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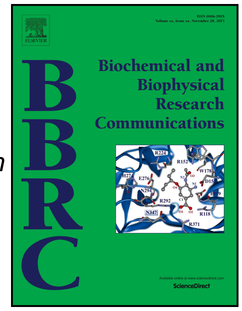

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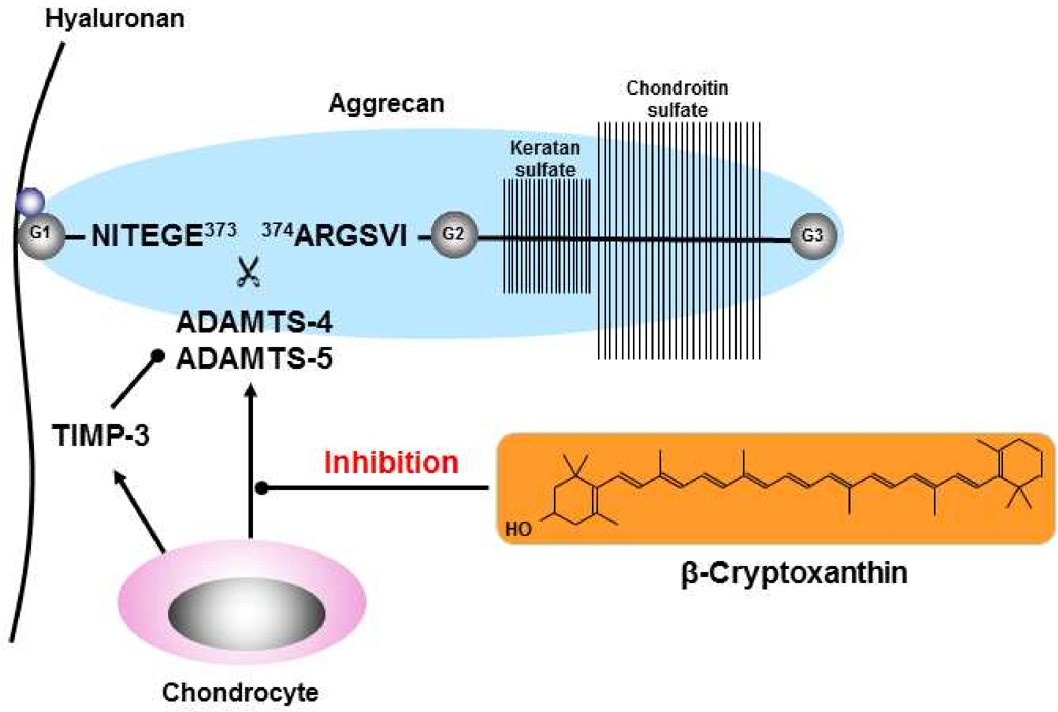
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G1
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TIMP-3
Inhibition
HO
β-Cryptoxanthin
Chondrocyte

Anti-arthritic actions of β-cryptoxanthin against the degradation of articular cartilage *in vivo* and *in vitro*

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ABSTRACT

An inverse correlation between the morbidity of rheumatoid arthritis and daily intake of β-cryptoxanthin has been epidemiologically shown. In this study, we investigated the effects of β-cryptoxanthin on the metabolism of cartilage extracellular matrix *in vivo* and *in vitro*. Oral administration of β-cryptoxanthin (0.1-1 mg/kg) to antigen-induced arthritic rats suppressed the loss of glycosaminoglycans in articular cartilage, which is accompanied by the interference of aggrecanase-mediated degradation of aggrecan. Inhibition of the interleukin 1α (IL-1α)-induced aggrecan degradation by β-cryptoxanthin was also observed with porcine articular cartilage explants in culture. β-Cryptoxanthin (1-10 μM) dose-dependently down-regulated the IL-1α-induced gene expression of aggrecanase 1 (ADAMTS-4) and aggrecanase 2 (ADAMTS-5) in cultured human chondrocytes. Moreover, β-cryptoxanthin was found to augment the gene expression of aggrecan core protein in chondrocytes. These results provide novel evidence that β-cryptoxanthin exerts anti-arthritic actions and suggest that β-cryptoxanthin may be useful in blocking the progression of rheumatoid arthritis and osteoarthritis.

