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*Original Article*

# Interleukin-4 Downregulates the Cyclic Tensile Stress-induced Matrix Metalloproteinases-13 and Cathepsin B Expression by Rat Normal Chondrocytes

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**Mechanical stress plays a key role in the pathogenesis of cartilage destruction seen in osteoarthritis (OA). We investigated the effect of cyclic tensile stress (CTS) on the anabolic and catabolic gene expression of rat cultured normal chondrocytes using the Flexercell strain unit. The effects of interleukin (IL)-4, a chondroprotective cytokine, on the changes in gene expression induced by CTS were also investigated. CTS (7% elongation at 0.5 Hz) for 24 h did not affect the expression of aggrecan and type II collagen, whereas CTS significantly upregulated matrix metalloproteinase (MMP)-13 and cathepsin B mRNA expression by chondrocytes. IL-1$\beta$ expression was also significantly upregulated by CTS up to 12 h. The upregulation of MMP-13 was observed at 3 h, which was earlier than that of IL-1$\beta$. Furthermore, pre-treatment with IL-4 (10 ng/ml) suppressed both MMP-13 and cathepsin B induction by mechanical stress, as well as CTS-induced IL-1$\beta$ expression. Our results suggest that IL-4 might have a therapeutic value in the treatment of OA by downregulation of mechanical stress-induced MMP-13 and cathepsin B expression by chondrocytes.**

**Key words:** IL-4, MMP, cathepsin B, mechanical stress, aggrecanase

Aggrecan and type II collagen are major extracellular matrix (ECM) components of articular cartilage [1]. Aggrecan is highly hydrated because of its negatively charged long polysaccharide chains, thus conferring flexibility to the cartilage to resist mechanical loads, such as compression, shearing, stretching stress and hydrostatic pressure. The degradation or loss of aggrecan is considered a critical early event of cartilage destruction, occurring initially at the joint surface and then progressing to the deeper zones. This is followed by degradation of collagen fibrils and mechanical failure of the tissue, which leads to osteoarthritis (OA) [2].

It is generally accepted that proteolytic enzymes are activated during the disease process [3] and contribute to the loss of ECM in OA [3, 4]. Of these, matrix metalloproteinases (MMPs)-1, 3, 8, 13 and 14 are reportedly responsible for destructive collagenolysis, and aggrecanases such as cathepsin B and ADAMTS (a disintegrin and a metalloproteinase

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domain with thrombospondin motifs) 1, 4, 5, 8, 9 and 15 are likely to be responsible for destructive aggrecanolysis [5].

MMP-13 has a particular role in cartilage degradation because it is expressed by chondrocytes, and it hydrolyzes type II collagen more efficiently than the other collagenases [6, 7]. MMP-13 is induced in response to the cytokines and growth factors usually found in arthritic joints. In OA, autocrine secretion of inflammatory cytokines such as IL-1 by chondrocytes stimulates MMP-13 expression and cartilage degradation in the absence of inflammatory cells [7, 8]. Furthermore, Wong *et al.* [9] reported that cyclic tension upregulated the MMP-13 via upregulation of the Cbfa1/MMP-13 pathway.

Cathepsin B, a lysosomal cysteine proteinase, can cleave aggrecan at a site close to that of MMP-3 (also known as stromelysin) [10, 11]. Mehraban *et al.* [12] reported that cathepsin B is upregulated in chondrocytes and synovial tissue in experimental OA at different time points representing disease progression. The histochemical and pathological distribution of cathepsin B in human OA cartilage suggested that it may be involved in the perpetuation of OA rather than in the initial assault on the cartilage [13, 14]. High levels of activity of extracellular cathepsin B has been found around clefts and in the zones of hypercellularity in OA cartilage [14]. Although the mechanism of upregulation of cathepsin B expression is still unclear, IL-1 reportedly upregulates intracellular cathepsin B by increasing its protein synthesis [12]. However, there is little information on the relationship between mechanical stress and cathepsin B expression.

