Tartrate-resistant acid phosphatase as a diagnostic factor for arthritis

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Abstract. Tartrate-resistant acid phosphatase (TRAP) is highly expressed in osteoclasts and chondroclasts. The present study investigated changes in TRAP activity after chondrocyte death and cartilage damage, and also evaluated the possible use of TRAP as a diagnostic factor in a model of osteoarthritis. We induced experimental osteoarthritis in beagle dogs and separated chondrocytes from articular cartilage using an enzyme probe. Chondrocyte death was induced by proteasome inhibition and TRAIL treatment, and levels of lactate dehydrogenase, reactive oxygen species (ROS), caspase activation and TRAP activity were measured in the chondrocytes and synovial fluid. Proteasome inhibition and TRAIL treatment significantly enhanced chondrocyte death via caspase-8 activation and ROS generation in the primary cultured canine chondrocytes. TRAP activity was highly increased in damaged chondrocytes, but was decreased by blocking chondrocyte death using caspase inhibition or an ROS scavenger. In the synovial fluid of osteoarthritic dogs, TRAP activity as well as caspase activation and ROS levels were higher than those in the normal joint. Our study demonstrated that TRAP is activated by apoptosis and oxidative stress in primary cultured chondrocytes and osteoarthritic joints and also suggests that TRAP may be used as a diagnostic biomarker for detection of cartilage-related diseases, including osteoarthritis.

Introduction

Tartrate-resistant acid phosphatase (TRAP) is a member of the ubiquitously expressed enzyme family of acid phosphatases (1). It first became known to hematologists as a cytochemical marker for hairy cell leukemia. Subsequently, TRAP enzymes were isolated from many mammalian sources, including bovine and rat spleen, human and rat bone, and human lungs and placenta (2,3). TRAP expression plays an important role in various cellular events, including signal transduction, activation, proliferation and differentiation (4). Recently, TRAP was reported to be released by osteoclasts into the circulation during bone resorption, and also to be the only known marker for osteoclast activity (5). In the synovium at sites of cartilage destruction in patients with rheumatoid arthritis (RA), TRAP-positive mononuclear and multinucleated cells are often noted. However, the exact levels and the functional role of TRAP in the destruction of articular cartilage and chondrocytes has not been well investigated.

The ubiquitin-proteasome protein degradation pathway plays an important role in regulating cell proliferation and death (6). Most recent studies have suggested that ubiquitin-proteasome-dependent proteolysis is also involved in apoptosis, although its precise role is controversial (7). Inhibitors of the proteasome can act through multiple mechanisms to arrest tumor growth, tumor spreading and angiogenesis. Also, previous studies showed that various proteasome inhibitors enhanced TRAIL-induced apoptosis in a variety of tumor cell lines (8-10). Several studies have characterized the function of the proteasome in chondrocytes. Human chondrocytes were found to be sensitive to proteasome-induced apoptosis (11), and proteasome inhibition was found to suppress growth plate proliferation and induce chondrocyte apoptosis (12). However, the proteasome inhibitor MG-132 protects articular chondrocytes *in vivo* and *in vitro* through induction of heat shock protein (13). These conflicting reports led us to elucidate the effects of proteasome inhibition and TRAIL in primary cultured canine chondrocytes.

In the present study, we generated an experimental model of osteoarthritis in beagle dogs and separated the chondrocytes from articular cartilage using an enzyme probe. We then investigated the activity of TRAP after chondrocyte death and cartilage degradation, raising the possibility of using TRAP as a diagnostic factor for arthritis.

Materials and methods

Osteoarthritis induction. Beagle dogs (n=20) with a mean (standard deviation, SD) age of 1.4 (0.4) years and a mean (SD) weight of 10.2 (1.4) kg were used. Right stifle joint medial arthrotomy was performed. The cranial cruciate and medial collateral ligaments were transected, and a medial meniscectomy was performed. All procedures involving the animal experiments were approved by the Standard

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Operation Procedure (SOP) of the Institutional Animal Care and Use Committee (IACUC), Korea.

Synovial fluid preparation. Synovial fluid was collected 4 weeks after induction of osteoarthritis. Briefly, experimental animals were sedated with acepromazine (Sedazect injection, Samwoo Pharm. Co. Ltd., Korea) at 0.2 mg/kg i.v. and placed in ventrodorsal recumbency with the right stifle joints flexed. Digital pressure was applied to the medial side of the straight patellar ligament. A 21-gauge spinal needle was inserted through the fat pad into the intercondylar space lateral to the straight patellar ligament. The maximum volume of synovial fluid was collected and did not include contamination with blood.

