Tartrate-resistant acid phosphatase, matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in early stages of canine osteoarthritis

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ABSTRACT: The aim of this study was to determine if the activity of tartrate-resistant acid phosphatase (TRAP) in the synovial fluid (SF) and serum can be used as a marker for diagnosing the early stages of osteoarthritis (OA). We also wished to determine if identifiable differences in the concentrations of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) could be detected in SF between normal joints and OA joints for the diagnosis of early OA. Ten skeletally mature beagle dogs underwent a unilateral surgical transection of the cranial cruciate ligament and medial meniscectomy. Five sham-operated beagle dogs were used as controls. The synovial fluid was collected in 1, 2 and 3 months and examined by western blotting for MMP-2 and ELISA for TIMP-2. The activity of TRAP in the SF and serum was measured using a spectrophotometer. In addition, the presence of TRAP positive cells in the synovium was identified by enzyme histochemistry. The level of the activity of TRAP and MMP-2 in the SF from the induced OA dogs was significantly higher than that of the control over a three-month period ($P < 0.05$). The TIMP-2 level in the SF was significantly lower in the induced OA dogs than in the control. However, there was no difference in TRAP activity in the serum. Histochemistry revealed a higher number of TRAP positive cells in the synovium from the induced OA dogs. Based on these data, we conclude that the activity of TRAP, MMP-2 and TIMP-2 in SF can be used as a biomarker to diagnose and monitor the early stages of OA.

Keywords: tartrate-resistant acid phosphatase; matrix metalloproteinase-2; tissue inhibitor of matrix metalloproteinase-2; osteoarthritis; synovial fluid

Osteoarthritis (OA) is one of the most common chronic musculoskeletal diseases that are characterized by the degeneration of the articular cartilage and synovitis (Malemud et al., 2003; Rosemann et al., 2007). OA affects 20% of the canine population over one year of age and is a common cause of lameness in dogs (Johnston, 1997).

There is no modifying treatment for OA currently available. Ideally, a treatment for preventing its progression should be applied in the early stage of the disease (Henrotin et al., 2005). However, an early diagnosis of OA is difficult in veterinary and human medicine because the diagnosis is routinely established on the basis of the clinical and radiographic changes that have already occurred in the later stages of the disease. Therefore, various studies have focused on identifying a biomarker which is mostly macromolecule or fragment released from the joint tissue, as indicators of normal biological process and pathogenic process in the serum or synovial

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fluid. They have made numerous attempts to apply this concept for diagnosing the early stage of OA, monitoring the treatment response and determining the prognosis of OA (Malemud and Goldberg, 1999; Hegemann et al., 2002; Budsberg et al., 2006).

Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs), as biomarkers, have been widely used in studies of rheumatoid arthritis (RA) and OA. MMPs are among the proteolytic enzymes that degrade the extracelluar matrix (ECM) of the cartilage and play a major role in the extracelluar matrix (ECM) turnover (Fox and Cook, 2001). TIMPs bind to the active site on the MMPs enzyme to form a 1:1 non-covalent complex that inhibits the activity of MMPs. In a normal joint, there is a slight excess of TIMPs relative to MMPs for the normal turnover and degradation of the cartilage matrix. In OA or RA joint, there is an imbalance of MMPs and TIMPs expression (Pay et al., 2002; Hegemann et al., 2003).

Tartrate-resistant acid phosphatase (TRAP) expressed by osteoclasts, macrophages and dendritic cells has been used for several years as a marker enzyme of bone-resorbing osteoclasts. Recent reports have shown that the synovial intima of OA contains a large number of TRAP-containing cells ($TRAP^+$ cells) such as dendritic cells and macrophage-like cells (Muir et al., 2005). It was demonstrated that TRAP positive cells play an important role in cytokine synthesis, in the expression of MMPs and cartilage damage (Tsuboi et al., 2003; Adamopoulos et al., 2006b). In another study, the synovial fluid of OA contains inflammation cells such as macrophages and osteoclasts and these cells come into direct contact with the cartilage and play a role in the joint destruction (Adamopoulos et al., 2006a). The results of these studies suggest that TRAP positive cells are related to the cartilage destruction and development of OA.

