UNIVERSITAT AUTŮNOMA DE BARCELONA
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THE HEMOSTATIC SYSTEM IN CANINE NEUROLOGY: STUDY OF THE FIBRINOLYTIC ACTIVITY IN CEREBROSPINAL FLUID IN NEUROLOGICAL DISORDERS AND EVALUATION OF TISSUE FACTOR EXPRESSION, FIBRIN/FIBRINOGEN DEPOSITION AND FIBRINOLYSIS IN CANINE GLIOMAS.

Memèria presentada per

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Sònia Añor Torres, professora titular del Departament de Medicina i Cirurgia Animals, de la Facultat de Veterinària, de la Universitat Autònoma de Barcelona,

CERTIFICA:

Que la tesi doctoral que porta per títol, **"THE HEMOSTATIC SYSTEM IN CANINE NEUROLOGY: STUDY OF THE FIBRINOLYTIC ACTIVITY IN CEREBROSPINAL FLUID IN NEUROLOGICAL DISORDERS AND EVALUATION OF TISSUE FACTOR EXPRESSION, FIBRIN/FIBRINOGEN DEPOSITION AND FIBRINOLYSIS IN CANINE GLIOMAS"**, de la qual és autor el llicenciat en Veterinària **Cristian de la Fuente Hernández** s´ha realitzat sota la seva direcció.

I per a que això consti, a efectes de ser presentada com a treball de Tesi per a optar al Grau de Doctor en Veterinària, signo el present certificat a Bellaterra, 27 de Març de 2014.

Sònia Añor Torres

"... Y dejemos que lo cierto sea lo que imaginamos..."

HDS

AGRAÏMENTS

Sovint, l´esforç que suposa compaginar les tasques clíniques amb la recerca no és prou reconegut i per això, aquesta tesis va dedicada a tots els clínics que ens endinsem en el món de la recerca.

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In memoriam

Dr. Lluís Monreal Bosch

ABBREVIATIONS

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CNS	Central nervous system
GME	Granulomatous meningoencephalomyelitis
NME	Necrotizing meningoencephalitis
NLE	Necrotizing leukoencephalitis
MUE	Meningoencephalitis of unknown etiology
SRMA	Steroid responsive meningitis-arteritis
CRP	C-reactive protein
MRI	Magnetic resonance imaging
IL	Interleukin
TF	Tissue factor
VEGF	Vascular endothelial growth factor
IVT	Intravascular thrombosis

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INTRODUCTION

INTRODUCTION

1. CANINE NEUROLOGICAL DISORDERS

Clinical veterinary neurology is one of the most evolving areas in veterinary medicine. The scientific knowledge in the field has been growing for the last two decades due to the increasing availability of advanced diagnostic tools, such as advanced imaging modalities and molecular and genetic testing. The medical progress and the greater demand from society to face and solve canine and feline neurological disorders have contributed to a better understanding of the underlying mechanisms of neurological diseases in veterinary patients. Despite this, the etiologic origin of some diseases remains still obscure, and clinical and therapeutic approaches are limited in some cases. Research in veterinary neurology is an example of multidisciplinary research that involves neuropathologists, geneticists, molecular biologists and neurologists among others to move forward in knowledge and understanding of neurological disorders.

There are many diseases affecting the nervous system of the canine species. From a clinical point of view and to make the clinical approach to the patient with neurologic impairment straightforward, neurologic disorders are grouped into different etiologic categories that include vascular, inflammatory/infectious, traumatic/toxic, congenital or anomalous, metabolic, idiopathic, neoplastic and degenerative disorders. Disorders within each category have a common typical clinical presentation regarding onset and progression of clinical signs over time. Among these disease categories, central nervous system (CNS) inflammatory disorders and neoplasms are currently being extensively studied because they include disorders of unknown etiology and disorders for which an effective therapeutic approach does not exist, thus they carry a poor prognosis. In addition, some canine diseases belonging to these groups are among the most common

disorders affecting the CNS in the canine species, and some of them have their human counterpart, thus research in this field may benefit both, human and veterinary patients.

I. Central nervous system inflammatory diseases

Inflammatory diseases of the CNS are among the most common causes of neurological dysfunction in the dog.[1] Canine inflammatory diseases of the CNS can affect the encephalon (encephalitis/meningoencephalitis), the spinal cord (myelitis/meningomyelitis) or the whole CNS (meningoencephalomyelitis). Clinical signs are usually acute and progressive and may reflect the location of the lesion within the CNS. On the other hand, dogs with meningitis can only exhibit pain without neurologic deficits unless the nervous parenchyma adjacent to the inflamed meninges is affected. Additional systemic clinical signs such as fever can occur, but they are not always present.

Inflammatory CNS diseases are grouped into two broad categories, those caused by a known infectious agent and those with a non-infectious aetiology.[2] The latter group encompasses the most common CNS inflammatory disorders affecting the canine species and are assumed to be caused by aberrant immune responses against the CNS.[3]

Within the non-infectious etiology group, diseases are named according to their histopathological features and the most common are: granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME) and necrotizing leukoencephalitis (NLE).

The main histopathological features of these disorders are:

- GME is an angiocentric, non-suppurative inflammatory process that predominantly affects the white matter of the brain and/or spinal cord. The inflammatory cell population, commonly composed of histiocytes and lymphocytes, is concentrated around blood vessels (perivascular cuffs). There are three established forms of the disease according to the extension of the affected areas in the CNS (disseminated, multifocal and focal). The origin of inflammatory cell population is currently unknown, but migration-maturation from blood-derived inflammatory cells is the presumed and widely accepted mechanism.[3,4] The etiopathogenesis of GME remains obscure, although a T cell-mediated delayed hypersensitivity reaction or autoimmune encephalitis induced by glial acid protein antibodies are proposed as possible underlying mechanisms.[5] Recent investigations to identify potential viral triggers have had negative results.[6] GME may represent up to 25% of all canine CNS diseases and although all breeds and both sexes can be affected, females and toy and terrier breeds are overrepresented.[3,7]

- NME and NLE have similar histopathological features and they are non-suppurative meningoencephalitides with different degrees of cerebral necrosis. The topographical distribution of the lesions is different in both diseases. In NME there is severe inflammation commonly affecting the leptomeninges and extending through the cortex to the subcortical white matter and corona radiata, whilst in NLE the cerebral cortex is relatively spared and lesions are located in the periventricular white matter and brainstem.[3,4] The etiopathogenesis of NME

and NLE is poorly understood but an autoimmune basis has been suggested.[3] NME and NLE have been described as breed-related diseases in the Pug, Maltese, Chihuahua, Yorkshire Terrier, Pekingese, West Highland White Terrier, Boston Terrier, Japanese Spitz, and Miniature Pinscher breeds. These are breeds with high predisposition for NME or NLE, but any canine breed can be affected by both disorders. Although a strong familiar inheritance pattern has been described for NME in Pug dogs, a simple Mendelian pattern could not be demonstrated suggesting that NME is a multifactorial disorder.[3] In addition, NME has been recently described in new canine breeds suggesting that the disease is not a breed-restricted disorder.[8] Because of the neuropathological similarity with viral meningoencephalitides and the presence of Mx proteins (proteins associated with viral disease) in brain tissue of dogs with NME, a viral origin has been considered but attempts to isolate any viruses from affected brain tissue have been unsuccessful.[9,10]

The antemortem diagnosis of a specific variant of non-infectious meningoencephalomyelitis in dogs (GME, NME or NLE) is often difficult because of the overlap in neurodiagnostic profiles of those disorders. The term meningoencephalitis of unknown etiology (MUE) is often used antemortem when histopathology is lacking and an infectious etiology has been ruled out.[2]

Immunosuppressive therapy is the current basis of treatment for MUE. Because MUE represents a broad spectrum of diseases, a gold-standard therapy has not been established. The prognosis of MUE in dogs has improved during the last years with combined immunosuppressive therapies, but it is commonly considered poor, especially for the

necrotizing variants. Research to elucidate the etiopathogenesis and/or to find alternative therapeutic strategies to block neuroinflammation is critical to improve survival times for these life-threatening disorders in dogs.

Another common non-infectious inflammatory disorder of the CNS of dogs is steroid responsive meningitis-arteritis (SRMA). SRMA is considered the most frequent meningitis in dogs and it is thought to be a systemic immune-mediated disorder with the main inflammatory lesions affecting the leptomeninges of the spinal cord and their associated vessels.[11]

The etiopathogenesis of SRMA is unknown but several studies suggest an immunopathologic mechanism because of the marked increase of IgA concentration in cerebrospinal fluid (CSF) and serum, an increased B-cell/T-cell ratio in peripheral blood and CSF, and a suggested Th2-mediated immune response.[11,12] Typically young-adult dogs are affected, and Bernese Mountain dogs, Beagles and Boxers are predisposed. The main clinical signs are spinal pain and fever, and they are commonly episodic.

CSF analysis is the most important diagnostic tool in this disease, and it typically shows a marked neutrophilic pleocytosis in acute cases. Mononuclear pleocytosis is reported in the protracted form of the disease and normal CSF results are possible due to the waxing and waning nature of the disease.[11,12] There is not a gold standard test for the diagnosis of SRMA although measurement of IgA concentrations in CSF and serum has been proposed for a long time as a specific biomarker. A recent study demonstrated that a combination of elevated IgA levels in the CSF and serum supports the diagnosis of SRMA with high sensitivity but very low specificity, thus the value of IgA as specific

biomarker of SRMA is questionable.[13] Although the prognosis is considered good in 60-80% of SRMA cases with appropriate treatment, relapses are frequent (up to 40%).[11]

The main differential diagnosis for acute SRMA is bacterial meningitis. Bacterial meningitis is much less frequent than SRMA in dogs but it can have the same clinicopathologic findings. Ruling out bacterial meningitis is challenging but essential because long-term immunosuppression is the mainstay treatment of SRMA, and the consequences of this in patients with bacterial meningitis can be fatal. Thus finding a specific biomarker for SRMA would be very valuable. In this direction, C-reactive protein (CRP), a major acute-phase protein synthesized by hepatocytes, has been measured in the CSF and serum of dogs with neurological disorders, and its usefulness as a biomarker for meningeal inflammation has been demonstrated. CRP concentration in canine CSF, although not specific, is an adjunctive tool in the diagnosis of SRMA, and serum CRP concentrations can be used to monitor response to treatment in these patients.[14-16]

II. Central nervous system neoplastic diseases

Brain tumors are a common cause of neurologic dysfunction in dogs, especially in animals over 5 years of age. Although the true incidence of brain tumors in dogs is unknown, according to the most recent necropsy-based studies the incidence of intracranial neoplasms in dogs range from 1% to 4.5 %, which is similar to the 2% frequency of brain tumors in human necropsies.[17,18] Meningiomas and glial cell tumors (astrocytoma, oligodendroglioma and mixed glioma) are the most commonly reported primary brain tumor types in dogs. Meningiomas arise from the leptomeninges and are

the most common primary brain neoplasms followed by gliomas, which arise from cells of neuroectodermal origin. Certain canine breeds, such as Boxers and Golden Retrievers, are especially prone to develop primary brain tumors and glial tumors are reported to be more prevalent in brachycephalic breeds.[17,19] Histological subtypes and grades of meningiomas and gliomas reported in dogs are very similar to those reported in humans although the occurrence of specific grades of malignancy within each tumor type is quite different.[19] For instance, dogs are more prone to develop malignant meningiomas than humans.[20]

Clinical signs are often chronic and progressive although they can acutely worsen if compensatory mechanisms of the brain to control intracranial pressure are exhausted. Clinical signs of primary brain tumors are usually focal and reflect their location within brain, although multifocal neurological deficits are possible because of mass effect, peritumoral vasogenic edema or tumoral infiltration of functionally distinct neuroanatomic areas.

The diagnosis of brain tumor in dogs usually relies upon diagnostic imaging because definitive histopathological diagnosis implies invasive procedures such as open brain biopsy for superficial lesions or stereotactic procedures for deep-seated brain lesions. The invasiveness of open brain biopsy and the limited availability of stereotactic systems for veterinary patients, as well as financial costs of these procedures make difficult to reach a definitive premortem diagnosis.

Although magnetic resonance imaging (MRI) has a high sensitivity to identify brain tumors in dogs, its specificity is low because some imaging findings of neoplasms are

shared with other neurological disorders such as inflammatory diseases, hematomas or non-neoplastic cysts.[21] Therefore veterinary neurologists deal often with brain masses suspected to be brain neoplasias. The scientific literature about brain tumor therapy in dogs clearly reflects this issue: much of the published data relates to broad groups of tumors or even masses without histological diagnosis or grading.[19] The consequence is that gold standard therapies for canine brain tumors are lacking.

Currently, conventional therapeutic approaches to brain tumors in dogs include (alone or combined): surgical resection/debulking, chemotherapy and radiation therapy. Prognosis is considered poor despite treatment especially in dogs with glial neoplasms.
Research is needed to further understand brain tumor pathophysiology and to design more effective therapeutic approaches.