Canine chondrocyte isolation. Cartilage was acquired from the articular surfaces of the knee joints of beagles (2-year-old females). The slices were chopped and incubated at 37˚C with 0.25% trypsin for 30 min, followed by 0.1% collagenase (Sigma-Aldrich) for 6 h in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml gentamycin and 100 μg/ml penicillin-streptomycin). Cells were filtered through a 100-μm cell strainer (Falcon, NJ), washed twice with PBS, and then seeded onto tissue culture flasks. N-Acetyl-Leu-Leu-Norleu-Al (ALLN, Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and used at a final concentration of 10 μM.

Cell viability test. Canine chondrocytes were seeded in 12-well plates, pre-treated with NAC (1 mM), Z-VAD-fmk (40 μM) and/or ALLN (10 μM) for 12 h, and then co-incubated with or without recombinant TRAIL protein (100 ng/ml) for an additional 12 h. Cell morphology was photographed with a camera attached to an optical microscope, and cell viability was determined using the crystal violet staining method as described elsewhere (14). Briefly, the cells were stained for 10 min at room temperature with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde), washed four times with water, and then dried. The cells were then lysed with 1% sodium dodecyl sulphate (SDS) solution, and the absorbance was measured at 550 nm. The cell viability was calculated based on the relative dye intensity compared to the controls.

Lactate dehydrogenase (LDH) assay. Canine chondrocytes plated in 6-well plates were pre-treated with ALLN for 12 h and then further incubated with recombinant TRAIL protein for 12 h. The culture media and synovial fluid were collected and used to measure LDH activity via a chemical colorimetric method. LDH activity was determined by measuring the level of pyruvic acid using a spectrophotometer (SpectraMax fluorometer with SoftMax software, Molecular Probes, USA), and the absorbance was determined at a wavelength of 490 nm.

Western blot assay. To prepare whole cell lysates, cells were harvested and resuspended in lysis buffer [25 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT) and protease inhibitor mixture]. Synovial fluid was diluted 10-fold with phosphate buffer. Equal amounts of lysate protein were also resolved on an 8-15% SDS-polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane. Immunoreactivity was detected through sequential incubation with horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Peibro). A caspase-8 antibody (AAP-118, Stressgen, Victoria, Canada) was used for the Western blot analyses.

Determination of reactive oxygen species (ROS). ROS levels were assessed using the oxidant-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA). Cells treated with chemicals for 12-24 h were washed twice with phosphate buffer and incubated with 10 μM $DCFH_2$-DA in sodium pyruvate containing DMEM medium for 1 h at 37˚C. After $DCFH_2$-DA incubation, cells were washed and further incubated in sodium-containing medium for 10 min to allow for desterification. Cells were then collected, and the fluorescence signals corresponding to intracellular ROS were monitored at 490 nm excitation and 525 nm emission using a fluorescence plate reader (SpectraMax fluorometer with SoftMax software).

TRAP activity assay. After incubation of the cells with chemicals for 12-24 h, supernatants were acquired from the wells, and synovial fluid was used for measurement of TRAP release. TRAP enzyme activity was assayed by adding samples (100 μl) to each well of 96-well plates. TRAP buffer (100 μl) containing 2.5 mM p-nitrophenyl phosphate (p-NPP), 0.1 M sodium acetate buffer (pH 5.8), 0.2 M KCl, 0.1% Triton X-100, 10 mM sodium tartrate, 1 mM ascorbic acid and 100 μM $FeCl_3$ was added to each well, followed by incubation for 1 h. The sodium tartrate in the TRAP buffer converts p-NPP to p-nitrophenol. The p-nitrophenol liberated after incubation at 37˚C for 1 h was converted into p-nitrophenolate by the addition of 50 μl of 0.9 M NaOH, and the absorbance was measured at 405 nm using a fluorescence plate reader. One unit of TRAP activity corresponds to 1 μM of p-nitrophenol liberated per min at 37˚C.

Statistical analysis. Data were expressed as the mean ± standard deviation (SD) and were compared using the Student's t-test and the ANOVAs Duncan test with the SAS statistical package. The results were considered significant at a P-value <0.05.

