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Cyclic compression loading alters osteoarthritis-related gene expression in three-dimensionally cultured human articular chondrocytes via a different mechanism than interleukin-1β induction

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ABSTRACT

Objectives: Mechanical and inflammatory stimuli are key factors in the pathophysiology of osteoarthritis (OA). However, the effects of mechanical stimulation on joint tissues and cells at the molecular level and the mechanisms of interaction after stimulation with inflammatory cytokines remains uninvestigated.
Methods: Three-dimensional cyclic compression loading (CCL) was applied to human articular chondrocytes, and the expression of OA-related genes was analyzed using reverse transcription quantitative real-time polymerase chain reaction. Additionally, the effects of CCL after the chondrocytes were stimulated with interleukin (IL)-1β were evaluated. A DNA microarray assay was used to compare changes in gene expression after chondrocytes were stimulated with IL-1β and CCL was applied, and to search for pathways that are affected by CCL.
Results: CCL of 40 kPa significantly upregulated the expression of IL-8, cyclooxygenase (COX)-2, nerve growth factor, matrix metalloproteinase (MMP)-1, and MMP-3. Transcription of IL-8, COX-2, and MMP-3 was synergistically promoted by CCL and IL-1β. The top 10 pathways enriched in the Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed genes were not common in either group, except for the "cytokine-cytokine receptor interaction". The "tumor necrosis factor signaling pathway" and the "nuclear factor-kappa B signaling pathway" in the IL-1β group and "cell cycle" and the "Hippo signaling pathway" in the CCL group were included.
Conclusions: Comprehensive gene expression analysis revealed that CCL-induced changes in gene expression were different to those induced by stimulation with IL-1β. Our results provide new insights into the involvement of mechanical stimulation in the pathogenesis of OA.

1. Introduction

Synovial joints, including the temporomandibular and knee joints, can develop osteoarthritis (OA), often accompanied by inflammation [1]. Synovial fluid exhibits elevated concentrations of inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α [2–4], which promote the expression of OA-related factors [5–7]. Moreover, treatments for OA such as nonsteroidal anti-inflammatory drugs and arthrocentesis relieve pain and inhibit disease progression [8].

Similar to inflammatory cytokine stimulation, mechanical stimulation is considered a part of the pathophysiology of OA. In healthy joints, physiological mechanical stimulation caused by mastication and locomotion contributes to tissue homeostasis [9]. However, under pathological conditions—wherein the anabolic and catabolic balance is disrupted—mechanical stimulation may lead to the progression of OA [10,11]. Understanding the contribution of mechanical stimulation to the development of OA is important, and studies on the effects of mechanical stimuli on joint tissues and cells have accumulated in recent years [12–14]. However, several questions remain unanswered. The

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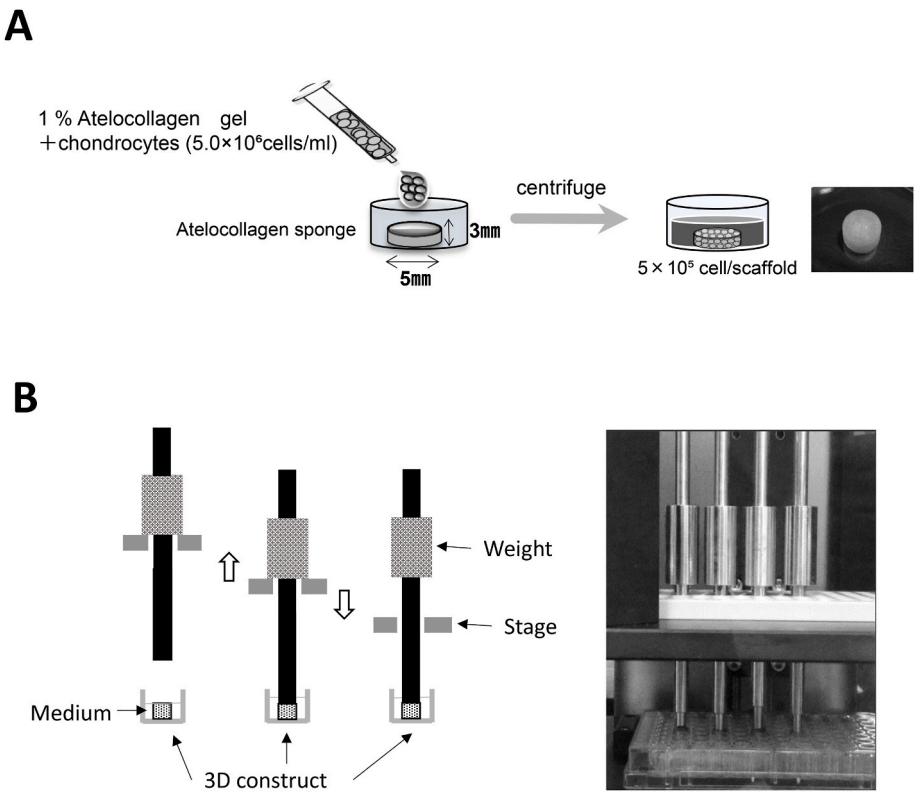


Fig. 1. Three-dimensional (3D)-cultured system and cyclic compressive loading (CCL) model.
(A) Three-dimensional cell scaffold constructs made using collagen scaffolds (Mighty® Koken Corp) (B) Cyclic load stimulator (CLS-5J-Z, Technoview, Osaka, Japan) in the incubator. Schematic representation of the cyclic load stimulator, cyclic-loaded samples.