Keywords*:* rheumatoid arthritis, osteoarthritis, carotenoid, extracellular matrix, aggrecanase, chondrocytes

1. Introduction

Degenerative joint diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) are characterized by cartilage destruction with a loss of its ability to resist compressive and tensile forces due to degradation of the extracellular matrix (ECM) [1-3]. Cartilage is a specialized connective tissue whose ECM is highly organized having major macromolecules including type II collagen, hyaluronan, and aggrecan [4]. Aggrecans are present in cartilage as large aggregates interacting with hyaluronan and link proteins, and they are highly hydrated due to the negatively charged polysaccharide chains attached to the core proteins. This provides the cartilage with its ability to resist compressive loads. On the other hand, type II collagen forms a fibrillar meshwork that provides the tissue with tensile strength. These macromolecules function to maintain the homeostasis as well as the structural integrity of cartilage. In RA and OA, degradation of cartilage ECM exceeds its synthesis due to elevated activity of proteolytic enzymes, of which aggrecanases and matrix metalloproteinases (MMPs) are considered to be the major effectors [1].

Increased aggrecanase-dependent aggrecan degradation has been reported to be detectable in RA and OA cartilage [2,3]. The expression of aggrecanse-1, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4, in OA cartilage has been reported to correlate with the Mankin score of the disease [5]. On the other hand, aggrecanase-2 (ADAMTS-5) is constitutively expressed in human cartilage, but the expression of ADAMTS-5 is transiently elevated in human cartilage treated with interleukin (IL)-1, which is an inflammatory cytokine for the aggravation of RA and OA [6]. Furthermore, the deletion of active ADAMTS-5 in mice has been reported to protect their joints from the destruction occurring in the antigen-induced RA model [7], or in the meniscus destabilization model of OA [8], suggesting that aggrecan degradation by ADAMTS-5 is crucial for the development of arthritis at least in those animal models. These observations allow us to speculate that aggrecanase may become a target molecule for the treatment of RA and OA. At the moment, however, clinically effective inhibitors of aggrecanases for RA and OA have not been developed [2,3].

Currently, treatments to targeting cytokines, including anti-tumor necrosis factor α (TNFα) antibodies (Infliximab and Adalimumab), soluble TNF receptor (Etanercept), anti-IL-6 receptor antibody (Tocilizumab) and IL-1 receptor antagonist (Anakinra), are widely used for RA [9], but these treatments have problems, especially in cost and the increased susceptibility to infection [10]. Therefore, therapeutics that can be safely used for a long term would be preferable. A few epidemiologic studies [11,12] have shown the inverse correlation between morbidity of RA and daily intake of β-cryptoxanthin, suggesting that β-cryptoxanthin may prevent the development of RA. β-Cryptoxanthin is a widely distributed carotenoid pigment in citrus fruits, and most abundant in *Citrus unshiu* Marcovich (mandarin-orange) [13]. Like other carotenoids, β-cryptoxanthin exhibits an anti-oxidative action. In addition, a portion of intestinally absorbed β-cryptoxanthin has been reported to be enzymatically converted to retinoids in intestine and liver by β-carotene-15,15'-oxygenase [14]. Furthermore, several biological effects, e.g., an inhibition of carcinogenesis [15], bone anabolic activity [16], and an interference with bone resorption [17], have been experimentally shown. However, the effects of β-cryptoxanthin on cartilage ECM metabolism have not been reported. In this study, we found the inhibitory effects of β-cryptoxanthin on cartilage degradation in antigen-induced arthritic rats. Using cultured human articular chondrocytes and synovial fibroblasts, we have demonstrated that β-cryptoxanthin prevents aggrecan degradation by oppositely modulating the gene expression of aggrecanases and that of aggrecan core protein.

2. Materials and methods

2.1 Antigen-induced arthritis model

Female Lewis rats (6 weeks age, body weight 180 to 200 g, Charles River Laboratory Japan, Kanagawa, Japan) were immunized 21 and 14 days before induction of antigen-induced arthritis (AIA) with 1 mL of a suspension containing 0.5 mL each of methylated bovine serum albumin (mBSA) dissolved in phosphate buffered saline (PBS) and Freund's complete adjuvant by multiple subcutaneous injections into both flanks of the animals according to the method of Andersson *et al*. [18]. AIA was induced by intra-articular injection of 0.1 mg mBSA in 0.05 mL of PBS into the knee joint cavity. β-Cryptoxanthin (0.1, 0.3, and 1 mg/kg) (purity ≥95%; Shikoku Yashima Pure Chemicals, Tokushima, Japan) was orally administered to rats once a day starting 1 day before till the 3rd day after AIA induction (Total of 4 days). On the day 4 after AIA induction, knee joints were dissected and fixed immediately for 2 days in 4% paraformaldehyde, and then decalcified in 22.5% formic acid/10% citric acid for 5 days followed by neutralization with 5% sodium sulfate for 1 day. Tissues were then embedded in paraffin, and the tissue sections (5 μm) mounted on slides were subjected to toluidine blue (pH 4.1)-staining for glycosaminoglycans and immunohistochemical analysis. The animals had free access to food and water according to the Guidelines of Experimental Animal Care issued by Prime Minister's Office of Japan. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Sciences.

