Examination of synovial fluid hyaluronan quantity and quality in stifle joints of dogs with osteoarthritis

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Objective—To determine the quantity (concentration) and quality (molecular weight) of synovial fluid hyaluronan with respect to presence and severity of osteoarthritis in stifle joints of dogs.

Animals—21 purpose-bred dogs and 6 clinically affected large-breed dogs (cranial cruciate ligament [CrCL] disease with secondary osteoarthritis).

Procedures—Research dogs underwent arthroscopic surgery in 1 stifle joint to induce osteoarthritis via CrCL transection (CrCLt; n = 5 stifle joints), femoral condylar articular cartilage groove creation (GR; 6), or meniscal release (MR; 5); 5 had sham surgery (SH) performed. Contralateral stifle joints (n = 21) were used as unoperated control joints. Synovial fluid was obtained from research dogs at time 0 and 12 weeks after surgery and from clinically affected dogs prior to surgery. All dogs were assessed for lameness, radiographic signs of osteoarthritis, and pathologic findings on arthroscopy as well as for quantity and quality of hyaluronan.

Results—Clinically affected dogs had significantly greater degrees of pathologic findings, compared with dogs with surgically induced osteoarthritis (ie, those with CrCLt, GR, and MR stifle joints), and with respect to lameness scores, radiographic signs of osteoarthritis, pathologic findings on arthroscopy, and synovial fluid hyaluronan concentration. Synovial fluid from stifle joints of dogs with surgically induced osteoarthritis had hyaluronan bands at 35 kd on western blots that synovial fluid from SH and clinically affected stifle joints did not.

Conclusions and Clinical Relevance—Synovial fluid hyaluronan quantity and quality were altered in stifle joints of dogs with osteoarthritis, compared with control stifle joints. A specific hyaluronan protein fragment may be associated with early pathologic changes in affected joints. (*Am J Vet Res* 2008;69:1569–1573)

Osteoarthritis is an important and costly problem in dogs, horses, and humans. In dogs, it is suggested that 20% of middle-aged and 90% of older dogs have osteoarthritis in 1 or more joints.[1] The cause of osteoarthritis is unknown, but it inevitably results in degradation of articular cartilage, leading to pain, inflammation, and loss of motion in the joint.[1] Because osteoarthritis is a widespread problem, veterinarians need a precise way to diagnose the disease early and accurately. Osteoarthritis is commonly diagnosed in the late and irreversible stages, where treatment can only be expected to decrease pain and slow progression of disease in a palliative manner.[2] By developing methods for earlier diagnosis of osteoarthritis, prevention or even curative treatment strategies to manage the disease become more realistic.

Biomarkers are being investigated intensively for early diagnosis, identification of patients at increased risk, and monitoring therapeutic strategies in osteoarthritis.[3] As such, a scheme for standardized classification of biomarkers was put forth by the Osteoarthritis Biomarker Network to categorize biomarkers under development into one or more of the following areas: burden of disease, investigative, prognostic, efficacy of intervention, and diagnostic.[3] In our laboratory, we are particularly interested in identification and validation of biomarkers that can detect early stages of osteoarthritis to provide accurate diagnostic and prognostic information prior to onset of clinical disease. The study reported here focuses on a potential marker for diagnosis and staging of osteoarthritis in synovial fluid. Synovial fluid was chosen for this research as it is known to have sensitive and rapid responses to joint insult and

Abbreviations

CrCL	Cranial cruciate ligament
CrCLt	Cranial cruciate ligament transection
GR	Femoral condylar articular cartilage groove creation
MR	Meniscal release
SH	Sham surgery

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injury, and these changes are primarily joint specific, which may be especially important in veterinary medicine. Hyaluronan was examined in this way because it is a major functional constituent of synovial fluid and articular cartilage, and it undergoes quantitative and qualitative changes in joint disease.[4,5]

