Inhibitory Effects of Pentosan Polysulfate Sodium on MAP-Kinase Pathway and NF-κB Nuclear Translocation in Canine Chondrocytes In Vitro

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ABSTRACT. Pentosan polysulfate sodium (PPS) has a heparin-like structure and is purificated from the plant of European beech wood. PPS has been used for the treatment of interstitial cystitis for human patients. Recent years, it was newly recognised that PPS reduce pain and inflammation of OA. The molecular biological mechanism of PPS to express its clinical effects is not fully understood. The purpose of the present study is to investigate a mechanism of action of PPS on inflammatory reaction of chondrocytes in vitro. It was evaluated that effects of PPS on interleukin (IL)-1β-induced phosphorylation of mitogen-activated protein kinases (MAPKs), such as p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), nuclear translocation of nuclear factor-kappa B (NF-κB), and matrix metalloproteinase (MMP)-3 production in cultured articular chondrocytes. As a result, in the presence of PPS existence, IL-1β-induced phosphorylation of p38 and ERK were certainly inhibited, while JNK phosphorylation was not affected. Nuclear translocation of NF-κB and MMP-3 production were suppressed by PPS pretreatment prior to IL-1β stimulation. In conclusion, it is strongly suggested that PPS treatment prevents inflammatory intracellular responses induced by IL-1β through inhibition of phosphorylation of certain MAPKs, p38 and ERK and then nuclear translocation of NF-κB in cultured chondrocytes. These PPS properties may contribute to suppressive consequence of catabolic MMP-3 synthesis. These data might translate the clinical efficacy as PPS treatment could inhibit the cartilage catabolism and related clinical symptoms of OA in dogs.

KEY WORD: canine, MAP kinase, NF-kB, osteoarthritis, pentosan polysulfate sodium.


In the treatment of canine osteoarthritis (OA), multiple therapeutic intervention tools are used for multimodal pain management, including systemic administration of non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular administration of hyaluronic acid, intravenous or intramuscular administration of disease-modifying osteoarthritis drugs (DMOADs), the use of oral nutraceutical agents, rehabilitation and weight management [20]. In particular, DMOADs are most likely to relief pain and other clinical symptoms directly related to joint pathologies [16].

It is thought that the pathophysiology of OA is related with the imbalance of reparative and degradation processes leads to loose aggrecans and collagen from extracellular matrix (ECM) of hyaline cartilage, resulted in articular cartilage degeneration [1, 15]. As catabolic enzymes of ECM, matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are playing major role in the process and in establishment of OA pathologies, to which DMOADs should target [19].

Pentosan polysulfate sodium (PPS) has a heparin-like structure and is extracted from the plant of European beech wood. In North America and Europe, PPS has been used for several ten years for the purpose of treatment of phlebitis and medical relief of pain or discomfort of interstitial cystitis in humans [8, 12, 22]. PPS has been recognised as treatment drug for Creutzfeldt-Jakob disease. It has been shown that intraventricular PPS administration prolonged the incubation period before the development of overt clinical signs and reduced the extent of abnormal prion protein deposition in the brain by animal experiment [13]. From the late 1990, the clinical effects of PPS for OA patients were recognized [8, 22]. Suppressive effects of PPS to OA progression in cruciate deficicancy dogs after surgical stabilization of stifle joints [3]. In humans, it was reported that PPS oral administration significantly reduced pain score and cartilage metabolism marker, C2C in sera from OA patients [22]. From the results of previous in vitro and in vivo studies, PPS would be qualified as DMOADs [8, 12, 21, 22]. However, molecular biological mechanism of PPS to cartilage protection is not fully understood.

The purpose of the current study is to investigate the mechanisms of action of PPS on inflammatory reaction of chondrocytes in vitro. Our hypothesis is that PPS would compromise directly to intracellular pathway of reactive process to induce catabolic reactions of chondrocytes, which could be seen in the inflammation of OA process.

MATERIALS AND METHODS

Cell culture: Canine chondrocytes were freshly isolated from articular cartilage of femoral head removed in three
dogs suffering Legg-Calve-Perthes disease. Cartilaginous tissue was dissected out from apparently healthy part of the specimen, minced, and then incubated for 4 hr at 37°C in 5% CO2 with serum free Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco-BRL, Paisey, U.K.) supplemented with 4 mg/ml of collagenase type IA (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100 IU/ml of penicillin and 0.1 mg/ml of streptomycin. Cells were suspended in DMEM supplemented with 10% Fetal bovine serum (FBS; Biomedical Inc., Auroha, OH, U.S.A.), 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Cells were incubated at 37°C in 5% CO2 to obtain monolayer adherent cultures. At confluence, cells were washed with phosphate buffered saline (PBS, pH=7.2), trypsinized and passaged. Cells between second and fifth passage were used for following experiments. Cell viability, assessed by trypan blue exclusion test, always exceeded 95%.

