Mechanobiological Signal Transduction in Differentiating Chondrocyte and New Configuration for Mechanical Stress Culture

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Mechanobiological Signal Transduction in Differentiating Chondrocyte and New Configuration for Mechanical Stress Culture

Ichiro Takahashi*[1], Taisuke Masuda[1], Fumihito Arai[2] *Member, IEEE*, Takahisa Anada[1], Toshio Fukuda[3] *Fellow, IEEE*, Osamu Suzuki[1] and Teruko Takano-Yamamoto[1]

[1]*Division of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry, Tohoku University Sendai, Japan
[2]Graduate School of Engineering, Tohoku University, Sendai, Japan
[2]Graduate School of Engineering, Nagoya University, Nagoya, Japan

Abstract- **Musculoskeletal system regulates and supports mammalian body movement by generating force and by resisting to the force exerted on the body. Skeletal tissues, bone, cartilage, muscle and tendon are mechano-responsive tissues consisted of osteoblasts, chondrocytes, myocytes and fibroblasts and their specific extracellular matrices (ECMs). Mechanical stress presents a variety of effects on the metabolism and differentiation state of these cells. During bone growth, growth plate cartilage plays a pivotal role in growth in length of bone by providing templates consisted of cartilage specific ECM, which is replaced by bone. Chondrocytes, form cartilage in synovial joints and growth plates, respond to compressive force by activating their metabolism and progressing differentiation, while tensile stimulation inhibits their differentiation. Mechanical stimulation is translated into intracellular signaling, which regulates the differentiation state and metabolism of chondrocytes. To analyze the mechanobiological response, we have been developing mechanical stress culture device. Here, we present the mechanical stress culture device and mechnobiological response of differentiating chondrocytes to stretch stimulation**

Keywords: chondrogenesis, mechnial stress, culture device

1. Introduction

Musculoskeletal tissues, bone, cartilage, muscle and tendon, form body supporting element for mechanical movement of mammalian body. These tissues are consisted of tissue specific cells, such as osteoblasts, osteocytes, chondrocytes, myocytes and fibroblasts. Generally, many different type of growth factors, cytokines and hormones regulate formation of these tissues. Recently, mechanical stimulation to the cells became to be considered as one of the key regulator of cellular metabolism and differentiation similar to those soluble factors described above.

Cyclical mechanical stress activates the metabolism of bone forming cells, osteoblasts and mechanical compressive stimulation enhances the metabolism of cartilage specific extracellular matrix (ECM) aggrecan [1]. At the same time, differentiation of chondrocytes is stimulated by physiological level of compressive force [2], while tension force inhibits their differentiation [3]. Thus, mechanical stimulation plays roles in the regulation of cellular phenotypic changes of skeletal tissue cells. During these processes, it could be considered that the mechanical stimulation is translated into biochemical signals. Indeed, stretch-activated Ca^{2+} channel is activated and c-fos, a transcriptional factor, and mitogen-activated protein kinases (MAPKs) are phosphorylated by mechanical stimulation, in fibroblasts and other cell types of skeletal tissues.

We have previously presented the mechanobiological response of chondrocytes for mechanical stretch stimulation where differentiation of chondrocytes is inhibited by stretch stimulation and developed of mechanical stress culture device [4-7]. Here, we describe the intracellular mechanobiological response of differentiating chondrocytes and new configuration of mechanical stress culture device for quantitative analysis under precisely controlled mechanical stimulation.

*Contacting author: Ichiro Takahashi, E-mail: takahasi@mail.tains.tohoku.ac.jp. This research has been supported by a Grant-in-aid (#17076001 and #18592228) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

2. Materials and Methods

2.1 Micromass culture

Cells derived from fore- and hind-limb buds of embryonic day 12 rat embryos were used for mechanobiological signal transduction analysis and, in addition, a chondrogenic cell line, ATDC-5 was used to configure the mechanical stress culture device in the present study. Micromass culture with 50μl drop of $2x10^5$ cells/ml of cell suspension was assembled on Flexer Cell plates (Figure 1) or mechanical stress culture device as described bellow. Primary cells were cultured with Dalbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100U/ml of penicillin and 100mg/ml of streptomycin) in 5%CO_2 incubator at 37℃ for up to 7 days. ATDC-5 cells were cultured with DMEM/F12 (50% each) medium supplemented with 10% fetal bovine serum and antibiotics as described avobe. Three days after assembling the culture, mechanical stimulation was applied.