Results

TRAP activity was increased upon chondrocyte death by combined treatment with proteasome inhibitors and TRAIL. We investigated the effects of combined treatment with proteasome inhibitors and TRAIL on primary cultured canine chondrocytes. Treatment of cells with ALLN alone or TRAIL alone did not induce cell death in chondrocytes, but co-treatment with ALLN and TRAIL induced >60% cell death (data not shown). Examination of cell morphology also supported this result (Fig. 1A). LDH levels in the culture media were increased by co-treatment with ALLN and TRAIL as compared to the negative controls, although ALLN

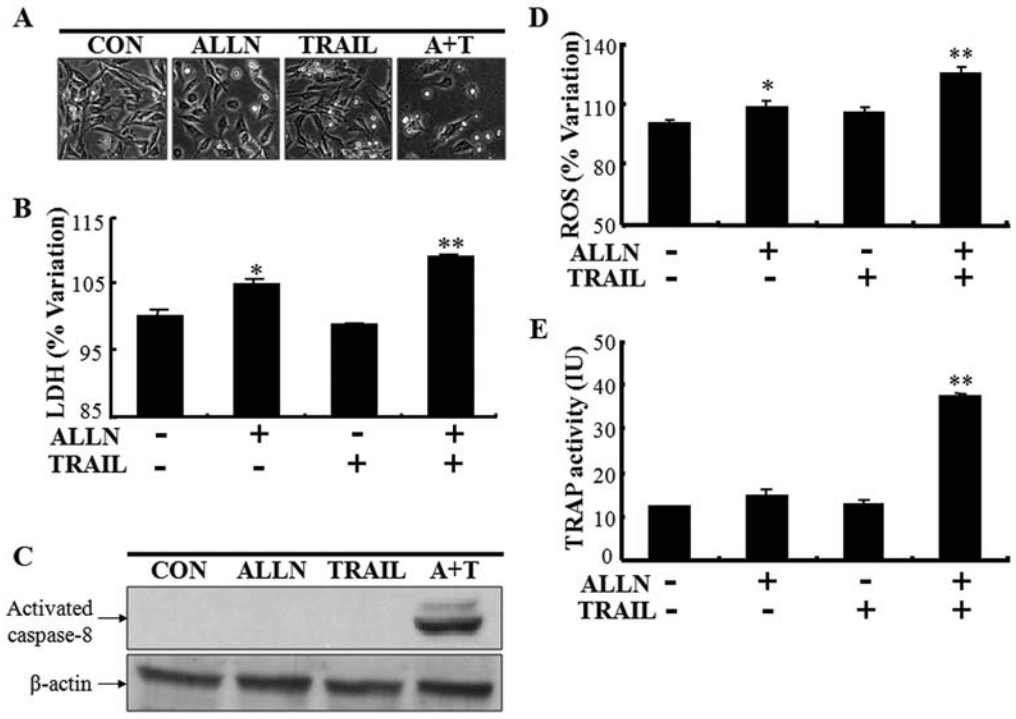

Figure 1. TRAP activity was increased upon chondrocyte death. (A) Canine chondrocytes seeded in a 12-well plate were pre-treated with ALLN (10 μM) for 12 h and then co-incubated with (A+T) or without recombinant TRAIL protein (100 ng/ml) for an additional 12 h. Cell morphology was photographed (x200). (B) LDH activity was determined by measuring the pyruvic acid level using a spectrophotometer, and absorbance was determined at a wavelength of 490 nm. (C) Whole cell lysates were prepared and then analyzed for apoptotic proteins by Western blotting. ß-actin indicates a non-specific protein band that was used to ensure equal protein loading. (D) The ROS level was measured using $DCFH_2$-DA. The fluorescence value for the control cells was set to 100%; fluorescence values relative to the control are presented. (E) TRAP activity was determined using p-NPP. The experiments were performed in triplicate at least twice. Bars indicate standard error. $^{**}P<0.01$, $^{*}P<0.05$ vs. the nontreated sample; Student's t-test.