Table 1
Primer sequences for real-time reverse transcriptase (RT)-polymerase chain reaction (PCR).

Genes	Primer sequences (5′-3′)	
GAPDH	(Forward)	TCTCTGCTCCTCCTGTTCGAC
	(Reverse)	GTTGACTCCGACCTTCACCTTC
IL-8	(Forward)	TGG CAG CCT TCC TGA TTT C
	(Reverse)	GGG TGG AAA GGT TTG GAG TAT G
IL-1β	(Forward)	CTTCGAGGCACAAGGCACAA
	(Reverse)	TTCACTGGCGAGCTCAGGTA
TNF-α	(Forward)	TGGGATCATTGCCCTGTGAG
	(Reverse)	GGTGTCTGAAGGAGGGGGTA
COX-2	(Forward)	AGGGTTGCTGGTGGTAGGAA
	(Reverse)	GGTCAATGGAAGCCTGTGATACT
NGF	(Forward)	CCAGTGGTCGTGCAGTCCAAG
	(Reverse)	TGTCCTGCAGGGACATTGCTCT
MMP-1	(Forward)	CCCAAAAGCGTGTGACAGTAAG
	(Reverse)	CTTCCGGGTAGAAGGGATTTG
MMP-3	(Forward)	CGTGAGGAAAATCGATGCAG
	(Reverse)	CTTCAGCTATTTGCTTGGGAAAG
MMP-9	(Forward)	AGTCCACCCTTGTGCTCTTC
	(Reverse)	TTTCGACTCTCCACGCATC
MMP-13	(Forward)	CTTCCCAACCGTATTGATGC
	(Reverse)	ACTTCTTTTGGAAGACCCAGTTC

IL, interleukin; TNF, tumor necrosis factor; COX, cyclooxygenase 2; NGF, nerve growth factor; MMP, matrix metalloproteinase.

relationship between mechanical stimulation and inflammatory cytokine stimulation, as well as the signaling pathways involved in mechanical stimulation remain elusive.

Monolayer-cultured chondrocytes and organ cultures of cartilage tissue are often used to study the mechanical stimulation of articular cartilage [15]. However, the characteristics and behavior of monolayer-cultured cells differ significantly from those observed in vivo; therefore, three-dimensional (3D) experimental culture systems are increasingly garnering attention. Although organ culture experimental systems for cartilage tissues offer the advantage of resembling in vivo environments, caution is necessary in terms of experimental reproducibility. Therefore, an experimental system was developed wherein cyclic compressive loading (CCL) was applied to 3D-cultured cells in atelocollagen scaffolds. This experimental system allows for the application of a variety of forces and can reproduce excessive stimulation that can be identified using cytotoxic markers. Several studies have investigated mechanical stimulation using this system [16–19].

In this study, we aimed to validate the gene expression changes induced by CCL in 3D-cultured human articular chondrocytes, including comparison with those induced by IL-1β, one of major inflammatory cytokines [20,21], and explore the signaling pathways involved in mechanical stimulation. We hypothesized that mechanical stimulation would induce gene expression changes in human articular chondrocytes that are qualitatively different from those induced by IL-1β stimulation; moreover, identifying relevant pathways would provide important insights into the pathogenesis of OA.

2. Material and methods

2.1. Cell culture of primary human chondrocytes

Human knee articular cartilage was aseptically obtained from five women aged 66–79 years who underwent total knee arthroplasty for OA. Cartilage specimens were harvested from the lateral femoral condyle, rinsed with phosphate-buffered saline, minced meticulously, and digested with 0.1 % collagenase in growth medium (Dulbecco's modified Eagle medium [DMEM], 10 % fetal bovine serum [FBS], and 1 % penicillin/streptomycin solution) at 37 °C for 6 h. The cells were then cultured in a growth medium and used at passages 4–6.

