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Tribological Maturation of Regenerated Cartilage was Inhibited by Using Chondrocyte Aggregates*

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Abstract

Cartilage tissue was regenerated using fibroin sponge seeded with chondrocyte aggregates. The aggregates were formed on the sponge by micro-folding culture, where chondrocytes were cultured on a substrate containing an array of pits of 100 μm diameter. The aggregate-derived cartilage tissue showed higher safranin O staining intensity and was positive for collagen type II by immunostaining. However, its friction coefficient was higher than that of the cartilage tissue regenerated using individual cells. Moreover, the surface of this aggregate-derived cartilage tissue showed weak positive staining for collagen type I. Thus, regeneration of the aggregate-derived cartilage tissue has the possibility of forming larger amounts of extracellular matrix from a limited number of chondrocytes. However, such tissue showed relatively poor tribological function possibly because of its immature functional layer structure.

Key words: Fibroin Sponge, Cartilage, Chondrocyte, Cell Aggregate, Friction Test

1. Introduction

Cell proliferation and efficient extracellular-matrix formation are important roles of cartilage regeneration using autologous chondrocytes as cell source. Cell aggregate (spheroid) culture has been advocated as a useful technique in regeneration medicine. It is believed that a cell aggregate has a three-dimensional structure and therefore can maintain a higher level of specific functions over a long period [1]. Therefore, regeneration of aggregate-derived cartilage tissue has the possibility of forming larger amounts of extracellular matrix from a limited number of chondrocytes.

Several methods for forming cell aggregates have been proposed, such as culture using a rotating wall vessel [2] and rotation culture [1]. Recently, researchers have reported several methods for cartilage regeneration using chondrocyte aggregates. In most experiments, cartilage was regenerated as scaffold-free tissue [3]. Chondrocyte aggregates were cultured under dynamic conditions, in which cells were suspended along the flow of the culture medium. However, these methods tend to require a large number of cells, and necrosis of cells may occur when the tissue is placed under static conditions. Micro-folding culture is another technique for forming cell aggregates by culturing cells on

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a substrate containing an array of pits. The advantage of this method is the ability to control cell-cell contact through the shape of the pits [4].

Fibroin sponge is one of the porous-type scaffolds made from refined silk fibroin and has been used for cartilage regeneration. It is mechanically robust enough to be fixed to the joint surface and can be designed to desired mechanical specifications including ultimate tensile strength, yields point and stiffness. Morita et al. demonstrated that the friction coefficient of the regenerated cartilage using the fibroin sponge was as low as that of natural cartilage in the early stage of the friction test [5]. This suggests that a hydrophilic layer was formed on the surface of the fibroin sponge by the synthesized extracellular matrix with an increase of culture time. Aoki et al. reported that chondrocytes proliferated in the fibroin sponge without losing their differentiated phenotype and a hyaline-like cartilage tissue was organized specifically on the sponge surface [6].

In this study, cartilage tissue was regenerated by seeding chondrocyte aggregates on fibroin sponge with the aim of increasing extracellular matrix formation. Our objective was to investigate the chondrogenic performance of chondrocyte aggregates in fibroin sponge.

2. Methods

2-1. Preparation of patterned culture substrate with array of pits

Figure 1 shows the fabrication process of a patterned culture substrate which has array of pits to form cell aggregates. Glass substrate (Matsunami Glass Ind., Ltd., Japan) treated by aluminum evaporation was coated with SU-8 2100 resist (MichoChem Corp., USA). Resist thickness was controlled approximately 100 μm by spin coating. After that, glass substrate was exposed under UV (Fig. 1(a)), then the pattern was developed by SU-8 developer (MichoChem Corp., USA) and rinsed with isopropanol (Fig. 1(b)). PDMS liquid solution of room temperature vulcanization (RTV) was prepared by mixing KE-106 and CAT-RG (Shin-Etsu Chemical Co., Ltd., Japan) at a rate of 10:1. The glass substrate was coated by the solution (Fig. 1(c)), placed in atmosphere under 1 kPa to remove air in the liquid, and placed on a hot plate at 80˚C. After 2 hours, PDMS was gently removed from the patterned glass substrate, and the patterned culture substrate which has array of pits of 100 μm depth and 100 μm diameter were obtained (Fig. 1(d)). The substrates were placed on polystyrene plastic cases (length: 68 mm, width: 39 mm, thickness: 15 mm in outer size) (As One Co., Japan).

