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ORIGINAL ARTICLE

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Interleukin-4 protects matrix synthesis in chondrocytes under excessive mechanical stress in vitro

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Abstract We examined the effect of interleukin-4 (IL-4) on matrix synthesis in chondrocytes under excessive mechanical stress in vitro. Chondrocytes from 7-day-old rat articular cartilage were incubated in the presence of rat IL-4 (0, 1, and 10 ng/ml) under a 5% CO_2 atmosphere for 36 h. Cyclic mechanical stress (0.5 Hz, 7% elongation) was loaded using a Flexercell strain unit for 12, 16, and 24 h. Levels of messenger RNA (mRNA) for aggrecan (AGG), type II collagen (CII), fibronectin (FN), and integrin-β1 (INTb1) were measured by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using GAPDH as the internal control. Mechanical stress for 16 h significantly decreased levels of mRNA for both AGG and CII ($P < 0.01$), but with rat IL-4 at doses of 1 or 10 ng/ml these levels recovered ($P < 0.05$). In addition, mRNA levels of FN and INTb1 were increased by IL-4 in chondrocytes under mechanical stress ($P < 0.05$). IL-4 protects chondrocytes loaded with excessive mechanical stress against degradation

Key words Chondrocytes · Fibronectin (FN) · Integrin-βl (INTb1) · Interleukin-4 (IL-4) · Mechanical stress

Introduction

Because articular cartilage is exposed to various magnitudes and cycles of mechanical stress, cyclic mechanical stimulation plays an important role in chondrocyte metabolism.[1] Many studies have shown that optimal mechanical stimulation maintains the structure and function of articular cartilage, whereas excessive mechanical force leads to a loss of cartilage and the onset of osteoarthritis (OA).[2–5]

Cartilage explants and cultured chondrocytes can be exposed to direct mechanical load, hydrostatic pressure, and stretching forces in vitro.[6–12] Some of these studies suggest that excessive mechanical stress both decreases matrix metabolism and changes protein biosynthesis.[6,7,10,12]

Interleukin-4 (IL-4), a 20-kDa glycoprotein, is a B cell growth and differentiation factor secreted by activated T cells that can suppress synthesis of the catabolic factor interleukin-1 (IL-1), tumor necrotic factor alpha (TNF-α), and the arachidonic acid metabolite prostaglandin E2 (PGE2), in human monocytes. In bovine articular cartilage explants, IL-4 inhibits cartilage breakdown.[13] Mechanically stimulated human articular chondrocytes release IL-4 and IL-4 from chondrocytes with subsequent autocrine/paracrine activity is likely to be part of an important regulatory pathway that maintains the structure and function of articular cartilage.[14]

To clarify the protective effect of IL-4 on matrix synthesis in vitro, we loaded rat chondrocytes with excessive mechanical stress in the presence of IL-4. Levels of mRNA for type II collagen (CII), aggrecan (AGG), fibronectin (FN), and integrin-β1 (INTb1) were quantified by real-time reverse transcription polymerase chain reaction (RT-PCR).

Materials and methods

Cells

Chondrocytes isolated from the knee joints of 7-day-old Wistar rats were digested with 0.1% α-chymotrypsin (Wako, Japan) in alpha-modified Eagle's medium (α-MEM) pH 7.4 and 0.2% type II collagenase (SIGMA Chemical, St Louis, MO, USA) following the method of Bruckner et al.[15] Chondrocytes (5×10^4/ml) were seeded in 25-mm Flexercell dishes coated with type I collagen (Flexcell International, McKeesport, PA, USA) and cultured in 3 ml α-MEM containing 10% fetal bovine serum (FBS, Sanko Junyaku, Tokyo, Japan) and 100 mg/l kanamycin. The Ethical Committee for Animal Experimentation at Ehime University (Ehime, Japan) approved the study protocol.

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Culture with IL-4

Chondrocytes were incubated in a 5% CO_2 humidified incubator at 37°C for 3 days. After washing with phosphate-buffered saline (PBS), the chondrocytes were incubated with recombinant rat IL-4 (Diaclone Research, Cedex, France) (0, 1, and 10 ng/ml) under a 5% CO_2 atmosphere for 36 h, and were then loaded with mechanical stress.

