

Increased levels of the 14-3-3 η and γ proteins in the synovial fluid of dogs with unilateral cranial cruciate ligament rupture

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Abstract

The present study investigated whether the 14-3-3 η and γ proteins, which are potent matrix metalloproteinase (MMP) stimulators, are detectable in the synovial fluid of dogs with cranial cruciate ligament rupture (CCLR). Synovial fluid samples from 7 dogs with unilateral CCLR and control samples from 4 dogs without a history of any joint inflammation or any other abnormalities underwent Western blot analysis for the 14-3-3 η , γ , and σ proteins as well as MMP-1 and MMP-3. Craniocaudal and lateral radiographic projections of the stifle joint were evaluated for the presence and severity of 13 specific radiographic markers of osteoarthritis and graded numerically. The Spearman method was used to detect any correlation between the 14-3-3- η level in the synovial fluid and the radiograph-based grade. The η isoform was present only in the samples from the dogs with CCLR. The levels of 14-3-3- γ , MMP-1, and MMP-3 were significantly higher in the samples from the dogs with CCLR than in the control samples ($P < 0.05$). However, there was no significant difference between the CCLR and control samples in the level of the σ isoform. The Spearman method showed a significant correlation between the 14-3-3- η level in the synovial fluid and the presence of either patellar osteophytes or lateral or medial (or both) condylar periarticular osteophytes ($P < 0.05$). The MMP stimulatory effect of the 14-3-3 η and γ isoforms may be the reason for the high levels of MMP-1 and MMP-3 observed. Thus, 14-3-3 proteins, especially the η isoform, may be important markers of osteoarthritis caused by CCLR.

Résumé

La présente étude visait à déterminer si les protéines 14-3-3 η et γ , qui sont de puissants stimulateurs des matrices de métalloprotéases (MMP), sont détectables dans le liquide synovial de chiens souffrant d'une rupture du ligament croisé cranial (CCLR). Des échantillons de liquide synovial provenant de 7 chiens avec un CCLR unilatéral et des échantillons témoins de 4 chiens sans histoire d'inflammation articulaire ou autre anomalie ont été analysés par immunobuvardage pour les protéines 14-3-3 η , γ et σ aussi bien que MMP-1 et MMP-3. Des radiographies cranio-caudales et latérales du grasset ont été évaluées pour la présence et la sévérité de 13 marqueurs radiographiques spécifiques d'ostéoartrite et notée numériquement. La méthode de Spearman a été utilisée pour détecter toute corrélation entre le niveau de 14-3-3- η dans le liquide synovial et le pointage basé sur la radiographie. L'isoforme η était présent seulement dans les échantillons provenant de chiens avec CCLR. Les niveaux de 14-3-3- γ , MMP-1 et MMP-3 étaient significativement plus élevés dans les échantillons provenant de chiens avec CCLR comparativement aux chiens témoins ($P < 0,05$). Toutefois, il n'y avait pas de différence significative entre les échantillons provenant des chiens CCLR et les animaux témoins pour l'isoforme σ . La méthode de Spearman a montré une corrélation significative entre le niveau de 14-3-3- η dans le liquide synovial et la présence de, soit, des ostéophytes patellaires ou d'ostéophytes péri-articulaires du condyle latéral ou médial (ou les deux) ($P < 0,05$). L'effet stimulant des isoformes MMP et des isoformes 14-3-3- η et γ pourrait être la raison pour les niveaux supérieurs observés de MMP-1 et MMP-3. Ainsi, les protéines 14-3-3, plus spécialement l'isoforme η , pourraient être des marqueurs importants de l'ostéoartrite causée par CCLR.

(Traduit par Docteur Serge Messier)

Introduction

The cranial cruciate ligament (CCL) restricts cranial translation of the tibia on the femur. The CCL also resists overextension and inward rotation, stabilizing the femur on the tibia. Rupture of the CCL in the stifle joint (knee) results in partial or complete joint instability, pain, and lameness. Torn ligaments retract and do not heal. If the injury is not treated, damage to connective tissues often

results in degenerative joint disease. Cranial cruciate ligament rupture (CCLR) is one of the most common orthopedic injuries in dogs and is the most common cause of degenerative disease in the stifle joint (1–3). Gross instability results from complete rupture and minor instability from partial rupture (4). Untreated animals will show some degenerative change in the affected joint within a few weeks and can have severe degenerative changes within a few months (4). Osteoarthritis, a disease prevalent in both humans and

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dogs, is common with CCLR (4) and is progressive, as mechanical abnormalities result in degradation of the joint, including the articular cartilage and subchondral bone (1,3).

