Osteoarthritis, also known as degenerative joint disease, is the end result of many articular disorders. It is a common arthropathy with radiographic features that include periosteal proliferation, deformity of the subchondral bone, and narrowing of joint spaces, mainly as a result of damage to articular cartilage. These changes are irreversible,1 are progressive, and result from joint instability or subluxation. The current treatment of osteoarthritis is aimed at slowing disease progression through alteration of pathobiological pathways.

Polysulfated glycosaminoglycan is a slow-acting disease-modifying agent that has been shown experimentally to slow and clinically ameliorate osteoarthritis.2-4 Polysulfated glycosaminoglycan is a semisynthetic polysulfated chondroitin sulfate (repeating sulfated residues of β, D-glucuronic acid and α, D-N-acetylglalactosamine linked by glycosidic β 1, 3 bonds) with approximately 3.25 sulfate groups/repeating disaccharide unit. The anionic charge and conformation of the drug are thought to enable its interaction with the active moiety in MMPs and thereby inhibit the activity of these enzymes. Results of in vitro and in vivo experiments have revealed the ability of supplemental PSGAG to diminish matrix molecule degradation3,5 and, in some studies, to enhance matrix molecule synthesis.6 The complete mechanism of the disease-modifying effects of PSGAG is not fully understood.

Cartilage oligomeric matrix protein is a macromolecule distributed abundantly in cartilage,7,8 synovi-
Cartilage oligomeric matrix protein is pentameric, consists of 5 globular domains that are attached to a central assembly domain by flexible strands, and belongs to the thrombospondin family. Fragments of COMP have been detected in the diseased cartilage, synovial fluid, and serum of patients with osteoarthritis and rheumatoid arthritis. We have already reported that synovial fluid COMP concentration in dogs with naturally developing and experimentally induced arthropathy is higher than in dogs with normal joints and measurement of serum COMP concentration can be useful when differentiating among arthropathies in dogs. Matrix metalloproteinases are fundamental to the pathogenesis of joint disease and the breakdown of articular cartilage matrix, which occurs in diseases such as osteoarthritis and rheumatoid arthritis and is thought to occur primarily as a result of enzymatic degradation. Purified COMP is a substrate for MMPs including interstitial collagenase (MMP-1), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), and gelatinase (MMP-2, and -9). Matrix metalloproteinase-2 and -9 have been examined for their possible role in joint disease in humans, dogs, and horses. Matrix metalloproteinase-2 and -9 exert a degradative action on collagens and proteoglycans in cartilage matrix and are able to degrade aggrecan in a similar manner to other MMPs, as well as cartilage link protein. Matrix metalloproteinase-2 and -9 concentrations are increased in synovial fluid from diseased joints. Matrix metalloproteinase-9 is produced by chondrocytes, peripheral blood monocytes, and neutrophils, and MMP-2 is produced by chondrocytes and synovial fibroblasts. Thus, MMP-2 and -9 are potential targets for therapeutic investigation in the treatment of joint disease. The purpose of the study reported here was to investigate the effects of treatment with PSGAG on serum COMP and CRP concentrations, serum MMP-2 and -9 activities, and lameness in dogs with arthropathy.

Materials and Methods

Animals and sample collection—Polysulfated glycosaminoglycan (5 mg/kg) was administered IM to 16 privately owned dogs that were brought to the Veterinary Teaching Hospital of Kagoshima University because of arthropathy (ostearthrosis) and had chronic lameness and osteophytes on radiographic evaluation of the affected joint and also to 5 clinically normal control dogs in our laboratory. Informed consent was obtained from owners prior to the use of dogs in the study. Polysulfated glycosaminoglycan administration was performed twice a week for 4 weeks for a total of 8 treatments. Blood samples were obtained just before PSGAG administration and the day after the final administration. After centrifugation, serum samples were stored at −80°C until analysis. Also, all dogs were subjected to physical examination, observation of limb use, and radiographic examination. The study was approved by the Kagoshima University Animal Care and Use Committee.