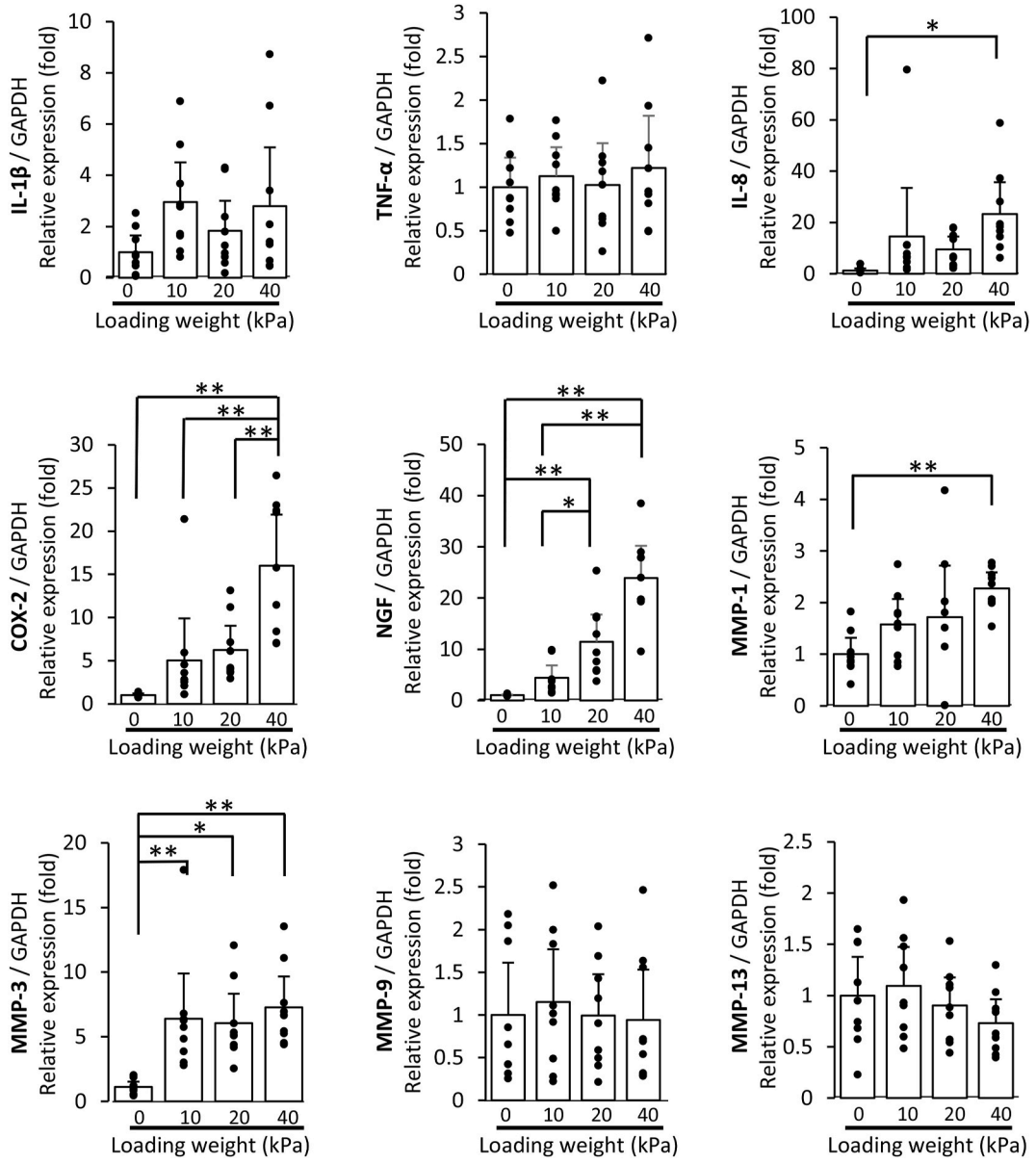


Fig. 2. Effect of cyclic compressive loading (CCL) stimulation on osteoarthritis (OA)-related factors gene expression by changing the loading weight.
CCL of 10, 20, and 40 kPa were applied to the constructs for 1 h at a rate of 0.5 Hz. RT-qPCR results for samples harvested 12 h after CCL. Data were obtained from three donors. *P < 0.05, **P < 0.01 RT-qPCR results. Means with 95 % confidence intervals (CI) from one independent experiment.

2.2. Three-dimensional-culture of human articular chondrocytes

Cultured cells (5×10^5/scaffold) were suspended in growth medium (DMEM, 10 % FBS, 1 % penicillin/streptomycin solution) and mixed with an equal volume of 1 % atelocollagen gel (AteloCell®; KOKEN CO., LTD., Tokyo, Japan) in an ice bath to produce a cell suspension in 0.5 % collagen solution. The cell suspension was incorporated into 5-mm-wide and 3-mm-thick collagen scaffolds (Atelocollagen Sponge, MIGHTY; KOKEN CO. LTD., Tokyo, Japan) by centrifuging at 500×*g* for 5 min. The cell-scaffold constructs were incubated at 37 °C for gelation to form 3D cell-scaffold constructs (Fig. 1A).

2.3. CCL experiments

The 3D-cultured chondrocytes were maintained in high glucose DMEM with 10 % FBS under free-swelling conditions at 37 °C and 5 % CO2 for three days. CCL was applied using a custom-designed CLS-5J-Z cyclic load bioreactor (Technoview, Osaka, Japan) (Fig. 1B). Loading experiments were conducted using metal plates and plastic culture dishes in HG-DMEM with 10 % FBS under a humidified incubator at 37 °C and 5 % CO2. CCL at 10, 20, and 40 kPa was applied to the constructs for 1 h at 0.5 Hz, and cultures were evaluated 12 h post-CCL. For IL-1β stimulation, 10 ng/mL IL-1β (Recombinant Human IL-1β/IL-1F2, R&D Systems, Minneapolis, MN) was added to the constructs and evaluated 12 h later. For combined stimulation, IL-1β was added immediately before applying 40 kPa CCL at 0.5 Hz for 1 h. For inhibition experiments, 5 μM verteporfin (SML0534, Sigma-Aldrich, St. Louis, MO) was added 6 h before applying 40 kPa CCL at 0.5 Hz for 1 h. Static cultures for 12 h served as controls.

2.4. Quantitative mRNA expression analysis

Total RNA was extracted from 3D constructs using PureLink™ RNA Mini Kit (Invitrogen, Waltham, MA). 200 ng of total RNA was reverse transcribed to cDNA using High-Capacity RNA-to-cDNA Kit™ (Applied Biosystems, Foster City, CA, USA). PCR was performed using Power