The pathogenesis of CCLR is not completely understood, and there are no effective methods for preventing the subsequent degeneration or slowing its progress (3,4). Most of the research into the etiopathogenesis of CCLR has focused on identifying factors that might lead to structural failure of the ligament (3). The concentrations of glycoaminoglycans and matrix metalloproteinases (MMPs) have been shown to be significantly increased in the joint fluid of dogs with CCLR (1,5).

Fibroblast-like synoviocytes (FLS) are present in the capsule, cartilage, CCL, and other tissues of the joint. The FLS, which account for about two-thirds of the synovium population, have a well-defined secretory system and express many degradative enzymes, including MMPs, cathepsins, and tartrate-resistant acid phosphatase (3,6–8). The MMPs, specifically MMP-1, 3, 8, 9, 10, 11, and 13, play an important role in tissue remodeling (3,9–11). They can be inactivated by specific inhibitors, such as tissue inhibitor of metalloproteinases (TIMP); TIMP-1, -2, -3, and -4 are present in most body fluids (3). Although the etiopathogenesis of CCLR in dogs is not well understood, large amounts of MMPs in the synovial fluid have been documented (3,6). An imbalance in the activation and inhibition of MMPs may be key to a shift in the MMP cascade from physiologic to pathologic (3,12): MMPs can accumulate in the tissues and fluid and play a prominent role in the degradation of tendons, ligaments, and articular cartilage (3,12–14). Numerous researchers have shown that MMP-1 and MMP-3 play an important role in joint damage (15), and both are used as biomarkers owing to their predictive validity for structural damage in arthritis (16). A significant increase in MMP-1 activity has been shown in experimental CCL transection in rabbits and sheep (3,17–20). Furthermore, a significant increase in MMP-1 activity and a decrease in TIMP-1 activity in humans and dogs with CCL injury have been reported (3,21,22). The main source of MMPs in synovial fluid is FLS. Some classic proinflammatory cytokines, such as interleukin-1 and tumor necrosis factor- α , have been shown to stimulate the synthesis of MMP-1 and stromelysins in FLS, synovial fibroblasts, and chondrocytes in vitro (23–25).

The 14-3-3 proteins are an abundant family of acidic dimeric molecules that have a wide range of functions. There are 7 known mammalian isoforms: β , γ , ϵ , η , σ , τ , and ξ (26). Since the discovery of the first 14-3-3 protein in 1967, members of this family have been repeatedly discovered on the basis of their biologic activities, primarily in signal transduction pathways (27). They have been identified as activators of tryptophan and tyrosine hydroxylase and protein kinase C (PKC) (28,29). Subsequent studies identified the 14-3-3 proteins as molecules that interact with PKC, Raf family members, and now more than 200 other intracellular proteins with critical biologic functions, including cellular response to DNA damage and cell cycle regulation (30–33). The finding in our laboratory that MMP-1 has a stimulatory effect on 14-3-3 for fibroblasts was the first indication of a relevant extracellular biologic function for this important family of proteins (25). Recently our laboratory found higher levels of 2 specific isoforms of 14-3-3 proteins (η and γ) in synovial fluid from a human patient with joint inflammation (9). These findings suggest that these proteins in serum and synovial fluid can be used as biomarkers for inflamed joints.

The present study asked whether 14-3-3 proteins are detectable in synovial fluid from other mammalian species, whether these proteins play any role in the pathogenesis of CCLR in dogs, and whether there is a difference in the levels of specific 14-3-3 isoforms in the synovial fluid of normal dogs versus those with CCLR.