Cyclic mechanical stress

Chondrocytes in the Flexercell dishes were loaded with cyclic mechanical stress using a Flexercell strain unit (Flexcell) under the following conditions: elongation 7%, 30 cycles/min, alternative loading, and relaxation for 1 s, following a method established previously, but with some modification.[16]

Total RNA extraction and cDNA synthesis

Total RNA extracted from cultured chondrocytes using Trizol (Gibco BRL, NY, USA) was dissolved in 0.1% diethylpyrocarbonate (DEPC) water and quantified by spectrophotometry at 260 nm (absorbance). Reverse transcriptase-PCR proceeded using a TaKaRa RNA PCR Kit Ver. 2.1 (TaKaRa Bio, Tokyo, Japan).

Primers

All primers were designed at the Japan Gene Research Laboratories (Tokyo, Japan). The sequences for primers are shown in Table 1.

Real-time quantitative PCR

Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. Reaction components were prepared to the end-concentration indicated: 9.4 μl water, 1.6 μl $MgCl_2$ (3 mM), 1.0 μl forward primer (0.5 μM), 1.0 μl reverse primer (0.5 μM), 2.0 μl LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics), and 5.0 μl sample. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-DNA Master SYBR Green I mix. To improve SYBR Green I quantification, a high-temperature fluorescence measurement was performed.[17] The LightCycler real-time PCR cycling conditions for all assays are listed in Table 2.

Table 1. Forward (for) and reverse (rev) primer sequences

Primer	Sequence (5′ → 3′)
GAPDH for	AGA ACG GGA AGC TCA CTG G
GAPDH rev	TCC ACC ACC CTG TTG CTG TA
Collagen II for	CCC AGA ACA TCA CCT ACC AC
Collagen II rev	GGT ACT CGA TGA TGG TCT TG
Aggrecan for	GAT GTC CCC TGC AAT TAC CA
Aggrecan rev	TCT GTG CAA GTG ATT CGA GG
Fibronectin for	GTC AAC TAC AAG ATC GGA GA
Fibronectin rev	GAT ACT CTT TCT GCC ACT GT
Integrin β1 for	AAT TGT AGC AGG CGT GGT
Integrin β1 rev	TCG GAT TGA CCA CAG TTG T

Statistical analysis

The results were examined by one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Histological findings

We cultured chondrocytes with or without IL-4 and loaded them with mechanical stress using a Flexercell strain unit for 24 h. We then examined the stressed cells located in the middle of the Flexercell dishes using a phase-contrast microscope. Without mechanical stress the chondrocytes were polygonal, and this was not altered by IL-4 (data not shown). After 24 h stimulation, chondrocytes that were not incubated with IL-4 gathered together and the cell density decreased (Fig. 1A). Cells in the presence of IL-4 (10 ng/ml) retained their original morphology (Fig. 1B).

Effect of mechanical stress on the matrix synthesis

To investigate the effect of mechanical stress on matrix synthesis in the cultured chondrocytes, levels of mRNA for AGG and CII were measured. Mechanical stress was loaded onto the chondrocytes for 0, 12, 16, and 24 h. The mRNA of AGG and CII was decreased significantly after 16 and 24 h stimulation (Table 3). mRNA expression of CII was significantly increased after 12 h stimulation, but it was significantly decreased after 16 and 24 h stimulation. There was no significant change in the mRNA expession of CII after 6 h stimulation (data not shown). These findings suggested that appropriate mechanical stress might enhance the synthesis of cartilage matrix.

Protective effect of IL-4 against the degradation of chondrocytes under excessive mechanical stress

The protective effect of IL-4 against the degradation of chondrocytes under excessive mechanical stress was examined. Several doses of IL-4 (0, 1, and 10 ng/ml) were added, and mechanical stress was loaded. mRNA levels of AGG in the chondrocytes were significantly increased after 12 and 16 h stimulation compared with levels with no treatment with IL-4, but after 24 h stimulation there was no significant difference (Fig. 2A). Furthermore, CII mRNA levels of chondrocytes treated with IL-4 at doses of 1 and 10 ng/ml

