Triamcinolone acetonide alters expressions of *matrix metalloproteinase-3* gene in primary human chondrocytes

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ABSTRACT

Intra-articular glucocorticoid (GC) injection such as triamcinolone acetonide (TA) is an ultimate pharmaceutical treatment for knee osteoarthritis (OA) patients who do not respond well to other pharmaceutical treatments. Effects of GCs on homeostasis of cartilage extracellular matrix (ECM) are not conclusive. In this study, we investigated whether TA altered expression of genes involved in digestion of collagens and proteoglycans. Chondrocytes isolated from cartilage of ten knee OA patients were treated with TA at 1 and 5 mg/ml. Reverse-transcription real-time polymerase chain reaction (real time RT-PCR) was used to evaluate expressions of genes involving ECM degradation (*matrix metalloproteinase* (*MMP*)*-1, MMP-3, MMP-13, a disintegrin and metalloproteinase with thrombospondin motives* (*ADAMTS*)*-5* and *tissue inhibitor of matrix metalloproteinase* (*TIMP*)*-3*). Analysis of mRNA expression using real time RT-PCR showed that *MMP*-3 mRNA expressions were significantly increased to 6.59 ± 5.2-fold (p = 0.022) and 5.43 ± 3.46-fold (p < 0.01) in chondrocytes treated with TA at 1 and 5 mg/ml, respectively, as compared to non-treated control. Levels of *MMP-1*, *MMP-13*, *ADAMTS-5*, and *TIMP-3* were not significantly changed. Whether TA-increased mRNA expressions of *MMP-3* in chondrocytes involves degradation of cartilage and accelerates OA progression need further investigations in order to provide information for physicians in TA treatment planning for OA patients.

Keywords: chondrocyte; osteoarthritis; glucocorticoid; triamcinolone acetonide; matrix metalloproteinase

INTRODUCTION

Pharmaceutical treatments for knee osteoarthritis (OA) aim to increase patients' quality of life by reducing pain and improving joint function. Several drugs taken by ingestion and intra-muscular injection have been used for reliving pain and reducing joint inflammation, and most of them have considerably low toxicity to the joints (Kon *et al.*, 2012). However, some patients do not respond to these drugs, as a result, intra-articular injections of glucocorticoids (GCs) are administered. Balance between benefits and adverse effects of GCs is controversial. Triamcinolone acetonide (TA) is a frequently chosen agent for intra-articular injection due to the extended duration of effects (Neustadt, 2001).

Homeostasis of articular cartilage is controlled by adequate production and degradation of extracellular matrix (ECM) components such as collagens and proteoglycans. Optimal ECM turnover is crucial for joint stability and function. High production of collagenases and proteinases from chondrocytes and synoviocytes leads to elevated degradation of cartilage matrix, which can progress to OA (Dewire, 2001). Genes involved in degradation of collagens and proteoglycans include *metalloproteinases* (*MMPs*), *a disintegrin and metalloproteinase* (*ADAMs*), *ADAM with thrombospondin motives* (*ADAMTSs*), and *tissue inhibitor of matrix metalloproteinases* (*TIMPs*).

MMP-1 and MMP-13 are collagenases that can cleave intact collagen fibrils; hence make them susceptible to cleavage by other proteolytic enzymes. MMP-3 is a stromelysin whose substrates are proteoglycans, fibronectin, elastin, and partially

digested collagens (Gepstein *et al.*, 2002). ADAMTS-4 and ADAMTS-5 are important aggrecanases in articular cartilage and are thought to be prime aggrecanases involved in OA. Levels of ADAMTS-4 in serum of patients with early stage knee OA were increased while the increased levels of ADAMTS-5, MMP-1, and MMP-3 were found in patients with intermediate and advanced stage OA compared with healthy participants (Li *et al.*, 2014a). Also, inhibition of *ADAMTS-4* and *ADAMTS-5*, but not *ADAMTS-1*, by siRNA reduced glycosaminoglycan aggrecan neoepitope (AGEG) release in both OA-cartilage explants and cytokine-induced normal cartilage (Song *et al.*, 2007). However, ADAMTS-5 has been suggested to be an important target for OA treatment because results from both *ex vivo* and *in vivo* have shown decrease in aggrecan secretion after applying monoclonal antibody against the ADAMTS-5 protein (Fosang, 2015). The ECM homeostasis is kept in balance between degradation and inhibition by proteins such as TIMPs. TIMP-3 is a strong inhibitor not only to MMPs, but also to ADAMTS-4 and ADAMTS-5 (Nagase *et al.*, 2006). TIMP-3 has been suggested to be an important MMPs regulator by an *in vivo* study (Nagase *et al.*, 2006). In OA cartilage, expressions of *TIMP-3* were decreased while *ADAMTS-5* expressions were increased in condylar cartilage of rats compared with healthy controls (Li *et al.*, 2014b).

