Effects of Mechanical Stimulation on Gene Expression of Articular Chondrocytes in Polylayer Culture

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MAEDA, S., YOSHIDA, M., HIRANO, H. and HORIUCHI, S. *Effects of Mechanical Stimulation on Gene Expression of Articular Chondrocytes in Polylayer Culture.* Tohoku J. Exp. Med., 2001, **193** (4), 301-310 — Mechanical loads are constantly being applied to articular cartilage. However, comparatively few studies have investigated the effects of mechanical stress on the gene expression of chondrocytes. Hence, the objective of the present study was to clarify the effects of mechanical stress on cultured chondrocytes. Articular chondrocytes of rabbits were isolated and placed in minimum essential medium eagle α modification containing 10% fetal bovine serum. The chondrocytes were cultured in polylayer in polypropylene tubes. After 48 hours, centrifugal stress (2.7 MJ) was applied once every 24 hours for 28 consecutive days. The mRNA expression of insulin-like growth factor-I (IGF-I) and cartilage-specific genes (type II collagen and aggrecan) in chondrocytes, cell count, wet tissue mass and protein content were subsequently measured. The results showed that the mRNA expression of the IGF-I gene for the stress groups was significantly greater than that of the control group from days 3 to 5 of incubation, as was the mRNA expression of the type II collagen gene from days 7 to 14 of incubation. However, there were no significant differences in the mRNA expression of the aggrecan gene between the control and stress groups. The cell count for the stress groups was significantly higher from days 7 to 28 of incubation, and the wet tissue mass and protein content were significantly higher from days 14 to 28 of incubation. These findings suggest that one of the mechanisms by which mechanical stress increases the number of cultured chondrocytes and the volume of extracellular matrix is to increase IGF-I expression at the early stage of incubation. ————— mechanical stress; chondrocyte; cell culture

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Recently, it has been found that insulin-like growth factor-I (IGF-I) and/or transforming growth factor- β (TGF- β) are important anabolic factors for cartilage tissue. Yaeger et al. (1997) have reported that synergistic action of TGF- β and IGF-I induced the expression of type II collagen and aggrecan genes in human articular chondrocytes. Matsumoto et al. (2000) have suggested that IGF, IGF-binding proteins and IGF receptor may play important roles in regulating cartilage metabolism. Reinecke et al. (2000) have reported that IGF-I increased IGF-I mRNA in the proliferative zone of cartilage tissue.

Mechanical loads are constantly being applied to articular cartilage. Excessive loading is thought to be one of the factors associated with osteoarthritis. However, there are still many unknowns in the relationship between mechanical stress and articular cartilage metabolism, in particular, the regulation of gene expression. In an attempt to clarify the effects of mechanical stress on cultured chondrocytes, we applied centrifugal stress to polylayer-cultured chondrocytes and subsequently quantified the mRNA expression of the IGF-I and cartilage-specific genes (type II collagen and the core protein of aggrecan).

MATERIALS AND METHODS

Materials

Minimum essential medium eagle alpha modification (α -MEM) and fetal bovine serum (FBS) were purchased from GIBCO BRL (Eggenstein, Germany). Trypsin 250, collagenase type IA; Lot27H6829, kanamycin monosulfate and L-ascorbic acid 6-palmitate were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Chondrocyte isolation

Four-week-old immature Japanese white rabbits were anesthetized using diethylether before the shoulder, knee and hip joints were

removed. Chondrocytes were isolated from the tissue with 0.1% ethylenediaminetetraacetic acid-disodium salt/0.1% trypsin solution (20 ml) digestion for 35 minutes, followed by 0.15% collagenase solution (20 ml) for 4 hours as described by Goto et al. (1999).

The number of chondrocytes was counted using a hemocytometer and the concentration of these cells was adjusted to 1.0×10^5 /ml using α -MEM containing 10% FBS, 60 μ g/ml kanamycin and 50 μ g/ml L-ascorbic acid. One ml of the resulting cell suspension was placed in 2 ml polypropylene tubes with a lid (Assist Co., Ltd., Tokyo) and centrifuged at $300 \times g$ for 5 minutes to prepare cell pellets that were then placed in an incubator for 48 hours (0.5% CO₂ at 37°C). The above procedures were carried out in a sterile manner.

Histological findings of the polylayer-cultured tissue

The polylayer-cultured tissue at day 14 of incubation was stained with toluidine blue.

Mechanical stimulation

Centrifugal stress was applied to cultured cells using a centrifuge at 37°C.