Preparation of cell extracts and Western blot analysis: Canine chondrocytes (1 × 10⁶ cells/well) were plated onto 6-well culture plates (Corning, Lowell, MA, U.S.A.) in serum free DMEM for 24 hr. Cells were pre-treated with 100 µg/ml of PPS (DS Pharma animal health Co., Ltd., Osaka, Japan) for 1 hr and then incubated with 10 ng/ml of recombinant human (rh) IL-1β (Wako) for 5, 15, 30, 60 or 240 min. Cells were washed with ice-cold Tris-Buffered Saline (TBS, pH=7.4) twice and lysed with 200 µl of RIPA buffer (Sigma, St. Louis, MO, U.S.A.).

Western blot analysis: Protein concentration of each sample was determined by Bradford protein assay using bovine serum albumin and aligned as 30 µg. Samples were denatured and subjected to SDS-PAGE using a 10% (w/v) polyacrylamide gels and electrotransferred into nitrocellulose membranes (Whatman, Dassel, Germany). The membranes were blotted with blocking solution, containing 10 mM Tris-HCl, 0.15 M NaCl, 0.1% (w/v) Tween-20, 1% (w/v) bovine serum albumin in TBS with 0.05% NaN₃ for 1 hr at room temperature. Membranes were incubated overnight at 4°C with primary antibodies for p38, phospho-p38, c-Jun N-terminal kinase (JNK), phospho-JNK, extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2, β-actin (Cell Signaling Technology, Danvers, MA, U.S.A.) or MMP-3 (Sigma-Aldrich) at a dilution of 1:1,000 in blocking solution, containing 5% (w/v) BSA, 10 mM Tris-HCl; pH 7.4, 0.15 M NaCl, 0.1% (w/v) Tween-20. Membranes were washed with Tween-TBS, containing 10 mM Tris-HCL; pH 7.4, 0.15 M NaCl, 0.1% (w/v) Tween-20 three times for 5 min each, incubated with anti-rabbit alkaline phosphatase conjugate IgG (Sigma) at a dilution of 1:1,000 in Tween-TBS. For detection, the NBT/BCIP system (Roche Applied Science, Mannheim, Germany) was used according to the manufacturer’s protocol.

Nuclear translocation of NF-kB: Canine chondrocytes (1 × 10⁵ cells) were cultured in 8-well culture slide (Becton Dickenson, Franklin Lakes, NJ, U.S.A.) in 500 µl of DMEM. Cells were pre-treated with 10 or 100 µg/ml of PPS for 24 hr then incubated with 10 ng/ml of rhIL-1β for 24 hr. Cells were washed with PBS and fixed with 4% paraformaldehyde (Wako) in PBS for 15 min at room temperature. The staining was performed by incubating with 10% normal goat serum (Sigma) in PBS for 1 hr followed by incubating with primary anti-human NF-kB p65 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) (1:100 dilution) for 1 hr in PBS with 1% normal goat serum, washing three times with PBS, incubating for 1 hr with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Sigma) (1:100 dilution) in PBS with 1% normal goat serum, washing three times with PBS, and finally mounting with aqueous mounting medium. The images were observed under a fluorescent microscope. Three different fields were randomly picked from each slide, 200 cells were counted and the rate of NF-kB nuclear translocation was calculated.

MMP-3 mRNA expression in canine chondrocytes by real-time RT-PCR: Canine chondrocytes (1 × 10⁶ cells) were plated onto 6-well culture plates in serum free DMEM for 24 hr. Cells were pre-treated with 10 or 100 µg/ml of PPS for 24 hr and then incubated with 10 ng/ml of rhIL-1β for 24 hr. Total RNA from chondrocytes was extracted using RNeasy Mini Kit® (QIAGEN, Germantown, MD, U.S.A.) according to the manufacturer’s protocol. Total RNA was quantified by spectrophotometry at 260 nm. RNA with a 260/280 nm ratio in the range 1.8–2.0 was considered high quality. Total RNA was reverse transcribed into cDNA with M-MLV RT kit (Takara Bio, Tokyo, Japan) according to manufacturer’s recommended procedures. Quantitative real-time PCR analysis was performed with KAPA SYBR® FAST qPCR kit (KAPA biosystems, Woburn, MA, U.S.A.). cDNA template with the amount of 2 µl was added to each 10 µl of premixure with specific primers. The mRNA level for the gene of interest was quantified as the percentage of that determined for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primers were MMP-3, forward, 5'-ATGGCATCCAGTCCCTGTAT-3'; reverse, 5'-AAAGAACAGGAACTCCTCCCC-3' and GAPDH, forward 5'-AAGGTTCATCCCTGAGCTGAA-3'; reverse 5'-GACCACCTGTCCTCAGTGT-3'.