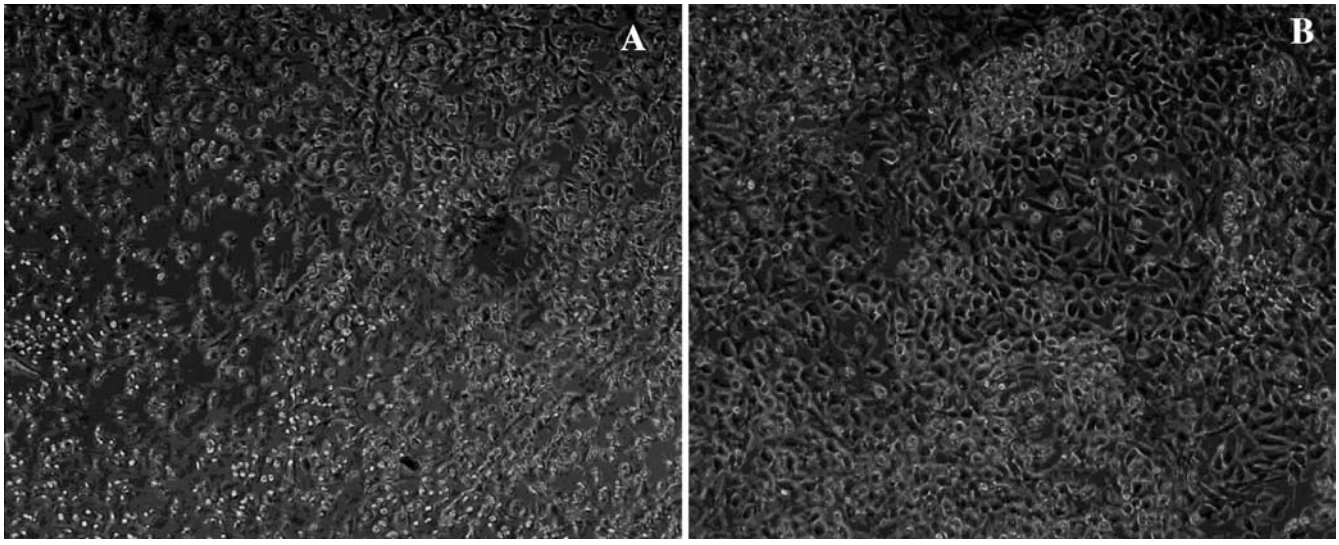

Fig. 1. Pathohistological findings of cultured chondrocytes loaded with mechanical stress. Cultured chondrocytes were loaded with mechanical stress consisting of 7% elongation at 0.5 Hz for 24 h without (**A**) and with (**B**) interleukin-4 (IL-4)

