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Characteristics of a Scaffold-Free Articular Chondrocyte Plate Grown in Rotational Culture

TOSHIHIRO NAGAI, M.D.,[1] KATSUKO S. FURUKAWA, Ph.D.,[2] MASATO SATO, M.D., Ph.D.,[1] TAKASHI USHIDA, Ph.D.,[3] and JOJI MOCHIDA, M.D., Ph.D.[1]

ABSTRACT

We investigated whether articular chondrocytes could form three-dimensional tissue-engineered cartilage in a rotational culture system without a scaffold. A suspension of chondrocytes derived from Japanese white rabbits was inoculated into a mold. Eight hours later, the cell suspension in the mold showed cell aggregation, forming a chondrocyte plate. The mold was removed, and the plate was cultured under static conditions. After 7 days of primary static culture, the plate was cultured under dynamic 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. Conversely, after 3 weeks of static culture, the plate gradually changed into an arch over that time. 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. Chondron units were detected with scanning electron microscopy. In contrast, a plate cultivated in static culture for 3 weeks was irregular in shape, and histological analysis indicated irregularly accumulated glycosaminoglycans. TUNEL-positive cells had increased significantly in the central region in 3-week static cultures, compared with those in 3-week rotational cultures. In this study, cartilaginous tissue in a scaffold-free environment has been produced. Significantly rotational cultures produce a construct, which is stable enough to be handled with surgical forceps after only 2 weeks of rotational culture. This system should be useful for implantation in the future.

INTRODUCTION

ARTICULAR CARTILAGE IS AN AVASCULAR TISSUE consisting of a sparse cell population with low mitotic activity, and its capacity for self-repair is also limited.[1,2] Articular cartilage lesions are frequently associated with disability and with such symptoms as joint pain, locking phenomena, and either reduced or disturbed function. Such lesions are generally believed to progress to severe forms of osteoarthritis.[2,3] Currently, prosthetic joint replacement is the usual clinical approach to treating severe and extended degeneration of the cartilage. However, complications associated with this treatment include infection and a loosening of the components over time.[4] Consequently, many cell therapies and types of tissue-engineered cartilage have been established to repair cartilage lesions.

A pellet culture system that allows cell–cell interactions, analogous to those that occur in precartilage condensation during embryonic development, has been reported as a way to prevent and reverse the phenotypic modulations of chondrogenesis *in vitro*. Because this system forms only one aggregate, this aggregate cannot be used to produce

[1]Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Isehara, Kanagawa, Japan.
[2]Departments of Bioengineering and Mechanical Engineering, School of Engineering, University of Tokyo, Tokyo, Japan.
[3]Division of Biomedical Material and Systems, Faculty of Medicine, Center for Disease Biology and Integrative Medicine, The University of Tokyo, Tokyo, Japan.

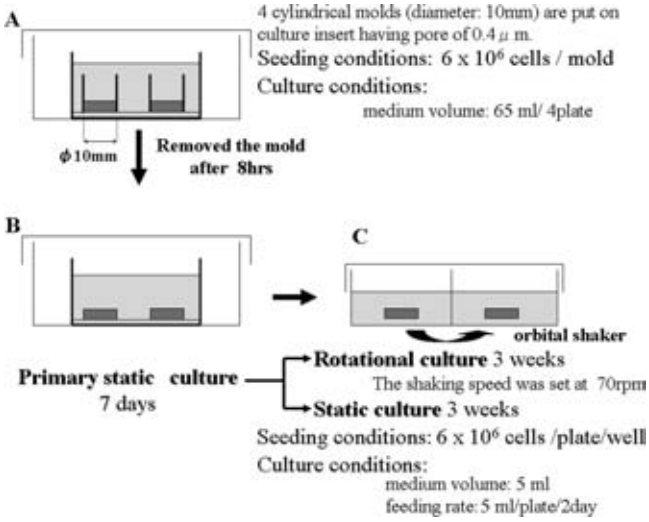

FIG. 1. (**A**) The first step, the cell suspension (chondrocytes) was inoculated in the mold. (**B**) After 8 h, the chondrocyte plate was formed and then the mold was removed. The chondrocyte plate was cultured under primary static culture for 7 days. (**C**) In the next step, the constructs were moved onto nonadherent dishes and which were then cultured under rotational culture and static culture conditions for 3 weeks.

