Matrix metalloproteinase and tissue inhibitor of metalloproteinase in serum and synovial fluid of osteoarthritic dogs

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Summary
To better understand the mechanisms responsible for the pathological processes of osteoarthritis (OA) and to potentially identify a profile of changes that could be predictive of early OA, matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in the synovial fluid and serum of normal and osteoarthritic dogs were examined. The concentration of MMP-1 in the synovial fluid of osteoarthritic dogs (0.62 ± 0.16), as measured by densitometry, was significantly higher than that found in control dogs (0.42 ± 0.19) (P = 0.03). The concentration of MMP-1 in the serum of osteoarthritic dogs (0.74 ± 0.16) was significantly less than that found in control dogs (0.87 ± 0.08) (P = 0.05). The concentration of TIMP-2 in the synovial fluid of osteoarthritic dogs (46.2 ± 21.9 ng/ml) was significantly less than that of control dogs (122.0 ± 66.5 ng/ml) (P = 0.009). The concentration of TIMP-2 in the serum of osteoarthritic dogs (116.2 ± 43.1 ng/ml) was not significantly different than that of control dogs (95.1 ± 94.4 ng/ml) (P = 0.554). In addition, a phospho-tyrosine immunoprecipitation and mass spectrometry were used to isolate and identify interferon-alpha in canine synovial fluid.

Keywords
Matrix metalloproteinase, synovial fluid, serum

Vet Comp Orthop Traumatol 2006; 19: 49–55

Introduction
Osteoarthritis (OA) is a complex disorder with a multitude of interacting biochemical and biomechanical factors involved in its pathogenesis. It has been estimated that OA affects as many as 20% of the canine population over one year of age (15). Since the degenerative processes that lead to OA begin long before clinical manifestations of the disease, early detection of OA is a challenge (9, 26). Recently, investigations have focused on finding serum or synovial fluid (SF) biomarkers that are useful to diagnose, monitor treatment or determine prognosis for patients with OA (9, 28).

Among the proteins that have received recent attention as putative biomarkers of OA are a group of tissue proteases, responsible for initiating cartilage matrix digestion (20). The matrix metalloproteinases (MMP) are a diverse family of zinc dependent endopeptidases that together are capable of degrading all the constituents of the extracellular matrix. The MMP are required for normal development and turnover of the articular cartilage extracellular matrix, but their elevated activity in many pathological conditions, such as arthritis, is said to lead to excessive destruction of the extracellular matrix (3, 4, 12, 20–22, 31). Among the MMP, MMP-1, which degrades the collagen component of the extracellular matrix, is highly important since it is expressed by chondrocytes, synoviocytes, fibroblasts, keratinocytes, endothelial cells, monocytes, and macrophages (2). MMP-1 synthesis can be increased by a variety of inflammatory cytokines involved in the development of OA, including tumour necrosis factor and interleukin-1 (2, 8, 11).

The proteolytic activity of the MMP is regulated by specific inhibitors known as tissue inhibitors of metalloproteinases (TIMP) (2). The N-terminal domain of the TIMP molecule competitively binds to the active site on the MMP enzyme to form a 1:1 non-covalent complex that abolishes MMP activity. TIMP-2 is the major TIMP expressed by fibroblasts, macrophages, and endothelial cells and it potently inhibits most MMP (2, 6, 30). In the healthy joint, balanced MMP and TIMP activities are probably important for normal turnover and degradation of cartilage matrix. Imbalances between MMP and TIMP expression may be a key event in the transition of MMP from physiological to pathological processes (5, 14, 17, 19, 30). Cartilage from humans affected with OA has increased MMP expression without any corresponding elevations in TIMP expression. Under these conditions, it is hypothesized that unregulated MMP activity may lead to excessive matrix degradation. (7). The possibility that suppression of abnormal MMP activity might alleviate OA joint pathology has prompted research into the development of selective MMP inhibitors for the treatment of human OA (1).