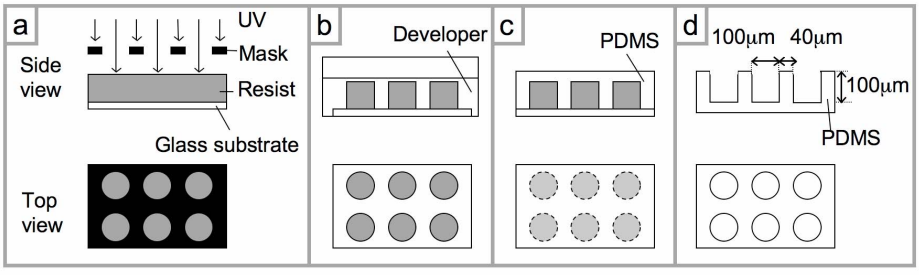

Fig. 1 The scheme for fabrication of patterned culture substrate with array of pits. Resist-coated glass substrate was exposed by UV with patterned mask (a). Patterns were developed by SU-8 developer (b). The patterned substrate was coated by PDMS liquid solution and placed on a hot plate at 80˚C (c). After 2 hours, PDMS was gently removed from the patterned glass substrate, and the patterned culture substrate which has array of pits of 100 μm depth and 100 μm diameters were obtained (d).

2-2. Cell culture

Articular cartilage tissues were harvested from the proximal humerus, distal femur and proximal tibia of 4-week-old Japanese white rabbits (Oriental Bio Service Co., Ltd., Japan). The cartilage tissues were diced into ~1 mm^3 segments after adherent connective tissues were removed from them, and chondrocytes were isolated by enzymatic digestion. Small cartilage segments were digested with 0.25% trypsin-EDTA (Nacalai Tesque, Inc., Japan) for 30 min in a temperature-controlled bath at 37˚C. After being rinsed twice with PBS and centrifuged at 1500 rpm for 5 min, they were enzymatically digested with solution prepared with Dulbecco's modified Eagle medium (DMEM) (Nacalai Tesque, Inc., Japan) not containing serum for 8 h at 37°C. A single-cell suspension was obtained, after being strained through cell strainer (BD Falcon™, USA) and washed twice with PBS. Cells were cultured on 100 mm diameter culture dishes (IWAKI Co., Ltd., Japan) with DMEM containing 10% FBS and 1% antibiotics at 37˚C under humidified atmosphere with 5% CO_2.

2-3. Aggregate formation

9.6 ml PBS was added in the plastic case with a patterned culture substrate, and placed in atmosphere under 1 kPa to remove air on the substrate. After that, 8.4 ml PBS in the case was replaced by Leubovits's L-15 Medium (L-15) (Invitrogen Co., USA).

Cells subcultured once were seeded on the case at a cell density of 2.8×10^6 per cm^2 (considering area of pits only). The cases were centrifuged at 1000 rpm for 2 min to place cells inside the pits. Culture condition was at 37˚C under humidified atmosphere. Average number of cells in each pit was counted with a phase contrast microscope IX71 (Olympus Co., Japan). Cells cultured on a 100 mm culture dishes were used as controls.

2-4. Evaluation of aggregate size by image processing

After 48 h, cells were collected by forceful pipetting and placed on 100 mm diameter culture dishes. Images were acquired by a CCD camera DP71 (Olympus Co., Japan) mounted on a phase contrast microscope. Each image was digitized in a scale of two values (black and white). From the images, the diameter of aggregate was measured by converting a pixel area into an equivalent circle diameter. Digitized particle images with equivalent circle diameters less than 40 μm were excluded from the analysis, since most of them were not aggregates but single cells.

