Effect of cyclic compressive loading on redifferentiation of human chondrocytes in three-dimensional cultured tissue

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Mechanical stress causes cartilage destruction in the temporomandibular joint (TMJ) and dedifferentiation of chondrocytes in TMJ cartilage. Although dedifferentiated chondrocytes redifferentiate in vitro under certain culture conditions with growth factors such as BMPs and TGF-β or mechanical loading, the effect of mechanical stress on redifferentiation of chondrocytes is unclear. We attempted to elucidate the effect of cyclic compressive loading on the redifferentiation of dedifferentiated human chondrocytes in three-dimensional (3D) culture. Our hypothesis was that cyclic compressive loads alter gene expression in an early stage of cartilage redifferentiation. Dedifferentiated human chondrocytes in monolayer were seeded onto a collagen scaffold to produce 3D tissue and were cultured with BMP-2 (500 ng/mL) and TGF-β3 (10 ng/mL) for 28 days. The effect of cyclic compressive loading of 20 kPa at 0.5 Hz for 1 hour per day on the 3D tissue was analyzed for Sox9, BMP2, Aggrecan and type II collagen gene expressions on days 1, 4 and 7.

Three-dimensional constructs with BMP-2 and TGF-β3 exhibited positive Safranin O staining on day 28. mRNA expression levels of Sox9 and Aggrecan gene were upregulated on day 4 with BMP-2 and TGF-β3, and type II collagen gene expression was also upregulated on day 7. Cyclic compressive loading on the 3D constructs further upregulated Sox9 and BMP2 gene expression on day 4, and BMP2 gene expression was also upregulated on day 7. We concluded that cyclic compressive loading alters gene expression during the early stage of cartilage redifferentiation. (J Osaka Dent Univ 2017; 51: 23-30)

Key words: Three-dimensional tissue; Human chondrocytes; Redifferentiation

INTRODUCTION

Although dynamic mechanical stimulation to cartilage differentiation has been reported to be important in cartilage formation and maintenance,¹³ excessive mechanical stress on the synovial joint causes cartilage degeneration and leads to joint destruction. Once hyaline cartilage is injured, it is difficult to repair or regenerate because there are no nerves or blood vessels. In temporomandibular joint disorder (TMD), excessive mechanical stress is known to cause synovitis and result in cartilage degeneration. In our previous reports, we noted that excess compressive stress on the three-dimensional (3D) tissue of human synovial cells induced inflammatory cytokines such as IL-6, IL-8, and upregulated MMPs gene expressions.⁴⁶

Chondrocytes in degenerated cartilage undergo dedifferentiation and lose their cartilage specific phenotype. Nevertheless, dedifferentiated chondrocytes redifferentiate in vitro under certain culture conditions with growth factors such as BMPs and
TGF-β, or mechanical loading. Furthermore, dynamic load stimulations such as hydraulic pressure or direct mechanical loading using a bioreactor have been reported to induce cartilage differentiation from undifferentiated cells with growth factors.\textsuperscript{7-10} The exact mechanism by which these mechanical stresses differentiate undifferentiated cells, or redifferentiate dedifferentiated cells is still unclear.

The purpose of this study was to elucidate the effect of cyclic compressive loading on redifferentiation of dedifferentiated human chondrocytes in 3D culture. Our hypothesis was that cyclic compressive loading alters gene expression at an early stage of cartilage redifferentiation.

**MATERIALS AND METHODS**

**Monolayer human chondrocyte culture**
Cultured human chondrocytes were supplied by Olympus RMS, Tokyo, Japan. These cells were harvested from human knee articular cartilage, and sub-cultured for one passage in monolayer, collected by trypsin digestion and washed in phosphate buffered saline before shipping. The supplied cells were resuspended in Dulbecco’s Modified Eagle Medium (DMEM; Gibco BRL, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies), 1% penicillin/streptomycin (Gibco BRL, Life Technologies) and cultured until passage 7.

**Generation of 3D tissue of human chondrocytes**
Three-dimensional cultured tissue of human chondrocytes was generated according to the previously described methods. Briefly, human cultured chondrocytes in monolayer were collected, resuspended at $1.0 \times 10^7$ cells/mL in DMEM, and then mixed with the same amount of Atelocollagen\textsuperscript{®} gel (Koken, Tokyo, Japan) on ice. The cell-collagen solution mixture was seeded into a collagen scaffold (Atelocollagen Sponge Mighty\textsuperscript{®}, Koken) in a 96 well cell culture plate by centrifugal force for 5 min at 500 G and incubated at 37°C for 1 hour to produce a 3D cell-scaffold construct ($5 \times 10^5$ cells/scaffold). After gelation, 3D cultured tissues were incubated in DMEM containing 10% FBS and 1% penicillin/streptomycin for 3 days at 37°C in 5% CO\textsubscript{2} atmosphere.