Materials and methods

Animals and experimental set-up

The dogs included in this study were scheduled to undergo a tibial plateau leveling osteotomy for unilateral CCLR. They were between 3 and 7 y old (mean \pm standard deviation, 5 ± 2). Control samples had been collected from 4 dogs that had no history of any joint inflammation or any other abnormalities and were undergoing euthanasia owing to accidental trauma. After informed consent was obtained, synovial fluid was collected with a 23-gauge syringe, clarified by centrifugation at $1500 \times g$ for 15 min, and stored in aliquots at -80°C for subsequent analysis. The samples were analyzed for the 14-3-3 η , γ , and σ proteins as well as MMP-1 and MMP-3 with the use of Western blotting. In parallel, blood samples were collected to determine the level of 14-3-3- η in the serum. Serum was separated from blood by centrifugation at $1500 \times g$ for 10 min, and stored in aliquots at -80°C . The University of British Columbia Ethics Committee approved the study.

Radiographic evaluation

The presence and severity of 13 specific radiographic markers of osteoarthritis were evaluated on craniocaudal and lateral radiograph images. The markers included patellar osteophytes, femoral trochlear groove periarticular osteophytes, lateral or medial (or both) condylar periarticular osteophytes, femoral subchondral sclerosis, distal femoral condylar remodeling, distal femoral subchondral cystic lucencies, femoral intercondylar notch osteophytes, proximal tibial periarticular osteophytes, proximal tibial subchondral sclerosis, proximal tibial subchondral cystic lucencies, central tibial plateau osteophytes, joint effusion or capsular thickening, and meniscal mineralization. Each marker was graded individually as 0, 1, 2, or 3, respectively, for absent, mild, moderate, or severe radiographic change (34).

Antibodies

Antibodies raised in rabbit against human 14-3-3- η , - γ , and - σ were generously provided by Dr. Alastair Aitken, School of Biomedical and Clinical Sciences, University of Edinburgh, Edinburgh, Scotland, and monoclonal antibodies against human MMP-1 and MMP-3 were purchased from R&D Systems, Minneapolis, Minnesota, USA. Secondary antibodies against mouse and rabbit IgG conjugated with horseradish peroxidase (HRP) were obtained from Bio-Rad Laboratories, Hercules, California, USA.

To verify if the antibodies against human 14-3-3- η , - γ , and - σ and against human MMP-1 and MMP-3 were able to detect these proteins in dogs, we tested synovial fluid samples from 3 of the dogs with CCLR, chosen at random, along with 3 different types of samples from humans as positive control: a keratinocyte lysate as a positive control for 14-3-3 proteins; a dermal fibroblast lysate as a positive

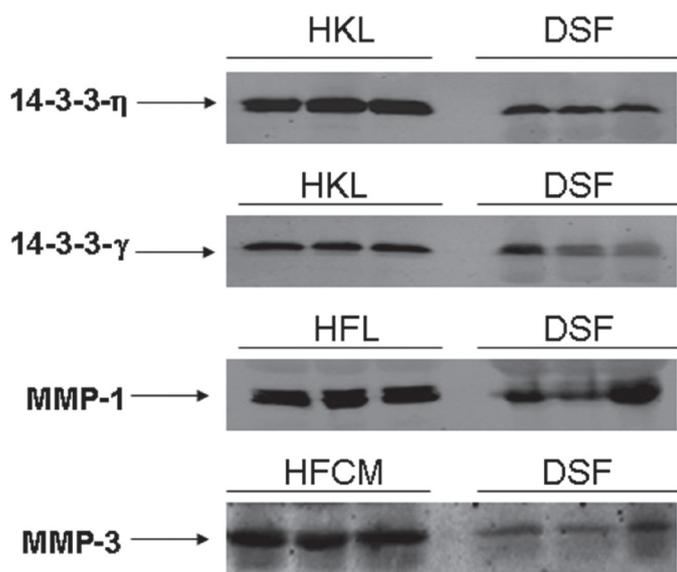


Figure 1. Detection by Western blot analysis with human antibodies of canine 14-3-3 and matrix metalloprotease (MMP) proteins in synovial fluid samples from 3 randomly chosen dogs with cranial cruciate ligament rupture (CCLR). Positive controls were as follows: for 14-3-3 proteins, a human keratinocyte lysate (HKL); for MMP-1, a human dermal fibroblast lysate (HFL); and for MMP-3, human fibroblast conditioned medium (HFCM). DSF — dog synovial fluid.

control for MMP-1; and fibroblast conditioned medium as a positive control for MMP-3. The first 2 lysates were established in our laboratory (9). The fibroblast conditioned medium was collected from the cultured fibroblasts established in our laboratory. Each experiment was done in triplicate with 3 different cell strains.