III. Canine glioma: a natural model for human disease

Human gliomas, especially high-grade gliomas, have fatal outcomes. For instance, the overall survival rate for human patients with glioblastoma (astrocytoma grade IV) is approximately 42% at 6 months and less than 5% at 24 months.[19] Surprisingly, prognosis for human patients with high-grade gliomas has not changed considerably over the past 20 years despite of extensive research in cancer therapy. The biological behavior, advanced imaging findings and histological features of canine gliomas are very similar to their human counterparts.[19] Despite these similarities, canine glioma as a potential animal research model has been seldomly used until recent times.[22-24]

Rodent xenograft models of disease are the basis of most novel therapeutic approaches in

humans. Although the usefulness of the information provided from rodent models is indisputable, the real scenario is characterized by failure of most novel therapies for high-grade gliomas to progress further than to preclinical or phase I clinical trials.[19] The limited impact of novel therapies in cancer patients during the last 20 years demonstrates the inherent weakness of these models systems. Spontaneous brain tumors in rodents are extremely rare and induced tumoral models may not appropriately reflect real disease or real tumor environment. Spontaneous gliomas in dogs have been proposed as intermediate step between rodent models and human clinical trials to test new therapies.[19] Because of the lack of gold standard therapies for dogs with brain tumors and the poor response of canine gliomas to conventional treatments, testing new therapeutic strategies in dogs would be ethically correct as long as potential benefits and acceptable adverse effects would have been previously documented.

In addition, dogs can also be a model to investigate genetic and environmental factors involved in the initiation and progression of tumors, as well as to map cancer genes in canine breeds predisposed to develop brain tumors. Therefore, both humans and dogs would benefit from such a research model.

2. CEREBROSPINAL FLUID ANALYSIS AS DIAGNOSTIC TOOL IN VETERINARY NEUROLOGY

CSF is an ultrafiltrate of plasma with very low protein content and relatively acellular. CSF is mainly produced by the choroid plexuses with some contribution of the ependymal lining cells within the ventricular system. CSF flows through the ventricular system and then into the subarachnoid space surrounding the entire CNS to provide

physical support of neural structures. CSF also contributes to the control of intracranial pressure, regulates the chemical environment of the CNS and participates as a vehicle in the intracerebral transport of biologically active substances. Because there is no lymphatic system in the brain, CSF has also an important role in excretion/removal of large proteins and cells.[25]

CSF analysis is an important tool for the diagnosis of neurological disorders, and should be collected when a CNS disorder is suspected unless contraindicated (e.g., increased intracranial pressure). Routine CSF analysis usually includes a total nucleated cell count, quantification of total protein content and a cell differential count. In addition, serology titers and/or PCR testing for infectious diseases are often performed in CSF samples. Despite its high sensitivity, the specificity of CSF analysis is very low to reach definitive diagnoses of different neurological disorders.[26] In a previous study, CSF analysis provided specific etiologic information in only 2% of the 256 included cases.[27] In most instances, CSF analysis only rules out some disease etiologic groups from the differential list. Moreover, a normal CSF analysis does not rule out a CNS disorder. For instance, it has been reported that CSF analysis is within normal limits in approximately 10% of CNS inflammatory disorders.[27] The need for new CSF biomarkers is evident and efforts to increase the specificity of CSF analysis for the diagnosis of CNS disorders are currently made by several researchers. This is an active research field in veterinary medicine and there are several recently published studies about the expression of acute phase response molecules in CSF and serum of neurological patients, as well as studies about the role of myelin basic proteins and TAU protein in the CSF as prognostic biomarkers in dogs with intervertebral disk disease.[14-16, 28-31]

Among the acute phase response proteins, CRP is becoming one of the most important biomarkers providing information on the existence and monitoring of inflammatory CNS diseases. CRP concentrations in the CSF of dogs with inflammatory CNS disorders are significantly higher, especially in dogs with SRMA, than in healthy dogs and those with other neurologic diseases such as disk disease, CNS neoplasias or idiopathic epilepsy.[14,28]

Studies in human beings demonstrate that there is a significant correlation between CRP concentrations in CSF and serum because this protein flows easily to the subarachnoid space, so systemic non-neurologic diseases can raise CSF CRP concentrations.[32,33] This may limit the specificity of CRP for the diagnosis of neurologic disorders. The two veterinary studies that tried to address this issue found opposite results.[14,28] The more recent of the two studies measured CRP concentrations by time-resolved immunofluorimetric assay and found a significant positive correlation between CSF and serum CRP concentrations, which has also been demonstrated in human studies.[28] The time-resolved immunofluorimetric assay is the most sensitive and accurate method to measure CRP in the CSF of dogs, and it can be considered as an alternative to ELISA or immunoturbidimetric assays.[28]

3. THE LINK BETWEEN COAGULATION AND INFLAMMATION

A delicate balance between coagulation and fibrinolysis exists physiologically to prevent hemorrhage or disseminated thrombosis. Inflammation can disrupt this balanced state towards a procoagulant scenario. Inflammation and coagulation are closely related processes, and an extensive crosstalk exists between these two systems.[34-36] Disseminated intravascular coagulation is the classic example of the relationship between inflammation

and coagulation.[37] Inflammation-induced coagulation activation results in intravascular fibrin formation that might lead to multiple organ dysfunction syndrome.

The three main known mechanism through which inflammation directly affects the hemostatic system include: activation of coagulation, downregulation of natural endogenous anticoagulants and inhibition of fibrinolysis.[35,36,38] Inflammation can activate coagulation by influencing both, primary and secondary hemostasis. A plethora of inflammatory mediators are released in response to tissue damage. Inflammatory cytokines are fundamental mediators of the immune system and among them, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are the main ones interacting with the hemostatic system. IL-6 stimulates the production of highly thrombogenic platelets, which easily aggregate providing the negatively charged phospholipid surface necessary for secondary hemostasis to occur. Moreover, IL-6 has an influence on secondary hemostasis by inducing tissue factor (TF; the initiator of the extrinsic pathway of the coagulation cascade) expression on monocytes and endothelial cells. TNF-α exerts its procoagulant action through induction of TF expression and inhibition of fibrinolysis by suppressing tissue plasminogen activator.[36]

Other inflammatory mediators that can have a procoagulant effect on secondary hemostasis include other inflammatory cytokines (e.g., IL-1), acute phase response proteins such as CRP, and complement activation. These inflammatory mediators initiate coagulation by upregulating TF expression on endothelial cells, monocytes and macrophages, but they can also inhibit fibrinolysis by enhancing the synthesis of plasminogen activator inhibitor-1, which acts as a potent inhibitor of tissue plasminogen activator.[36]

Inflammatory mediators can also downregulate the production of the natural endogenous anticoagulants: antithrombin, protein C and tissue factor pathway inhibitor.[36] All these mechanisms unbalance the hemostatic system toward a procoagulant state leading to thrombin generation and subsequent clot formation.

The extensive crosstalk between inflammation and coagulation is not unidirectional because the activation of the coagulation system yields proteases that induce signaling pathways to modulate the inflammatory response through specific cell receptors known as protease-activated receptors.[36,38] Some components of the coagulation system such as thrombin, TF-factor-VIIa complex and fibrin/fibrinogen have proinflammatory properties that promote cytokine synthesis and induce leukocyte migration. Conversely, there is evidence that natural endogenous anticoagulants (TF pathway inhibitor, antithrombin and C-protein) have inherent anti-inflammatory properties to directly minimize the inflammatory process.[36] If cross-linked fibrin is not removed efficiently, the inflammatory response is enhanced and perpetuated through intracellular signaling pathways regulated by fibrin.[39] Thus inflammation activates coagulation and coagulation modulates the inflammatory response. The current knowledge suggests that most components of the coagulation cascade have proinflammatory properties and targeting proinflammatory aspects of coagulation without affecting coagulation itself might be a novel therapeutic approach to inflammatory disorders.[40]

4. D-DIMERS AS SPECIFIC MARKERS OF SYSTEMIC AND LOCAL FIBRINOLYTIC ACTIVITY

Fibrinolysis is the process of removal of fibrin clots and it is an essential component of normal hemostasis. The fibrinolytic system is responsible for removal of fibrin formed into the blood stream to avoid capillary microthrombosis and tissue ischemia. Fibrinolysis can be assessed measuring plasma D-dimer concentrations. D-dimers are specific degradation products of cross-linked fibrin generated by the action of plasmin. D-dimers are sensitive markers of fibrinolytic activity in humans and animals and consequently, markers of previous clot formation. Thus, D-dimer measurement is a reliable tool for the diagnosis of thromboembolic diseases.[41]

In addition to systemic fibrinolytic activity, the fibrinolytic pathways can be locally activated in different body compartments. The fibrinolytic activity in different body fluids has been studied in humans since 1958.[42] Local fibrinolytic activity activation has been demonstrated to increase under pathological conditions in synovial fluid, pleural fluid, peritoneal fluid, cerebrospinal fluid and aqueous humor among others.[43-47] Fibrinolysis in these fluids is responsible for the destruction of fibrin formed in body cavities in order to restore homeostasis after inflammatory insults and to avoid the deleterious effects of excessive inflammation-induced fibrin deposition.

The fibrinolytic activity in CSF has received special attention in human medicine. Normal human CSF contains very low quantities of fibrinolytic enzymes, but the fibrinolytic activity increases under pathologic conditions.[48] Although the origin of physiological and disease-induced fibrinolysis in the CNS remains controversial, the bidirectional

relationship between inflammation and coagulation (and consequently with fibrinolysis) points to the intrathecal fibrinolytic system as a target for research of several CNS diseases. In this direction, the D-dimer concentration has been used together with other parameters to assess the fibrinolytic activity in human CSF, and it has been advocated as a useful tool to differentiate between idiopathic subarachnoid hemorrhage and iatrogenic hemorrhage from traumatic spinal puncture in human beings.[49]

The significance of the D-dimer CSF concentration as diagnostic tool for infectious meningitides and neoplastic diseases in people has also been studied. D-dimers have been suggested as markers of CNS neoplastic involvement and as diagnostic adjuvant tools for bacterial meningitides. A proteome study of human CSF following traumatic brain injury has pointed to the fibrin/fibrinogen degradation products as trauma-associated makers.[50-54] Activation of the fibrinolytic system in the CNS occurs to destroy any fibrin formed in order to avoid fibrin-related detrimental effects. Evidence supports that fibrin deposition correlates with neuropathology because fibrin, a pleiotropic protein, can activate cellular mechanism and signaling pathways that contribute to neuroinflammation, neurodegeneration and neoplastic proliferation.[39,55]

In veterinary medicine, local fibrinolytic activity has been demonstrated by measuring D-dimer concentrations in peritoneal and synovial fluids in horses, and in canine aqueous humor.[56-59] These studies have demonstrated that there is local fibrinolytic activity in several body fluids and that D-dimer concentration is significantly higher in severe inflammatory disorders affecting these compartments. It is worth to mention that D-dimer concentration in synovial fluid has been proved to be more sensitive than routine synovial analysis for the diagnosis of non-infectious joint disease, and the potential role of D-

dimers as specific biomarkers of septic arthritis is promising.[57,58]

There is no published data about the fibrinolytic activity in the CSF of veterinary patients, except for one small-scale study (conference abstract) about the effect of blood contamination on canine CSF D-dimer concentrations.[60] The results of this study were in agreement with findings in humans and suggested that the CSF D-dimer concentration was not affected by iatrogenic blood contamination and that intrathecal fibrinolytic activity is low to non-detectable in the CSF of healthy subjects.

Because of the role of the hemostatic system in the pathophysiology of nervous system diseases, the intrathecal fibrinolytic activity in veterinary patients with neurologic disorders is worth to study.

5. THE HEMOSTATIC SYSTEM IN CANCER BIOLOGY

A close association between malignant disorders and blood coagulation dysfunction has been long recognized in human medicine. Since the first descriptions by Trousseau in 1865 of migratory thrombosis in patients that later manifested a visceral malignancy (the so-called Trousseau syndrome), coagulation dysfunctions of different nature and magnitude have been recognized affecting up to 50% of cancer patients.[61]

Solid tumors are dependent on angiogenesis to grow beyond a few millimeters.[61] Several studies have demonstrated that some components of the hemostatic system influence angiogenesis facilitating tumor growth and metastasis.[63,64] Thus research has focused on the extravascular activation of the coagulation and fibrinolytic systems to elucidate their

role in tumor biology. Currently, some components of the hemostatic system are considered not only as blood clotting proteins but also as pleiotropic proteins that are important for tumor progression and hence potential therapeutic targets.[61,64,65] In this direction, TF, fibrin/fibrinogen and the fibrinolytic system have been focus of research in human medicine. Increased TF expression has been observed in many human tumor types.[66] Tumor cells express TF and it is suggested that tumor cells can also trigger TF synthesis by adjacent host cells.[61] Investigations about the role of TF in tumoral biology have demonstrated that TF triggers cellular signaling pathways that induce tumoral growth, angiogenesis and metastasis.[67-70] TF switches angiogenesis on through upregulation of vascular endothelial growth factor (VEGF) and downregulation of negative angiogenic factors.[71]

Regarding brain tumors, human gliomas overexpress TF and its expression is associated with the histologic grade of malignancy and vascularity.[72-74] TF upregulation in human glioblastomas is also associated with significant reductions in patient survival.[75]

With the advances in knowledge on the role of TF in cancer biology, two new treatments for human brain tumors that show promising results in rodent xenograft models have been recently published. The first one is a new antibody-drug conjugate that has been synthetized to target TF and has been demonstrated to have potent activity against a broad spectrum of solid tumors.[76] The second one is a monoclonal antibody specifically designed to block TF signaling without influencing the coagulation cascade, and it has been shown to reduce tumor cell invasiveness in a glioma model.[75]

There are some investigations on angiogenic factors in canine primary brain tumors, but these studies have focused in the expression of VEGF or their associated tyrosine kinase receptors.[77-79] In veterinary medicine, there is only one study evaluating TF expression in canine tumors. This recent study has demonstrated TF expression in different canine tumor cell lines, particularly in those derived from epithelial tumors, but there is no information about TF expression in canine gliomas.[80]

As mentioned before, fibrin/fibrinogen can activate signaling pathways to enhance and perpetuate the inflammatory response. The evidence shows that these components of the coagulation system also play a role in tumor biology.[39] Fibrin/fibrinogen deposition provides a provisional extracellular matrix that facilitates angiogenesis and binding of growth factors (such as VEGF), and promotes cell adhesion, proliferation and migration. Fibrin/fibrinogen may also act as a barrier between tumor and host that may interfere with an adequate immune response.[81-83]

Fibrinogen is not normally present in the CNS and can only reach the brain parenchyma through vascular damage or blood-brain barrier disruption. Increased vascular permeability may allow fibrinogen to reach the brain parenchyma. VEGF is known to be a potent factor that increases vascular permeability and it has been associated with the presence of peritumoral vasogenic edema in human CNS tumors.[84] VEGF is highly expressed in canine gliomas, but an association with peritumoral edema was not found in a previous study that addressed this issue comparing VEGF mRNA expression with presence and degree of peritumoral edema on MRI images.[78] As the authors of such study discussed, the low number of tumor samples with available MRI images included probably influenced the results.

