that this enzyme is involved either directly or indirectly in the pathophysiology of this disease.[90] Recently, by use of zymography, canine high-grade osteosarcomas were found to have significantly more MMP 2 and MMP 9 activity when compared to normal tissues.[92] Also, mast cell tumors have been evaluated in a similar fashion and found to have increased amounts of active MMP 2 and active MMP9.[93]

Methods of Detecting/Analysis of MMPs

Zymography

MMPs have been evaluated in many types of tissues and fluids by various means. Zymography, substrate-incorporated SDS-PAGE, a technique that is used extensively in the study of MMPs. This process allows the identification of individual MMPs and can provide a semiquantitative amount of each enzyme present.[94] Zymography is also beneficial because it demonstrates MMPs in their various forms (both active and latent) on the same gel based on their molecular weights.[94] In this technique, a substrate such as gelatin is copolymerized with acrylamide in the running gel.[95] The enzymes are separated based on their migration in an electric field under non-reducing conditions. [96] The use of SDS for denaturation non-enzymatically activates MMPs, which allows for the identification of the active and pro forms of the enzymes.[79,94,96] Next, a detergent,

such as Triton X 100, is used to displace SDS from proteins allowing the enzymes to be re-activated.[96] The gels are then incubated in a calcium chloride containing enzyme buffer, which enhances their activity. The gelatin in the gels is then stained with Coomasie blue. Areas of clearing correspond to areas of gelatin degradation, which indicates enzyme activity.[94]

This process is sensitive enough to identify picogram quantities of MMPs. The sensitivity of zymography can be increased even more by lengthening incubation times.[81,94] Based on finding MMP 9 in CSF of patients with both viral meningitis and multiple sclerosis only with zymography and not ELISA, zymography is considered more sensitive.[53,84] The detection limit for MMP 2 was 2 pg with 48 hours of incubation (vs. 10-20 pg after 18 hours of incubation).[94]

Zymography can be used to semi-quantify enzymes using densitometry of the areas of clearing in the gel. This technique requires an imaging system to measure the enzyme bands.[94] Arbitrary units are assigned to both the degree of gelatin degradation as well as the width of the zone of clearing.[56] While controversial, many believe computer scanning can identify more faint bands of clearing that can not be visualized directly.[53,84] The intensity of the bands has been shown to correlate with MMP concentrations measured by ELISA in human samples of CSF.[53] Unfortunately, it has been shown by Leppert and others that MMP 9 has 25 times more activity against gelatin than MMP 2, which may make direct comparison between bands of clearing on the same gel problematic.[81]

The use of enzyme inhibition provides further proof that these enzymes are indeed MMPs.[55] For example canine MMP 2 and MMP 9 in osteosarcomas has been evaluated and compared to human MMP standards by first using zymography and then by inhibiting the activity of the enzymes using TIMP 2 and EDTA.[92] Others studies have used the addition of chelating agents EDTA or phenanthroline to the enzyme buffer to again inactivate the enzyme. Comparing untreated and treat gels will then help to verify the enzymes in question are calcium dependent, their activity is inhibited by chelation.[97]

Naturally occurring inhibitors of MMPs can complicate MMP identification when using an enzyme activity assay. However, the process of zymography separates MMPs

from their low molecular weight inhibitors (TIMPs). These inhibitors are normally present as part of the mechanism of control of MMPs. However TIMP levels may be altered in with various diseases. This allows the identification of the MMP and is another advantage of zymography.[94] Furthermore, reverse zymography can be used to identify TIMPs. In this procedure regular zymography is performed and then the gels are incubated in a buffer containing MMPs. All gelatin is degraded by the MMPs except in the portion of the gel where the TIMPs are located. Therefore these areas are the only regions that are stained.[98]

ELISA

A one-step sandwich enzyme immunoassay using monoclonal antibodies has been developed for human MMP 9 and has been used to evaluate human plasma.[99] ELISAs for many different MMPs are now commercially available. They have the advantage of being specific for individual MMPs, providing quantification of MMPs in a relatively small amount of time, and not being altered by TIMP binding.[100] Unfortunately, ELISA does not allow the identification of activated and latent MMPs. While zymography is more sensitive, it is only semiquantitative. This is because an MMP that is complexed or degraded shows a different pattern of migration and would not be included in the measurement of a particular band. For this reason ELISA cannot be directly compared with zymography.[84] The combination of ELISA and zymography may be helpful when used together.

Normal values

There is considerable variation in the amounts of MMP 2 and MMP 9 that have been reported in human CSF. In one study, MMP 2 levels were found to range from 14.6 to 183.2 ng/ml in normal human CSF when measured by ELISA. In the same study, patients with viral infections had MMP-2 levels ranging from 1.8 to 134 ng/ml.[53] In a group of normal CSF samples taken from humans there was a median MMP 2 concentration of 32.7 ng/ml.[53] All normal samples of CSF found to have no MMP-9 by zymography, were evaluated by ELISA and found to be negative (detection limit 0.8 ng/ml). In patients that had CSF positive for MMP 9 (33%) as determined by the more sensitive technique of zymography, the levels ranged from 1-87 ng/ml.[53] In one group of patients with MS, MMP 2 was found to be present in levels between 0.51 ng/ml (the

detection limit of the assay) and 1.95 ng/ml.[84] The samples were not quantified because no differences were found between in MMP 2 levels in the normal group when compared to patients with MS.[84]

MMP's in CNS disease

Based on studies evaluating normal human subjects MMP 2 is constituitively expressed in CSF and in CNS tissues, while significant quantities of MMP 9 are produced only as the result of pathological processes.[78] In the normal human brain, MMP 2 expression can be detected by in both neurons as well as unidentifiable cells in the perivascular space.[64] Others have found MMP expression in microglia, oligodendrocytes and astrocytes.[66] Membrane type MMP, another class of MMP, activates latent MMP 2 produced by astrocytes during normal physiologic processes such as growth and tissue remodeling.[78] MMP 2 is expressed in human and animal CSF in a latent form. However, based on studies in lab animals where MMP 2 was injected intracerebrally, active MMP 2 is capable of disrupting the BBB.[60]

Contrary to earlier studies, most authors agree that no MMP 9 is present in the CSF of healthy individuals.[53,56,101-105] Using immunocytochemistry it has been shown that MMP 9 is not normally present in CNS parenchyma. However, it may be found in neutrophils within the CNS vasculature and some white matter microglial cells.[64,80] TIMP-1, an inhibitor of MMP 9, is constituitively expressed in normal human CSF.[105]

MMPs have multiple roles in diseases of the CNS. The inactive form of MMP 9 is found in the CSF in a variety of nervous system diseases of humans and lab animals including Multiple Sclerosis, viral meningitis, Lyme disease, and bacterial meningitis.[53,54,56,84,101,102,106,107] A common feature of these diseases is disruption of the BBB and migration of leukocytes into the CNS. It has been postulated that MMP 9 is involved in the disruption of the BBB.[103,106] In addition, MMP 9 may be involved with the migration of white cells across blood vessels.[103] Other MMP pro-inflammatory actions include destruction of myelin and tumor necrosis factor alpha release.[77] These activities may be mediated through MMP release by leukocytes and brain tissue during pathologic processes like inflammation or even metastasis. MMP activity may result in

edema and hemorrhage as a result of disruption of CNS vasculature. This was demonstrated using MMP inhibitors in lab animals.[76,108]

The degradation of the blood-nerve or blood-brain-barrier secondary to MMPs 2 and 9 allows immune cells to enter the nervous system from the blood through the disruption of the subendothelial basement membrane.[81,109] In normal human white matter it has been shown that microglial cells were immunoreactive for MMP 9, which may allow rapid production and activation during disease.[80] An in vitro study using rat astrocytes showed that these cells produce MMP 2 and MMP 9 that can be stimulated by cytokines such as interleukin 1 and inhibited by such cytokine altering drugs as corticosteroids.[110] In cases of bacterial meningitis where 27 children were followed over time with repetitive neurological examinations and CSF analysis, a positive correlation was found between increasing MMP 9 concentrations and TNF alpha levels as well as residual neurological damage.[105]

