Development of Cartilaginous Tissue in Chondrocyte-Agarose Construct Cultured under Traction Loading

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Abstract— **In this study, chondrocytes isolated from bovine cartilage tissue were seeded in agarose gel and resultant chondrocyte-agarose constructs, a well-established experimental model to examine the effect of mechanical loadings on the chondrocyte metabolism, were cultured with a traction loading on the construct surface to examine its effect on the regeneration of the cartilaginous tissue by chondrocytes. Custom-designed mechanical loading equipment was developed to apply the traction loading on the upper surface of constructs being cultured in the CO_2 incubator. After 2 or 3 weeks culture, quantities of glycosaminoglycan (GAG) molecules that proteoglycan, and type II collagen were determined, and immunofluorescent staining of keratin sulfate, a type of GAG, and type II collagen was performed to verify the chondrocyte biosynthesis of extra cellular matrix (ECM) and characterize the structure of elaborated cartilaginous tissue by confocal laser scanning microscopy (CLSM). Results indicated that the traction loading enhance ECM biosynthesis in the surface region of constructs and collagen rich layer covered with GAG rich superficial layer was formed in the articulation surface. Results of quantification for ECM molecules indicated that the production of type II collagen and GAG was more significant outside the slide track compared with inside the slide track.**

Keywords— ***chondrocyte, ECM, cartilage tissue, traction loading,***

I. Introduction

In synovial joints of mammals, the articular cartilage covers bony both ends. Cartilage tissue is formed by chondrocytes, cells embedded in cartilage tissue, and the extracellular matrix (ECM) produced from chondrocytes. Type II collagen and proteoglycan are the principal components of cartilage ECM. Type II collagen is fibrous protein, cartilage tissue is strengthened by a 3 dimensional network of collagen fibrils which resists tension and shear force. The biological body cartilage has structural anisotropy. Type II collagen grows parallel to surface in the surface layer, and it grows vertically at deep part. The proteoglycan is glycoprotein, and it is made from the glycosaminoglycan (GAG), which is polysaccharide chain assembly, and core protein. It makes possible by such structure not only simple load support, but also shock absorption, low friction, and abrasion quality. However, since a blood vessel does not exist, self-repair capacity is scarce, and self-recovery is difficult.

Currently, artificial joint replacement, mosaicplasty, etc. are used to reconstruct damaged synovial joints. But, these medical treatments have problems, such as a limitation of joint lifetime, and the cartilage tissue engineeringis expected to be a novel medical treatment of joint diseases. However, the regenerated cartilage does not have sufficient dynamic function compared with the normal articular cartilage. Then, research for the improvement in functionality is necessary for the cartilage tissue engineering. It turn out that the quantity of type II collagen produced from chondrocytes increases, when the dynamic load is given to the regenerated cartilage tissue model under cultivation. However, the load which arises in a living body is not the simple in fact. The cell is exposed to the dynamic stress field.

In this study, traction load is given with a roller to the cartilage tissue model surface under cultivation, and the influence is investigated.

II. Method

A. Culture

Cartilage tissue was harvested from the metacarpal-phalangeal joint of steers and cut into small pieces using a surgical knife. Then, tissue was digested by 5 mL of 25 units/mL protease solution for 3 hours and subsequently by 30 mL of 200 units/mL collagenase solution for 18 hours at 37 °C. After removal of residual tissue, chondrocytes were isolated from the digested solution by centrifugal separation. The isolated cells were washed twice by using sterile culture medium to remove residual enzyme, and finally re-suspended in 5 mL of fresh culture medium. The number of viable chondrocytes in this suspension was measured by the

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trypan blue assay. The cell suspension was diluted to have a cell concentration of 2 x 10^7 cells/mL and mixed with 2 w/v% agarose solution to have 1 w/v% agarose solution with cell concentration of 10^7 cells/mL. The cell-agarose solution was directly poured into specially designed culture dishes made from polycarbonate and gelled at 4 °C for 30 minutes to have a chondrocyte-agarose construct with a diameter of 18 mm and a height of 2.5 mm (Fig.1).