Classical cytological indicators of inflammation are absent in OA. However, there is ample evidence of inflammation in this disease process, such as the presence of inflammatory mediators and activation of the JAK-STAT pathway that involves the Janus kinases (JAK) and their downstream signal transduction, and activation of transcription (STAT) activities (19, 32). Inter-
feron-alpha is a glycoprotein with immunomodulatory, antiproliferative, and antiviral properties that helps to maintain the delicate balance of the immune system, and plays a pivotal role in modulating the inflammatory response and cellular apoptosis (25, 27, 29). Interferon alpha binds to and activates its receptor, which subsequently activates proteins in the JAK-STAT signal transduction pathway. Proinflammatory cytokines bind and activate membrane receptors that in turn activate intracellular kinases, leading to a cascade of intracellular phosphorylation events. While the complete role of interferon-alpha has yet to be elucidated, it is believed that a signalling cascade, which involves the activity of a protein-tyrosine phosphatase, may play an important role in promoting IFN-alpha-induced apoptosis (10). Protein phosphorylation has multiple cellular effects including transcription activation of genes that encode inflammatory proteins, such as adhesion molecules, tumour necrosis factor alpha, interleukin 1B, and iNOS (32). Reversible phosphorylation of certain protein residues, especially tyrosine, is a major regulatory step in many cytosolic signalling events, such as cellular growth, migration, and survival, and abnormal tyrosine phosphorylation states have been linked with many human diseases including cancer, diabetes, hypertension, and cardiac hypertrophy (16). The role of phosphorylation in OA is currently not understood. Identification of the proteins that are phosphorylated in patients with OA will provide opportunities to better understand the key signalling signal-transduction pathways involved in the pathogenesis of OA and identify additional therapeutic agents.

In order to better understand the pathogenic mechanisms of OA and to identify possible biomarkers for early OA, we determined MMP-1 and TIMP-2 concentrations and the presence of phosphoproteins in the SF and serum of normal dogs and dogs with OA. We hypothesized that there are differences in MMP-1 and TIMP-2 concentrations in the SF and serum of dogs with OA when compared to normal dogs, and that phosphoproteins in the SF may be useful biomarkers for canine OA.

### Materials and methods

#### Sample population

Client-owned dogs that were patients of the Kansas State University Veterinary Medical Teaching Hospital were recruited for the study. The Institutional Animal Care and Use Committee approved this research protocol and informed owner consent was obtained prior to participation in this study. The dogs participating in the study had not been treated with any non-steroidal anti-inflammatory drugs or slow-acting disease-modifying agents (e.g., chondroitin sulphate, hyaluronic acid, polysulphated glycosaminoglycan) for a minimum period of two weeks or 30 days, respectively, prior to inclusion in the study. Nine dogs that were found to be completely normal by physical and orthopaedic examination, were enrolled in the control group. Radiographs of one randomly chosen stifte obtained after sedation with hydromorphone (0.08 mg/kg IV) and acepromazine (0.02 mg/kg) were examined to ensure normality. Blood was collected via venipuncture for serum samples and then SF was aseptically aspirated from the designated joint using a 22-gauge needle and a 3 ml syringe. Nine client-owned dogs that were present for surgical repair of a ruptured cranial cruciate ligament (CCL) were enrolled in the OA group. On physical examination, each dog had evidence of CCL instability, and had radiographic evidence of joint effusion and OA. While the dogs were under general anaesthesia and were being prepared for CCL surgery, serum and SF samples from the OA stifte were collected using the same technique as for the control group. Serum and SF samples of at least 2,000 µL and 500 µL, respectively, were considered acceptable for use in the study. The SF samples were diluted 1:10 in sterile water to aid in ease of sample handling. Serum and SF from both groups of dogs were centrifuged at 4°C for five minutes at 5,000 rpm, treated with 1 µl protease inhibitor per 1000 µl sample, and frozen at -80°C until processing.