Fibrin/fibrinogen deposition has been demonstrated in primary and metastatic brain neoplasms.[84] In veterinary medicine, fibrin/fibrinogen deposition has been described in different canine tumors, but information is lacking regarding CNS tumors.[86,87]

The fibrinolytic system is critical for tumor progression and is considered a regulator of tumor angiogenesis.[61,83,88] However, the relationship between the fibrinolytic system and angiogenesis is not fully understood because of the coexistence of both, pro- and anti-angiogenic properties of some of their components.[61] For instance, fibrin fragment E is a potent angiogenic factor that enhances the effect of VEGF.[88] On the other hand, the induction of plasminogen cleavage releases proteolytic fragments, such as angiostatin, that are potent angiogenic inhibitors.[61]

Research on the role of the hemostatic system in cancer biology has demonstrated that glioma progression is dependent of, or at least benefits from, a procoagulant environment. On the other hand, it is also known that hypoxia is critical for glioma progression.[89] Glioma cells surrounding necrotic foci, also known as *pseudopalisading cells*, are hypoxic and secrete angiogenic factors promoting microvascular hyperplasia. Glioblastoma (WHO grade IV) is distinguished histopathologically from anaplastic astrocytoma (WHO grade III) by the presence of either necrosis or microvascular hyperplasia and both features can usually be identified.[90,91]

Although the mechanisms that induce necrosis and the ensuing hypoxia-induced tumor progression have not been fully elucidated, intravascular thrombosis (IVT) within gliomas has been proposed as a possible mechanism.[89] IVT has been investigated in human CNS malignancies and it is more frequent in glioblastomas than in other CNS

tumors, so it has been proposed as a potential marker of aggressive behavior for anaplastic astrocytomas.[92] There is no information regarding the occurrence of IVT in canine gliomas.

HYPOTHESES

HYPOTHESES

1. The intrathecal fibrinolytic activity is activated in neurological disorders in dogs.

    a) The fibrinolytic activity is more severe in inflammatory neurological disorders because of the relationship between inflammation and coagulation.

    b) The D-dimer concentration in the CSF is a useful biomarker of meningeal inflammation.

2. TF is expressed in canine glioma cells and TF expression is associated with the malignancy grade of these tumors.

3. Fibrin/fibrinogen deposition and extravascular activation of the coagulation and fibrinolytic systems occurs within the tumor stroma in canine glioma.

4. IVT occurs in canine gliomas and it is a histological feature linked to glioma type and malignancy grade.

OBJECTIVES

OBJECTIVES

1. To assess the fibrinolytic activity in the CSF of dogs with different neurological disorders by measuring D-dimer concentrations in paired blood and CSF samples.

2. To investigate the usefulness of CSF D-dimers as biomarkers of meningeal inflammation compared with that of CRP, a molecule that is currently being studied as a biomarker of some neurological diseases, especially inflammatory disorders.

3. To investigate TF expression in canine gliomas by means of immunohistochemistry and to correlate the results with histologic type and malignancy grade.

4. To identify fibrin/fibrinogen and D-dimer deposits in canine gliomas by means of immunohistochemistry, to characterize their distribution and to correlate the results with histologic type and malignancy grade.

5. To investigate the occurrence of IVT in canine gliomas by means of immunohistochemistry, and to evaluate its usefulness as a potential marker of histologic type or malignancy grade.

PUBLISHED ARTICLES

To fulfill the requirements of this PhD thesis the following scientific studies were designed and performed:

Study #1: C. de la Fuente, L. Monreal, J. Cerón, J. Pastor, J. Viu, S. Añor. **Fibrinolytic activity in cerebrospinal fluid of dogs with different neurological disorders.** Journal of Veterinary Internal Medicine 2012;26:1365-1373.

Study #2: C. de la Fuente, M. Pumarola, E. Blasco, F. Fernández, J. Viu, S. Añor. **Immunohistochemical evaluation of tissue factor, fibrin/fibrinogen and D-dimer deposits in canine glioma.** The Veterinary Journal (2014), doi:10.1016/j.tvjl.2014.03.021

Study # 1

Fibrinolytic activity in cerebrospinal fluid of dogs with different neurological disorders

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Fibrinolytic Activity in Cerebrospinal Fluid of Dogs with Different Neurological Disorders

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Background: Fibrinolytic activity in cerebrospinal fluid (CSF) is activated in humans by different pathologic processes.

Objectives: To investigate fibrinolytic activity in the CSF of dogs with neurological disorders by measuring CSF D-dimer concentrations.

Animals: One hundred and sixty-nine dogs with neurological disorders, 7 dogs with systemic inflammatory diseases without central nervous system involvement (SID), and 7 healthy Beagles were included in the study. Dogs with neurological disorders included 11 with steroid-responsive meningitis-arteritis (SRMA), 37 with other inflammatory neurological diseases (INF), 38 with neoplasia affecting the central nervous system (NEO), 28 with spinal compressive disorders (SCC), 15 with idiopathic epilepsy (IE), and 40 with noninflammatory neurological disorders (NON-INF).

Methods: Prospective observational study. D-dimers and C-reactive protein (CRP) were simultaneously measured in paired CSF and blood samples.

Results: D-dimers and CRP were detected in 79/183 (43%) and in 182/183 (99.5%) CSF samples, respectively. All dogs with IE, SID, and controls had undetectable concentrations of D-dimers in the CSF. CSF D-dimer concentrations were significantly ($P < .001$) higher in dogs with SRMA than in dogs with other diseases and controls. CSF CRP concentration in dogs with SRMA was significantly ($P < .001$) higher than in dogs of other groups and controls, except for the SID group. No correlation was found between blood and CSF D-dimer concentrations.

Conclusions and Clinical Importance: Intrathecal fibrinolytic activity seems to be activated in some canine neurological disorders, and it is high in severe meningeal inflammatory diseases. CSF D-dimer concentrations may be considered a diagnostic marker for SRMA.

Key words: Intrathecal fibrinolysis; D-dimer; C-reactive protein; SRMA.

Fibrinolysis is the removal process of intravascular fibrin clots and it is an essential component of normal hemostasis. D-dimers are specific indicators of fibrinolysis and sensitive markers of systemic fibrinolytic activity in humans[1] and animals.[2]

Inflammation and coagulation are closely related processes, and there is an extensive crosstalk between these 2 systems.[3–8] Activation of coagulation by inflammatory processes can result in intravascular fibrin formation that could lead to organ failure. In addition, activation of the coagulation system yields proteases that induce signaling pathways to modulate the inflammatory response through specific cell receptors.[6] Thus, inflammation activates coagulation, and coagulation also affects the inflammatory response.

Normal human CSF contains very low concentrations of fibrinolytic enzymes, but the fibrinolytic activity increases in pathologic conditions to minimize the detrimental effects of fibrin deposition.[9] Although the origin of physiological and disease-induced fibrinolysis in the central nervous system (CNS) remains controversial, the relationship among inflammation, coagulation, and fibrinolytic activity in CNS diseases is worth studying. CSF D-dimer concentrations have been used to assess fibrinolytic activity in the CSF of humans with CNS infectious, neoplastic, traumatic, and vascular diseases.[10–15] In addition, D-dimers in the CSF have been proposed as markers of meningeal inflammation,[16,17] and as a useful tool to differentiate between idiopathic subarachnoid hemorrhage and traumatic spinal tap.[18–20] Furthermore, in human medicine, the role of fibrin in CNS diseases has been recently redefined not only as a blood-clotting protein but also as a factor that regulates inflammatory and regenerative cellular responses in neurodegenerative diseases.[21] Finally, some studies point to the coagulation system as being critical for tumor progression and as an effective cancer therapy target.[22,23]

There is no information about the fibrinolytic activity in the CSF of veterinary patients, except

Abbreviations:

CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
IE	idiopathic epilepsy
INF	other inflammatory neurological diseases
NEO	neoplasia affecting the central nervous system
NON-INF	noninflammatory neurological disorders
SCC	spinal cord compressive disorders
SID	systemic inflammatory diseases without CNS involvement
SRMA	steroid-responsive meningitis-arteritis
TNCC	total nucleated cell count
TR-IFMA	time-resolved immunofluorimetric assay

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for 1 study performed in healthy dogs about the effect of blood contamination on CSF D-dimer concentrations.[a]

C-reactive protein, a major acute-phase protein synthesized by hepatocytes, has been measured in the CSF and serum of dogs with neurological disorders, and its usefulness as a biomarker for meningeal inflammation has been demonstrated. CRP concentration in canine CSF, although not specific, is an adjunctive tool in the diagnosis of SRMA, and serum CRP concentrations can be used to monitor response to treatment in these patients.[24–26] Time-resolved immunofluorimetric assay (TR-IFMA) has been recently validated in canine CSF samples to measure CRP concentrations. TR-IMFA has been shown to have a high sensitivity, so very small amounts of CRP can be detected in canine CSF samples.[27]

We hypothesized that the intrathecal fibrinolytic activity is activated in disease conditions and that the activation is more marked in neurological inflammatory disorders due to the relationship between inflammation and coagulation.

The main purpose of this study was to assess the fibrinolytic activity in the CSF of dogs with different neurological disorders by measuring D-dimer concentrations in paired blood and CSF samples. In addition, the usefulness of CSF D-dimers as biomarkers of meningeal inflammation was compared with that of CRP, a molecule that is currently being studied as a biomarker of some neurological diseases, especially inflammatory disorders.

Materials and Methods

Study Population

In this prospective study, dogs examined by the Neurology Service of the Hospital Clínic Veterinari (UAB) that had a CSF analysis performed as part of their workup between October 2007 and April 2011 were included. Dogs presented for different neurological signs, and were distributed into 7 groups according to final diagnosis: (1) steroid-responsive meningitis-arteritis (SRMA), (2) other inflammatory neurological diseases (INF), (3) neoplasias primarily arising or secondarily involving the CNS (NEO), (4) spinal cord compressive disorders (SCC), (5) noninflammatory neurological disorders (NON-INF), (6) idiopathic epilepsy (IE), and (7) systemic inflammatory diseases without CNS involvement (SID).

In the SRMA group, diagnosis was reached on the basis of signalment, history, physical and neurological examinations, complete blood work, cervical spinal radiographs, presence of neutrophilic pleocytosis in the CSF with absent microorganisms, and resolving clinical signs with immunosuppressive treatment. Eight dogs were additionally sampled at 6 weeks after treatment initiation (1st monitoring). The treatment protocol used was one previously described.[28]

Diagnostic procedures in all dogs within the INF, NEO, and IE groups consisted of physical and neurological examinations, complete blood work, thoracic radiographs, abdominal ultrasound, magnetic resonance imaging, and CSF analysis. Some dogs in the INF group had also infectious agent testing and histopathology, which allowed further classification into a specific meningoencephalitis (infectious, necrotizing meningoencephalitis, granulomatous meningoencephalitis) or meningoencephalitis of unknown origin. Electrophysiology was also performed in cases of acute idiopathic polyradiculoneuritis. The NEO group included histologically confirmed neoplasms and highly suspected neoplasms on the basis of MRI findings and CSF analysis.

In the SCC group, diagnosis was reached on the basis of signalment, history, physical and neurological examinations, complete blood work, thoracic radiographs, abdominal ultrasound, myelography/magnetic resonance imaging (or both), and CSF examination. Furthermore, the diagnosis was confirmed in all dogs that were surgically treated.

In the NON-INF group, diagnosis was reached on the basis of signalment, history, physical and neurological examinations, complete blood work, thoracic radiographs, abdominal ultrasound, myelography/magnetic resonance imaging (or both), and CSF analysis. Electrophysiology was also performed in dogs with diseases involving peripheral nervous system.

The SID group included dogs with clinical signs that mimicked neurological signs (spinal pain), but that finally had a diagnosis of systemic inflammatory disease without CNS involvement. These dogs had a CSF analysis performed as part of the diagnostic protocol and also had several other diagnostic tests, including complete blood work, urinalysis, synovial fluid analysis, thoracic radiographs, abdominal ultrasound, spinal column radiographs/magnetic resonance imaging (or both), and infectious agent testing.

Dogs were excluded from the study if any anti-inflammatory or immunosuppressive treatment had been given before presentation, or the diagnostic tests were not conclusive and a definitive or highly presumptive diagnosis could not be reached.

Additionally, blood and CSF samples were obtained from 7 control healthy Beagles. Animal care and experimental procedures involving these dogs were approved by the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (authorization reference number: 5,719).