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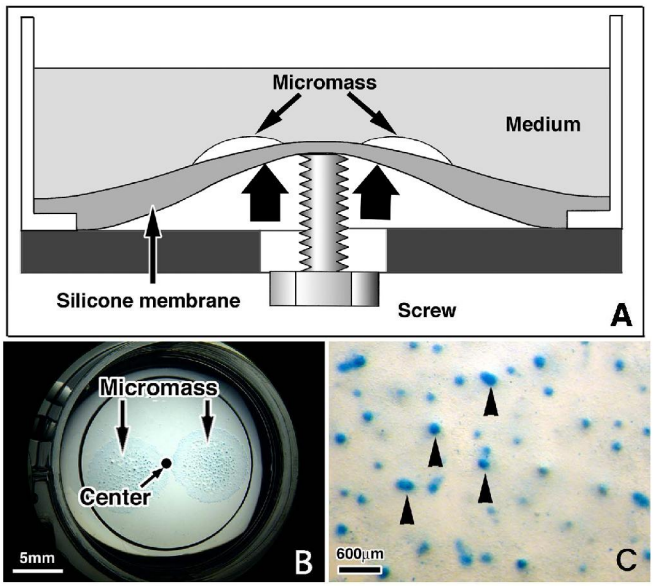

Figure 1. Mechanical stretch stimulation using Flexer Cell plate is illustrated in (A), overview of the plate with micromass culture (B) and magnified and alcian blue stained chondrogenic nodules (C, arrowheads) were indicated.

2.2 Mechanical stress loading culture devices

Basic concept of polydimethylsiloxane (PDMS)-made chamber for cell and tissue culture was developed, in order to apply precisely and quantitatively controlled mechanical stimulation to the cells as shown in figure 2.

A mechanical stress culture device was composed of an outer PDMS chamber and an inner PDMS chamber with 100μm-thick PDMS membrane with cell culture surface. Outer PDMS chamber has the mutually separate pits (•5mm) which were covered by PDMS membrane of inner PDMS chamber. Cell culture surface of the inner chamber underwent hydrophilic treatment adjacent to the pits (• 5-8mm), and the treated surface was coated by fibronectin (Sigma MO. USA).

These pits were connected with the tube for decompression. Thus, this PDMS chamber was designed so that the cells inoculated on the PDMS membrane would be strained by the decompression of the pits.

The regulating system for decompression is controlled by an air actuator as shown in figure 3. Cyclically controlled strain can be loaded to the cells plated on the pits of the device.

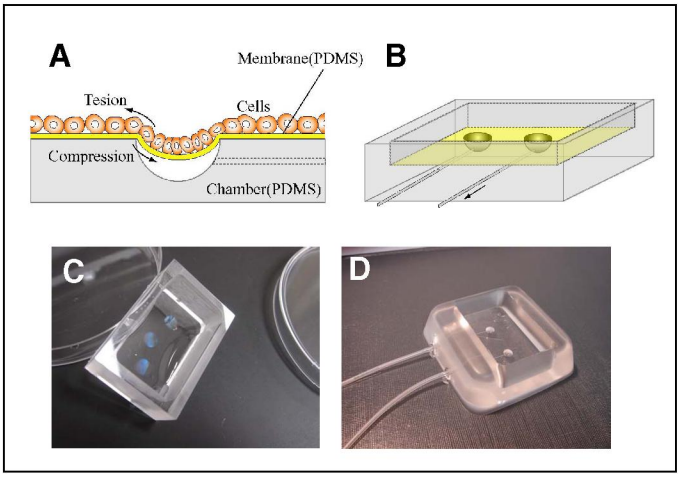

Figure 2. Schematic diagram of the concept of the mechanical stress culture devise (A). Cells attached on the PDMS membrane are strained by decompression of the pits. PDMS membrane (100μm-thick) adhered to acrylic frame is subjected to hydrophilic treatment (bluish spots in C). The treated surface was coated by fibronectin. The pits of PDMS chamber are connected with the tube of the pump (B and D).