Several studies have shown that GCs had both protective and harmful effects on cartilage by reducing mRNA expressions of proteolytic enzymes, and MMPs inhibitors, respectively. Several studies reported that glucocorticoids (TA, dexamethasone, and methylprednisolone acetate) protected against loss of glycosaminoglycan and type II collagen in cytokine-challenged animal cartilage and suppressed expression of the *MMP-1*, *MMP-13*, *MMP-3*, and *TIMP-3* genes. However, no expression changes were reported in non-challenged samples (Garvican *et al.*, 2010; Lu *et al.*, 2011; Caron *et al.*, 2013). Another study also reported the reduction effect of dexamethasone on *TIMP-3* expression in cytokine-induced primary chondrocytes from both bovine and human (Su *et al.*, 1996). Nevertheless, expressions of mRNA involved in ECM degradation have not been studied in chondrocytes from OA patients. Furthermore, the *in vivo* study in healthy horses intra-articular injected with TA showed an elevation in ECM degradation, levels of aggrecan turnover, and collagen cleavage biomarkers (chondroitin sulphate 864 epitope, keratin sulphate epitope, and collagenase cleavage neoepitopes 1 and 2) in synovial fluid (Celeste *et al.*, 2005). According to these studies, effects of TA on expressions of these proteolytic enzymes and *TIMP-3* are still inconclusive. As a result, primary chondrocytes from patients with knee OA were used in this study to simulate the pathophysiology condition in the OA cells.

The objective of this study is to determine effects of TA on expressions of collagenase and aggrecanase genes (*MMP-1*, *MMP-13*, *MMP*-3, and *ADAMTS-5*) and an inhibitor *TIMP-3* in human OA chondrocytes.

MATERIALS AND METHODS

Ethical approval and sample collection

Collection of cartilage samples and study procedure were approved by the Human Ethics Committee of Thammasat University (MTU-ED-OT-4 – 099/57). All participants received full information referring to study objectives and possible side effects, and provided signed informed consents. Cartilage samples of ten OA patients (age 40 – 70 years old, with Kellgren-Lawrence grade 3 – 4 radiographic knee OA) who underwent knee replacement surgery were collected at Department of Orthopaedic Surgery, Faculty of Medicine, Thammasat University.

Isolation of primary chondrocytes

The protocol for chondrocyte isolation was adjusted from the Goldring method (Picot, 2005). Articular cartilage samples were cut with sterile surgical blades into size smaller than 5×5 mm^2 and put into clean 50 ml tubes. Then, they were washed twice with 5 ml phosphate-buffered saline (PBS) and digested with 5 ml of 0.25% trypsin (Gibco, Billings, MT, USA) for 30 min at 37 °C. After another washing with PBS, 5 ml of 1% protease (Invitrogen, Carlsbad, CA, USA) was added and the samples were incubated for 30 min at 37 °C. The cartilage was then washed and digested with 5 ml of 1% collagenase (Gibco) at 37 °C for at least 8 hrs. After incubation with the collagenase, the possibly contaminated monocytes would have been eliminated (Morsy *et al.*, 2005) and other non-adhering lymphocytes would have been washed out. The released chondrocytes were passed through cell strainer with 70-µm diameter into new 50 ml tubes and centrifuged at 1,000 $\times g$ for 5 min for cell collection. The cell pellet was washed with PBS and re-suspended with culture media (Dulbecco's modified Eagle's medium/F12 with 10% foetal bovine serum (Gibco) and 1% antibiotic-antimycotic reagent (Gibco). The cell suspension was transferred to a cell

culture flask and cultured in an incubator containing 5% CO_2 at 37 °C until they reached confluence. The chondrocytes were characterised by their fibroblast-like morphology and by detecting mRNA expressions of *Type II Collagen* (*COL2A1*) by reverse transcription PCR and agarose gel electrophoresis in a preliminary study. Results from the preliminary study showed that expressions of *COL2A1* mRNA were detected in all samples.