tissue-engineered cartilage.[5] Therefore, various materials have been used as scaffolds. A scaffold provides a three-dimensional structure both for *in vitro* chondrocyte proliferation and to control the shape of the regenerated cartilage for implantation. However, the development of such scaffolds tends to be very complex, involving many biocompatibility issues.[3,6] As a result, there are still no totally satisfactory methods available for clinical use.[7]

To date, only a few scaffold-free approaches have been available for three-dimensional cultivation.[6,8–10] We have chosen a rotational culture system, using dense dissemination in a mold, in which cell aggregation is induced and a three-dimensional chondrocyte plate is eventually formed. Suitable tissue-engineered cartilage was constructed using an orbital shaker, which makes it easy to create a rotational flow in the medium, thereby inducing the appropriate shearing stress on the chondrocyte plate.

The object of the present study was to investigate whether rabbit articular chondrocytes could form tissue-engineered cartilage without a scaffold or any additional growth factors, using a rotational culture system, and to evaluate the optimum conditions for this construction.

MATERIALS AND METHODS

Source and monolayer culture of cells used for seeding

Articular cartilage slices from knee and shoulder joints were obtained from 4-week-old male Japanese white rabbits weighing about 1 kg ($n = 10$). These cartilage slices were minced and then digested in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco-Invitrogen, Carlsbad, CA) containing 0.4% (w/v) actinase E (Kaken Pharmaceutical, Tokyo, Japan) for 1 h and DMEM/F12 containing 0.016% (w/v) bacterial collagenase P (Roche Diagnostics GmbH, Mannheim, Germany) for 3 h. The digested tissue was passed through a cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) with a pore size of 70 μm. The filtered substitute was centrifuged at 1500 rpm for 5 min to separate the cells. The washed pellet was resuspended in medium consisting of DMEM/F12, 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 0.25 μg/mL fungizone (Gibco), and 50 μg/mL ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan). Chondrocytes were seeded on 500 cm^2 square dishes at 1×10^4 cells/cm^2 at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in air and 95% humidity. After approximately 1 week, primary passage (P 0) cells were detached using 0.05% trypsin/EDTA (Gibco) for 20–30 min at 37°C. The cells were centrifuged, washed three times, and then counted in a hemocytometer. The cells were resuspended in medium, and then placed into square dishes at a concentration of 1×10^4 cells/cm^2 for three passages.

Primary static culture

We harvested every chondrocyte after passages 1 to 3 in culture. Thereafter, we adjusted the cell suspension to a density of 1.0×10^7 cells/mL in DMEM/F12 supplemented with 20% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, and 50 μg/mL ascorbic acid. Four cylindrical molds (diameter, 10 mm; height, 10 mm; Iwaki, Tokyo, Japan) were then each put onto a culture insert, with a pore size of 0.4 μm (Corning Costar Japan, Tokyo, Japan). At first, 13 mL of culture medium was added under the culture insert. Subsequently, 13 mL of medium was added onto the culture insert, around the molds. The surrounding culture medium was checked to ensure that it did not enter the mold from the bottom. Next, 600 μL of the cell suspension (6×10^6 cells) was inoculated into the mold, and the cells were observed to settle 5–20 min later. Thereafter, another 40 mL (approximately) of culture medium was added to the culture insert dish until the molds were completely filled with medium (Fig. 1A). The addition of the medium after gravity-assisted cellular sedimentation prevented cell leakage from the top of the mold. The culture insert was placed in a dish measuring 150 mm in diameter (Becton Dickinson Labware), without a culture insert dish cover. Eight hours later, the cell suspension in the mold showed cell aggregation, producing a chondrocyte plate, and the mold was then removed. The chondrocyte plate was cultivated in primary static culture for 7 days until it formed a regular cylindrical shape under dynamic culture conditions (Fig. 1B). The dishes were placed in a humidified 5% CO_2 incubator at 37°C.

Rotational culture system

In the rotational culture studies, all constructs were grown in a primary static culture and were then cultured under dynamic conditions using a rotational culture system, as follows (Fig. 1C). The chondrocyte plate was moved into a nonadherent, six-well dish with a medicine spoon. Then, a rotational culture system using an orbital shaker was used. The construct was cultured at a speed of 70 rpm for 3 weeks. The culture medium was completely replaced every 2–3 days. The dishes were placed in a humidified 5% CO_2 incubator at 37°C.