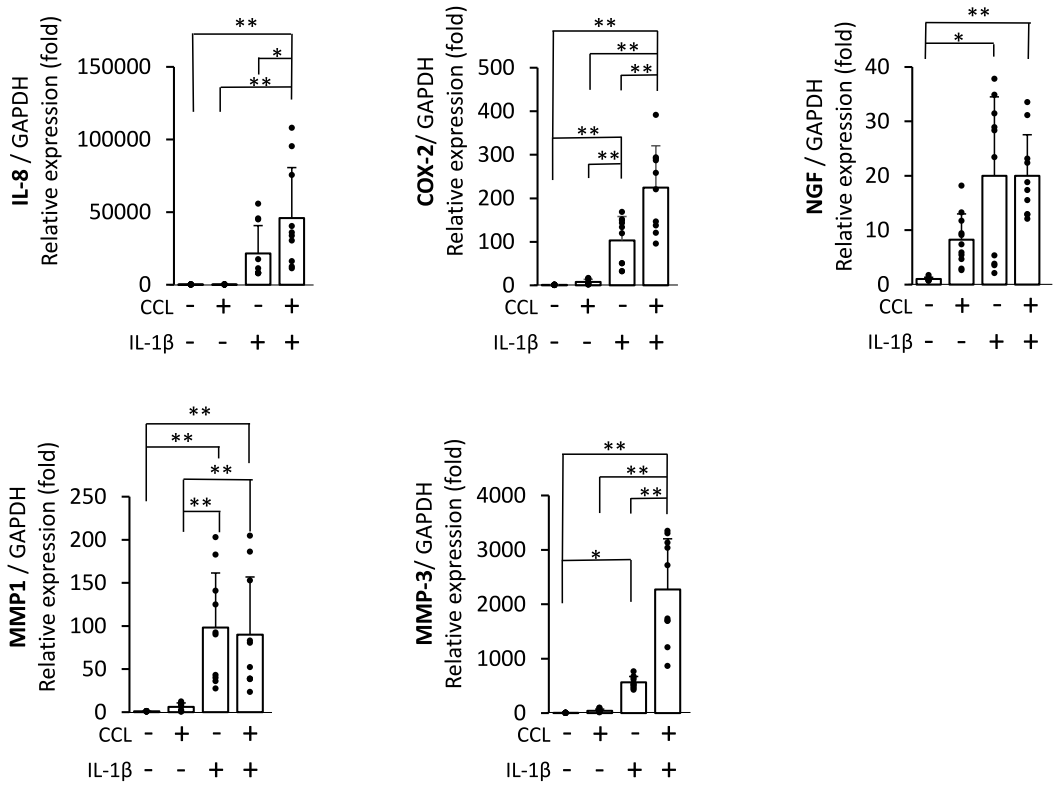


Fig. 3. Changes in gene expressions changes by simultaneous stimulation of IL-1β and cyclic compression loading (CCL).
CCL is applied at 40 kPa, 0.5 Hz for 1 h. In the group, simultaneous stimulation with IL-1β and CCL, IL-1β is added immediately before the application of CCL. RT-qPCR results using samples harvested 12 h after CCL or IL-1β stimulation. Data were obtained from three individual donors. *P < 0.05, **P < 0.01 RT-qPCR results. Means with 95 % confidence intervals (CI) from one independent experiment.

SYBR™ Green Master Mix™ (Applied Biosystems) on a QuantStudio™ 7 Flex Real-Time PCR System™ (Applied Biosystems). Primer sequences are provided in the supplemental data (Table 1). Data were analyzed using the ΔΔCT method, normalized to GAPDH, and expressed relative to the control group.

2.5. Quantitative PGE2, IL-8 and MMP-3 protein analysis

Enzyme immunoassays measured prostaglandin E2 (PGE2), IL-8, and matrix metalloproteinase-3 (MMP-3) concentrations in the culture supernatant using homogeneous time-resolved fluorescence human PGE2 and IL-8 assay kits (CIS Bio International, Saclay, France) and MMP-3 assay kit (PerkinElmer Co., CA). Supernatant from control, 40 kPa CCL, and 40 kPa CCL + IL-1β groups were compared, and protein concentration ratios were calculated.

2.6. Microarray analysis

DNA microarray was performed using three samples for each group (control, IL-1β, and CCL groups). The G3 Human Gene Expression Microarray 8 × 60 K v3 was used for the DNA microarray analysis. All hybridized microarray slides were scanned using a DNA microarray scanner (Agilent scanner, Agilent Technologies, Santa Clara, CA). Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Analysis using the Linear Models for Microarray Analysis package in Bioconductor identified 28,524 genes. Differentially expressed genes (DEGs) were selected with a p-value <0.05 and an absolute log fold-change >2. Heatmaps of the differentially expressed genes were generated using heatmap.2 in R. Principal component analysis (PCA) was also conducted In R. Enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was performed using DAVID (https://david.ncifcrf.gov/), utilizing Fisher's exact test and applying a false discovery rate (FDR) < 0.05. Gene Set Enrichment Analysis (GSEA) was performed using both the KEGG database and the hallmark gene sets from the Molecular Signatures Database (MSigDB, http://software.broadinstitute.org/gsea/msigdb/index.jsp). The microarray data have been deposited in the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) under general accession number GSE 193185.