Lameness score—All dogs were evaluated for lameness and weight bearing on the basis of criteria reported previously with some modifications. The lameness scale was as follows: 1 = standing with weight bearing and walking normally; 2 = standing with weight bearing normally and slight lameness while walking; 3 = standing with weight bearing normally and severe lameness while walking; 4 = incomplete weight bearing at standing and lameness while walking; and 5 = non-weight bearing at standing and also during walking.

Preparation of canine COMP—Canine COMP was purified by use of a method described elsewhere. Briefly, 10 g (wet weight) of frozen cartilage slices that was obtained from normal hip joints of an adult dog euthanatized for reasons unrelated to orthopedic disease was milled in liquid nitrogen to obtain a fine particulate. Powdered tissue were suspended in 10 volumes of 4M guanidine hydrochloride in 50mM sodium acetate buffer (pH, 5.8) containing proteinase inhibitors (10mM EDTA, 100mM 6-aminohexanoic acid, 5mM benzamidine HCl, and 5mM N-ethylmaleimide), homogenized, and extracted at 4°C for 24 hours with gentle stirring. After centrifugation (17,000 g at 4°C for 30 minutes), supernatant was collected, filtered, and dialyzed against a solution containing 7M urea, 20mM Tris HCl (pH, 8.0), 5mM EDTA, 1mM benzamidine, and 2mM N-ethylmaleimide. This solution was applied to an anion-exchange column. Material bound to the column was eluted with a linear gradient of 0 to 1.00M NaCl in the same buffer. A peak fraction eluted between 0.2 and 0.3M NaCl; therefore, those fractions were pooled to provide a COMP-rich fraction, which was dialyzed against distilled water. The preparation was considered to include cartilage COMP on the basis of analysis by use of SDS-PAGE and western immunoblot analysis that used a monoclonal antibody to human cartilage COMP (ie, 17C10). The preparation was subsequently lyophilized and used as a canine COMP standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in accordance with the Laemmli protocol. Nonreduced and reduced COMP molecules were applied to gradient (4% to 12%) gels. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 or electrotransferred onto polyvinyldene difluoride membranes in Tris-glycine buffer (25mM Tris HC1, 192mM glycine [pH, 8.3], and 20% methanol) at 50 V overnight at 4°C. The transferred membrane was blocked and then analyzed by use of a murine monoclonal antibody (ie, 17C10) diluted 1:1,000 in 2% skim milk in Tris-buffered saline (0.9% NaCl) solution containing 0.05% Tween 20. Positive binding was detected by use of alkaline phosphatase–conjugated goat anti-mouse IgG antibody diluted 1:10,000 in 2% skim milk in Tris-buffered saline solution containing 0.05% Tween 20 and developed by use of 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium as a substrate.

Serum COMP analysis—An inhibition ELISA for canine COMP was performed with the method described elsewhere. Fifty microliters of purified canine COMP antigen in a coating buffer (20mM sodium carbonate, 20mM sodium bicarbonate, sodium azide [pH, 10]) was placed into each well of 96-well ELISA plates, incubated for 2 hours at room
temperature (approx 25°C), and incubated overnight at 4°C. Canine COMP standards were prepared as serial 2-fold dilutions in PBS solution containing 0.05% Tween 20 (pH, 7.0). Samples of serum were diluted in the same buffer. Aliquots (70 µL) of diluted standards (final dilutions, 40,000 to 156.3 ng/mL) and serum samples (final dilutions, 1:10 and 1:20) were mixed with the same volume of monoclonal antibody 17C10 (final dilution, 1:20,000 in PBS solution containing 0.05% Tween 20 [pH, 7.0]) and then incubated overnight at 4°C.