Static culture

To investigate the differences between rotational culture and static culture, we also generated a chondrocyte plate under static conditions, which were maintained for the whole culture period. In the static culture studies, all constructs were generated in primary static cultures and were then cultured under static culture conditions, as follows (Fig. 1C). The chondrocyte plate was moved to a nonadherent dish and was then cultured under static culture conditions for 3 weeks. The culture medium was completely replaced every 2–3 days. The dishes were placed in a humidified 5% CO_2 incubator at 37°C.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

The samples were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin, and 4-μm-thick sections were prepared. Histological staining was performed with safranin O to visualize glycosaminoglycans. The sections were deparaffinized for immunohistochemistry using standard procedures. The sections were treated with 0.005% proteinase (type XXIV; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C before incubation with primary antibodies. After the slides were washed with phosphate-buffered saline (PBS), the endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15–20 min at room temperature. The sections were rinsed with PBS and then incubated for 30 min at room temperature with normal goat serum, diluted 1:20 in PBS. The primary mouse monoclonal antibody directed against human collagen types I and II (Daiichi Fine Chemical, Toyama, Japan) was diluted 1:200 in PBS–1% bovine serum albumin (BSA; Sigma). The slides were incubated overnight at 4°C, washed 10 times with PBS, and then incubated at room temperature in a humidified chamber for 1 h with biotin-conjugated goat anti-mouse secondary antibody diluted 1:100 in PBS–1% BSA. The slides were then treated with horseradish peroxidase (HRP)–labeled streptavidin (streptavidin–HRP) for 1 h. Finally, they were soaked for 2–4 min in a 0.05% solution of diaminobenzidine (DAB) in Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide, until the color developed. At the end of the immunohistochemical procedures, the slides were counterstained with Mayer's hematoxylin to improve the visualization of the cells.

Scanning electron microscopy

The samples were fixed in 2.5% glutaraldehyde (TAAB, Berkshire, UK)/0.1 mol/L phosphate buffer (pH 7.2) at 4°C for 6 h and postfixed in 1% osmic acid/0.1 mol/L phosphate buffer at 4°C for 2 h. They were then dehydrated in a graded ethanol series (50%, 70%, 80%, 90%, and 100%). The samples were immersed in *t*-butyl alcohol (Wako) and frozen at −20°C. The samples were then freeze-dried by the Inoue and Osatake method,[11] then spatter coated with gold (JFC-1100E; JEOL, Tokyo, Japan) and observed by scanning electron microscopy (SEM; JSM-840; JEOL).

Apoptosis

The DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) was used to detect apoptotic cells in paraffin-embedded samples on the basis of DNA strand breaks. The manufacturer's protocol was followed to deparaffinize the samples using standard procedures. The slides were washed with PBS, fixed in 4% PFA solution at room temperature for 15 min, washed with PBS again, and treated with proteinase K (20 μg/mL in PBS) for 30 min at room temperature. The slides were then washed with PBS and fixed in 4% PFA at room temperature for 5 min, before being washed again with PBS. Biotinylated nucleotides were incorporated at the 3′-OH at the DNA ends using a recombinant terminal deoxynucleotidyl transferase (rTdT) reaction mix enzyme. The slides were treated with the rTdT reaction mix enzyme at room temperature for 1 h in a humidified chamber. After the slides were washed with PBS, the endogenous peroxidase activity on the slides was blocked with 0.3% hydrogen peroxide in PBS for 3–5 min at room temperature. After they were again washed with PBS, the slides were treated with streptavidin–HRP for 30 min and then soaked in 0.05% DAB containing 0.005% hydrogen peroxide for 2 min. At the end of this procedure, the slides were counterstained with Mayer's hematoxylin.

The number of TUNEL-positive cells was assessed by imaging under a light microscope. The spatial distribution of TUNEL-positive cells within constructs cultivated for 3 weeks was assessed with image analysis of TUNEL-stained cross sections. Areas measuring 0.04 mm^2 were randomly chosen and classified as either central areas or peripheral areas, depending on whether the area was more or less than 0.1 mm from the surface of the construct, respectively. The number of apoptotic cells in each area was determined by counting the TUNEL-stained nuclei. The average cell density per square millimeter in at least four different constructs was calculated from 10 central or peripheral areas.

