Gelatinase activity in synovial fluid and synovium obtained from healthy and osteoarthritic joints of dogs

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Objective—To determine matrix metalloproteinase (MMP) activity in synovial fluid (SF) obtained from the joints of dogs with degenerative joint disease (DJD) secondary to various underlying conditions.

Sample Population—35 samples of SF obtained from 18 clinically normal (control) dogs and 34 samples of SF obtained from 17 dogs with DJD; dogs with DJD were from 2 populations (client-owned dogs and research dogs that had DJD secondary to the lysosomal storage disease mucopolysaccharidosis VII).

Procedure—MMP activity in samples of SF was semiquantitatively examined by use of gelatin or casein zymography. Western blot analysis was performed by use of antibodies for MMP-2 and MMP-9. In addition, in situ MMP activity was observed in sections of synovial membrane obtained from healthy and osteoarthritic joints.

Results—Samples of SF from osteoarthritic joints had higher MMP-2 activity and dramatically increased MMP-9 activity, compared with values for healthy joints. Substrate-overlay analyses indicated minimal gelatin-degrading activity in synoviocytes obtained from control dogs, whereas greater activity was seen in osteoarthritic synoviocytes, with additional activity in the underlying tissue.

Conclusions and Clinical Relevance—Higher MMP-2 activity and dramatic increases in MMP-9 activity were associated with the osteoarthritic state, even though MMP-2 activity was detected in healthy joints. This study expands information on MMP production in SF of osteoarthritic joints in other species and documents the similarity of MMP activity patterns regardless of the cause of DJD. (*Am J Vet Res* 2003;64:1225–1233)

Osteoarthritis is a complex disease process of articular cartilage that is associated with a variable degree of synovitis resulting from natural aging as well as trauma or disease.[1] Irrespective of the underlying cause, osteoarthritis is the cumulative result of aberrant homeostatic biochemical mechanisms within a joint. Evidence implicates intra-articular synthesis of **matrix metalloproteinases (MMPs)** in damaged cartilage.[2,3] The MMP family is a group of zinc-dependent endopeptidases involved in the turnover of the extracellular matrix.[4] At least 20 vertebrate MMPs have been identified and characterized as to preference for specific substrates.[4,5] Regulation of MMP activity is modulated by low-molecular-weight local inhibitors and **tissue inhibitors of MMPs (TIMPs)**, as well as by the production of the proteinase as a proenzyme that requires cleavage of the N-terminal peptide for activation.[5]

Matrix metalloproteinases and their TIMPs are believed to play an important role in normal turnover and degradation of cartilage matrix. Dysregulation of MMPs, their inhibitors, or both, can be a key event in the transition of the role of MMPs from physiologic to pathologic conditions.[6-8] Qualitative and quantitative differences in MMPs stratified within the complex layers of cartilage tissue suggest that damage is initiated by particular MMPs interacting with other factors in the immediate surroundings. The orchestration of damage caused by each MMP within the layers of articular cartilage and supporting subchondral tissues is a key event for mediating osteoarthritis. However, it remains to be determined whether changes in MMPs are initiators of joint disease or are a result of disease progression. Identification of specific roles for MMPs could aid in developing treatments to stop destruction of cartilage matrix. In addition to serving as targets for pharmacologic agents aimed at ameliorating cartilage destruction, MMPs may serve as useful markers for diagnosing and monitoring the progression of osteoarthritis.

The purpose of the study reported here was to determine MMP activity in **synovial fluid (SF)** obtained from joints of dogs with osteoarthritis secondary to various orthopedic diseases, including dysplasia of the elbow and hip joints, osteochondrosis dissecans, rupture of the cranial cruciate ligament, and **mucopolysaccharidosis VII (MPS VII)**. In this study, zymographic examination of the SF from osteoarthritic dogs was undertaken to determine whether the underlying cause of DJD influenced MMP activity. In addition, MMP activity during the progression of DJD was determined by examining, over time, 2 joints from a single dog with progressive osteoarthritis secondary to dysplasia of the elbow joint. Finally, synovial membranes obtained from osteoarthritic and healthy (con-

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trol) dogs were examined to determine in situ gelatinase activity.

Materials and Methods

Sample population—Samples of SF were obtained from joints of clinically normal dogs and joints of dogs with osteoarthritis, some of which were owned by clients. Research protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania; all research animals were housed in approved University Laboratory Animal Research facilities. Informed consent was obtained from all owners prior to participation of their dogs in this study.

Synovial samples (n = 35) were obtained from 18 clinically normal dogs being used for studies unrelated to this project. Mixed-breed dogs (13 sexually intact females and 5 sexually intact males) that ranged from 0.25 to 7.75 years of age (mean, 2.71 years) were used. Orthopedic examination of these dogs did not reveal any abnormalities. Cytologic examination of SF obtained from these control dogs was performed, when sample volume permitted, to rule out arthropathies in the control population. In addition, approximately half of the control dogs were euthanatized (in accordance with their primary protocol) during the period of the study reported here, and joints were examined to confirm the lack of gross evidence of pathologic changes. Samples of SF obtained from clinically normal dogs were included in the study only when SF analysis was performed or the joints were examined during necropsy and found to be free of gross evidence of pathologic changes.

Synovial samples (n = 19) were obtained from 11 client-owned osteoarthritic dogs that had been examined by personnel in the orthopedic service at the Veterinary Hospital of the University of Pennsylvania to determine the cause of lameness. Dogs included 6 Labrador Retrievers, 1 Greater Swiss Mountain Dog, 1 Akita, 1 Basset Hound, and 2 mixed-breed dogs (3 spayed females, 4 sexually intact males, and 4 neutered males) that ranged from 0.6 to 9.0 years of age (mean, 3.10 years).