Stroke

MMP 9 is expressed in most microvascular endothelial cells while MMP 2 is not, suggesting that MMP 9 plays a role in inflammatory disease states.[80] For this reason MMP 9 might have a significant role in CNS ischemic events such as stroke.[64] In acute ischemic lesions, MMP 9 expression is greatly elevated in invading neutrophils and macrophages, indicating that this MMP is involved in the processes of secondary tissue injury and vasogenic edema as a result of leukocyte invasion.[64] One theory to explain some of the actions of MMP 9 has been taken from the link between an increased risk of hemorrhage and the use of tissue plasminogen activator (t-PA) for acute stroke lesions. Tissue-plasminogen-activator activates MMPs and therefore might enhance vascular disruption leading to hemorrhage.[64]

Based on tissue samples taken from patients with ischemic brain injuries, after a stroke, MMP 9 activity is greater than MMP 2 within the CNS.[58] While MMPs 2 and 9 are present in normal plasma, based on the disproportionate rise in CNS MMP 9 compared to MMP 2 in these cases, the increase in MMP 9 is probably not due to enzyme leakage but rather secondary to MMP 9's role in the pathophysiology of stroke.[58] However, MMP 2 is also involved in this type of disease process. In a focal ischemia model in the brain of primates, there is a significant increase in latent MMP 2 within one

hour following middle cerebral artery occlusion and reperfusion, which also correlates with the extent of neuronal injury.[111]

MMPs may have more of a role than just destruction of the BBB following CNS ischemic injuries. After a collagenase induced injury to rat brains, an increased amount of MMP 9 was present. Unfortunately the source of MMP 9 in this study was unknown.[112] However, this study supports the enzyme's role in not only BBB breakdown, but also ECM disruption that leads to edema and cell death.[112] Also following hemorrhagic transformation following an ischemic injury, latent MMP 9 levels are increased.[111] In this type of disease process the use of a broad spectrum MMP inhibitor BB-1101 reduces secondary brain edema.[113]

Inflammatory Diseases

MMPs are important in inflammatory diseases of the CNS. Altered MMP profiles have been found in the CNS of patients with Lyme disease, HIV, and bacterial meningitis.[54,103,105,106] Patients with neuroborreliosis were found to have increased MMP-9 levels in the CSF.[54] MMP mediated destruction of the ECM may promote the spread of organisms such as B. burgdorferi in the CNS.[54] Other infectious diseases show alterations in MMP expression. In children with bacterial meningitis, 97% of patients had MMP 9 in their CSF. However, levels of MMP 2 and TIMP 2 were not increased.[105] There is also similar MMP expression in patients with viral meningitis.[53] In these cases the neutrophil count correlated with MMP-9 levels while mononuclear cells did not.[53] Patients with HIV also show MMP-9 activity that correlates with CSF pleocytosis and an increased CSF-to-serum albumin ratio.[103] It is speculated that MMPs are involved in the pathogenesis of neurological complications of HIV.[103] MMP 9 is increased in Human T cell lymphotrophic virus type 1 infected human and rat glial cells, while MMP 2 expression is not changed.[114] HTLV-1 is the agent that causes a chronic myelopathy (HTLV-1 associated myelopathy). The exact role of MMPs in this disease process is probably similar to the activity of MMP 9 in other diseases in that this enzyme damages the BBB, but might also have a role in CNS ECM destruction.[114]

CSF MMP levels are also altered in animal infectious CNS diseases. Rabbits with pneumococcal meningitis have increases in CSF protein and cell count that correlates with

increases in MMP-9 levels.[107] MMP-9 is also present in the CSF of rats with experimentally induced meningococcal meningitis.[106] The administration of the MMP inhibitor BB-94 (Batimastat) decreases the BBB permeability in these rats, but WBC counts are not significantly different.[106] Based on these results, MMP 9 inhibition does not inhibit WBC migration into the CNS.

Immune Mediated Diseases

Immune mediated inflammatory diseases also show changes in MMP expression. MMP-9 has increased activity in the blood of human patients with Guillain-Barre syndrome and increasing levels correlates with the severity of neurological deficits.[115] Also, human chronic inflammatory neuropathies have significant MMP 2 and MMP 9 activity, mediated through T cells.[109] In experimental autoimmune neuritis in mice, an experimental model of Guillain-Barre syndrome, MMP 9 is elevated in the T lymphocytes that surround blood vessels.[116] This suggests a role for MMP 9 in the disruption of the blood-nerve barrier due to its presence in inflammatory cells.

MMPs are involved in the pathogenesis of multiple sclerosis (MS) and the murine inflammatory CNS disease, experimental autoimmune encephalomyelitis.[56,117] MMPs 2 and 9 may have two roles in MS, disruption of the BBB allowing invasion by inflammatory cells and the destruction of myelin.[84] In addition to destroying myelin basic protein, the matrix component of the nerve sheath is also destroyed.[117] MMP 9 levels correlate with gadolinium contrast enhancement on MRI, a common method of evaluating breakdown of the BBB.[102]

Cells that produce MMP 9 in MS include astrocytes, microglia and endothelial cells.[117] Invading white cells, in particular monocytes and lymphocytes, may also release MMPs that are found in perivascular cuffs around CNS vasculature.[64,117] One group suggested that mononuclear cells are the source of MMP 9 in this disease, based on an increase of MMP 9, but not MMP 2, in CSF.[84] Others have reported that MMP 9 is found mostly in macrophages in acute MS lesions, but then later it is found only in neutrophils.[64]

Further support of the importance of MMPs in this disease is seen in patients with multiple sclerosis where elevated levels of MMP 9 have been found in the serum as well

as the CSF.[84,118] Serum may leak across a disrupted BBB and account for some of the increase in MMP 9 found in the brain.[84] Expression of MMPs in MS is not constant. A difference in MMP activity occurs based on the age of the MS lesion.[64] Acute lesions have more MMP 2 than those that are more chronic. [64]

There is some debate about the exact role of MMPs in MS patients. MMP 2 most likely has a role in the pathophysiology of MS because it can degrade the intrinsic membrane protein of myelin, myelin basic protein (MBP). Based on the work of Chandler and others using Hamster ovary cell produced MMPs, MMP 2 is 100 times more able to degrade MBP than MMP 9.[117] Therefore, MMP 9 may be a marker of MS, but may not be the most destructive component of the disease.[64]

CSF MMP 9 levels do not correlate with the clinical severity of MS.[84] Also, there are variations in CSF MMP 9 expression based on the type of MS present. MMP 9 activity is present in 100% of patients with relapsing-remitting multiple sclerosis but in only 57% of patients with primary progressive multiple sclerosis.[84] In the relapsing and remitting form of the disease MMP 9 levels correlate with the IgG index, which is supportive of chronic intrathecal immunoglobulin production.[84] The presence of MMP-9 in CSF may be a sensitive indicator of neuroinflammatory disease, but it is not specific for a particular type of disease such as relapsing-remitting MS or meningitis. This makes differentiation of the two diseases difficult by MMP analysis alone.[84]

CNS Neoplasia

MMPs are involved in CNS neoplastic processes including gliomas and meningiomas.[57,119-121] MMP2 and MMP9 levels correlate with activity of brain tumors in some studies.[72] These enzymes may have multiple functions in neoplasia including the destruction of the ECM, which allows tumor cells to invade and metastasize within the CNS, in addition to promoting angiogenesis.[72,121,122] In health, the CNS extracellular matrix contains the glycoprotein versican and glial hyaluronate binding protein (GHAP) both of which inhibit the migration of neurons, cell attachment and axonal growth.[72]