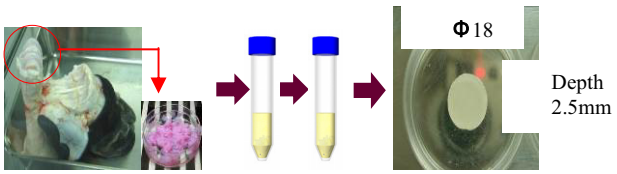


Fig.1 How to make a model

Bottom of the culture dish consisted of polyethylene porous membrane to keep the cell viability in the relatively large construct by securing the material transportation between the culture medium and chondrocyte-agarose construct. A culture medium used in this study is Dulbecco's modified eagle medium (DMEM) supplemented with 20vol% fetal bovine serum, L-glutamine, penicillin, amphotecerin-B, streptomycin, HEPES and 2mM of L-Ascorbic Acid 2-Phosphate (A2P). Culture media was exchanged every 24 hours.

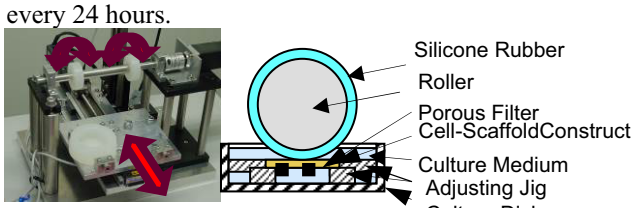


Fig.2 traction culture machine

During the culture period, the cyclic traction loading was applied to the surface of chondrocyte-agarose construct by using custom-designed rolling-sliding loading culture system. The detailed configuration of the loading system is shown in Fig.2. This equipment consists of upper oscillating plastic roller and lower reciprocating specimen stage. Vertical movement and oscillation of the roller and horizontal reciprocation of the stage were independently driven by three AC servomotors which were controlled by PC through a control board. The chondrocyte-agarose construct fitted into the culture dish was mounted on the specimen stage and the roller was rolled over its upper surface with a defined slip/roll ratio to apply the traction loading to the construct. Since the polypropylene roller was much harder than the construct, the roller surface was covered with silicone rubber sheet to avoid the wear of construct. The silicone rubber was exchanged every day. The loading equipment was installed in a CO_2 incubator and the traction loading was applied to the construct at 1 Hz for 12 hours a day during the culture period of 2 to 3 weeks. Another construct with same dimensions was cultured in CO_2 incubator at the same time under same conditions without traction loading and used as control.

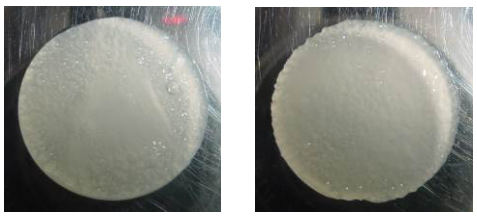
B. Experiment

Amount of type II collagen and, GAG contained in the model after cultivation, and the amount of GAG diffused in the culture medium which exchanges every day is evaluated. Cylindrical specimens with a diameter of 5 mm harvested from cultured specimen for quantitation assays. On the other hand, whenever a culture medium exchanges, it extracts 1 ml of culture media, and it preserved them in frozen storage. After crushing a specimen using a homogenizer, it added to the buffer which contains sodium acetate, EDTA, and cysteine hydrochloride to potassium phosphate buffer, agarase, and papain. Subsequently it was kept at 37 ℃ overnight. Centrifugal separation was performed after cooling to room temperature. The supernatant fluid was used for the evaluation of GAG quantity, and the sediment was used for the evaluation of type II collagen amount. For the quantitative assay of GAG, optical density was measured after dyeing in the DMMB. For the assay of type II collagen, it melted in the alkaline solution and measured optical density after dyeing in the Sirius red.