Analysis of gene expression

The chondrocyte plate was stored in liquid nitrogen before analysis of collagen types I and II, aggrecan, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene expression. The frozen constructs were pulverized with a Cryo-Press (Microtec Nition, Chiba, Japan) in liquid nitrogen before the addition of TRIzol reagent (Invitrogen). Total RNA was isolated with TRIzol reagent and the manufacturer's suggested protocol. It was then further purified using DNaseI (TaKaRa Biotechnology, Shiga City, Japan) and RNase inhibitor (Toyobo, Osaka, Japan). Absorbance at 260 and 280 nm was measured for RNA quantification and quality control. RNA samples were then reverse transcribed to cDNA using oligodT primers and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA), followed by the specific amplification of matrix-specific genes. The upstream and downstream primer sequences, respectively, for each set of primers were as follows: GAPDH, 5′-TCACCATCTTCCAGGAGCGA-3′ and 5′-CACAATGCCGAAGTGGTCGT-3′; type II collagen-α1 chain, 5′-AACACTGCCAACGTCCAGAT-3′ and 5′-CTGCAGCACGGTATAGGTGA-3′; aggrecan, 5′-GTGGGCGGTGAGGAGGACATCAC-3′ and 5′-GGGCCGGGTGGCCTCTTCAGTC-3′; type I collagen, 5′-TTGGTGCTGCAGGAGCAC-3′ and 5′-ACGGTTTCCATGTTTGCCAG-3′.

Polymerase chain reaction (PCR) amplification was performed using a two-step protocol that included a 10-min pre-PCR heating step at 95°C to activate Ampli*Taq* Gold DNA polymerase (Applied Biosystems), followed by 40 cycles of denaturing at 95°C for 15 s and annealing at 60°C for 1 min. The PCR products were separated electrophoretically using nondenaturing 1.2% Tris-borate/EDTA pH 8.3 (TBE) polyacrylamide gels, and stained with ethidium bromide. The gels were scanned under UV light with the Densitograph system (Atto Biotechnologies, Tokyo, Japan).

Statistical analyses

The numbers of TUNEL-positive cells assessed histomorphometrically in the constructs grown in either rotational or static cultures were compared using Student's *t* test.

RESULTS

Macroscopic appearance and properties

Eight hours after the inoculation of chondrocytes in the mold, the cells formed a three-dimensional chondrocyte plate, without becoming scattered. After primary static culture for 7 days, the chondrocyte plate could be collected in a spoon while maintaining the form of a circle. It was then moved into the rotational culture system (Fig. 2A). When the chondrocyte plate was pinched with tweezers, it could not maintain its shape (Fig. 2B). However, after rotational culture for 3 days, it did not lose its regular cylindrical shape, even when pinched with tweezers (Fig. 2C). After 2 weeks of rotational culture, the chondrocyte plate was considered to be sufficiently stable to be handled with surgical pincers. The chondrocyte plate measured about 1.1 mm in thickness after 2 weeks and about 1.4 mm after 3 weeks' cultivation