Gene expression analysis

Primary chondrocytes were trypsinised and counted with haemocytometer. Chondrocytes at 10^5 cells per well were added into a 6-well plate and cultured for 48 hr. Then, TA (LBS. Laboratory, Watthana, Bangkok, Thailand) at 0, 1, and 5 mg/ml were added to each well in replicates, and incubated for 48 hr. The concentrations used in this study were based on concentrations used in clinical practice and were adjusted according to surface area of one well (Dragoo *et al.*, 2012). A recent study by our research group found that TA concentrations of 1 and 5 mg/ml do not significantly induce cell death but induce different expressions in genes involving oxidative stress (Suntiparpluacha *et al*., 2016). The chondrocytes were then collected for RNA extraction using RNeasy® Mini Kit (Qiagen, Stanford, VA, USA) following the company's instruction. Concentration of extracted RNA was evaluated using UV-Vis spectrophotometer at 260 nm. For reverse transcription, 500 ng of total RNA was mixed with nuclease-free water and *AccuPower*® RT Premix (Bioneer, Seoul, South Korea), and reverse transcription process was performed according the manufacturer's protocol. Real-time polymerase chain reaction (PCR) was performed using 2x QPCR Green Master Mix, LRox (Biotech rabbit, Henningsdorf, Germany) with a StepOnePlus™ Real-time PCR System (Applied Biosystems, Waltham, MA, USA) for evaluation of *MMP-3*, *MMP-13*, and *TIMP-3*. For analysis of *MMP-1* and *ADAMTS-5*, real-time PCR was carried out in a CFX96™ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Conditions for real-time PCR were: initial activation at 95 °C for 2 min, and amplification for 33 cycles of denaturation at 95 °C for 5 s and annealing/extension at 56 – 62 °C for 15 s. Efficiencies of real-time PCR condition were between 95% – 105%. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as a reference gene for normalisation. The relative quantification ($2^{-\Delta\Delta Cq}$) method was used for comparing normalised expressions of the target genes to their non-treated controls. Primer sequences for all gene studied are shown in Table 1. Primers for amplifying *MMP*-1, *TIMP-3*, and *GAPDH* were designed with NCBI Primer Blast (Ye *et al.*, 2012) and Primer 3 programmes (Koressaar and Remm, 2007; Untergrasser, 2012).

Statistical analysis

Differences between treatment groups were analysed using one-way Analysis of Variance (ANOVA) with Dunnett T3 post-hoc analysis by SPSS program version 13.0 (SPSS Inc., Chicago, IL, USA).

Table 1 Primers for real-time PCR

Gene	Primer sequence (5′ → 3′)	Amplicon size (bp)	Annealing temperature (°C)
MMP-1	Forward - GCTGCTTACGAATTTGCCGA Reverse - CATCATACCTCCAGTATTTGTTAGC	217	59
MMP-3	Forward - CTTTTGGCGCAAATCCCTCAG Reverse - AAAAGAACCCAAATTCTTCAA	404 (Nganvongpanit *et al.*, 2009)	56
MMP-13	Forward - TCGCGTCATGCCAGCAAATTCCAT Reverse - TTCTTCCCCTACCCCGCACTTCTG	116 (Kotepui *et al.*,2012)	62
ADAMTS-5	Forward - AGGAGCACTACGATGCAGCTATC Reverse - CCCAGGGTGTCACATGAATG	73 (Corps *et al.*, 2008)	59
TIMP-3	Forward - TGACAGGTCGCGTCTATGAT Reverse - GGCAGGTAGTAGCAGGACTT	151	56
GAPDH	Forward - GAAGGTGAAGGTCGGAGTC Reverse - GAAGATGGTGATGGGATTTC	237	58

RESULTS

Expressions of *MMP-3*, but not *MMP-1*, *MMP-13*, *ADAMTS-5* and *TIMP-3*, were altered by TA