Semi-quantification of mRNA expression

Total RNA was isolated from each sample using a Fast RNATM Kit-Green (BIO101, Vista, CA, USA). The concentration of the isolated total RNA was adjusted to 0.1 μ g/ml. After reverse transcription, the expression of mRNA was semi-quantified by real-time PCR using a SYBRTM Green PCR Core Reagents Kit (PE Applied Biosystems, Tokyo) and measuring with ABI PRISMTM 7700 (PE Applied Biosystems). Table 1 shows the primers used in the present study.

Cell count measurement

To each sample, 1000 μ l of a 0.10% collagenase solution prepared using α -MEM was added. Pumping was carried out 30 times

using a needle (18 gauge) and syringe (2.5 ml), and the sample was then kept warm at 37°C for 3 hours under constant stirring. Then, 500 μ l of the resulting cell suspension was centrifuged at $300\times g$ for 5 minutes. After removal of the supernatant, the precipitated cells were rinsed twice. The number of rinsed cells was determined using a hemocytometer. The same cell suspension was used to determine the cell count by measuring fluorescence intensity using a Cell Counting Kit-F (Wako Pure Chemical, Osaka).

Wet tissue mass measurement

Each sample was placed on a piece of filter paper (each paper was cut into 8 equal pieces, TOYO ROSHI 5A, Tokyo) and then placed in a desiccator for 48 hours. The weight of the sample before and after desiccation was measured.

Protein content measurement

Each sample was washed twice using phosphate buffered saline (PBS) (—), and was stored frozen after the addition of 500 μ l of PBS (—). The frozen sample was thawed at 37°C for 5 minutes, and pumping was carried out 30 times using a syringe (2.5 ml) and needles (23, 22 and 18 gauge, in this order) to prepare a cartilage homogenate. The protein content of the homogenate was determined by the Lowry-Folin technique (Lowry et al. 1951).

Mechanical stimulation of cells in tube cultures

In Group A (control group), centrifugal stress was not applied during the incubation period (Fig. 1a). In Groups B and C, the intensity of centrifugal stress was set at 0.9 and 1.8 MJ, respectively. In groups D, E, F and G, centrifugal stress was set at 2.7 MJ. In addition, in Groups D and E, the frequency of

Fig. 1. Mechanical stress techniques for tube cultures.

Centrifugal stress; ▲: 0.9 MJ, ○: 1.8 MJ, ●: 2.7 MJ, ↑: culture media exchange, ↓: sampling

centrifugation was set at 24 or 48 hours, respectively. Furthermore, in Groups D, F and G, the timing of centrifugal stress was applied at days 2–14, days 2–7 and days 8–14, respectively. At day 14 of incubation, the mRNA expression of the type II collagen, cell count and wet tissue mass were determined. Culture media were exchanged every 72 to 96 hours.

Effects of mechanical stimulation on tube cultures

The mRNA expression of the IGF-I, type I and type II collagen, aggrecan and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined. Also, cell count, wet tissue mass, and protein content were measured. Centrifugal stress was applied every 24 hours starting from day 2 of incubation. Sampling was performed at days 0, 3, 5, 7, 14, 21 and 28 of incubation. Culture media were exchanged every 72 to 96 hours (Fig. 1b).

Statistical analysis

Data were expressed as the mean $\pm$ s.d. A multiple comparison test Bonferroni/Dunn (Dunn's procedure as a comparison procedure) was used, and *p* values of less than 0.05 were

considered significant.

RESULTS

Histological findings of the polylayer-cultured tissue

Histological staining with toluidine blue was observed in the matrix of polylayer-cultured tissue, called metachromasia, at day 14 of incubation (Fig. 2).

Establishing a technique for mechanical stimulation of tube cultures

Effects of the intensity of mechanical stimulation (I). Increases in the intensity of centrifugal stress applied to cultured cells significantly increased the mRNA expression of the genes studied when compared to the control (Fig. 3a). When compared to the control group, increases in the intensity of centrifugal stress applied to cultured cells also significantly increased the cell count, except in Group B (Figs. 3b and 3c). Increases in the intensity of centrifugal stress applied to cultured cells significantly increased the wet tissue mass, except for Group B when compared to the control (Fig. 3d).

Effects of the frequency of mechanical stim-

Fig. 2. Histological findings of the polylayer-cultured tissue
Histologic staining with toluidine blue was observed in the matrix of polylayer-cultured tissue, called the metachromasia, at day 14 of incubation. Magnification $\times 200$.

