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Scaffold-free cartilage tissue by mechanical stress loading for tissue engineering

Katsuko S Furukawa, Masato Sato, Toshihiro Nagai
Stephanie Ting, Joji Mochida and Takashi Ushida
*University of Tokyo, Tokai University
Japan*

1. Introduction

Articular cartilage has a limited repair capacity due to its low mitotic activity, avascular nature, and sparse cell population. Osteoarthritis, a painful condition of the joints, has affected over 200 million people worldwide. Cell-based tissue engineering is emerging as a promising clinical option to address tissue and organ failure by implanting biological substitutes for the compromised tissue.

Various dynamic stresses such as shear flow, hydrostatic pressure, and direct compression exist in living bodies and are thought to contribute to cartilage formation. A rotational culture can provide such a mechanical load in a simple manner. In this chapter, we introduce our recent scaffold-free cartilage tissue engineering approaches using such a rotational culture.

2. Articular cartilage

Articular cartilage is a form of hyaline cartilage that envelopes the surface of diarthrodial joints in the human body to distribute the force and allow smooth movement with minimal friction between two bones. Diarthrodial joints are joints where there is a lot of motion between opposing bones; for example, the ankle, knee, hip, shoulder, or elbow joints. The joints can bear very large compressive loads and has a very low coefficient of friction (0.005 to 0.05). Unlike other connective tissues, cartilage does not contain any blood vessels, nerves, or lymphatics. Therefore, it receives nutrients through convection from the surrounding synovial fluid, or diffusion, which is aided by the pumping action created during compression of the cartilage. Cartilage is made up of chondrocytes embedded in a rich matrix of collagen and proteoglycans (Fig. 1).

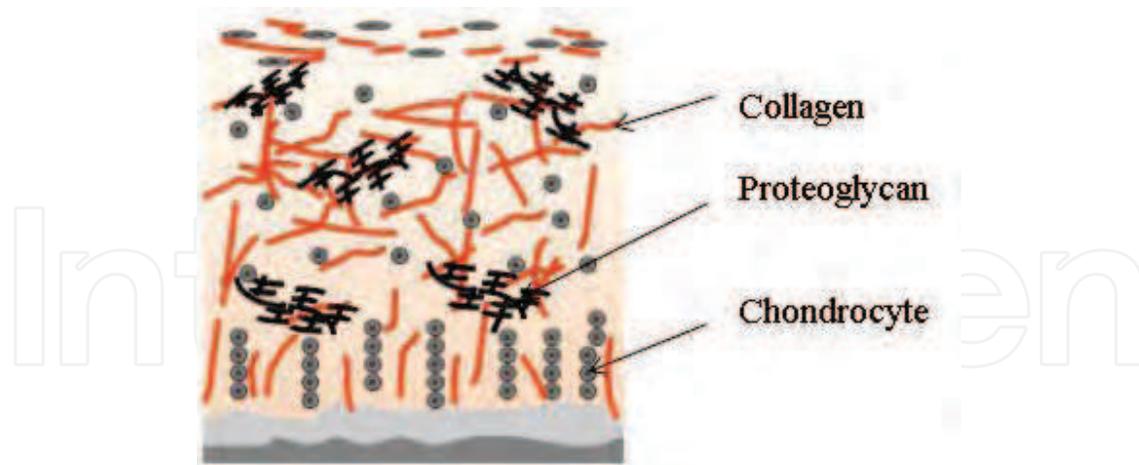


Fig. 1. Cartilage composition

Cells in articular cartilage, which are known as chondrocytes, account for only 5% of the volume of the cartilage. Approximately 70-80% of the articular cartilage is composed of water, whereas the solid phases are composed primarily of type II collagen and aggrecan, a chondroitin and keratan sulfate proteoglycan (Table).

Water	66-79%
Solid	21-34%
Minerals	
Residual ash	5-6%
Organic materials	
Collagen	48-62%
Glycosaminoglycan	8-15%
Protein	14-23%
Hyaluronic acid	<1%
Sialic acid	<1%
Lipid	<1%
Glycoprotein	<1%

Table 1. Biochemical composition of articular cartilage

Collagen forms a network of fibrils, which resist the swelling pressure generated by the proteoglycans. Aggrecan, because of its tendency to non-covalently interact with hyaluronic acid, forms huge aggregates that become trapped in the collagen network. Because of their numerous negatively charged sulfate groups, these proteoglycan aggregates attract cations, which in turn bring in water to minimize differences in osmotic pressure. Thus, type II collagen and proteoglycans create a swollen, hydrated tissue that resists compression. Therefore, the articular cartilage functions as load bearing, shock absorbing, and friction reducing material in diarthrodial joints.

Cartilage tissue has only a small capacity for self-repair. The avascular nature, the sparse cell population, and the low mitotic activity of the chondrocytes severely limit the natural healing of these cartilage defects (Campbell et al., 1969; Mankin et al., 1982; Ushida et al., 2002). Many

patients with damaged cartilage due to trauma or diseases such as osteoarthritis and osteochondrosis dissecans are existing in the world. The degradation of cartilage eventually leads to osteoarthritis, which is the thinning of the articular cartilage resulting in a bone-to-bone joint, causing pain and a reduction in motion. In this disease, there is often the loss of proteoglycans, which leads to decreased compressive stiffness of the tissue. When osteoarthritis reaches the subcondral bone, an ingress of cells from the bone marrow will generate fibrocartilage repair. This leads to a tissue that has lower proteoglycan and high levels of type I collagen. Fibrocartilage is mechanically insufficient as it cannot withstand the normal physiological loads and wear stresses on the knee during work or sports. In very severe cases, both bones of the joint may grow into one another, causing them to fuse together. The joints most commonly affected by osteoarthritis include the hips, knees, wrists, between the fingers, and between the vertebrae.

3. Current treatment for osteoarthritis

Current therapies include transplantation of healthy host cartilage or implantation of artificial prosthetic devices. However, problems remained in these treatments. The amount of donor tissue for transplantation is limited, and durability of the prosthetic devices (Fig. 2) is not good by wear debris or adhesive breakdown at the host/prosthesis interface. For these reasons, there is considerable interest in developing cell therapies and tissue-engineered cartilages to treat damaged cartilage (Brittberg et al., 1994; Freed et al., 1994a, Freed et al., 1994b, Freed et al., 1994c).