#### Protein concentration

Total protein concentrations of SF and serum samples were measured spectrophotometrically with a commercially available micro BCA protein assay reagent kit using bovine serum albumin as the standard.

#### MMP-1 detection

The SF and serum samples containing equivalent amounts (2 µg) of total protein were incubated in Laemmeli sample buffer (LSB) with B-mercaptoethanol, heated to 90°C for five minutes and subjected to SDS polyacrylamide gel electrophoresis on 4 – 20% tris-glycine gradient gels. A sample of human MMP-1 protein (3 µl) was loaded in LSB, heated to 90°C for five minutes and run on each gel as a control. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane by electrophoretic transfer in a tank system with plate electrodes. The membranes were blocked for one hour at 25°C in 5% non-fat dried milk dissolved in TBS-Tween, and then incubated with mouse monoclonal MMP-1 antibody in 5% non-fat dried milk dissolved in TBS-Tween in a 1:1,000 volume-to-volume dilution. After incubation, membranes were washed four times with 0.1% TBS-Tween, and then incubated for one hour at room temperature in a horseradish peroxidase-labeled secondary antibody diluted 1:10,000 in TBS with 0.1% Tween. After four washes with TBS-Tween, bands of immunoreactivity were developed by addition of Pico chemiluminescent substrate and exposed to radiographic film.

#### TIMP-2 detection

The SF and serum samples were assayed for free TIMP-2 and TIMP-2 complexed with active forms of MMP by means of a commercially available TIMP-2 Biotrack Assay kit. Briefly, 100 µl of prepared standard and
100 µl samples containing 600 µg total protein were incubated at 25°C for one hour with 100 µl of anti-TIMP-2 peroxidase conjugate. Wells were aspirated, washed and then incubated at 25°C for 30 minutes with 100 µl of tetramethylbenzidine hydrogen peroxide. This reaction was stopped by the addition of 1.0 M sulphuric acid, and optical density was measured at 450 nm. The sample concentrations were determined from a standard curve and were reported in ng/ml.

**Phosphotyrosine immunoprecipitation**

Synovial fluid and serum samples containing 200 µg total protein were incubated with immobilized protein G. The samples were then centrifuged at 10,000 rpm for five minutes at 4°C and the pellets were discarded. Next, mouse monoclonal phosphotyrosine antibody was added to the sample at a 1:100 dilution. The samples and antibody were rocked for 18 hours at 4°C. Immobilized protein G was added at a 1:10 dilution and rocked for 2 hours at 4°C. The samples were then centrifuged at 10,000 rpm for five minutes and the supernatant was discarded. The pellet was washed three times in TBS-Tween at 4°C. The LSB was added in a 1:1 dilution and allowed to sit at room temperature for 30 minutes. The samples were centrifuged at 10,000 rpm for five minutes and the pellet was discarded. The supernatant was heated to 90°C for five minutes and loaded on a 4–20% tris-glycine gradient gel and subjected to SDS polyacrylamide gel electrophoresis. Proteosilver was used to stain the gels for protein band detection. Bands were detected at 20, 50, and 75 kDa. Each band was excised. Gel plugs were enzymatically digested using trypsin and the subsequent peptides identified using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). A National Center for Biotechnology Information database search was performed using the ProFound search engine by entering the tryptic digest products. The quality criteria encompassed a protein mass range of 15 to 20 kDa, a protein isolectric point range of 2 to 14, a charge state of MH+, and a monoisotopic mass tolerance of 100 ppm.