Coated wells were washed, and 100 µL of the inhibition mixture was added to each well. Plates were incubated for 1 hour at room temperature and then for 1 hour at 4°C. After incubation, plates were washed and 100 µL of alkaline phosphatase-conjugated goat antimuscle IgG (diluted 1:10,000 in PBS solution containing 0.05% Tween 20 was added to each well. Plates were incubated again and then washed, and 100 µL of glycine buffer (0.1M glycine, 1mM MgCl₂, and 0.02% NaN₃, pH 10.4) containing 1 mg of p-nitrophenyl phosphate/mL was placed in each well, and the color was allowed to develop for 1 hour. Absorbance was read with an ELISA plate reader at a wavelength of 405 nm. A semilogarithmic standard graph was constructed in which log₁₀ concentrations of COMP were plotted against absorbance value. The concentration of antigenic COMP in serum was calculated with the linear portion of the standard curve corresponding to the reliable range of COMP concentration in the assay.

Gelatin zymography and densitometry—Gelatin zymography was performed by use of a protocol described previously. Serum MMP-2 and -9 activities were analyzed on 7.5% polyacrylamide gels copolymerized with gelatin (1 mg/mL). After electrophoresis of a mixture of 5 µL of serum (diluted 3:100 with distilled water) and 5 µL of zymography buffer (4% sodium dodecyl sulfate, 20% glycerol, 1% bromophenol blue, and 2.5M Tris-HCl [pH, 6.8]), the sodium dodecyl sulfate was removed from the gels by several washes with 2.5M Tris-HCl [pH, 6.8]. Serum MMP-2, 67 kd; pro-MMP-9, 92 kd; and active MMP-9, 83 kd) by use of software packages. Actual measurements for samples were converted to values relative to the MMP-2 and -9 standards. To determine the intra- and interassay variability, fresh aliquots of serum were thawed and 10 measurements were repeated on a single plate (1 gel) for 10 consecutive working days (ie, total of 10 gels).

**Serum CRP analysis**—A commercially available kit was used to measure canine serum CRP concentrations. Thirty microliters of serum was added to 0.5 mL of buffer solution, and an antibody solution was added after a 3-minute incubation. Canine serum CRP concentration was analyzed by use of a light-scattering system.

**Statistical analysis**—All quantitative data were expressed as median (interquartile range) values. Differences in data before and after treatment were analyzed by use of the Wilcoxon signed rank test, and comparisons of data from forelimbs versus hind limbs were analyzed by use of the Mann-Whitney U test. Values of P < 0.05 were considered significant.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Osteoarthritic dogs</th>
<th>Control dogs</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
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<tr>
<td>COMP (µg/mL)</td>
<td>18.32 (7.50–24.23)</td>
<td>13.84 (6.09–22.48)</td>
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<tr>
<td>MMP-2†</td>
<td>1.24 (0.78–2.17)</td>
<td>1.25 (0.42–2.55)</td>
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<tr>
<td>MMP-9†</td>
<td>1.25 (0.77–3.38)</td>
<td>1.48 (0.12–3.28)</td>
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<tr>
<td>LS (1 to 5)</td>
<td>3.2 (1 to 5)</td>
<td>1 (1 to 4)</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.05 (0.0–0.8)</td>
<td>0.10 (0.0–1.05)</td>
</tr>
</tbody>
</table>