2.3 Immunohistochemical staining

For immunohistochemical analysis of aggrecan fragments, paraffin sections (5 μm) were deparaffinized and heated by microwave oven in 10 mM citrate buffer (pH 6.0) to antigen retrieval. Prior to the reaction with antibody, paraffin sections of the joint tissues were incubated with 0.1 units/mL of chondroitinase ABC (Seikagaku, Tokyo, Japan) and 0.1 units/mL of keratanase (Seikagaku) in 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl at 37°C for 1 h to digest the polysaccharide chains of aggrecan. Tissue sections were reacted with the antibody that recognizes the aggrecanase-cleaved C-terminal neoepitope amino acid sequence GGNITEGE of aggrecan core protein (Thermo Fisher Scientific, Kanagawa, Japan) as a primary antibody overnight at 4°C followed by a reaction with horseradish peroxidase-conjugated goat anti-rabbit IgG (Nichirei Biosciences, Tokyo, Japan) as a secondary antibody for 30 min at room temperature. After incubation with the secondary antibody, the sections were stained with 3,3-diaminobenzidine (Sigma Chemical, St. Louis, MO). For the control staining, non-immune rabbit IgG was used instead of the primary antibody. Positive immunohistochemical staining in a constant area of articular cartilage was calculated using a computer software for imaging analysis, Lumina vision (Mitani, Fukui, Japan).

2.4 Cell culture

Normal human articular chondrocytes (Cambrex Bio Science Walkersville, Walkersville, MD) were encapsulated in alginate beads and cultured in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) (Invitrogen, Carlsbad, CA) in the presence of 10% fetal bovine serum (FBS) (Thermo ELECTRON, Melbourne, Australia) and antibiotics [100 units/ml of penicillin G (MP Biomedicals, solon, OH) and 100 μg/ml of streptomycin sulfate (Meiji Seika, Tokyo, Japan)]. Briefly, chondrocytes were suspended in 1.2% alginate/0.15 M NaCl at the density of 4 x 10^6 cells/mL, and the cell suspension was dropped into 102 mM $CaCl_2$ solution under stirring to form alginate beads. The alginate beads were washed several times with 0.15 M NaCl, and then cells embedded in alginate beads were cultured for 7 days in DMEM-F12 with 10% FBS, the antibiotics, and 50 μM ascorbic acid-2 phosphate in 24-multiwell plates (4 beads/well), and then treated for 6 days with IL-1α (10 ng/ml) (R&D Systems, Minneapolis, MN) and/or β-cryptoxanthin (1 to 10 μM) in DMEM-F12 with 0.2% lactalbumin hydrolysate (LAH) (Sigma Chemical), the antibiotics and 50 μM ascorbic acid-2 phosphate. β-Cryptoxanthin was dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical), and the final concentration of DMSO was 0.1 % in all cultures. The harvested culture medium and cell lysate were stored at -20 °C until use.

2.5 RNA extraction and quantitative real-time reverse transcription (RT)-PCR

The total RNA (1 μg) isolated from cells using Isogen (Nippon Gene, Tokyo, Japan) was subjected to RT reaction using a QuantiTect Reverse Transcription kit (Qiagen, Tokyo, Japan) according to the manufacturer's instruction. An aliquot of the RT reaction products (an equivalent of 25 ng of total RNA) was subjected to real-time PCR using a QuantiTect SYBR Green PCR kit (Qiagen) and a QuantiTect Primer Assays [Cat No. QT00032949 for human ADAMTS-4, Cat No. QT00011088 for human ADAMTS-5, Cat No. QT00001365 for human aggrecan core protein, and Cat No. QT00079247 for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] (Qiagen). PCR was performed using ABI PRISM 7000 sequence detection system (Applied Biosystems, Tokyo, Japan) under the following conditions, denature at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Relative expression level was calculated by $\Delta\Delta C_T$ methods with the C_T value of GAPDH.

2.6 Measurement of aggrecanase activity in porcine cartilage explants

Aggrecanase activity was determined using porcine cartilage explant culture as previously described [19]. Total glycosaminoglycan (GAG) released from the cartilage explants into the conditioned media was measured using a modification of the dimethylmethylene blue assay [20].