**Western blot analysis for Interferon-alpha and phosphotyrosine**

In order to confirm the presence of interferon-alpha in the samples and to determine if the interferon was tyrosine-phosphorylated, an interferon immunoprecipitation was performed. Synovial fluid samples from two dogs with ruptured CCL, each containing 200 µg total protein, were incubated with immobilized protein G. The samples were then centrifuged at 10,000 rpm for five minutes at 4°C and the pellets were discarded. Next, human polyclonal interferon-alpha antibody was added to the samples at a 1:100 dilution. The samples and antibody were rocked for 18 hours at 4°C. Immobilized protein G was added at a 1:10 dilution and rocked for two hours at 4°C. The samples were then centrifuged at 10,000 rpm for five minutes and the supernatant was discarded. The pellets were washed three times in TBS-Tween at 4°C. LSB was added in a 1:1 dilution and allowed to sit at room temperature for 30 minutes. The samples were centrifuged at 10,000 rpm for five minutes and the pellets were discarded. The supernatants were heated to 90°C for five minutes and loaded in two lanes of a 4–20% tris-glycine gradient gel and subjected to SDS polyacrylamide gel electrophoresis. Proteins were then transferred to a PVDF membrane by electrophoretic transfer in a tank system with plate electrodes. Membranes were blocked for one hour at 25°C in 5% non-fat dried milk dissolved in TBS-Tween. One lane was separated and then incubated with primary polyclonal antibody raised against interferon-alpha of human origin in a 1:1,000 volume-to-volume dilution. The other lane was incubated with mouse monoclonal phosphotyrosine antibody in a 1:1,000 volume-to-volume dilution. After incubation, membranes were washed four times with 0.1% TBS-Tween, and then incubated for one hour in a horseradish peroxidase-labelled secondary antibody diluted 1:10,000 in TBS with 0.1% Tween. After four washes with TBS-Tween, bands of immunoreactivity were developed by addition of Pico chemiluminescent substrate and exposed to radiographic film.

**Data analysis**

Western blots were scanned using digital imaging and the relative intensity of bands was determined by densitometry using image analysis software. The values obtained were normalized to a maximum value of 1.0. Data for the MMP-1 concentrations in serum and SF were not normally distributed and therefore were compared using independent group T-Test analysis. Data for the TIMP-2 concentrations in serum and SF were normally distributed and therefore were compared using the Mann-Whitney U Test. All of the values are reported as mean ± standard deviation. Significance was defined as P < 0.05.

**Results**

There were four castrated males and five spayed females in the control group. Breeds represented were mixed breed (n=4), Labrador retriever (n=2), boxer (n=1), German shorthair pointer (n=1), and Brittany spaniel (n=1). The mean age and weight of dogs in the control group were 5.4 years (range, 3 to 6.5 years) and 28.5 kg (range, 19 to 35 kg), respectively. There were three castrated males, five spayed females, and one intact male in the osteoarthritic group. The breeds represented were Labrador Retriever (n=3), Mixed Breed (n=3), Rotweiller (n=1), Golden Retriever (n=1), and Newfoundland (n=1). The mean age and weight of osteoarthritic dogs were 4.9 years (range, 3–6 years) and 32.4 kg (range, 22 to 42 kg). Subsequent to collection of samples in the OA...
group, CCL rupture was confirmed by arthrotomy in all of the dogs.

**Protein concentrations**

The total protein concentrations in SF in osteoarthritic dogs (16.9 ± 6.4 µg/µl) and in control dogs (12.7 ± 6.4 µg/µl) were not significantly different (P=0.099). Similarly, the total protein concentrations in serum in osteoarthritic dogs (19.3 ± 2.9 µg/µl) and in control dogs (20.8 ± 1.7 µg/µl) were not significantly different (P = 0.089).

**MMP-1**

The concentration of MMP-1 in the SF of osteoarthritic dogs (0.62±0.16) was significantly higher than that found in the control dogs (0.42±0.19) (P=0.03) (Figs. 1, 2). The concentration of MMP-1 in the serum of osteoarthritic dogs (0.74±0.16) was significantly lower than that measured in the control dogs (0.87±0.08) (P=0.05) (Figs. 2, 3).