Fig. 3. Establishing a technique for mechanical stimulation of tube cultures.

I: Effects of the intensity of mechanical stimulation (a-d). II: Effects of the frequency of mechanical stimulation (e-h). III: Effects of the timing of mechanical stimulation (i-l). Bars and vertical lines are means $\pm$ S.D. ($n=6$). The asterisks indicate significant difference at (**) $p < 0.01$. All data were obtained from the same rabbit.

ulation (II). When the frequency of centrifugal stress applied to cultured cells was set at 24 hours, there were significant differences in the mRNA expression, cell count and wet tissue mass compared to the control group. When the frequency of centrifugal stress applied to cultured cells was set at 48 hours, there was no significant difference in mRNA expression (Figs. 3e, 3f, 3g and 3h). However, there were significant differences between Groups D and E.

Effects of the timing of mechanical stimula-

tion (III). When centrifugal stress was applied up to day 7 of incubation, there were no significant differences in the mRNA expression of the type II collagen. However, when centrifugal stress was applied from days 8 to 14, the mRNA expression of the type II collagen increased significantly (Fig. 3i). In all groups, the cell count and wet tissue mass increased significantly (Figs. 3j, 3k and 3l).

Based on the above findings, the intensity and frequency of centrifugal stress was estab-

TABLE 1. *The primers used in PCR and cDNA product size*

Gene	Primer sequence (5' to 3')	Product size (bp)
IGF-I	F, TCTTCAGTTCGTGTGTGGAGACA; R, AAGCAGCACTCATCCACGATG.	104
Collagen $\alpha 1$ (II)	F, ACCATCAACGGTGGCTTCCA; R, CGGGAGGTCTTCTGTGACGG.	323
Collagen $\alpha 2$ (I)	F, ACACAAGGAGTCTGCATGTCTA; R, TCACCAGCAGGTCCTTGGA.	406
Aggrecan	F, TGAGGAGGGCTGGAACAAGT; R, GGCAGCTGGTAATTGCAGGG.	311
GAPDH	F, ACTGGCGTCTTCACCACCAT; R, AAGGCCATGCCAGTGAGTGAGCTT.	404

Fig. 4. Effects of mechanical stimulation on mRNA expression, cell count, wet tissue mass, and protein content in rabbit chondrocytes in tube culture.

(a): IGF-I mRNA. (b): Type II and type I collagen mRNA. (c): Aggrecan mRNA. (d and e): cell count. (f): wet tissue mass. (g): protein content.

Values are means $\pm$ s.d. (IGF-I on days 0-7 only, $n=10$, the others, $n=6$). The asterisks indicate significant difference at (**) $p < 0.01$, and (*) $p < 0.05$.

○, control group; ●, stress group; △, control group, type I collagen; ▲, stress group, type I collagen.

lished at 2.7 MJ and 24 hours, respectively.

Effects of mechanical stimulation on tube cultures

After conducting an experiment using the same set of samples, there were significant differences in the mRNA expression between the control and stress groups at days 3 and 5 of incubation ($n=6$). Since there were some fluctuations in data, we conducted another experiment ($n=4$). Hence, mRNA expression was analyzed using a total of ten samples at days 0 to 7. The mRNA expression of the IGF-I for the stress groups peaked at day 5 of incubation. The mRNA expression of the IGF-I increased for the stress groups, and there were significant differences between the control and stress groups at days 3 and 5 of incubation (Fig. 4a).

The mRNA expression of the type II collagen was high at day 0 of incubation, but it quickly decreased and reached the minimum level at day 3. Then, its mRNA expression increased up to day 14 of incubation for both the control and stress groups. The mRNA expression of the type II collagen for the stress groups was significantly greater than that for the control group at days 7 and 14 of incubation.

No mRNA expression of the type I collagen was found in the control or stress groups throughout the incubation period (Fig. 4b).

The mRNA expression of the aggrecan quickly decreased starting at day 0 of incubation and reached a minimum level at day 3. Its mRNA expression increased up to day 7 of incubation for both the control and stress groups. There were no significant differences in the mRNA expression of the aggrecan between the control and stress groups (Fig. 4c).

The cell count for the control and stress groups increased from day 0 of incubation and plateaued at day 14 of incubation. After day 7, the cell count for the stress groups was significantly greater than that for the control

group (Figs. 4d and 4e).

The wet tissue mass for the control and stress groups increased from day 0 of incubation and reached a plateau at day 14. Thereafter, the wet tissue mass for the stress groups was significantly greater than that for the control group (Figs. 4f).