Human malignant gliomas have an increased level of MMP 2 when compared to normal brain tissue.[119] This enzyme exists in the endothelium of migrating and dividing

cerebral vessels, suggesting a role for angiogenesis and tumor invasion.[119] In another study of human gliomas, it was found that MMP 2 was upregulated and it was suggested that this enzyme played a role in invasion by tumor cells and angiogenesis.[122] MMP expression in human gliomas has been inconsistent; a recent study found MMP 2 activity to be more elevated and only faint bands of MMP 9 were found with zymography.[121]

MMP expression is correlated with the prognosis and aggressiveness of some CNS neoplastic diseases. In cases of human pediatric neuroblastoma, MMP 2 activation by MT-MMP1, a membrane type MMP 1, was correlated with the stage of the disease and resulted in a worse outcome.[123] Active MMP 2 is not present in normal tissues. Increased MMP 9 activity is present in human glioblastomas when compared to meningiomas and astrocytomas.[124] This suggests that MMP 9 plays a large role in the invasiveness of this aggressive type of tumor.[124]

The CSF of patients with primary and metastatic brain tumors was found to have proMMP-9 in addition to the proMMP-2 present in control samples.[72] In some forms of CNS neoplasia the form of MMP present may be suggestive of a particular disease process. For instance activated MMP-2 may be an indicator of meningeal carcinomatosis.[72] Therefore, analysis of CSF may serve to aid in diagnosis and detection of tumor recurrence, prognostication, as a target for therapy.[72] No correlation was found between MMP levels and glucose and protein quantities in these types of cases.[72]

CSF Cell Count and MMP Levels

The relationship between CSF cell counts and MMP levels is unresolved as various studies have found conflicting results. Leukocytes produce MMPs, however, other cells within the CNS, including endothelial and glial cells, produce MMPs and therefore may contribute to CSF levels of these enzymes. Possible reason for variations reported in MMP production may be the result of the type of disease present as well as the stage of the disease. While MMPs play in the breakdown of the BBB and the migration of WBCs into the CNS, basic questions still remain about The exact relationship between CSF MMP levels and CSF leukocyte counts. This area clearly warrants further investigation.

Some have reported that the CSF cell count and MMP 9 levels do not correlate in diseases like bacterial meningitis, MS or optic neuritis.[101,105] However, a correlation between increased MMP9 and a pleocytosis in CSF has been found in multiple CNS diseases such as MS, Lyme disease and viral meningitis.[53,54,56,84,125] In a recent study of patients with various neurological disorders, MMP9 was present in all CSF samples with cell counts greater than greater than 4/μl.[125] Blood brain barrier disruption alone, determined by the albumin quotient, did not result in large elevations of MMP9 in these patients.[125] However a pleocytosis alone does not account for increased MMP activity because in some patients with relapsing-remitting MS with normal CSF cell counts (<4/μl), measurable levels of MMP 9 in CSF can be present.[84]

Multiple studies have confirmed the relationship between pleocytosis and MMP levels. However, it may be difficult to make correlations between other parameters in CSF. This may be because much of MMP 9 found in CSF is the product of leukocytes and not from the serum secondary to BBB disruption. There was no correlation between the albumin quotient and CSF MMP9 levels or the CSF/serum ratio of MMP9 and the albumin quotient in patients with various CNS inflammatory diseases.[125] Also, MMP 9 levels in CSF have not correlated with the albumin quotient in some cases of MS.[84] In contrast, some have reported that CSF parameters have variable correlation with MMP activity. In patients with HIV, MMP 9 levels correlated with a pleocytosis and albumin quotient, but not with CSF total protein.[103] A correlation exists between MMP9 and CSF total protein of the CSF in cases of bacterial meningitis.[106]

The type of white cell present and type of disease may influence MMP expression in CSF. This is evident in bacterial meningitis patients that have significantly higher MMP 9 concentrations than patients with multiple sclerosis or viral meningitis.[53,84,105,125] MMP 9 levels correlate with the number of neutrophils present in the CSF.[53] Using techniques to isolate white cells from the blood of patients with various inflammatory diseases it has been shown that granulocytes and monocytes/macrophages produce a larger amount of MMP9 than lymphocytes.[125] Therefore, the type of inflammation may directly affect CSF MMP9 levels. Inflammatory diseases that have predominantly lymphocytic inflammation may have

lower levels of MMP9 than diseases with granulocytic or granulomatous inflammation. The role of leukocytes is still unclear. In an animal model of meningitis in rats, inhibition of MMPs decreased BBB disruption but granulocytes still entered the CSF.[106] The duration of a disease such as bacterial meningitis and the particular etiologic agent involved may not affect MMP 9, TIMP1 or TNFalpha expression.[105]

Therapeutic Considerations

With such a wide range of pathologic properties, the inhibition of MMP function has been a major area of research. Inhibitors of MMPs are being evaluated for use in many different diseases. Currently there are clinical trials for synthetic MMP inhibitors for rheumatoid arthritis, and various forms of neoplasia including prostate and small cell lung carcinomas.[77] Unfortunately, little long term information about the effects of these compounds is available and there is concern about the potential side effects of the broad spectrum inhibitors of MMPs because of their importance in normal physiologic activities.[77]

MMP inhibitors are potentially valuable in diseases of the CNS. Experimental autoimmune encephalomyelitis (EAE) has been shown to be improve in a dose-dependent manner with inhibition of MMPs using a hydroxamate inhibitor.[126] The use of this drug resulted in a decrease in inflammation and subsequent demyelination. In another study of chronic relapsing EAE, multiple MMP inhibitors decreased glial scarring, demyelination, and expression of cytokines in the CNS.[127] Because T cells produce MMP 2 and MMP 9, inhibition of T cell MMP production might inhibit T cell migration across the subendothelial basal lamina, a potential component of T cell mediated autoimmune diseases.[81] Based on recent data that MMP9 is elevated in patients with viral meningitis, synthetic inhibitors of MMPs may play a role in the therapy of viral meningitis again by inhibiting inflammation.[53]

Tetracycline and doxycycline are known inhibitors of MMP2 and MMP9.[104] These drugs were found to inhibit the activity of MMP 2 and MMP 9 in human CSF. A higher concentration of doxycycline was required to produce an inhibitory effect.[104] In an in vitro swine model of aneurysmal disease, doxycycline decreased the degradation of elastin and the activity of MMP 9.[128] The dose of doxycyline needed to achieve the

levels used in vitro is possible to achieve by oral administration of the drug.[128] The mechanism of action of doxycycline is the nonselective inhibition of MMPs through binding of active zinc sites and an inactive calcium site, which inhibits the function of the enzyme through a change in conformation.[128] There are a number of compounds that inhibit MMP function by chelation of calcium or zinc ions. For instance, the angiotensin inhibitor Captopril, used to treat congestive heart failure, may be effective against gliomas in humans through chelation of zinc.[69]

Another group of MMP inhibitors is the hydroxamic acid group. This is a group that is considered to be broad spectrum inhibitors based on their ability to decrease all classes of MMP proteolysis.[84] Members of this group include marimastat and batimastat.[129] Extensive research has been done with regard to the efficacy of these drugs in neoplastic processes such as metastasis and tumor cell invasion.