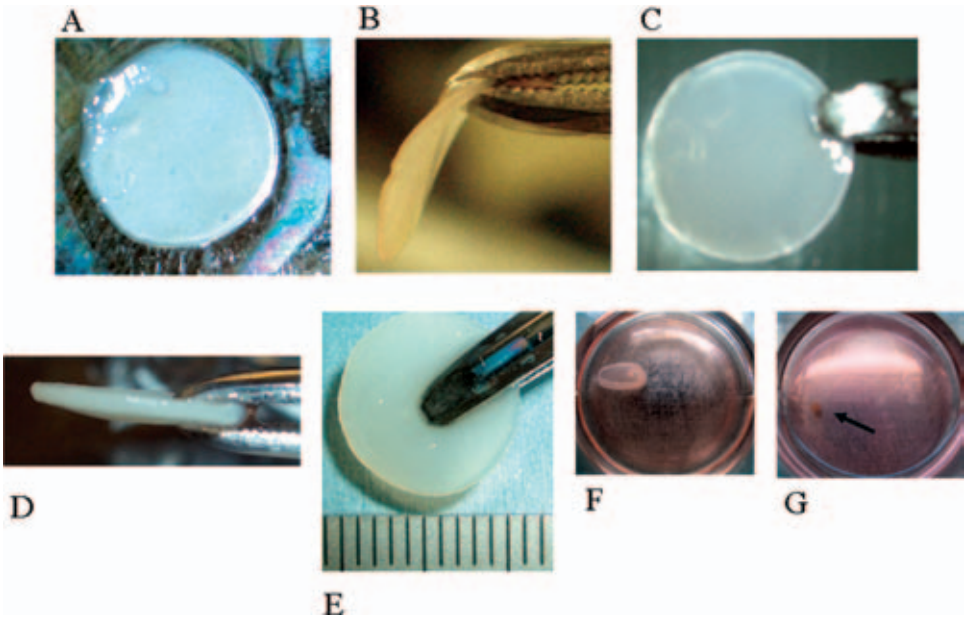

FIG. 2. Macroscopic appearance of the chondrocyte plate that formed after 7 days of primary static culture (**A, B**). Subsequently, a chondrocyte plate formed after 3 days (**C**) and 3 weeks (**D, E**) of rotational culture. The 1-mm scale is shown below. The macroscopic appearance of chondrocyte plate that formed after rotational culture if primary static culture was less than 4 days (**F, G**). It is shown with an arrow. Color images available online at www.liebertpub.com/ten.

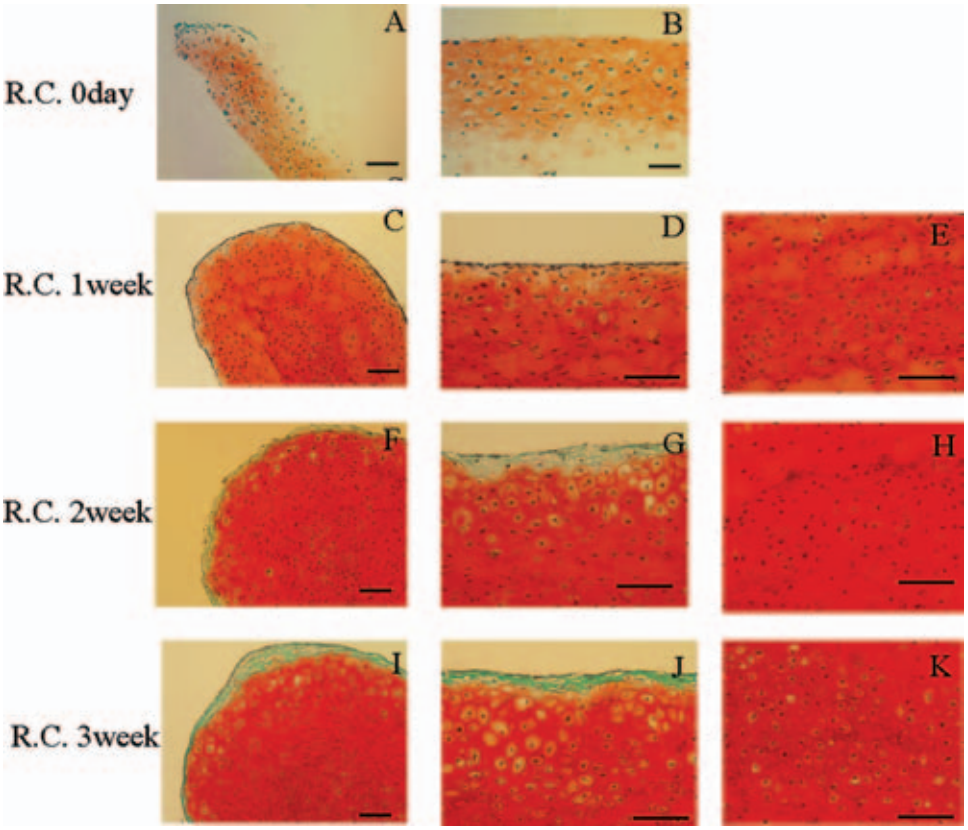

FIG. 3. Safranin O–stained paraffin sections of a chondrocyte plate that formed after 7 days of primary static culture (**A, B**). Subsequently, other sections of the chondrocyte plate formed after 1 week (**C–E**), 2 weeks (**F–H**), and 3 weeks (**I–K**) of rotational culture. (**A, C, F, I**) The construct reveals a dense matrix in the central regions with fibroblastic-like cell layers in the peripheral regions. (**B, D, G, J**) The peripheral region that was not stained in safranin O forms elongated fibroblastic-like cells. (**E, H, K**) The central region demonstrates a dense matrix containing spherical-shaped chondrocytes. Scale bar: 100 μm. Color images available online at www.liebertpub.com/ten.