2.7 Detection of aggrecan fragments

Aggrecan fragments caused by aggrecanase were analyzed by Western blotting using a mouse monoclonal antibody [BC-3] against the N-terminal neoepitope ARGSV of aggrecan generated by aggrecanase (a gift from Dr. C. Hughes from University of Cardiff), as described previously [21]. Immunoreactive aggrecan neoepitope (ARGSV) was visualized with enhanced chemiluminescence-Western-blotting detection reagents (GE Healthcare Bio-Sciences, Tokyo, Japan) using an Image Analyzer LAS-1000 Plus (GE Healthcare Bio-Sciences) according to the manufacturer's instructions.

2.8 Statistical analysis

A one-way ANOVA was performed using StatView version 5.0 (SAS Institute, SAS Campus Drive Cary, NC) for the data analysis. Independent student's *t*-test was applied for pair comparisons, and Fisher's PLSD *post-hoc* test was performed for multiple comparisons. P-value less than 0.05 was considered significant difference.

3. Results

3.1 Anti-arthritic action of β-cryptoxanthin in antigen-induced arthritic rats

To investigate the anti-arthritic action of β-cryptoxanthin, AIA rats were used as an arthritic disease model [18], and the knee joints were histologically evaluated. Poorly stained signals by toluidine blue were detected in AIA rats compared with untreated control rats, indicating the loss of glycosaminoglycans (GAG) (Fig. 1A *vs.* B). Orally administered β-cryptoxanthin interfered with the loss of GAG in a dose-dependent manner (Figs. 1C-E *vs.* B). Immunohistochemical staining with the antibody recognizing the C-terminal neoepitope NITEGE sequence of aggrecan showed an increased aggrecanase-dependent fragmentation of aggrecan in AIA rat, which were largely localized with chondrocytes (Fig. 1F *vs.* G). The treatment of the AIA rats with β-cryptoxanthin suppressed the generation of NITEGE fragments (Figs. 1H-J *vs.* G). Imaging analysis of immunostained NITEGE fragments revealed that β-cryptoxanthin dose-dependently decreased aggrecan degradation in AIA rats (Fig. 1K). These observations suggested that β-cryptoxanthin exerts an anti-arthritic action through interference with the aggrecanase-mediated aggrecan degradation.

3.2 β-Cryptoxanthin blocks IL-1α-induced aggrecan degradation in porcine cartilage

Next, we examined whether β-cryptoxanthin affects aggrecan degradation in cartilage stimulated with IL-1α. As shown in Fig. 2A, β-cryptoxanthin dose-dependently suppressed the IL-1α-augmented GAG release from porcine articular cartilage. Western blot analysis also revealed that β-cryptoxanthin decreased the amount of N-terminal neoepitope ARGSV of aggrecan generated by the IL-1α-induced aggrecanase activity (Fig. 2B). There were no detectable aggrecan fragments produced by MMPs under these experimental conditions (Fig. S1). These observations suggest that β-cryptoxanthin blocks the aggrecan degradation in cartilage by decreasing aggrecanase activity.

3.3 Effects of β-cryptoxanthin on the gene expression of ADAMTSs-4 and -5, and aggrecan core protein in human articular chondrocytes

To clarify the effect of β-cryptoxanthin on the gene expression of ADAMTSs-4 and -5 in articular chondrocytes, human articular chondrocytes encapsulated in alginate beads were treated with IL-1α in the presence or absence of β-cryptoxanthin. As shown in Fig. 3A, β-cryptoxanthin suppressed the IL-1α-induced gene expression of ADAMTSs-4 and -5 in a dose-dependent manner (*upper* and *lower panels*, respectively). Tissue inhibitor of metalloproteinases 3 (TIMP-3) inhibits the enzymatic activity of ADAMTSs-4 and -5 [22], but there were no significant changes in the mRNA level of TIMP-3 in the β-cryptoxanthin-treated chondrocytes (Fig. 3B). On the other hand, IL-1α slightly decreased the mRNA levels of aggrecan core protein in chondrocytes, but β-cryptoxanthin augmented the gene expression of aggrecan core protein above the level of the control chondrocytes (Fig. 3C). Taken together, these results suggest that β-cryptoxanthin protects cartilage from degradation by suppressing the gene expression of ADAMTSs-4 and -5 and augmenting that of aggrecan core protein in human articular chondrocytes.

4. Discussion

Aggrecanases play central roles in both physiological and pathological catabolism of cartilage matrix [2,3], and ADAMTS-4 is considered to participate in the degradation of ECM molecules in joint tissues, including those in cartilage and ligaments. Therefore, the suppression of ADAMTS expression is considered to be an effective way to block the cartilage destruction in RA and OA [2,3].