Drugs that modulate the immune system have mechanisms that involve MMPs. High doses of corticosteroids decrease transcription of MMPs. High doses of methylprednisolne have been shown to reduce MMP 9 levels in the CSF of patients with multiple sclerosis after 3 days.[102] Similarly, the beneficial effect of interferon beta for multiple sclerosis may be mediated by MMP inhibition.[84] In cases of bacterial meningitis it has been suggested that the use of corticosteroids may decrease neurological damage because MMP 9 expression is down regulated.[105] Selective inhibition of MMP 9 may aid in decreasing vasogenic edema and CNS tissue damage.[64] Neutral proteinase inhibitors such as epsilon aminocaproic acid have been used for inflammatory CNS diseases and their mechanism of action may be through the inhibition of the MMP activator plasmin.[116]

Sample Considerations and Storage

The storage of CSF is an important consideration, especially for zymography, because the activity of these enzymes may be altered by repeated freeze/thaw cycles or by storage at inappropriate temperatures.[72] It is recommended that samples should only be frozen once before zymography.[72] Samples of human CSF can be stored at 4°C for up to 7 days following lumbar puncture, with no apparent alterations in MMP activity.[54]

CSF samples are generally stored at -70°C, but others have stored CSF samples at -20°C without problems.[106]

Blood contamination of CSF samples is also a factor because MMP 2 and MMP9 are found in normal human blood serum.[125] However, samples with blood contamination have been used in at least one study, but an effort was made to use the tube with the least amount of blood. This same study found that adding as much as 5% normal human serum to CSF made no difference in gelatinase activity.[54] Another consideration for falsely altering MMP activity is the possibility of white blood cells contributing to MMP production. Some have excluded CSF samples with WBC counts > 5/mm3.[72] Others have used centrifugation of samples before storage.[106] Previous studies in veterinary medicine of canine MMPs have evaluated synovial fluid for gelatinases after centrifugation and subsequent storage of the supernatant at -20°C.[88]

Objectives and Hypothesis

It is evident that while there is a considerable amount that can be learned from the routine analysis of CSF, the potential to gain more information exists. MMPs have been evaluated in canine arthritis and neoplasia, but information about MMPs in normal or diseased canine CSF is not known.[92,106] This study hopes to demonstrate MMP expression in normal canine CSF. Future research may show that MMP expression and activities are altered by CNS disease. This information may aid in the diagnosis of different canine neurologic diseases. Understanding MMP function in the canine CNS may also help to gain an understanding of some of the diseases of the canine CNS. This knowledge may also lead to the use of MMP inhibitors as an aid in therapy.

This study will establish the presence or absence of MMPs 2 and 9 in normal canine CNS. In addition, the form in which these enzymes are present, activated, proenzyme, or complexed, will be characterized. An attempt will be made to determine the quantities of MMPs 2 and 9. Any correlation between CSF indices (WBC count, protein) and MMP levels will be evaluated. It is hypothesized that pro MMP2 will be constituitively expressed in normal canine CSF, while MMP9 activity will not be present. The CSF will be evaluated using a combination of gelatin zymography and ELISA. The genetic similarity of MMPs between various species will allow the use of commercially available human products. Human MMP 2 and MMP 9 standards will be used for

references in zymography since these MMPs have similar molecular weights when compared to canine MMPs.[88-92] Also, due to the similarity in MMP structure, polyclonal antibodies against human MMPs will be used for ELISA. [89]

Chapter 3: Characterization of MMP 2 and MMP 9 in Normal Canine CSF

Introduction

Cerebrospinal Fluid (CSF) analysis remains one of the most common diagnostic aids used to evaluate diseases of the central nervous system. CSF analysis, with rare exceptions, generally allows the distinction of the disease into inflammatory and non-inflammatory categories. However, CSF rarely provides a specific diagnosis.[6] It was found that in one third of dogs with either inflammatory or infectious diseases of the CNS, the specific cause of inflammation could not be identified by analysis of CSF.[25] New methods of gaining diagnostic information from CSF are under investigation. For instance, interleukin-8 has been evaluated in dogs with steroid responsive meningitis-arteritis and found to play a role in chemotactic activity of leukocytes into the CNS.[52] In human medicine, a group of enzymes, referred to as matrix metalloproteinases (MMPs), have been implicated in a variety of CNS diseases. Both CSF and tissue concentrations of these enzymes are altered in many neurologic diseases including viral meningitis, amyotrophic lateral sclerosis, multiple sclerosis, brain tumors, and cerebral ischemia.[53-58,72] Characteristic patterns of MMP expression can aid in both diagnosis and prognosis of specific diseases in humans. Furthermore, extensive research has been done concerning the role these enzymes play in the pathogenesis of disease and the potential for MMP inhibition as a means of therapy.

Matrix metalloproteinases have also been identified in dogs. Specifically, canine MMP2 and MMP9 have been described.[88-92] Tissues that have been evaluated for MMPs include canine synovial fluid, the brains of dogs that displayed clinical signs of an Alzheimer's type disease, myocardium taken from dogs with cardiomyopathy, osteosarcomas, and mast cell tumors.[88,90-93]

The most common means of evaluating samples for the presence of MMPs is zymography. Utilizing substrate-incorporated sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis, zymography allows identification of individual MMPs based on their enzymatic activity. It can be used to provide a semiquantitative

assessment of each enzyme's activity.[94] Zymography has the added benefit of being able to identify both the enzyme as well as the proenzyme form in the same assay.[94]

In humans, it is known that proMMP 2 is constituitively expressed in the CSF and in the brain, while MMP 9 is present only during pathological processes.[78] In the CNS, many types of cells including neurons, microglia, oligodendrocytes, and astrocytes produce MMPs.[66] Peripheral blood leukocytes may also contribute to increases in CNS MMP levels during disease.

This study was designed to characterize both pro and active forms MMPs 2 and 9 in normal canine CSF. This study will serve as a baseline for future studies of CSF taken from dogs with diseases of the CNS.

Materials and Methods

Dog selection

Twenty-three clinically normal dogs that were used. All dogs were terminal animals from other projects that did not impact the nervous system.

CSF Collection

CSF samples were collected from the cerebellomedullary cistern while the animal was under general anesthesia (Isoflo, Abbott Laboratories, North Chicago, IL) immediately prior to euthanasia. CSF was analyzed within 20 minutes of collection. The numbers of red blood cells and nucleated cells was determined with a hemocytometer. A differential cell count was done following cytocentrifugation and Wright's staining. CSF protein and glucose concentrations were determined using colorimetric analysis on an Olympus AU400 (Olympus America, Inc., Melville, NY). After analysis, samples were divided into small aliquots and stored at -70°C until the assays were performed.

CSF samples were considered normal if the total nucleated blood cell count was less than 6 per microliter, the red blood cell count less than 25 per microliter, and the total protein concentration less than 26 mg/dl.

Histopathology

The animals were killed with an overdose of barbiturate anesthesia (Beuthanasia-D Special, Schering-Plough Animal Health Corporation, Union, NJ). The brains were

removed and placed in 10% neutral buffered formalin. Following fixation, the brains were sectioned and the cerebral cortex, hippocampus, midbrain, and the cerebellum/ brainstem were embedded in paraffin. Hematoxylin and eosin (H&E) stained sections were then evaluated by light microscopy to establish that the dogs were free of disease.

Zymography

Zymography was performed with modifications to the procedure described by Rosenberg et al. 1996.[102] Briefly, minigels were made using the Biorad Miniprotean II system (Bio-Rad Laboratories, Hercules, CA). The resolving gel consisted of 1.74 ml high performance liquid chromatography (HPLC) grade water, 45 µl 10% sodium dodecyl sulfate (SDS), 1.5 ml 30% acrylamide/0.8% bisacrylamide, 0.94 ml 1.88 M Tris, and 0.3 ml gelatin (15 mg/ml Sigma G-2500 Type A Porcine skin, Sigma Chemical Company, St. Louis, MO). The gel was polymerized by addition of 22 µl 10 % ammonium peroxodisulfate (APS) and 2.2 µl N,N,N',N'-tetramethylethylenediamine (TEMED). Following polymerization, a stacking gel consisting of 500 µl 30% acrylamide stock, 500 µl 1.25 M Tris-HCl, pH 6.8, 50 µl 10% SDS (BDH), and 3.9 ml HPLC-grade water. The gel was polymerized with 25 µl 10% ammonium persulfate (APS), and 2.5 µl TEMED. The reservoir buffer consisted of 30 ml 10X electrode buffer, 3 ml 10% SDS, and 267 ml distilled water. The gels were then loaded with CSF and 2X sample buffer for a total volume of 10 µl (5 µl CSF). The sample buffer consisted of 128 µl 1.25M Tris HCl pH 6.8, 200 µl 10% SDS, 80 µl glycerol and 40 µl saturated Bromphenol blue. Gelatinase zymography standards (0.1mg/ml) for human MMP2 and MMP9 (Chemicon International Inc., Temecula, CA) were diluted 1000:1 with HPLC water and then diluted with sample buffer as for the CSF. A tissue homogenate of canine osteosarcoma (gift form Dr. Susan Lana, Colorado State University), recognized to contain canine MMP 2 and 9, was also evaluated by gelatin zymography. The gels were electrophoresed at a constant voltage of 200 V, (limit 250V, current 75 mA, power 40W) for about 90 minutes. Following electrophoresis the gels were rinsed and then gently shaken in 2.5% Triton X 100 for 15 minutes, washed, and then shaken in Triton X 100 for 30 minutes. They were then rinsed and incubated in enzyme buffer which consisted