Statistical analysis: Each data set was expressed as mean ± standard deviation. All data was statistically analyzed by performing the Mann-Whitney U test, using a commercial statistical software (StatView 5.0, SAS institute Inc., Cary, NC, U.S.A.) to determine significant differences between groups. P values less than 0.05 were considered significant.

RESULTS

Effect of PPS on p38, ERK1/2 and JNK MAPK activation: As shown in Fig. 1, phosphorylation of p38, ERK and JNK were all elevated in chondrocytes treated with 10 ng/ml of rhIL-1β alone at 5, 15 and 15 min of stimulation time, respectively. Phosphorylation of p38 and ERK were inhibited by 100 mg/ml of PPS, while JNK remained constant despite PPS pretreatment prior to rhIL-1β stimulation.

Effect of PPS on NF-kB translocation induced by rhIL-1β: Under control conditions, a diffuse cytoplasmic staining was seen and the average percentage of NF-kB positive nuclei cells was 9.5% (Figs. 2A and 3). On the other hand, as shown in Fig. 3, 78.2% of chondrocytes incubated with rhIL-
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1β had a nuclear staining pattern, indicating NF-κB nuclear translocation (P<0.05) (Fig. 2B). Preincubation with 10 and 100 µg/ml PPS reduced NF-κB translocation rate to 55.6 (P=0.054 compared to rhIL-1β alone) and 30.2% (P<0.05 compared to rhIL-1β alone), respectively (Figs. 2C and 3).

Effect of PPS on MMP-3 mRNA expression in chondrocytes induced by rhIL-1β: As shown in Fig. 4, in chondrocytes stimulated by 10 ng/ml of rhIL-1β, the MMP-3 mRNA expression was upregulated three times higher than normal condition. PPS alone had little effect on MMP-3 mRNA expression in chondrocytes, while PPS downregulated MMP-3 mRNA expression induced by rhIL-1β in a concentration-dependent manner.

Effect of PPS on MMP-3 protein expression in chondrocytes induced by rhIL-1β: As shown in Fig. 5, the MMP-3 protein expression was increased after 24 hr of stimulation with 10 ng/ml of rhIL-1β. PPS alone had little effect on MMP-3 protein expression in chondrocytes. Effect of rhIL-1β was inhibited by PPS preincubation in a concentration-dependent manner.

DISCUSSION

It was shown that in vitro cultures chondrocytes up-regulated mRNA expression and increased synthesis of MMP-3 by stimulation with 10 ng/ml rhIL-1β. Interleukin-1β could stimulate overproduction of several catabolic molecules, including MMPs [2], thus thought to play an important role in manifestation of OA pathologies [4, 6, 10, 11, 17, 18]. These results indicated that rhIL-1β could induce inflammatory reaction in cultured chondrocytes used in the present study. Further experiments were therefore done with these IL-1 stimulated chondrocytes for later investigation of intracellular mechanism, which may be interfered by PPS.

In this present study, IL-1 induced MMP-3 synthesis from cultured chondrocytes were certainly reduced by PPS treatment prior to inducing inflammation. This interference was seen and confirmed in mRNA and protein expression of MMP-3. In the course of inflammation, IL-1β binds to its specific cell-surface receptor IL-1 receptor R1, of which expression was increased on osteoarthritic chondrocytes and synoviocytes in human patients [2]. After IL-1 binding to specific receptors, nuclear reaction to synthesize a series of inflammation-related products through mitogen activated protein kinases (MAPKs) pathways [14, 19]. In the present study, phosphorylation of three MAPKs, including ERK, p38, and JNK, were induced by IL-1 stimulation with/without PPS treatment.

Phosphorylation of all three MAPKs in cultured chondrocytes was confirmed by rhIL-1β-stimulation, while PPS pretreatment caused inhibition of rhIL-1β-induced phosphorylation of p38 and ERK. Phosphorylation of JNK was however unchanged regardless of the presentation of PPS in the culture media. Pentosan polysulfate sodium has a kind of glycosaminoglycan, of which structural feature would be like heparins or some other glycosaminoglycans, having somehow electrical or physical affinity to surface receptors. This might cause the competitive inhibition of the binding between IL-1 to its receptors. The essential question was raised where PPS could act as a suppressor to intracellular reaction of inflammation. The results of this study might clearly explain that PPS was not affecting IL-1 binding to its cell surface receptors. In the present study, IL-1 induced JNK phosphorylation was fully completed. This fact could indicate that PPS did not affect both IL-1 binding and JNK phosphorylation at the same time. Pentosan polysulfate could act its anti-inflammatory role through binding certain receptor apart from IL-1Rs or direct effects after migrating into target cells.