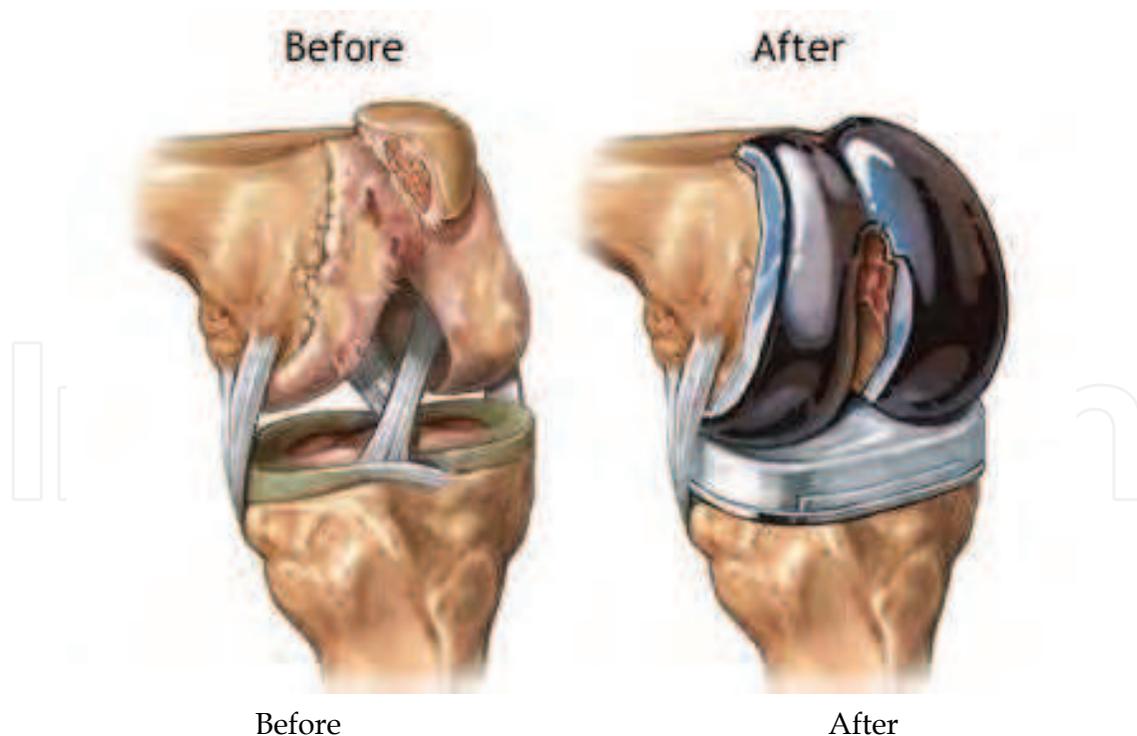


Fig. 2. Knee joint replacement prosthesis
([http:// assets.aarp.org/external_sites/adam/html/2/9494.html](http://assets.aarp.org/external_sites/adam/html/2/9494.html))

Still, many problems remain in cartilage tissue engineering using cells. When grown in two-dimensional culture, adult articular chondrocytes are capable of proliferation, promoting researchers to use cultured autologous chondrocytes to accelerate cartilage regeneration. However, the proliferating chondrocytes gradually lose their differentiated phenotype (von der Mark et al., 1977; Benya et al., 1982, Fig.3), as indicated by the loss of synthesis of aggrecan and type II collagen and the increase of synthesis of type I collagen (Fig. 4, Chen et al., 2003, Ushida et al., 2004). Therefore, in order to form a tissue-engineered cartilage using proliferated chondrocytes with a dedifferentiated phenotype, it is thought that reexpression of the chondrogenic phenotype should be induced before implantation.

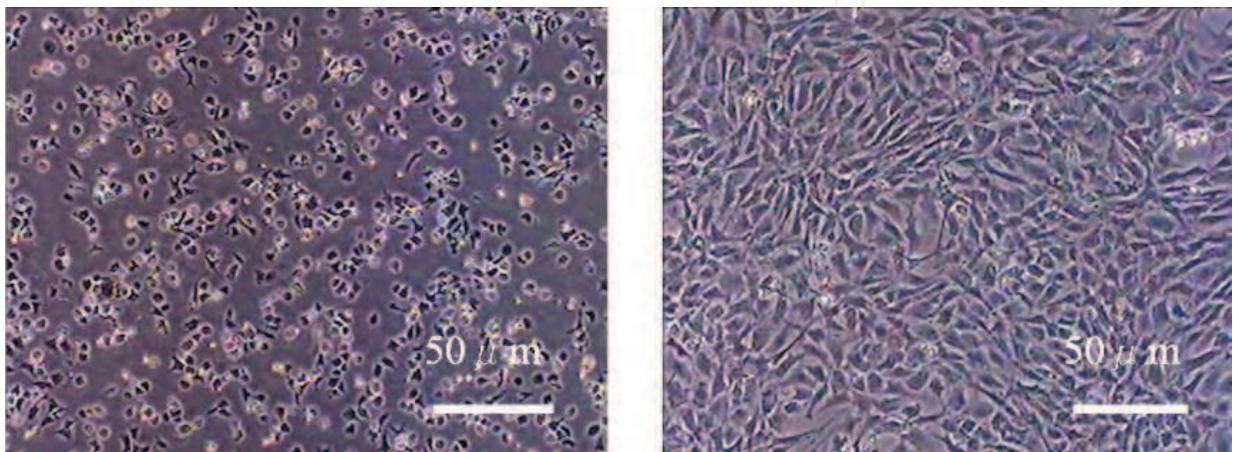


Fig. 3. Chondrocytes in 2-dimensional culture. Left: Primary chondrocytes, right: dedifferentiated chondrocytes

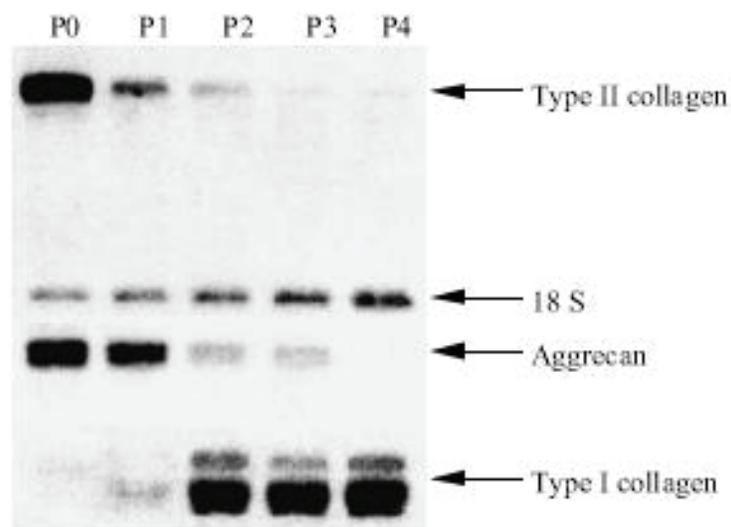


Fig. 4. Northern blot analysis of the mRNA encoding type I collagen, Type II collagen, and aggrecan of bovine chondrocytes cultured in monolayer for 0, 1, 2, 3 and 4 passages (Chen et al., 2003, Ushida et al., 2004).

To date, "pellet" culture systems (Fig. 5) under static conditions have been reported as a method for preventing and reversing the phenotypic modulation of chondrogenesis *in vitro* (Hay et al., 2000; Johnstone et al., 1998; Lunstrum et al., 1999; Mackay et al., 1998; Oberlender et al., 1994; Pittenger et al., 1999; Tracy et al., 1994; Yoo et al., 1998).

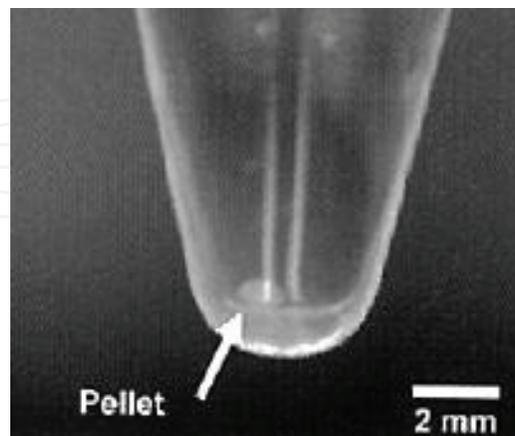


Fig. 5. Pellet culture by dedifferentiated chondrocytes (Tsuchiya et al., 2005).

This culture system allows cell-cell interactions analogous to those that occur in condensation during embryonic development. Although the system facilitates chondrogenic differentiation from dedifferentiated chondrocytes or mesenchymal stem cells with high reproducibility, its clinical application to tissue engineering has not been successful thus far. Because the "pellet" culture system forms only one small cell-aggregate per tube by a centrifugator, it is difficult to yield sufficient numbers of pellets with the differentiated phenotype. Therefore, a cartilage tissue with a large size in which cell-cell interactions exist, as in a pellet culture, without using a scaffold was tried to form for tissue engineering.