The Basset Hound was donated to the University of Pennsylvania for the purpose of inclusion in this study. The dog was examined at 8, 11, 13, 16, and 19 months of age; the dog was neutered after the second examination. Radiographs, computed tomography, and arthroscopy by use of a 2.7-mm rigid arthroscope[a] via a standard medial approach to the elbow joint were performed at each examination. Detailed comparison of the results of 3 imaging modalities used in this dog has been reported elsewhere.[9] Some of the arthroscopic images used in that report were used to illustrate the relationship between SF gelatinase activity and the macroscopic state of **degenerative joint disease (DJD)**.

Samples of SF were obtained from the client-owned dogs after they had been anesthetized for surgical correction of the inciting cause of the osteoarthritis. Cytologic examination of SF was performed on samples obtained from osteoarthritic joints, when sample volume permitted, to rule out inflammatory joint disease. Macroscopic evidence of disease in the joint from which the sample was obtained was confirmed during arthrotomy or arthroscopy.

Samples of SF were obtained from 15 joints of 6 additional dogs with documented osteoarthritis that were included in the study. These dogs had MPS VII. Dogs with MPS VII typically develop severe DJD and are unable to walk by 6 months of age.[10-12] They were all mixed-breed dogs (1 sexually intact female and 5 sexually intact males) that were 0.4 to 1.1 years of age (mean, 0.58 years).

Samples of SF—Samples of SF were obtained from 19 aseptically prepared joints of client-owned dogs with known DJD after the dogs were anesthetized prior to surgical procedures (arthroscopy or arthrotomy) for correction of the inciting cause of the osteoarthritis; these included samples obtained from the Bassett Hound, which were obtained while the dog was anesthetized during examinations. For comparison, SF from the clinically normal dogs without DJD was obtained while the dogs were anesthetized or immediately after the dogs were euthanatized. Synovial fluid with evidence of gross blood was considered contaminated and excluded from the study. Samples of SF were divided into aliquots and stored frozen at –70°C until analyzed.

Gelatin zymography—Samples of SF were thawed and centrifuged in a microcentrifuge[b] at 735 × *g* for 10 minutes at 4°C to remove potential contaminating blood components. Protein quantitation of SF was performed by use of a modified protein assay[c] conducted in accordance with the manufacturer's instructions.

Sixty micrograms of total protein per sample of SF was resuspended in Laemellis nonreducing sample buffer and loaded into each lane of a 12% Tris-glycine precast gel containing 0.1% of gelatin or β-casein, in accordance with the manufacturer's instructions.[d] Following electrophoresis, gels were placed in 1% Triton X-100 on a rocking platform at room temperature (25°C) for 30 minutes to renature proteins and remove SDS; gels were incubated overnight at 37°C in 100mM Tris-HCl (pH, 8.0) containing 1mM $CaCl_2$, with or without 20mM EDTA. After incubation, gels were stained with 0.5% Coomassie brilliant blue stain. Gelatin degradation was evident as clear zones in the Coomassie-stained gels.

Matrix metalloproteinases were identified on the basis of molecular weight by comparison to standard molecular mass markers[e] and by comparing activity in gelatin and β-casein substrates. Digital images of the polyacrylamide gels were captured by use of a bioimager[f] and associated imaging software. Zymographic appearance was subjectively scored for the degree of gelatin degradation at specific molecular weights. Scoring was conducted as follows: absent, no detectable clear zone in the Coomassie blue-stained gelatin substrate; mild, faint clear zone in the gelatin substrate; moderate, increased evidence of a clear zone in the gelatin substrate; and marked, distinct clear zone in the gelatin substrate. To enhance printed visibility, all data were represented after black-to-white inversion of the digital images,[g] whereby proteinases appeared as dark bands on a pale background.

Western blotting—Samples containing equivalent amounts of total protein concentrations were loaded in Laemmelis sample buffer with β-mercaptoethanol and subjected to SDS polyacrylamide gel electrophoresis on 4–20% tris-glycine gradient gels or 8–16% Tris-glycine gradient gels.[h] A sample (0.5 µg) of purified human MMP[i,j] was included on each immunoblot as a control sample. Samples were transferred to a nitrocellulose membrane[k] and blocked for 2 hours at 25°C in 3% nonfat dried milk dissolved in PBS solution. Blots were incubated with primary polyclonal antibody raised against human MMP-2[l] or -9[m] in 1% nonfat dried milk in PBS solution, and bands of immunoreactivity were developed by addition of luminol reagent.[n] Blots were exposed to autoradiography film.[o]

In situ gelatinase activity of synovial membranes—A method of fluorescent substrate overlay used by our laboratory group in another study[13] permits detection of in situ proteinase activity in sections of intact frozen tissues. Proteinases in each tissue section are able to cleave the substrate and convert it to a fluorescent form. Synovial membranes from MPS VII-affected dogs with osteoarthritis and control dogs without macroscopic evidence of osteoarthritis were frozen and embedded in media.[p] Transverse sections (6 µm thick) were immediately overlaid with 20 µL of rhodamine-labeled β-casein[q] (4 mg/mL in 10mM Tris [pH, 7.8]) or 20 µL of fluorescein isothiocyanate-labeled gelatin[r]

(2 mg/mL in 50mM Tris [pH, 7.6]). Coverslips were applied, and samples were incubated in a humidified chamber at 37°C for 3 hours. Areas of proteolytic activity observed during microscopic examination were compared with areas on serial sections inhibited by use of 1,10-phenanthrolene monohydrate. Images were captured by use of a fluorescent microscope[s] and associated analyzer software.[t]

Statistical analysis—Comparison of total protein concentration among groups was performed by use of a Student 2-tailed *t* test with unequal variance.[u] Values of $P < 0.05$ were considered significant.