The effects of TA on expressions of proteolytic enzyme genes (*MMP-1*, *MMP-13*, *MMP-1*, *ADAMTS-5*) and their inhibitor (*TIMP-3*) in chondrocytes were analysed by real-time PCR. When treated with TA, expressions of *MMP-3* gene increased significantly as compared with non-treated chondrocytes (6.59 ± 1.64 fold for 1 mg/ml TA (p = 0.022) and 5.43 ± 1.09 fold for 5 mg/ml TA (p < 0.01)). No statistical difference in mRNA levels of *MMP-3* between cells treated with 1 and 5 mg/ml TA was observed (Figure 1). Addition of TA did not significantly alter mRNA expressions of *MMP-1* (0.83±0.37-fold for 1 mg/ml TA and 1.63±0.76-fold for 5 mg/ml TA), *MMP-13* (2.03 ± 0.86-fold for 1 mg/ml TA and 1.91 ± 0.78-fold for 5 mg/ml TA), *ADAMTS-5* (2 ± 0.55-fold for 1 mg/ml TA and 2.29 ± 0.51-fold for 5 mg/ml TA), and *TIMP-3* (1.37 ± 0.32-fold for 1 mg/ml TA and 1.33 ± 0.39-fold for 5 mg/ml TA) (Figure 1).

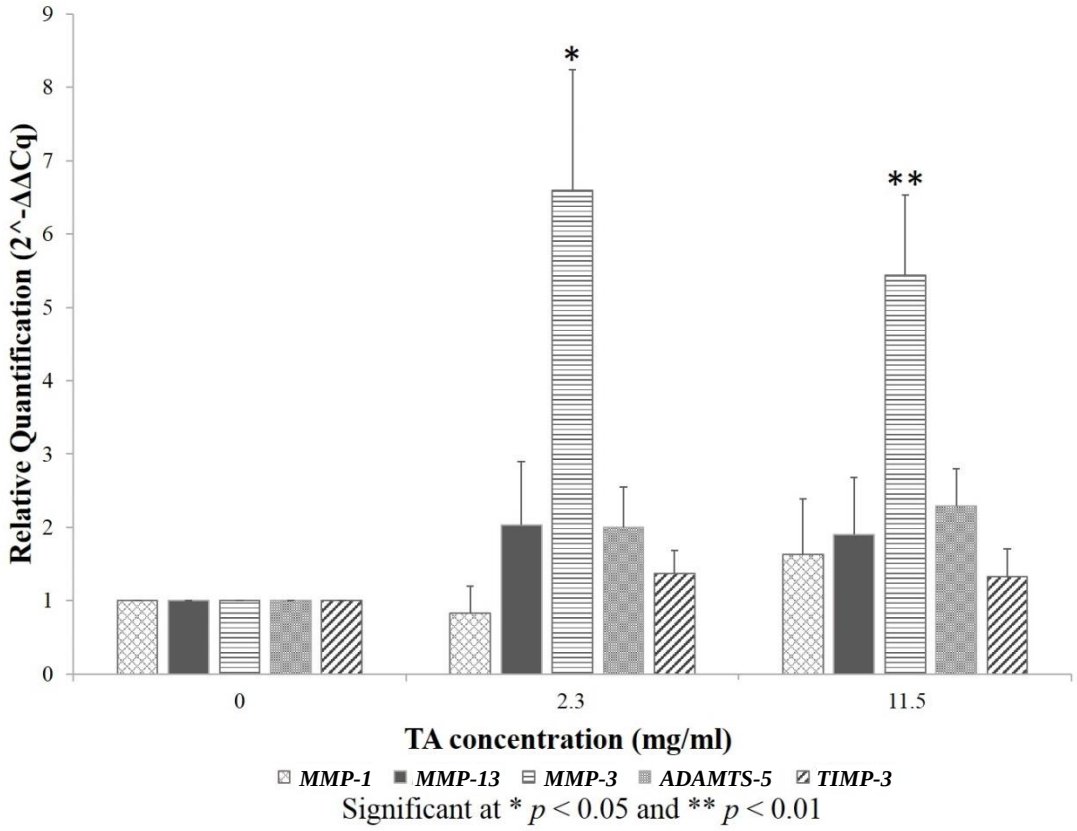

Figure 1 Relative mRNA levels of *MMP-1*, *MMP-13*, *MMP-3*, *ADAMTS-5*, and *TIMP-3* in TA-treated chondrocytes compared with non-treated controls