2-5. Preparation of fibroin sponge scaffold

The fibroin sponge scaffold used for cartilage regeneration was manufactured as reported previously [7]. Briefly, silk fibroin aqueous solution was prepared from Bombyx mori silkworm cocoons by dissolving them in a lithium bromide solution. After addition of water-miscible organic solvent, the silk fibroin protein solution was frozen to produce phase separation for 24 h and then the sponge was formed by freeze drying. After removal of water-miscible organic solvent, the fibroin sponges were stored in water or PBS at 4˚C. The average pore diameter was approximately 80 μm.

2-6. Regeneration of cartilage tissue

Autoclaved fibroin sponge scaffold specimens with 8 mm diameter and 1 mm thickness were used for cartilage tissue regeneration. 40 μl cell suspensions containing 5.0×10^5 cells were seeded onto the top of the fibroin sponge in 24 well culture plate (IWAKI Co., Ltd., Japan). Cell density of the suspension containing aggregates was adjusted based on initial cell number seeded on the patterned culture substrate, assuming that cells don't proliferate without adhesion to the substrate. As a control, single cells were seeded on the fibroin sponge. After 3 h incubation at 37˚C under humidified atmosphere with 5% CO_2, 1.5 ml DMEM with 10% FBS, 1% antibiotics and 1% vitamin C was added to each well

and incubated at 37˚C under humidified atmosphere with 5% CO_2. The medium was replenished every 2, 3 days.

2-7. Histologic evaluations.

At 14 and 28 days in culture, regenerated cartilage tissues were fixed in 4% paraformaldehyde, then in 10% neutral buffered formalin and embedded in paraffin. Specimens were sectioned about 7 μm thick in the sagittal plane. Safranin O staining and collagen types I and II immunostaining were carried out for the histologic evaluation of regenerated cartilage tissue.

2-8. Measurement of Friction coefficient

To evaluate mechanical performance of regenerated cartilage tissue, friction test was performed according to the method of Yamamoto et al. [8] at 14 and 28 days in culture. The friction force between regenerated cartilage tissue and flat stainless steel (Ra: 0.06 μm) was measured by using the experimental apparatus shown in Fig. 2. Figure 3 shows the result of kinetic friction coefficient between bovine articular cartilage (diameter: 6 mm,

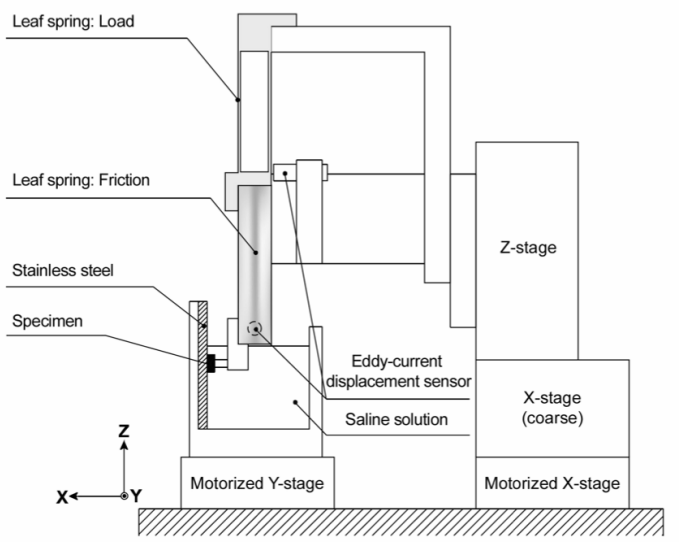

Fig. 2 A schematic drawing of the experimental apparatus for friction test. Reported by Yamamoto et al. [8].