Therefore, we hypothesized that the activity of TRAP in the synovial fluid (SF) and serum may be higher in patients with OA than in normal patients, and can be used as a biomarker for the detection of the early stage of OA. In order to test this hypothesis, the activity of TRAP in the SF and serum from the dogs with induced OA was examined and compared with that from normal dogs. We also wished to determine if identifiable differences in the concentrations of MMP-2 and TIMP-2 could be detected in SF between normal dogs and dogs with induced OA for the diagnosis of early OA.

To our knowledge, there is no report on the use of the activity of TRAP in the SF as a biomarker for OA.

MATERIAL AND METHODS

Experimental animals

Fifteen beagle dogs, aged 1–2 years (average 1.5 ± 0.3 years), weighing 9–11 kg (average 10.1 ± 1.6 kg), were used in this study. The dogs were housed in individual cages and received standard diet and water *ad libitum*.

Ten beagle dogs (experimental group) were given intravenous lactated Ringer's solution (10 ml/kg/h). The surgical area was shaved and a prophylactic antibiotic, cephalexin (Methilexin Inj®, Union Korea Pharm. Co. Ltd., Korea) 25 mg/kg, intravenous therapy (*i.v.*) was administered 1 h before surgery. The experimental animals were premedicated with atropine sulphate (Atropin Sulfate Inj®, Dai Han Pharm. Co. Ltd., Korea) 0.05 mg/kg, *s.c.*, anaesthesia was induced using propofol (Anepol Inj®, Hana Pharm. Co. Ltd., Korea) 6 mg/kg, *i.v.* and was maintained with enflurane and oxygen delivered endotracheally. The experimental animals were positioned on the dorsal recumbency and draped. The right stifle joint arthrotomy was performed. The cranial cruciate ligament transection and medial menisectomy were carried out according to standard procedures. During surgery, care was taken to prevent bleeding and soft tissue damage as much as possible. After surgery, the synovium, fasciae and skin were sutured. The postoperative treatment was administered with butophanol (Butopan Inj®, Hana Pharm. Co. Ltd., Korea) 10 mg/kg, intramuscular therapy (*i.m.*) every 12 h for three days, and cephalexin (Methilexin Inj®, Union Korea Pharm. Co. Ltd., Korea) 25 mg/kg, *i.v.* every 12 h for four days.

Five beagle dogs (control group) underwent a sham procedure in which the stifle joint was fully opened and the cranial cruciate ligament and meniscus were exposed.

Collection of synovial fluid and serum

The synovial fluid was collected from the right stifle joint by arthrocentesis three times in monthly intervals. The largest possible volume of synovial fluid was collected and care was taken to prevent

contamination with blood. The synovial fluid was centrifuged at 4°C for 10 min at 12 000 rpm. The supernatant was stored at –80°C until assayed.

Blood samples were collected three times in monthly intervals. The blood was transferred to sterile screw-capped tubes without anticoagulant, left at the room temperature for the coagulation of blood, and centrifuged for 10 min at 1 500 rpm. The supernatant serum was stored at –80°C until assayed.

Tartrate-resistant acid phosphatase assay in synovial fluid and serum

The activity of TRAP in the stifle synovial fluid and serum was determined using a biochemical assay in 96-well plates, with *p*-nitrophenylphosphatate (pNPP) (Sigma Chemical Co., St Louis) as the substrate (Lang et al., 2001). All the reagents for this assay were purchased from a commercial supplier. In order to reduce the viscosity and improve the pipetting accuracy, the synovial fluid samples were digested with 50 IU/ml of *Streptomyces* hyaluronidase (Sigma Chemical Co., St. Louis) for 30 min at 37°C before the assay. Before the assay, the serum samples were also incubated without *Streptomyces* hyaluronidase for 30 minutes at 37°C. The purpose of incubating the serum and synovial fluid was to inactivate the TRAP activity from the erythrocytes. All the synovial fluid and serum samples were diluted 1:4 in 0.9% NaCl prior to analysis. 0.9% NaCl was used as the negative control. 100 µl synovial fluid and serum samples were added to 100 µl of the reaction mixture so that the final incubation medium contained 2.5mM pNPP (ditris salt), 0.1M sodium acetate buffer, pH 5.8, 0.2M KCl, 0.1% Triton X-100, 10mM sodium tartrate, and the reducing agents ascorbic acid (1mM) and FeCl3 (100M). The activity of TRAP was determined using a 200 µl volume of the final incubation medium per well plate for 1 h at 37°C. The reaction was quenched by adding 50 µl of 0.9M NaOH to each well. The absorbance was measured at 405 nm using a spectrophotometer (Cess UV 90c, Bioteck Co., US). The pNPP was converted to *p*-nitrophenol. The amount of *p*-nitrophenol production was calculated through a comparison with a standard curve for *p*-nitrophenol solutions (Sigma Chemical Co., St Louis). One unit (IU) of TRAP activity was defined as the amount of enzyme required to hydrolyze 1 µmol of pNPP per minute at 37°C. The samples were diluted further and remeasured if the activity of TRAP exceeded the range of the standard curve.