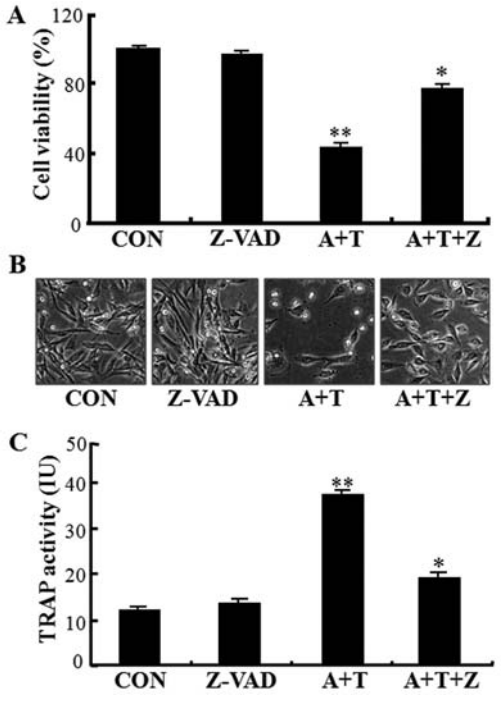

Figure 2. Increased chondrocyte death and TRAP activity, after co-treatment of cells with a proteasome inhibitor and TRAIL, were inhibited by Z-VAD-fmk. (A) Cell viability was determined by the crystal violet staining method. Viability of control cells was set as 100%, and viability relative to the control is presented. (B) Images of cell morphology (x200). (C) TRAP activity was determined using p-NPP. The experiments were performed in triplicate at least twice. Bars indicate standard error. $^{**}P<0.01$, $^{*}P<0.05$ vs. the untreated sample; Student's t-test.

administered alone resulted in a low level of LDH release from the extracellular chondrocytes (Fig. 1B). To address the mechanism by which proteasome inhibition enhances TRAIL-induced apoptosis in canine chondrocytes, we examined caspase-8 activation and ROS generation. Western blotting showed that caspase-8 was significantly activated after combined treatment with ALLN and TRAIL (Fig. 1C). Pre-treatment with ALLN increased ROS levels, and significant ROS generation was also induced by co-treatment with TRAIL (Fig. 1D). These results showed that chondrocyte death by proteasome inhibition and TRAIL treatment occurs through the caspase pathway and ROS generation.

In order to examine the release of TRAP, we exposed canine chondrocytes to ALLN for 12 h and then treated them with recombinant TRAIL protein for an additional 12 h. TRAP activity was only slightly increased by ALLN treatment, while the combined treatment with ALLN and TRAIL significantly enhanced TRAP activity (Fig. 1E). This result suggests that TRAP is released after chondrocyte death by combined treatment with proteasome inhibitors and TRAIL.

TRAP activity was inhibited by caspase inhibition and ROS scavenging. We tested whether the pan-caspase inhibitor Z-VAD-fmk could inhibit ALLN/TRAIL-mediated death to chondrocytes, and whether TRAP activity was correlated with caspase-related cell death. After pre-treatment with Z-VAD-fmk (40 μM) for 1 h, canine chondrocytes were exposed to ALLN (10 μM) for 12 h and then treated with recombinant TRAIL (100 ng/ml) for an additional 12 h. Z-VAD-fmk

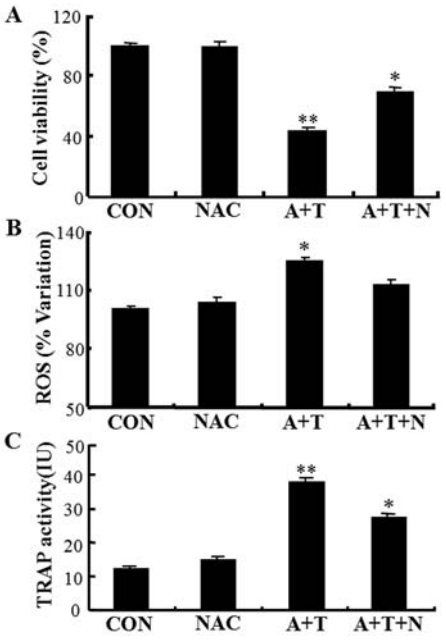

Figure 3. Chondrocyte death and TRAP activity, after co-treatment with the proteasome inhibitor and TRAIL, were inhibited by NAC. (A) Cell viability was determined by crystal violet staining method. Viability of control cells was set as 100%, and viability relative to the control was presented. (B) The ROS level was measured using DCFH-DA. The fluorescence of control cells was set as 100%; fluorescence values relative to the control are presented. (C) TRAP activity was determined using p-NPP. The experiments were performed in triplicate at least twice. The bar indicated standard error. **P<0.01, *P<0.05 vs. untreated sample; Student's t-test.

completely blocked the induction of chondrocyte death (Fig. 2A). Cell morphology supported these findings (Fig. 2B). Furthermore, ALLN/TRAIL-induced TRAP activity was inhibited by pre-treatment with Z-VAD-fmk (Fig. 2C).