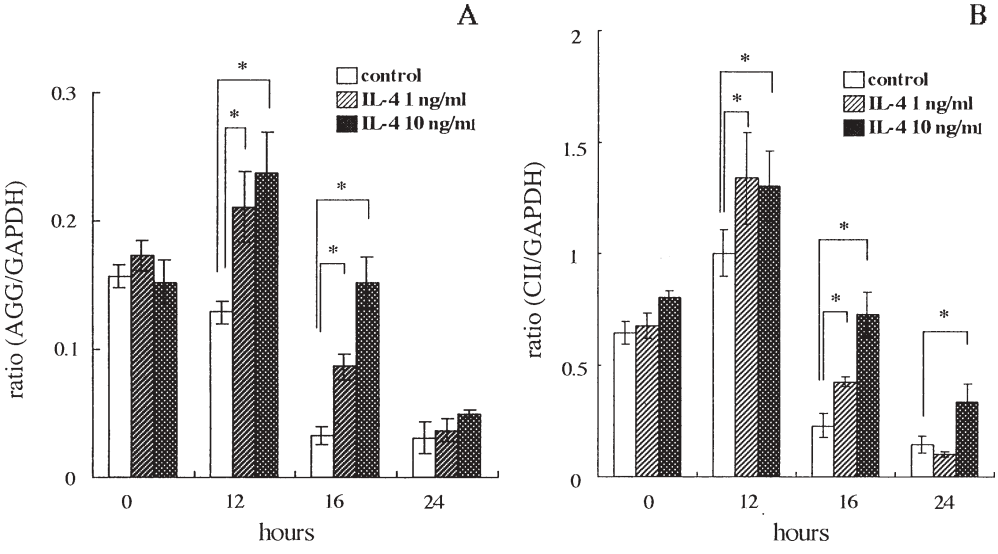

Fig. 2. Protective effect of IL-4 on matrix synthesis in rat chondrocytes loaded with mechanical stress. Cultured chondrocytes were loaded with mechanical stress as described in the legend to Fig. 1 for 0, 12, 16, and 24 h. Aggrecan (*AGG*) (**A**) and type II collagen (*CII*) (**B**) mRNAs in chondrocytes were measured by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using control GAPDH. Each experiment was repeated six times. $*P < 0.05$

Table 2. LightCycler real-time PCR cycling condition of all assays

Denaturation program: 95°C/10 min				
PCR: gene	Assay steps: Temperature (°C)/incubation time(s)			
	Denaturation	Annealing	Extension	Fluorescence acquisition
GAPDH	95/15	60/10	72/12	82/1
Type II collagen	95/15	62/10	72/8	84/1
Aggrecan	95/15	60/10	72/10	86/1
Fibronectin	95/10	62/10	72/7	83/1
Integrin beta 1	95/10	62/10	72/6	80/1
Melting curve program: 60°–95°C with a heating rate of 0.2°C/s and continuous fluorescence measurements				
Cooling: 40°C				

PCR, polymerase chain reaction

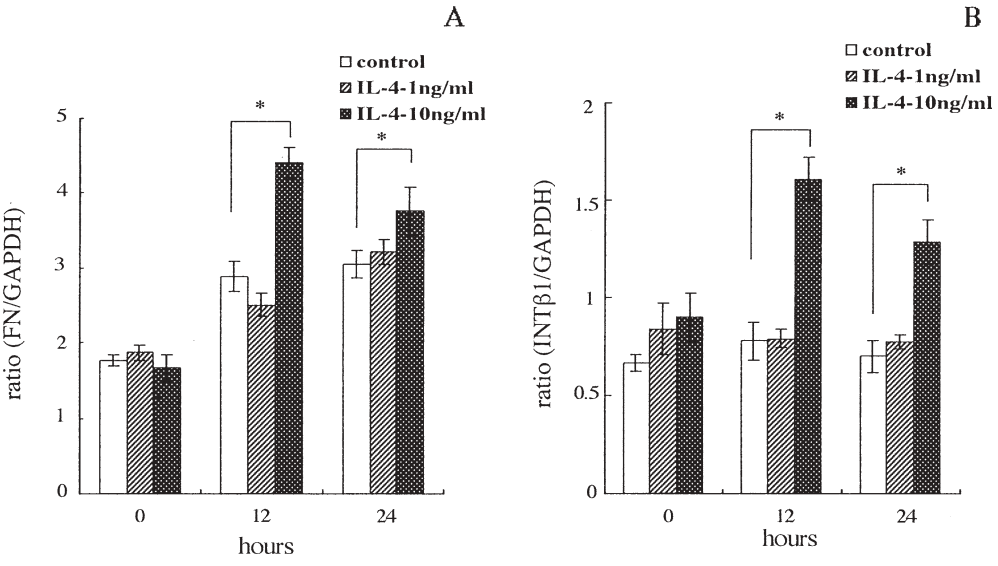

Fig. 3. The involvement of fibronectin (*FN*) and integrin-β1 (INTb1) in the matrix metabolism of rat chondrocytes loaded with mechanical stress in protecting against degradation. Mechanical stress, 7% elongation, 0.5 Hz, was loaded onto cultured chondrocytes which were stressed as described in the legend to Fig. 2 for 0, 12, and 24 h. Messenger RNAs for FN (**A**) and INTb1 (**B**) in chondrocytes were measured by quantitative RT-PCR using control GAPDH. Each experiment was repeated six times. $*P < 0.05$

Table 3. Degradation of chondrocytes by mechanical stress

mRNA ratio	Duration (h)			
	0 (control)	12	16	24
AGG/GAPDH	0.157 ± 0.157	0.120 ± 0.104	0.033 ± 0.03*	0.031 ± 0.024*
CII/GAPDH	0.646 ± 0.17	1.003 ± 0.239*	0.23 ± 0.130*	0.147 ± 0.114*

AGG, aggrecan; CII, type II collagen
Each experiment was repeated 6 times
Values represent the mean ± SE
$*P < 0.01$, comparison with control

also increased significantly under stimulation for 12 or 16 h (Fig. 2B). No effect was found on levels of mRNAs for AGG or CII with IL-4 and without mechanical stress (data not shown).

