Direct and indirect markers of cartilage metabolism in synovial fluid obtained from dogs with hip dysplasia and correlation with clinical and radiographic variables

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Objective—To compare activities of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and matrix metalloproteinase (MMP)-3 and contents of sulfated glycosaminoglycan (S-GAG) in joint fluid obtained from dogs with hip dysplasia (HD) and clinically normal dogs, evaluate correlations among these markers in joint fluid obtained from dogs with HD, and evaluate correlations between each marker and clinical and radiographic variables.

Animals—26 dogs with HD (clinical group) and 43 clinically normal Beagles (control group).

Procedure—Joint fluid was aseptically collected from the hip joints of all dogs. For each dog in the clinical group, age, duration of lameness, radiographic osteoarthritis (OA) score, and Norberg angle in each affected joint were recorded. Activities of IL-1β, IL-6, TNF-α, and MMP-3 and S-GAG contents were measured. Values were compared between groups by use of Mann-Whitney *U* tests, and the Spearman rank correlation test was used to evaluate correlations among markers and between each marker and clinical or radiographic variables.

Results—Values of all markers were significantly higher for the clinical group, compared with values for the control group. There was a moderate positive correlation between lameness duration and IL-6 activity and a strong negative correlation between the Norberg angle and IL-1β activity.

Conclusions and Clinical Relevance—Analysis of our results indicated that there was a significant increase in markers of OA in dogs with HD. Activities of IL-1β and IL-6 in joint fluid of dogs with HD may be influenced by the severity of laxity in the hip joint and lameness duration, respectively. (*Am J Vet Res* 2005;66:2028–2033)

Hip dysplasia (HD) is a developmental disease primarily affecting medium- and large-breed dogs; it is characterized by instability and incongruity of the hip joint.[1] Components of HD include varying degrees of laxity of surrounding soft tissues, joint instability, malformation of the femoral head and acetabulum, and **osteoarthritis (OA)**.[2]

Osteoarthritis is a slowly progressive disorder of synovial joints. It is characterized by a loss of balance between synthesis and degradation of articular cartilage constituents. This leads to subsequent destruction of joint cartilage, remodeling of the underlying bone, formation of osteophytes, and variable degrees of synovitis.[3,4] Diagnosis of OA is routinely made on the basis of radiographic changes that are only visible relatively late in the disease process. Early detection of OA may provide improved opportunities to identify the underlying cause of the OA and allow clinicians to decrease the rate of or halt the progression of OA. There are several reports[5–7] on markers in joint fluid that could be used for early detection of OA. However, to our knowledge, no suitable, specific, and sensitive markers have been identified.

Markers of cartilage metabolism in joint fluid have been subdivided into 2 classes (direct and indirect markers).[8] Examples of direct markers are proteoglycans and related molecules,[9,10] epitopes of chondroitin sulfate (ie, 3-B-3 and 7-D-4),[11] and collagen type II C-propeptide (chondrocalcin).[a] Indirect markers of cartilage metabolism include proinflammatory cytokines (**interleukin [IL]**-1,[12] **tumor necrosis factor [TNF]**, and IL-6[13,14]) and proteinases[15] common to many tissues and cell types. There are few reports about changes in the concentration of these markers in joint fluid obtained from dogs with OA, and to our knowledge, there have been no reports on evaluation of these markers in joint fluid from dogs with HD.

The purpose of the study reported here was to compare IL-1β, IL-6, TNF-α, and **matrix metalloproteinase (MMP)**-3 activities and contents of **sulfated glycosaminoglycan (S-GAG)** in joint fluid obtained from the hip joints of dogs with HD and clinically normal dogs. In addition, we evaluated correlations among these markers as well as between the markers and clinical or radiographic variables.