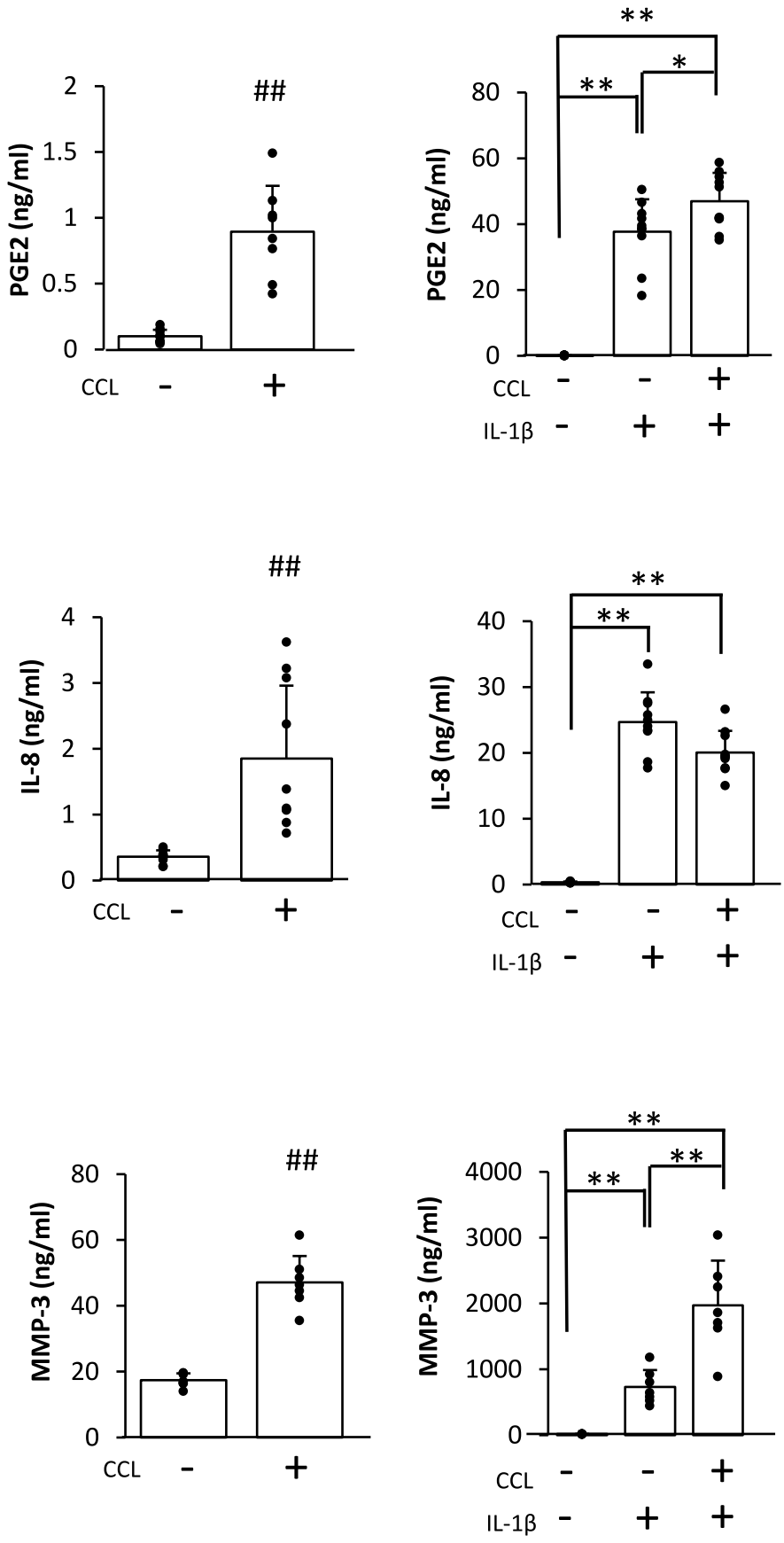
2.7. Statistical analysis

To analyze the results of RT-qPCR and enzyme immunoassays, the statistical significance of differences between two groups was determined using Student's t-test, and the statistical significance of differences among multiple groups was determined by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A significance level of 95 % with a p-value of 0.05 was set for all statistical tests. R. software was used for all statistical analyses.

3. Results

3.1. Effect of CCL on the expression of OA-related genes

CCL at 40 kPa significantly upregulated IL-8, COX-2, NGF, MMP-1, and MMP-3 expression (Fig. 2). COX-2 and NGF expression was load-dependent. No significant differences were observed in IL-1β, TNF-α, MMP-9, or MMP-13 gene expression between groups. In the presence of IL-1β, 40 kPa CCL significantly increased IL-8, COX-2, and MMP-3 expression compared to IL-1β stimulation alone (Fig. 3). NGF and MMP-1 expression did not significantly change under these conditions.



(caption on next page)

Fig. 4. Changes in protein expression by cyclic compressive load (CCL) and simultaneous stimulation with interleukin (IL)-1β and CCL.
Results using supernatants from samples collected 12 h after CCL or IL-1β stimulation. Data were obtained from three donors. (#) indicates comparisons between the CCL and control groups (#P < 0.05, ##P < 0.01) by *t*-test. (*) indicates comparisons between IL-1β group, CCL + IL-1βgroup and control group (*P < 0.05, **P < 0.01) by ANOVA followed by Tukey's post hoc test. Bars represent the mean (SD).