4. Scaffold-free cartilage model by dynamic rotational culture

By using a high porous simple mold, scaffold-free cartilage tissue of arbitrary shapes was tried to fabricate (Furukawa et al., 2008). Various dynamic stresses such as shear flow, hydrostatic pressure, and direct compression exist in living bodies and are thought to contribute a scaffold-free rotational culture provided such a mechanical load in a simple manner. Thus, it was demonstrated that a scaffold-free cartilage tissue with a large size and arbitrary shape for tissue engineering could be regenerated without any scaffolds, by making cell-cell interaction and mechanical stress loading for tissue engineering.

When a cylindrical glass mold with a diameter of 1 cm was used, dedifferentiated chondrocytes of 1.5×10^7 cells were inoculated into the mold using a commercially available culture-insertion film with pores of 0.4 μm . Oxygen and nutrition can diffuse from the lower and upper sides of cells. After 8 hours of cell inoculation, the mold was removed from the membranous surface, and cell plates were left on there with the same shape as the mold. After 24 hours' static culture for shape stabilization, scaffold-free tissue was moved to a 6-well culture plate without any shape changing adding 5 ml of differentiation medium, and was begun on rotational culture. In this point, the scaffold-free plate was very fragile; therefore, displacement of the tissue to the rotational culture plate was done with great care. A disk-like cartilage tissue was formed by 3 weeks' culture as shown in Fig. 6.

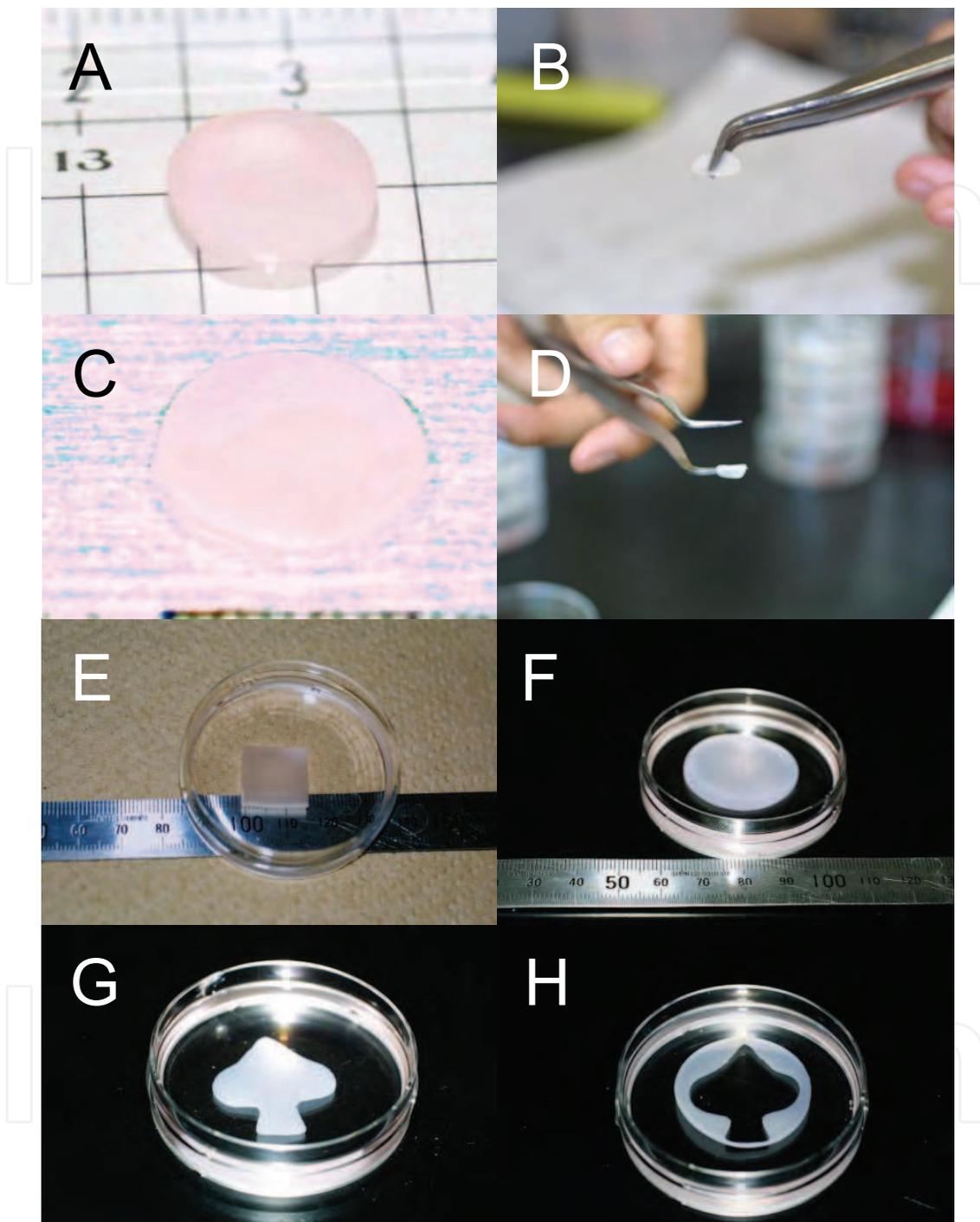


Fig. 6. Appearance of the scaffold-free cartilage tissue after 3-week culture. A, B, E-H: Rotational culture, C, D: static culture. A-D, F: mold shapes were circle. E: Mold shape was square. G: Spade shape formed by stamping out with a metal mold. H: F-C.

When the cartilage plate without any scaffold cultured under shear flow conditions (rotational culture condition) was grasped, the shape of the plate with a disk-like form was maintained (Fig. 6B). In contrast, the cartilage plates cultured under static conditions

changed to a dogleg shape when grasped (Fig. 6D); often they could not keep their shape for 3 weeks if we changed the medium. Therefore, it was obvious that mechanical conditions played an important role in the mechanical properties of a scaffold-free cartilage tissue. When a square mold was used, a square-shaped cartilage plate was formed under shear flow conditions (Fig. 6E). The formation of a comparatively large cartilage tissue was also possible as shown in Fig. 6F. Furthermore, it was possible to form and cut it into spade shapes, i.e., arbitrary shapes, by stamping out with a metal mold (Fig. 6G, 6H).

The thickness, diameter, and volume of cartilage tissue formed by a glass mold with a diameter of 1 cm at the start point of culture were measured. The thickness of the cartilage tissue continued to increase for the 3-week rotational culture (Fig. 7A). The diameter increased slightly for 1 week, then remained the same (Fig. 7B). The volume continued to increase for the 3-weeks culture (Fig. 7C). Similar results were obtained in control static culture. To confirm the viability of the chondrocytes in the cartilage tissue, the scaffold-free cartilage with a diameter of 1 cm cultured under shear flow conditions was stained with calcein-AM and propium iodide. Necrosis in the center of the tissue was not observed; the majority of the cartilage cells in the central part of the cartilage tissue were understood to be living. Therefore, it was suggested that the chondrocytes included in the cartilage tissue were alive and maintained their growth for 3 week *in vitro*.