Hyaluronan is synthesized by chondrocytes and synovial fibroblasts in articular cartilage and synovial intima, respectively.[2] In the cartilage matrix, hyaluronan forms the backbone of proteoglycan aggregates that are interwoven with collagen to create the unique structure of hyaline cartilage.[6] Synovial fluid hyaluronan provides joint lubrication and helps limit inflammation, pain, and cartilage degradation[7] as well as acts as a shock absorber and allows the joint to move in a smooth, nearly frictionless manner.[2] Alterations in joint biomechanics and physiologic characteristics as a result of insult, injury, or aging have direct effects on the quantity and quality of hyaluronan.[2,7,8] When hyaluronan in synovial fluid or cartilage is abnormal in either respect, joint functions are further compromised, exacerbating progression toward irreversible osteoarthritis.[2,5] Osteoarthritic joints in dogs are reported to have decreases in synovial fluid hyaluronan molecular weight and concentration as a result of fragmentation of hyaluronan and insufficient production of hyaluronan.[2] Theses findings have encouraged investigators to study the effects of hyaluronan on joint function and pursue hyaluronan supplementation for treatment of osteoarthritis. To our knowledge, however, little has been reported regarding the use of hyaluronan as an osteoarthritis biomarker. One study[8] investigated serum and synovial fluid hyaluronan as a potential diagnostic tool in dogs but did not find it useful as studied because of metabolic effects on serum hyaluronan concentrations and substantial overlap among disease states for synovial fluid concentrations. Therefore, we decided to focus on synovial fluid hyaluronan quality so as to avoid systemic influences on protein concentration and composition and to ascertain the importance of molecular weight subcategories in assessment of joint health. The objective of the study reported here was to determine the quantity (concentration) and quality (molecular weight) of synovial fluid hyaluronan with respect to presence and severity of osteoarthritis in stifle joints of dogs. We hypothesized that synovial fluid hyaluronan quantity and quality would be significantly altered in stifle joints of dogs insulted by surgical induction of osteoarthritis and in stifle joints of dogs with osteoarthritis resulting from CrCL disease, compared with synovial fluid from unaffected control stifle joints.

Materials and Methods

Animals—All procedures were approved by the institutional animal care and use committee. Twenty-one purpose-bred hound dogs (mean body weight, 23.3 kg; age range, 2 to 4 years) were used for surgical induction of osteoarthritis. Dogs were kept in individual kennels and allowed an acclimation period prior to study initiation that included 15 minutes of on-leash exercise 5 d/wk. Six large-breed adult dogs (mean body weight, 24.8 kg; age range, 2 to 6 years) examined at the University of Missouri Veterinary Medical Teaching Hospital and with a diagnosis of CrCL disease with secondary osteoarthritis in 1 stifle joint were used with informed owner consent to obtain clinical data and samples.

Procedures—Assessment of limb function was performed by 2 investigators blinded to dog number, group, and clinical signs by use of a categoric[9] and visual analogue scale for lameness. All dogs were assessed for lameness the day before surgery, and research dogs were assessed at 4, 8, and 12 weeks after surgery.

Craniocaudal and mediolateral radiographic views of the stifle joint were obtained in standardized fashion the day prior to surgery for all dogs and again at 12 weeks after surgery for research dogs. Radiographs were scored by 1 investigator blinded to dog number, group, and clinical signs by use of a subjective scoring system described in previous studies.[10,11] Nine regions of the stifle joint were evaluated and given a score from 0 to 3 on the basis of severity of radiographic findings observed. Therefore, each stifle joint could have a score ranging from 0 to 27. Stifle joints scored from 0 to 4 were considered normal. Stifle joints scored from 5 to 9 were considered to have mild osteoarthritis, stifle joints scored from 10 to 18 were considered to have moderate osteoarthritis, and stifle joints scored from 19 to 27 were considered to have severe osteoarthritis.

All dogs were premedicated, anesthetized, and prepared for aseptic surgery of 1 stifle joint. Prior to surgery, arthrocentesis of the stifle joint intended to receive surgery was performed. Synovial fluid was aspirated from each stifle joint by use of a 1.5-in 18-gauge needle and syringe, such that all synovial fluid that could readily be aspirated with redirection of the needle was obtained. The synovial fluid was then divided into aliquots and frozen at –70°C until needed.