Western blot analysis

A 5- μ L aliquot from each sample of synovial fluid was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE); 12% (wt/vol) acrylamide gel was used for the 14-3-3 proteins and 10% gel for the MMPs. The separated proteins were electrotransferred onto polyvinylidene fluoride membranes (Millipore, Bedford, Massachusetts, USA). Nonspecific proteins on the membranes were blocked in 5% skim milk powder in phosphate-buffered saline (PBS) with 0.05% Tween 20 overnight at 4°C. Immunoblotting was performed with use of the rabbit antibodies against human 14-3-3- η , - γ , and - σ (1:1000 dilution) and the monoclonal antibodies against human MMP-1 and MMP-3 (1:250 dilution). The membranes were then incubated with the appropriate HRP-conjugated secondary antibodies (1:2500 dilution). Immunoreactive proteins were visualized with the Western blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, California, USA). The positive controls were those described above. Ponceau-S staining was used for all membranes to ensure that the loading control was even (images not shown).

Detection of 14-3-3- η in serum versus synovial fluid

To determine whether the 14-3-3- η level in synovial fluid reflects the level in serum from the same dogs, the synovial fluid samples from the 7 dogs with CCLR were pooled, as were the samples

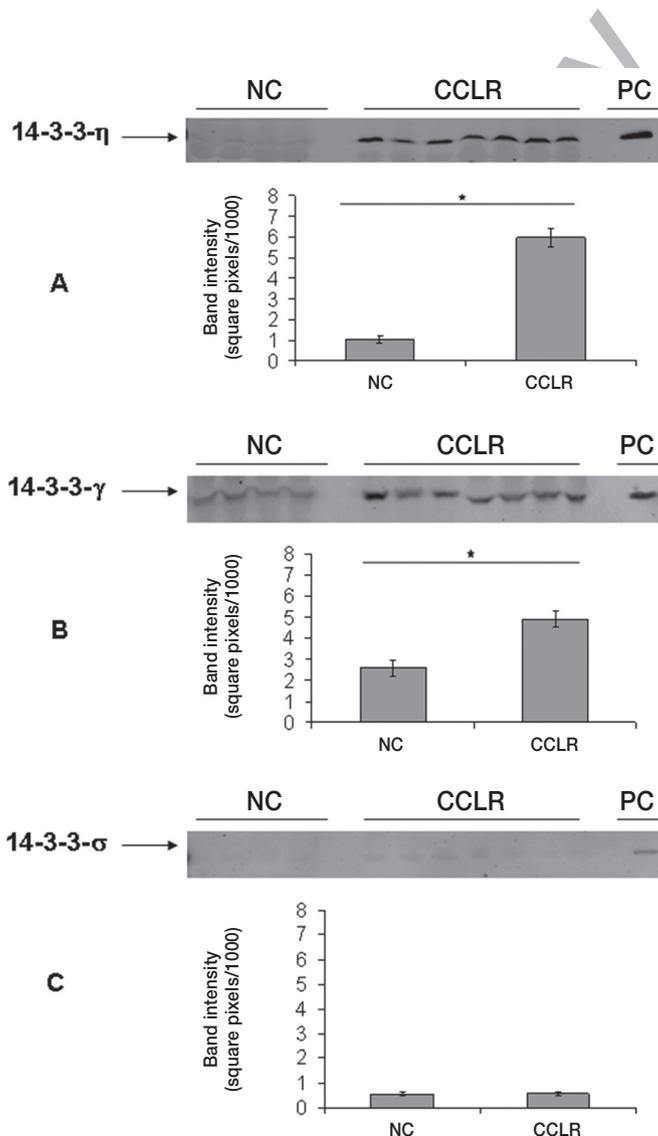


Figure 2. Presence and levels of 14-3-3- η , - γ , and - σ , as determined by Western blot analysis, in the synovial fluid of 4 dogs without a history of joint inflammation or other abnormalities [negative controls (NC)] and 7 dogs with CCLR. The intensity of the bands for each protein was quantified by densitometry. PC — positive control. The asterisks indicate a significant difference between the mean values; the bars indicate the standard deviation.