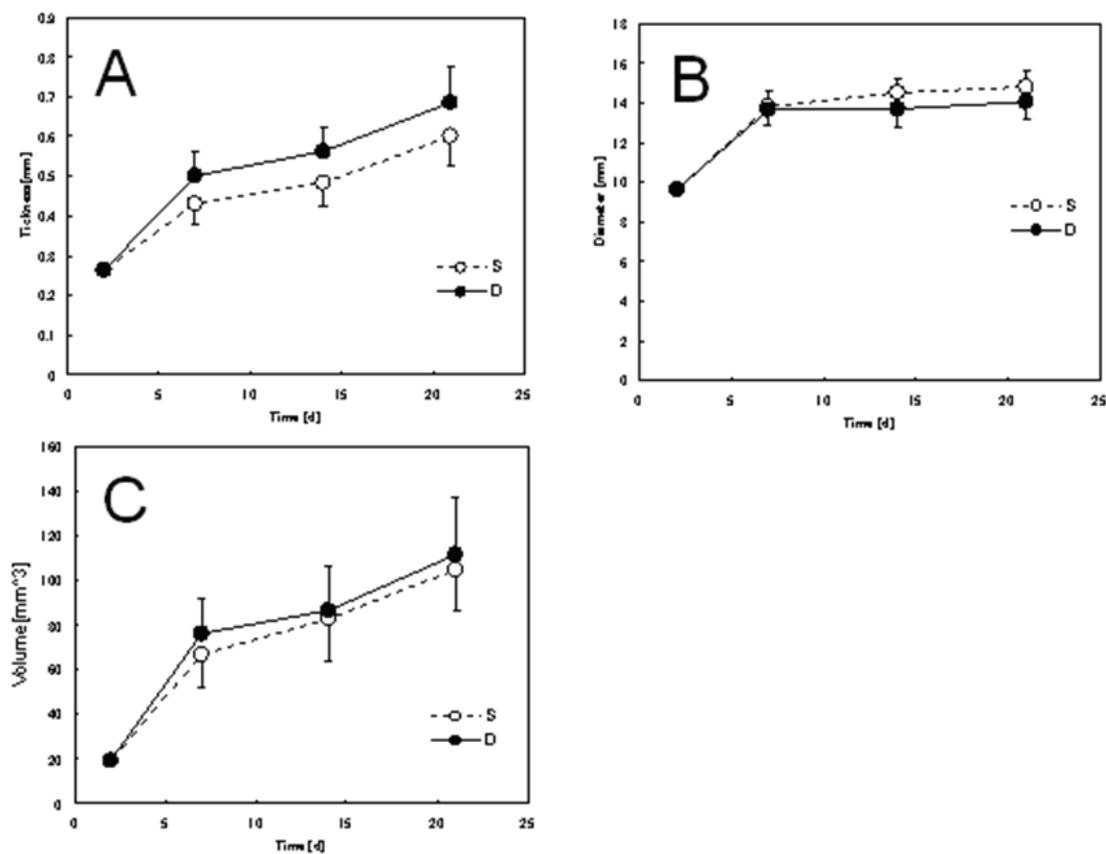


Fig. 7. Time changes in thickness, diameter, and volume of the cartilage tissue for 3-week rotational culture. D: Dynamic (rotational) culture of the cartilage tissue, S: Static culture. A) Thickness of the cartilage plate was measured by a mechanical spectrometer (AGS-G, Shimazu, Kyoto, Japan), mean \pm SE (n=4). B) Diameter was measured by a caliper square. C)

Volumes of the cartilage plate were calculated from the data of the thickness and the diameter of the cartilage plates, independently.

To investigate the existence of proteoglycans, the cartilage tissue with a diameter of 1 cm was stained with safranin-O and toluidine blue. The cartilage tissue cultured under dynamic conditions (rotational culture) for 3 weeks showed strong staining properties (Fig. 8B and 8D) compared with that of the static culture (Fig. 8A and 8C). In rotational culture, cells aligned horizontally to the surface were observed (Fig. 9B). In contrast, cartilage tissue cultured under static conditions did not have such alignment; instead, cells near the surface had a round shape without any orientation (Fig. 9A).

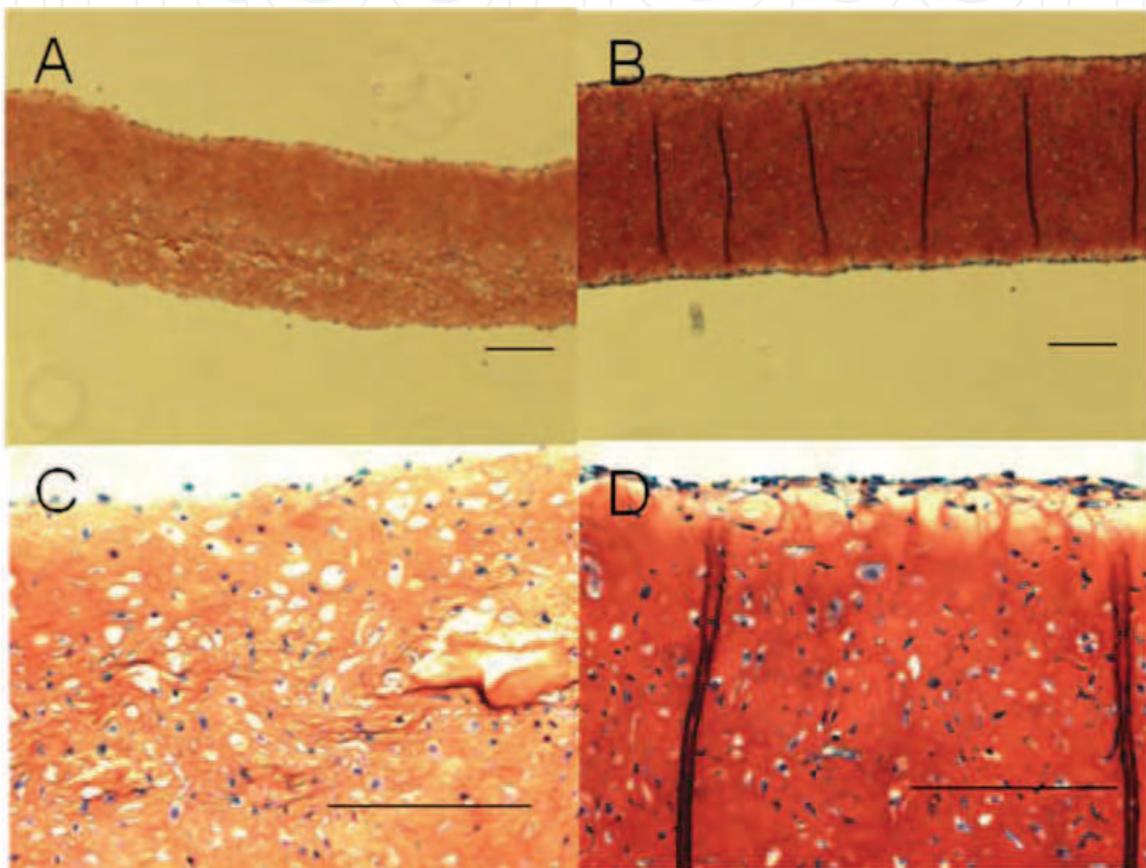


Fig. 8. Distribution of proteoglycan was investigated. A and C) Scaffold-free cartilage tissue cultured under a static condition without flow was stained with safranin-O after cultivation of 3 weeks. B and D) Scaffold-free cartilage tissue cultured by rotational culture was stained with safranin-O after 3 weeks. Scales, 200 μ m. Magnifications of objective lens, A, B; x4, C, D; x20.

The edge of the cartilage tissue cultured under static conditions was not smooth while that of rotational culture was extremely smooth. In cartilage tissue by rotational culture, there were parts, near the surface, not stained with safranin-O (Fig. 3B and 3D). The results by toluidine blue were similar to the results of safranin-O (data was not shown). The cartilage tissue formed by rotational culture presented metachromasia, and the color was stronger than those of the static cultures. The tendencies of the cell shape and tissue formation were also similar to those of safranin-O.