(Fig. 2D), with a regular cylindrical shape and the macroscopic appearance of cartilage (Fig. 2E). The chondrocyte plate was made without loss of its regular cylindrical shape, regardless of the number of cellular passages (two or three passages). However, the shape of the chondrocyte plate did change, undergoing deformity or contraction after rotational culture, if the primary static culture had been maintained for less than 4 days (Fig. 2F, G). On the other hand, after 3 weeks of static culture, the plate gradually changed into an arch or stretched over that time.

Histology

Histological staining revealed a high cell density and substantial matrix accumulation. Safranin O staining showed that the matrix mainly consisted of glycosaminoglycans. After primary static culture for 7 days, the chondrocyte plate showed an extracellular matrix stained uniformly with safranin O. Significantly, the chondrocytes were distributed uniformly in the plate (Fig. 3A, B). After 1 week of rotational culture, the chondrocyte plate was intensely stained with safranin O, and thus contained an abundant extracellular matrix in the center of the construct (Fig. 3C, E). The shape of the chondrocytes in the chondrocyte plate was spherical, and differentiation was maintained. However, after 1 week of cultivation, the construct grown in rotational culture had only one layer of different cells at the surface. The chondrocytes of the peripheral region had lost their spherical shape and had become more elongated, similar to fibroblast-like cells (Fig. 3D). This layer also lacked any specific staining for proteoglycans. During the 3 weeks of rotational culture, the central region of the chondrocyte plate became thicker and was more strongly stained with safranin O in all cross sections (Fig. 3F, H, I, and K). The peripheral region, which did not stain with safranin O, increased progressively. However, after rotational culture for 2–3 weeks, the peripheral region had only 2–3 cell layers (Fig. 3G, J).

The chondrocyte plates constructed from chondrocytes harvested after two or three passages were strongly stained with safranin O in the central regions after rotational culture for 3 weeks (Fig. 4A, C, D, and F). At the same time, the peripheral region showed only 2–3 cell layers, which were not stained with safranin O (Fig. 4B, E).

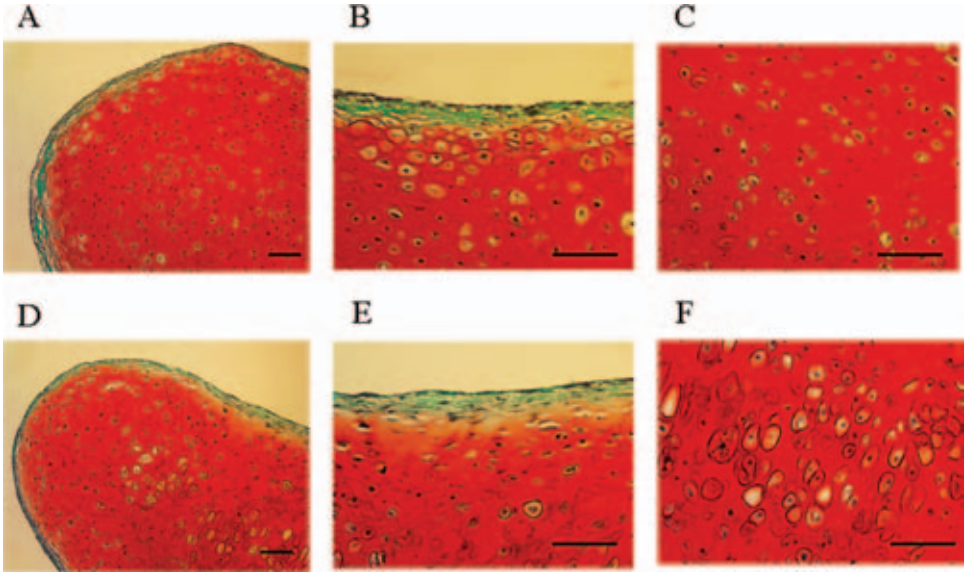

FIG. 4. Safranin O–stained paraffin sections of the chondrocyte plate formed by chondrocytes, which were harvested after 2 (**A–C**) or 3 passages (**D–F**) after 3 weeks of rotational culture. Scale bar: 100 μm. Color images available online at www.liebertpub.com/ten.