N-acetylcysteine (NAC) is a ROS scavenger and an anti-inflammatory or anti-oxidant agent. We tested whether NAC could inhibit the effects of combined treatment with ALLN and TRAIL, and whether induction of TRAP activity by both treatments was correlated with the production of ROS. After pre-treatment with NAC (1 mM) for 1 h, canine chondrocytes were exposed to ALLN (10 μM) for 12 h and then treated with recombinant TRAIL protein for an additional 12 h. NAC treatment reduced ALLN/TRAIL-induced chondrocyte death (Fig. 3A). Pre-treatment with NAC did not change ROS levels. However, ROS generation induced by the combination treatment with ALLN and TRAIL was reduced by pre-treatment with NAC (Fig. 3B). ALLN/TRAIL-induced TRAP activity was also inhibited by pre-treatment with NAC (Fig. 3C). These results suggest that TRAP activity increases extracellularly upon chondrocyte death.

TRAP activity in osteoarthritic synovial fluid was higher than that in the normal joint. To examine the macroscopic damage to articular cartilage by experimentally induced OA, the articular cartilage of femoral condyles was observed. Cartilage damage was found on the tibial plateau of the experimental joints when compared to the control joint. The cartilage of

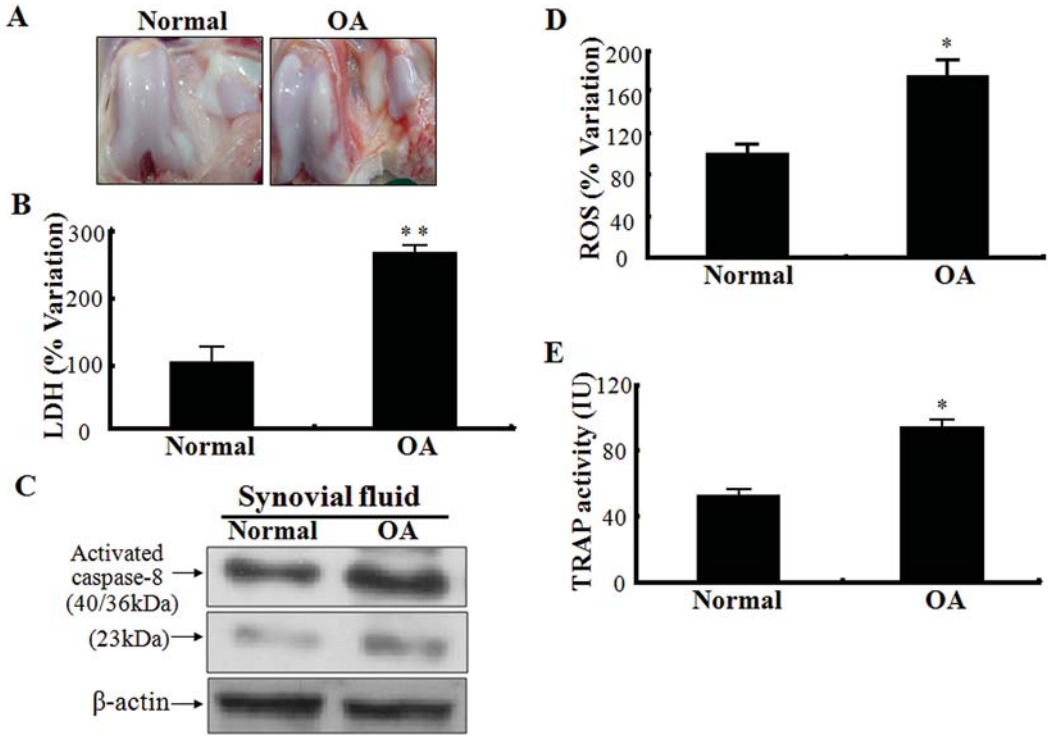

Figure 4. TRAP activity in osteoarthritic synovial fluid was higher than in the normal joint. (A) Photomicrograph of the articular cartilage of femoral condyles in normal and osteoarthritic joints. (B) The synovial fluid was collected at 4 weeks after induction of osteoarthritis. LDH activity was determined by measuring pyruvic acid levels with a spectrophotometer. (C) Proteins were electrophoretically resolved using an 8-15% SDS gel, and then apoptotic proteins were detected by Western blotting. ß-actin indicates a non-specific protein band used to ensure equal protein loading. (D) The ROS level was measured using $DCFH_2$-DA. The fluorescence value for control cells was set as 100%; fluorescence values relative to the control are presented. (E) TRAP activity was determined using p-NPP. Experiments were performed in triplicate at least twice. Bars indicate standard error. **P<0.01, *P<0.05 vs. normal sample; Student's t-test.