Materials and Methods

Animals—Two groups of dogs were included in the study. There were 26 dogs in the clinical group (11 sexually intact males, 3 neutered males, 10 sexually intact females, and 2 spayed females). Dogs in the clinical group had a mean body weight of 27.4 kg and mean age of 17.4 months. The

Received December 17, 2004.
Accepted May 12, 2005.
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Presented in part at the 14th Annual American College of Veterinary Surgeons Symposium, Denver, October 2004.
The authors thank Drs. Shuichi Tsuchida and Ryozo Akuzawa for technical assistance.
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most commonly represented breeds were Labrador Retriever (n = 6) and Golden Retriever (6). Forty-three clinically normal Beagles were in the control group (12 males and 31 females). All of these dogs were sexually intact. Dogs in the control group had a mean body weight of 11.3 kg and mean age of 24 months. Dogs were included in the control group on the basis of lack of abnormalities detected during orthopedic examination and evaluation of pelvic radiographs. This study was approved by the Committee on Bioethics of Nippon Veterinary and Animal Science University.

Dogs with HD (clinical group)—Dogs evaluated for HD at the animal medical center at Nippon Veterinary and Animal Science University were considered for inclusion in the study. Inclusion criterion for the clinical group was diagnosis of HD during both orthopedic and radiographic examinations.[16] Clinical findings considered compatible with HD included lameness of the pelvic limbs and signs of pain when the affected joint was extended. Radiographic findings considered compatible with HD included mild to severe osteophytosis and subluxation of the femoral head. Other orthopedic and neurologic diseases, such as rupture of the cranial cruciate ligament and degenerative lumbosacral stenosis, were excluded on the basis of results for orthopedic, neurologic, and radiographic examinations.

Information for each dog (age and body weight) was recorded, and duration of lameness of the affected limb was ascertained from information provided by the owners. Ventrodorsal and lateral radiographs of the hip joint with the hip in an extended position were scored for severity of OA by use of a scoring system established in another study.[17] Scores for this system ranged from 0 (no signs of OA) to 4 (signs of severe OA). The Norberg angle was measured for each affected hip joint of each dog by use of a ventrodorsal radiographic view.[18]

Collection of joint fluid—Samples of joint fluid were obtained from 36 hip joints of dogs with HD. Samples of joint fluid were collected by aseptic percutaneous arthrocentesis in dogs anesthetized for arthrocentesis or during surgery immediately prior to arthrotomy. In 10 dogs that had bilateral HD, joint fluid was collected from both hip joints. The amount of joint fluid removed was the maximal amount that could be obtained without excessive aspiration or repeated attempts. Synovial fluid was obtained from hip joints of clinically normal dogs in a manner similar to that used for the clinical group of dogs.

All samples of joint fluid were centrifuged at 6,000 $\times$ *g* for 10 minutes at 4°C to separate cells and debris. Supernatant was harvested and stored at –80°C until assays were performed.

Bioassay for IL-1β activity—Activity of IL-1β in samples of synovial fluid was determined by use of a cytotoxicity bioassay that used the human melanoma subclone A375S2.[b] The cytotoxicity bioassay was performed as described elsewhere.[19,20] Briefly, 100-µL volumes of serial 2-fold dilutions of joint fluid or recombinant human IL-1β[c] in minimal essential medium[d] were added to 96-well flat-bottomed tissue culture plates.[e] Then, 2×10^3 A375S2 cells (100 mL/well) were added on top of the samples, and plates were incubated for 92 hours at 37°C and 5% carbon dioxide.

After incubation, cytotoxic effects were measured colorimetrically by use of tetrazolium salt. Briefly, 50 µL of a solution of 2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide[f] (1 mg of solution/mL of RPMI 1640 medium containing 20 µL of phenazine methosulfate[g] [0.383 mg/mL]) was added to each well. After incubation for an additional 4 hours at 37°C and 5% carbon dioxide, color was measured at 450 nm by use of a microplate reader.[h] Activity of IL-1β was determined by comparison with the standard curve for recombinant human IL-1β. All samples were assayed in triplicate.