Results

Animals—Orthopedic examination, cytologic examination of SF, or gross examination of the synovial joint did not yield evidence of arthropathies in any of the 18 clinically normal (control) dogs. All dogs in the control population were sexually intact mixed-breed dogs. Four (22%) of the control dogs were < 1 year old, whereas 8 of 17 (48%) osteoarthritic dogs were < 1 year old (3/11 client-owned dogs and 5/6 dogs with MPS VII).

Cytologic examination was performed on SF samples obtained from 9 of 17 osteoarthritic dogs to rule out evidence of suppurative arthropathy. Of 11 client-owned dogs, all underwent arthrotomy or arthroscopy for surgical treatment of the primary disease. All of these dogs had DJD secondary to various conditions, including 3 with dysplasia of the elbow joint (2 of which had bilateral disease), 3 with rupture of the cranial cruciate ligament (including 1 with a concurrent torn medial meniscus), 3 with osteochondrosis dissecans, 1 with hip dysplasia, and 1 with histiocytic sarcoma of the stifle joint.

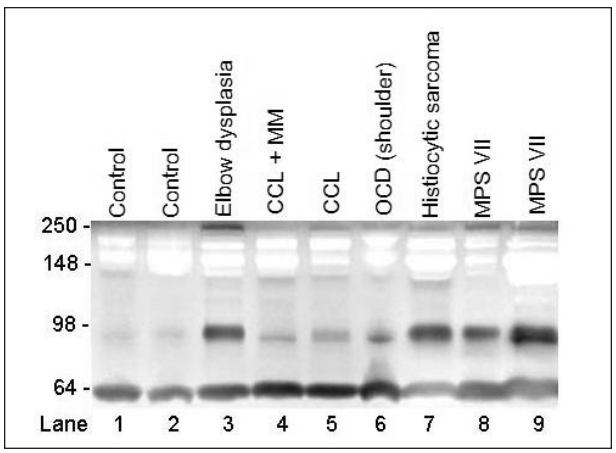

Figure 1—Zymogram of representative activity of matrix metalloproteinase (MMP)-9 and -2 in synovial fluid (SF) obtained from joints of dogs. Equivalent amounts of total protein (60 µg/lane) from SF of 2 dogs with healthy joints (control; lanes 1 and 2) and 7 dogs with osteoarthritis secondary to various disease states (lanes 3 to 9) were electrophoresed in gelatin containing polyacrylamide gel and developed for visual examination. Osteoarthritic dogs are classified on the basis of the underlying pathologic condition of the joint from which the SF was obtained (lane 3, elbow dysplasia; lane 4, ruptured cranial cruciate ligament [CCL] with a torn medial meniscus [MM]; lane 5, ruptured CCL without a torn MM; lane 6, osteochondritis dissecans [OCD]; lane 7, histiocytic sarcoma of the stifle joint; and lanes 8 and 9, mucopolysaccharidosis VII [MPS VII]). Values for molecular weight standards are indicated on the left side of the figure (64, 98, 148, and 250 kd, respectively). Notice the prominent bands of gelatinolytic activity at approximately 62 (ie, MMP-2), 92 (MMP-9), and 225 kd. To enhance printed visibility, all data represent black-to-white inversion of the digital images such that proteinases appear as dark bands on a pale background.

Total protein concentrations in SF samples—Total protein concentrations in SF were significantly ($P < 0.001$) higher in the 17 osteoarthritic dogs (mean ± SD, 27.1 ± 8.1 µg/µL), compared with concentrations for the 18 control dogs (16.1 ± 8.8 µg/µL). There was not a significant ($P = 0.6$) difference in protein concentration in SF obtained from osteoarthritic dogs with MPS VII, compared with concentrations in the client-owned dogs that had DJD attributable to other pathologic conditions.

MMP activity in SF samples—To determine whether MMP activity in SF of osteoarthritic dogs differed from MMP activity in clinically normal dogs, SF samples were subjected to polyacrylamide gel electrophoresis under nonreducing conditions. Zymographic patterns were observed in SF from clinically normal and osteoarthritic dogs (**Fig 1**). A band of enzyme activity at approximately 62 kd was detected in SF from all samples (joints of osteoarthritic and control dogs), whereas a band of enzyme activity at approximately 92 kd was more prominent in SF samples from osteoarthritic joints. Although all samples were compared by use of gelatin zymography in which equal concentrations of total protein were loaded in each lane, loading equivalent volumes of SF per lane did not alter our conclusion that the band of activity at approximately 92 kd was greater in samples from osteoarthritic joints (data not shown). When samples were subjected to zymography by use of β-casein (a substrate designed to highlight activity of serine proteinases), we did not detect substantial bands (data not shown). In addition, activity for degradation of gelatin and β-casein attributable to other MMPs (collagenase or stromelysin) was not apparent on our zymograms (data not shown).