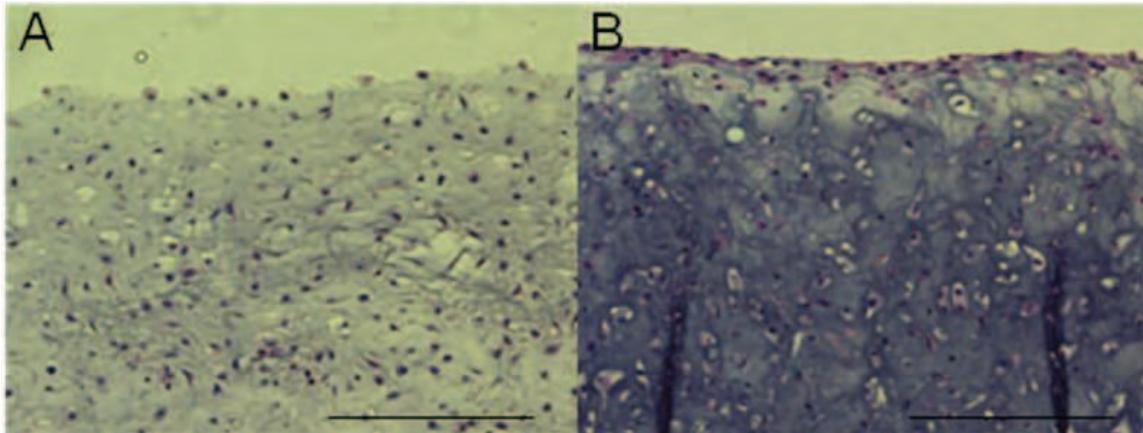


Fig. 9. Tissue cultured by static (A), and rotational conditions (B) was stained with HE in order to observe cell distributions. A: Magnification of objective lens= $\times 5$, B: Objective lens= $\times 20$. Scales, 200 μ m.

Immunohistochemical analysis showed that type II collagen protein was abundantly distributed in scaffold-free cartilage tissue cultured by rotational culture (Fig. 10A). On the other hand, cartilage tissue formed by static culture showed inhomogeneous distribution of type II collagen protein (Fig. 10B).

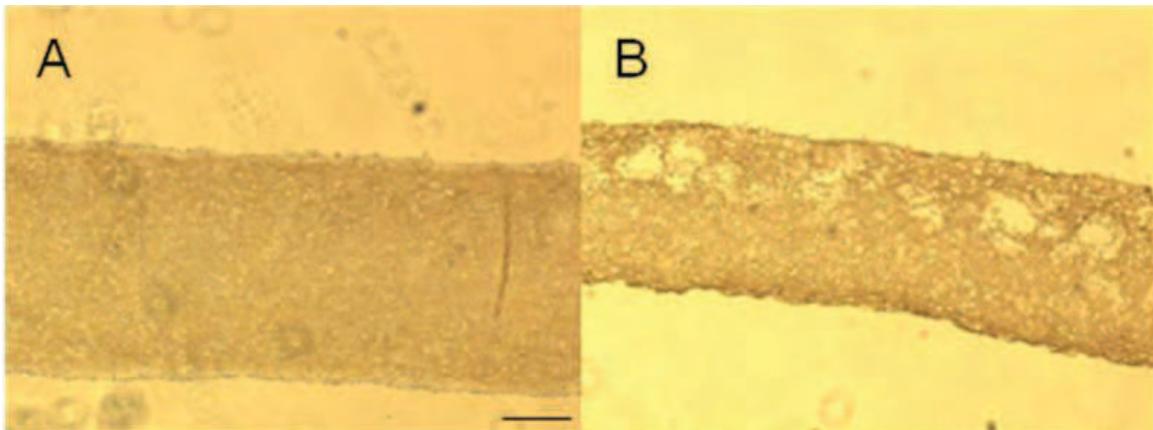


Fig. 10. Cartilage tissue by 3-week culture was stained with type II collagen antibody. A) Cartilage tissue cultured by rotational conditions. B) Cartilage tissue cultured by static conditions. Objective lens: $\times 5$, Scales; 200 μ m.

The cartilage tissue formed by 3 weeks' rotational culture had scarcely any type I collagen protein (Fig. 11A). In static culture, type I collagen protein appeared at the edge of the cartilage tissue as shown in brown color (Fig 11B).

The amounts of proteoglycan and total collagen protein secreted were quantitatively investigated. As shown in Fig. 12, rotational culture significantly increased the protein production. The rotational culture was effective in terms of matrix production per DNA contents in the cartilage tissue with a diameter of 1 cm when compared to static culture. There were statistical differences between dynamic and static cultures (PG/DNA; $p < 0.05$, CN/DNA; $p < 0.01$).

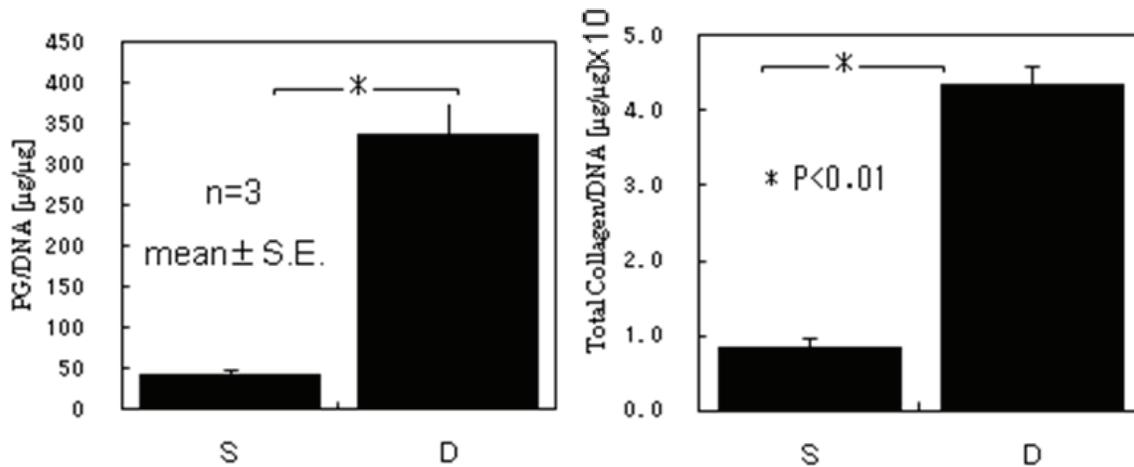


Fig. 12. Proteoglycan and total collagen protein expressed in the cartilage tissue were measured by DMMB method and hydroxyproline assay. S) Static culture of the cartilage tissue, D) Dynamic (rotational) culture of the cartilage tissue.

The expression levels of mRNA of type II collagen and proteoglycan in cartilage tissue cultured under shear flow conditions were higher than that of static culture (Fig. 13). On the other hand, type I collagen mRNA was expressed at a lower level in the cartilage tissue under rotational culture than in static cultures.

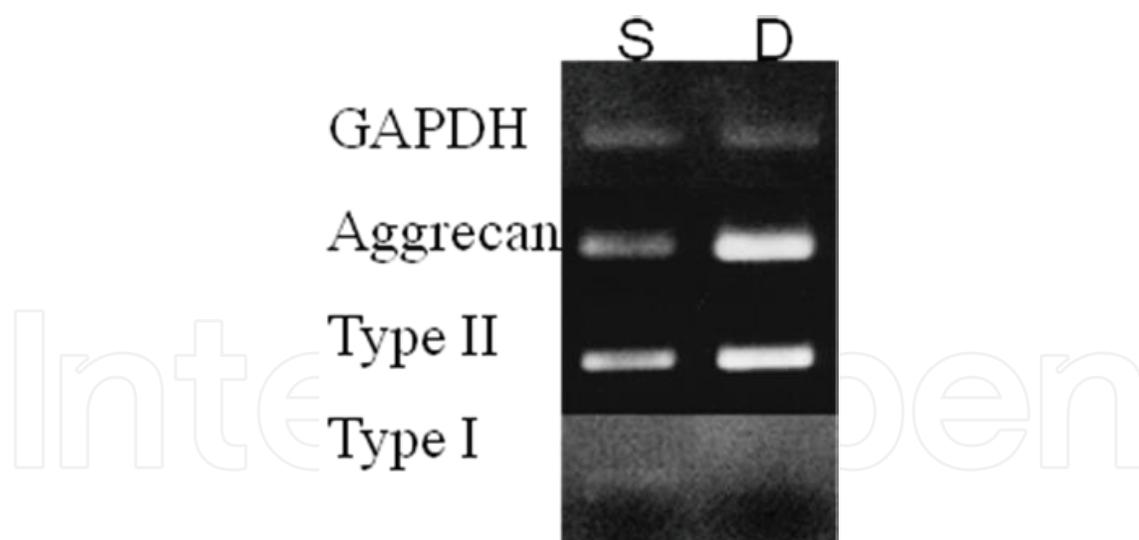


Fig. 13. Gene expression of GAPDH, aggrecan, type II collagen, and type I collagen of the tissue was investigated using RT-PCR methods. S) Static culture of the cartilage tissue, D) Rotational culture of the cartilage tissue.