All dogs underwent arthroscopic surgery of 1 stifle joint (right stifle joint for dogs with surgically induced osteoarthritis; affected stifle joint for dogs with osteoarthritis resulting from CrCL disease) by use of a craniolaterally directed camera and craniomedial instrument portals. One of 4 surgical procedures was performed on dogs with surgically induced osteoarthritis; contralateral stifle joints of dogs with surgically induced osteoarthritis served as unoperated control joints, and clinically affected stifle joints were examined, thus creating 6 (ie, CrClt, GR, MR, SH, clinically affected, and control) groups of stifle joints for comparison.

The CrCLt stifle joints (n = 5) underwent complete transection of the CrCL by use of a No. 11 scalpel blade. Transection was confirmed by visual observation and palpation of cranial tibial translocation. For creation of GR stifle joints (n = 6), a sterile ring curette (3 mm inner diameter) was placed through the instrument portal and used to make two 6- to 8-mm-long full-thickness grooves in the cartilage on the weight-bearing portion of the medial femoral condyle. Grooves were measured with a calibrated arthroscopic probe. For creation of MR stifle joints (n = 5), a sterile meniscal push knife was inserted through the instrument portal. The knife was used to create a complete radial transection of the meniscus at the caudal horn junction with the caudal meniscotibial ligament. Complete meniscal transection was confirmed by arthroscopic inspection. The SH stifle joints (n = 5) underwent a procedure in which a cali-

brated probe was inserted through the instrument portal. The CrCL, femoral condyle, and medial meniscus were touched with the probe. Unoperated contralateral stifle joints of research dogs were used as control stifle joints (n = 12), and affected stifle joints of dogs with osteoarthritis resulting from CrCL disease (6) were assessed and treated arthroscopically as deemed clinically appropriate by a single attending clinician.

Arthroscopic evaluation of stifle joints was performed in standardized fashion. All articular surfaces of the patella, femur, and tibia were examined and scored with respect to degree of articular cartilage damage by use of the International Cartilage Repair Society cartilage injury classification system.[12] Meniscal pathologic change was also arthroscopically assessed and described in terms of nature, extent, and location.

Twelve weeks after surgery, research dogs were euthanatized by IV administration of an overdose of phenytoin and pentobarbital. Radiography and aseptic arthrocentesis were performed on both stifle joints of each dog as already described.

To quantitatively assess hyaluronan content in synovial fluid of dogs in this study, the concentration of hyaluronan in the synovial fluid was measured by use of a commercially available ELISA.[a] This is a competitive ELISA where the colorimetric signal is inversely proportional to the amount of hyaluronan in the sample. For our study, samples were diluted 1:4,000 in diluent. The samples were then processed and analyzed according to manufacturer's instructions. Concentration of hyaluronan is reported as mg/mL.

To qualitatively assess hyaluronan content in synovial fluid of dogs in this study, western blot gels[b] were used. Samples were then reduced by use of sample buffer[c] and 2-mercaptoethanol and heated at 70°C for 5 minutes. Samples were loaded into the wells and connected to a power supply[d] at 125 V and 70 mA for 90 minutes. Transfer was then completed in a unit[e] at 18 V and 110 mA for 130 minutes. Blocking was performed after that for 30 minutes with 30 mL of Tris-buffered saline (0.9% NaCl) solution containing 0.05% Tween and 1.5 g of nonfat dry milk. Then 30 µL of primary antibody (mouse anti-rat proteoglycan hyaluronan IgG monoclonal) was added, and the blot was incubated at 4°C overnight. The antibody was obtained from the Developmental Studies Hybridoma Bank developed under auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa. The following day, the blot was washed 3 times with the Tris-buffered saline solution containing Tween and then incubated on a shaker at 24°C with cell-signaling anti-mouse IgG horseradish peroxidase–linked secondary antibody. After 1 hour, the blot was washed 3 times with the Tris-buffered saline solution containing Tween. Blots were incubated with enhanced chemiluminescence solution containing the cell-signaling reagent[f] and peroxide to produce a signal. Bands were detected by use of hyperfilm-enhanced chemiluminescence film (8 × 10 inch) at 30 seconds and 1 minute and developed by use of a commercial film processor. Once films were developed, bands were labeled and analyzed for size and distribution of bands.