Sample Collection and Handling

Blood samples were collected by venipuncture from the jugular or cephalic veins into 1-mL tubes containing 3.8% sodium citrate. Blood samples were centrifuged at 2,000 × *g* for 10 minutes and plasma was separated within 1 hour of sample collection. CSF was collected aseptically, under general anesthesia, by puncture at the cerebellomedullary cistern or the lumbar cistern. The puncture site was closest caudally to neurolocalization, except for dogs with suspected inflammatory cervical lesions and control dogs, which had all cisternal taps. CSF was analyzed immediately for total nucleated cell count (TNCC), red blood cell count, and protein concentration. CSF was considered normal when the TNCC was <5 cells/μL, the total protein concentration was <25 mg/dL (cerebellomedullary cistern) or <45 mg/dL (lumbar cistern), and the red blood cell count was <4,000 cells/μL.[29] Plasma and CSF samples were aliquoted, stored, and frozen at –70°C until assayed.

D-Dimers

Blood and CSF D-dimer concentrations were determined in duplicate by a quantitative immunoturbidimetric latex agglutination assay[b] with commercial reagents and controls[c] according to the instructions provided by the manufacturer. The assay had been previously used to assess local fibrinolytic pathway activation in different biological fluids of canine[d] and equine origin.[30,31] Results were reported in ng/mL.

CRP

C-reactive protein was measured in blood and CSF samples, as previously described,[27] using a TR-IFMA method.[e–k] Results were reported in mg/L.

Statistical Analysis

Data analysis was performed by using a commercial software (SPSS for Windows, version 18.0).[1] Frequencies and percentages were used for qualitative variables. For descriptive analysis of age of dogs, TNCC, red blood cell count, and CSF total protein concentration, median and minimum and maximum values were used. D-dimer and CRP concentrations, which were not normally distributed, were expressed as median and interquartile range (25–75th percentile). For inferential analysis, a 1-way rank ANOVA with Bonferroni adjustment for multiple comparisons among groups was used. The Spearman's rank correlation coefficient was used to compare results of the main variables in CSF and blood. The Wilcoxon signed rank test was performed for SMRA pre- and posttreatment comparisons. Significance was set at $P < .05$ for all tests.

Results

Dogs Included in the Study

One hundred and eighty-three dogs were included in the study: 11 SRMA, 37 INF, 38 NEO, 28 SCC, 15 IE, 40 NON-INF, 7 SID, and 7 control dogs. One hundred and thirteen dogs were male (62%) and 70 were female (38%). Ages ranged from 0.3 to 14 years (median 6 years). The breed distribution reflected the hospital's referral population with mixed-breeds and 38 different pure breeds represented (Table 1).

SRMA

Five of 11 dogs were male (45%) and 6 were female (55%). Ages ranged from 0.3 to 1 year (median 0.9 years).

INF

Twenty-four of 37 dogs were male (65%) and 13 were female (35%). Ages ranged from 1 to 10 years (median 5 years). Inflammatory disorders included were 19 meningoencephalitis of unknown origin, 7 granulomatous meningoencephalitis, 3 necrotizing meningoencephalitis, 2 bacterial meningomyelitis, 2 acute idiopathic polyradiculoneuritis, and one of each of the following: idiopathic eosinophilic meningoencephalitis, *Prototheca* meningoencephalitis, distemper virus encephalitis, and trigeminal neuritis. In this group, 44% of the final diagnoses were confirmed by histopathologic examination.

NEO

Seventeen of 38 dogs were male (45%) and 21 were female (55%). Ages ranged from 4 to 14 years (median 10 years). Histopathologically confirmed neoplasias included 7 meningiomas (5 intracranial; 2 spinal), 10 intracranial gliomas, 3 peripheral nerve sheath tumors (1 cranial nerve; 2 spinal nerve), and one of each of the following: intracranial primary lymphoma, intracranial multicentric lymphoma, pituitary carcinoma, invasive nasal neuroblastoma, and multicentric plasma cell tumor. Suspected neoplasias on the basis of MRI findings and CSF analysis included 6 intracranial meningiomas,

Table 1. Breed distribution of dogs included in the study.

Breed	SRMA (n = 11)	INF (n = 37)	NEO (n = 38)	SCC (n = 28)	IE (n = 15)	NON-INF (n = 40)	SID (n = 7)
German Shepherd	–	2	1	1	4	4	1
Boxer	5	2	8	–	–	3	–
Cocker Spaniel	–	1	4	5	–	3	–
French Bulldog	–	6	4	1	2	2	–
Yorkshire Terrier	–	3	1	3	–	1	1
Golden R	–	–	2	–	1	3	–
Beagle	3	–	1	2	–	–	–
Mixed breed	1	9	7	5	2	12	1
Other (n = 2)	–	BC, WHWT, SHT	PB, BT	D, LR	LR	WHWT	–
Other (n = 1)	BMD, RW	SH, RW, AST, P, CT, M, PCH, LR	BD, AM, EB, AST, MP, G	P, CT, GD, RW, WP, SH, FB	SH, EB, MS, BD	SHT, SH, S, W, MP, JR, AST, RW, BD, D	CTE, BZ, GD, PW

SRMA, steroid-responsive meningitis-arteritis; INF, other inflammatory neurological diseases; NEO, neoplasias affecting the central nervous system; SCC, spinal cord compressive disorders; IE, idiopathic epilepsy; NON-INF, noninflammatory neurological disorders; SID, systemic inflammatory diseases without CNS involvement.

Breeds: Alaskan Malamute (AM), American Staffordshire Terrier (AST), Belgian Tervueren (BT), Bernese Mountain dog (BMD), Burdeaux dogue (BD), Border Collie (BC), Borzoi (BZ), Cairn Terrier (CTE), Cotton de Tulear (CT), Dobermann (D), English Bulldog (EB), Fila Brasileiro (FB), Great Dane (GD), Griffon (G), Jack Russel (JR), Labrador Retriever (LR), Maltese (M), Miniature Pinscher (PCH), Miniature Poodle (MP), Miniature Schnauzer (MS), Pitbull (PB), Portuguese water dog (PW), Pug (P), Rottweiler (RW), Samoyed (S), Sharpei (SH), Shih Tzu (SHT), Siberian Husky (SH),Weimaraner (W), West Highland White Terrier (WHWT), Whippet (WP).

4 intracranial gliomas, and one of each of these: pituitary neoplasia, medulloblastoma, and vertebral bone neoplasia. In this group, 66% of the final diagnoses were confirmed by histopathologic examination.

SCC

Twenty-one of 28 dogs were male (75%) and 7 were female (25%). Ages ranged from 0.8 to 14 years (median 7.5 years). This group included 19 dogs with Hansen type I intervertebral disk disease, and 9 dogs with caudal cervical spondylomyelopathy. In this group, 75% of the final diagnoses were surgically confirmed.

IE

Twelve of 15 dogs were male (80%) and 3 were female (20%). Ages ranged from 1.5 to 11 years (median 4 years). All dogs in this group had an onset of epileptic seizures between 1 and 5 years, normal interictal neurological examination and unremarkable bloodwork, CSF analysis, and brain MRI.

Non-INF

Twenty of 40 dogs were male (50%) and 20 were female (50%). Ages ranged from 0.5 to 13 years (median 7.5 years). This group included 10 noncompressive traumatic spinal cord injuries, 13 cerebrovascular accidents, 6 fibrocartilaginous embolisms, 4 idiopathic vestibular syndromes, 3 suspected degenerative myelopathies, and one of each of the following: congenital hydrocephalus, Jack Russell Terrier hereditary ataxia, cerebellar abiotrophy, and Dancing Doberman disease.

SID

Six of seven dogs were male (85%) and one was a female (15%). Ages ranged from 0.3 to 4 years (median 1 year). Final diagnoses in this group were 6 nonerosive immune-mediated polyarthritis and 1 immune-mediated polymyositis. All these dogs had normal CSF analysis.

Control Dogs

These were 7 male, 1-year-old Beagle dogs with blood and CSF analysis results within normal ranges.

Routine CSF Analysis

Results of complete CSF analysis were available for 163/183 dogs. Red blood cell counts were <4,000/μL in all samples, and only 11/163 samples had red blood cell counts between 501 and 3,900/μL. Partial CSF analysis results (only total protein concentration) were available for 20/183 dogs and 15 of these had increased total protein concentrations. Five dogs with partial CSF analysis that had CSF total protein concentration within normal ranges were not included in either the normal CSF or altered CSF analysis groups for statistical comparisons, because of lack of CSF cell counts. Thus, finally, 47/178 (26.4%) dogs had normal CSF analysis and 131/178 (73.6%) had altered CSF (either cell count, protein concentration, or both). Results of CSF analysis for each group of dogs are shown in Table 2.

Correlations between CSF D-Dimer and CRP Concentrations with CSF Analysis Results

Cerebrospinal fluid D-dimer and CRP concentrations in dogs with altered CSF analysis were significantly ($P < .001$ and $P = .002$, respectively) higher than those of dogs with normal CSF analysis. There were significant, but weak correlations between D-dimer concentrations and TNCC (rho = 0.352, $P < .01$), and D-dimers and red blood cell count (rho = 0.184, $P = .019$). A moderate correlation was found between D-dimers and total protein content (rho = 0.552, $P < .001$). CSF CRP concentrations were significantly but weakly correlated with TNCC (rho = 0.255, $P = .01$), red blood cell count (rho = 0.221, $P = .005$), and total protein content (rho = 0.232, $P = .002$).

Comparison of CSF and Blood D-Dimer Concentrations

All dogs in the control, IE, and SID groups had undetectable D-dimer concentrations in the CSF. Twenty of 37 dogs in the INF group, 23/38 dogs in the NEO group, and 20/40 in the NON-INF group had detectable concentrations of D-dimers in the CSF. In the SCC group, 5/28 dogs had detectable concentrations of D-dimers in the CSF, and the two with the highest values (210 ng/mL and 65 ng/mL, respectively) had a xanthochromic CSF. All dogs in the SRMA group had detectable concentrations of D-Dimers in the CSF, and the values for this group were significantly ($P < .001$) higher than those of other groups and control dogs (Table 3). Dogs in the SID group had the highest blood D-dimer concentrations, but no significant differences were found between groups and controls (Table 3). No correlation was found between blood and CSF D-dimer concentrations.

Comparison of CSF and Blood CRP Concentrations

C-reactive protein concentrations were detected in the CSF of 182/183 dogs. CRP concentration was significantly ($P < .001$) higher in the CSF of dogs with SRMA than in dogs from other groups and controls, except for dogs with SID. In fact, the CSF CRP concentration in the SID group was significantly higher than in the SCC ($P = .038$) and the control groups ($P = .012$). No significant differences were observed between any other group and controls (Table 3). The CRP blood concentration was significantly higher in the SRMA group than in all other groups and controls, except for the SID group. The blood CRP

Table 2. Cerebrospinal fluid results for dogs in each group (n = 183).

Variable	CD (n = 7)	SRMA (n = 11)	INF (n = 37)	NEO (n = 38)	SCC (n = 28)	IE (n = 15)	NON-INF (n = 40)	SID (n = 7)
TNCC (cells/μL)	0 (0–3)	649 (60–7290)	12 (0–2,900)	1 (0–129)	0 (0–9)	0 (0–4)	0 (0–46)	0 (0–3)
Total protein (mg/dL)	20.2 (17.9–23.4)	130.7 (39.4–573)	62 (18.2–1,500)	41.4 (14.4–392)	71 (21–226)	22.7 (13–30.8)	50.2 (13.5–293.6)	17.5 (13–44)
RBC (cells/μL)	0 (0–0)	45 (0–732)	5 (0–1,600)	2 (0–3,900)	0 (0–400)	0 (0–105)	5 (0–3,600)	1 (0–122)
Puncture site	C (n = 7)	C (n = 11)	C (n = 29) L (n = 8)	C (n = 32) L (n = 6)	L (n = 28)	C (n = 15)	C (n = 24) L (n = 16)	C (n = 4) L (n = 3)

C, cerebellomedullary cistern; CD, control dogs; IE, idiopathic epilepsy; INF, other inflammatory neurological diseases; L, lumbar cistern; NEO, neoplasia affecting central nervous system; NON-INF, noninflammatory neurological disorders; RBC, red blood cells; SCC, spinal cord compressive disorders; SID, systemic inflammatory disease without CNS involvement; SRMA, steroid-responsive meningitis-arteritis; TNCC, total nucleated cell count.

Data are expressed as median (range). Cerebrospinal fluid cell counts available from 163/183 dogs. Cerebrospinal fluid total protein concentrations available from all dogs included in the study.

Table 3. D-dimer and C-reactive protein concentrations in cerebrospinal fluid and blood.

Variable	CD (n = 7)	SRMA (n = 11)	INF (n = 37)	NEO (n = 38)	SCC (n = 28)	IE (n = 15)	NON-INF (n = 40)	SID (n = 7)
(CSF D-dimer) (ng/mL)	0 (0–0)	283[a] (217–872)	4 (0–39)	6.3 (0–41)	0 (0–0)	0 (0–0)	2 (0–15.9)	0 (0–0)
(Blood D-dimer) (ng/mL)	0 (0–67)	83 (16–244)	64.5 (6.5–146.2)	26 (0–135)	110 (19.6–168.1)	61 (0–120)	52 (8–146.4)	122.5 (61–2,064)
(CSF CRP) $\times 10^{-3}$(mg/L)	1.6 (0.6–9.6)	645.1[b] (256.9–3,146)	13 (4.7–82.1)	13.3 (6–98.3)	6.3 (2.5–17)	7 (2.7–8.8)	11.2 (4–56.2)	43.5[c] (30.1–193.5)
(Blood CRP) (mg/L)	4 (3–11.3)	220.8 [b] (85–327.1)	5.2 (4–42.1)	7.7 (4–27.4)	5.7 (3.9–15.2)	10 (4.7–16.9)	11.7 (3.6–45.1)	179[c] (46.9–392.2)

CD, control dogs; CRP, C-reactive protein; CSF, cerebrospinal fluid; IE, idiopathic epilepsy; INF, other inflammatory neurological diseases; NEO, neoplasias affecting central nervous system; NON-INF, noninflammatory neurological disorders; SCC, spinal cord compressive disorders; SID, systemic inflammatory diseases without CNS involvement; SRMA, steroid-responsive meningitis-arteritis.