Bioassay for IL-6 activity—Activity of IL-6 in samples of synovial fluid was determined by use of a proliferative bioassay that used the IL-6–dependent murine hybridoma cell line 7TD1.[i] The proliferative bioassay was performed as described elsewhere.[20,21] Briefly, 100-µL volumes of serial 2-fold dilutions of joint fluid or recombinant human IL-6[j] in RPMI 1640 medium[k] were added to 96-well flat-bottomed tissue culture plates.[e] Then, 5×10^3 7TD1 cells (100 mL/well) were added on top of the samples, and plates were incubated for 68 hours at 37°C and 5% carbon dioxide. After incubation, proliferation was measured colorimetrically by use of tetrazolium salt. Activity of IL-6 was determined by comparison with the standard curve for recombinant human IL-6. All samples were assayed in triplicate.

Bioassay for TNF-α activity—Activity of TNF-α in samples of synovial fluid was determined by use of a cytotoxicity bioassay that used WEHI 164 clone 13 murine fibrosarcoma cells.[l] The cytotoxic effects were bioassay was performed as described elsewhere.[13,14,20,22] Briefly, 100-µL volumes of serial 2-fold dilutions of joint fluid or recombinant human TNF-α[m] in RPMI 1640 medium[k] were added to 96-well flat-bottomed tissue culture plates.[e] Then, 5×10^4 WEHI 164 cells (100 mL/well) were added on top of the samples by use of RPMI 1640 medium containing actinomycin D (1 µg/mL/well), and plates were incubated for 20 hours at 37°C and 5% carbon dioxide. After incubation, cytotoxic effects were measured colorimetrically by use of tetrazolium salt. Activity of TNF-α was determined by comparison with the standard curve for recombinant human TNF-α. All samples were assayed in triplicate.

Assay of MMP-3 activity—Activity of MMP-3 in samples of synovial fluid was measured by use of a fluorogenic substrate.[23,n] The MMP assay was performed in samples of synovial fluid diluted 2- and 5-fold with the addition of an

Table 1—Measurements of markers of cartilage metabolism in samples of synovial fluid obtained from the hip joints of clinically normal dogs (control group) and dogs with hip dysplasia (clinical group).

	Control group			Clinical group		
Marker	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**
IL-1β (pg/mL)	490.0	14.5	830.0	2,010.0*	460.0	20,400.0
IL-6 (pg/mL)	184.0	24.0	808.0	834.0*	13.6	14,080.0
TNF-α (pg/mL)	105.3	14.5	368.0	600.0*	63.4	2,880.0
MMP-3 (nM/L)	41.7	14.9	68.9	155.5*	33.2	431.5
S-GAG (µg/mL)	12.1	6.3	30.3	41.3*	12.3	154.7

*Value differs significantly ($P < 0.05$; Mann-Whitney *U* test) from the median value for the control group.
IL = Interleukin. TNF = Tumor necrosis factor. MMP = Matrix metalloproteinase. S-GAG = Sulfated glycosaminoglycan.

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EDTA-free general proteinase inhibitor cocktail[o] to prevent conversion of the fluorogenic substrate by proteinases other than MMPs. All dilutions were prepared in assay buffer[23] (50mM Tris HCl [pH, 7.5], 0.15M NaCl, 10mM $CaCl_2$, 0.05% Brij 35, and 0.02% NaN_3). For this assay, MMP-3 activity was converted to amounts of active enzyme by use of a standard curve for human MMP-3.[p]

Assay of S-GAG content—A microplate dimethylmethylene blue dye–binding assay, as described elsewhere,[24] was performed. Contents of S-GAG in joint fluid were interpolated as equivalents of a bovine nasal cartilage proteoglycan standard[q] by use of a linear standard curve.

Statistical analysis—Significant differences for each measurement of joint markers between the clinical group and control group were evaluated by use of the Mann-Whitney *U* test. In the clinical group, correlations among markers (IL-1β, IL-6, TNF-α and MMP-3 activity, and S-GAG contents) and between each marker and clinical or radiographic variables were evaluated by use of the Spearman rank correlation. For all comparisons, values of $P \le 0.05$ were considered significant.

Results

Control group versus clinical group—The mean volume of joint fluid obtained from dogs in the clinical group was 3.8 mL (range, 2.4 to 6.0 mL). In the control group, the mean volume obtained was 0.4 mL (range, 0.2 to 0.6 mL).