Data analysis—To test our hypothesis, data from all assessments were compared for significant ($P < 0.05$) differences by use of an ANOVA or rank sum tests with post hoc analyses. Correlation between lameness assessment systems was determined by use of Spearman rank order correlation analysis.

Results

All research dogs had no detectable lameness prior to surgery. At 4 weeks after surgery, CrCLt-treated dogs were significantly ($P = 0.02$) more lame than all other groups. At 8 and 12 weeks, CrCLt- and MR-treated dogs were significantly more lame than GR- and SH-treated dogs. Lameness in clinically affected dogs measured at the time of hospital admission was compared with research dogs measured at 12 weeks after surgery. Clinically affected dogs were significantly more lame than GR- and SH-treated dogs but not significantly different from CrCLt- and MR-treated dogs (power = 0.3). Visual analogue scale and categoric lameness scoring revealed a strong ($r = –0.976$) and significant ($P < 0.001$) negative correlation.

All study dogs had normal radiographic appearances of both stifle joints prior to study initiation. At 12 weeks, the degree of radiographic signs of osteoarthritis was significantly higher in CrCLt stifle joints than all other study groups, except MR stifle joints. Radiographic signs of osteoarthritis in MR stifle joints were significantly ($P < 0.001$) higher than in SH and control stifle joints. Clinically affected dogs had radiographic osteoarthritis scores that were significantly ($P < 0.001$) higher than all study groups.

Evaluated by arthroscopy, GR and MR stifle joints had significantly more pathologic changes in articular cartilage in terms of amount and severity than all other groups. The CrCLt stifle joints had significantly more articular cartilage damage than SH and control stifle joints, which had no articular cartilage damage. The clinically affected dogs had a large degree of variability in amount and severity of articular cartilage damage. Osteoarthritic stifle joints of clinically affected dogs had significantly more articular cartilage damage than SH and control stifle joints. No significant differences in articular cartilage damage were found between osteoarthritic stifle joints of clinically affected dogs and CrCLt stifle joints or between GR and MR stifle joints (power < 0.2).

To test the hypothesis of the present study, only 12-week synovial fluid samples from the experimental and control stifle joints were analyzed and compared with the single time point clinical samples. The time 0 synovial fluid samples were used as part of another aspect of this research that did not focus on comparison with clinically affected dogs. The ELISA data revealed high amounts of synovial fluid hyaluronan in control stifle joints as well as stifle joints with surgically induced osteoarthritis (**Figure 1**). Osteoarthritic stifle joints of clinically affected dogs had a significantly ($P = 0.043$) lower concentration of synovial fluid hyaluronan than all other groups. There were no significant ($P = 0.4$) differences in concentration of synovial fluid hyaluronan among stifle joints with surgically induced osteoarthritis and control stifle joints at the 12-week time point.

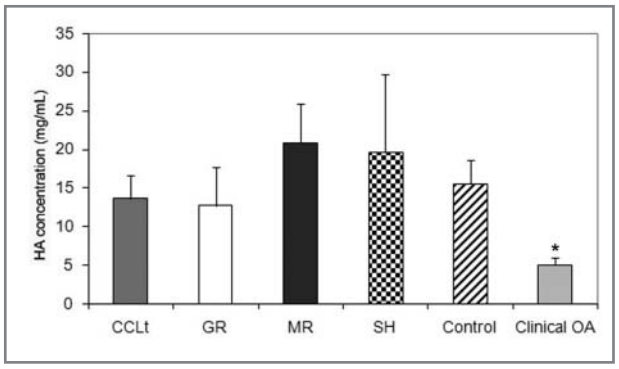

Figure 1—Mean ± SD concentrations of hyaluronan in synovial fluid from stifle joints of dogs in each group in this study obtained at 12 weeks after surgery or at the time of surgery for clinically affected dogs. *Significantly ($P < 0.05$) different from all other groups. Clinical OA = Osteoarthritic stifle joint of clinically affected dogs.