of 50 mM Tris pH 7.5, 200 mM NaCl, 5mM CaCl2, and 0.02% Brij-35 (30% w/v) (Sigma Diagnostics, St. Louis, MO), in 1 liter total volume of distilled water. Each gel was incubated in enzyme buffer for 18-24 hrs at 37 °C. Following incubation the gels were rinsed and stained with Coomasie Blue (CBB) stain consisting of 0.58 grams CBB, 200ml methanol, 4 ml acetic acid 1%, and 196 ml water for one hour. Following staining, the gels were destained with 10% acetic acid initially for 30 minutes, and then again for about 2 hours in fresh solution of 10% acetic acid. The gels were then washed with distilled water and allowed to dry. Enzyme activity was evaluated by comparing the distance of migration with commercial standards.

ELISA

To quantitate the amount of proMMP 2 present, a commercially available Bindazyme ProMMP-2 Enzyme Immunoassay Kit (The Binding Site, Birmingham, England) was utilized according to the manufacturer's instructions. The kit contained polyclonal antibodies against human proMMP 2. Canine CSF samples (25 µl) were diluted with 75 µl of sample diluent, and each sample was run in triplicate. A curve was generated using human standards provided in the immunoassay kit. Recombinant canine MMP 2 (gift from Dr. Barbara Kitchell, University of Illinois) was also evaluated to document the activity of canine MMP 2. The curve generated was used to estimate the amount of proMMP 2 in each CSF sample. Using the average of the optical densities a linear equation was generated using Microsoft Excel (Microsoft Systems, Seattle, WA). Each optical density was used in the linear regression equation and this value was multiplied by the dilution factor. The mean of these three numbers was used as the concentration of MMP 2 in ng/ml.

Results

CSF Analysis

All dogs were considered normal based on histopathological examination. The mean total nucleated cell count (WBC) per microliter was 0.96, with a range of 0-3 WBC/µl. The standard deviation for the WBC count was 1.02. The mean red blood cell count (RBC) was 3.65 RBC per microliter with a range of 0-21 RBC/µl and a standard deviation of 5.34. The mean total protein concentration was 12 mg per deciliter with a

range of 8-17 mg/dl and a standard deviation of 2.15. The cytology of the samples varied. However, predominantly mononuclear cells, either lymphocytes or monocytes were present.

Zymography

Using gelatin zymography, all samples showed distinct areas of clearing that corresponded in size to the commercial human standard proMMP2 (68 kDa) (Figure 3-1). A canine osteosarcoma sample with high levels of MMP 2 and MMP 9 activity was used as a canine control sample (Figure 3-2).[92] Other bands were present, however these were much more faint and varied in location when compared to the standard. A faint and somewhat variable band was present at the region that corresponded to the standard activated form of MMP 2 (62 kDa). There were no areas of clearing that corresponded to the commercial proMMP 9 (88 kDa). Other bands were not identified specifically.

Figure 3-1 Gel with normal canine CSF

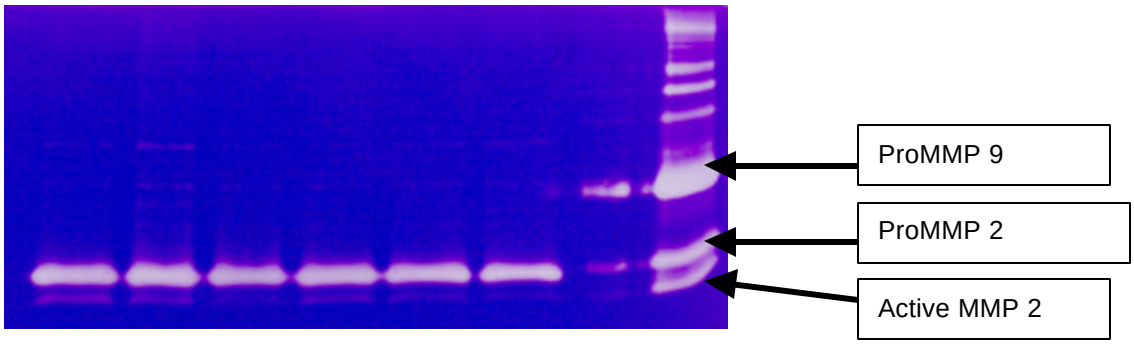
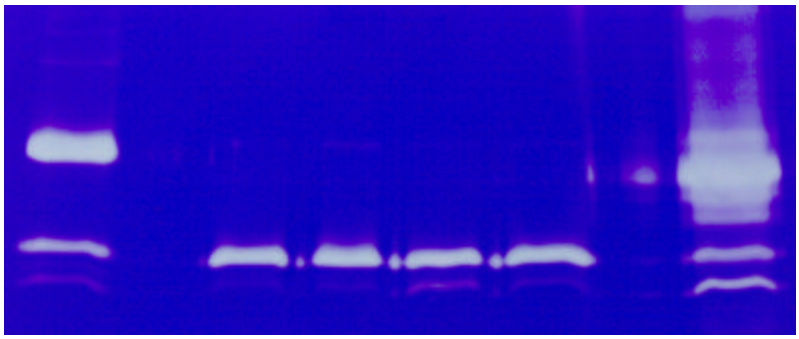


Figure 3-2 Gel with Osteosarcoma Sample



Standard CSF Osteosarcoma

ELISA

All samples also were positive for proMMP2 using a polyclonal ELISA. A recombinant canine MMP2 product was used as a control. The mean concentration of ProMMP 2 was 5.61 ng/ml with a range of 3.36-10.83 ng/ml and a standard deviation of 1.92. Correlation between ProMMP 2 concentrations and both white blood cell numbers or total protein concentration was attempted. Linear regression revealed no significant correlations between proMMP 2 concentration and neither white blood cell count ($R2 = 0.000003$) nor protein concentration ($R2 = 0.150$). (Figures 3-3 and 3-4)