Involvement of IL-4 in the expression of FN and INTb1 by chondrocytes under mechanical stress

To investigate the involvement of IL-4 in the expression of FN and INTb1 by chondrocytes under mechanical stress, we measured the levels of FN and INTb1 mRNAs in chondrocytes. Neither of these were significantly changed in the absence of mechanical stress. However, levels of mRNA for FN in chondrocytes increased significantly after both 12 and 24 h of mechanical stimulation with IL-4 at a dose of 10 ng/ml (Fig. 3A). INTb1 mRNA levels in chondrocytes that were not affected by excessive mechanical stress were significantly increased by IL-4 at a dose of 10 ng/ml accompanied by both 12 and 24 h of mechanical stimulation (Fig. 3B). No effect was found on levels of mRNAs for FN or INTb1 with IL-4 and without mechanical stress (data not shown).

Discussion

Published reports indicate that applied stress at various degrees of magnitude and duration lead to an increase or decrease of matrix synthesis in articular cartilage.[6,8,18–20] This study found that CII mRNA expression increased in cultured chondrocytes loaded with mechanical stress consisting of 7% elongation and 0.5 Hz for 12 h, but was not changed after only 6 h (data not shown). We also found that both AGG and CII mRNA expression decreased in cultured chondrocytes loaded for over 16 h. These findings suggest that specific mechanical loading protocols may be required to optimally promote the repair and regeneration of damaged cartilage. Toyoda et al.[11] reported that collagen synthesis increases significantly under cyclic tensile loading, although proteoglycan synthesis does not. Their report and our observations support the notion that cyclic tensile loading plays an important role in the metabolism of CII, but not of AGG, in chondrocytes.

This study showed that AGG and CII mRNA expression in cultured chondrocytes incubated with IL-4 did not change significantly in the absence of mechanical stress. Under excessive stress, however, mRNA expression of these matrix components did not decrease compared with that in the absence of IL-4. These results indicated that

receptors for IL-4 on chondrocytes might be presented under mechanical stress, and that IL-4 protects against cartilage degradation under excessive mechanical stimulation. Interleukin-4 potently inhibits cartilage degradation in chondrocytes stimulated with chondrodegenerative agents.[13] Millward et al.[14] reported that IL-4 from chondrocytes with autocrine/paracrine activity is likely to be part of an important regulatory pathway in the maintenance of articular cartilage structure and function.

How IL-4 protects matrix degradation against excessive mechanical stress remains unclear. We have shown that levels of mRNA for FN and INFb1 were increased by excessive mechanical stress in the presence of IL-4. Homologous dimers, each with chains of about 2500 amino acids, are folded into six domains to comprise FN. Individual domains are specialized for binding to cartilage matrix molecules.[21,22] Levels of FN are increased under degenerative cartilage conditions such as OA.[23,24] However, the function of FN in normal articular cartilage is unknown. Steinmeyer et al.[10] reported that intermittent loading modulates and partly mediates the biosynthesis and release of FN. Wong et al.[12] found that cyclic mechanical stress is associated with increased FN synthesis. We showed that mechanical stimulation alone significantly increased FN mRNA.

Integrin-$\alpha5\beta1$ is an adhesion molecule on the cell surface that can bind to FN via the RGD sequence of FN. The interaction between integrin-$\alpha5\beta1$ and FN is key to the adhesion and spread of chondrocytes.[25] We conclude that the matrix network of cartilage is stabilized and strengthened by the production of matrix substrates FN and INFb1 in chondrocytes exposed to IL-4.

Since anti-IL-4 receptor treatment abrogated the protective effect of IL-4 against chondrocyte degradation, the response was IL-4-specific (data not shown).

These findings suggested that IL-4 might protect articular cartilage from damage caused by mechanical stress and therefore could be a useful therapeutic agent for treating arthritic disorders.

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