from the 4 control dogs. Serum from the blood samples collected in parallel were also pooled. The synovial fluid or serum samples were loaded at various volumes (1, 2.5, and 5 μ L per lane) on an SDS-PAGE gel and subjected to Western blot analysis with use of the 14-3-3- η antibody.

Statistical analysis

The levels of the 14-3-3 and MMP proteins were quantified by densitometry. Statistical analysis was performed with the SPSS 11 program for Windows (SPSS, Chicago, Illinois, USA). The means of the groups were compared with Student's *t*-test. For detection of any correlation between the 14-3-3- η level and the radiograph-based scoring system parameters, the Spearman method was used. Differences were considered statistically significant when the *P*-value was < 0.05.

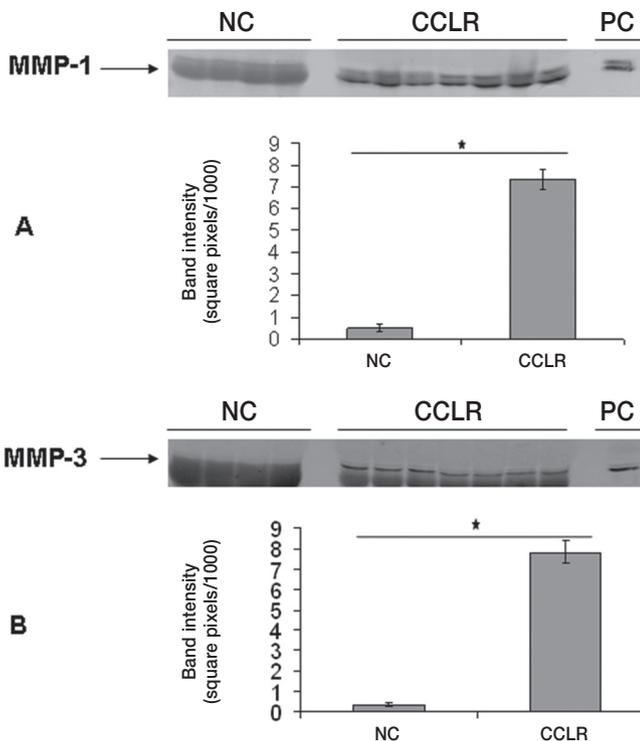


Figure 3. Presence and levels of MMP-1 and MMP-3, as determined by Western blot analysis, in the synovial fluid of the control dogs and the dogs with CCLR.

Results

Western blot analysis with human antibodies against 14-3-3- η and - γ and against MMP-1 and MMP-3 detected the corresponding canine proteins in synovial fluid samples from a randomly chosen subset of the dogs with CCLR (Figure 1) and thus confirmed that the human antibodies could be used for the experiments.

Subsequent Western blot analysis after SDS-PAGE showed that 14-3-3- η was present only in the synovial fluid samples from the dogs with CCLR and not the samples from the control dogs ($P < 0.05$) (Figure 2A). Compared with the control dogs, the dogs with CCLR had significantly more 14-3-3- γ ($P < 0.05$) in their synovial fluid (Figure 2B). Interestingly, 14-3-3- σ was present in extremely low levels in the synovial fluid of both groups of dogs (Figure 2C).

Owing to previous studies (9,35) showing that some 14-3-3 isoforms stimulate MMP-1 and MMP-3 expression, the presence of these proteins was also sought by Western blot analysis. The levels of both MMP-1 and MMP-3 were found to be significantly greater in the synovial fluid samples from the dogs with CCLR than in the samples from the controls ($P < 0.05$) (Figure 3).

As shown in Figure 4, the levels of 14-3-3- η were significantly greater in the pooled samples of synovial fluid than in the pooled samples of serum ($P < 0.05$) from the dogs with CCLR.