**TIMP-2**

The concentration of TIMP-2 in the SF of osteoarthritic dogs (46.2 ± 21.9 ng/ml) was significantly lower than that measured in the control dogs (122.0 ± 66.5 ng/ml) (P = 0.042) (Fig. 4). The concentration of TIMP-2 in the serum of osteoarthritic dogs (116.2 ± 43.1 ng/ml) and control dogs (95.1 ± 94.4 ng/ml) were not significantly different (P = 0.337) (Fig. 4).
Distinct bands were evident at 20, 50 and 75 kDa on gels loaded with SF immunoprecipitated with phosphotyrosine antibody (Fig. 5). The bands were excised and subjected to MALDI-TOF MS and a series of peptide masses were determined for each protein. The protein at 75 kDa could not be identified due to small quantity. A database search was performed using these masses. The protein at 50 kDa was most consistent with an immunoglobulin and good homology between the protein at 20 kDa and bovine interferon was found with 20% sequence overlap (Fig. 5). Western blotting with the interferon alpha antibody was performed on the immunoprecipitated SF samples to confirm the presence of interferon-alpha in the sample and determine if it was tyrosine phosphorylated. Bands of immunoreactivity were detected at 20 kDa for the SF samples. Therefore, western blots revealed the presence of INF-alpha in SF and serum of dogs with OA and control dogs, but when the band detected at 20 kDa was probed with the phosphotyrosine antibody, western blotting failed to demonstrate that the interferon was phosphorylated at the tyrosine residue (Fig. 6).

### Discussion

The current study sought to determine if identifiable differences could be detected in the concentrations of MMP-1 and TIMP-2 between normal dogs and dogs with OA for potential diagnostic, prognostic, and therapeutic purposes. Utilizing western blotting and ELISA techniques, we evaluated MMP-1 and TIMP-2 concentrations, respectively, in the SF and serum of dogs with and without stifle OA. We identified elevated MMP-1 concentrations and reduced TIMP-2 concentrations in SF and decreased MMP-1 concentrations in serum in dogs with OA, compared to dogs devoid of OA. This study supports previous reports that documented cartilage degradation in OA occurring in conjunction with an imbalance of MMP and TIMP (5, 17, 23). In both dogs and humans, increased cytokine activity has been associated with increased amounts of both MMP and TIMP in SF and cartilage, but the relative ratio of MMP:TIMP was found to be reduced (7, 13, 17, 20). One in vitro investigation suggested that the increased MMP:TIMP ratio occurs in human rheumatoid arthritis because inflammatory cytokines may have little to no effect on production of TIMP while strongly increasing...
MMP production by synovial fibroblasts (18). Another study found decreased synthesis of TIMP-1 and TIMP-2 in cartilage explants of humans with OA while MMP production was increased (19).

A reduction in serum MMP-1 in dogs with OA was unexpected. Investigators working in an in vitro system reported an increase in the production of MMP-1 by OA cartilage at both the mRNA and the protein level, and it was suspected that an over-expression of an unidentified serum factor was responsible for enhancing MMP-1 gene expression (24). As we found that MMP-1 concentrations were increased in SF, but decreased in serum of osteoarthritic dogs, it may be postulated that MMP-1 was leaving the systemic circulation and entering the SF. However, there are not any reports available of data derived from canine or other species that support this theory, and the current belief is that the MMP-1 in SF is produced locally by synoviocytes and chondrocytes (2). Our findings support the use of MMP-1 in serum as a possible biomarker for OA in the dog, but it is currently unknown if the decrease in serum MMP-1 is specific for OA.

Western blotting as performed in this study was not strictly quantitative. Densitometry is a semi-quantitative analysis that allowed us to detect differences in the amount of MMP-1 present relative to the total amount of protein in the sample. The antibody used to detect MMP-1 recognizes both the active and inactive forms of the protein. It specifically binds to MMP-1 and does not cross-react with other MMP family members. Although the canine cross-reactivity of the antibody used in this study to detect MMP-1 has not been investigated, there is excellent sequence homology between species with cross-reactivity for human, mouse, rat, rabbit, and porcine MMP-1 (4). An ELISA that recognizes total MMP-1, free MMP-1 and that complexed with inhibitors is available. This assay would have allowed for MMP-1 quantification. Unfortunately, this MMP-1 assay is species specific and does not cross-react with canine MMP-1 (Salinardi, B. Unpublished data).