IL-4 is an anti-inflammatory cytokine, like IL-10 and IL-13, which is known to suppress pro-inflammatory cytokine production and activities [3]. Previous studies have demonstrated the anti-inflammatory properties of IL-4 in synovial tissue or in models of arthritis. In OA synovial tissue, IL-4 inhibits the production of tumor necrosis factor-$\alpha$ and IL-1$\beta$ [15]. In cartilage, IL-4 acts as an anti-inflammatory cytokine through the downregulation of IL-1-induced MMP-1 and -3 production [16, 17] and upregulation of tissue inhibitor of metalloprotease (TIMP)-1 [17, 18]. Local overexpression of IL-4 protects cartilage from MMP-induced destruction by preventing the activation of pro-MMPs during immune complex-mediated arthritis [19]. These results suggest that IL-4 is likely to have the potential to antagonize catabolic mediators involved in cartilage destruction. Interestingly, it has been reported that IL-4 also acts as an anti-inflammatory cytokine against mechanical stimulation in an autocrine manner via type II receptors in chondrocytes [20].

In the current study, we examined the *in vitro* effect of IL-4 on mechanical stress-induced MMP-13 and cathepsin B expression by rat normal chondrocytes. To investigate the mechanism of action of IL-4 on chondrocyte gene expression, the expression of IL-1$\beta$ at mRNA and protein levels were also examined up to 24 h after the application of mechanical stress. The results of the current study suggested that IL-4 may exhibit a beneficial role as a therapeutic agent against OA, at least in part, by downregulation of CTS-induced MMP-13 and cathepsin B expression by chondrocytes.

## Materials and Methods

***Chondrocyte culture.*** Articular cartilage obtained from the femoral condyle of 7-day-old Wistar rats was aseptically dissected, and chondrocytes were isolated by digestion of cartilage specimens in 0.1% $\alpha$-chymotrypsin (Wako, Osaka, Japan) and 0.2% collagenase (SIGMA, Tokyo, Japan) following the method of Bruckner *et al.* [21]. Chondrocytes were seeded in six-well plates ($5 \times 10^4$/ml) coated with type I collagen (BioFlex collagen 1 culture plate; Flexcell International, McKeesport, PA, USA), cultured in 3 ml $\alpha$-minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin, and incubated in a 5% $CO_2$ humidified incubator at 37 °C for 3 days before the start of the experiments.

***Exposure of chondrocytes to CTS.*** Cells were grown to confluence on the flexible surface of the BioFlex plates and exposed to cycles of stretch and relaxation using a computer-driven, vacuum-operated, stress-providing instrument (Flexercell Strain Unit FX-2000; Flexcell International). The culture plate bottoms were deformed in a cyclic manner (1 s on, 1 s off; 0.5 Hz), resulting in a 7% elongation of the diameter of the flexible surface. Cell morphology with or without CTS was examined using a phase-contrast microscope.

***IL-4 treatment.*** To investigate the effect of

IL-4 on CTS-induced gene expression, chondrocytes were incubated in $\alpha$-MEM without FBS with rat recombinant IL-4 (rrIL-4) (R&D, McKinley Place, MN, USA) (0 or 10 ng/ml) under a 5% $CO_2$ atmosphere for 24 h, and were then loaded with CTS.

***Real-time polymerase chain reaction (PCR) for the quantitative detection of each type of mRNA.*** After the exposure of the cells to the CTS for 3, 6, 12, 24 and 36 h total RNA was isolated using ISOGEN (Nippon Gene, Toyama, Japan) and the concentration and purity were assayed by spectrophotometer. The RNA was reverse-transcripted using ReverTra Ace-$\alpha$ (TOYOBO, Tokyo, Japan). The sequences of primers for type II collagen, aggrecan, cathepsin B, MMP-3, MMP-13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 1. The primer for IL-1$\beta$ was purchased from Search-LC (Heidelberg, Germany).

Real-time quantitative PCR reactions were performed on a Lightcycler (Roche Diagnostics, Mannheim, Germany) using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) as recommended by the manufacturer. The final expression value was calculated by dividing the expression level of aggrecan, MMP-3, MMP-13, cathepsin B and IL-1$\beta$ mRNA by the expression level of GAPDH, and each value at 0 h was set as 1.

***IL-1$\beta$ enzyme assay.*** After the exposure of the cells to the CTS for 3, 6, 12, 24 and 36 h, the concentration of IL-1$\beta$ in the supernatant was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Endogen, IL, USA) and following the manufacturer's recommendations.

***Statistical analysis.*** The results were examined by one-way analysis of variance (ANOVA) using StatView (SAS Institute Inc., version 5). A value of $p < 0.05$ was considered to indicate a statistically significant difference.