To obtain quantitative data of the mechanical properties of the cartilage tissue, a stress-strain curve (Fig. 14B) was obtained using dumbbell-shaped culturing samples (Fig. 14C) prepared by stamping out with a metal mold (Fig. 14A). The values of calculated rupture strength and Young's modulus from the data of Fig. 13C significantly increased in cartilage

tissue cultured under shear flow conditions. There were statistically significant differences in the values between dynamic and static cultures. The tactile impression of the cartilage tissue by rotational culture was like a plastic coaster.

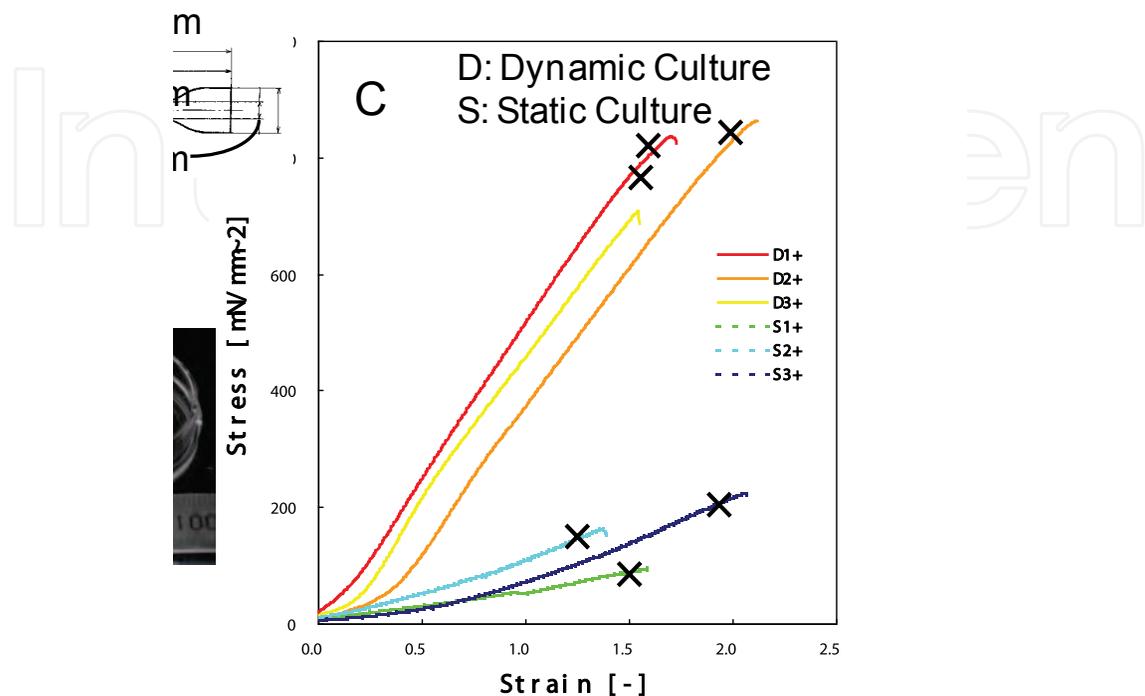


Fig. 14. Mechanical properties of the cartilage tissue. A) Shape of the mold for evaluating the mechanical properties of cartilage tissue. B) Stress-strain curve was investigated. D1, D2 and D3 are data of the samples cultured by rotational culture. S1, S2 and S3 are those of samples cultured under static conditions. C) Dumbbell-shaped samples were prepared for the mechanical evaluations of the cartilage tissue.

5. *In vivo* implantaion experiments of scaffold-free cartilage tissues cultured under dynamic rotational conditions

For animal transplantations of a scaffold-free cartilage tissue cultured under shear flow conditions, the tissue by dedifferentiated chondrocytes derived from Japanese white rabbits was tried to form (Nagai, Furukawa et al, 2008; Sato et al., 2008). After 7 days weeks of rotational culture, the plate was cultured under dynamnic conditions, using rotational culture. After 2-3 weeks of rotational culture, the chondrocyte plate maintained a constant form and was considered stable enough to be handled with surgical pincers (Fig. 15). Conversely, after 3 weeks of static culture, the plate gradually changed into an arch over that time (data was not shown). Histological and immunohistochemical evaluations indicated that the plate had cartilaginous qualities in terms of cell distribution and organization and the production of glycosaminoglycans and type II collagen in rotational cultures. The chondrocyte-plates measuring about 1.1 mm in thickness after 2 weeks and about 1.4 mm after 3 weeks of cultivation had a regular cylindrical shape and the macroscopic appearance of cartilage. The chondrocytes-plate was thus able to be made

without losing its regular cylindrical shape regardless of the cellular passage number (Fig.15).

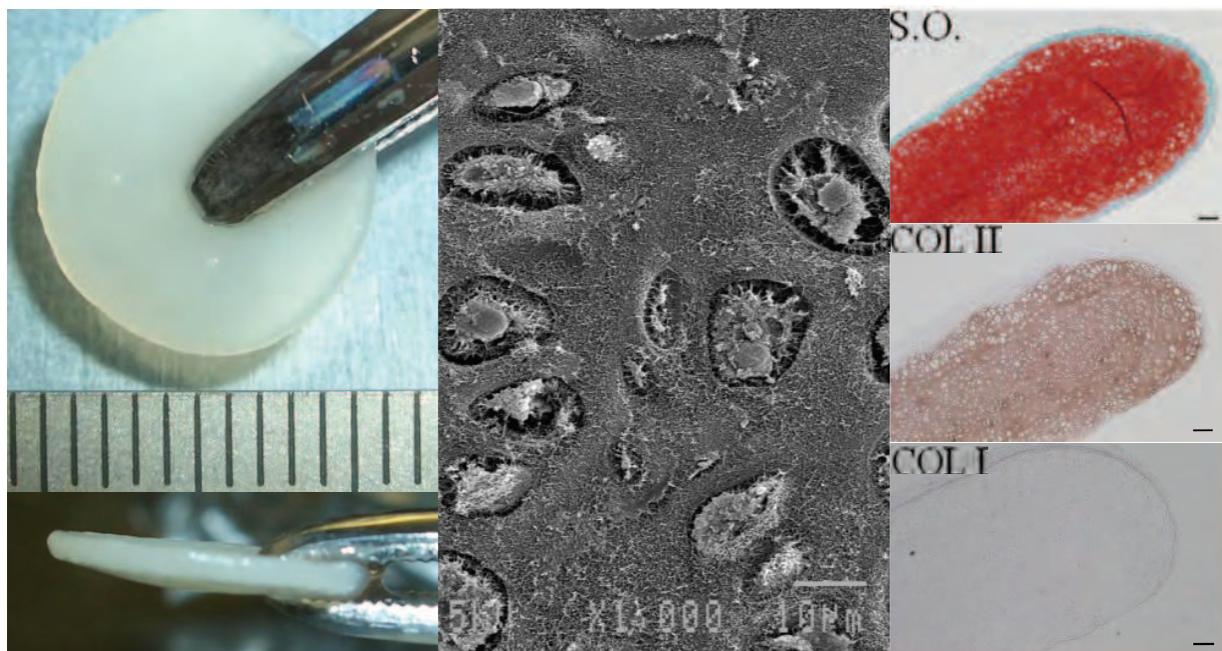


Fig. 15. Scaffold-free cartilage tissue by dedifferentiated rabbit chondrocytes cultured under dynamic rotational culture conditions. Left columns were macroscopic appearances. Center was the scanning electron microscopic appearance. Right columns show the cross-sections stained with safranin-O (S.O.), collagen type II (COL II) and collagen type I (COL I) antibodies.