Matrix metalloproteinase-2 assay in the synovial fluid

The synovial fluid containing (40 µg) the total protein was incubated with B-meraptoethanol in a Laemmeli sample buffer (LSB). The resulting fluid was heated to 90°C for 5 min and subjected to SDS polyacrylamide gel electrophoresis on 4–20% tris-glycine gradient gels. The proteins were then transferred to polyvinylidene difluride (PVDF) membranes by electrophoretic transfer in a tank system with plate electrodes. The membranes were blocked with 5% non-fat dried milk dissolved in TBS-Tween for 1 h at 25°C, and then incubated with mouse monoclonal MMP-2 (Chemicon, CA, USA) in TBS Tween in a 1:1 000 volume to volume dilution. After incubation, the membranes were washed four times with 0.1% TBS Tween, and then incubated in a horseradish peroxidase-labelled secondary antibody diluted 1:10 000 in TBS with 0.1% Tween for 1 h at room temperature. After four washes with TBS Tween, the bands of immunoreactivity were developed by adding a pico chemiluminescent substrate (Pierce Biotechnology Inc, Rockford, IL, USA) and exposed to a radiographic film.

Assay of tissue inhibitor of metalloproteinase-2 in the synovial fluid

The synovial fluid samples were assayed to determine the free TIMP-2 and TIMP-2 complexed with the active forms of MMP using a commercially available TIMP-2 Biotrack Assay kit (Amersham Biosciences Inc, Piscataway, NJ, USA) Briefly, 100 µl of the prepared standard and 100 µl of the samples were incubated at 25°C for 1 h with 100 µl of anti-TIMP-2 peroxidase conjugate. The wells were then aspirated, washed and incubated again with teramethylbenzidine for 30 min at 25°C. The reaction was stopped by an addition of 1.0M sulphuric acid, and optic density was measured at 450 nm within 30 minutes. The sample concentrations were determined from a standard curve and are reported as ng/ml.

Histochemical examination of joint tissues for tartrate-resistant acid phosphatase

The experimental animals were sacrificed at 12 weeks to evaluate OA after experimental sur-

gery. The synovium and cartilage from the hind limbs were collected for histochemical staining.

Histochemical staining specific to TRAP was performed on 15 µm frozen sections of all the synovium specimens. Briefly, all the reagents for histochemical staining were obtained from a commercial supplier (Sigma Chemical Co., St. Louis, MO, USA). A solution of naphthol AS-BI phosphate was prepared by dissolving 25 mg of naphthol AS-BI phosphate in 2.5 ml of *n,n*-dimethylformamide to which 40 ml of 0.05M Tris-maleate buffer (pH 5) was added. A solution of hexazotized pararosanaline was prepared by dissolving 80 mg of pararosaniline hydrochloride in 1.6 ml of distilled water to which 0.4 ml of hydrochloric acid was added. This solution was mixed with an equal volume of 4% sodium nitrite immediately before use. The final reaction mixture for histochemical staining was prepared by adding 4 ml of the hexazotized pararosanaline solution together with 50mM sodium-potassium tartrate to the naphthol AS-BI phosphate solution. The final reaction mixture (pH 5) was filtered prior to use. The sections were incubated in the reaction mixture at 37°C for 1 h, rinsed in distilled water, counterstained with Mayer haematoxylin, and mounted. For each batch of slides, a negative control was prepared by omission of the naphthol AS-BI phosphate. All of the synovium specimens were examined by light microscopy to identify the cells containing TRAP.