Immunoblots incubated with antisera developed against MMP-2 and -9 confirmed the identity of the bands at approximately 62 and 92 kd, respectively (**Fig 2**). The band at approximately 62 kd in the immunoblots was consistent with the reported size for activated canine MMP-2, and the band at approximately 92 kd was consistent with the reported size for latent, canine MMP-9.[14-18] Purified human antigen controls for latent MMP-2 (68 kd) and latent MMP-9 (92 kd) confirmed the identity of the immunoreactive bands. Incubation of SF with **aminophenylmercuric acetate (APMA)**, an agent used to activate latent forms of MMP, resulted in conversion of the 92-kd band to a doublet of approximately 92 and 88 kd, confirming that the increase in MMP-9 activity in SF from osteoarthritic joints was mostly latent metalloproteinase (**Fig 3**). In contrast, APMA activation of the single band believed to represent the activated form of MMP-2 did not result in conversion to a doublet, confirming that the MMP-2 was already in the active form. Therefore, these bands represented active MMP-2 and latent MMP-9, respectively, as determined on the basis of the apparent molecular weight, specificity for the gelatin substrate, sensitivity to EDTA (data not shown), antigen recognition, and results for APMA activation.

Degree of gelatin degradation in the zymograms was subjectively scored as absent, trace, moderate, and marked. Samples from control and osteoarthritic dogs contained moderate or marked activity attributable to MMP-2; however, a higher percentage of SF samples from osteoarthritic joints had marked activity (**Fig 4**). In contrast, amounts of MMP-9 activity were absent or barely detectable in control samples but were clearly increased in SF from osteoarthritic joints (Fig 1). Underlying disease did not appear to affect the MMPs detected, and there did not appear to be an association between the amount of MMP activity in the SF and age or sex of the dogs. Even though some control samples contained trace amounts of MMP-9 activity, the highest amounts detected in the control samples did not exceed the lowest amounts evident in SF obtained from osteoarthritic joints. Samples of SF that had marked MMP-9 activity (band at approx 92 kd) also had distinct bands of activity at approximately 125 and 225 kd.

Although SF from several dogs that had torn cranial cruciate ligaments, dysplasia of the elbow joint, and DJD secondary to MPS VII was analyzed, a consistent pattern did not emerge to correlate activity of MMP-9 with a specific primary disease. Thus, increased amounts of MMP-9 activity were correlated with osteoarthritis, but absolute amounts of MMP-9 activity varied among affected dogs (Fig 1).

Gelatinase activity in SF obtained during the progression of DJD—Samples were obtained from both elbow joints of a male Bassett Hound with bilateral dysplasia at intervals of approximately 3 months; these samples were used to examine the effect of progression of DJD over time on gelatinase activity in SF. At the same time points, arthroscopy was performed to correlate disease progression in this dog with MMP activity in SF. The MMP activity in SF obtained from both

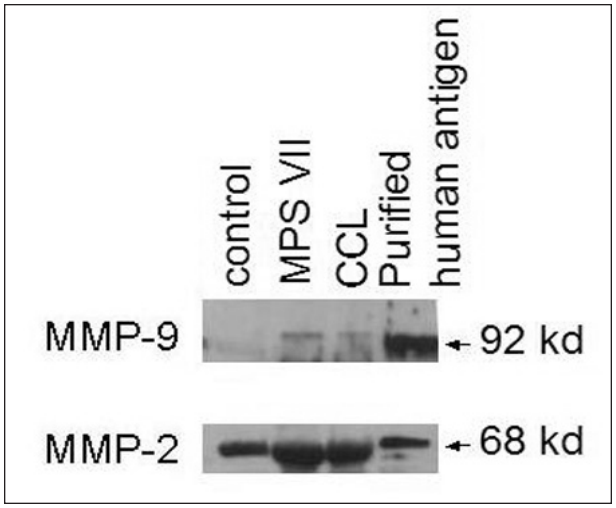

Figure 2—Immunoblot of MMP-9 and -2 in SF samples obtained from healthy and osteoarthritic joints of dogs. Equivalent amounts of total protein (60 and 30 μg/lane for MMP-9 and -2, respectively) in SF were subjected to immunoblot analysis by incubation with polyclonal antibodies raised against human recombinant MMP-9 (top panel) and human recombinant MMP-2 (bottom panel). A sample of 0.5 μg of the corresponding purified human antigen for latent MMP-2 (68 kd) and latent MMP-9 (92 kd) was included on each immunoblot as a control sample. Values for molecular weight standards are provided on the right. *See* Figure 1 for key.

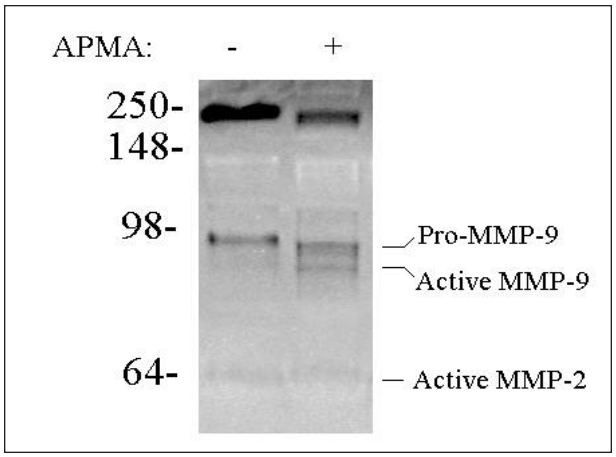

Figure 3—Latency status of gelatinases in SF obtained from an osteoarthritic joint of a dog with MPS VII. Prior to zymographic analysis, the SF was incubated without (–) or with (+) 5mM aminophenylmercuric acetate (APMA) for 30 minutes at 37°C to activate latent proteinases. Samples were then electrophoresed in gelatin containing polyacrylamide gel and developed for visual examination. Values (in kilodaltons) for molecular weight standards are provided on the left. Pro-MMP-9 = Latent form of MMP-9.