Immunohistochemistry

Immunohistochemical evaluation indicated that, after primary static culture for 7 days, type II collagen was expressed in the chondrocyte plate, whereas type I collagen was not (Fig. 5A, B). Type II collagen was produced uniformly around the cells.

After rotational culture for 1–3 weeks, type II collagen was strongly expressed in the central region of the chondrocyte plates, in a region similar to the safranin O–stained area (Fig. 5C, E, and G). The area in which type II collagen was expressed could be clearly distinguished from the peripheral region. Type I collagen was not expressed in the central region. In contrast to the peripheral region, type I collagen was expressed in the most superficial couple of cell layers, which neither stained with safranin O nor expressed type II collagen (Fig. 5D, F, and H).

Histological and immunohistochemical comparison of the chondrocyte plates in static culture

The chondrocyte plates were affected by the cultivation conditions, as shown in Figure 6A and B. The constructs in static culture were thin and irregular in shape (Fig. 6A), and glycosaminoglycan accumulated irregularly in the chondrocyte plate (Fig. 6C, E). The peripheral region of the constructs in static culture was not smooth, but opened in pores (Fig. 6D). Moreover, the central region of the chondrocyte plate in static culture was irregularly positive for type II collagen (Fig. 6F, G), but was not positive for type I collagen (Fig. 6H). The constructs grown in rotational culture contained more glycosaminoglycan than those grown in static culture. The constructs grown in rotational culture were also thick and contained a continuous cartilaginous extracellular matrix over their cross sections, and smooth peripheral region formed a capsule around the central region (Fig. 6B).

TUNEL labeling and cell counting

TUNEL-positive cells were observed in the static culture after 3 weeks, mainly in the central region of the chondrocyte plate (Fig. 7B, E). Conversely, only a few of these cells were seen in the chondrocyte plates after 3 weeks of rotational culture (Fig. 7A, D). A significantly larger number of TUNEL-positive cells, present in both the central and peripheral areas, were observed in samples grown in static culture than in those grown in rotational culture (Fig. 7C, F).

Scanning electron microscopy

When a cell suspension was inoculated in a mold, cell aggregation was observed 8 h later by SEM (Fig. 8A). After 7 days in a primary static culture, a chondrocyte plate formed with a 1 or 2 μm extracellular matrix of the scaffold with a structure similar to that of honeycomb (Fig. 8B). After 3 weeks of rotational culture, SEM revealed the formation of chondron units, consistent with the native chondrocyte arrangement (Fig. 8C, D). The chondron units were also observed in the constructs after 3 weeks of static culture (Fig. 8E).

PCR analysis of mRNA expression

RT-PCR analysis of mRNA expression demonstrated increasing expression of type II collagen and aggrecan after 2 weeks of rotational culture. However, the levels of type II