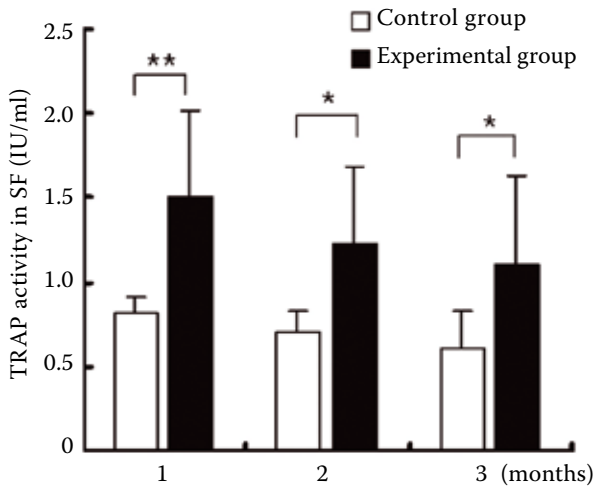

Figure 1. The level of TRAP activity in the synovial fluid from the experimental and control group. Significant differences were observed between the experimental and control group. The data is reported as the mean ± standard deviation (SD). Unpaired Student's *t*-test: significant difference (*$P < 0.05$, **$P < 0.01$)

Calculations and statistics

The Western blots were scanned using digital imaging and the relative intensity of the bands was determined by densitometry (Bio-Rad Laboratories Inc, Hercules, CA, USA). The values obtained were normalized to a minimum value of 100. Student's *t*-test was used to compare the data of the experimental and control joints. *P*-values less than 0.05 were considered significant. The data is expressed as the mean ± standard deviation (SD).

RESULTS

Tartrate-resistant acid phosphatase assay in synovial fluid and serum

The level of TRAP activity in the SF from the experimental group in 1, 2, and 3 months after inducing OA was 1.5 ± 0.50 (IU/ml), 1.23 ± 0.44 (IU/ml) and 1.10 ± 0.51 (IU/ml), respectively. These values were significantly higher than the levels in the control group [0.81 ± 0.10 (IU/ml), 0.7 ± 0.12 (IU/ml) and 0.6 ± 0.21 (IU/ml), respectively) ($P < 0.05$)] (Figure 1). The TRAP activity in the serum from the experimental group in 1, 2, and 3 months was 2.66 ± 1.77 (IU/ml), 2.18 ± 0.97 (IU/ml) and 2.34 ± 1.12 (IU/ml), respectively and 2.5 ± 0.53 (IU/ml), 2.32 ± 0.74 (IU/ml) and 2.14 ± 0.71 (IU/ml) in the control group, respectively. There was no significant difference between the experimental and control group.

Matrix metalloproteinase-2 assay in the synovial fluid

The level of MMP-2 in the SF from the experimental group in 1, 2 and 3 months after the induction of OA was 167 ± 10, 158 ± 11 and 148 ± 9, respectively, when these values were significantly higher than those of the SF from the control group (117 ± 11, 115 ± 9 and 117 ± 11, respectively) ($P < 0.05$) (Figure 2).

Assay of tissue inhibitor of metalloproteinase-2 in the synovial fluid

The level of TIMP-2 in the SF from the experimental group in 1, 2 and 3 months after inducing OA was 82 ± 43 (ng/ml), 78 ± 31 (ng/ml) and 91 ±

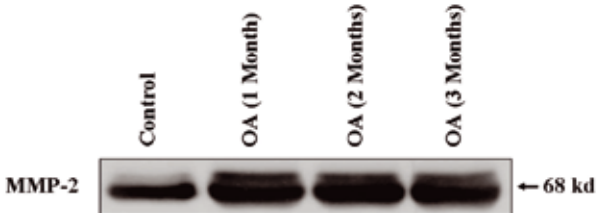

Figure 2. Western blot demonstrating MMP-2 in the synovial fluid at different experimental periods after the induction of OA. The SF of sham-operated group was used as a control