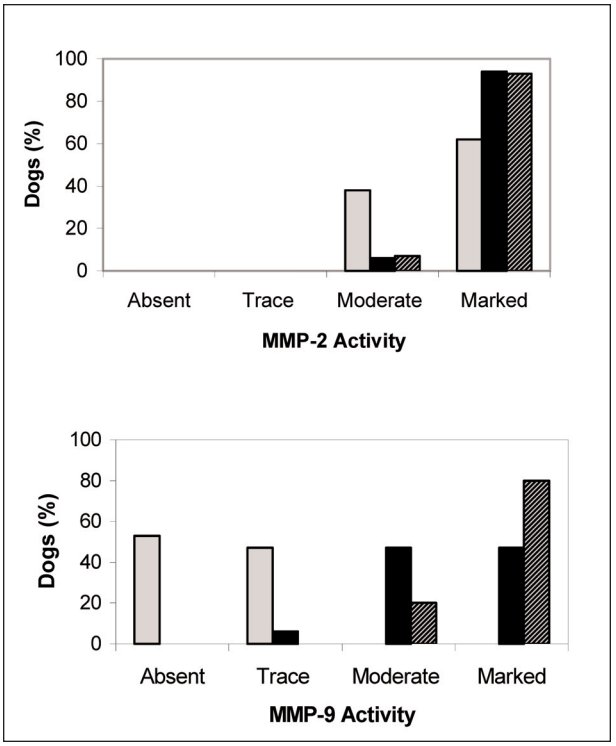

Figure 4—Graphic representation of the distribution of MMP-2 (top) and MMP-9 (bottom) activity in SF samples obtained from joints of clinically normal dogs (control; gray bars), client-owned dogs with osteoarthritis attributable to various conditions (black bars), and dogs with osteoarthritis attributable to MPS VII (diagonal-striped bars). Equal amounts of total protein were assayed under nonreducing conditions, and degree of gelatin degradation at specific molecular weight sizes was scored for MMP-9 and -2 activities as follows: absent, no detectable clear zone in the Coomassie blue-stained gelatin substrate; mild, faint clear zone in the gelatin substrate; moderate, increased clear zone in the gelatin substrate; and marked, distinct clear zone in the gelatin substrate.

elbow joints in this dog was measured by use of gelatin zymography (**Fig 5**). At each time point, high amounts of MMP-9 activity (band at 92 kd) were detected, which contrasted with results for a representative sample of SF obtained from a clinically normal dog. However, there did not appear to be a correlation between increased MMP-9 activity within the SF samples and disease progression within the joints (**Fig 6**).

Arthroscopy of the right dysplastic elbow joint confirmed incongruity of the joint and a **fragmented medial coronoid process (FMCP)** that had been suspected based on imaging studies (Fig 6). The elbow joint did not have evidence of osteochondrosis dissecans or an ununited anconeal process. Examination of arthroscopic images revealed exposure of subchondral bone at the FMCP and an increase in synovitis and cartilage fibrillation over time. Despite progression of synovitis and articular cartilage degeneration seen in these arthroscopic images, high amounts of MMP-9 activity in SF obtained from this joint did not continue to increase over time. Arthroscopic examination of the left elbow joint of the same dog did not reveal evidence of abnormalities in the region of the medial coronoid process of the ulna or lesions indicative of osteochondrosis dessicans. Mild degenerative changes (cartilage fibrillation and synovial hypertrophy) were detected during the arthroscopic examinations performed at 8 and 11 months of age. These degenerative changes had progressed substantially by 16 months of age. The MMP-9 activity was increased in the SF sample obtained at 16 months, compared with the value for the sample obtained at 11 months. However, it should be mentioned that MMP activity at 16 months was similar to that detected at 8 months (Fig 5).

MMP activity in synovial membranes—In situ MMP activity in the synovial membranes of clinically normal dogs and dogs with DJD was measured by use of a fluorescent gelatin-substrate-overlay technique. This assay exploits the fact that fluorescence of the substrate is quenched when in its native conformation, but cleavage by proteinases in the tissue sections causes the substrate to fluoresce.

Representative images of synovial membranes were obtained (**Fig 7**). Images of synovium obtained from a control dog did not have gross evidence of DJD and revealed minimal detectable MMP activity in the synovial membrane. In contrast, dogs with DJD had high MMP activity at the surface of the synovial membranes, within synoviocytes, and, to a lesser degree, within the extracellular matrix of the underlying connective tissue. A general MMP inhibitor (ie, 1,10-phenanthrolene) included during the incubation of the tissue with the labeled substrate dramatically decreased activity in these sections, confirming that fluorescence was attributable to MMP activity. A similar experiment conducted by use of rhodamine-labeled β-casein as a substrate did not reveal appreciable enzyme activity in synovial membranes obtained from clinically normal dogs or dogs affected with osteoarthritis, suggesting that serine proteinases in synovial membranes did not play a major role in the diseased membranes (data not shown).

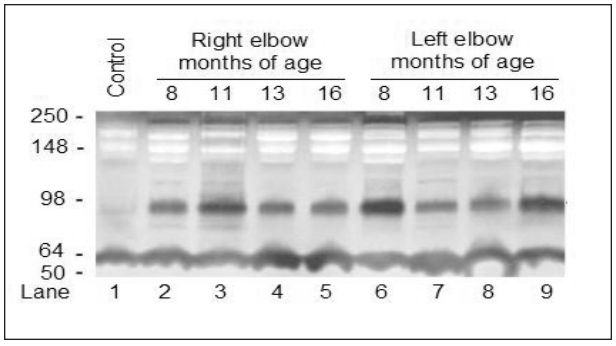

Figure 5—Zymographic analysis of MMP-9 and -2 activity in SF obtained during serial examinations of a Bassett Hound with bilateral incongruity of the elbow joints, a fragmented medial coronoid process (FMCP) of the right ulna, and an ununited anconeal process of the left ulna. Zymographic analysis was used to evaluate SF samples obtained from each elbow joint when the dog was 8, 11, 13, and 16 months old and an SF sample from a healthy dog (control, lane 1). Molecular weight standards are indicated on the left side of the figure (50, 64, 98, 148, and 250 kd, respectively). Notice the prominent bands of gelatinolytic activity at approximately 62 (ie, MMP-2), 92 (MMP-9), and 225 kd.