Values expressed as: median (interquartile range). Analyses were performed by the 1-way rank-ANOVA with Bonferroni's adjustments.

[a]Significantly different from all groups and control dogs in the same row ($P < .001$).

[b]Significantly different from all groups and controls except for SID group in the same row ($P < .001$).

[c]Significantly different from controls in the same row ($P < .05$).

Table 4. C-reactive protein and D-dimer concentrations in dogs with steroid-responsive meningitis-arteritis at the moment of diagnosis and 6 weeks after treatment initiation.

Variable	$SRMA_{diagnosis}$ (n = 11)	$SRMA_{6\ weeks}$ (n = 8)
(CSF D-dimer) (ng/mL)	283 (217–872)	0[a] (0–0)
(Blood D-dimer) (ng/mL)	83 (16–244)	4.25[a] (0–50.3)
(CSF CRP) $\times 10^{-3}$ (mg/L)	645.1 (256.9–3,146)	0.6[a] (0.2–2.9)
(Blood CRP) (mg/L)	220.8 (85–327.1)	3.5[a] (3–5.1)

CRP, C-reactive protein; CSF, cerebrospinal fluid; SRMA, steroid-responsive meningitis-arteritis.

Values expressed as: median (interquartile range). Analyses were performed by Wilcoxon signed rank test.

[a]Significantly different in the same row ($P < .05$).

concentration in the SID group was significantly higher than that in the SCC ($P = .013$), NON-INF ($P = .036$), and control groups ($P = .009$). No significant differences were observed between any other group and controls (Table 3). There was a significant correlation between the CRP concentrations in blood and CSF (rho = 0.652, $P = .01$).

Correlation between D-Dimer and CRP Concentrations in CSF

There was a significant, but moderate correlation between the concentrations of D-dimers and CRP in CSF (rho = 0.457, $P < .001$).

Follow-up CSF and Blood Samples in the SRMA Group

Cerebrospinal fluid and blood samples from 8/11 SRMA dogs taken 6 weeks after treatment initiation were available. All dogs were in clinical remission and had a CSF analysis within normal range (median TNCC = 2 cells/μL, range: 0–4 cells/μL; median total protein = 21.2 mg/dL, range: 15.4–24.4 mg/dL). Six weeks after treatment initiation, CSF D-dimer concentrations were undetectable in all dogs. Concentrations of D-dimers and CRP in CSF and blood were significantly ($P < .05$) lower than those observed in the same dogs before treatment (Table 4).

Discussion

The results of this study demonstrate that an intrathecal fibrinolytic pathway is activated in the CSF of dogs with some neurological disorders, especially in dogs with SRMA. Our study also demonstrates that the CSF fibrinolytic activity can be measured, and suggests that the local activation of fibrinolysis is not linked to a concurrent systemic hyperfibrinolysis. The results support the use of CSF D-dimer concentrations as a potentially useful diagnostic marker of SRMA.

D-dimers are specific degradation products of cross-linked fibrin generated by the action of plasmin, thus any increase in CSF D-dimer concentration should be a specific indicator of active intrathecal fibrinolysis. In fact, the D-dimer test is being used as a sensitive marker of local fibrinolytic activity in humans and animals, and it is suggested that the fibrinolytic pathways are locally activated to avoid the detrimental effects of fibrin deposition in different body compartments.[d,9–13,30,31] Although the kinetics of D-dimers in the CSF of humans and animals are unknown, due to the short half-life of these products in serum and the fast turnover of CSF, a raised CSF D-dimer concentration might be indicative of recent hypercoagulation and hyperfibrinolysis.

Cerebrospinal fluid endogenous fibrinolysis has been studied in humans under normal and pathologic conditions and it is reported that normal human CSF contains very low to undetectable concentrations of fibrinolytic enzymes and D-dimers.[9] Our study suggests that the situation is similar in dogs, because no D-dimers were detected in the CSF of healthy control dogs or in dogs with SID.

Under pathologic conditions, the CSF fibrinolytic activity can be strongly activated in humans, and raised CSF D-dimer concentrations have been observed in some inflammatory,[32] infectious,[12] traumatic,[15] neoplastic,[13] and vascular CNS diseases.[14,33] In our study, D-dimers were detected in the CSF of all dogs in the SRMA group, and in some dogs of the INF, NEO, NON-INF, and SCC groups. No D-dimers were detected in the CSF of idiopathic epileptic dogs. Our results indicate that intrathecal fibrinolysis is activated in some dogs with different pathologic conditions affecting the CNS, as it happens in humans.

The highest CSF D-dimer concentrations were found in dogs with CNS inflammatory and neoplastic disorders (Table 3). In inflammatory diseases, the relationship between inflammation and coagulation could explain the local activation of the fibrinolytic pathways, as it occurs in systemic inflammatory disorders, which cause increased blood D-dimer concentrations. In dogs with SRMA, the elevated CSF D-dimer concentration could also be related to blood vessel inflammation, increased risk of subarachnoid bleeding, and clot formation. Interestingly, 17/20 dogs with inflammatory disorders (including some dogs with histologically confirmed diagnosis) did not have detectable CSF D-dimer concentrations, which suggests that intrathecal fibrinolysis was not activated in these animals. A potential explanation for this could be that some inflammatory CNS diseases in dogs affect mainly deep parenchymal regions, without or with minimal meningeal involvement. Thus, it is possible that lesion localization has an influence on D-dimer concentrations in the CSF.

In neoplastic disorders, either the inflammatory response induced by the tumor or associated hemorrhages could trigger local fibrinolytic activity. Interestingly, tissue factor, an enzyme cofactor involved in activation of coagulation is overexpressed in a wide variety of human tumors including CNS neoplasias,[22,23] and has been recently reported in canine tumor cell lines.[34] Although there is no information about the overexpres-

sion of tissue factor in canine CNS neoplasms, tissue factor-mediated coagulation activation may play a role in local fibrinolytic activity in these cases.

Half of the dogs in the NON-INF group had detectable concentrations of D-dimers in the CSF. Dogs in this group were mainly animals with noncompressive traumatic spinal cord injuries and cerebrovascular accidents, and most of these had detectable CSF D-dimers, as it occurs in humans with acute cerebrovascular disease and traumatic brain injury.[14,15]

Only 5/23 dogs in the SCC group had detectable D-dimers in the CSF. Of these, the two with the highest values had a xanthochromic CSF. Thus, local activation of coagulation and fibrinolysis resulting from subarachnoid hemorrhage was thought to be the most likely explanation.

A traumatic spinal tap could also cause an increase in the CSF D-dimer concentration. A previous study performed on healthy dogs did not find statistically significant variations in CSF D-Dimer concentrations before and after contamination with peripheral blood, and found that CSF red blood cell counts <4,000 cells/μL did not have any influence on CSF D-dimer concentration.[a] None of the samples included in the study had a red blood cell count above the upper limit of contamination reported previously, thus it seems unlikely that iatrogenic blood contamination could influence our results. In fact, the D-dimer test has been described as a useful tool to differentiate traumatic spinal tap from subarachnoid hemorrhage in humans.[19,20]

Blood D-dimer concentrations were measured in all dogs, and no statistically significant differences were found between groups. No correlation was detected between blood and CSF D-dimer concentrations either. Only 15/183 dogs included in the study had blood D-Dimer concentrations above the reference ranges for healthy dogs,[2] and only 5 of these 15 dogs had detectable D-dimer concentrations in the CSF. Furthermore, dogs in the SID group had the highest blood D-dimer values, but no fibrinolytic activity in the CSF. These findings suggest that there is intrathecal fibrinolytic activity, and that this is independent from the blood fibrinolytic activity, as it occurs in humans.[9]

When multiple comparisons between groups were performed, dogs in the SRMA group had marked and significantly higher CSF D-dimer values than dogs from all other groups. The source of fibrinolytic enzymes in the CNS remains controversial, and 2 main mechanisms have been proposed: direct leakage through a damaged blood–brain barrier, and existence of an endogenous fibrinolytic system in which endothelial cells of the brain and meninges appear to be the source of fibrinolytic activity.[9] Both mechanisms could play a role in SRMA: direct damage to the meninges and meningeal arteries can alter the blood-CSF barrier allowing direct leakage of fibrinolytic enzymes into the CSF, and severe meningeal inflammation could trigger the endogenous fibrinolytic system. The findings of our study are in agreement with those from human studies, in which fibrin degradation products in the CSF are significantly higher in patients with neurological disorders involving the meninges, and they are correlated with the severity of meningeal inflammation.[16,17]

C-reactive protein, a major acute phase reactant, has recently been described as a biomarker of neurological diseases, especially inflammatory CNS disorders. CRP has been measured in the CSF of dogs with different neurological disorders and has been proposed as a useful marker for the diagnosis of SRMA.[24–26] For this reason, CRP was also measured in CSF and blood samples of the same patients to assess the relationship between inflammation and coagulation. In our study, CRP was detected in 182/183 CSF samples, which demonstrates the high sensitivity of the TR-IFMA method compared with that of other techniques previously reported.[27] Our study showed that CRP is present in the CSF of healthy dogs, a fact that has not been previously reported. In addition, dogs with SID had increased CSF CRP concentrations. Although the CRP concentration in the CSF of SRMA dogs was significantly higher than that of dogs in other neurological disease groups and controls, there were no significant differences between the CSF concentrations of CRP in SRMA and SID dogs. There was a good and significant correlation between CSF and blood CRP concentrations, which is in agreement with previous reports.[27,35] In the SID group, 6/7 dogs were diagnosed of nonerosive immune-mediated polyarthritis, a disease that can occur occasionally in conjunction with SRMA.[28,36] In these animals (that had normal CSF analyses), the increased CSF CRP concentrations could be thought to be due to occult SRMA, because of the waxing and waning course of the disease. In the authors' opinion, a more likely explanation is that CRP passes easily from blood to CSF, so that CSF CRP concentration is directly affected by the CRP blood concentration. Previous studies have found increased CSF CRP concentrations in dogs with sepsis without CNS involvement,[24] and the same findings have been described in people with bacterial infections (patients with pneumonia and urinary tract infections).[35] Thus, the CRP concentration in the CSF seems to be directly affected by the blood CRP concentration, and this would limit its diagnostic specificity for neurological diseases affecting the CNS.[24,35]

Cerebrospinal fluid D-dimer concentrations had a significant but moderate correlation with CSF CRP concentrations. In addition, when CSF D-dimer and CRP concentrations were measured in 8/11 dogs with SRMA 6 weeks after treatment initiation (dogs free of clinical signs), no fibrinolytic activity was detected in any posttreatment initiation CSF sample, and significantly lower concentrations of CRP were detected in the CSF and blood of these dogs. Our findings suggest that CSF D-dimer concentration can be a reliable marker of meningeal inflammation that is not affected by systemic disease, as it occurs with CRP, so CSF D-dimer concentration might be an adjunctive

diagnostic marker for SRMA. Further studies are necessary to evaluate its potential value and to compare it with that of currently used parameters, such as CSF TNCC, total protein, and IgA. In addition, the potential diagnostic value of CSF D-dimers for meningitides of different etiologies remains unknown, but deserves further study.

Our study had some limitations, namely the low number of samples and the heterogeneity of diseases in some groups that could influence some statistical results. Nevertheless, the data presented demonstrate that the intrathecal fibrinolytic system is activated in some canine neurological disorders, especially in SRMA. Future investigations should be carried to elucidate the relevance of the coagulation and fibrinolytic systems, their role as potential therapeutic targets and the value of D-dimers as biomarkers of certain CNS diseases.

Footnotes

[a] Rossmeisl JH, Troy GC, Inzana KD, et al. Normal and blood-contaminated canine cerebrospinal fluid D-dimer concentrations. J Vet Intern Med 2002;16:369 (abstract)
[b] Miniquant, Biopool, Trinity Biotech, Wicklow, Ireland
[c] Miniquant-1, Biopool, Trinity Biotech
[d] Escanilla N, Leiva M, Peña MT, Monreal L. Aqueous humor fibrinolytic activity in dogs with ocular disease. Vet Ophthalmol 2010;13:354 (abstract)
[e] Sulfo-NHS-Biotin, Pierce Biotechnology Inc, Rockford, IL
[f] DELFIA streptavidin microtitration strips, PerkinElmer, Wallac Oy, Turku, Finland
[g] DELFIA/AutoDELFIA wash concentrate, PerkinElmer
[h] DELFIA assay buffer, PerkinElmer
[i] DELFIA Eu-labeling kit, PerkinElmer
[j] DELFIA enhancement solution, PerkinElmer
[k] VICTOR^2 1420 multilabel counter, PerkinElmer
[l] SPSS Inc, Chicago, IL

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Study # 2

Immunohistochemical evaluation of tissue factor, fibrin/fibrinogen and D-dimer deposits in canine glioma

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Immunohistochemical evaluation of tissue factor, fibrin/fibrinogen and D-dimers in canine gliomas

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ABSTRACT

In human gliomas tissue factor (TF) is overexpressed associated with the grade of malignancy and influences tumour biology. Intra-tumoural fibrin/fibrinogen deposition and activation of the fibrinolytic system also play a role in tumour cell proliferation and angiogenesis. The first aim of the present study was to investigate TF expression and the presence of fibrin/fibrinogen and D-dimers in canine glioma samples, graded according to the World Health Organization (WHO) classification of tumours of the central nervous system. The second aim was to investigate the occurrence of intravascular thrombosis (IVT) in canine gliomas, as a potential histological marker of glioma type or grade of malignancy. Immunohistochemical studies, using antibodies against TF, fibrin/fibrinogen and D-dimers were performed on 24 glioma samples, including 15 oligodendrogliomas, 6 astrocytomas and 3 mixed gliomas. Immunohistochemical data were statistically analysed to determine whether there was any relationship between glioma type and grade of malignancy. All gliomas were moderate to strongly positive for TF and the staining score was significantly higher ($P = 0.04$) in high-grade (III and IV) than in low-grade gliomas. Intra-tumoural fibrin/fibrinogen deposition was detected in all tumour biopsies assessed, and D-dimers were detected in 17/24 gliomas. IVT was a frequent finding, but was not linked to a specific glioma type or malignancy grade. TF expression, fibrin/fibrinogen deposition, extravascular fibrinolytic system activation and IVT occur in canine gliomas. Canine glioma might be a suitable model for studying coagulation and fibrinolysis as potential therapeutic targets for human gliomas.