the medial tibia plateau was fibrinoid and showed erosion (Fig. 4A). Additionally, LDH levels, activation of caspase-8, ROS generation, and TRAP activity were significantly increased in OA synovial fluid (Fig. 4B-E). These findings indicate that factors related to cell damage are found in OA synovial fluid and that TRAP activity increases in the synovial fluid following destruction of articular cartilage by OA

Discussion

Apoptosis is an evolutionarily conserved form of programmed cell death (PCD) involved in normal physiological processes related to growth, development and homeostasis (15). Distinct from necrosis, apoptosis is characterized by chromatin condensation, nuclear fragmentation, cell shrinkage, plasmalemma blebbing and apoptotic body formation (16). Several therapeutic applications of anti-apoptotic agents have already been explored for neurodegenerative diseases, ischemia/reperfusion injury and autoimmune disorders. The key role of caspases in the initiation and execution of apoptosis make them prime targets for apoptosis modulation. Fewer reports are available regarding the prevention of apoptosis in chondrocytes. Nuttall *et al* reported that apoptosis was induced in immortalized human chondrocytes and inhibited by the pan-caspase inhibitor z-VAD-fmk (17). D'Lima *et al* reported that caspase inhibition with z-VAD-fmk significantly reduced the percentage of cells undergoing apoptosis (18). ROS plays a crucial role in cell proliferation and destruction. ROS generation causes inflammation of articular cartilage and apoptosis of chondrocytes, finally resulting in arthritis. In the present study, TRAIL and ALLN individually did not induce cell death, but combined treatment resulted in cell death via caspase-8 activation and ROS generation. Such cell death effects were completely inhibited by treatment of the cells with the pan-caspase inhibitor z-VAD-fmk and with the ROS scavenger NAC. Taken together, our data indicate that combined treatment of canine chondrocytes with proteasome inhibitors and TRAIL enhances cell death by oxidative stress via ROS generation and the caspase pathway, the key regulator of the TRAIL-induced cell death pathway.

In recent years, markers of articular cartilage metabolism have been widely sought. Promising targets include matrix metalloproteinase-1 (MMP-1), MMP-3 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-2). MMP-1 and -3 both belong to the proteinase family, and TIMP-2 is an inhibitor of MMP-1. These molecules were thought to play a significant role in the process of cartilage damage (19,20). TRAP has been used as a cytochemical marker for osteoclasts and for clinical detection of osteoporosis and bone metastasis in breast cancer (21). Our study showed that TRAP activity was significantly enhanced by combined treatment of canine chondrocytes with ALLN and TRAIL. This increased activity was inhibited by pre-treatment with a pan-caspase inhibitor and ROS scavenger. This suggests that TRAP is released into the extracellular environment (culture media) upon the death of canine chondrocytes.

Synovial fluid-derived markers are used to monitor tissue degradation in human and animal joint diseases such as rheumatoid arthritis and osteoarthritis, and the estimation of synovial fluid volume aids in the quantification of joint biomarkers. Activated synovium can release proteases and cytokines which in turn accelerate the deterioration of adjacent cartilage lesions (22). Our study revealed a difference in LDH levels between the control group and the experimental osteoarthritis group. Caspase activation and ROS production in the osteoarthritic synovial fluid were significantly higher than that in the control joint. These data suggest that changes in apoptotic factors might be related to inflammation and can be observed in the synovial fluid. These changes might be related to the natural history of the disease and might thus be helpful for the detection of patients at risk of rapidly progressing disease. Additionally, TRAP activity in the osteoarthritic synovial fluid was higher than that of the control joint, indicating that damage to articular cartilage and chondrocytes results in increased TRAP activity.

In summary, the present study examined whether TRAP can be used as a biomarker for the detection of OA. Our results show that combined treatment of canine chondrocytes with a proteasome inhibitor and TRAIL significantly enhanced cell death via caspase-8 activation and ROS generation, and that the combined treatment significantly enhanced TRAP activity in the extracellular environment. The enhanced cell death and TRAP activity was inhibited by pre-treatment of the cells with a caspase inhibitor and a ROS scavenger. Caspase activation, ROS levels, and TRAP activity in the osteoarthritic synovial fluid were higher than in the control joint. This suggests that TRAP was released upon chondrocyte death caused by cartilage degradation, and that TRAP may be used as a biomarker for the detection of joint-related diseases including osteoarthritis.

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