Discussion

Analysis of results of the study reported here supports the hypothesis that there are higher activities of gelatinases in SF collected from joints of dogs with DJD than in SF obtained from healthy joints, indicating important parallels between osteoarthritis in humans and dogs.[19-22] Although MMPs have been implicated in a multitude of pathologic conditions, they have had limited use as prognostic markers, because most stud-

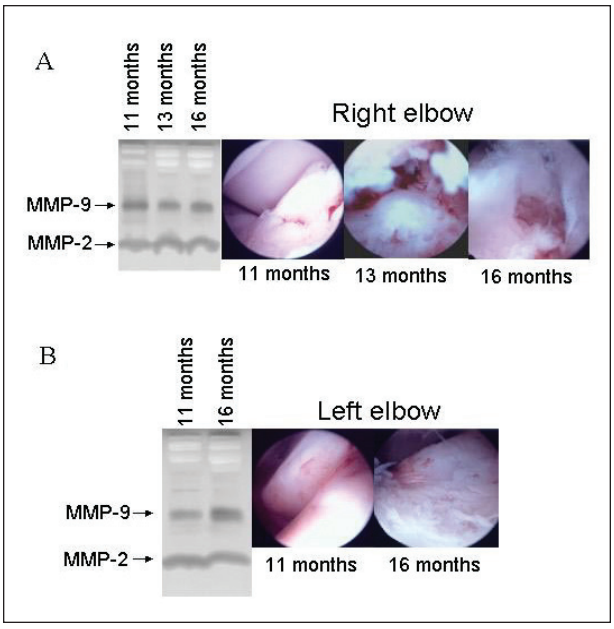

Figure 6—Comparison of gelatinase activity in samples of SF and gross pathologic changes evident during arthroscopic examination of the dysplastic right (A) and left (B) elbow joints of a Bassett Hound over time. Notice exposure of the subchondral bone at the FMCP in the right elbow joint. Arthroscopic images of the left elbow joint do not reveal evidence of abnormalities in the region of the medial coronoid process of the ulna or lesions of osteochondrosis desiccans but do reveal increasing synovitis and cartilage fibrillation over time, similar to that seen in the right elbow joint.

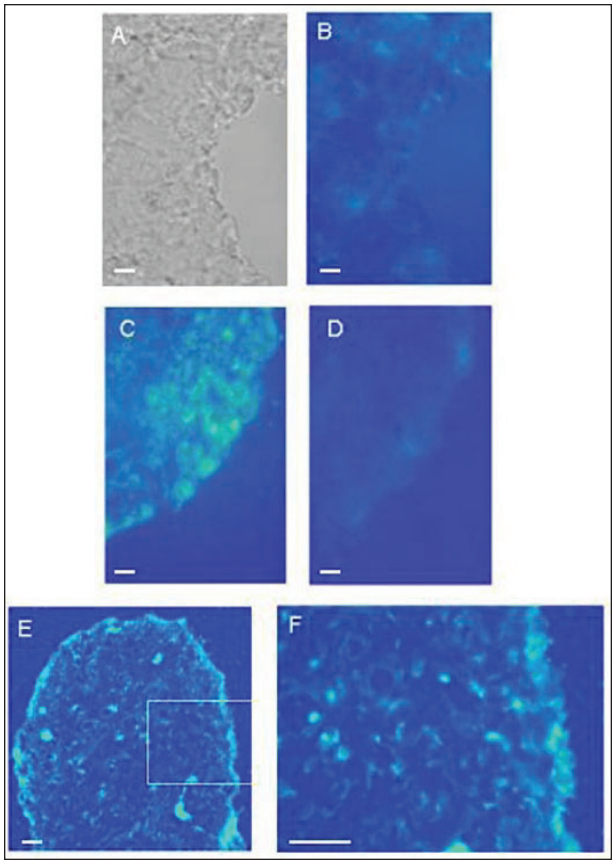

Figure 7—In situ metalloproteinase activity in sections of synovial membranes obtained from the stifle joints of a dog that did not have gross evidence of joint disease (panels A and B) and from dogs with degenerative joint disease (DJD) secondary to MPS VII (panels C and D) and a ruptured CCL (panels E and F). In situ proteinase activity is evident as fluorescence in intact fresh-frozen sections of synovial membranes. Fluorescein isothiocyanate-labeled gelatin is converted into its fluorescent form following digestion by proteinases within the tissue sections. These areas of proteolytic activity were captured by use of fluorescence microscopy. Notice that phase (A) and fluorescent (B) images from healthy synovial membranes have little MMP activity in synovial lining cells or deeper tissues. In contrast, proteinase activity is seen in synovial lining cells and deeper tissues of both dogs with DJD. Proteinase activity was confirmed to be metalloproteinases by the dramatic decrease in fluorescence seen with the addition of a general MMP inhibitor (1,10-phenanthrolene) to the tissue section during incubation of the tissue (D), compared with fluorescence for the same tissue incubated without the inhibitor (C). The area indicated in the inset of panel E is magnified and shown in panel F; notice that the MMP activity in synoviocytes and underlying extracellular matrix can be seen in this magnified view. Bar = 50 µm.