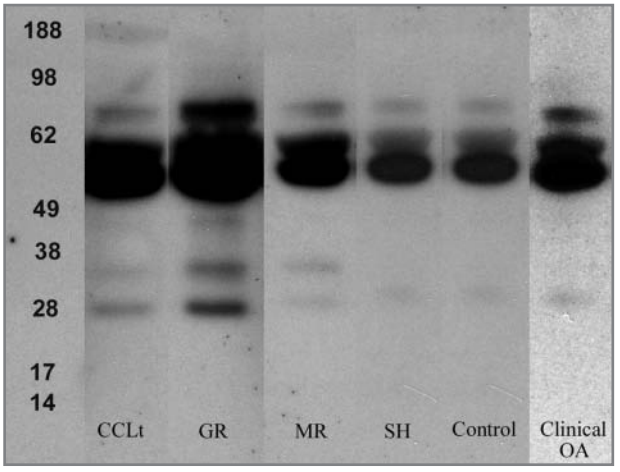

Figure 2—Western blot of hyaluronan in synovial fluid from stifle joints of dogs in each group in this study. Representative lanes from each group have been combined from multiple blots. A band is evident at approximately 35 kd in CrCLt, GR, and MR lanes that was not evident in any lanes for the other groups. Numbers represent values in kilodaltons. *See* Figure 1 for remainder of key.

Western blot analysis was used to examine the quality of the hyaluronan in the synovial fluid. Bands staining for the hyaluronan antibody were consistently seen at 70 kd and also 50 kd and 28 kd.[13] Subjectively, staining intensity differences were observed among groups that varied among samples. No attempt to quantify these differences was made in the present study. Importantly, synovial fluid from stifle joints with surgically induced osteoarthritis (ie, CrCLt, GR, and MR) consistently had a band at approximately 35 kd that synovial fluid from SH and control stifle joints as well as synovial fluid from osteoarthritic stifle joints of clinically affected dogs did not (**Figure 2**). A band at approximately 45 kd was observed in western blot analysis of synovial fluid from GR stifle joints; however, this was not consistently found among samples.

Discussion

In this study, we found that synovial fluid hyaluronan quantity and quality were altered in stifle joints from clinically affected dogs with secondary osteoarthritis from cruciate ligament disease, compared with stifle joints from research dogs with surgical insults performed to evaluate various causes of secondary osteoarthritis and with unoperated stifle joints. Synovial fluid hyaluronan quantity was most severely affected in the clinically affected dogs. Synovial fluid hyaluronan quality was most differentially affected in stifle joints with surgically induced osteoarthritis. We did not find significant differences in hyaluronan quantity or quality among the various forms of surgically induced osteoarthritis on the basis of our sample number, timing, and method. Importantly, lameness, radiographic signs of osteoarthritis, and cartilage pathologic data revealed changes over time and among groups that will be critical for determining the clinical usefulness of synovial fluid hyaluronan as an early diagnostic and prognostic biomarker of osteoarthritis.

We reported a mean synovial fluid hyaluronan concentration of 15.3 mg/mL in normal stifle joints of dogs included in this study. This concentration is considerably higher than described for human knee joints, which has been reported to be 2 to 3 mg/mL in normal joints[14] and 0.17 to 1.32 mg/mL in diseased joints.[15] Our clinically affected dogs with osteoarthritis of the stifle joint had a mean synovial fluid hyaluronan concentration of 1.16 mg/mL, which is similar to the previously reported concentration of 0.9 mg/mL in stifle joints of dogs with osteoarthritis of the stifle joint.[8] The differences in synovial fluid hyaluronan concentration between dogs and humans could be the result of anatomic, metabolic, or functional differences in species as well as differences in ages of study subjects. In addition, a much larger difference in concentration was found between control and affected stifle joints for dogs, compared with humans. This difference may be related to age and species differences as mentioned as well as differences in nature, severity, and temporal progression of osteoarthritis between dogs and humans. During the early stages of osteoarthritis, synovial fluid hyaluronan concentration is reported to increase as a result of elevated synthesis in response to various pathologic stimuli.[14] As the disease progresses, hyaluronan concentrations in synovial fluid decrease to amounts below normal as synthesis is overcome by degradation and loss.[2,7,8] Data from this study match this temporal progression of hyaluronan concentration in that 2 of the stifle joint groups with surgically induced osteoarthritis had numerically higher hyaluronan concentrations at 12 weeks after insult, whereas osteoarthritic stifle joints of clinically affected dogs had significantly lower synovial fluid hyaluronan concentrations than all other groups. Taken together, these data suggest that synovial fluid hyaluronan concentration alone may not have clinical usefulness as a biomarker for early diagnosis of osteoarthritis in dogs.