Introduction

The role of the haemostatic system in cancer biology has long been recognised and several studies have demonstrated that some of these components influence angiogenesis, facilitating tumour growth and metastasis (Langer and Bokemeyer, 2012). Research has focused on the extravascular activation of the coagulation and fibrinolytic systems in order to elucidate their role beyond the intravascular compartment. Thus, the biological actions of some components of the haemostatic system are currently considered to be not only associated with blood clotting but also as pleiotropic proteins that are important for tumour progression (Wojtukiewicz et al., 2001; Langer and Bokemeyer, 2012). Fibrin/fibrinogen and tissue factor (TF; the initiator of the extrinsic coagulation cascade) are currently under investigation, to determine whether an activated coagulation cascade in the tumour microenvironment might be an effective therapeutic target (Liu et al., 2011).

TF expression in human solid tumours was first reported some time ago (Callander et al., 1992). More recently it was shown that TF triggers cellular signalling pathways that can induce tumour growth, angiogenesis and metastasis (Mueller and Ruf, 1998; Hjortoe et al., 2004; Yu et al., 2005; Versteeg et al., 2007). TF is overexpressed in human gliomas and the degree of TF expression is associated with the histological grade of malignancy and vascularity (Hamada et al., 1996; Guan et al., 2002; Thaler et al., 2013). TF expression has also been demonstrated in different canine tumour cell lines, particularly in those derived from epithelial tumours (Stokol et al., 2011), but there is little information about TF expression in canine gliomas.

Extravascular activation of the coagulation cascade, within the tumour microenvironment, leads to fibrin/fibrinogen deposition, which provides a provisional extracellular matrix to facilitate angiogenesis, binding of growth factors (such as vascular endothelial growth factor/VEGF) and promoting cell adhesion, proliferation and migration. Fibrin/fibrinogen might also act as a barrier between tumour and host that could interfere with an anti-tumour immune response (Dvorak et al., 1983; Simpson-Haidaris and Rybarczyk, 2001; Staton et al., 2003). Fibrin/fibrinogen deposition has been demonstrated in the majority of human tumour types, including primary

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and metastatic brain neoplasms (Bardos et al., 1996). There are few reports describing fibrin/fibrinogen deposition in canine tumours (McEvoy et al., 1996; Golombiewski et al., 1997), but evidence is lacking regarding expression in central nervous system (CNS) tumours.

The components of the fibrinolytic system and fibrin degradation-related fragments also play a role in tumour angiogenesis and are critical for tumour progression (Bootle-Wilbraham et al., 2001; Wojtukiewicz et al., 2001; Staton et al., 2003). However, the relationship between fibrinolysis and angiogenesis is not fully understood because of the coexistence of both pro- and anti-angiogenic properties of some of their components (Wojtukiewicz et al., 2001). For example, fibrin fragment E is a potent angiogenic factor that enhances the effect of VEGF (Bootle-Wilbraham et al., 2001), whereas cleavage of plasminogen releases proteolytic fragments, such as angiostatin, which are potent inhibitors of angiogenesis (Wojtukiewicz et al., 2001). Although the influence of D-dimers on angiogenesis is unknown, these represent specific degradation products of cross-linked fibrin and can be used as specific markers of fibrinolysis. A recent study has demonstrated that there is fibrinolytic activity in the cerebrospinal fluid of dogs affected with glioma (de la Fuente et al., 2012).

The aim of the present study was to use immunohistochemistry to identify and characterise TF expression, as well as fibrin/fibrinogen and D-dimer deposition in canine gliomas. Since intravascular thrombosis (IVT) has been proposed as the underlying mechanism that leads to hypoxia-induced tumour progression in human gliomas (Tehrani et al., 2008), the secondary aim was to investigate the occurrence of IVT in canine gliomas, as a potential histological marker of glioma type or grade of malignancy.

Materials and methods

Study population

The veterinary neuropathology database of the Universitat Autònoma de Barcelona was used to identify dogs affected with brain neoplasia between 2001 and 2013. Inclusion criteria for the study were that a histopathological diagnosis of brain glioma had been made, and that there was archival tissue available that was suitable for immunohistochemical studies. All samples were reviewed by an ECVP Board-certified pathologist (MP), who classified and graded all cases according to the World Health Organization (WHO) classification of CNS tumours (Louis et al., 2007).

Oligodendroglial tumours were classified as grade II (oligodendroglioma) or grade III (anaplastic oligodendroglioma), and astrocytomas were classified as grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) or grade IV (glioblastoma). Mixed glial tumours were graded as either II or III. Gliomatosis cerebri was considered a grade III astrocytic tumour (Stoica et al., 2011).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (3 μm) were dewaxed with xylene and rehydrated in descending concentrations of ethanol. For antigen retrieval, tissue sections were boiled in bain-marie (96–98 °C) for 20 min with 0.01 M citrate buffer (pH 6.0) (TF, D-dimer) or treated with 0.1% trypsin in distilled water for 20 min (fibrin/fibrinogen). Endogenous peroxidase activity was suppressed with 3% H_2O_2 for 40 min. Slides were blocked with goat serum for 1 h. TF immunolabelling was performed using a monoclonal rabbit anti-human antibody (ab151748, Abcam) at a dilution of 1:400. The positive control tissue consisted of sections of canine mammary gland carcinoma, a neoplasia with high TF expression (Stokol et al., 2011).

Fibrin/fibrinogen immunoreactivity was assessed using a polyclonal rabbit anti-human fibrin/fibrinogen antibody (A0080, Dako) at a dilution of 1:10,000 as described previously (Cotovio et al., 2007). This antibody reacts with fibrin, fibrinogen and fibrinogen fragments D and E. To verify immunoreactivity with canine fibrin/fibrinogen, sections from a brain with intra-parenchymal haemorrhages were assessed as a positive control. D-dimer immunolabelling was performed with a monoclonal mouse anti-D-dimer antibody (ABS 015-22-02, Thermo Scientific) as described previously, but at a 1:20,000 dilution (Carretón et al., 2013). Immunohistochemistry for TF and fibrin/fibrinogen was carried out using an EnVision+ System-HRP (DAB) Rabbit Kit (Dako), with an EnVision+ System-HRP (DAB) Mouse Kit (Dako) used for D-dimers. The chromogen substrate used was 3, 3′diaminobenzidine (Dako) and counterstaining was performed with haematoxylin (Merck). Negative control tissue sections from the same specimens were identically processed, replacing the specific primary antibody with an isotype-control IgG of the same species and concentration as the primary antibody.

The intensity of TF staining was graded, based on the estimated proportion of the tumour cell population showing positive staining for TF (Hamada et al., 1996): negative (score of 0; no positive cells detected); weakly positive (score of 1; <50% positive tumour cells); moderately positive (score of 2; ≥50% positive tumour cells with weak intensity); strongly positive (score of 3; ≥50% positive tumour cells with strong intensity). Fibrin/fibrinogen and D-dimer immunoreactivity were graded using a modification of a scoring system described previously (Cotovio et al., 2007). Briefly, 20 high power fields (HPF; 40× objective lens) were evaluated within each tumour, then fibrin/fibrinogen or D-dimer deposition was graded as follows: no staining; 0, <10% positive fields; 1, 11–25% positive fields; 2, 26–50% positive fields; 3, 51–75% positive fields; 4, ≥76% positive fields with staining in an almost continuous pattern.

IVT was assessed in canine gliomas, by evaluating the presence of thrombosed vessels within each tumour. A thrombosed vessel was one that was occluded by a deposit of D-dimers. The number of thrombosed vessels was counted in 20 HPF in triplicate. The results, expressed as mean and standard deviation of thrombosed vessels per HPF, were compared for each histological type, malignancy grade and TF scores.

Statistical analysis

Data analysis was performed using commercial software (SPPS, version 18.0). Frequencies and percentages were used for qualitative variables and range for age of dogs. Quantitative results for variables not normally distributed are expressed as median and 25th–75th percentiles. For inferential analysis, the chi-square test was used to compare the scores of each stain in different glioma types and malignancy grades. One-way ANOVA with Bonferroni adjustment was used to detect differences between the mean of thrombosed vessels in each glioma type, malignancy grade and TF score. Significance was set at $P < 0.05$ for all tests.

Results

Study population

Twenty-four dogs diagnosed with brain gliomas met the inclusion criteria. Dogs were aged between 4 and 15 years (median 8.5 years). Six dogs had grade II oligodendrogliomas, nine dogs had grade III oligodendrogliomas, one dog had a grade II astrocytoma, four dogs had grade III astrocytomas (including one dog with gliomatosis cerebri), one dog had a grade IV astrocytoma (glioblastoma), one dog had grade II mixed glioma and two dogs had grade III mixed gliomas.

Tissue factor in canine gliomas

Immunoreactivity to TF was detected in all 24 glioma specimens, and was moderate (score 2; 14/24) or strongly positive (score 3; 10/24) as shown in Table 1. The pattern of TF expression was diffuse and homogeneous in 6/24 specimens (two grade II oligodendrogliomas, two grade III oligodendrogliomas, one grade IV astrocytoma and one grade II mixed glioma). Most gliomas showed a heterogeneous pattern of TF immunostaining, characterised by a lack of or low expression of TF in the periphery of the tumour, and a progressive increase of staining close to the tumour core.

Expression of TF was marked in the tumour cell population surrounding the microvasculature and around necrotic and haemorrhagic areas (Figs. 1A and B). Scattered clusters of tumour cells with angiocentric distribution and strong TF immunolabelling were visible in all specimens, even in gliomas with a moderate staining score (score 2). At the cellular level, TF immunolabelling was usually stronger at the cell surface than in the cytoplasm of glial cells. No TF immunoreactivity was observed in the adjacent normal brain tissue, except for scattered reactive astrocytes, surrounding the tumour, which showed strong TF expression.

No statistical differences were found in TF scores, comparing different glial tumour histological types (oligodendrogliomas *versus* astrocytomas *versus* mixed gliomas) or between different malignancy grades, within a specific glioma type. However, when all gliomas were grouped according to grade into high (grades III and IV) and low-grade (grade II) gliomas, the TF score was significantly greater for high-grade gliomas ($P = 0.04$).

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Table 1
Number of samples and tissue factor (TF) score for each glioma type and WHO grade.

Tumour type (WHO grade)	Number of samples	TF score[a]				TF score median (25th–75th percentiles)
		0	1	2	3	
Oligodendroglioma (II)	6	–	–	5	1	2 (2–2)
Oligodendroglioma (III)	9	–	–	3	6	2 (2–3)
Astrocytoma (II)	1	–	–	1	–	N/A
Astrocytoma (III)	4	–	–	2	2	2.5 (2–3)
Glioblastoma (IV)	1	–	–	–	1	N/A
Mixed glioma (II)	1	–	–	1	–	N/A
Mixed glioma (III)	2	–	–	2	–	2 (2–2)

N/A, not applicable.
[a] Expression of TF was classified as follows: (0) negative; (1) weakly positive with <50% positive tumour cells; (2) moderately positive with ≥50% positive tumour cells with weak intensity; (3) strongly positive with ≥50% positive tumour cells with strong intensity.

Fibrin/fibrinogen deposits in canine gliomas

Fibrin/fibrinogen deposits were detected in all glial tumours studied, with 14/24 gliomas having a score ≥3, whereas 5/24 samples scored as 0 (Table 2). Fibrin/fibrinogen labelling was only visualised extracellularly (Fig. 1C). Extracellular deposits showed a fibrillary pattern, surrounding tumour cells with an amorphous pattern in necrotic areas when present. In gliomas with haemorrhagic areas, fibrin/fibrinogen deposition was pronounced, within the haemorrhagic foci. No fibrin/fibrinogen deposits were seen in normal brain parenchyma. There were no significant differences in fibrin/fibrinogen scores comparing the different glioma histological types, between different malignancy grades within a specific glioma histological type, or between low-grade (grade II) and high-grade (grade III and IV) gliomas.