As a preliminary *in vivo* implantation experiment of the scaffold-free cartilage tissue cultured under dynamic rotational culture conditions, one-week and 2-week plates were transplanted to the total thickness-defect model in patellar grooves of rabbits. At 4 weeks after implantation, fibrous tissue ad replaced most of the 1-week plates, and many inflammatory cells were seen in the subchondral bone. Alternatively, 2-week plates maintained their shape and integrated with the host cartilage (Fig. 16).



Fig. 16. Preliminary *in vivo* implantation experiments. Histological analysis with safranin O and Masson's trichrome at 4 weeks. Left picture in the 1 week chondrocyte plate insertion group and right in the 2 week chondrocyte plate insertion group (scale bars=1mm).

At 4 weeks after implantation, according to macroscopic observation, the chondrocyte plate insertion group seemed to show better results in terms of the integration of host cartilage, and the defects repaired by the plate were smoother than in the noninsertion group (Fig. 17, Nagai et al., 2008). In the chondrocyte plate insertion group (CP), the repair site appeared to

be filled with cartilaginous tissues, which were strongly stained with safranin O (Fig. 17), positive for type II collagen (Fig. 17), and negative for type I collagen (data was not shown). Lateral integration of the chondrocyte plate was well bounded at both sides of host cartilage. Basal integration of the plate was also good. Each implanted chondrocyte plate was clearly visible but was thinner than before implantation (650-700 μm thick). The interfacial adhesion between the plate and the lower portion of the repair tissue contained hypertrophic chondrocytes that were remodeling the subchondral bone. Structural integrity of the plate was shown by the beginning of columnar organization of rounded chondrocytes. No infiltration of inflammatory cells within the subchondral bone was seen. Alternatively, in the noninsertion group, the defects were filled with mainly fibrous tissue concealing the lower portion of the repair tissue. The lower portions of the regions stained with safranin O were positive for type II collagen but not for type I.

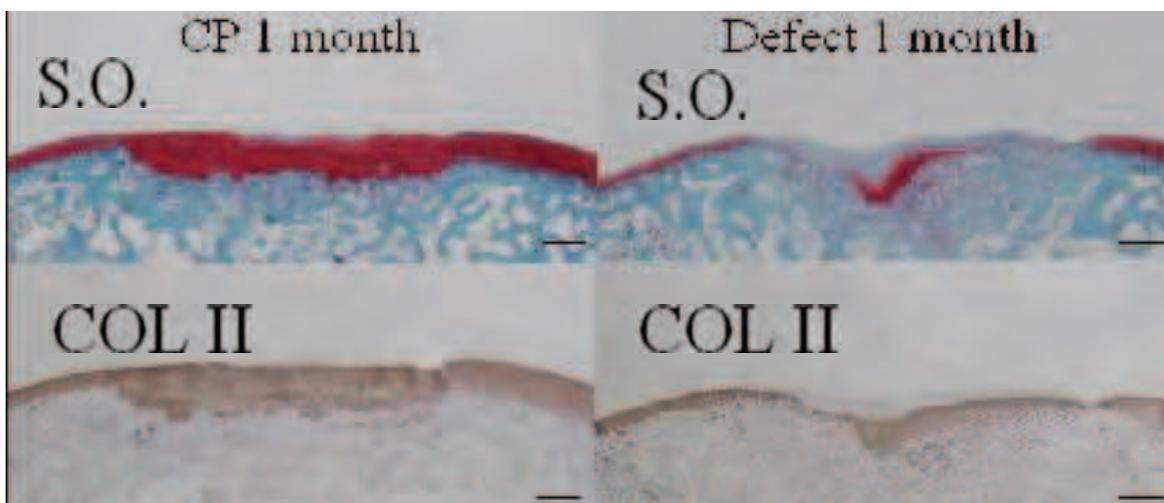


Fig. 17. Macroscopic and histological analysis of the in vivo study for safranin O and for type II collagen. Macroscopic observations on femoral chondyles at 4 weeks after surgery. In the chondrocyte plate group (CP), the defect seemed to be better regarding the integration of host cartilage than in the noninsertion control group (Defect). Scale bars=250 μm

6. Perspectives for the scaffold-free cartilage formation cultured under dynamic rotational conditions

The scaffold-free cartilage tissue by rotational culture was proven to have better mechanical, biochemical, and histochemical properties than that of scaffold-free cartilage tissue under static conditions. During a 3-week culture, cartilage tissue composed of dedifferentiated chondrocytes and extracellular matrices was formed, without use of a scaffold. Based on our analysis of tissue growth after 3 weeks, the scaffold-free cartilage tissue by rotational culture was able to assume arbitrary shapes according to the mold. If shear force is never loaded onto the cartilage tissue, the mechanical properties become extremely weak; hence, the rotational culture may be an essential factor in terms of shape control of scaffold-free cartilage tissue with an arbitrary shape. From the view point of clinical medicine, although we must obtain detailed data on differences for scaffold-free cartilage formation of factors such as cellular species, culture period, passage number, number of cells inoculated to a mold and so on, this work should suggest an evocative concept for future research.

Many papers have reported on high-cell-density cultures in which cells contact, creating cell-cell interactions, to promote differentiation from dedifferentiated chondrocytes (Koch, 2002) and/or stem cells such as mesenchymal stem cells (Denker, 1999). Moreover, it is also known that cartilage differentiation is promoted when cells condense in the stage of generating (Summerbell, 1972; Thorogood, 1975). Furthermore, it is also reported that the mechanical stresses existing under physiological conditions such as a walking (Hodge, 1986) regulate the differentiation of chondrocytes (Smith, 2000, Matsuda, 2003). Therefore, these backgrounds may support the reason why dramatic facilitatory effects in differentiation occurred by loading of shear flow (rotational culture) to scaffold-free cartilage tissue.

As a mechanism of the effect of rotational culture on the differentiation of dedifferentiated chondrocytes and the increase in the mechanical properties, involvements of shear stress, oxygen and nutrition diffusion were considered. Comparing the scaffold-free cartilage tissue cultured under static conditions with that under dynamic conditions, the static-culture cartilage tissue had surface irregularities and was not smooth; the exterior surface seemed to be open. On the other hand, a smooth tight membrane existed on the surface area of the scaffold-free cartilage tissue cultured under dynamic conditions (rotational culture). The cells in the membrane aligned horizontal to the surface. The appearance of this smooth layer was considered to have enabled accumulation of an extracellular matrix such as proteoglycan and collagen in the cartilage tissue. As a result, the method may have succeeded at tissue formation with abundant matrices like cartilage and improvement of mechanical properties. It is speculated that this smooth membrane layer appeared only in the dynamic culture and is due to the direct effect of shear flow. The reason why the membranous layer appeared only in the dynamic culture system was not clear, but it is obvious that cartilage cells can detect mechanical stresses such as shear stress, hydrostatic pressure, direct compression, etc., and that the stresses promote the production and deposition of a matrix similar to that in cartilage. There is no doubt that a low shear force exists in the rotational culture. This shear force may stimulate scaffold-free cartilage tissue to differentiate without cell damage. Therefore, it was suggested that a combination of mechanical stress and scaffold-free cartilage tissue composed solely of chondrocytes at the start point of culture may well become a suitable model for tissue-engineered cartilage.