## Results

***Effect of CTS on type II collagen and aggrecan mRNA expression in the cultured chondrocytes.*** The chondrocytes had a polygonal morphology, which was not altered by CTS after 24 h (Fig. 1A and B). To determine whether or not CTS (7% elongation, 0.5 Hz) alters the gene expression of anabolic factors, we performed real-time quantitative PCR for type II collagen and aggrecan. Fig. 2A shows the changes in type II collagen mRNA levels in the cell layer when chondrocytes were maintained for 0 to 36 h with or without CTS. The level of type II collagen mRNA was upregulated slightly during the course of incubation up to 36 h. CTS had a tendency to decrease the expression of type II collagen, but the differences did not reach significance (Fig. 2A). Aggrecan mRNA expression was upregulated fourfold at 12 h without CTS and gradually decreased later (Fig. 2B). There was no significant difference in aggrecan mRNA expression between the cells with and without CTS.

***Effect of CTS on MMP-13 and cathepsin B mRNA expression in the cultured chondrocytes.*** To determine whether or not CTS induces the gene expression of the catabolic factors MMP-13 and cathepsin B, we performed real-time quantitative PCR at each time point after the application of CTS. The level of MMP-13 mRNA was significantly increased (10–25 fold) after 3 h CTS, and the increased expression was continued at 6, 12 and 24 h after mechanical stimulation, and decreased to the control level at 36 h (Fig. 3A). Cathepsin B mRNA expression significantly increased (2.5–3.2 fold) after 24 h stimulation, and decreased to the control level

**Table 1** Oligonucleotide primer sequences

| | Upstream | Downstream |
|---|---|---|
| Type II collagen | 5'-CCC AGA ACA TCA CCT ACC AC-3' | 5'-GGT ACT CGA TGA TGG TCT TG-3' |
| Aggrecan | 5'-GAT GTC CCC TGC AAT TAC CA-3' | 5'-TCT GTG CAA GTG ATT CGA GG -3' |
| MMP-13 | 5'-GCT TTC CCC GTG TCC TCA AA-3' | 5'-TGA CCT GGG ATT TCC AAA AFA G-3' |
| Cathepsin B | 5'-TTG GGT TCA GCG AGG ACA TA-3' | 5'-TCA GCA GAC ACC TCC ACA TT-3' |
| GAPDH | 5'-AGA ACG GGA AGC TCA CTG G-3' | 5'-TCC ACC ACC CTG TTG CTG TA-3' |

after 36 h stimulation (Fig. 3B).

***Effect of IL-4 on CTS-induced MMP-13 and cathepsin B.*** At 24 h, aggrecan mRNA expression was not affected by CTS. IL-4 pre-treatment did not influence the expression of aggrecan mRNA (Fig. 4A). MMP-13 expression was increased by CTS and significantly downregulated by IL-4 pre-treatment (Fig. 4B). Cathepsin B mRNA was significantly increased by CTS, and decreased by IL-4 pre-treatment at 24 h (Fig. 4C).

IL-1$\beta$ mRNA was upregulated at 24 h after starting stimulation. IL-4 pretreatment downregulated CTS-induced IL-1$\beta$ expression by 24 h (Fig. 5A). The level of the IL-1$\beta$ protein was also upregulated