The ELISA used in this study to determine TIMP-2 concentrations recognizes both free TIMP-2 and that complexed with the active form of MMP. The immunoreactivities of the assay for free TIMP-2 and TIMP-2 complexed with active MMP-1 are 100% and 330%, respectively. This ELISA does not recognize TIMP-1 or TIMP-3, therefore the cross-reactivity towards other TIMP is very low. The ELISA used for TIMP-2 is capable of quantifying TIMP-2 with a sensitivity defined as two standard deviations above the mean optical density of 20 zero standard replicates, 3.0 ng/mL. The TIMP-2 peptide epitopes used to raise antibodies for the ELISA are conserved across species. Therefore, the assay should recognize TIMP-2 from all species, including the human, mouse, rat, dog, guinea pig, and cow (4, 6).

Several sources of variability exist when performing a study of this nature. The chronicity and severity of the disease could not be standardized in these client-owned animals as it could have been in experimental animals. Although all of the animals in this study were affected with CCL rupture and stifie OA, they had varying degrees of arthritic changes radiographically. As radiographic changes are considered end-stage changes of OA, we can not come to a conclusion about the value of MMP-1 and TIMP-2 in the diagnosis of early OA. It is also difficult to control the medications administered to treat osteoarthritis, prior to sample collection. To our knowledge, prior to enrollment in the study, non-steroidal anti-inflammatory drugs or slow-acting disease-modifying agents had not been administered to any of the dogs for two weeks or 30 days respectively. Also, the animals were adult and within a narrow age range to alleviate any potential effect of age on MMP concentrations. In humans, it has been shown that serum concentrations of MMP-3, but not MMP-1 are significantly increased with age (19).

Although MMP and TIMP concentrations in SF indicate the presence of OA in a joint, an understanding of these changes is critical to our comprehension of the pathogenesis of OA. In order to address some of the possible underlying mechanisms of OA, we examined SF for the presence of phosphorylated proteins. Stimulation of protein phosphorylation is one mechanism by which cytokines produce an inflammatory effect. In this study, we determined the complement of tyrosine-phosphorylated proteins in the SF using an anti-phosphotyrosine antibody. The antibody immunoprecipitated three proteins but only two were able to be identified. One protein was canine immunoglobulin G, which was not unexpected given the inflammatory changes found in SF from dogs with OA. However, the finding that canine INF-alpha was immunoprecipitated by the anti-phosphotyrosine antibody was unexpected. The amino acid sequence of canine INF-alpha (gi:47076420) is known and a comparison with the human sequence revealed an interesting difference. Canine INF-alpha contains three tyrosine residues and one of these is predicted to be subject to phosphorylation. However, subsequent experiments failed to demonstrate INF-alpha phosphorylation under these conditions, prompting the hypothesis that canine INF-alpha co-immunoprecipitates with a Tyr phosphoprotein in the SF, perhaps the 75-kD protein (p75) identified in our P-Tyr immunoprecipitation experiments.

In this study we found significant differences between control and OA dogs in the serum and SF concentrations of MMP-1 and TIMP-2. It would be ideal if a pattern could be identified from this data that would allow detection of canine OA by serum or SF assays. However, despite statistical significance, there was considerable overlap between groups. Nevertheless, the results of this study broaden our knowledge of MMP and TIMP in osteoarthritic dogs and suggest the necessity for further studies to explore MMP targeting as therapy for canine OA. This study also documented the presence of non-tyrosine-phosphorylated interferon-alpha in canine SF which appeared to be associated with a protein that is phosphorylated at the tyrosine residue. More detailed knowledge of the modulating factors, as well as the major regulators involved in OA, has and will continue to generate new insights into OA pathophysiology and may aid in the accurate identification of effective therapeutic targets in the treatment of OA.
References


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