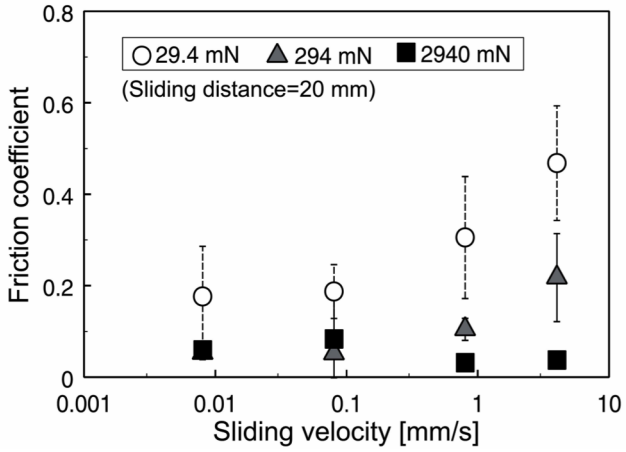

Fig. 3 Lubrication properties of bovine articular cartilage for flat stainless steel (0.06 μm Ra) in saline solution. Sliding velocity: 0.008, 0.08, 0.8, 8 mm/s (logarithmic plot), Normal load: 29.4, 294, 2940 mN (n=3, mean±SD.). Reported by Yamamoto et al. [8].

thickness: 1 mm) and the stainless steel measured by the apparatus (sliding velocity: 0.008, 0.08, 0.8, 8 mm/s, normal load: 29.4, 294, 2940 mN). The friction coefficient clearly showed a positive correlation to the sliding velocity under low normal load, despite the coefficient exhibited significantly high values. It seems that the frictional performance under low normal load is affected by hydration properties on the cartilage surface. Considering those characteristics and of the stiffness on regenerated cartilage tissue, the experimental conditions of continuous reciprocal sliding and of normal load were determined as follows: the sliding velocity was 0.8 mm/s; the normal load was 29.4 mN.

3. Results

Immediately after seeding, chondrocytes were trapped uniformly in the pits of the patterned culture (Fig. 4(A)). The number of cells in each pit was 19.7 (SD 4.5) (n=30). After 48 h of culture, the chondrocytes formed aggregates in the pits (Fig. 4(B)). The mean diameter of an aggregate was mean 57.4 (SD 10.5) μm (n=30).

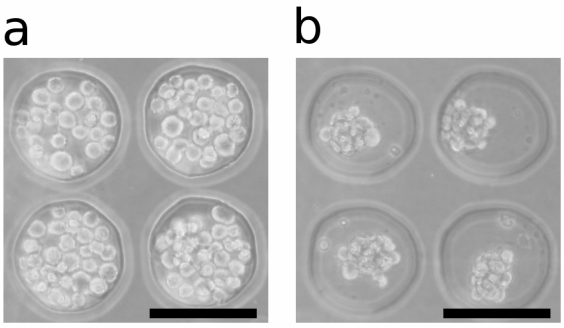

Fig. 4 Morphology of chondrocyte cultured on the patterned culture substrate with array of pits. Chondrocytes were seeded on the substrate (a), and chondrocyte aggregate was formed in the pit after 48 hours in culture (b). Scale bar=100 μm.

The results of the histological examination of the regenerated cartilages subjected to safranin O, and collagen types I and II immunostaining are shown in Fig. 5. The tissue surface at 28 days of culture was smoother than that at 14 days of culture for both the cartilage tissue regenerated from chondrocyte aggregates (AGG group) (a4) and that regenerated from individual cells (CON group) (a2). Safranin O-stained (a3, a4) and collagen type II-immunostained tissues (b3, b4) of the AGG group appeared more chromatic than those of the CON group (a1, a2, b1 and b2, respectively). The surface of the tissues of the AGG group was weakly stained with safranin O compared with the deeper part of the same tissues or the surface of the tissues of the CON group. The anti-collagen type I-immunostained tissues are shown in Fig. 5(c), where the tissue surface of the AGG group (c3, c4) was slightly positively stained while almost no staining was observed in the CON group (c1, c2, respectively).

Figure 6 shows transitions of the friction coefficient of the regenerated cartilage tissues. The friction coefficient at 28 days of culture (b) was smaller than that of 14 days of culture (a) in both the AGG and CON groups. Comparing the results between the AGG and CON groups, we found that the friction coefficient in the AGG group tended to be higher than that in the CON group.