**Three-dimensional tissue of human chondrocytes cultured with BMP-2 and TGF-β3**
The three-dimensional cultured tissue was transferred to a 24 well plate and incubated in the chondrogenic medium with BMP-2 and TGF-β3. This was designated as day 1. The chondrogenic medium contained 500 ng/mL BMP-2 (Peprotech, Rocky Hill, NJ, USA) and 10 ng/mL TGF-β3 (Peprotech) in DMEM supplemented with 10^{-7} M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/mL ascorbate-2-phosphate (Sigma-Aldrich), 40 μg/mL proline (Sigma-Aldrich), 100 μg/mL sodium pyruvate (Sigma-Aldrich), 50 mg/mL ITS Pre-mix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL BSA, 5.35 μg/mL linoleic acid) (BD Biosciences, St. Louis, MO, USA).\textsuperscript{11, 12} Control medium was used without BMP-2 and TGF-β3. The medium was replaced every 3-4 days for 28 days (Fig. 1 A).

![Fig. 1 Cartilage differentiation protocol. (A) The 3D constructs were cultured in chondrogenic medium or control medium, and gene expression was then analyzed during the early stage of cartilage differentiation. (B) The 3D constructs were examined for the effect of cyclic compressive loading during the early stage of cartilage differentiation. After changing the chondrogenic medium, the constructs were exposed to cyclic compression loading for one week, and analyzed at 1, 4 and 7 days.](http://example.com/figure1.png)
Cyclic loading of 3D human chondrocytes

Cyclic compression load was applied using a cyclic loading bioreactor (CLS-7 J® Technoview, Osaka, Japan) according to the previously described method.4,6 Before dynamic loading, 3D tissue was washed twice with 200 μL of 1 × phosphate-buffered saline (PBS), washed once with 100 μL of DMEM of FBS free and 100 μL of chondrogenic medium was added. The 3D tissue medium was changed to chondrogenic medium, and cyclic compressive loading was applied in a 96 well dish after incubation for 3 days. In this study, cyclic compression was applied to 3D constructs at 0 (no loaded) or 20 kPa at 0.5 Hz for 1 hour per day for 7 days from day 1 to day 7. Three-dimensional tissue was collected and analyzed on days 1, 4 and 7 at 6 hours after loading (Fig. 1 B).

Quantitative mRNA expression analysis of Sox 9, BMP2 and Aggrecan genes

The collected 3D constructs were processed for extraction of total RNA using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and PureLink™RNA Kit (Invitrogen). Single strand cDNA was synthesized by transcriptase using High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Foster City, CA). The mRNA expression of the specimens were quantitatively determined by real-time RT-PCR using TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific) (Table 1) and TaqMan® Array Cards (Thermo Fisher Scientific) (Table 2).

Histology

The three-dimensional cultured tissues were rinsed at 7, 21 and 28 days with PBS and fixed in 4% paraformaldehyde paraffinized sections which were then prepared for Safranin-O (SO).

Statistical analysis

Data from the experimental groups were analyzed by Tukey-Kramer and the Student-t test. Statistical significance was established at the p<0.05 level.

RESULTS

The effect of BMP-2 and TGF-β3 on redifferentiation of 3D tissue of dedifferentiated chondrocytes

The mRNA expression levels of Sox9 and Aggrecan in chondrogenic medium were significantly higher than those in control medium on day 4, and these gene expression levels remained higher than the contro thereafter until day 28. While mRNA expression levels of Collagen II a 1 in the control medium were minimal, those in the chondrogenic medium were upregulated on day 7 and were significantly higher on and after day 7 (Fig. 3). In addition, the morphological characteristics of the cells derived from human cartilage were fibroblast-like and spindle shaped (Fig. 2 A). Three-dimensional tissue of human chondrocytes did not show any SO staining on day 7 (not shown), while those on day 21 demonstrated positive staining for SO only at the edge of the tissue (Figs. 2 C and E). The stain-

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### Table 1  Primer sequences

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<td>Collagen II a 1</td>
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<td>Sox9</td>
<td>5-CAGTACCAGACTGCACAC-3</td>
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<tr>
<td>BMP2</td>
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### Table 2  Microarray analysis

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<td>Collagen II a 1</td>
<td>Hs 00264051_m1</td>
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</table>
Effect of cyclic compressive loading on redifferentiation

The Sox9 mRNA expression level in 20 kPa on day 4 was statistically significantly higher than that in 0 kPa (Fig. 4). BMP2 mRNA expression levels in 20 kPa on days 4 and 7 were upregulated compared with samples in 0 kPa. The mRNA expression level of the Aggrecan gene in 0 kPa increased significantly on day 7 compared with that on days 1 and 4, although the expression levels in 0 kPa and 20 kPa did not show any significant difference. The mRNA expression of Collagen II a 1 was not detectable in either group (not shown).
Fig. 3 Gene expression of Sox9, Aggrecan and Collagen II a1 during the early stage of cartilage differentiation at days 4, 7, 14, 21 and 28 after a change to the chondrogenic medium or the control medium (*p<0.05, **p<0.01).