Representative lateral and craniocaudal plain film radiographs of the affected stifle from the dogs with CCLR were taken, and are shown in Figure 5. The Spearman method detected statistically significant correlations ($P < 0.05$) between the 14-3-3- η level and

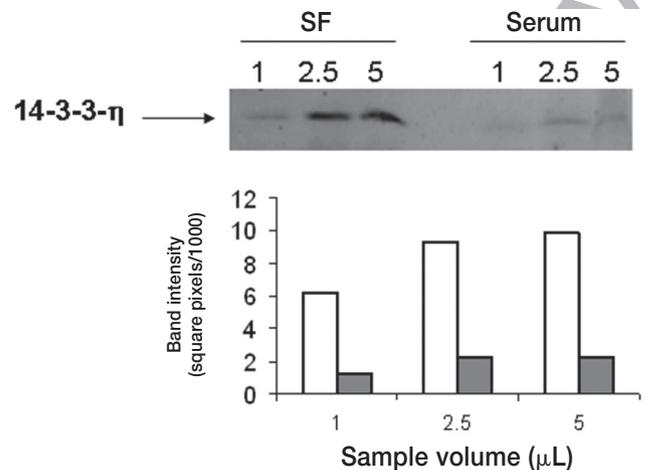


Figure 4. Presence and levels of 14-3-3- η in pooled synovial fluid (SF; white bars) and serum (grey bars) from the dogs with CCLR. Each lane contained a different volume of pooled SF or serum. The SF values are significantly greater than the serum values ($P < 0.05$).

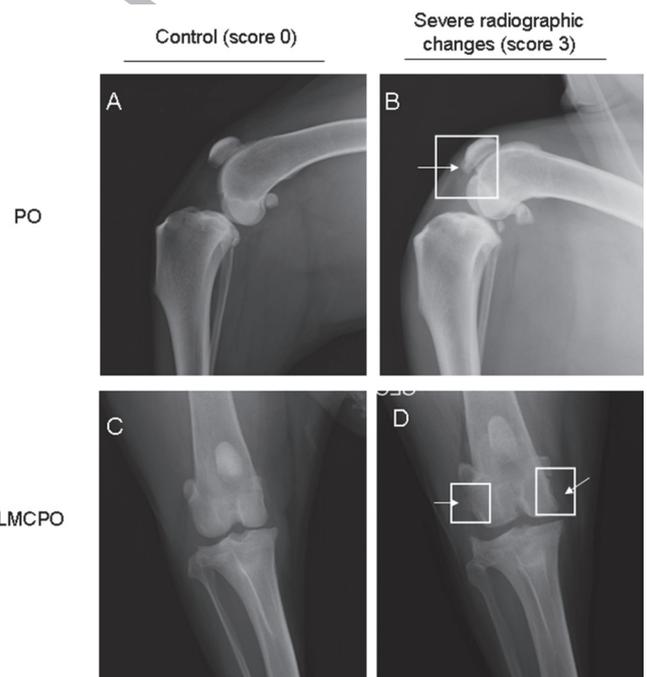


Figure 5. Lateral (A and B) and craniocaudal (C and D) radiographs from the stifle joint of dogs with (CCLR). A and B were graded as 0 and 3, respectively, according to the presence and severity of patellar osteophytes (PO; arrow). C and D were graded as 0 and 3, respectively, according to the presence and severity of lateral or medial (or both) condylar periarticular osteophytes (LMCPO; arrows).

patellar osteophytes (Table I and Figure 5B) and lateral or medial (or both) condylar periarticular osteophytes (Table I and Figure 5D).

