

Synovial fluid matrix metalloproteinase-2 and -9 activities in dogs suffering from joint disorders

Kohei MURAKAMI¹⁾, Shingo MAEDA¹⁾, Tomohiro YONEZAWA¹⁾ and Naoaki MATSUKI¹⁾*

¹⁾Department of Veterinary Clinical Pathobiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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ABSTRACT. The activity of matrix metalloproteinase (MMP)-2 and MMP-9 in synovial fluids (SF) sampled from dogs with joint disorders was investigated by gelatin zymography and densitometry. Pro-MMP-2 showed similar activity levels in dogs with idiopathic polyarthritis (IPA; $n=17$) or canine rheumatoid arthritis (cRA; $n=4$), and healthy controls ($n=10$). However, dogs with cranial cruciate ligament rupture (CCLR; $n=5$) presented significantly higher pro-MMP-2 activity than IPA and healthy dogs. Meanwhile, dogs with IPA exhibited significantly higher activity of pro- and active MMP-9 than other groups. Activity levels in pro- and active MMP-9 in cRA and CCLR dogs were not significantly different from those in healthy controls. Different patterns of MMP-2 and MMP-9 activity may reflect the differences in the underlying pathological processes.

KEY WORDS: canine rheumatoid arthritis, cranial cruciate ligament rupture, dog, idiopathic polyarthritis, matrix metalloproteinase

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There are two major categories of joint diseases in the dog: inflammatory arthropathies and degenerative diseases [12]. Canine inflammatory arthropathies include primarily bacterial infective arthritis and immune-mediated polyarthropathy, which is a commonly recognized syndrome in dogs. Immune-mediated polyarthropathy is further divided into two broad categories, non-erosive and erosive, based on whether bony destructive changes are present on the radiographs [2]. The most common non-erosive and erosive forms in dogs are, respectively, idiopathic polyarthritis (IPA) and canine rheumatoid arthritis (cRA). The underlying pathogenesis of these disorders is not fully understood. On the other hand, the most common degenerative joint disease in dogs is osteoarthritis, and cranial cruciate ligament rupture (CCLR) is the major cause of osteoarthritis in dogs [7].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases and are produced as inactive zymogens that are activated in physiological conditions by a cascade of proteolytic reactions [29]. MMPs degrade extracellular matrix components and contribute to tissue remodeling. Among MMPs, the gelatinase subfamily, which consists of MMP-2 and MMP-9, can degrade non-matrix proteins, such as cytokines and chemokines, thereby regulating the activity of these proteins [13, 17, 18, 28, 31, 32, 35]. Therefore, MMP-2 and MMP-9 play a crucial role in tissue repair, angiogenesis and, in particular, inflammation [24].

In humans, several types of arthritis are known to increase

MMP-2 and/or MMP-9 in synovial fluid (SF) [9, 19, 25]. In dogs, high levels of SF MMP-2 were observed in CCLR [3, 20, 26], and one report described elevated SF MMP-9 levels in seven dogs with polyarthropathy [5]. However, no study has compared the activity of MMP-2 and MMP-9 in inflammatory (e.g., IPA) versus degenerative (e.g., CCLR) arthropathy or in non-erosive (e.g., IPA) versus erosive (e.g., cRA) polyarthropathy. This study describes the differences in SF MMP-2 and MMP-9 activity in dogs with IPA, cRA or CCLR, as well as in healthy dogs.

Seventeen client-owned dogs with IPA (0.8–13.9 years of age, median 9.8 years; 2.1–9.2 kg body weight, median 4.6 kg; intact males [$n=3$], neutered males [$n=3$], intact females [$n=6$] and spayed females [$n=5$]), four dogs with cRA (3.3–12.7 years of age, median 6.6 years; 2.0–5.0 kg body weight, median 4.5 kg; neutered male [$n=1$], intact female [$n=1$] and spayed females [$n=2$]) and five dogs with CCLR (8.3–14.2 years of age, median 12.4 years; 5.2–31.6 kg body weight, median 13.8 kg; intact male [$n=1$] and spayed females [$n=4$]) were included in the present study. These dogs were referred to the University of Tokyo Veterinary Medical Center between 2012 and 2015. The diagnosis of IPA was made according to a previous description [22]. Definitive diagnosis of cRA was performed if: 1) erosive polyarthropathy was confirmed by synovial fluid examination of multiple joints and by a radiographic assessment of the joints, and 2) infectious arthritis was excluded by cytologic examination and/or bacterial culture of SF. CCLR was diagnosed on the basis of a cranial drawer sign and/or tibial thrust during the initial physical examination. Surgical confirmation was obtained in all dogs with CCLR. As healthy controls, 10 beagle dogs (0.8–8.0 years of age, median 4.0 years; intact males [$n=4$] and intact females [$n=6$]) that had no evidence of disease were included. Dogs that had received glucocorticoid treatment within a week prior to the diagnostic tests were excluded from the study.