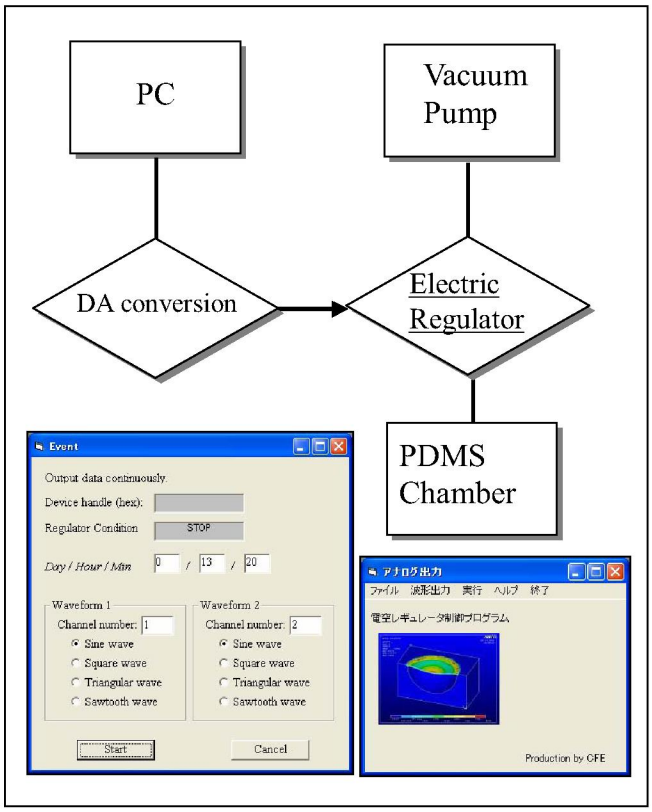

Figure 3. The flowchart of regulating system for micro decompression of the mechanical stress culture device is indicated. First, single 39% strain is loaded for the cultures until 12 hours, after it is released until 12hours.

2.3 Application of mechanicals stress

Chondrogenic differentiation of the cells was assayed by alcian blue staining in the culture. Cultures were primarily maintained for 3 days prior to mechanical stimulation. For Flexer cell plate culture system, thereafter, stepwise stretch was applied every 24 hours for 4 days. First stimulation gave 12% strain to the culture and 8% additional strain was loaded to the culture at every activation of the system. In our newly developed mechanical stress culture devise, after the primary culture period, cyclic stress with 12 hours on and off cycle was loaded for 4 days.

2.4 ERK inhibition assay

MEK1 (5.0μM), MEK1/2 (10.0μM) inhibitors (PD98059 or U0126, Cell signaling technology, MA, USA) or DMSO was added to the culture 1 hour prior to the mechanical stimulation. After 60 min of stimulation, cultures were harvested for western blotting analysis.

2.5 Inhibited protein production assay for ERK activation.

Cycloheximide (10μg/ml, Sigma, MO, USA), which was an inhibitor for protein synthesis, was added to the culture 60 min prior to the stretch stimulation. After 60 min of stimulation, cultures were harvested for western blotting analysis.

2.6 Alcian blue staining

After the culture period, cells were fixed in 2% acetic acid in 95% ethanol for 10 min, hydrated in the graded series of ethanol, rinsed in phosphate-buffered saline (PBS, pH 7.4) and stained with 0.5% alcian blue solution for over night at 4℃. Then the cultures were rinsed thoroughly in PBS, and the area of alcian blue positive cartilaginous nodules was measured.