Discussion

Our previous study showed that 2 specific isoforms of 14-3-3 proteins (η and γ) are detectable in the synovial fluid of human patients with inflamed joints (9). There was a significant correlation between the quantities of these proteins and those of MMP-1 and MMP-3 (9). As there are no commercially available antibodies

Table I. Correlation between the 14-3-3- η protein level in the synovial fluid of 7 dogs with cranial cruciate ligament rupture and radiographic markers of osteoarthritis

Radiographic marker	Spearman correlation coefficient	P-value
Patellar osteophytes	0.898	0.006*
Femoral trochlear groove periarticular osteophytes	0.599	0.155
Lateral or medial (or both) condylar periarticular osteophytes	0.879	0.009*
Femoral subchondral sclerosis	0.668	0.101
Distal femoral condylar remodeling	0.692	0.119
Distal femoral subchondral cystic lucencies	0.661	0.106
Femoral intercondylar notch osteophytes	0.661	0.106
Proximal tibial periarticular osteophytes	0.580	0.172
Proximal tibial subchondral sclerosis	0.430	0.335
Proximal tibial subchondral cystic lucencies	0.567	0.184
Central tibial plateau osteophytes	0.458	0.301
Joint effusion or capsular thickening	0.735	0.134
Meniscal mineralization	0.535	0.216

* Significant correlation.

against canine 14-3-3 and MMP proteins, we first conducted some experiments to show that human antibodies are able to detect these canine proteins. As shown in Figure 1, the sizes of the 14-3-3 and MMP bands detected in the samples of synovial fluid from dogs with CCLR were identical to those in samples from humans, which indicates that the human antibodies can be used to address the questions explored in this study.

The joint tissues of patients, canine or human, with rupture of the cranial (or, in humans, anterior) cruciate ligament express many degradative enzymes, especially MMPs, which play an important role in the pathogenesis of this disease (3,21,36). It has been documented that the 14-3-3 β , σ , and η proteins have potent MMP-1 and MMP-3 stimulatory effects on fibroblasts (9,35). Also, it has been suggested that increasing levels of glycoaminoglycans (indicators of joint tissue degradation) in the synovial fluid of dogs with CCLR correlate with increased levels of MMPs in the synovial fluid and joint tissue (3,5). The previous results in humans and those provided here suggest that the MMP-1 and MMP-3 stimulatory effects may be due to 14-3-3- η (9). Although the number of synovial fluid samples examined was limited in this study, the statistical analysis still showed significant differences in the levels of 14-3-3- η and - γ between the CCLR and control samples.

The results of this study support previous findings of high levels of the 14-3-3 η and γ isoforms in synovial fluid from patients with joint inflammation (9). The present findings also support previous results showing a direct correlation between levels of 14-3-3- η , MMP-1, and MMP-3 (9). However, this is the first study showing a high level of these proteins in the synovial fluid of dogs with CCLR.

Although it has been shown that 14-3-3 proteins are primarily intracellular, under certain pathological conditions they can be found in tissue fluid (37,38). The mechanism by which these proteins are released into the synovial fluid is not completely understood. However, it is known that keratinocytes release 14-3-3 proteins via exosomes (38,39). It is not clear why synovial fluid samples from

inflamed joints contain such high levels of 14-3-3- η and - γ but not other isoforms, such as σ (9,40). It is possible that this reflects differential expression of 14-3-3 isoforms by cells within the synovium. The present study, as with previous studies in humans, demonstrated very low levels of the 14-3-3- σ isoform in the synovial fluid of dogs with CCLR.

Radiographs are extensively used to evaluate joint disease. However, in this study only 2 of the 13 radiographic markers of osteoarthritis correlated with the level of 14-3-3- η . Osteophytosis may become apparent radiographically as early as 14 d after CCLR (34). Biomechanical instability creates stresses across articular surfaces that, in turn, generate inflammation because of the poor resistance of hyaline cartilage to shear mechanical stress. Subsequently there is more inflammation and a higher radiographic score in CCLR cases (41).

As the MMPs have a significant effect on joint inflammation and injury (22), it may be that 14-3-3- η and - γ induce FLS to produce high levels of MMPs (42), especially MMP-1 and MMP-3. These proteins can cause extracellular matrix degradation leading to the joint erosion seen in many patients with severe arthritis. This action may influence cell structures in the CCL and might be one of the pathways in the pathogenesis of CCLR.

In conclusion, this study has demonstrated that 14-3-3 proteins, especially the η isoform, can be readily detected in the synovial fluid of dogs with CCLR. This protein might be useful as an early diagnostic marker in some conditions involving joint inflammation, such as CCL weakness and rupture. It may also be considered a predisposing factor in some cases of CCLR.

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