The protein content for the control and stress groups increased from day 3 of incubation and plateaued at day 14. Thereafter, the protein content for the stress groups was significantly greater. The protein content at day 0 of incubation was below the detection limit for the Lowry-Folin technique ($50 \mu\text{g/ml}$) (Fig. 4g).

DISCUSSION

A number of techniques involving compression, stretching, bending or distention have been devised as a means to apply mechanical stress to cultured cells (Suh et al. 1999; Brown 2000). These previous studies applied stress to monolayer cultures. In the present study, in order to replicate as closely as possible the intensity of mechanical loading on the human knee and hip joints in vitro, a technique for applying mechanical stress to the polylayer cultures has been established.

The results of the present study show that as the intensity and frequency of centrifugal stress increased, the mRNA expression of the type II collagen increased.

The polylayer-cultured chondrocytes grew into the cartilage tissue. That is, histological findings show that the staining with toluidine blue, called metachromasia, was observed in the matrix of cartilage tissue at day 14 of incubation. In addition, the mRNA of type II collagen was expressed for 28 days, while the mRNA of type I collagen, indicating dedifferentiation, was not expressed during the whole period of incubation.

The cartilage matrix is deformable. The chondrocytes divide and secrete more matrix.

The cells depend on the surrounding matrix, which is usually made by the cell itself. Thus, a cell can create an environment that then reinforces its differentiated state. Furthermore, the extracellular matrix that a cell secretes forms part of the environment for its neighbor cells in the same way (Alberts et al. 1994).

On a Petri dish coated with basal membrane extracellular matrix, the proliferation and differentiation of chondrocytes was strongly facilitated, and this effect was more pronounced than when a dish was coated with collagen alone (Kato and Gospodarowicz 1985). In general, cultured cells undergo the following phases after inoculation: lag phase, logarithmic growth phase, stationary phase and death phase. Kato et al. (1988) developed a technique for culturing chondrocytes in centrifuge tubes, and documented that chondrocytes in centrifuge tubes started to proliferate 2 days after inoculation and became stationary 12 days after inoculation. They also divided the synthesis of proteoglycan into three stages: mitotic stage (days 0–10), matrix-maturation stage (days 10–16) and hypertrophic stage (days 16–25). Chondrocytes in centrifuge tubes undergo terminal differentiation following the mitotic and matrix-maturation stages, and become hypertrophic to induce matrix calcification (Kato and Iwamoto 1990).

The secretion of IGF-I in chondrocytes has been confirmed (Jansen et al. 1989), and the mRNA expression of the IGF-I gene is elevated in osteoarthritic cartilaginous tissue (Middleton and Tyler 1992; George et al. 1993; Dore et al. 1995). In cultured chondrocytes, increases in the mRNA expression of the type II collagen (Yaeger et al. 1997; Nixon et al. 1998) and the synthesis of proteoglycans (McQuillan et al. 1986; Maor et al. 1993; Takigawa et al. 1997; Yaeger et al. 1997; Martin and Buckwalter 1998; van Osch et al. 1998) have been reported. In addition, there is an optimal IGF-I concentration for inducing cell proliferation, and lower

or higher concentration than the optimal cannot sufficiently induce cell proliferation (Nixon et al. 1998).

The relationship between mechanical stress and cartilage-specific genes in cultured chondrocytes reportedly involves an elevation of the mRNA expression of the aggrecan in monolayer cultures (Suh et al. 1999). However, in the present study, there were no marked differences in the mRNA expression of the aggrecan in the polylayer cultures. This is attributable to the fact that the mRNA expression of the aggrecan in cultures was about 1.67 times higher than that in monolayer cultures (data not shown), and this appears to be the upper limit of aggrecan expression.

Joint lubrication (Mow and Soslowsky 1991) and healthy subchondral bones (Nixon et al. 1998) have been shown to be involved in articular cartilage repair. The results of the present study show that cells stimulated by mechanical stress also can play some role in the maintenance of articular cartilage.

In the present study, the cell count, wet tissue mass and protein content peaked at day 21 of incubation for both the control and stress groups. When compared to the control group, the mRNA expression of the IGF-I and the type II collagen for the stress groups was significantly higher at days 3–5 and days 7–14 of incubation, respectively. These results suggest that the activity of IGF-I in the early stage of incubation can be one of the factors to play a role in the mechanical stress-induced increases in cell count and extracellular matrix volume of cultured chondrocytes.

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