D-dimer deposits and IVT in canine gliomas

D-dimer immunostaining was apparent in 17/24 gliomas, with 13 samples demonstrating both extravascular and intravascular deposits, two samples having only extravascular deposits and two others having only intravascular deposits. Ten of 24 gliomas had a

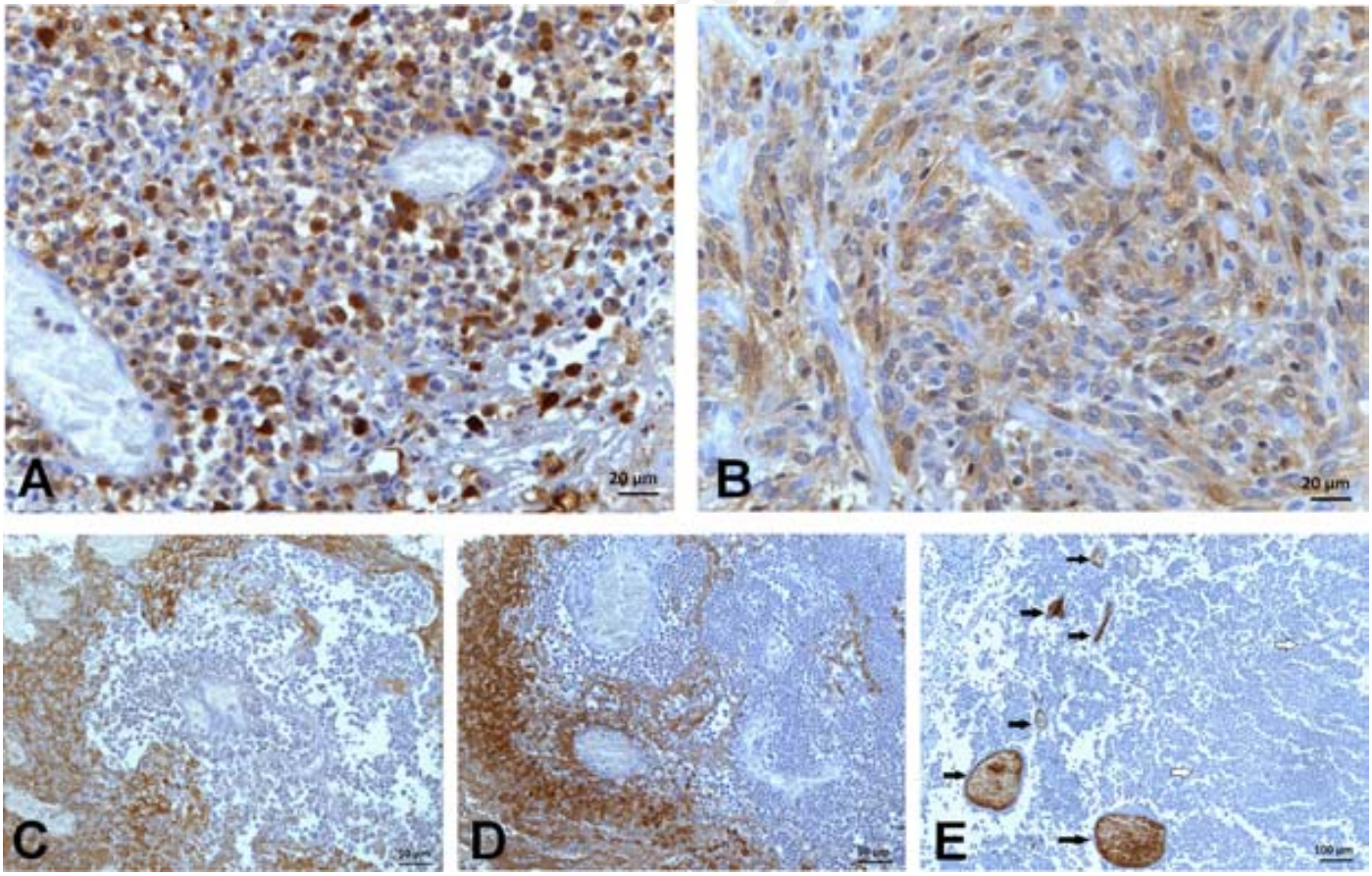

Fig. 1. (A) Tissue factor (TF) immunohistochemistry. Positive staining for TF (brown) in tumour cells of a dog affected with an anaplastic oligodendroglioma (score 3); scale bar 20 µm. (B) Positive staining for TF (brown) in tumour cells of a dog affected with an anaplastic astrocytoma (score 3); scale bar 20 µm. (C) Fibrin/fibrinogen immunohistochemistry. Fibrin/fibrinogen deposition (brown) in the oligodendroglioma of the dog in (A) (grade 4); scale bar 50 µm. (D) D-dimer immunohistochemistry. D-dimer deposition (brown) in the tumour of the dog in (A) (score 4); scale bar 50 µm. Note the negative intravascular immunostaining in the microvasculature. (E) Example of multiple vascular occlusion and distension by D-dimer deposits (brown) in a dog affected with an anaplastic oligodendroglioma; scale bar 100 µm. Note the coexistence of thrombosed (black arrows) and non-thrombosed (white arrows) vascular structures and the absence of extravascular D-dimer deposition.

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Table 2
Number of samples and fibrin/fibrinogen scores for each glioma type and WHO grade.

Tumour type (WHO grade)	Number of samples	Fibrin/fibrinogen score[a]					Fibrin/fibrinogen score median (25th–75th percentiles)
		0	1	2	3	4	
Oligodendroglioma (II)	6	1	–	–	4	1	3 (3–3)
Oligodendroglioma (III)	9	2	–	1	4	2	3 (2–3)
Astrocytoma (II)	1	–	–	–	–	1	N/A
Astrocytoma (III)	4	1	–	1	1	1	2.5 (1.5–3.25)
Glioblastoma (IV)	1	–	1	–	–	–	N/A
Mixed glioma (II)	1	–	1	–	–	–	N/A
Mixed glioma (III)	2	1	–	1	–	–	1 (0.5–1.5)

N/A, not applicable.
[a] Slides with absent staining or <10% positive fields received a score of 0; slides with 11–25% positive fields received a score of 1; slides with 26–50% positive fields received a score of 2; slides with 51–75% positive fields received a score of 3; and slides with ≥76% positive fields with staining in an almost continuous pattern received a score of 4.

D-dimer score ≥3 (Table 3). Extravascular D-dimer staining coincided with that for fibrin–fibrinogen, and it was particularly marked within and surrounding haemorrhagic foci and necrotic areas (Fig. 1D). Seven of 24 tumours were negative for D-dimer immunostaining, but only two of these were scored as 0 for fibrin/fibrinogen immunostaining. No D-dimer immunolabelling was detected in normal brain parenchyma. There were no significant differences in D-dimer scores comparing different histological types of glioma, between different malignancy grades within a specific histological type, or between low-grade (grade II) and high-grade (grades III and IV) gliomas.

IVT was detected in the microvasculature of 15/24 glioma specimens (Fig. 1E). The mean numbers of thrombosed vessels per HPF were 1.4 ± 1.5 in oligodendrogliomas, 0.4 ± 0.4 in astrocytomas and 1 ± 1.7 in mixed gliomas (Table 3). No statistically significant differences were seen in terms of the prevalence of thrombosed vessels comparing different histological types of glioma, between different malignancy grades within a particular histological type, or between high-grade (grades III and IV) and low-grade (grade II) gliomas. Higher TF scores were not associated with greater numbers of thrombosed vessels per HPF. No thrombosed vessels were detected in normal brain parenchyma of any sample.

Discussion

Several studies have redefined the biological functions of TF, beyond its classical role as an initiator of the extrinsic coagulation pathway. There is evidence that TF can promote angiogenesis in tumours through increased production of VEGF and down-regulation of negative angiogenic factors (Zhang et al., 1994). VEGF is overexpressed in canine gliomas, and its expression correlates with grade of malignancy (Rossmeisl et al., 2007). TF expression has been demonstrated in different canine tumour cell lines supporting the hypothesis that TF could be involved in the biological behaviour of canine tumours, as is the case in humans (Stokol et al., 2011).

We investigated the expression of TF in canine gliomas, which has not been reported previously in the veterinary literature. The results of our immunohistochemical analysis of canine glioma tissue are similar to those reported in human studies (Hamada et al., 1996; Guan et al., 2002; Thaler et al., 2013). It was demonstrated that canine gliomas expressed TF, and that the intensity of immunolabelling was stronger in high-grade tumours. Significant differences in TF scores were only found when tumours were grouped into high-grade and low-grade gliomas, and no differences were found between different malignancy grades of the same histological type. It is possible that significant differences would become apparent within a particular glioma type, if a larger sample size was available for analysis.

Although astrocytes are thought to be the primary source of TF in the CNS, normal human brain parenchyma is negative for TF immunolabelling (Eddleston et al., 1993; Akassoglou and Strickland, 2002). In the present study, normal brain tissue surrounding each neoplasm was consistently negative for TF expression, but some peritumoural immunoreactive astrocytes were evident. This finding is consistent with results of a recent study in human gliomas (Thaler et al., 2013). A potential limitation of the present study could be the use of an anti-human TF antibody. This could reduce the sensitivity of the technique, because the canine TF extracellular domain shares only 72% identity at the amino acid level with human TF (Stokol et al., 2011) and this might reduce the antibody affinity with its target antigen. However, all canine glial tumours were positive for TF immunolabelling, although it is possible that TF scores were

Table 3
Number of samples and D-dimer scores for each glioma type and WHO grade. Median of D-dimer scores and mean ± SD of thrombosed vessels per high power field (HPF) are also shown.

Tumour type (WHO grade)	Number of samples	D-dimer score[a]					D-dimer score median (25th–75th percentiles)	Mean thrombosed vessels/HPF
		0	1	2	3	4		
Oligodendroglioma (II)	6	2	1	–	1	2	2 (0.25–3.75)	1.6 ± 1.9
Oligodendroglioma (III)	9	3	–	1	1	4	3 (0–4)	1.3 ± 1.4
Astrocytoma (II)	1	1	–	–	–	–	N/A	0[b]
Astrocytoma (III)	4	–	–	2	2	–	2.5 (2–3)	0.4 ± 0.2
Glioblastoma (IV)	1	–	1	–	–	–	N/A	0.2[b]
Mixed glioma (II)	1	–	1	–	–	–	N/A	2.9[b]
Mixed glioma (III)	2	1	1	–	–	–	0.5 (0.25–0.75)	0 ± 0

N/A, not applicable.
[a] Sections with absent staining or <10% positive fields received a score of 0; sections with 11–25% positive fields received a score of 1; slides with 26–50% positive fields received a score of 2; sections with 51–75% positive fields received a score of 3; and sections with ≥76% positive fields with staining in an almost continuous pattern received a score of 4.
[b] Results from one sample only.

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underestimated in some samples, potentially leading to a degree of bias.

Extravascular fibrin/fibrinogen deposition was frequently observed in the gliomas we studied, and was considered significant in most cases, similar to reports for human gliomas (Bardos et al., 1996). Fibrin/fibrinogen are not normally present in the CNS and can only reach the brain parenchyma through vascular damage or disruption of the blood–brain barrier, consistent with the lack of detection of fibrin/fibrinogen deposition in control brain tissue. The implications of fibrin/fibrinogen deposition are numerous in CNS pathology, as there is evidence that they play a role in neuroinflammation, neurodegeneration and tumour biology (Wojtukiewicz et al., 2001; Akassoglou and Strickland, 2002). The antibodies used to detect fibrin/fibrinogen are not specific for cross-linked fibrin and can also react with fibrinogen and fibrinogen fragments D and E. Thus, the results of fibrin/fibrinogen immunostaining should be interpreted in combination with those of D-dimer immunostaining to confirm the presence of cross-linked fibrin.

Extravascular deposition of D-dimers in tumours was located in similar areas as fibrin/fibrinogen, but D-dimers were not detected in approximately one-third of gliomas while fibrin/fibrinogen deposition was present in all those tested. The presence of D-dimers demonstrates that some amount of fibrin/fibrinogen immunostaining relates to cross-linked fibrin, indicating that there is local extravascular activation of the coagulation system in canine gliomas. Furthermore, their presence indicates that there is extravascular activation of the fibrinolytic system in most gliomas, as has been reported previously (de la Fuente et al., 2012). Further interpretation of these findings is difficult, since fibrinolysis can be potentiated or inhibited in CNS disease, and the precise role of the fibrinolytic system in glial neoplasia remains to be elucidated (Wojtukiewicz et al., 2001; Staton et al., 2003). Fibrin formation and deposition, as well as fibrinolysis, are time-dependent processes; thus several factors, such as time from diagnosis to death/euthanasia or treatments received (unknown data) could have biased the results of our study. Prior use of corticosteroids could decrease fibrinogen deposition, through reduction of the pathologically increased permeability of the blood–brain barrier (Platt et al., 2005).

In humans, IVT is more frequent in glioblastoma than in other CNS tumours, and has been proposed as a potential marker of an aggressive phenotype in anaplastic astrocytomas (Tehrani et al., 2008). In the present study, IVT was observed in most gliomas, but was not linked to a particular type of glioma, grade of malignancy or TF score. D-dimer immunostaining was preferred by the authors over fibrin/fibrinogen immunostaining to assess IVT, since D-dimers are sensitive markers of previous clot formation. IVT occurs in canine gliomas, but further studies with larger number of tumours are necessary to elucidate whether IVT could be useful as a histological marker of aggressive behaviour.

The main limitation of our study was the relatively low number of glioma samples available for some groups and this could have influenced the outcome. However, the research findings presented here support the need for further investigations into the role of the haemostatic system in canine gliomas.

Conclusions

TF is expressed in canine gliomas and the degree of TF expression is greater in high-grade gliomas. Fibrin/fibrinogen and D-dimer deposition in canine gliomas suggest that there is intra-lesional activation of the coagulation and fibrinolytic systems in this type of tumour. These findings support the need for further studies to elucidate the role of the haemostatic system as a potential therapeutic target for brain gliomas.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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DISCUSSION

The results of the studies presented here provide new information on the role of the hemostatic system components in canine neurologic disorders and address an interesting and not previously investigated aspect of brain tumor biology in veterinary medicine. In addition, the findings of the first study support the use of CSF D-dimers as specific biomarkers of SRMA in dogs.