Figure 3-3

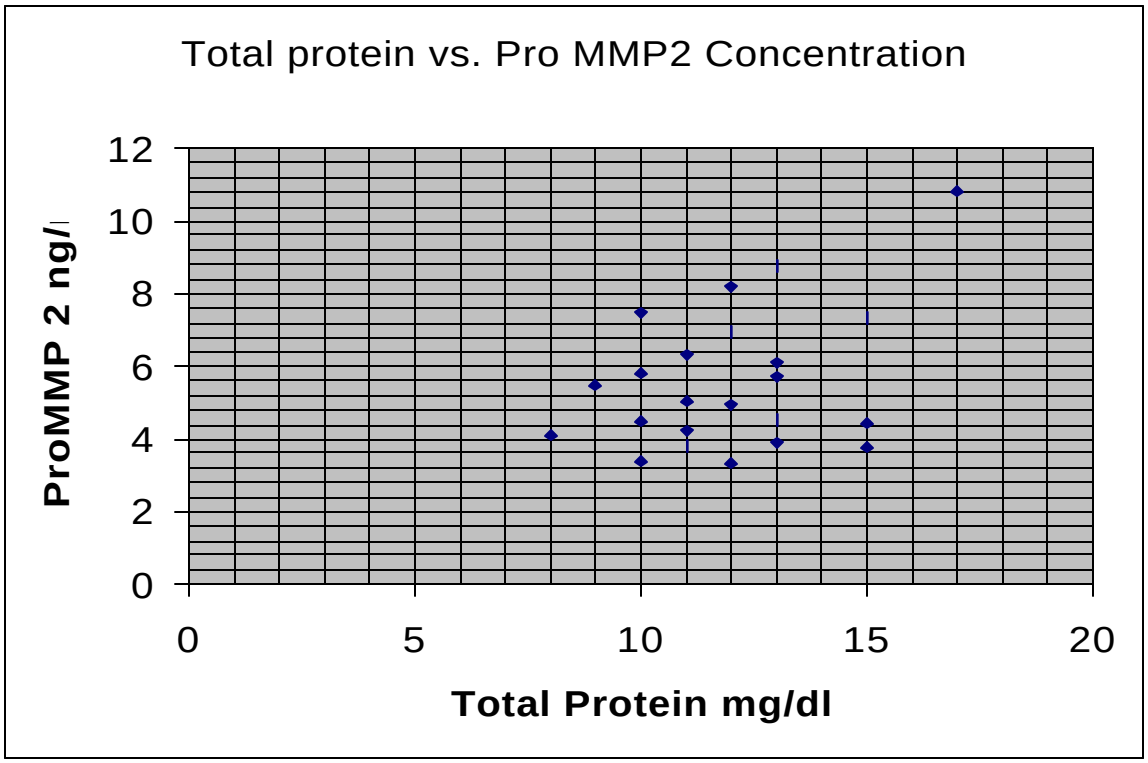
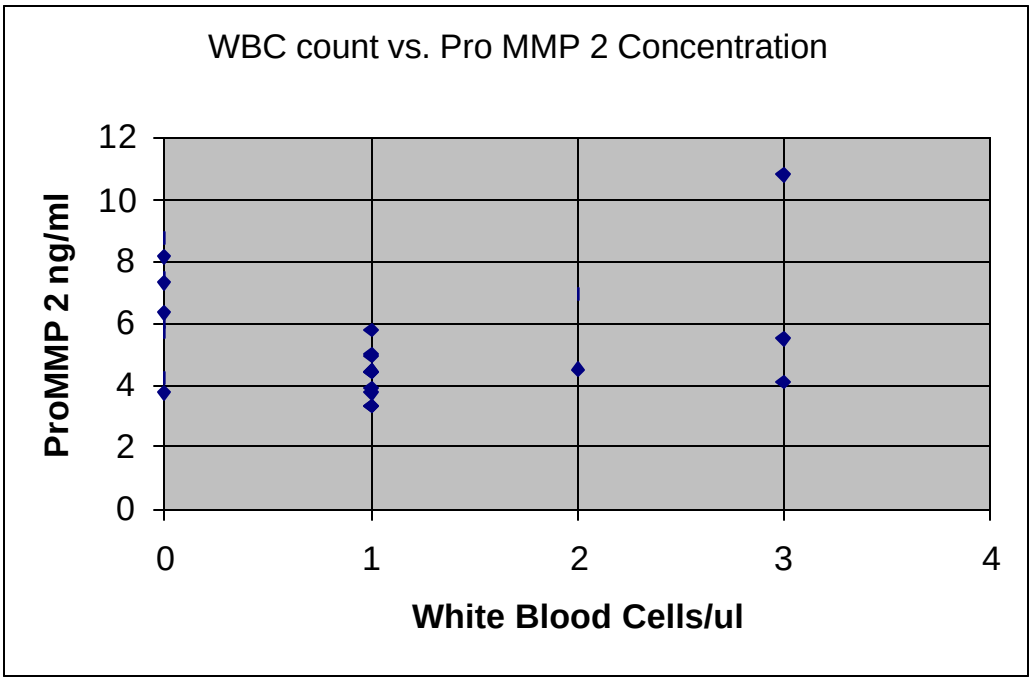

Total protein vs. Pro MMP2 Concentration
ProMMP 2 ng/l
12
10
8
6
4
2
0
0
5
10
15
20
Total Protein mg/dl

Figure 3-4


WBC count vs. Pro MMP 2 Concentration
ProMMP 2 ng/ml
12
10
8
6
4
2
0
0
1
2
3
4
White Blood Cells/ul

Histopathology

No significant histological abnormalities were detected in any of the dogs used in this study.

Discussion

The proenzyme form of MMP 2 was present in the cerebrospinal fluid of normal dogs. The active form of MMP 2 also appeared to be present in some normal samples, but at a very low activity level. The proenzyme form of MMP 9 was not present in the CSF of control dogs. No correlation was found between the CSF white blood cell count or between the CSF total protein concentration and proMMP 2 concentration. However, the relative homogenous character of the CSF samples may have made any correlations difficult to detect.

These data will serve as a baseline for future studies of matrix metalloproteinases in dogs. In addition, a consistent method of performing discontinuous zymography using dilutions of canine CSF specimens has been developed by modifying the method described by Rosenberg et al. 1996.[102] This method produces distinct bands that can be compared with commercially available standards or from known samples. There did not appear to be a significant difference in the migration of MMP 2 and MMP 9 proteins from human standards, canine osteosarcoma samples, and normal canine CSF. This is not surprising since these enzymes are highly conserved between species.[89]

Multiple faint areas of clearing were evident in both canine CSF and canine osteosarcoma samples that were not observed in the commercial standard. Based on the distance of migration, these were large molecular weight molecules greater than 88 kDa in mass. These unknown bands could be dimmers of MMP 2 or MMP9, MMP: Tissue inhibitors of MMPs (TIMP) complexes, or other gelatinases. Other studies have reported that bands not corresponding to known MMP standards could be other gelatinases or TIMPs.[54,98] Various methods have been used to verify that areas of clearing are truly indicative of gelatinase activity. One technique is to incubate samples with calcium chelating agents such as EDTA or phenanthroline in the enzyme buffer which inhibits enzyme activity.[97] This was not done in the present study. However, based on ELISA results pro MMP 2 appears to be present in substantial amounts.

An ELISA for MMP 9 was not used in the present study. Since zymography is considered a more sensitive technique and MMP 9 was not present in zymograms the ELISA would be less helpful.[53,84] This will be developed in future studies with MMP 9-positive zymograms.

Zymography appears to be useful for the evaluation of CSF because it requires very small amounts of CSF, 5-10 µl per sample. This is helpful because only limited amounts of CSF are acquired from some clinical cases. Furthermore, the enzymes appear to be relatively stable and can be stored at -70°C for long periods without loss of their activity. Many of these samples were stored for more than one year.

Zymography has been used as a semi-quantitative assay for evaluating MMPs. However, this was not done in the present study. For quantification using zymography, densitometry is performed and then arbitrary units of activity are applied.[56] This would not be useful in determining the total amount of enzyme present since zymography is only a measure of enzyme activity. It would also not be useful in comparing the quantity of two different enzymes because each MMP has different activity levels. MMP 9 has 25 times more activity against gelatin than MMP 2.[81] Therefore, quantification by direct comparison of bands of clearing is not helpful.