After the culture period, thin slices with the thickness of about 300 to 500 μm were collected from the cultured construct and stained immunofluorescently to observe the morphological characteristics of cartilaginous tissue elaborated in the construct by chondrocytes. Each slice was rinsed with phosphate buffered saline (PBS) and immersed in PBS containing 1 w/v% bovine serum albumin (BSA) for 30 minutes. Then the slice was immersed in the PBS containing primary antibodies for type II collagen and keratan sulfate which is a type of glycosaminoglycan (GAG) chain consistent of proteoglycan molecules for 90 minutes to label each molecule, respectively. After washing by PBS, the labeled slices were immersed in PBS containing secondary antibodies for 60 minute and finally distributions of labeled type II collagen and GAG in the construct were captured by using the confocal laser scanning microscopy after washing 2 times by PBS.

III. Result and Discussion

Figure 4 shows image of model surfaces after cultivation. The model cultured under the traction loading has clear sliding track on its surface. Therefore, specimens for analyses were divided into three groups, inside the slide track, outside the track, and control.



Friction Control

Fig.4 The reproduction cartilage tissue model

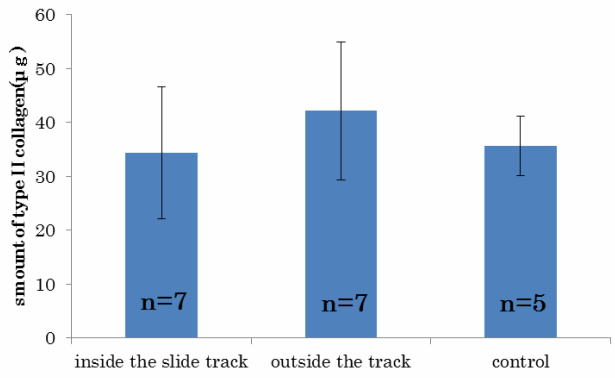


Fig.5 amount of type II collagen in the model

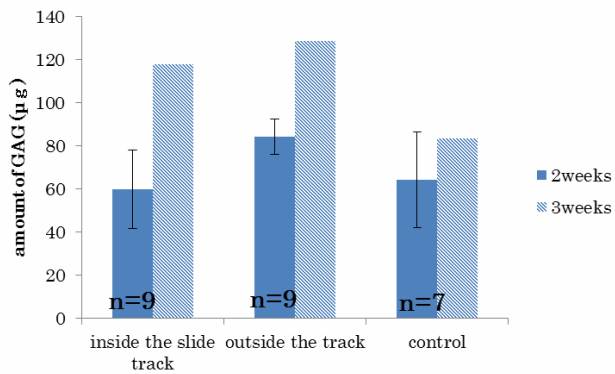


Fig.6 amount of GAG in the model

Figure 5 and 6 are results of quantitation assays for type II collagen and GAG, respectively. Although any significant difference could not be found in the type II collagen assay, the amount of collagen tends to be higher at the outside the sliding track in the traction loaded model. The collagen amount outside the track was also relatively higher compared with the control. Similar results could be found in the GAG assay and GAG amount outside the sliding track was higher than that inside the sliding track and also than the GAG amount in the control. Moreover, the amount of GAG diffused in the culture medium was larger for the traction loaded model compared with the control (Fig.7).

These results might be indicating that the traction loading applied to the model surface stimulate chondrocytes cultured in agarose construct and uplegulate the biosynthesis of ECM molecules. The traction loading on the sliding track also induce some deformation outside the sliding track. In this case, chondrocytes outside the sliding track experienced tensile or shear strains, while chondrocytes inside the sliding track were exposed to larger strains containing compression. Such difference in the strain field may be responsible for the difference of ECM biosynthesis and resultant ECM amount among the positions in the traction loaded model.