We believed it was important to examine the quality (ie, molecular weight) of the hyaluronan in this study because these changes may be more sensitive to early pathologic changes in a joint in osteoarthritis and therefore more useful as a clinical biomarker for diagnosis of disease than previously determined.[8] The structure of hyaluronan is altered during the development of osteoarthritis. The molecular weight of hyaluronan in

normal human synovial fluid is 7×10^6 and decreases to 3 to 5×10^6 in degenerative joints, corresponding to structural changes in the synovial fluid.[14] In our study, synovial fluid from normal and affected stifle joints had multiple bands found on western blots assessing hyaluronan concentrations at various molecular weights. Bands at 70, 60, 50, and 28 kd were consistently observed in all groups. These data are consistent with those reported in another study[13] that found hyaluronan bands at 70 kd and then after purification cleaved into 50- and 27- kd bands. As part of our method for western blot analysis, synovial fluid samples were reduced for primary antibody to bindings. Sample reduction leads to the development of protein fragments (ie, lower molecular weight hyaluronan). While fragmentation may occur ex vivo as part of sample processing, in vivo fragmentation of hyaluronan is also reported to occur as a result of pathologic changes in the joint.[15] This fragmentation may be the result of alterations in synthesis, posttranslational modifications, degradation, or any combination of these.[15] Importantly, in our study, 1 fragmentation band was found to be consistently different among synovial fluid samples of control stifle joints and stifle joints with surgically induced osteoarthritis. This band at 35 kd may represent a form of the hyaluronan protein that is a result of early pathologic processes associated with initiation of osteoarthritis. If correct, this protein could serve as a valuable biomarker for diagnosis of osteoarthritis. We are currently pursuing further characterization of this protein for future prospective assessment of its importance as an osteoarthritis biomarker.

There are certainly some important limitations of this study that should be considered when interpreting the data. One such limitation was the duration of study with respect to the surgically induced osteoarthritis. Although the data suggest that the 12-week endpoint was adequate for early changes associated with osteoarthritis to be evident on the basis of functional, radiographic, arthroscopic, and synovial fluid changes in dogs, it may not have been long enough to allow for differences in hyaluronan quantity to mimic those of clinical patients admitted for treatment of osteoarthritis of the stifle joint secondary to CrCL disease. Therefore, we cannot be certain that any of these surgically induced forms of osteoarthritis truly mimic the early stages of secondary osteoarthritis in the stifle joints of clinically affected dogs or which type of surgical insult may be most appropriate for study of osteoarthritis biomarkers in dogs. Lastly, the number of subjects per group was not high enough for us to consistently reach powers > 0.8 in our statistical analyses. Therefore, data reported that revealed no significant differences among groups in this study must be interpreted with caution.

The data from this study allowed us to accept our hypothesis that synovial fluid hyaluronan quantity and quality would be altered in stifle joints of dogs insulted by surgical induction of osteoarthritis and in osteoarthritic stifle joints of clinically affected dogs resulting from CrCL disease, compared with control stifle joints. Synovial fluid hyaluronan concentration may not be helpful for early diagnosis of osteoarthritis, but a protein fragment of hyaluronan observed on the western blot may be associated with early pathologic changes in a joint. Further study of this fragment to determine whether or not this protein could serve as a biomarker for early diagnosis of osteoarthritis is warranted.

a. Echelon Biosciences Inc, Salt Lake City, Utah.
b. NuPAGE Novex Bis-Tris gels, Invitrogen Life Technologies, Carlsbad, Calif.
c. NuPage LDS sample buffer, Invitrogen Life Technologies, Carlsbad, Calif.
d. Invitrogen power supply, Invitrogen Life Technologies, Carlsbad, Calif.
e. BioRad cassette, BioRad, Hercules, Calif.
f. LumiGlo reagent, Cell Signaling Technology, Beverly, Mass.

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