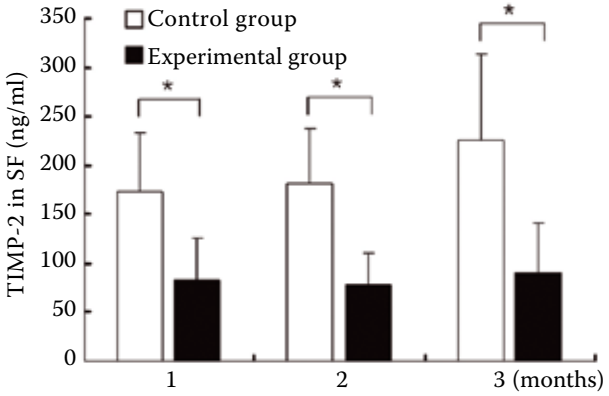

Figure 3. TIMP-2 in the synovial fluid from the experimental and control group. The values were obtained from a standard curve and are recorded in ng/ml. The data is reported as the mean ± standard deviation (SD). Unpaired Student's *t*-test: significant difference (*$P < 0.05$)

50 (ng/ml), respectively. These values were significantly lower than the levels measured in the control group [173 ± 60 (ng/ml), 181 ± 56 (ng/ml) and 224 ± 87 (ng/ml) in 1, 2 and 3 months, respectively] ($P < 0.05$) (Figure 3).

Histochemical examination of joint tissues for tartrate-resistant acid phosphatase

Figure 4 shows the representative histology sections. The synovial tissue from the experimental group showed synovial inflammation with many TRAP positive cells observed in the synovium. In contrast, there were few TRAP positive cells observed in the synovium from the control group.

DISCUSSION

This study measured the TRAP activity, MMP-1 and TIMP-2 in the SF and serum from the OA-induced dogs (experimental group) and the sham-operated dogs (control group). The results showed significant increases in the levels of TRAP activity in the SF as well as the presence of the increased number of $TRAP^+$ cells in the synovium of OA-in-

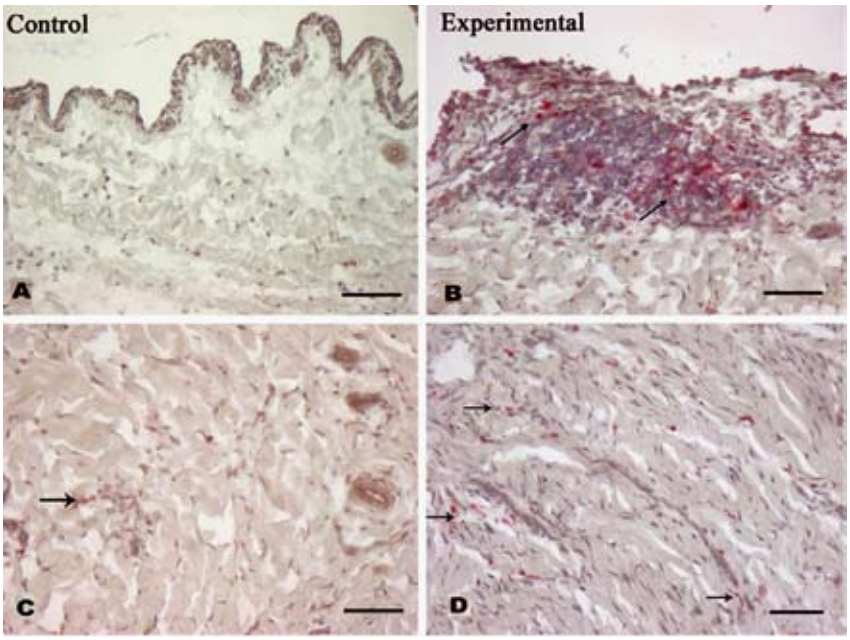

Figure 4. Photomicrographs of a transverse frozen section of the synovium. The cells stained red for TRAP (black arrows) can be observed in the synovium (A, B, C and D). An increase in the number of $TRAP^+$ cells was observed in the synovium of the experimental group (B and D) compared with the synovium of the control group (A and C). Bar = 100 μm

duced dogs in the early stage of OA. We also found the elevated MMP-2 concentrations and reduced TIMP-2 concentrations in SF from the experimental group compared to the control group. These findings supported the hypotheses of the present study and suggest that the TRAP activity, MMP-2 and TIMP-2 can be used as biomarkers for diagnosing the early of stage OA.