The expression of ADAMTSs-4 and ADAMTS-5 mRNAs is induced by IL-1, TNFα, the combination of IL-1 and oncostatin M, or transforming growth factor-β [6,23], whereas n-3 fatty acids [24] and fibroblasts growth factor 2 [6] suppress of the expression of ADAMTS-4 and ADAMTS-5, respectively. A few natural organic compounds have been reported to block the inflammatory cytokine-induced production of ADAMTSs in chondrocytes or synovial fibroblasts. For example, triptolide from the Chinese herb, *Tripterygium wilfordii* Hook F suppresses the expression of ADAMTS-4 in chondrocytes [25], and nobiletin, a citrus polymethoxy flavonoid, blocks the expression of ADAMTSs-4 and -5 in collagen-induced arthritic mice model [26]. β-Cryptoxanthin described here is a novel natural compound that prevents cartilage destruction both *in-vitro* and *in-vivo* models of arthritis. The efficacy of the orally administered β-cryptoxanthin in blocking cartilage destruction in the rat AIA model suggests its potential as an anti-arthritic agent in OA and RA.

Regarding the control of enzymatic activity of aggrecanases, TIMP-3 has been reported to be an important cartilage protectant due to the inhibition of ADAMTSs-4 and -5 activity [22]. In this study, we have demonstrated that β-cryptoxanthin suppressed the IL-1α-induced gene expression of ADAMTSs-4 and -5 in human chondrocytes, but it did not alter the gene expression of TIMP-3 in human chondrocytes. On the contrary, we found that β-cryptoxanthin augments the gene expression of aggrecan core protein in human chondrocytes. Aggrecan is the most abundant proteoglycan in cartilage, and aggrecanase-mediated degradation of aggrecan is observed at the initial phase of cartilage destruction [1-3], which makes collagen fibrils more susceptible to collagenases [27]. Therefore, the augmentation of aggrecan biosynthesis shifts cartilage metabolism towards the anabolic state. Together, these results indicate that β-cryptoxanthin prevents cartilage from catabolism and helps to maintain homeostasis of the cartilage tissue.

It has been reported that the serum concentration of β-cryptoxanthin is approximately 0.1 to 0.2 μM, whereas more than 1 μM is detectable in Japanese inhabitants who usually take *Citrus unshiu* Marcovich [28,29]. Thus, our findings support the epidemiological studies reporting an inverse correlation between the morbidity of RA and the daily intake of β-cryptoxanthin. In contrast, the daily intake of other carotenoid pigments does not correlate with the incidence of RA [11,12]. We have presented preliminary data demonstrating that β-carotene and astaxanthin suppressed the gene expression of ADAMTS-4, but not that of ADAMTS-5, while β-cryptoxanthin decreased the mRNA level of both ADAMTSs-4 and -5 in cultured human synovial fibroblasts (Fig. S2). Furthermore, since ADAMTS-5 has been shown to be the major aggrecanase, at least in mouse arthritis models [7,8], the blocking effect of β-cryptoxanthin on ADAMTS-5 expression may be a key factor related to the morbidity of RA.

Orally administered β-cryptoxanthin has been reported to be partially converted to retinoids by β-carotene-15,15'-oxygenase [14], which thereby exhibits provitamin A activity. Although retinoic acid has been reported to up-regulate the expression of ADAMTS-5 mRNA [30], we have found that all-trans retinoic acid (1 μM) did not influence the gene expression of ADAMTS-5 but suppressed that of ADAMTS-4 in human synovial fibroblasts (Fig. S3). Furthermore, we conclude that β-cryptoxanthin is the molecule that suppresses ADAMTS-5 expression, but further experiments are necessary to clarify the transcriptional regulatory mechanism of β-cryptoxanthin.