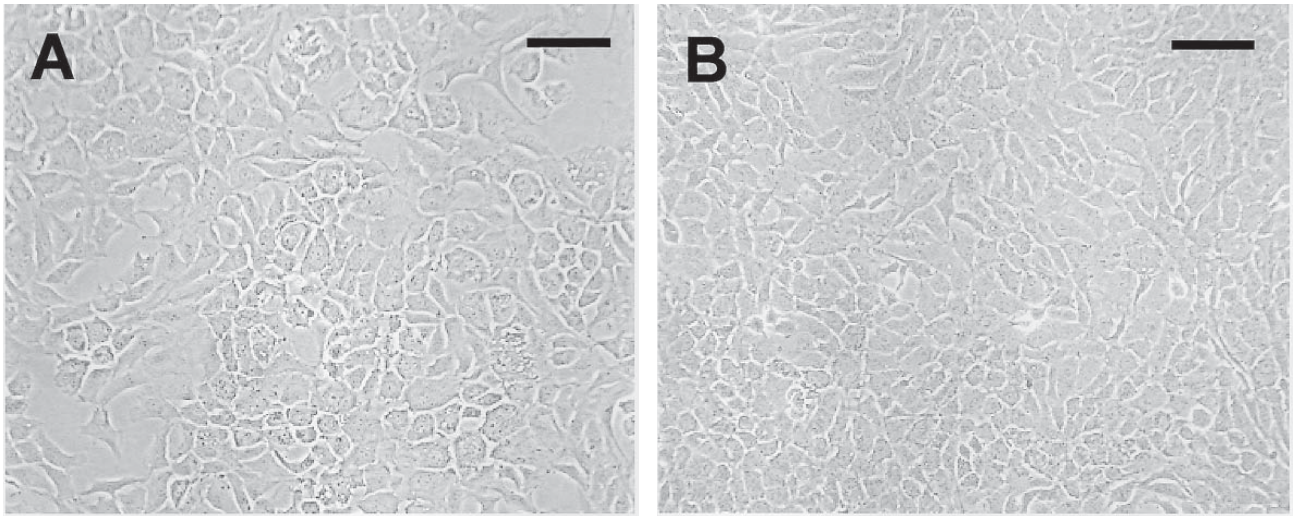


Fig. 1 A. Cell appearances under phase contrast microscopy of cultured chondrocytes with (B) or without (A) CTS (7% elongation at 0.5 Hz) for 24 h. Bar = 100 $\mu$m

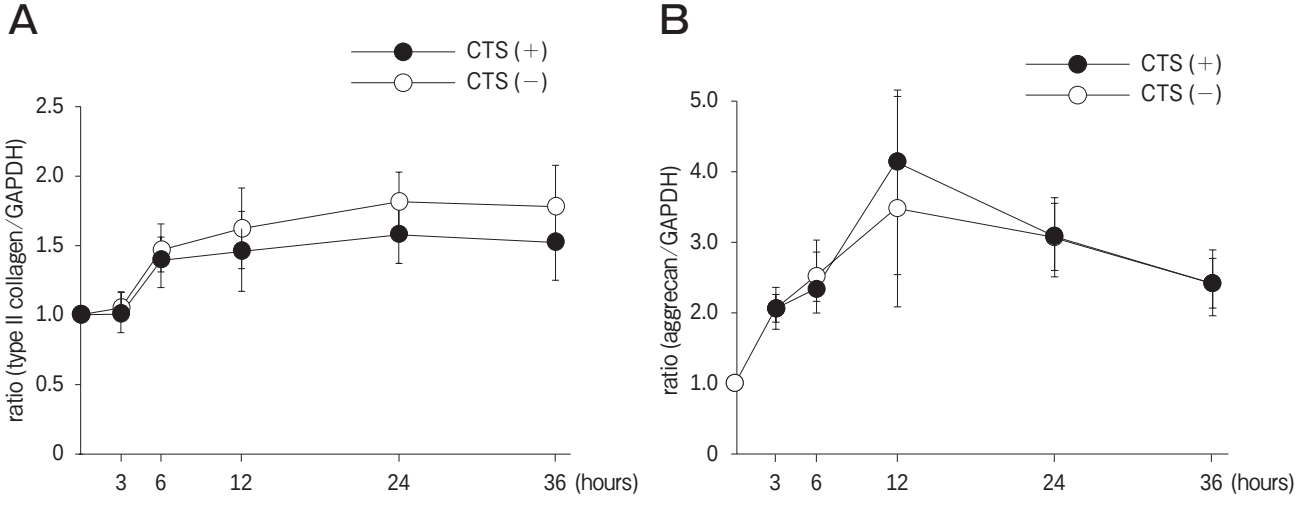


Fig. 2 Effects of CTS (7% elongation at 0.5 Hz) on type II collagen (A) and aggrecan (B) mRNA expression by rat femoral condyle chondrocytes. The changes in mRNA levels in the chondrocytes of type II collagen and aggrecan were measured by real-time quantitative PCR using control GAPDH. Data were shown as the mean ± standard deviation of triplicate determinations. The experiments were repeated 4 times and obtained similar results.

at 24 h after mechanical stimulation. IL-4 pre-treatment countered its upregulation (Fig. 5B).