2.7 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

After the culture period, total RNA was isolated from cultures using RNeasy mini kit (Qiagen, Hilden, Germany) by following manufacturer's instruction. They were reverse transcribed and subjected to semi-quantitative RT-PCR of ERK-1/2 and type II collagen gene, which is a cartilage specific ECM molecules. The PCR condition used in the present study was described in our previous study [4].

2.8 Western blotting analysis

Protein samples were isolated from the cultures 30, 60, and 90 min, and 2, 3, 6 and 12 hours after the beginning of mechanical stimulation. After the concentration of the samples were determined by using BCA protein assay kit (Pierce Biotechnology, IL, USA), they were separated on 2% SDS-PAGE, transblotted onto nitrocellulose membrane, and subjected to western blot analysis of ERK-1/2, JNK-1/2/3 and p38 MAPK. Primary antibodies used were for both phosphorylated and non-phosphorylated MAPKs (Cell Signaling Technology, MA, USA). All primary antibodies were diluted at 1:1000. HRP-conjugated anti-rabbit IgG secondary antibodies were used for chemiluminescent detection by using chemiluminecsence detection kit (Pierce Biotechnology, IL, USA). Optical density of the bands were quantified by using ImageJ software (NIH, MD, USA).

3. Results and Discussion

3.1 Intracellular signaling activated by mechanical stretch (Figures 4 and 5)

Chondrogenic differentiation of the cells were observed in the culture, assayed by alcian blue staining and by RT-PCR for type II collagen expression, a cartilage specific ECM gene without any mechanical stimulation. Chondrogenic differentiation was inhibited at day 4 after stretch stimulation started as shown in figure 4. Alcian blue positive nodules and their area were fewer in the stretched culture than that in non-stretched culture. Thus, chondrogenic differentiation of rat limb bud cells was inhibited by stepwise stretch stimulation.

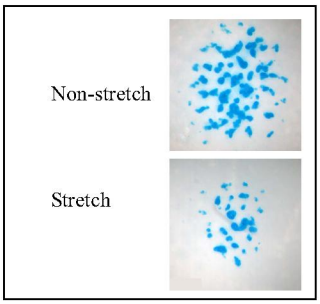

Figure 4. Alcian blue positive nodules were fewer in the stretched culture than that in the non-stretched culture.

Intracellular signaling through various MAPKs (ERK-1/2, JNK-1/2/3, and p38 MAPK) was analyzed by western blotting analysis. Phosphorylation of ERKs was transiently and directly up-regulated (5-fold in ERK-1 and 2-fold in ERK-2), 1 hour after the stretch stimulation (figure 5), while other MAPKs were not (data not shown). Phosphorylation of ERK increased again after 6 hours of stimulation. On the other hand, JNK-1/2/3 and p38 MAPK did not change their phosphorylation level throughout 12 hours of experimental period. In addition, gene expression level of ERK-1/2 did not change throughout the experimental period by semi-quantitative RT-PCR analysis. Taken together, mechanical stretch directly activated ERK-1/2, but not JNKs and p38 MAPK.

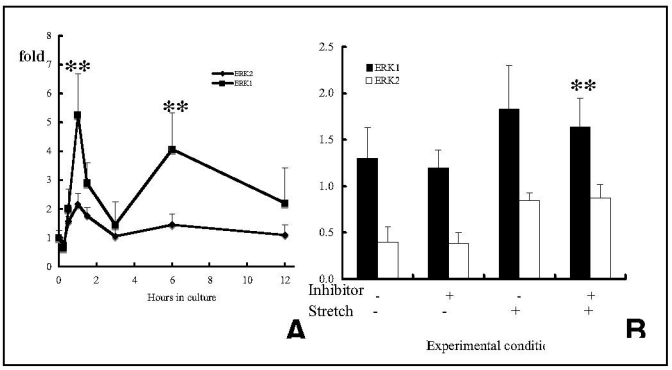

Figure 5. Time course change in phosphorylation of ERK-1 and ERK-2 after stretch stimulation is indicated in (A). This activation of ERK was not dependent on protein production, because ERK-1/2 were activated even with cycloheximide supplementation in the medium (B). **: P<0.01