ies address expression of the protein or mRNA, which is not necessarily indicative of the activity of MMPs. To obtain strict quantitative information amenable to statistical analysis, MMP substrate-degradation assays are the analysis method of choice; however, analysis of small sample volumes, such as were obtained from the synovial joints of our control dogs, precluded use of those assays. Zymography provides important information about the identity of the proteinases and the relative amounts of the enzymes, allowing direct comparison of activity between samples, as in the study reported here. However, strict quantitative analysis of gelatin zymography is fraught with problems, including the unavailability of proper canine standards to act as control samples (at the time this study was conducted), the need to establish a linear range of detectable substrate digestion, and the inclusion of inactive enzymes in the quantifiable bands. Even though APMA can be used to activate latent MMPs in a sample before zymographic analysis, it also degrades activated enzymes, thereby precluding accurate quantitation of total enzyme activity in a given sample.[23]

Nevertheless, analysis of our data clearly revealed that a higher percentage of osteoarthritic joints contained marked MMP-2 activity, compared with activity in control joints, whereas latent MMP-9 was dramatically increased in the osteoarthritic state. These data agree with reports describing increased MMP-9 synthesis in osteoarthritic but not normal cartilage from humans[24] and the expression of MMP-2 in normal and arthritic joints in dogs,[25] horses,[26] and humans.[8,27,28]

As would be expected with inflammation, protein concentrations in SF obtained from osteoarthritic dogs were significantly higher than those in SF obtained from control dogs. Although gelatin zymography performed in the study was adjusted on the basis of equal amounts of total protein, equivolume loading did not change any of our conclusions. Because total protein concentrations were significantly greater in dogs with DJD, compared with concentrations in control dogs, equivolume loading would have increased the amount of osteoarthritic SF loaded per well; therefore, equivolume loading may have enhanced differences in gelatinase activity between the 2 groups.

Analysis of our data suggests that reporting results adjusted on the basis of equal amounts of total protein may, in fact, underestimate differences in MMP-9 activity between SF obtained from control and osteoarthritic dogs. In addition, this study revealed a dramatic increase in gelatinase activity in synovial membranes obtained from osteoarthritic dogs, compared with values for samples from clinically normal dogs, by use of a new technique not previously used to determine gelatinase activity in canine synovial membranes.

A higher percentage of samples from osteoarthritic dogs had marked MMP-2 activity relative to samples from control dogs (Fig 4). Similarly, equine MMP-2 activity and human MMP-2 mRNA were increased in samples from osteoarthritic animals, compared with values for samples from control animals,[24,26,29] but MMP-2 activity was not increased in SF samples obtained from dogs with rheumatoid arthritis.[25] Matrix metalloproteinase-2 has been implicated as having a homeostatic role in normal joints, and it also functions in removal of abnormal wear products from the extracellular matrix.[24,27,30]

Along with the approximately 92-kd band for MMP-9, we detected bands of approximately 125 and 225 kd in SF from osteoarthritic joints (Fig 1, 5, and 6). Investigators in another study[31] identified a band of 110 to 130 kd in human samples, which represents MMP-9 complexed with TIMP-1; this band can be seen in some of the SF samples we obtained from osteoarthritic joints. It has been proposed that a band of 225 to 240 kd in equine and canine SF is a dimer of MMP-9 formed when the MMP-9 concentration is in

excess of the TIMP-1 concentration.[25,29,31] Formation of this dimer may reflect controlled activation of MMP-9, because once the dimer has been formed, it can be more readily activated by stromelysin, unlike the monomer of 110 to 130 kd for the complex of MMP-9 and TIMP.[31] Even though detection of the band at approximately 225 kd indicates that the mechanism for activation of MMP-9 was favored in the osteoarthritic dogs of our report, the inconsistent detection of the band at 110 to 130 kd for the complex of MMP-9 and TIMP that represents the classic storage form of inactivated MMP-9 further underscores the aberrant activation mechanism in SF obtained from dogs with osteoarthritis.

Within joints of horses, MMP-2 and -9 are secreted by synovial fibroblasts and chondrocytes, and MMP-9 is also secreted by blood monocytes and polymorphonuclear cells in animals with chronic synovitis and rheumatoid arthritis.[29,32-34] Even though most of the soluble MMP-9 found in SF from osteoarthritic joints is in the latent form, the increase in production of this gelatinase associated with the osteoarthritic state results in more gelatinase available for activation. Most likely, MMP-9 released by subsynovial cells is soluble and free to diffuse to structures, such as the extracellular matrix, underlying the synovial membrane,[35] as suggested by analysis of data for our gelatinase overlay (Fig 7). This soluble MMP-9 could then be activated by various proteinases, including MMP-1, -2, -3, and -7, as well as trypsin, tissue kallikrein, and cathepsin G.[36,37]

To characterize MMP activity in SF obtained from osteoarthritic dogs, we selected dogs with DJD secondary to various underlying pathologic conditions. We recorded variability of the magnitude of MMP-9 activity between dogs with the same underlying disease process and, therefore, did not see a direct relationship between the specific underlying cause of the DJD. The MMP-9 activity within a joint likely reflects the overall amount of inflammation within that joint at any given time and may vary, despite the development of more permanent, progressive changes within the joint.