As a cartilage tissue engineering, autologous chondrocytes transplantation was tried (Brittberg et al., 1994). But problems in this method due to loss of viability in the transplanted cells and the difficulty of fixing chondrocytes into a defect remained. To improve the cell loss, Wakitani et al. embedded allograft articular chondrocytes in collagen gel (Wakitani et al., 1989; 1996). However, it was difficult that the collagen gel keeps the shape with cells. Currently biodegradable scaffolds either synthetic or naturally derived ones, are applying to control the shapes. But the phenotype control of chondrocytes in the scaffold was not enough. On the other hand, scaffold-free cartilage tissues formed by methods using alginate gel (Matsuda, 2003; Stoddart et al., 2006a), agarose gel (Kelm et al., 2004), rotational wall vessel (Marlovits, 2003), special system (Maini-Varlet, 2001; Grogan, 2003; Wang, 2004; Park, 2006), and a suspension culture of aggregating chondrocytes (Furukawa, 2003; Tsuchiya, 2005) are reported, and the phenotype of chondrocytes in the scaffold-free cartilage was possible to control, but these methods could not freely form cartilage tissues into arbitrary shapes. Furukawa et al (2008) succeeded in the formation of cartilage tissue into arbitrary shapes using two kinds of techniques. Rotational culture realized cartilage tissue formation with arbitrary shape. It was thought

that the induction of mechanical properties by rotational culture contributed to the shape retention. In addition, the next point was based on a simple mold system. For oxygen and nutrition supply from the lower and upper sides of the cartilage tissue, a culture insertion with a highly porous film (pores 0.4 μ m in diameter) was used as a basement support at the only start point of tissue formation. Chondrocytes were not able to pass through the porous film. When a mold was set on the porous film, cartilage tissues of arbitrary shapes were easily induced. Because to adhere cells each other, a large amount of oxygen and nutrition may be needed. If we use normal culture dish or plastic rigid plate without pores at this point, all cells died and did not form tissue. In order to design complicated three-dimensional (3-D) molds for shapes such as a total knee cartilage, design of 3-D molds will be needed in the future, but this is a realizable technology.

To compare the mechanical properties of tissue engineered cartilages cultured by static and dynamic conditions, tensile tests were carried out. It was proven that the loading of mechanical stress increased the mechanical properties of the tissue by using the method. Therefore, it was understood that improvement of the mechanical properties realized formation of tissue engineered cartilage tissue with arbitrary shapes from the analysis. When the tensile testing data of scaffold-free cartilage tissue cultured under dynamic shear flow conditions to bovine native cartilage of 5 month old (Williams, 2001) were compared, the value of rupture strength was 58%, and Young's modulus was 6.4%. On the other hand, the values of scaffold-free tissue cultured under static conditions compared to the native cartilage tissue were 11, and 1.2%, respectively. These data suggested that the scaffold-free cartilages formed under dynamic and static culture conditions had inferior tensile properties to native cartilage tissue. Shear stress, oxygen and nutrition diffusion may reinforce the mechanical properties of scaffold-free cartilage by rotational culture. From the study, it was not clear which one has most prominent effect on mechanical properties of them. On the other hand, many researchers had already reported that the positive effects of mechanical stresses such as shear flow (Freed et al., 1994a, Freed et al., 1994b, Freed et al., 1994c, Vunjak-Novakovic et al., 1996), hydrostatic pressure with flow (Allemann et al., 2000, Mizuno et al., 2002), hydrostatic pressure without flow (Smith et al., 1996, Smith et al., 2000, Angle et al., 2003, Toyoda, et al., 2003, Kawanishi et al., 2007), dynamic compression (Elder et al., 2001), microgravity (Freed et al., 1997) on extracellular matrix production of chondrocytes. It was obvious that extracellular matrix production increase the mechanical properties of tissue engineered cartilage. Therefore, probably shear stresses by rotational culture also reinforce the mechanical properties of our scaffold-free cartilage tissue.

Recently, Elder et al reported an effect of hydrostatic pressures, and Stoddart et al (2006b) did an effect of of compressive force, on the formation of scaffold-free cartilage tissue from immature primary bovine chondrocytes. According to their studies, constant hydrostatic pressures of 5 and 10 MPa were more effective than oscillatory ones. The cyclical load of compressive force increased the chondrogenic protein and mRNA productions. However we can not directly compare the effects of the dynamic rotation, hydrostatic pressure and compression on matrix production of scaffold-free cartilage tissues, because the cell population was different; Elder et al and Stoddart et al. used immature chondrocytes (differentiated chondrocytes), and Furukawa et al used dedifferentiated bovine chondrocytes. The hydrostatic pressure and compressive force loading machines have complicated structures. Therefore, they have a higher risk of bacteria contaminations into their systems. In addition, the complicated structures take a great deal of time and effort to

load the mechanical stresses on tissue-engineered constructs. In contrast, in dynamic rotational culture, a simple and straightforward procedure is enough to load a mechanical stress on scaffold-free cartilage tissues. For clinical treatment of cartilage diseases, usages of a simple structured machine and protocol are important point for the success, therefore, the dynamic rotational culture will facilitate future clinical trials.

According to conventional tissue engineering, the mass balance between the disappearance (hydrolysis) of a biodegradable scaffold and the production/deposition of extracellular matrix from cells inoculated to the scaffold has been thought to be important for regulation of shape (Freed, 1997; Furukawa, 2002). The cartilage tissue which constituted of chondrocytes and ECM produced by inoculated dedifferentiated chondrocytes has arbitrary shapes, and did not need any scaffold to control the shape. In scaffold-free cartilage tissue examined by this study, although the thickness of the cartilage tissue increased linearly with time, a change in a transverse direction was not observed (constant). As a result, it turned out that the volume of the cartilage tissue also increased linearly with time. Therefore, a quantitative prediction of the tissue growth and shape change by a calculation may be possible. So, the model may point out the possibility for a new tissue design by shape control not only by using a biodegradable scaffold, but also by using a mold with an arbitrary shape and calculation of the cartilage growth.

From the view point of the clinical medicine, our scaffold-free cartilage tissue cultured by shear flow conditions was thought as a suitable model, because the scaffold-free cartilage tissue had promising biochemical, mechanical properties as a tissue-engineered cartilage tissue. In addition, it was cultured by medium without special expensive factors such as transforming growth factor and bone morphologic growth factor etc. The useless of special growth factors not only will prevent the tissue-engineered cartilage tissue from a risk of carcinogenesis, but also realize cost-cutting of medical expenses. Then, transplantation of biomaterial, a kind of foreign body, to our body was not needed in our approach. Therefore, it was thought that the barrier to the promotion of clinical trial is lower.

7. Conclusion

In conclusion, a scaffold-free cartilage tissue with arbitrary shapes and a large size with promising biological, mechanical properties was formed. The scaffold-free cartilage loaded mechanical stress based on a high porous simple mold system may become tools of not only therapies for diseases such as osteoarthritis and osteochondrosis dissecans as a tissue-engineered cartilage, but also clarification of the mechanisms of cartilage formation and therapeutic treatment instead of conventional pellet culture system.

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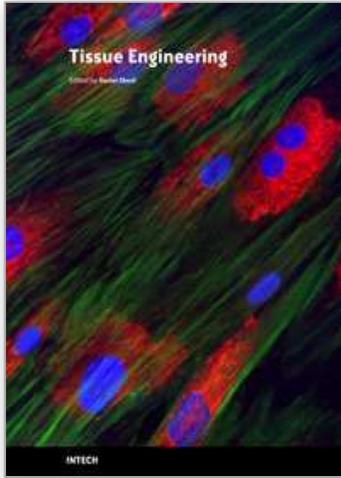
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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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www.intechopen.com

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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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