In conclusion, we have provided novel evidence that β-cryptoxanthin exerts chondroprotective actions by inhibition of aggrecan degradation by suppressing ADAMTSs-4 and -5 expression and augmenting aggrecan synthesis *in vivo* and *in vitro*. Our study sheds light on the molecular mechanism behind the epidemiologic observation that daily intake of β-cryptoxanthin reduces the morbidity of RA.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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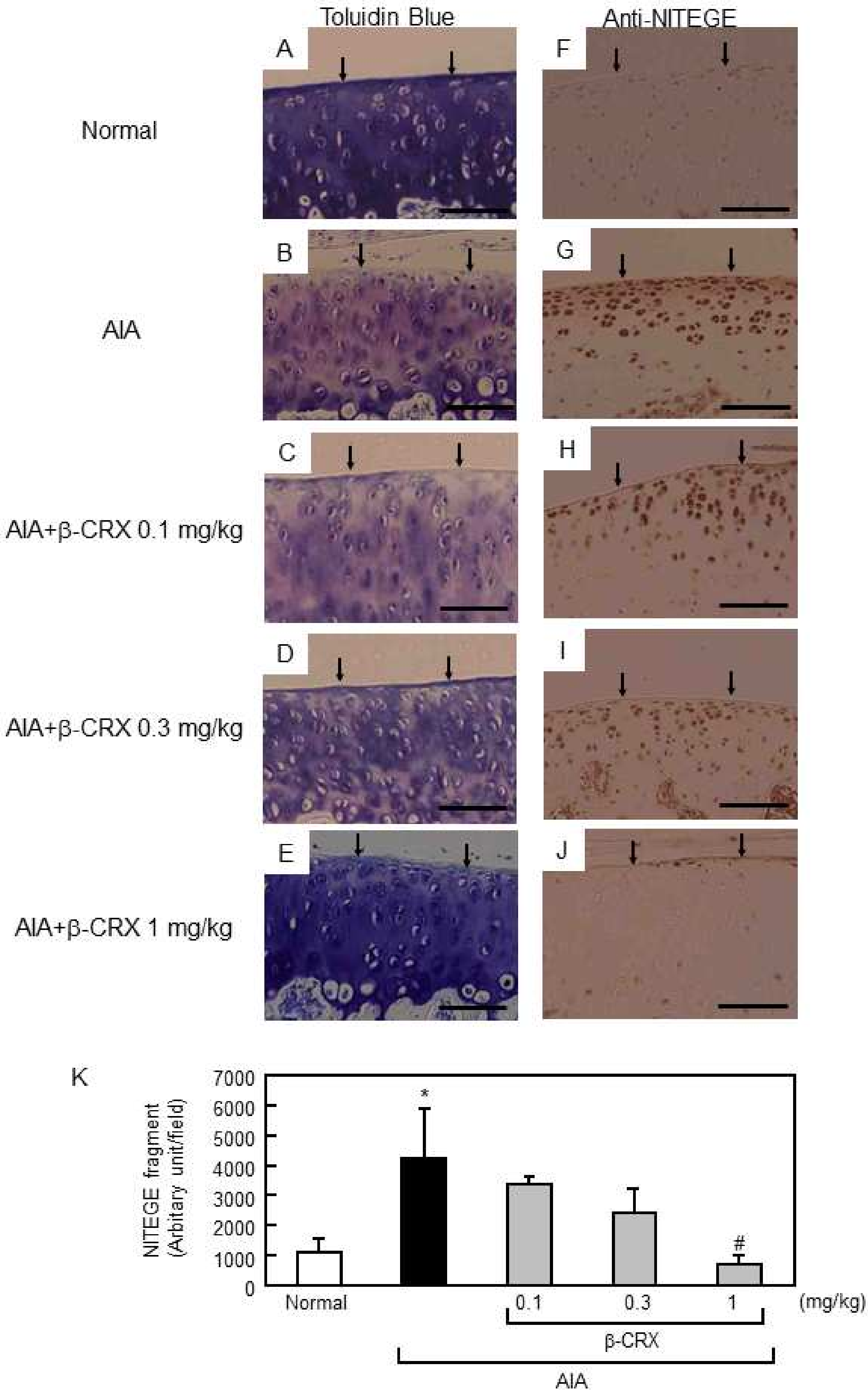
Figure legends

Fig. 1. Effects of β-cryptoxanthin on cartilage destruction in AIA rats. β-Cryptoxanthin (β-CRX) (0.1, 0.3, and 1 mg/kg) was orally administrated to rats once a day starting from 1 day before till the 3rd day after AIA induction. Knee joints were dissected, and the tissue sections (5 μm) mounted on slides were subjected to toluidine blue (pH 4.1)-staining and immunohistochemical analysis using the antibody that recognize the aggrecanase-cleaved C-terminal neoepitople amino acid sequence NITEGE of aggrecan core protein. [A]-[E]: toluidine blue (pH 4.1)-staining and [F]-[J]: immunohistochemical staining for the aggrecanase-cleaved fragments with anti-NITEGE antibody. [K]: Positive immunohistochemical staining in a constant area of articular cartilage was calculated by imaging analysis, and data represent as mean ± SEM for 3 animals. Arrows indicate the surface of cartilage. Bars indicate 100 μm. *, significantly different from control (Normal) ($p<0.05$). $^{\#}$, significantly different from AIA treatment ($p<0.05$).