Median, minimum, and maximum measurements of markers in joint fluid were summarized **(Table 1)**. There were significant differences between the control group and clinical group for all markers evaluated in the study. Mean IL-1β bioactivity for the control group (458 pg/mL) was significantly ($P < 0.001$) lower, compared with the mean value for the clinical group (3,265 pg/mL). Mean IL-6 bioactivity for the control group (284 pg/mL) was significantly ($P < 0.001$) lower, compared with the mean value for the clinical group (1,966 pg/mL). Mean TNF-α activity for the control group (131 pg/mL) was significantly ($P < 0.001$) lower, compared with the mean value for the clinical group (959 pg/mL). Mean MMP-3 activity for the control group (41.3nM/L) was significantly ($P < 0.001$) lower, compared with the mean value for the clinical group (197.3nM/L). Mean S-GAG content for the control group (14.9 µg/mL) was also significantly ($P < 0.001$) lower, compared with the mean value for the clinical group (51.7 µg/mL).

Correlations among markers in joint fluid—Correlations among markers in joint fluid were summarized **(Table 2)**. There were 2 moderate negative correlations. Bioactivity of TNF-α and MMP-3 activity were significantly correlated (r, –0.490; $P = 0.050$), and MMP-3 activity and S-GAG contents were also significantly correlated (r, –0.446; $P = 0.032$). No other significant correlations were detected among markers in joint fluid.

Correlations between markers in joint fluid and clinical and radiographic variables—Mean and range values for clinical and radiographic variables of the clinical group were calculated. Mean values were determined for age (17.4 months; range, 7 to 104 months), body weight (27.4 kg; range, 18.0 to 46.3 kg), lameness duration (13.1 weeks; range, 1 to 56 weeks), radiographic score (1.8; range, 0 to 4), and the Norberg angle (61.3°; range, 10° to 104°).

Relationships between clinical and radiographic variables and markers of cartilage metabolism in joint fluid were determined for the clinical group **(Table 3)**. A moderate positive correlation (r, 0.444; $P = 0.047$) was identified between lameness duration and IL-6 activity. Two moderately weak negative correlations were identified between age and S-GAG content (r, –0.383; $P = 0.030$) and between body weight and MMP-3 activity (r, –0.417; $P = 0.041$). A relatively strong negative correlation (r, –0.546; $P = 0.017$) was

Table 2—Correlation coefficient for comparisons among various markers of cartilage metabolism in samples of synovial fluid obtained from the hip joints of dogs with hip dysplasia.

	Marker comparison	*r*	*P*
IL-1β	IL-6	–0.076	0.761
	TNF-α	0.144	0.577
	MMP-3	0.302	0.200
	S-GAG	–0.138	0.546
IL-6	TNF-α	–0.242	0.291
	MMP-3	–0.376	0.145
	S-GAG	0.380	0.097
TNF-α	MMP-3	–0.490	0.050*
	S-GAG	0.248	0.267
MMP-3	S-GAG	–0.446	0.032*

*Correlation was significant ($P \le 0.05$; Spearman rank correlation).
See Table 1 for remainder of key.

Table 3—Correlations between markers of cartilage metabolism in joint fluid and clinical and radiographic variables.

	IL-1β		IL-6		TNF-α		MMP-3		S-GAG	
Variable	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Age	0.123	0.584	0.194	0.375	0.037	0.864	–0.245	0.229	–0.383	0.030*
Body weight	0.038	0.866	–0.032	0.885	0.077	0.717	–0.417	0.041*	–0.197	0.264
Lameness duration	–0.233	0.310	0.444	0.047*	–0.181	0.406	–0.207	0.321	–0.139	0.439
Radiographic score	0.074	0.747	0.381	0.106	–0.238	0.312	0.093	0.663	–0.201	0.280
Norberg angle	–0.546	0.017*	–0.126	0.584	–0.150	0.512	0.054	0.796	–0.251	0.169

*Correlation was significant ($P \le 0.05$; Spearman rank correlation).
See Table 1 for remainder of key.