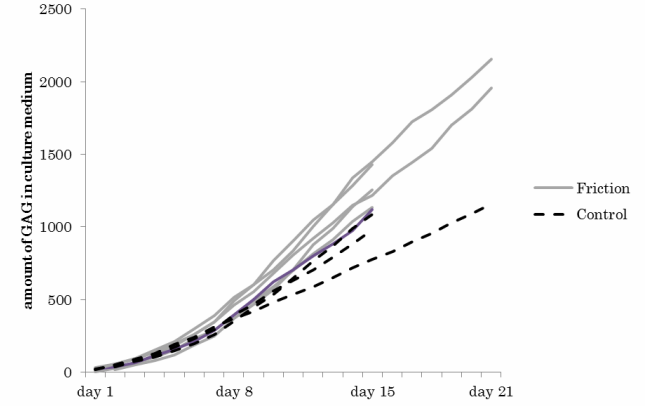
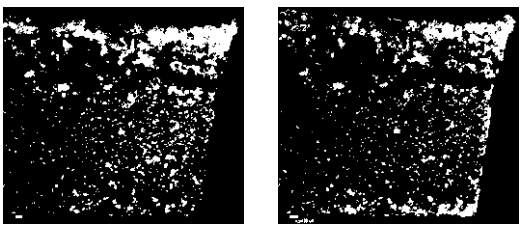
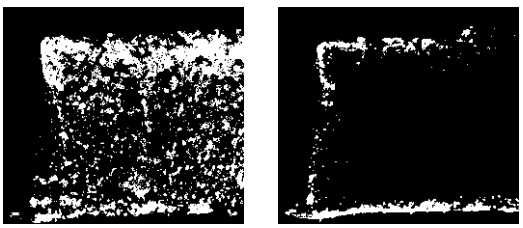


Fig.7 amount of GAG in culture medium



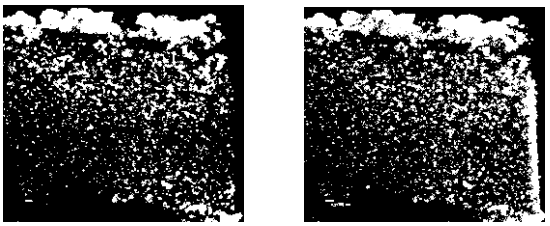
Type II collagen GAG(keratin sulfate)

Fig.8 Friction, inside the slide track



Type II collagen GAG(keratin sulfate)

Fig.9 Friction, outside the sliding track



Type II collagen GAG(keratin sulfate)

Fig.10 control

Results of fluorescence observation were shown in Fig.8 to Fig. 10. Some differences can be found in the distribution of type II collagen between the traction loaded model and control. In the traction loaded model, the collagen density was much higher near the surface compared with the center and deep zone of the model. Such an inhomogeneous distribution of collagen fibers could not found in the control.

GAG molecules tend to be accumulated near the free surface both in the traction loaded model and the control. However, it looks like the convergence of GAG molecules at the sliding surface is more significant for the traction loaded model. These results indicating that the traction loading on the model surface induce not only the increase of ECM biosynthesis but also the inhomogeneous distribution of ECM molecules which is the very important characteristic of natural articular cartilage.

IV. Conclusions

Chondrocytes isolated from bovine cartilage tissue was seeded in agarose gel and resultant chondrocyte-agarose construct was used as the regenerated cartilage tissue model. Influences of the traction load given by a roller on the biosynthesis of ECM could be confirmed from results of two to three weeks culture experiments. The traction loading stimulate the metabolism of chondrocytes and upregulate the biosynthesis of Type II collagen and GAG. However, the amount of elaborated ECM molecules was depended on the position in the loaded model and more ECM was accumulated outside the sliding track. These results may be brought by the inhomogeneous strain distribution caused by the traction loading at the surface. The inhomogeneous distribution also exerted the inhomogeneous structure in the loaded model.

Therefore, the traction loading on the surface may have a potential to make the structural anisotropy like a natural articular cartilage in regenerated cartilage tissue.

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