*CORRESPONDENCE TO: MATSUKI, N., Department of Veterinary Clinical Pathobiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. e-mail: amki@mail.ecc.u-tokyo.ac.jp

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SF samples were collected at the diagnosis (IPA and cRA) or surgery (CCLR). In dogs with IPA or cRA, SF samples were obtained by arthrocentesis of the affected joints, usually the carpus, the hock and the stifle joints. In dogs with CCLR or healthy controls, SF samples were obtained from the stifle joint. The samples with visible blood contamination were discarded. SF samples were stained with Wright-Giemsa solution for cytological analysis and/or examined by hematology analyzer (PocH-100i; Sysmex, Kobe, Japan). Using these analyses, the samples were confirmed to have increased neutrophil counts in dogs with IPA and cRA or to have no or mild increase of mononuclear cells in dogs with CCLR. The remainder of the SF was centrifuged (4°C, 5,000 × g and 10 min), and the supernatants were stored at -30°C until use. The stored aliquots of SF underwent total protein determination using a Bradford protein assay kit (Protein Assay; Bio-Rad Laboratories, Hercules, CA, U.S.A.).

MMP-2 and MMP-9 activity was determined in SF samples by gelatin substrate zymography as described previously [30], with some alterations. Briefly, gelatin (Wako Pure Chemical Industries, Osaka, Japan) was incorporated into 8% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gels at a final substrate concentration of 2.0 mg/ml. Samples (total protein, 5 µg/lane) were diluted two-fold in loading buffer (500 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS and 0.2% bromophenol blue). A PageRuler Unstained Protein Ladder (molecular weight kDa; Thermo Fisher Scientific, Hertfordshire, UK; 5 µl/lane) was used to determine the size of the activity bands. A commercially available zymography marker (MMP marker; Life Laboratory, Yamagata, Japan; 5 µl/lane) was run on each gel as an inter-assay standard as well as a positive control. Next, duplicated samples were loaded onto the gels and underwent electrophoresis. Following electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 for 1 hr at room temperature and subsequently incubated for 20 hr at 37°C in enzymatic activation buffer (50 mM Tris-HCl, 200 mM NaCl and 5 mM CaCl₂, pH 7.6). The gels were fixed, stained with Coomassie brilliant blue G-250 (2.5 g/l) in distilled water, methanol and acetic acid (4.5: 4.5: 1) for 1 hr and destained in distilled water, methanol and acetic acid (8.75: 0.5: 0.75) until the zones of proteolysis had cleared. The bands were quantified using an image analyzer system (Gel Doc EZ Imager; Bio-Rad Laboratories) and software (Image Labs; Bio-Rad Laboratories). The activity of each band in 5 µl of the standard was defined as 1 arbitrary unit, respectively.

Statistical analysis was performed using a statistical software package (Prism 5; GraphPad Software, La Jolla, CA, U.S.A.). The activity of pro-MMP-2, and pro- and active MMP-9 among the groups was assessed by one-way ANOVA, followed by Tukey-Kramer multiple comparison tests. A value of $P < 0.05$ was considered significant.

All of the SF samples contained prominent gelatinolytic bands with apparent molecular masses of 66 and 92 kDa, which corresponded to pro-MMP-2 and pro-MMP-9, respectively (Fig. 1). In some dogs, there was an additional area of gelatinolysis at approximately 82 kDa, which was assumed to be an active form of MMP-9 based on the molecular weight. There was no obvious band of the active form of

MMP-2 in the present study.

The SF samples were analyzed via densitometry of each of the three bands of activity and converted into arbitrary units (Fig. 2). As for pro-MMP-2, there was no difference in its activity in IPA, cRA and healthy dogs. However, SF pro-MMP-2 activity was significantly higher in CCLR dogs than in IPA dogs and healthy controls (both $P < 0.01$). In IPA dogs, the activity of pro-MMP-9 was significantly increased compared to cRA, CCLR and healthy dogs (all $P < 0.01$). IPA dogs also exhibited significantly higher levels of active MMP-9 than healthy controls ($P < 0.05$). Dogs with cRA showed a slightly higher catalytic activity of pro-MMP-9 than CCLR dogs and healthy controls, but this activity was not significant. The activity of active MMP-9 in cRA dogs was not different from that in other groups. The activity of pro- and active MMP-9 in CCLR dogs was similar to that in healthy controls.

In the present study, the activity of pro-MMP-2 in dogs with IPA was similar to that in healthy controls. However, dogs with IPA showed notably increased activity of latent and active MMP-9 in the SF. MMP-2 is produced by stromal cells in the synovial sublining layer [11], but is known to be relatively unresponsive to most growth factors and cytokines [15]. By contrast, MMP-9 is secreted by neutrophils, macrophages [8] and synovial cells [1], and its expression is induced by inflammatory cytokines [27]. Several inflammatory cytokines, including interleukin-1 β and tumor necrosis factor- α , are implicated in the pathogenesis of IPA [10]. Thus, increased activity of MMP-9 in SF from IPA dogs would reflect increased inflammatory cytokines compared to cRA, CCLR and healthy dogs. Furthermore, because MMP-9 knockout mice show reduced severity of antibody-induced arthritis, MMP-9 is considered to have stimulatory effects on arthritis [14]. Upregulation of MMP-9 in SF of dogs with IPA may be a useful target for additional therapeutic strategies.