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identified between the Norberg angle and IL-1β activity. No other significant correlations were detected between clinical variables and markers in joint fluid.

Discussion

In the study reported here, mean values for activity of all 3 cytokines (IL-1β, IL-6, and TNF-α), MMP-3 activity, and S-GAG contents in joint fluid from dogs with HD were significantly higher, compared with mean values for dogs in the control group. Our study also detected a few correlations between markers in joint fluid and clinical and radiographic variables.

It is generally believed that IL-1 and TNF have important roles in cartilage metabolism, specifically in cartilage destruction.[3,4,8,25] The role of IL-6 in cartilage metabolism is still ambiguous, although it has been suggested[26] that IL-6 functions to upregulate production of tissue inhibitors of metalloproteinases. Investigators in 1 study[14] reported that IL-6 and TNF-α activities in joint fluid obtained 3 months after transection of the cranial cruciate ligament were significantly higher, compared with cytokine activities in joint fluid obtained from the contralateral nontransected joints. In that report, the authors proposed that inflammatory cytokines may respond to cartilage lesions and contribute to the progression of OA through actions on chondrocytes. Results of those studies are consistent with results for our study of dogs with HD, which revealed that there were increasing bioactivities of the cytokines IL-1β, IL-6, and TNF-α in dogs with HD.

Activity of MMP-3 for the clinical group was significantly higher than activity for the control group. This is consistent with results of studies[27,28] in dogs with experimentally induced OA. It is believed that MMP-3 plays an important role in cartilage matrix destruction because it functions by degrading a number of cartilage components and is critical for the activation of other MMPs.[29] The MMP family consists of zinc endopeptidases that are structurally and functionally related, and analysis of data indicates a major role for them in the processes of OA and rheumatoid arthritis.[29] The degradation of extracellular cartilage matrix by MMPs depends on an increase in the biologically active forms. Although additional studies are needed, analysis of our results may suggest an increase in cleavage and degradation of cartilage matrix by MMP-3 in dogs with HD.

The clinical group had significantly higher S-GAG contents in synovial fluid, compared with contents for the control group. This result is also consistent with results in other reports.[10,30] In cartilage with OA, there are increases in synthesis and degradation of cartilage matrix by chondrocytes.[31] Cleavage of proteoglycans, glycosaminoglycans, or both into fragments is believed to be initiated by several proteases, which results in the accumulation of these breakdown products in synovial fluid.[32] Analysis of our results potentially supports an upregulation of cartilage metabolism in dogs with HD that includes degradation and synthesis of cartilage matrix.

Ranges of measurements in the study reported here differ slightly from results in other studies.[13,14] Possible explanations are that the technique for aspiration of joint fluid and methods for measuring each component differ. However, our results that activities of inflammatory cytokines in the clinical group were higher than those in the control group are consistent with results in other reports, and the differences between our study and other studies are small. Although comparison of samples of joint fluid obtained from clinically normal dogs of the same breed with similar body weight would have been ideal, this was not logistically possible for our study; thus, adult Beagles served as the control group. Additional studies are needed to compare measurements of these markers in dogs with HD or OA to those of dogs with rheumatoid arthritis, septic arthritis, or other causes of OA, such as trauma to joints.

The study reported here included the analysis of correlations among markers IL-1β, IL-6, TNF-α, MMP-3, and S-GAG in joint fluid obtained from dogs with HD. There were 2 significant negative correlations (between TNF-α bioactivity and MMP-3 activity and between MMP-3 activity and S-GAG content). One possible explanation for the correlation between TNF-α bioactivity and MMP-3 activity is that TNF-α is considered to be a catabolic mediator that has multiple functions[4,33] and that other mediators of OA, such as nitric oxides,[34] substance P, prostaglandin E_2,[35] other cytokines,[33] tissue inhibitors of metalloproteinases,[4,33] and other proteases,[29] may interact in cartilage metabolism during the progression of OA. Analysis of our results did not enable us to definitively conclude whether only TNF-α activity was attributed to MMP-3 activity in dogs with HD.