The first study of this PhD thesis was designed to evaluate the D-dimer test to assess CSF fibrinolytic activity, and to determine whether the D-dimer CSF concentration could be used as a biomarker of inflammation. Inflammation activates coagulation and coagulation also affects the inflammatory response. Consequently, to avoid the detrimental effects of inflammation-induced fibrin deposition, the fibrinolytic system must be activated to restore homeostasis. D-dimers, as specific markers of previous clot formation, might be also considered indirect biomarkers of inflammation. In this direction, previous studies in veterinary medicine have reported the use of the D-dimer test to assess local fibrinolytic activity in peritoneal and synovial fluids in horses and aqueous humor in dogs, and have demonstrated that high D-dimer concentrations are associated with inflammatory disorders.[56-59] Although further investigations are required to determine cut-off values, the mentioned studies support the role of D-dimers as specific biomarkers of disease in the equine species for severe gastrointestinal disorders and septic joint disease.[56,57] In fact, D-dimer concentrations in synovial fluid of horses are most sensitive than the total white cell count to detect joint disease.[58]

The results of study #1 confirm the first hypothesis of this thesis and demonstrate that an intrathecal fibrinolytic pathway is activated in the CSF of dogs with some neurological disorders, and that this activation can be measured by means of the D-dimer test. On the

other hand, intrathecal fibrinolytic activity was not detected in the CSF of healthy control dogs or in the CSF of dogs with systemic inflammatory diseases without CNS involvement. These findings are I agreement with those reported in human medicine. In humans, normal CSF contains very low levels of fibrinolytic enzymes, but the CSF fibrinolytic activity increases in CNS pathologic conditions.[48] As expected, dogs with SRMA, other inflammatory disorders and neoplastic disorders did have the highest CSF D-dimer concentrations, but the SRMA group was the only one with significantly higher CSF D-dimer concentrations than those of dogs from all other groups and controls. D-dimers were also detected in the CSF of some dogs belonging to non-inflammatory disorder group thus, activation of the intrathecal fibrinolytic system is not restricted to inflammatory and neoplastic disorders, and this is in agreement with results derived from human studies.[93,94]

The results also suggest that local activation of fibrinolysis is not linked to a concurrent systemic hyperfibrinolysis because there was no correlation between CSF and blood D-dimer concentrations. In addition, the results obtained in dogs with systemic inflammatory diseases without CNS involvement (dogs high blood D-dimer concentrations and absent CSF D-dimers) also support that there is intrathecal fibrinolytic activity independent from blood fibrinolytic activity. In contrast, the results suggest that CSF CRP concentrations seem to be directly affected by blood CRP concentrations, and this would limit the diagnostic specificity of CRP for neurological diseases affecting the CNS.

Although SRMA is an inflammatory disease, dogs with SRMA were grouped independently from other inflammatory disorders to evaluate D-dimer concentrations as a

possible specific biomarker of this disease. The reason for this was that in human medicine, fibrin degradation products significantly increase in neurological disorders involving the meninges and their concentrations are correlated with the severity of meningeal inflammation.[95,96] SRMA is an inflammatory disease involving the leptomeninges and meningeal arteries, so high D-dimer concentrations were expected to be found in the CSF of dogs with this disease. Moreover, standard monitoring of dogs with SRMA is based on follow-up CSF sampling and analysis, which made possible to evaluate the effect of anti-inflammatory treatment on CSF D-dimer concentrations. It is worth to mention that no fibrinolytic activity was detected in any CSF sample of SRMA dogs after initiation of immunosuppressive treatment. The results of study #1 suggest that the CSF D-dimer concentration can be a reliable marker of meningeal inflammation that is not affected by systemic disease, as it occurs with CRP. Thus CSF D-dimer concentration might be a diagnostic marker for SRMA. Although the CSF D-dimer concentrations of dogs with SRMA were much higher than those of dogs in other groups, further studies with a large number of SRMA cases are necessary to determine diagnostic cut-off values and to determine sensitivity and specificity of the D-dimer test compared with those of currently used CSF parameters, such as total nucleated cell count, total protein concentration and IgA.

The results of study #2 also support that there is activation of a local fibrinolytic system in some neoplastic disorders. This second study demonstrated, by means of D-dimer immunohistochemistry, the presence of fibrinolytic activity in most of the gliomas included in the study.

Although intrathecal fibrinolytic activity was detected in most samples from the

inflammatory and neoplastic groups in study #1 and from most gliomas included in study #2, there was a subset of dogs in both studies in which fibrinolytic activity was not detected. Potential explanations for this could be: 1) fibrinolysis can be potentiated or inhibited during the inflammatory response as well as in neoplastic disorders. The exact role of the fibrinolytic system in glial neoplasias remains to be elucidated;[61,83] 2) fibrin formation and deposition, as well as clot lysis, is a time-dependent process, thus sampling time could influence the results in both studies; 3) the kinetics of D-dimers in the CSF of dogs are unknown; 4) some inflammatory CNS diseases in dogs affect mainly deep parenchymal regions, without or with minimal meningeal involvement. Thus, it is possible that lesion localization has an influence on the D-dimer concentrations in CSF; 5) because the retrospective nature of study #2, treatments received (unknown data) before death/euthanasia could have biased the results of the study.

Regarding the fibrinolytic activity detected in the CSF of some dogs with neoplasias in study #1, the authors hypothesized that the possible mechanisms that might induce local fibrinolytic activity could be the inflammatory response induced by the tumor and the TF-mediated activation of coagulation. At the time of writing and publication of study #1 there was no information about TF expression in nervous system tumors in dogs and the hypothesis was based on human medicine studies. The results of study #2 have confirmed that TF is overexpressed in canine gliomas, thus TF-mediated coagulation activation may trigger CSF fibrinolytic activity in dogs with gliomas.

The results of study #2 demonstrated that TF expression is higher in canine high-grade gliomas than in low-grade gliomas, as it occurs in humans.[72-74] Moreover, all gliomas included in the study, independently of histologic type or malignancy grade, expressed

TF and these findings support the potential role of TF in canine glioma biology. This is the first study in veterinary medicine that looks into the expression of TF in gliomas and supports the relationship between the hemostatic system and cancer as a research field in veterinary medicine. Study # 2 demonstrated that canine gliomas develop in a procoagulant environment where TF factor is overexpressed, fibrinogen gains access to the tumor parenchyma and cross-linked fibrin is formed and deposited. Thus it demonstrated not only that there is activation of coagulation system within gliomas, but also the presence of intratumoral fibrinolysis by means of D-dimer immunolabeling.

The low number of glioma samples in some groups has probably impeded finding significant differences of TF expression between different malignancy grades of the same histologic type. However, there seems to be a trend to higher TF scores in grade III versus grade II oligodendrogliomas, so it is possible that differences would arise with a larger sample size.

D-dimer immunolabeling, used as specific marker of previous clot formation, allowed also the evaluation of IVT in canine gliomas. D-dimer immunolabeling demonstrated to be useful in a previous veterinary study that evaluated the occurrence of lung and kidney thromboembolism in canine heart worm disease.[97] The results of study #2 do not support the occurrence of IVT as a specific histologic marker of any glioma type or grade in dogs, as it occurs in human medicine.[92] Despite this, IVT was detected in most gliomas and may play a role in hypoxia-induced tumor progression as proposed, but further interpretations of this findings are difficult and deserve further investigations.[89] Again, the small sample size, in particular samples of the more malignant glioblastomas, possible preclude from finding significant differences in the occurrence of IVT between different

canine glioma types.

The results of this PhD thesis confirm that the hemostatic system is involved in the pathophysiology of some canine neurologic diseases and provide a new perspective of research, especially on CNS inflammatory and neoplastic disorders in dogs. This PhD thesis also evidences the need for future investigations on the hemostatic system as a potential source of novel biomarkers and therapeutic strategies for canine CNS disorders. In this direction, the results suggest that the CSF D-dimer concentration may be considered a diagnostic marker of SRMA. On the other hand, canine gliomas could be a suitable natural model of human gliomas to study the coagulation and fibrinolytic systems as potential therapeutic targets, because similar findings have been found in both species.

Future investigations could also include looking into the potential expression of TF and other hemostatic system related molecules in meningiomas, the most common primary brain tumor in the canine species. Dogs are prone to more malignant meningiomas compared to humans, so prognosis is usually considered guarded to poor in this species and new treatment strategies are necessary. One study in human medicine demonstrated that TF expression correlates with the proliferative ability of meningiomas, which would support researching this area in dogs.[98] These investigations would provide important insights into canine meningioma pathophysiology and treatment.

CONCLUSIONS

CONCLUSIONS

1. The CSF fibrinolytic activity in dogs can be measured by means of D-dimer concentrations.

2. The intrathecal fibrinolytic pathway is activated in the CSF of dogs with some neurological disorders, especially in dogs with SRMA.

3. Local activation of fibrinolysis in the CSF is not linked to a concurrent systemic hyperfibrinolysis.

4. CSF D-dimer concentrations are a potential diagnostic marker of SRMA.

5. TF is expressed in canine gliomas and the degree of TF expression is higher in high-grade gliomas than in low-grade gliomas.

6. Fibrin/fibrinogen and D-dimer deposition in canine gliomas suggest that there is intra-tumoral activation of the coagulation and fibrinolytic systems.

7. IVT is present in most gliomas, but it is not linked to a particular type of glioma or grade of malignancy.

8. IVT may play a role in hypoxia-induced glioma progression in dogs.

9. Because similar findings are described in human gliomas, canine gliomas could be a suitable natural model of human glioma to study the coagulation and fibrinolytic systems as potential therapeutic targets.

SUMMARY

Veterinary neurology is one of the most evolving areas in veterinary medicine. Recent medical advances and the greater demand from society to face neurological disorders in veterinary patients have contributed to a better understanding of the underlying mechanisms of neurological diseases in pets. Despite this, the etiologic origin of some diseases still remains obscure, and diagnostic and therapeutic approaches are limited in some cases, so research is needed to solve these problems.

Studies in human beings have demonstrated the relationship between the hemostatic system and the inflammatory response as well as the influence of hemostatic system molecules in cancer biology.

Neither the activation of the hemostatic system as part of canine brain tumor pathophysiology, nor the intrathecal fibrinolytic activity in dogs with neurologic disorders had been previously studied. D-dimers are specific cross-linked fibrin degradation products and their presence confirms previous coagulation system activation. Previous studies in human beings and animals demonstrated that local fibrinolytic activity can be measured by means of D-dimer concentrations in different biological fluids.

In this direction, this PhD thesis confirmed that intrathecal fibrinolytic activity is activated in some canine neurologic disorders, independently of systemic fibrinolytic activity. A significant rise in cerebrospinal fluid D-dimer concentration occurs in dogs with steroid responsive meningitis-arteritis, suggesting that CSF D-dimer concentrations might be potential diagnostic markers of this disease.

This PhD thesis also addressed the relationship between the hemostatic system and canine glioma biology and demonstrated that canine gliomas overexpress tissue factor, the extrinsic pathway activator of the coagulation cascade, and that there is intratumoral activation of the coagulation and fibrinolytic systems.

In conclusion, the results of this PhD thesis confirm that the hemostatic system is involved in the pathophysiology of some canine central nervous system diseases and provide a new perspective to perform further research, especially on inflammatory and neoplastic disorders of the canine central nervous system.

RESUMEN

La neurología es una de las especialidades veterinarias que más ha evolucionado en los últimos años. Los avances médicos junto a la demanda de la sociedad para diagnosticar y tratar enfermedades neurológicas en perros han contribuido a una mayor compresión de la fisiopatología de estas enfermedades. Aún así, la etiología de ciertas enfermedades sigue siendo desconocida, lo que limita el diagnóstico y tratamiento en muchos casos. La necesidad de nuevas líneas de investigación es clara.

Estudios en medicina humana han demostrado la relación existente entre el sistema hemostático y la respuesta inflamatoria, así como la influencia que ejercen los componentes de dicho sistema en la biología tumoral.

Ni la posible relación del sistema hemostático con los tumores cerebrales ni la actividad fibrinolítica intratecal han sido estudiados en la especie canina. Los D-dímeros son productos de degradación de la fibrina entrelazada y su presencia es específica de la activación de la cascada de la coagulación. Estudios en humanos y en animales han demostrado que la actividad fibrinolítica local puede medirse mediante la determinación de la concentración de D-dímeros en diferentes fluidos corporales.

Esta tesis doctoral ha confirmado que existe activación de la actividad fibrinolítica intratecal en algunas enfermedades neurológicas de los perros y que ésta es independiente de la actividad fibrinolítica sistémica. El incremento significativo de las concentraciones de D-dímeros en el líquido cefalorraquídeo de perros con meningitis-arteritis que responde a cortisona sugiere que la concentración de D-dímeros puede considerarse un biomarcador para dicha enfermedad.

Esta tesis doctoral aborda la relación entre el sistema hemostático y el glioma canino y demuestra que el factor tisular, el iniciador de la vía extrínseca de la cascada de la coagulación, se sintetiza en gliomas caninos. Además, demuestra la activación intratumoral de los sistemas de la coagulación y de la fibrinólisis.

En conclusión, los resultados de esta tesis doctoral confirman que el sistema hemostático está involucrado en la fisiopatología de algunas enfermedades del sistema nervioso central canino y establece la necesidad de nuevas líneas de investigación, especialmente en enfermedades inflamatorias y neoplásicas del sistema nervioso central de los perros.

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