Translocation of NF-κB into nuclei was seen after IL-1 stimulation in cultured chondrocytes. After 24 hr of rhIL-1β stimulation, a nuclear staining pattern was seen with anti-NF-κB antibodies. PPS alone had little effect on NF-κB translocation. Effect of rhIL-1β was inhibited by PPS preincubation in a concentration-dependent manner. The essential question was raised where PPS could act as a suppressor to intracellular reaction of inflammation. The results of this study might clearly explain that PPS was not affecting IL-1 binding to its cell surface receptors. In the present study, IL-1 induced JNK phosphorylation was fully completed. This fact could indicate that PPS did not affect both IL-1 binding and JNK phosphorylation at the same time. Pentosan polysulfate could act its anti-inflammatory role through binding certain receptor apart from IL-1Rs or direct effects after migrating into target cells.

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FIGURES

Fig. 1. Effects of PPS on rhIL-1β-induced p38, ERK and JNK MAPK phosphorylation. Phosphorylated kinases were showed as P-p38, P-ERK and P-JNK. Western blotting analysis showed p38, ERK and JNK phosphorylation at the rhIL-1β stimulation time of 5, 15 and 15 min, respectively. Those phosphorylations were significantly inhibited by PPS.

Fig. 2. Immunofluorescence staining representing NF-κB immunopositive nuclei in chondrocytes. Panel A: in control cells, a diffuse cytoplasmic staining was seen with anti-NF-κB antibodies. Panel B: in rhIL-1β stimulated chondrocytes, nuclear staining pattern was seen, indicating NF-κB nuclear translocation. Panel C: in PPS preincubated chondrocytes, a significant diminution of NF-κB nuclear translocation by rhIL-1β stimulation to chondrocytes was seen. Scale bar: 20 µm.
stimulation, most of NF-κB was observed at nucleus. On the other hand, in the circumstance of PPS existence prior to rhIL-1β, NF-κB was mostly observed at cytoplasm. This migration was certainly decreased by PPS treatment. Intracellular MAPKs phosphorylation in the process of inflammation will result in the activation of nuclear factors to catalyze the reaction to synthesize various resultants. Setting out of reaction of NF-κB and activator protein-1 (AP-1) could be proved by the intranuclear presence of these factors. One of these factors, NF-κB is activated by the phosphorylation of p38 or ERK, while another factor AP-1 is stimulated by the phosphorylation of JNK [5, 9]. Here in this study, phosphorylation was compromised by PPS, translocation of NF-κB was therefore investigated thereafter. This interference of its migration of this factor could prove suppressive effects of PPS on specific MAPKs, p38 and ERK. It is also likely to indicate the suppressive mechanisms of MMPs production and under inflammatory conditions. Previous reports showed the direct effects of PPS to inhibit aggreganase [21]. The same theoretical explanation may be applied on suppressive effects of PPS on ADAMTS production. Suppressive properties of PPS on intranuclear presence of AP-1 under inflammatory condition were reported (personal communication). If it is proved as fare results, inhibition of AP-1 activation was promised without JNK suppression. This might be suggestive that PPS could suppress not only certain MAPKs but also nuclear factors as a direct manner.

For clinical administration for dogs, PPS is usually used with 3 mg/kg s.c. once weekly for 4 weeks. Peak blood concentration is thought to be approximately 10 µg/ml or less [3, 7]. Thus, the dose we used in this study is higher than the dose for clinical use. Moreover, since our study is based on only in vitro study, it is necessary to consider a real reaction in vivo study. However, previous reports showed the inhibitory effect of PPS on TNF-α induced NF-κB activation in vivo [23], supporting the PPS efficacy in vitro.

In conclusion, it is strongly suggested that PPS treatment prevents inflammatory intracellular responses induced by IL-1 through inhibition of phosphorylation of certain MAPKs, p38 and ERK and then nuclear translocation of NF-κB in cultured chondrocytes. These PPS properties may contribute to suppressive consequence of catabolic MMP-3 synthesis. These data might translate the clinical efficacy as PPS treatment could inhibit the cartilage catabolism and related clinical symptoms of OA in dogs. Although farther study is needed, one of mechanisms of actions of PPS as a DMOAD could be explained through its suppressive effects on intracellular MAPK phosphorylation, while further investigation is necessary to have complete elucidation for this.

REFERENCES


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