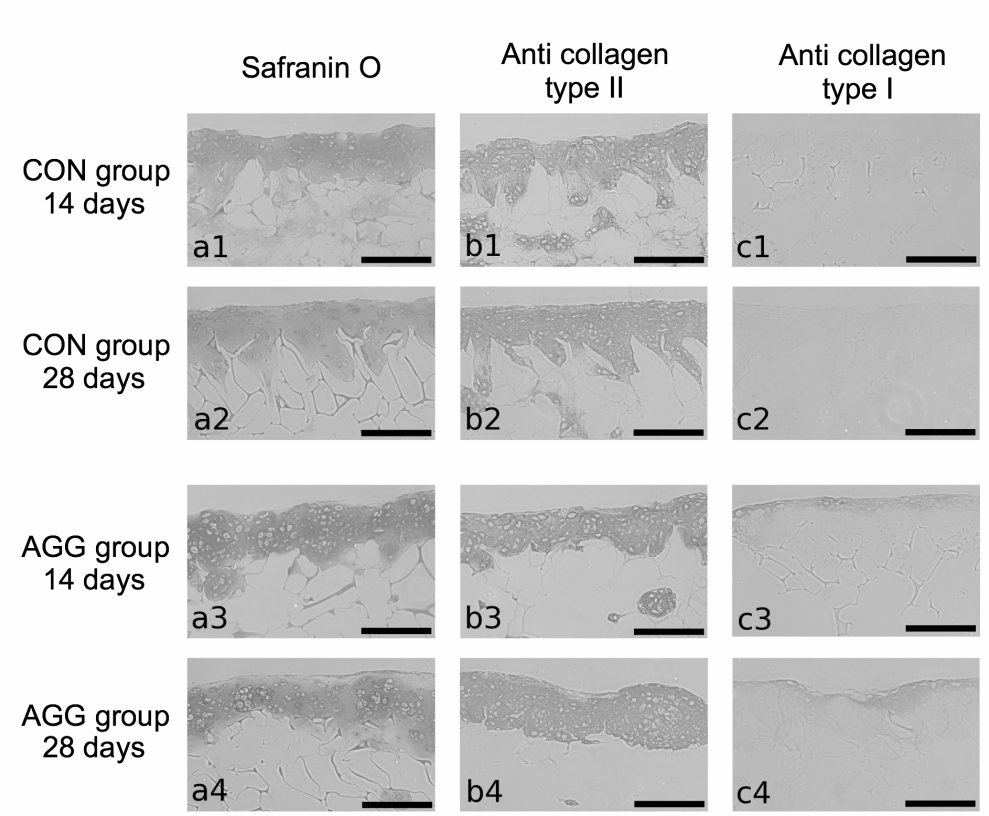

Fig. 5 Sections of regenerated cartilage stained with safranin O, anti collagen type II and type I. Safranin O stained view of the AGG group (a3, a4) and collagen type II immunostained view of the AGG group (b3, b4) showed relatively higher chromatic appearance compared to the CON group (a1, a2, b1, b2). The surface of the AGG group (c3, c4) was slightly positively stained while almost no staining was observed in the CON group (c1, c2). Scale bar = 200 μm.

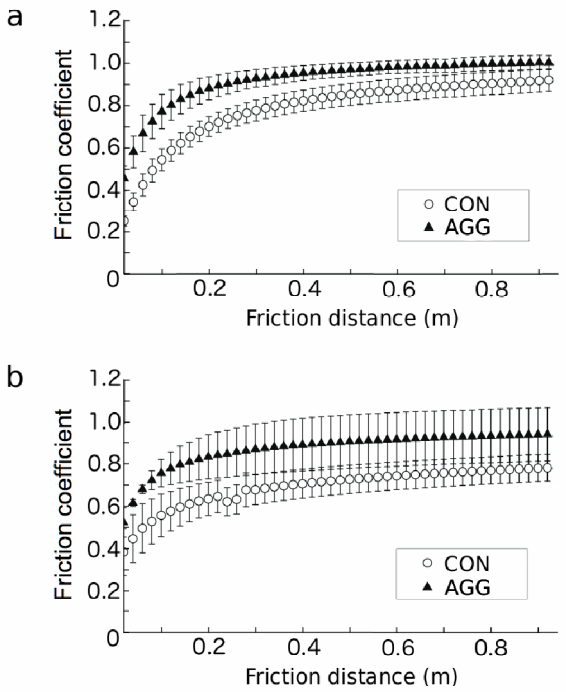

Fig. 6 Friction coefficient of regenerated cartilage. Culture periods were 14 (a) and 28 (b) days. AGG group showed higher friction coefficient than CON group either at 14 days and 28 days in culture.