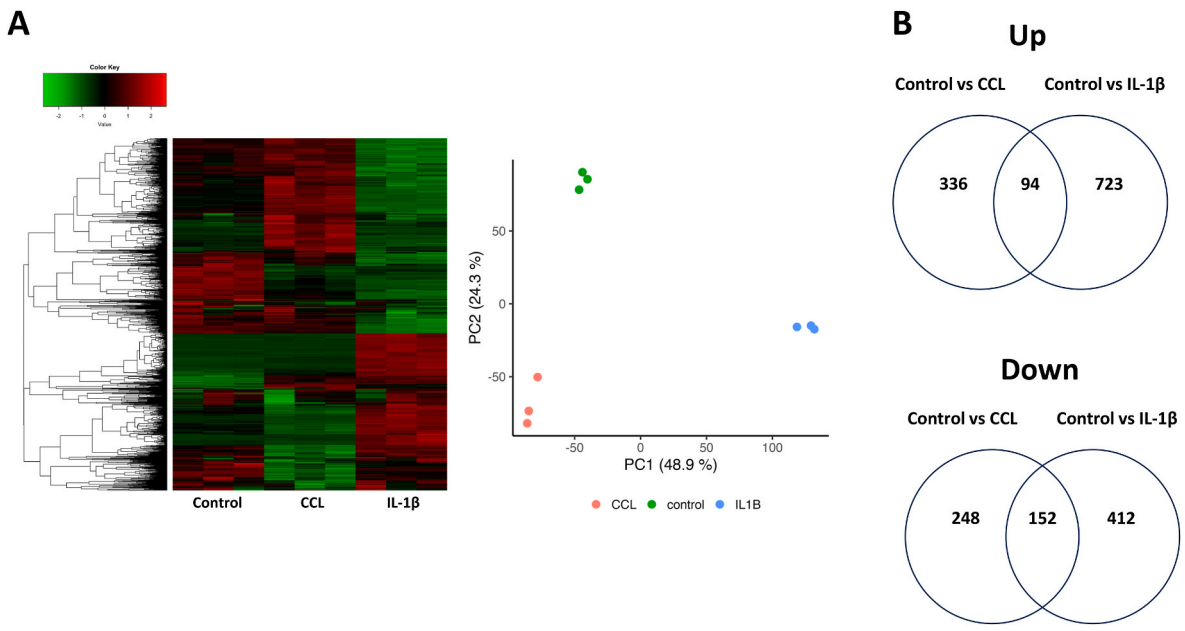


Fig. 5. Comprehensive analysis of gene expression changes associated with cyclic compression loading (CCL) and interleukin (IL)-1β stimulations.
(A) Heatmap representation of differentially expressed genes (DEGs) within the CCL, IL-1β, and control groups. Principal component analysis (PCA) plot of all detected probes of each group. (B) Venn diagrams show genes upregulated or downregulated in comparison with the control group.

3.2. Effect of CCL on IL-8, PGE2, and MMP-3 secretion

IL-8, PGE2, and MMP-3 levels in the culture supernatant significantly increased 12 h after 40 kPa CCL stimulation (Fig. 4). In the presence of IL-1β, 40 kPa CCL significantly increased COX-2 and MMP-3 protein secretion compared to IL-1β alone. IL-8 secretion did not significantly differ between the combined stimulation and IL-1β-only groups.

3.3. Comprehensive analysis of IL-1β and CCL-induced gene expression changes

Analysis of the GSE193185 dataset identified 1,965 DEGs out of 28,524 genes. Heatmaps and principal component analysis (PCA) of the top 1,000 variable genes clearly distinguished CCL from IL-1β groups (Fig. 5A). Venn diagrams showed limited overlap between groups, with 94 upregulated and 152 downregulated shared DEGs (Fig. 5B).

KEGG pathway enrichment analysis revealed non-overlapping top 10 pathways between groups, except for the "cytokine-cytokine receptor interaction" pathway (Fig. 6A). The IL-1β group showed enrichment in the "TNF signaling pathway" and "NF-κB signaling pathway," while the CCL group showed enrichment in the "cell cycle," "TGF-β signaling pathway," and "signaling pathways regulating pluripotency of stem cells." GSEA indicated a stronger enrichment of the "inflammatory response" hallmark gene set in the IL-1β group compared to the CCL group. The IL-1β group's top genes included chemokines and interleukins, whereas the CCL group's top genes were related to TGF-β and EGF (Fig. 6B).

3.4. Effect of verteporfin, an inhibitor of YAP/TAZ, on the promotion of gene expression by CCL

CCL-induced expression of COX-2, NGF and MMP-3 was significantly reduced 5 μM verteporfin (Fig. 7). Verteporfin did not significantly affect IL-8 or MMP-1 expression.

4. Discussion

This study provides novel insights into the effects of CCL on human articular chondrocytes. CCL significantly potentiated OA-related gene expression, particularly in the presence of IL-1β, and corresponding increases in protein secretion were confirmed. Comprehensive gene expression analysis demonstrated distinct profiles between CCL and IL-1β stimulation. The Hippo signaling pathway is implicated in CCL-induced gene expression of OA-related factors.

In 3D-cultured human articular chondrocytes, CCL led to the upregulation of multiple OA-related genes. The application of CCL to human synovial cells upregulates the gene expression and protein production of COX-2 and MMP-3 [17,18]. Evidence indicates that mechanical stimulation increases NGF expression. Specifically, monolayer-cultured bovine articular chondrocytes exhibited increased NGF expression under tensile loading and mouse cartilage explants exhibited enhanced NGF protein production in response to compressive loading [22,23]. NGF expression has been confirmed in the articular cartilage, synovium, and subchondral bone of OA model mice and human OA joints. Its pivotal role in pain and the link between NGF and mechanical stimuli have become topics of significant interest [24–27].