DISCUSSIONS

Since OA is caused by imbalance between construction and destruction of cartilage ECM, understanding the effects of GCs on expressions of these proteinases and their inhibitors can be crucial to suggest plans for OA patients. We intended to evaluate the effects of TA on expressions of collagenase, aggrecanase, and inhibitor of matrix metalloproteinase genes, *MMP-1*, *MMP-13*, *MMP-3*, *ADAMTS-5*, and *TIMP-3*, in primary chondrocytes isolated from OA patients, which represented the cells under pathological condition. The results showed the significant increase only in *MMP-3* expressions in TA-treated cells compared with non-treated control. The unchanged expressions of *MMP-1*, *MMP-13*, *ADAMTS-5*, and *TIMP-3* after TA treatments observed in this study were consistent with the results from Garvican *et al.* (Garvican *et al.*, 2010) who reported that changes in expressions of these genes were observed only in cytokine-challenged cartilage treated with glucocorticoids.

Expressions of these proteolytic enzymes are relatively low in normal condition, but they can be

elevated by certain stimulations (Nagase *et al.*, 2006). Several studies using chondrocytes or cartilage explants treated with pro-inflammatory cytokines to reflect an inflammatory state in knee joints and to study effects of GCs on attenuating the cytokines' effects. Results from those studies showed that GCs decreased mRNA expressions of *MMP-1*, *MMP-13*, *MMP-3*, and *TIMP-3* but further increased mRNA expressions of *ADAMTS-5* in cytokine-induced cartilage (Su *et al.*, 1996; Busschers *et al.*, 2010; Garvican *et al.*, 2010; Lu *et al.*, 2011). In a particular study, mRNA expressions of *MMP-1* and *MMP-3* were significantly deceased when IL-1α-stimulated primary bovine chondrocytes were treated with TA (0.1–50 μM) (Sadowski and Steinmeyer, 2002). In this study, while pro-inflammatory cytokines were not used to stimulate the primary chondrocytes, the cells used were taken from OA patients in order to reflect the patients' pathological states. As a result, effects of TA on gene expressions in this study were not consistent with the mentioned reports. One sample was excluded from analysis of *MMP-1* mRNA expressions due to the suspiciously high mRNA levels (52-fold and 15-fold for TA at 1 mg/ml and 5 mg/ml, respectively) and a lack of information about OA status that might explain the variation in this particular sample. Increased mRNA expressions of *MMP*-3 observed in this study were in agreement with results from the *in vivo* study using tendons of rats injected with TA and prednisolone at Achilles tendon-calcaneus junction for one week (Muto *et al.*, 2014). Our results showed that the *MMP-3* mRNA levels in chondrocytes treated with TA at 1 mg/ml were higher than those in chondrocytes treated with 5 mg/ml of TA, but with no significant difference. Regulation of *MMP-3* mRNA expressions by TA might be different from the collagenases (*MMP-1* and *MMP-13*), and aggrecanase (*ADAMTS-5*). One possible pathway might be via p38 (Sondergaard *et al.*, 2010). However, further study is required to determine pathways of TA in regulating expressions of proteolytic enzymes in order to select or develop pharmaceutical agents that can attenuate this adverse effect of TA.

Limitation in this study was that the experiments were performed on monolayer chondrocytes, whilst chondrocytes in the joint are embedded in three-dimensional matrix of cartilage. The wide-range variation of gene expression found in this study might be caused by other stromal cell contamination, as there was intra-articular chronic inflammation in OA patients. Further study in protein levels of MMP-3 and activities of MMP-1, MMP-3, MMP-13, and ADAMTS-5 should be performed in order to determine whether TA injection can potentially lead to further cartilage destruction.

TA increased expressions of *MMP-3* but did not alter expressions of collagenases (*MMP-1*, *MMP-13*), *ADMTS-5*, and *TIMP-3* in primary chondrocytes from OA patients. Whether TA-increased mRNA expressions of *MMP-3* in chondrocytes involve degradation of cartilage and accelerate OA progression need further investigations in order to elucidate the effect of TA used in treatment of OA patients.

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