A similar reason for a correlation between TNF-α and MMP-3 activities may be acceptable for the correlation between MMP-3 activity and S-GAG content in joint fluid. The S-GAG content in joint fluid is believed to be a result of the metabolism of cartilage matrix, which consists of synthesis and degradation. It is difficult to determine whether S-GAGs in joint fluid are metabolites from catabolic (degradative) or anabolic (synthetic) reactions in articular cartilage. Because it is generally believed that other mediators, such as cytokines, proteases, and growth factors, are involved in these reactions of chondrocytes, analysis of our results did not enable us to conclude that the S-GAG content in joint fluid was directly related to MMP-3 activity. Increased S-GAG content in joint fluid obtained from the clinical group could have been a result of anabolic activity. Histologic evaluation of articular cartilage is needed to determine the anabolic reaction of chondrocytes.

In the study reported here, we investigated correlations between markers of matrix metabolism in joint fluid and clinical and radiographic variables. Age, body weight, and lameness duration were selected as clinical variables, and radiographic score and the Norberg angle were selected as radiographic variables. A weak negative correlation was detected, which suggested that S-GAG content in joint fluid decreased with age of dogs. One possible explanation for decreased S-GAG content with aging is that various functions of chondrocytes in cartilage metabo-

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lism may be altered during aging in dogs with HD. Additional studies are required to confirm whether altered metabolism of cartilage exists in dogs of various ages with HD by evaluating the synthetic and degradative functions of chondrocytes.

A weak negative correlation was detected in our study between body weight and MMP-3 activity. This correlation was not consistent with our hypotheses, and the mechanism for it is unknown.

A significant positive correlation between lameness duration and IL-6 activity was detected in the study reported here. Determination of lameness duration on the basis of data collected from the owners has the potential to be inaccurate. Because HD in dogs is a progressive process,[1,2] it is likely that the lameness duration may have been underestimated, compared with the actual duration of disease in these dogs. Regardless, IL-6 activity in joint fluid obtained from dogs with OA that developed after HD may be continually influenced by lameness duration. Several reports[36–39] have indicated a relationship between IL-6 and clinical signs. In 1 study,[36] investigators reported that the IL-6 concentration was positively correlated with the severity of synovitis as scored by use of arthroscopy in human patients with OA, and they concluded that IL-6 content in joint fluid may be useful as an indicator of the extent of synovitis. In another report,[39] investigators reported that IL-6 content was correlated with pain elicited during joint movements. The positive correlation between lameness duration and IL-6 activity in the study reported here may be explained on the basis of a potential increase in sensitivity of IL-6 activity to altered cartilage metabolism.

The Norberg angle has been used to measure the degree of laxity of the hip joint in dogs with normal and dysplastic hip joints.[40–42] A Norberg angle of ≥ 105° has been considered compatible with a normal hip joint.[43] In the study reported here, we detected high IL-1β activities in dogs with Norberg angles < 105°. Analysis of this correlation suggests that IL-1β activity in joint fluid in dogs with hip laxity increases in proportion to the severity of laxity in the hip joint. In addition, catabolic effects of IL-1β on cartilage may be upregulated in dogs that have various degrees of hip subluxation. To confirm this finding, additional studies are needed to evaluate relationships between IL-1β activity in joint fluid and other diagnostic methods for evaluating laxity of the hip joint in dogs.

In the study reported here, inflammatory cytokine activities were also detected in joint fluid obtained from the control group. Studies[13,14,44] in dogs and humans have revealed inflammatory cytokines in normal joints. We concluded that activities of inflammatory cytokines in joint fluid obtained from the control group reflected physiologic mechanisms of metabolism.

Analysis of results of the study reported here confirmed that there was secondary osteoarthritis after HD that can be detected by altered activities and concentrations of the markers evaluated. This suggests that IL-1β and IL-6 activities in joint fluid may be influenced by the severity of laxity in the hip joint and lameness duration, respectively.

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o. Protease inhibitor cocktail, Sigma Chemical Co, St Louis, Mo.
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q. Bovine nasal cartilage proteoglycan, ICN Biomedicals Inc, Aurora, Ohio.

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