MMP-2 and MMP-9 can digest not only denatured collagen (gelatin) and Type-IV collagen but also other extracellular matrix components, including fibrillar collagen I and II [4, 33] and aggrecan, which exist primarily in the cartilage [23]. Therefore, these two enzymes play a key role in joint destruction in human rheumatoid arthritis [34]. Surprisingly, in the present study, we observed similar activity of pro-MMP-2 in dogs with IPA and cRA. In addition, we found significantly lower SF pro-MMP-9 activity in dogs with cRA than in dogs with IPA. These data indicate that MMP-2 and MMP-9 may not contribute directly to the destructive changes in cRA.

Previous studies confirmed increased MMP-2 activity in SF obtained from the joints of CCLR dogs [3, 20]. By contrast, the activity of MMP-9 in SF from CCLR dogs is controversial. Previous reports showed high levels of MMP-9 in SF from CCLR dogs using a commercial human-MMP-9 ELISA kit [26] and increased gene expression of MMP-9 in CCL tissues [20] or in synovial cells in SF [21]. On the other hand, Boland and colleagues recently reported that MMP-9 activity in SF was not significantly different in CCLR-affected joints and stifle joints of normal dogs based on antibody-based enzyme assay [3]. As for MMP-9 activity in SF, our data are consistent

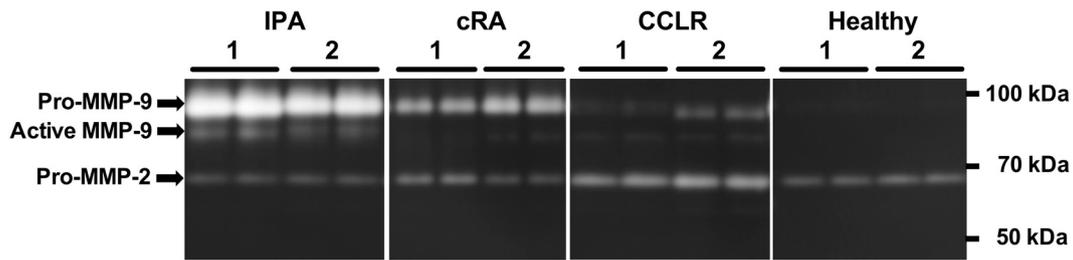


Fig. 1. Representative zymograms of synovial fluid samples from dogs with idiopathic polyarthritis (IPA), canine rheumatoid arthritis (cRA) or cranial cruciate ligament rupture (CCLR) and healthy controls. Each sample was analyzed in duplicate. Three distinct gelatinolytic activities (66, 82 and 92 kDa) were frequently observed in IPA dogs. MMP, matrix metalloproteinase.

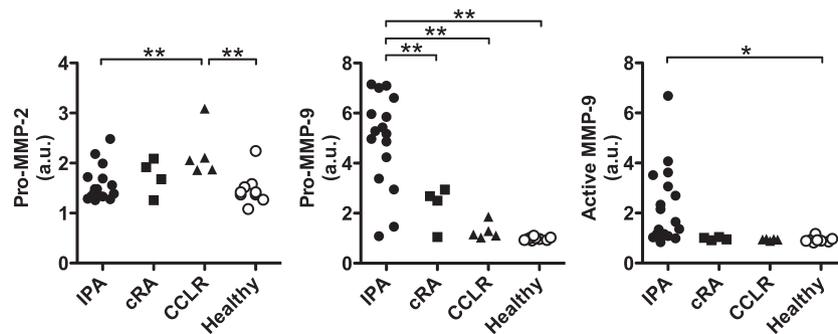


Fig. 2. Comparison of pro-matrix metalloproteinase-2 (pro-MMP-2), pro-MMP-9 and active MMP-9 activity in synovial fluid of idiopathic polyarthritis (IPA) joints, canine rheumatoid arthritis (cRA) joints, cranial cruciate ligament ruptured (CCLR) joints and joints from healthy dogs. * $P < 0.05$, ** $P < 0.01$, Tukey–Kramer multiple comparison test. a.u. arbitrary unit.

with the findings presented by Boland *et al.* The difference of the activity levels of MMP-9 between the current study and the published studies might be based on different measuring methods. In human patients with arthritis, the presence of the latent and activated forms of MMP-9 in SF is reflected in the inflammatory condition of the joints [16]. Although increased proinflammatory cytokines in SF of dogs with CCLR were reported [6], low activity of pro- and active MMP-9 in SF from CCLR dogs indicates that inflammation present in the joints of CCLR dogs was not severe.

The present study had some limitations. First, we investigated only a small number of SF samples. Second, the dogs in the present study were not completely balanced for age, sex and body weight. It was also different in joints that SF samples were obtained from. These differences might influence MMP-2 and MMP-9 activity.

In conclusion, the present study revealed the differences in MMP-2 and MMP-9 activity among canine arthropathies. Our data suggest that MMP-9 in SF was involved in the pathogenesis of cRA and, in particular, of IPA. However, MMP-2 and MMP-9 in SF may not contribute directly to the destructive joint changes seen in cRA.

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