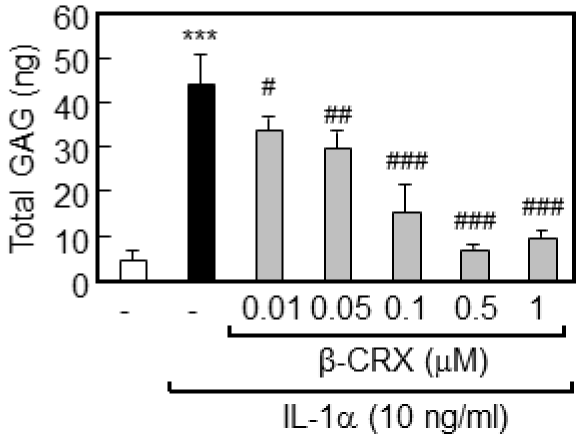
Fig. 2. β-Cryptoxanthin interferes with IL-1α-induced aggrecan degradation in cultured porcine cartilage explants. Porcine articular cartilage explants were treated with IL-1α (10 ng/ml) in the presence or absence of β-cryptoxanthin (β-CRX) (0.01 to 1 μM) for 48 h. [A]: total GAG released into the conditioned medium was measured by the DMMB assay as described in the text. [B]: aggrecan fragments generated by aggrecanase activity were detected by Western blot analysis using BC-3 antibody against N-terminal neoepitope ARGSV of aggrecan generated by aggrecanase. Data represent the mean ± SEM. ***, significantly different from the untreated control cells ($p<0.001$). $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$, significantly different from the cells treated with IL-1α ($p<0.05$, 0.01, and 0.001, respectively).

Fig. 3. Effect of β-cryptoxanthin on the gene expression of ADAMTSs-4 and -5, TIMP-3, and aggrecan core protein in human articular chondrocytes. Human chondrocytes embedded in alginate beads at the 5th passage were treated for 6 days with IL-1α (10 ng/ml) in the presence or absence of β-cryptoxanthin (β-CRX) (1, 5, and 10 μM). Total RNA was subjected to quantitative real-time RT-PCR for ADAMTSs-4 and -5 [A], TIMP-3 [B], and aggrecan core protein [C] as described in the text. Relative expression is shown by taking IL-1α-treated cells (lane 2) as 1 after normalized by GAPDH mRNA. Data represent the mean ± SEM for 3 independent experiments. * and ***, significantly different from the untreated cells ($p<0.05$ and 0.001, respectively). $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$, significantly different from the cells treated with IL-1α ($p<0.05$, 0.01, and 0.001, respectively).