*Significant (P < 0.05) difference between osteoarthritic and control dogs before treatment. †Significant (P < 0.05) difference between before and after treatment in osteoarthritic dogs. ‡Values relative to the MMP-2 and -9 standards. LS = Lameness score.
Serum COMP concentration—Serum COMP concentrations in osteoarthritic dogs were significantly (P = 0.019) higher (18.32 [interquartile range, 7.50 to 24.23] µg/mL) than in control dogs (12.06 [10.16 to 14.54] µg/mL) before treatment and decreased significantly (P < 0.001) after PSGAG treatment in osteoarthritic dogs (13.87 [6.09 to 22.48] µg/mL), compared with before treatment (Table 1). Serum COMP concentrations in 7 dogs with forelimb lameness before (20.7 [15.20 to 24.23] µg/mL) and after (16.65 [11.62 to 22.48] µg/mL) treatment were significantly higher than values in 9 dogs with hind limb lameness before (15.05 [7.50 to 23.84] µg/mL; P = 0.009) and after (12.32 [6.09 to 17.36] µg/mL; P = 0.016) treatment (Figure 2). Also, in the 12 osteoarthritic dogs that had an improvement in lameness score, serum COMP concentrations before and after treatment were 16.29 (7.50 to 24.23) µg/mL and 12.52 (6.09 to 17.36) µg/mL, respectively, which represented a significant (P = 0.028) decrease in response to treatment. In the 4 osteoarthritic dogs that had no improvement in lameness score, serum COMP concentrations before and after treatment were 20.98 (15.05 to 23.84) µg/mL and 19.6 (15.20 to 24.23) µg/mL.
adjunctive treatment for osteoarthritis and modifying the progression of degenerative changes in canine hip dysplasia. In our study, an improvement in lameness scores was detected in 12 of 16 osteoarthritic dogs. Improvements in lameness became evident from the fourth administration of PSGAG onward, and no adverse effects were observed.

Cartilage oligomeric matrix protein is a prominent noncollagenous component of cartilage, contributing to approximately 1% of the wet weight of articular tissue. Collagenous structure breakdown in cartilage tissue accompanies degradation of COMP and increases of COMP fragment concentrations in synovial fluid. Fragments of COMP have been detected in diseased cartilage, synovial fluid, and serum of patients with primary osteoarthritis, synovitis, and rheumatoid arthritis. Measurements of synovial fluid and serum COMP concentrations in dogs indicate that COMP concentrations can be used to diagnose cartilage degradation in arthropathy; serum COMP concentrations increase after increases in synovial fluid COMP concentrations in experimentally induced synovitis. In our study, the pretreatment serum COMP concentrations in dogs with osteoarthritis were significantly higher than in control dogs. Serum COMP concentrations in osteoarthritic dogs decreased significantly after PSGAG treatment, along with an improvement in lameness. Also, serum COMP concentrations were significantly higher in dogs with forelimb lameness than in dogs with hind limb lameness, and the response to PSGAG treatment for forelimb lameness was less pronounced than for hind limb lameness. Our results indicate that destruction of collagen in cartilage in osteoarthritic dogs was inhibited by PSGAG administration and that the potential effect of PSGAG would be more evident in hind limb lameness.

Matrix metalloproteinase-2 and -9 are collagenolytic enzymes; MMP-2 is formed constitutively with matrix metalloproteinase-9 activities would provide a useful biomarker of changes in cartilage matrix turnover after treatment. Matrix metalloproteinase-9 processes fragmented matrix components that are induced in response to tissue injury and inflammation. Results of previous studies indicate that MMP-2 and -9 activities in synovial fluid are high in joints with osteoarthritis and rheumatoid arthritis and can be inhibited by PSGAG. However, for our results, only serum MMP-9 activity in dogs with forelimb lameness was significantly higher than in dogs with hind limb lameness after treatment. The finding that serum MMP-9 activity did not decrease after PSGAG treatment indicates that concentrations or activities of other inflammatory mediators (eg, transforming growth factor-β1) continued to be high in affected joints, despite PSGAG treatment. Serum COMP concentration is significantly associated with joint inflammation and synovitis. In our study, although the difference was not significant, the value of serum CRP concentration in dogs with forelimb lameness was higher than in dogs with hind limb lameness before and after treatment. In terms of the proportion of body weight borne by forelimbs and hind limbs in dogs, forelimbs carry a greater load. Therefore, forelimbs might be more susceptible to mechanical stress and joint inflammation and thus more difficult to improve with PSGAG treatment.
Results of our study indicate that PSGAG might be effective for improving cartilage COMP turnover and joint inflammation and that the observed changes reflect serum COMP concentrations. Lameness scores were improved in parallel with changes in serum COMP concentrations. We conclude that IM administration of PSGAG is effective for inhibition of COMP degradation with improvement in lameness and that one of the mechanisms of action of PSGAG is inhibition of COMP degradation.

References