## Discussion

In the investigation of the chondrocyte responses to mechanical stimuli, the results might vary according to the type of mechanical stress (compression, tension and shear). In addition, the frequency, duration and magnitude of mechanical force all affect cellular responses, and are important determinants of the ultimate fate of the articular cartilage. Agarwal *et al.* [22, 23] reported that mechanical strain of low magnitude results in upregulation of proteoglycan and type II collagen synthesis that is drastically inhibited in inflamed joints. By contrast, mechanical strain of high

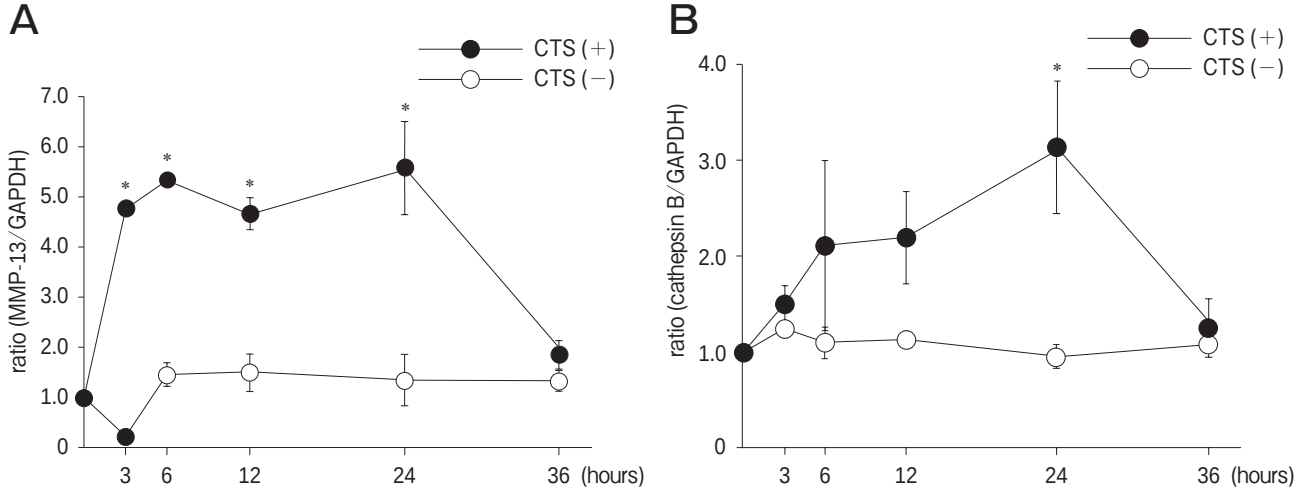


**Fig. 3** Effects of CTS (7% elongation at 0.5 Hz) on MMP-13 (**A**) and cathepsin B (**B**) mRNA expression by rat chondrocytes. The changes in the mRNA levels of MMP-13 and cathepsin B in the chondrocytes were measured by real-time quantitative PCR using control GAPDH. Data were shown as the mean ± standard deviation of triplicate determinations. (*$p < 0.05$) The experiments were repeated 4 times and obtained similar results.