Fig. 4 Cyclic compressive load was applied to the 3D tissues. Gene expression levels of Sox9, BMP2 and Aggrecan were analyzed at days 1, 4 and 7 using quantitative RT-PCR. All mRNA expression levels were divided by that of GAPDH and the value at each time point was calculated by the relative quantity value based on 0 kPa on day 1. Statistical significance was determined by Tukey-Kramer (*p<0.05, **p<0.01).
DISCUSSION

We demonstrated that 3D tissue of human dedifferentiated chondrocytes exhibited redifferentiation by BMP-2 and TGF-β3 supplementation on day 28, and that the cyclic compressive load of 20 kPa in an early stage of redifferentiation upregulated the expression of Sox9 and BMP2 genes at day 4. Cell differentiation or redifferentiation is closely associated with tissue development and tissue repair, and gene regulation is crucial in these processes. It has been known that the environmental conditions of both monolayer and 3D cells play an important role in gene regulation, and that gene regulation in 3D is important in vivo because cells in living tissue are surrounded by extracellular matrix (ECM) and form 3D structures.

It appears that experiments of tissue development, repair and regeneration in 3D are significant clinically, especially in cartilage, which is well known to form 3D structures with abundant ECM. Indeed, many studies have reported cartilage differentiation of undifferentiated cells in 3D culture such as high-density culture, pellet culture, and 3D culture with PEGT/PBT-based, or collagen I based scaffolds. Adachi also reported redifferentiation of human chondrocytes using Atelocollagene gel culture. In our study presented here, we for the first time demonstrated that 3D human dedifferentiated chondrocytes made of mechanically-reinforced collagen scaffold exhibited redifferentiation, suggesting the possibility of usage of this material and cells for cartilage regenerative medicine.

In this study, staining for SO was negative on day 7 (data not shown), became positive on day 21 only at the edge of the 3D tissue, and then became more evident on day 28 in chondrogenic medium with BMP-2 and TGF-β3. However, samples from control medium without BMP-2 and TGF-β3 did not show any positive staining for SO. Expression levels of Sox9 and Aggrecan genes were upregulated as early as on day 4 in chondrogenic medium, and these high levels of expression were maintained until day 28. This suggests that BMP-2 and TGF-β3 treatment induces cartilage differentiation by transcriptional level at an early phase, and that cartilaginous ECM production and deposition, such as proteoglycan (PG), which is positive for SO, becomes evident on day 21 and thereafter.

Since this study and the other studies showed that it takes 3-4 weeks for undifferentiated mesenchymal stem cells (MSCs) or human dedifferentiated chondrocytes to accomplish cartilage differentiation or redifferentiation, it might be of significance to enhance these processes and to reduce the time for cartilage differentiation or redifferentiation by alteration of gene regulation clinically. In our study, the cyclic compression of 20 kPa in the early stage upregulated the expression levels of the Sox 9 and BMP2 genes at day 4. Sox9 has been reported to be an important factor for the expression of Col II, Col IX, and Aggrecan. In addition, Sox9 modifies the target molecule and histone code, and promotes cartilage differentiation in the early stage of differentiation. Our study suggested that cyclic compressive load in an early stage of cartilage differentiation increases the expression of Sox9 and may promote cartilage differentiation.

In past studies, various mechanical load stimulations, such as hydrostatic pressure, dynamic compression and bioreactor, were used for cartilage differentiation and the enhancement of Sox9, Aggrecan, and Collagen II a 1 gene expressions and the promotion of cartilage differentiation. Increases in glycosamino glycan (GAG) and PG have been reported. All this evidence suggests that certain dynamic stimulation could promote cartilage differentiation or redifferentiation. The compressive load applied in this study was 20 kPa. From our previous study this compressive load induced less than 5% deformation in 3D tissue, which we assume is a force similar to that of physiological occlusion loaded on the TMJ.

It has been reported that compressive load on cartilage tissue activated Alk 5 and Smad 2/3, which are TGF-β3-specific signals, and that Smad-3 promoted cartilage differentiation by the potentiation of transcription activity with Sox9. In the present study, the compressive load of 20 kPa may have activated Sox9 gene expression through
the same signaling mechanism. However, the exact signaling pathways that induce Sox9 and BMP2 expression by compressive load are not clear. Further studies are needed. One limitation of this study is that it is not clear whether or not gene expressions upregulated by compressive load in an early phase of redifferentiation promote production of cartilaginous ECM in the late phase of redifferentiation. In addition, it is not clear what type of compressive load is most suitable, because this study tested only a 20 kPa load at 0.5 Hz for 1 hour per day; other conditions were not examined.

CONCLUSION

In conclusion, dedifferentiated human chondrocytes in 3D tissue exhibited redifferentiation at 28 days of culture with BMP2 and TGF-β3. The cyclic compressive load of 20 kPa in the early stage of redifferentiation upregulated the expression levels of the Sox9 and BMP2 genes.

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