3.2 Mechabiological signal in the chondrognensis is mediated by ERKs.

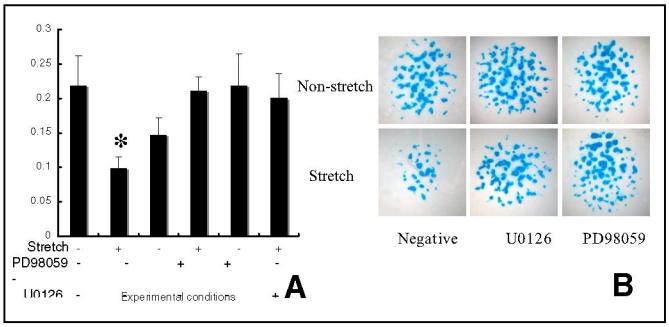

Figure 6. Inhibitors of ERK-1 and ERK-2 rescued the stretch mediated inhibition of chondrogenesis. Semi-quantitative RT-PCR analysis (A) and alcian blue staining (B) show the rescued chondrogenesis under the stretch stimulation. *: P<0.05

While gene expression of type II collagen decreased by 4 days of mechanical stepwise stretch stimulation, inhibition of MEK-1/2 or MEK-1 activities by inhibitors (U0126 or PD98059) rescued the gene expression of type II collagen. At the same time, formation of alcian blue positive chondrogenic nodules were also rescued by the inhibitors (figure 6). Thus, inhibition of ERK phosphorylation by inhibitors (U0126 or PD98059) rescued chondrogenic differentiation of embryonic limb bud cells under mechanical stimulation using Flexcer Cell plates-based culture device (figure 6).

Taken all together, it was demonstrated that the mechanical stepwise stretch inhibits chondrogenesis through intracellular signaling via ERK-1/2 but not othe MAPKs.

3.2 New configuration of mechanical stress culture devise

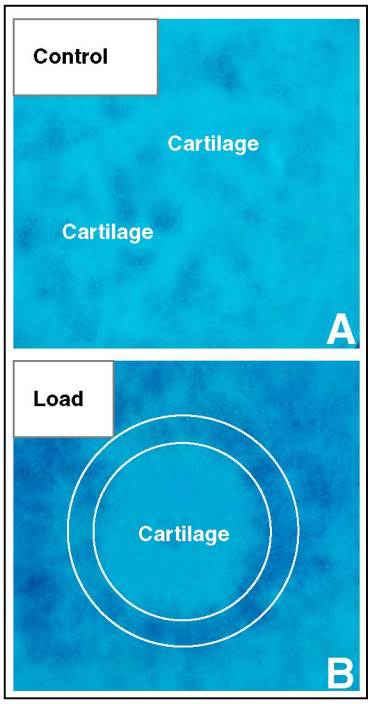

Figure 7. Newly designed stress culture devise inhibited chondrogenic differentiation of ATDC-5 cells where stretch stimulation loaded. On the other hand, compressive stimulation enhanced chondrogenic differentiation, when assayed by alcian blue staining. Alcian blue positive areas focused in the inner circle in (B) where compressive stress loaded when compared to (A) without mechanical stimulation. Almost no cartilage formation was found in the area between outer and inner circle (B), where tensile stress loaded.

Newly designed mechanical stress culture device inhibited chondrogenic differentiation of ATDC-5 cells and embryonic rat limb bud cells where stretch stimulation was loaded. In our previous study [8], finite element analysis revealed that compressive stress is loaded on the surface of PDMS membrane near the center of the pit, while tensile stress was loaded around the margin of inside of the pit. Thus, newly developed stress culture devise also exhibited the biological consequence of mechanobilogical response of chondrogenic differentiation of cells.

Taken all together, chondrogenic differentiation could be inhibited by mechanical stretch stimulation through ERK signaling. The newly designed mechanical stress culture device would be useful for further mechanobiological analysis.

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