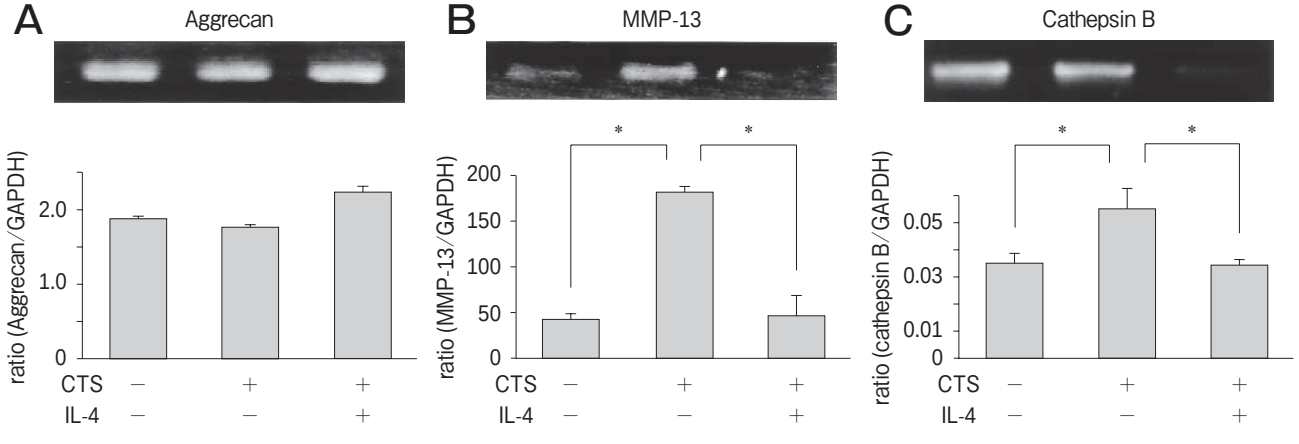


**Fig. 4** Effect of pre-treatment of IL-4 (10 ng/ml) on chondrocyte gene expression after 24 h CTS (7% elongation at 0.5 Hz). Expressions of mRNA for aggrecan (**A**), MMP-13 (**B**) and cathepsin B (**C**) were examined by real-time quantitative PCR. MMP-13 mRNA expression was significantly upregulated by CTS, and IL-4 pre-treatment countered this effect ($p < 0.01$). The experiments were repeated 3 times and obtained similar results.

magnitude is pro-inflammatory and initiates cartilage destruction while inhibiting matrix synthesis. In the current study, we applied 7% elongation, using 0.5 Hz of CTS on monolayer cultured chondrocytes from young animals. No significant differences were noted in the expressions of aggrecan and type II collagen mRNA between stimulated and unstimulated chondrocytes after CTS, whereas the expression of MMP-13 and cathepsin B was significantly upregulated at 24 h. MMP-13 and cathepsin B was downregulated at normal level at 36 h. The reason of this is unclear. These results suggested that 7% elongation, using 0.5 Hz CTS, of monolayer chondrocytes was not an anabolic stimulus and therefore did not increase the expression of anabolic factors, but rather increased that of catabolic factors.

The precise mechanism of MMP-13 in response to the mechanical stimuli has not been well elucidated. Cbfa1 is known to be involved in the expression of its target MMP-13, and Cbfa1 -/- mice show an absence of MMP-13 expression. Previous reports showed that CTS, but not in hydrostatic pressure, upregulated the expression of Cbfa1, the transcription factor with a fundamental role in bone formation, as well as that of MMP-13 in bovine cartilage [9]. Although we failed to examine the changes in Cbfa1 expression after CTS, the Cbfa1/MMP-13 pathway might contribute to the early upregulation of MMP-13 mRNA expression by rat chondrocytes.

Cathepsin B is a lysosomal cysteine proteinase which is likely to be involved in the progression of OA owing to its activity as an aggrecanase. The mechanism of cathepsin B expression by chondrocytes has not been fully elucidated, but IL-1 seems to play a critical role in cathepsin B induction. Baici *et al.* [24] reported that the accumulation of cathepsin B in intracellular granules was regularly stimulated by a factor of 2–4 in the presence of IL-1$\beta$ in rabbit articular chondrocytes. In the current study, we demonstrated the synergic upregulation of IL-1$\beta$ and cathepsin B after CTS between 12 and 24 h, suggesting the regulation of cathepsin B expression by IL-1$\beta$.

IL-4 pre-treatment significantly countered the mechanical stress-induced upregulation of MMP-13 and cathepsin B in the current study. In the rheumatoid synovium, IL-4 plays an anti-inflammatory role by reducing the production of IL-1$\beta$ by 2.3 fold and increasing that of the IL-1 receptor antagonist (IL-1Ra) by 2.8 fold [25]. The synchronized downregulation of cathepsin B and IL-1$\beta$ by IL-4 pretreatment might be caused by the inhibitory effect of IL-4 on CTS-induced IL-1$\beta$ expression.

Deschner *et al.* reported that high magnitude tensile stress generates signals that employ nuclear factor