4. Discussion

The articular cartilage serves as an effective bearing surface aiding lubrication and reducing wear, both of which are known to be important factors for promoting the long-term functioning of healthy joints. The interaction between the collagen network, proteoglycan and water is responsible for its excellent mechanical properties. Although its lubrication mechanism has not yet been elucidated, several studies in this area have already been undertaken. Naka et al. previously measured the friction coefficients of cartilage specimens with intact and wiped surfaces, and demonstrated that the hydration of the cartilage surface layer and proteoglycan found in this layer affects lubrication [9]. Jurvelin et al. estimated the thickness of the surface layer affecting the lubrication mechanism to be within 200-500 nm [10]. In the present study, not only histological analysis but also the friction test was performed in order to evaluate the tribological function of the regenerated cartilage tissue in terms of maturation of the functional layer structure. The friction coefficient of the aggregate-derived tissue (AGG group) was higher than that of the tissue regenerated using individual cells (CON group) under the experimental conditions of a 0.8 mm/s sliding velocity and a 29.4 mN normal load. As shown in Fig. 3, the frictional performances under low normal load are associated with some interfacial properties such as macromolecular interactions and hydration on the tissue surface in the case of bovine articular cartilage. Therefore, the results of the friction tests in this experiment would have some association with superficial layer formation, rather than the deformation and weeping of tissue.

Although the friction coefficient of the aggregate-derived cartilage tissue was higher than that of the tissue regenerated using individual cells, histological evaluation demonstrated that the former tissue showed positive immunostaining for collagen type II and higher staining intensity with safranin O than the latter tissue. It was also shown that the tissue surface of the AGG group was poorly stained with safranin O and positively immunostained for collagen type I. These results suggest that some interference in the maturation of the surface layer structure occurred in the AGG group.

It is well known that collagen type I, which has less water than collagen type II, is seen in fibrocartilage. Dedifferentiated chondrocyte shifts from the synthesis of collagen type II to collagen type I. This chondrocyte differentiates again when they form aggregate. It is reported that cells in an aggregate tend to keep its differentiated phenotype. However, faint expression of collagen type I at the surface of the tissue in the AGG group suggests that dedifferentiated chondrocytes were seen at the surface of the cartilage regenerated from aggregates. Complex interaction of differentiation and redifferentiation could be occurred in the process of matrix formation. The mechanism was not clear, but there is a possibility that the aggregates inhibited formation of functional tribological structure at the surface.

As a scaffold, some of the advantages of the fibroin sponge are that it favorably promotes chondrocytes proliferation while maintaining chondrogenic expression, and it facilitates hyaline-like tissue regeneration on the sponge surface. In this regard, researchers have studied the interactions between chondrocytes and the fibroin surface. Yamamoto et al. investigated the adhesive force of chondrocytes to the fibroin surface. They suggested that chondrocytes on the fibroin substrate express dynamic membrane changes during the initial period, and that some specific cascades are likely to be affected by the interaction between adhesive proteins or chondrocyte secretions and the fibroin surface. In this study, the chondrocytes in the AGG group would have less interaction with the fibroin surface than those in the CON group, since the cells in the AGG group were seeded on the fibroin sponge in the form of an aggregate, where cells adhered to each other. Thus, it could be thought that the interaction between the chondrocytes and the fibroin

surface has an important role in the formation of the functional surface layer during cartilage regeneration in the fibroin sponge.

5. Conclusion

In this study, cartilage tissue was regenerated using fibroin sponge and chondrocyte aggregates and biomechanical functions of the tissue were investigated. The cartilage tissue regenerated using chondrocyte aggregates showed higher staining intensity with safranin O and positive immunostaining for collagen type II. However, the friction coefficient of this tissue was higher than that of the cartilage tissue regenerated using individual cells. Moreover, the surface of the aggregate-derived tissue showed weak positive immunostaining for collagen type I.

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