Although SF from osteoarthritic dogs consistently had increases in MMP-9 activity, compared with activity in SF from control dogs, MMP-9 activity did not increase over time in conjunction with progression of disease in the Basset Hound that had dysplasia of the elbow joints. However, conclusions based on MMP data for the SF obtained from 2 joints of only 1 dog must be interpreted with caution. Other investigators[22] found a correlation between damage to osteoarthritic cartilage detected during arthroscopy and the activity of serine proteinases and TIMP-1 in SF obtained from the knee in humans. Analysis of these data suggests that regulation of serine proteases may differ from that for gelatinases and underscores the necessity for more comprehensive study of gelatinase activity during disease progression in dogs with osteoarthritis.

Evidence for induction of other MMP species (collagenases or stromelysin) was not found for any of the underlying disease processes, as determined on the basis of a lack of appropriate-sized bands on the zymograms. Furthermore, we did not see any association between age or sex and the pattern of gelatinase activity in the SF. Using a fluorogenic substrate that is degradable by many classes of MMPs, investigators in another study[38] found that MMP activity in SF obtained from normal joints of adult horses was not correlated with age once maturity was attained; however, SF obtained from joints of juvenile horses contained significantly higher MMP activity than did SF obtained from the adult horses. Similar to findings in the study reported here, investigators in that study[38] found that SF from osteoarthritic adult horses had increased MMP activity, compared with values for clinically normal adult horses. Brama et al[38] hypothesized that the gradual decrease in MMP activity in clinically normal maturing horses reflected a decline in metabolic activity resulting from cessation of growth and the accompanying decrease in cartilage remodeling. Discrepancies between data for immature dogs and horses could reflect differences in the amount of athletic activity, size differences between the 2 species, or differences in the methods used, because larger volumes of SF could be obtained from the joints of horses, which allowed quantitative assays for a broader range of MMP species.

Several difficulties exist when performing a prospective study to examine the importance of gelatinase activity in DJD of animals examined at a veterinary teaching hospital. Chronicity of the arthritis at the time of initial examination may vary, and it may not be possible to control the medications administered to treat DJD prior to the initial examination. All dogs in the study reported here had evidence of lameness for at least 1 month prior to initial examination at our facility, a time frame during which substantial changes can be detected in a number of SF biomarkers, including MMPs.[21,39-44] In addition, approximately a third of our osteoarthritic dogs had been treated with nonsteroidal anti-inflammatory agents (4 with carprofen[v] and 1 with aspirin on an as-needed basis) during the 6-week period prior to the study. There did not appear to be an obvious correlation between gelatinase activity and the use of anti-inflammatory medications in this study, and analysis of results of other studies[45,46] suggests that use of such medications is unlikely to influence results reported in our study. Although carprofen can reduce the progression of early structural changes in animals with experimentally induced osteoarthritis, there is no effect on the amount of collagenase and general MMP activity in osteoarthritic cartilage, compared with values for placebo-treated dogs.[45,46] Because of the large variation in the specific type of medication as well as the duration of administration, effects of these agents on gelatinase activity in SF could not be specifically analyzed. Development of therapeutic agents with increased specificity against specific MMP activity will likely lead to the development of new treatment options.

To the authors' knowledge, the study reported here is the first investigation implicating a role for gelatinases in dogs with osteoarthritis, regardless of the underlying cause of DJD. The increase in MMP-9 activity parallels that in horses with osteoarthritis.[26,29,47] In the study reported here, healthy joints contained low amounts of gelatinase-degrading activity (SF and syn-

ovial membrane); in contrast, osteoarthritic joints consistently had higher amounts of gelatinase activity. These results support the hypothesis that activation of gelatinases is coincident with joint disease and unrelated to the underlying cause of the DJD. Therefore, a common mechanism of MMP induction during the disease state provides potential therapeutic targets to halt or diminish the progression of DJD.

[a]2.7-mm Storz rigid arthroscope, Karl Storz, Goleta, Calif.
[b]Biorad microcentrifuge, Bio-Rad Laboratories, Hercules, Calif.
[c]Modified Lowry protein assay, Pierce Endogen, Rockford, Ill.
[d]12% Tris-glycine precast gel containing 0.1% of gelatin or β-casein, Invitrogen, Carlsbad, Calif.
[e]SeeBlue 2 prestained molecular weight standards, Invitrogen, Carlsbad, Calif.
[f]Phase III Bioimager, Phase III, Malvern, Pa.
[g]Adobe Photoshop 5.5,Adobe Systems Inc, San Jose, Cal.
[h]8–16% Tris-glycine gradient gels, Invitrogen, Carlsbad, Calif.
[i]Purified human MMP-2, Chemicon International Inc, Temecula, Calif.
[j]Purified human MMP-9, Chemicon International Inc, Temecula, Calif.
[k]Protran nitrocellulose membrane, Schleicher & Schuell, Keene, NH.
[l]Rabbit anti-human MMP-2 polyclonal antibodies, Chemicon International Inc, Temecula, Calif.
[m]Rabbit anti-human MMP-9 polyclonal antibodies, Chemicon International Inc, Temecula, Calif.
[n]Luminol reagent, Santa Cruz Biotechnology, Santa Cruz, Calif.
[o]X-Omat Blue film, VWR, Bridgeport, NJ.
[p]OCT media, Sakura Finetek, Torrance, Calif.
[q]Rhodamine-labeled β-casein, EnzChek gelatinase/collagenase assay kit, Molecular Probes, Eugene, Ore.
[r]Fluorescein isothiocyanate-labeled gelatin, EnzChek gelatinase/collagenase assay kit, Molecular Probes, Eugene, Ore.
[s]Nikon E600, Nikon Inc, Melville, NY.
[t]Phase III Image Pro analyzer software, Phase III, Malvern, Pa.
[u]Microsoft Excel, Microsoft Corp, Redmond, Wash.
[v]Rimadyl, Pfizer Animal Health, Exton, Pa.

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