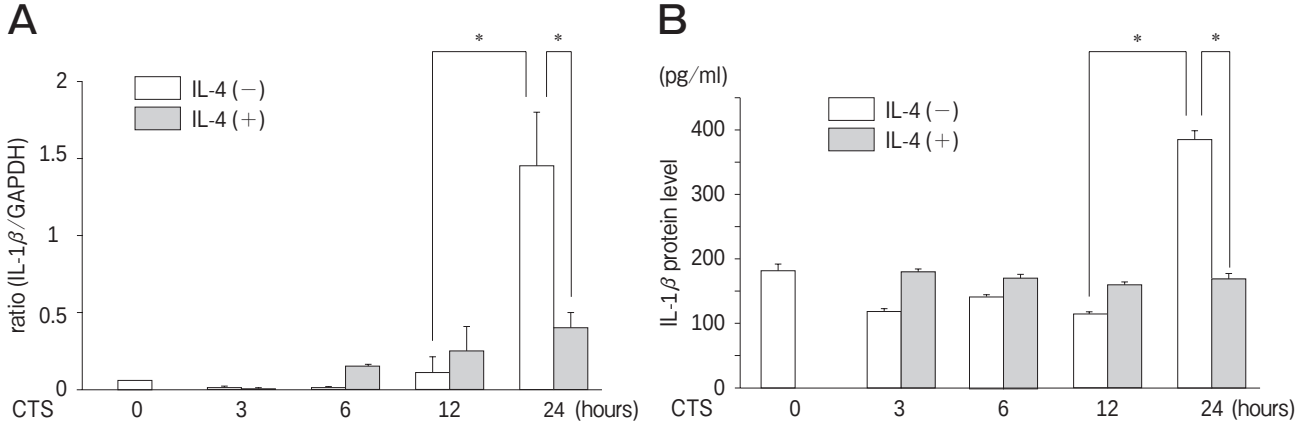


**Fig. 5** Effects of CTS (7% elongation at 0.5 Hz) on IL-1$\beta$ mRNA expression by rat chondrocytes. (**A**) The changes in mRNA levels in chondrocytes of IL-1$\beta$ were measured by real-time quantitative PCR using control GAPDH (clear bar). IL-4 pre-treatment (10 ng/ml) at each time point under CTS is shown by a dark bar. IL-1$\beta$ mRNA was upregulated by CTS after 24 h and was downregulated by IL-4. ($p < 0.01$) (**B**) Effects of CTS (7% elongation at 0.5 Hz) on IL-1$\beta$ protein expression by rat chondrocytes. The changes in protein levels in chondrocytes of IL-1$\beta$ were measured by ELISA. IL-4 pre-treatment (10 ng/ml) at each time point under CTS is shown by a dark bar. IL-1$\beta$ protein was upregulated by CTS after 24 h and was downregulated by IL-4. ($p < 0.01$) Data are shown as the mean ± standard deviation of triplicate determinations. The experiments were repeated 3 times and obtained similar results.

kappa B (NF-$\kappa$B), a transcription factor that regulates numerous pro-inflammatory genes, including that encoding MMP-13 [26]. As MMP-13 was downregulated by IL-4 pre-treatment at 24 h after CTS induction, MMP-13 downregulation was also partly associated with the downregulation of IL-1$\beta$, which increases the activity of NF-$\kappa$B. However, MMP-13 upregulation by CTS was noted at a relatively early time point of CTS induction (3 h), followed by upregulation of IL-1$\beta$. Because IL-4 might affect the IL-1-independent upregulation of MMP-13, it would be interesting to investigate the effect of IL-4 on CTS-induced activation of transcription factors, such as Cbfa1.

The effects of IL-1 are inhibited *in vitro* and *in vivo* by natural inhibitors such as IL-1 receptor antagonist by blocking its interaction with cell surface receptors. Inhibition of IL-1 has been proven to result in amelioration of osteoarthritis-like pathology in osteoarthritic culture model [27]. In the current study, we failed to examine the IL-1$\beta$ -independent effect of IL-4 on cathepsin B expression by CTS, with the blockage of IL-1$\beta$ by exogenous IL-1Ra. It was reported that IL-1$\beta$ antagonist (a single intraarticular injection of 50 or 150 mg) had no analgesic effect during 3 months of follow-up in a first randomized placebo-controlled trial [28]. These findings and the results of the current study suggest that benefit of IL-4 on the mechanical stress-induced expressions of catabolic factors might be limited to the early degenerative change of cartilage, and in the later stage of OA, it might be difficult to suppress all MMPs and other enzymes involved in the cartilage destruction even by the total suppression of IL-1$\beta$ by IL-1Ra.

In conclusion, the current study demonstrated that MMP-13 and cathepsin B are upregulated by CTS (7% elongation, 0.5 Hz for 24 h) in rat normal chondrocytes. Pre-treatment with IL-4 effectively inhibited the CTS-induced expression of MMP-13 and cathepsin B, which might have been partly caused by the regulation of CTS-induced IL-1$\beta$ expression by IL-4. Further study would be needed to explore the role of IL-4 on the changes in mechanical stress-induced transcriptional gene regulation in chondrocytes.

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