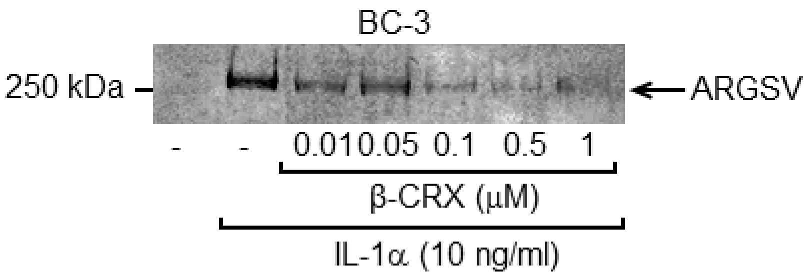


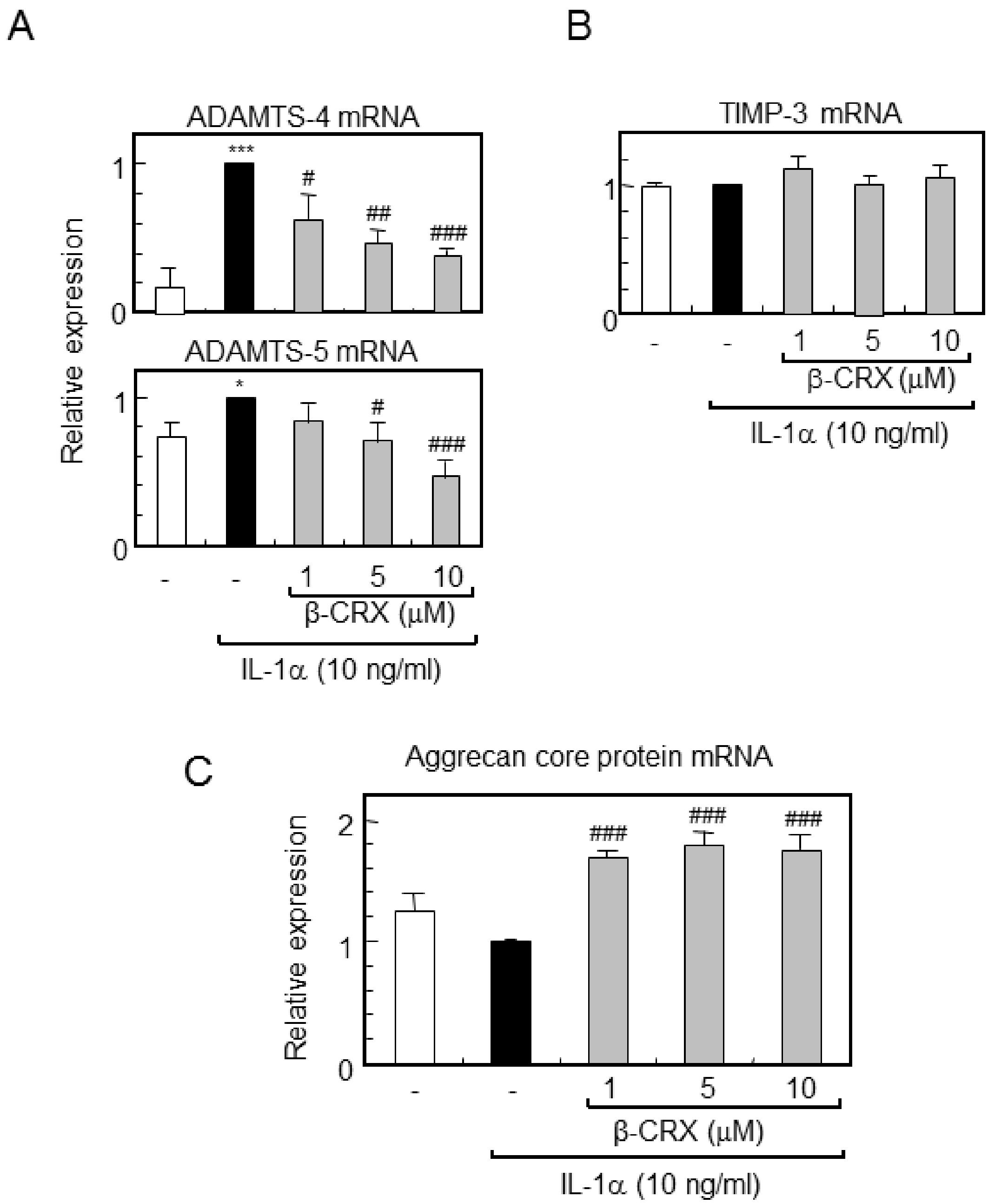
Toluidin Blue
Anti-NITEGE
A
F
Normal
B
G
AIA
C
H
AIA+β-CRX 0.1 mg/kg
D
I
AIA+β-CRX 0.3 mg/kg
E
J
AIA+β-CRX 1 mg/kg
K
NITEGE fragment (Arbitary unit/field)
7000
6000
5000
4000
3000
2000
1000
0
*
#
Normal
0.1
0.3
1
(mg/kg)
β-CRX
AIA

A


60
50
40
30
20
10
0
Total GAG (ng)
***
#
##
###
###
###
-
-
0.01 0.05 0.1 0.5 1
β-CRX (μM)
IL-1α (10 ng/ml)

B


BC-3
250 kDa
ARGSV
-
-
0.01 0.05 0.1 0.5 1
β-CRX (μM)
IL-1α (10 ng/ml)


A
ADAMTS-4 mRNA
***
#
##
###
ADAMTS-5 mRNA
*
#
###
Relative expression
1
0
1
0
-
-
1
5
10
β-CRX (μM)
IL-1α (10 ng/ml)
B
TIMP-3 mRNA
1
0
-
-
1
5
10
β-CRX (μM)
IL-1α (10 ng/ml)
C
Aggrecan core protein mRNA
###
###
###
Relative expression
2
1
0
-
-
1
5
10
β-CRX (μM)
IL-1α (10 ng/ml)

Highlights

- β-Cryptoxanthin blocks the aggrecan degradation in cartilage by decreasing aggrecanase activity *in vivo*.
- β-Cryptoxanthin down-regulates the expression of aggrecanase 1 (ADAMTS-4) and aggrecanase 2 (ADAMTS-5) in human chondrocytes.
- β-Cryptoxanthin augments the expression of aggrecan core protein in human chondrocytes.