The proenzyme form of canine MMP 2 was quantified using an ELISA. This not only provided an estimate of the amount of enzyme present, but also verified the bands identified using zymography were MMP 2. This commercially available assay consistently identified pro MMP 2 in the unknown CSF samples. The range of values obtained in this study (3.36-10.83 ng/ml) were somewhat similar to what has been reported in humans, 0.51 ng/ml (the detection limit of the assay) and 1.95 ng/ml.[53,84]

The ELISA for MMP 2 is useful because samples can be analyzed in 4-5 hours, whereas zymography, requires 2-3 days. A potential disadvantage of ELISA is the volume of CSF required for each well (25 µl). This volume was required in order to obtain an optical density that would fall within the curve generated by the standards supplied with the kit. This is only a concern if the volume of CSF is limited in a particular case. This kit has not been validated for use with samples from dogs or with CSF. Also, based on the more sensitive zymography results, MMP 9 was absent from the CSF samples.[53,84,94]

The results of this study may be subject to various questions. One is the fact that the samples were not centrifuged to remove all WBCs, which can produce MMPs. This is unlikely to be a problem since the samples were relatively acellular (mean WBC 1/ul). This concern was addressed in a human study only when the cell count was greater than 5 WBC per microliter.[72] Alterations in MMP activity may have resulted if there was variation in the time between collection of the sample and freezing the sample at –70° C. However, it has been reported that CSF samples can be refrigerated for up to 7 days with no significant change is activity.[54] Any sort of freeze thaw cycle can potentially decrease MMP activity, and every attempt was made to try and prevent multiple freeze thaw cycles.[72]

In summary, using gelatin zymography and a commercially available ELISA, proMMP 2 was found in normal canine CSF while MMP 9 was absent. Expression of active MMP 2 was variable. This information will provide a baseline for future studies of canine CSF.

Chapter 4: Future directions

This study has established that MMP 2 is constituitively expressed in normal canine CSF while MMP 9 is not. Zymography has proven to be useful in the analysis of MMPs in canine CSF. In addition, ELISA provides a means of quantifying proMMP 2. Other methods of analyzing MMPs would be useful, especially an ELISA for MMP 9. There are many basic questions about MMP expression in the canine nervous system regarding the origin of these enzymes and the effect of collection techniques. In addition, the clinical application of MMP function may be very useful in various diseases.

Does the site of collection of CSF influence the levels of MMPs that may be found? In humans, CSF is generally obtained from a lumbar puncture, which is not always the case in dogs. Sample collection in dogs is based on the lesion localization and the skill of the clinician. It has been demonstrated in dogs that collection of CSF is best done caudal to the site of the expected lesion as a result of the flow of the fluid.[51] Lumbar samples tend to contain more iatrogenic hemorrhage, are smaller in volume, and can be technically more difficult to obtain.[15,51] While it is unlikely that there would be dramatic alterations in MMP expression between the cerebellomedullary cistern and a lumbar tap, CSF from this more caudal site needs to be characterized. Normal variations in MMP levels may be present considering that other CSF constituents vary depending on the site of collection. For instance, protein concentrations in CSF taken from a lumbar tap are generally higher than CSF obtained from the cerebellomedullary cistern.[7]

The effect of blood contamination on CSF MMP levels is not known. This is an important consideration in CSF collection because blood contamination is unavoidable in some cases. MMP 2 and MMP9 are found in normal human serum.[125] One study found that adding as much as 5% normal human serum to CSF made no difference in gelatinase activity.[54] The normal MMP 2 and 9 levels in canine blood must first be evaluated. This may aid in determining the effect of blood contamination on CSF MMP levels.

In the present study cells were not removed from CSF. The effect that centrifugation of cells would have on CSF MMP analysis is unknown. This may be important due to the normal expression of MMPs by white cells. Some studies have excluded CSF samples with WBC counts > 5/mm3.[72] Other researchers have

centrifuged samples before storage.[106] Previous studies in veterinary medicine of canine MMPs have evaluated synovial fluid for gelatinases by centrifugation and subsequent storage of the supernatant at -20°C.[88] The number of white blood cells in CSF directly correlates with MMP levels, and could therefore affect MMP levels.[125]

The source of MMP's in canine CSF is unknown. Cells that express MMPs include leukocytes, glial cells, neurons, and endothelial cells.[69,76,80,84] When alterations occur in MMP expression, what cells are responsible for these changes? T cells and macrophages have been shown to secrete MMPs 2 and 9, which are involved in breakdown of the subendothelial basement membrane and their subsequent migration out of the vessels.[81,84] The differences in MMP expression may be evident in some forms of diseases that occur in the dog. These will be discussed in later paragraphs.

Clinical Applications

There are multiple diseases that occur in the nervous system of the dog that are not well characterized. In particular, neuroinflammatory diseases that show marked CNS pathology such as granulomatous meningoencephalitis (GME), pug dog encephalitis, yorkie/maltese encephalitis, and steroid responsive meningitis/arteritis have unknown etiologies. I think that it is possible that MMPs may have involvement in these diseases. Other possible areas of investigation are neurodegenrative diseases such as degenerative myelopathy and CNS neoplasia that may have associations with MMPs. These diseases currently are not well characterized and require more study. Knowledge of MMP function in these diseases may provide insight into the pathogenesis of these diseases.

Would a disease such as GME that results in a marked perivascular inflammation and BBB disruption show alterations in MMP 2 and 9 expression? In varying concentrations the inactive form of MMP 9 is found in the CSF in a variety of nervous system diseases of humans including multiple sclerosis, viral meningitis, Lyme disease, and bacterial meningitis.[54,56,84,101,102,106] Also, MMP 9 expression is found with bacterial meningitis models in rabbits and rats. [106,107] A common feature of these diseases is disruption of the BBB and migration of leukocytes into the CNS. MMP 9 may be involved in the movement of white cells across blood vessels and in disruption of the BBB.[103,106]

MMP levels could be measured in dogs with GME. In these cases serum and CSF levels of MMP 2 and MMP 9 could be evaluated at the time of the initial presentation and then compared to normal dogs. It has been shown that in patients with multiple sclerosis, elevated levels of MMP 9 have been found in the CSF and serum, suggesting that MMPs are involved in the disruption of the BBB through production by invading leukocytes.[84,118] This is further supported by increased production of MMPs by leukocytes found in perivascular cuffs.[64] Subsequently, patients with GME could then have serum and CSF monitored after treatment with corticosteroids or other drugs that inhibit MMP function, such as doxycycline.[128]

It has been shown that the stage of some diseases may have an effect on MMP expression. There are alterations in MMP expression in multiple sclerosis dependent on the type and phase of disease.[84,118] With MS, there are many changes in MMP expression that occur in relation to the white blood cell count, the phase of the disease (acute, chronic), and with therapy such as immunosuppressive doses of corticosteroids.[102] A difference in MMP expression occurs based on the age of the MS lesion. MMP 2 is expressed mostly by leukocytes in perivascular cuffs around blood vessels, with expression relating to the length of time that the lesion has been present.[64] Older lesions have less MMP 2. Also, MMP 9 is found mostly in macrophages in acute MS lesions. Otherwise MMP 9 is found only in neutrophils.[64] In GME cases the lesions could be evaluated for MMP 2 and 9, in an attempt to characterize MMP presence around the vasculature and in invading leukocytes.

The type of white cell present and type of disease may influence MMP activity in CSF. Bacterial meningitis patients have significantly higher concentrations than patients with multiple sclerosis or viral meningitis.[53,84,105] Therefore, would there be differences in a disease such as SRM that is primarily neutrophilic compared to a disease like GME that is primarily mononuclear? MMP 9 levels have been found to correlate with the number of neutrophils present in the CSF, which may be explained by the fact that this type of inflammatory cell secretes MMP 9.[53] Therefore, the type of inflammation may directly effect CSF MMP 9 levels. Inflammatory diseases that have predominantly lymphocytic inflammation may have lower levels of MMP 9 than diseases

with granulocytic or granulomatous inflammation. Granulocytes and monocytes/macrophages produce more MMP 9 and than lymphocytes.[125]

Although the presence of MMP-9 in CSF is a sensitive indicator of inflammatory processes in humans, it is not specific for a particular type of disease.[84] Therefore it is unlikely that MMP 9 detection would aid in making the diagnosis of a specific disease. It may be useful to use MMP analysis to serve as a marker of inflammation in the various neuroinflammatory diseases of dogs. This may also aid in monitoring therapy. Furthermore, while a profile of MMP activity for individual diseases is unlikely to provide a specific diagnosis, this information may further support a diagnosis, especially in cases such as GME where the CSF may be within normal limits or show minimal alterations.