Some studies have highlighted the effects of simultaneous stimulation of inflammatory cytokines and mechanical stimulation on the expression of OA-related genes. Centrifugal pressure on mouse osteoblasts under IL-1β significantly promoted the transcription of CXCL2 and CCL2 [28]. Cyclic compressive load on bovine articular cartilage cultured with IL-6 and TNF-α promoted the gene expression of COX-2 and ADAMTS5 in a load-dependent manner [29]. We found that in the presence of IL-1β, CCL notably enhanced the expression of IL-8, COX-2, and MMP-3 compared with IL-1β stimulation alone, a finding that may indicate part of the pathophysiology of OA. In contrast, no significant

A

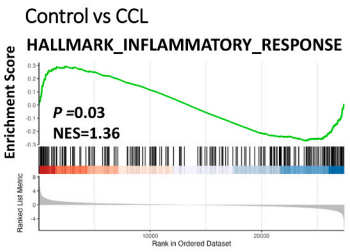
Top10 KEGG Enrichment of genes

Term	*P*-Value
control vs CCL	
hsa04060:Cytokine-cytokine receptor interaction	1.25E-08
hsa04110:Cell cycle	6.68E-05
hsa05200:Pathways in cancer	2.89E-04
hsa05224:Breast cancer	3.44E-04
hsa04390:Hippo signaling pathway	6.94E-04
hsa04550:Signaling pathways regulating pluripotency of stem cells	8.16E-04
hsa04080:Neuroactive ligand-receptor interaction	0.001097
hsa04928:Parathyroid hormone synthesis, secretion and action	0.001821
hsa04978:Mineral absorption	0.00641
hsa04350:TGF-beta signaling pathway	0.006572

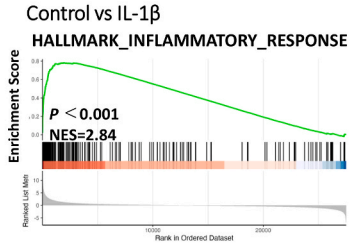
Top10 KEGG Enrichment of genes

Term	*P*-Value
control vs IL-1β	
hsa04060:Cytokine-cytokine receptor interaction	1.76E-27
hsa04061:Viral protein interaction with cytokine and cytokine receptor	1.54E-19
hsa04668:TNF signaling pathway	1.03E-14
hsa04657:IL-17 signaling pathway	6.84E-11
hsa04064:NF-kappa B signaling pathway	8.48E-10
hsa05323:Rheumatoid arthritis	8.46E-09
hsa04062:Chemokine signaling pathway	6.77E-08
hsa04020:Calcium signaling pathway	1.55E-07
hsa04978:Mineral absorption	1.64E-06
hsa04024:cAMP signaling pathway	8.91E-06

B



Gene Symbol	Rank in Gene list
INHBA	12
PROK2	106
SERPINE1	114
EREG	177
LIF	201
KCNJ2	202
HAS2	214
GPR183	223
HBEGF	331
DCBLD2	363



Gene Symbol	Rank in Gene list
IL23A	1
CCL20	2
CXCL3	3
IL1B	4
CSF3	5
CXCL8	6
CXCL6	7
CCL5	8
CXCL2	12
CCL3	13

Fig. 6. Enrichment pathway analysis and gene set enrichment analysis (GSEA) delineate the expression differences between the CCL and IL-1β groups.
(A) Enrichment pathway analysis comparing the CCL and IL-1β groups using the Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. (B) GSEA delineating the expression differences between the CCL and IL-1β groups, focusing on the inflammation-associated HALLMARK gene set. NES stands for normalized enrichment score.

changes in NGF or MMP-1 expression were observed upon CCL stimulation. These results indicate that each gene is differentially regulated by multiple stimuli, and this fact complicates the pathophysiology of OA.

Comprehensive gene expression analysis revealed distinct differences in the changes in gene expression between the CCL and IL-1β stimulation groups. These differences were particularly pronounced for the expression of inflammation-related genes. Interleukin-1β stimulates the expression of these factors in cell cultures, organ models, and various animal models [30–33]. In our model, IL-1β stimulation led to a significant upregulation of most inflammation-related factors. Conversely, although the expression of some inflammation-related factors increased in response to CCL, we also observed a decrease in the expression of other genes within this category. Additionally, CCL significantly enriched pathways related to the "cell cycle," "TGF-beta signaling pathway," "Signaling pathways regulating pluripotency of stem cells," and "Hippo signaling pathway." Studies have suggested the involvement of mechanical stimulation in cell and tissue anabolic processes [34–36]. Our study revealed that CCL not only promoted the expression of OA-related genes, but was also involved in anabolic processes, suggesting the complexity of the effects of mechanical stimulation on chondrocytes. The Hippo signaling pathway is a representative pathway that responds to mechanical stimulation. This is reportedly involved in inflammation and substrate decomposition by controlling NF-κB signaling in chondrocytes [37]. Our comprehensive gene expression analysis also suggested that the Hippo signaling pathway responds to CCL and is involved in inflammation and substrate decomposition by controlling NF-κB signaling in chondrocytes. Notably, the inhibition experiments revealed that the expression of COX-2, NGF, and MMP-3 was partly controlled by the Hippo signaling pathway. In addition, the Hippo signaling pathway is regulated by inflammatory cytokines, including IL-1 [38,39]. Therefore, it is desirable to accumulate knowledge about this pathway from the perspective of OA treatment.

This study has some limitations. First, articular cartilage exists as part of the complex joint environment within the human body, and the results observed in an artificial in vitro setting may not perfectly reflect the in vivo responses. The cell viability results and other studies suggest that excessive mechanical stimulation can be applied to articular cartilage-derived cells in patients with OA [16–19]. However, further verification using ex vivo and in vivo animal models is required. Second, this study focused exclusively on CCL as a mechanical stimulation. Articular cartilage experiences various types of mechanical forces, including tensile and shear stresses, which should be investigated in future studies. Third, this study only used chondrocytes collected from patients with OA. Healthy chondrocytes exhibit characteristics that differ from OA chondrocytes, such as increased gene expression of MMPs and COL I in primary culture [41]. Single-cell RNA sequencing analysis also revealed differences in gene expression profiles between normal patellofemoral articular cartilage and patellofemoral articular cartilage in patients with OA [42]. The results of this study provide new insights into the pathogenesis of OA in terms of the response of degenerative articular chondrocytes; however, further research on normal articular cartilage is warranted.