TRAP is a member of the iron-containing purple acid phosphatase. TRAP enzymes are commonly found in the activated cells of macrophages, dendritic cells and osteoclasts. TRAP can produce reactive oxygen species (ROS) through Fenton's reaction, where the redox-active ferrous iron reacts with hydrogen peroxide to generate highly destructive hydroxyl radicals. The ROS generated by TRAP are capable of collagen degradation (Halleen et al., 2006). However, the role of TRAP in the development of the joint disease is still unclear. In this study, we clearly showed that the level of TRAP activity in the SF from the experimental group was significantly higher than in the control group. Furthermore, a higher number of TRAP positive cells was seen in the synovium of the experimental group but few cells or none were seen in the control group. Histochemistry for TRAP produced results was consistent with those of an earlier study which reported an increase in the number of $TRAP^+$ cells in the synovium from humans with arthritis and dogs with osteoarthritis secondary to the naturally occurring rupture of the cranial cruciate ligament (Konttinen et al., 2001; Muir et al., 2005). These results suggest that an increase in the number of $TRAP^+$ cells in the synovium is related to the development of OA. The increased TRAP activity in the SF appears to be due to the presence of $TRAP^+$ cells in synovium.

Whilst there was a significant difference in TRAP activity in the SF from the experimental and control group, there was no significant difference in TRAP activity in the serum from the experimental and control group. This might be so because the TRAP activity was leaving the SF and entering the blood through the lymphatic vessels but either it was not absorbed at all or insufficient amounts to alter the TRAP activity in the blood were absorbed.

There are two problems of measuring the TRAP activity in the SF. One is the very high viscosity of the SF, which is difficult to sample accurately. The viscosity is affected by the high concentration of hyaluronic acid. Therefore, a method was developed to take an accurate SF volume using hyaluronidase, which had an excellent effect in reducing the viscosity. However, it is important to confirm that this method does not affect the TRAP activity. In our preliminary research, the TRAP activity was measured in the SF with and without hyaluronidase and there were no differences between the SF with and without hyaluronidase (unpublished data). The other problem is that the blood can affect the TRAP activity because erythrocytes can release TRAP unrelated to the $TRAP^+$ cells in the joint tissue. Therefore, SF without blood was used in this study. In addition, the SF was incubated at 37°C for 30 min to inactivate the TRAP released from the erythrocytes (Janckila et al., 2002). Overall, this method is considered adequate for measuring the TRAP activity in the SF.

MMPs derived from chondrocytes, synoviocytes, fibroblasts, monocytes and macrophages play a major role in the cartilage degradation. The proteolytic activity of MMP is regulated by specific inhibitors which are known as TIMPs. The balance between the MMPs and TIMPs is tightly controlled in healthy joints. However, in OA, the amount of MMPs exceeds the TIMPs, resulting in the excessive extracellular matrix degradation of the articular cartilage (Burrage et al., 2006). TIMP-2 is a major TIMP expressed by fibroblasts, macrophages and endothelial cells and potently inhibits most MMPs. In this study, the significant increase in the MMP-2 levels with a concomitant decrease in the TIMP-2 levels in the SF from the experimental group indicated a discrepancy in the balance of these two enzymes, suggesting a high rate of metabolic turnover in the articular cartilage. These findings are in agreement with previous reports (Manicourt et al., 1994; Salinardi et al., 2006).

In summary, we have shown the change in the levels of TRAP activity, MMP-2, TIMP-2 in the SF as well as the increased number of $TRAP^+$ cells in the synovium of OA-induced dogs in the early stages of OA. This suggests that the TRAP activity, MMP-2 and TIMP-2 can be used as biomarkers for both diagnosing and monitoring the early stages of OA.

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