Other diseases may also have MMP involvement. In Steroid Responsive Meningitis (SRM) of the dog, a profound neutrophilic pleocytosis is found initially.[31,130] MMP 9 levels have been found to correlate with the number of neutrophils present in human CSF, which may be explained by the fact that this type of inflammatory cell secretes MMP9.[53] There may be significant differences in MMP expression in cases of SRM when compared to GME.

Canine degenerative myelopathy has been a frustrating disease to diagnose and treat. At this time definitive diagnosis requires histopathology of the spinal cord, which is not possible antemortem. Diagnosis of the disease is always presumptive and relies on signalment, history, clinical signs, imaging such as a myelogram or MRI and a noninflammatory CSF profile. While degenerative myelopathy is not an inflammatory disease, MMPs may still play a role. MMPs are known to be present in neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease.[55,91,131-133] The characterization of MMPs in dogs with degenerative myelopathy may aid in supporting the diagnosis of this disease and may be useful in developing effective therapy. Epsilon amino caproic acid, a protease inhibitor, has been anecdotally shown to have a positive effect in these types of cases. Neutral protease inhibitors such as epsilon aminocaproic acid have been used for inflammatory CNS diseases and their mechanism of action may be through the inhibition of plasmin, which then activates proMMPs.[116]

Intervertebral disc disease is a very common clinical problem in veterinary medicine. MMPs are involved in the pathogenesis of this disease in humans.[134-136] Through analysis of tissue homogenates, cervical and lumbar degenerate discs have been shown to have increases in MMP activity.[136] A correlation was found between the degree of disc degeneration and MMP 2 and MMP 9 activities in both the pro and active forms when disc samples from human patients with intervertebral disc disease were analyzed.[134] While this disease is relatively easy to diagnose and treat in dogs, it can still have devastating consequences. The investigation of MMP activity in intervertebral disc disease may eventually aid in therapy following acute herniation by lessening the secondary injury that occurs with spinal cord compression and vascular compromise. Another goal of therapy by MMP inhibition may be to slow the progression of some forms of the disease, such as the slow herniation that occurs with type II disc disease. An additional use might be to decrease the recurrence of disc herniation in the chondrodystrophic dog by the long-term administration of drugs that inhibit the protease activity of MMPs.

The expression of MMPs in neoplastic diseases of the CNS in humans is well documented. Expression of MMPs in the CSF is altered with CNS neoplasia. Currently in veterinary patients CSF analysis in cases of suspected neoplasia is generally not helpful, except in the very rare cases where tumor cells are seen. CSF analysis can be nonspecific or have a variety of changes.[37] Collection of CSF in cases where brain tumors are present may provide additional risk due to herniation secondary to increased intracranial pressure. Presently some are less willing to analyze CSF because it provides little information and increases risk for the patient. MMP recognition could aid in the diagnosis of different disorders such as neoplasia by serving as a marker of disease, as an aid in monitoring response to therapy, and for determining prognosis. Also, MMP inhibition may play a role in preventing or at least slowing the spread of certain CNS tumors.

Therapeutic Considerations

Inhibitors of MMPs are being evaluated for use in many different types of diseases. In diseases of the CNS, MMP inhibitors are potentially valuable. Currently, clinical trials are underway for synthetic MMP inhibitors of rheumatoid arthritis, and

various forms of neoplasia including prostate and small cell lung carcinomas.[77] Unfortunately, little long-term information is available and there is concern about the potential side effects of the broad-spectrum inhibitors of MMPs due to their systemic activities.[77] However, there are some inhibitors that are already used commonly in veterinary medicine. It might be useful to evaluate the effects of these drugs on the various diseases mentioned previously.

The animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), has been shown to improve in a dose-dependent manner with inhibition of MMPs using a hydroxamate inhibitor.[126] Positive effects were noted with demyelination and inflammation. In another study of chronic relapsing EAE, multiple inhibitors of MMPs were shown to decrease glial scarring and demyelination.[127]

Would veterinary patients respond to therapy that inhibits MMP function? Diseases such as GME have been difficult to treat and other therapies would be helpful. Multiple drugs for MMP inhibition exist and some drugs such as doxycycline, are already used in veterinary medicine for other purposes. The addition of these types of drugs may allow for a different approach to treating these diseases. For instance, MMP inhibition might allow the use of a lower dose of corticosteroids, which might lessen side effects.

Corticosteroids are known inhibitors of MMP expression and this may account for some of the effectiveness of this class of drug in inflammatory diseases.[102] High doses of corticosteroids decrease transcription of MMPs and have been shown to decrease MMP9 levels in the CSF of patients with multiple sclerosis.[102] High doses of methylprednisolne have been shown to reduce gelatinase B levels after 3 days.[102] In addition to corticosteroids, the beneficial effect of interferon beta for multiple sclerosis may be mediated by MMP inhibition.[84] Selective inhibition of MMP 9 may aid in decreasing vasogenic edema and CNS tissue damage.[64]

Tetracycline and doxycycline in various concentrations are known inhibitors of MMP2 and MMP9.[104] These drugs were found to inhibit the activity of MMP 2 and MMP 9 in human CSF.[104] The mechanism of action is through chelation of calcium. There are a number of compounds that inhibit MMP function by chelation of calcium or

zinc ions. For instance Captopril, which is commonly used for its zinc chelation of angiotensin converting enzymes, may be effective against gliomas in humans.[69]

The hydroxamic acid group enzyme inhibitors stop MMP proteolysis.[84] Members of this group include marimastat and batimastat.[129] These drugs are considered to be broad-spectrum inhibitors, meaning that they can inhibit all classes of MMPs. Extensive research has been done with regard to the efficacy of these drugs in neoplastic processes such as metastasis and tumor cell invasion.

In summary there are multiple questions left to answer regarding MMP expression in the canine nervous system. Analysis of MMPs in various diseases of the dog may provide useful information about pathophysiology and potential therapies.

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Appendix 1-1

Total cell count:

Using capillary pipettes, one side of a hemocytometer is charged with undiluted CSF. All cells within the 9 primary cells are counted and the number is multiplied by 1.1. This equals the total cell count per mm3

Differential Cell Count/Cytology:

Centrifugation

CSF sample is centrifuged at 1000 rpm for 5 minutes. The supernatant is removed and the pellet at the bottom of the tube is added to one drop of serum and a smear is made.

Filtration

Using a clear membrane filter with a pore size < 5 mm, a mixture of 1 ml CSF and 2 ml of 40% ethanol are put through a filter using either a syringe or gravity. After filtration the filter placed in 95% alcohol for two minutes, followed by staining of the filter.

Sedimentation

A sedimentation chamber is fixed to a slide. CSF (1 ml) is added to the chamber for 25 minutes. This allows the cells to fall to the slide. The fluid is then removed and the slide is stained after it is air dried.

Cytocentrifugation

With this technique a centrifuge is used to concentrate cells on a slide in a method similar to a sedimentation chamber.

Vita

Robert Loring Bergman was born on January 27, 1972 in New Orleans, Louisiana. He attended West Virginia University in Morgantown, WV from 1990-1993. He graduated from the University of Georgia College of Veterinary Medicine in 1997. Following veterinary school, he completed a one year rotating internship in small animal medicine and surgery at the University of Georgia, Veterinary Teaching Hospital. In 1998 he started a neurology and neurosurgery residency at Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia. In July he will join the faculty at Texas A&M University college of veterinary medicine.