5. Conclusions

To summarize, CCL promoted the transcription of OA-related genes such as IL-8, COX-2, NGF, MMP-1, and MMP-3 in 3D cultured human articular chondrocytes, and the expression of some of these genes was further increased by simultaneous stimulation with IL-1β and CCL. Comprehensive gene expression analysis revealed that CCL-induced changes in gene expression were not similar to those induced by IL-1β stimulation. Furthermore, the Hippo signaling pathway is involved in

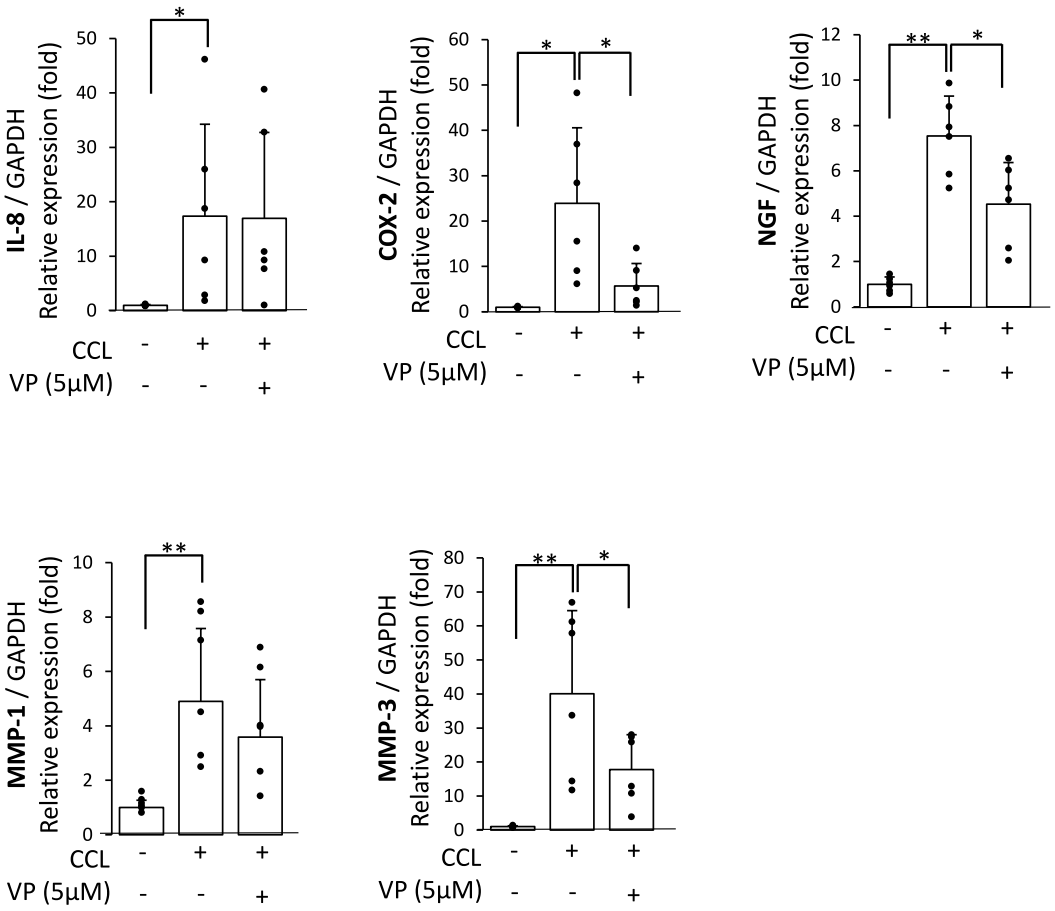


Fig. 7. Effect of Verteporfin, an inhibitor of YAP/TAZ, on the promotion of gene expression by CCL.
CCL is applied at 40 kPa, 0.5 Hz for 1 h. Evaluation of mRNA expression 12 h after CCL treatment. Data were obtained from two donors. Means with 95 % confidence intervals (CI) (*) indicate comparisons among the CCL, verteporfin (VP) group and control groups (*P < 0.05, **P < 0.01) by ANOVA followed by Tukey's post hoc test. Bars represent the mean (SD).

the promotion of the gene expression of several OA-related factors by CCL. These findings provide a valuable foundation for understanding OA pathology.

Ethical approval

This study utilized cells derived from human articular cartilage and received approval from the Osaka University Institutional Ethical Committee (approval IDs 19144 and 23187). Written informed consent was obtained from all participants, and all procedures adhered to relevant guidelines and regulations.

Author contributions

Minami Hikida: Data acquisition, analysis, and interpretation; drafted and critically revised the manuscript. Takashi Kanamoto: Contributed to conception, design, data acquisition, analysis, and interpretation, and drafted and critically revised the manuscript. Yoshihito Tachi: Drafted and critically revised the manuscript Kosuke Ebina: Drafted and critically revised the manuscript Masahiro Nakajima: Drafted and critically revised the manuscript Ken Nakata: Contributed to conception, design, drafted and critically revised the manuscript All authors provided their final approval and agreed to be accountable for all aspects of this study.

Declaration of competing interest

This study was conducted as a collaborative project between Osaka University and Interstem Co. Ltd. and was financially supported by Interstem Co. Ltd. K.N. received research grants from Interstem Co., Ltd. Y. Tachi is an employee of Interstem Co., Ltd. M. Hikida, T. Kanamoto, and M. Nakajima declare no competing interests.

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