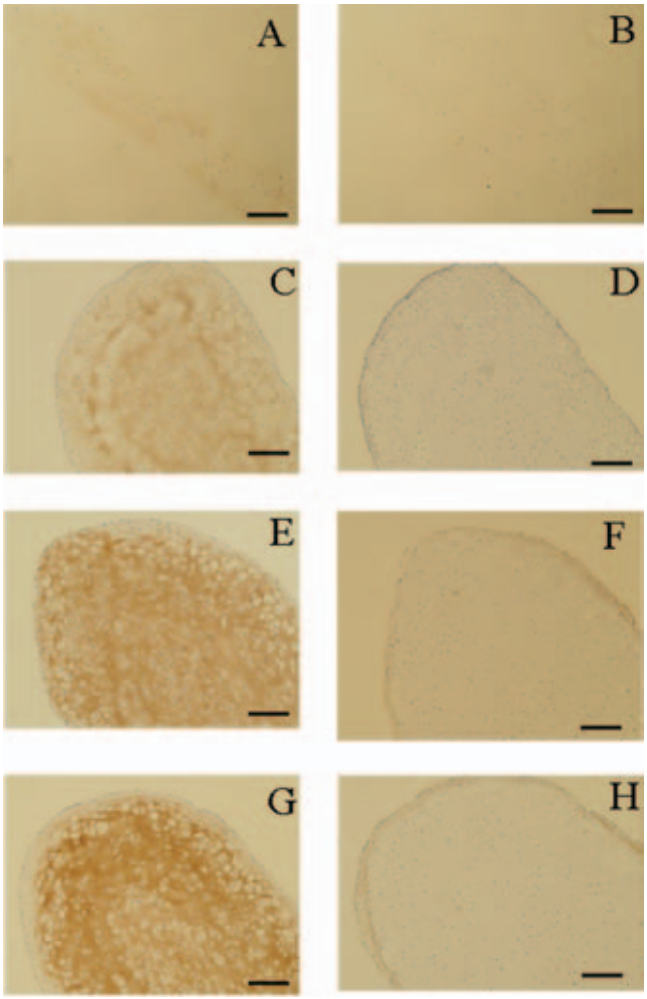

FIG. 5. Immunohistochemistry of paraffin sections of a chondrocyte plate that formed after 7 days of primary static culture (**A, B**). Subsequent sections of the chondrocyte plate formed after 1 week (**C, D**), 2 weeks (**E, F**), and 3 weeks (**G, H**) of rotational culture. (**A, C, E, G**) The central regions of the constructs were stained with type II collagen antibody but not by type I collagen antibody. (**B, D, F, H**) The peripheral regions of the constructs were stained with type I collagen antibody but not by type II collagen antibody. Scale bar: 100 μm. Color images available online at www.liebertpub.com/ten.

collagen and aggrecan mRNAs appeared to decline after 2 weeks of rotational culture. In contrast, the expression of type I collagen was observed to decrease after 3 weeks of rotational culture (Fig. 9). On the other hand, the expression of type I and II collagen mRNA was expressed in the plate under 3 weeks of static culture (data not shown).

DISCUSSION

In this study, we constructed three-dimensional tissue-engineered cartilage in a scaffold-free manner from rabbit articular chondrocytes using a rotational culture system without any additional growth factors. The constructs contained abundant proteoglycans and type II collagen. After 2 weeks of rotational culture, the constructs were stable enough to be handled with surgical forceps. Further, the constructs did not lose their cylindrical shape during rotational culture for 3 weeks.

In monolayer culture, cells are forced to grow in one place under space-limiting conditions. This is consequently an obviously artificial growth environment compared with development *in vivo*. Monolayer cultures of chondrocytes have the disadvantage of producing matrices that differ from those produced *in vivo*.[12] Moreover, chondrocytes previously maintained in monolayers have been reported to revert to a chondrocyte phenotype when cultured in agarose gel, synthesizing matrix-associated products. Therefore, various artificial materials have been used as scaffolds to maintain a chondrogenic phenotype and to support three-dimensional structures. However, the production of such scaffolds is a complex procedure involving many biocompatibility issues.[3]

To solve these problems, a few studies have attempted to construct tissue-engineered cartilage without a scaffold. Mainil-Varlet *et al.*[6] constructed scaffold-free tissue-engineered cartilage using a static bioreactor system. Marlvoits *et al.*[8] also constructed scaffold-free tissue-engineered cartilage that maintained its phenotype using a rotational wall vessel in an environment of microgravity. However, the disadvantage of their methods is that large numbers of cells ($2–3\times10^7$ cells) were required for their studies. The macroscopic and histological appearance of the cartilaginous tissue surface produced in a static, closed, and scaffold-free bioreactor system is irregular, like the cartilaginous tissue surface that we constructed in 3 weeks of static culture. The cartilage engineered in a scaffold-free environment has a polygonal rounded morphology resulting from the use of the rotational wall vessel, which is a dynamic culture. In other words, one of the disadvantages of this system is that it is impossible to control the shape of the engineered cartilage.

In this study, we were able to make tissue-engineered cartilage as a chondrocyte plate with a constant shape, using a rotational culture system, which is a dynamic culture. Further, the peripheral region of the tissue-engineered cartilage that we constructed in this rotational culture system was smooth. The chondrocytes of the peripheral region lost their rounded shape and were more elongated and orientated parallel to the surface, as is the *in vivo* cartilage surface. We were also able to produce a chondrocyte plate with a comparatively small number of cells (6×10^6 cells).

In a preliminary experiment, the form of the chondrocyte plate was deformed or contracted, when the plate was cultured in a rotational culture system without sufficient primary static culture (Fig. 2F, G). It is thought that cell–cell interactions are strong, which is one of the major causes of the contraction of these chondrocyte plates.

It has been suggested that it is important that the extracellular matrix is constructed after a sufficient period of

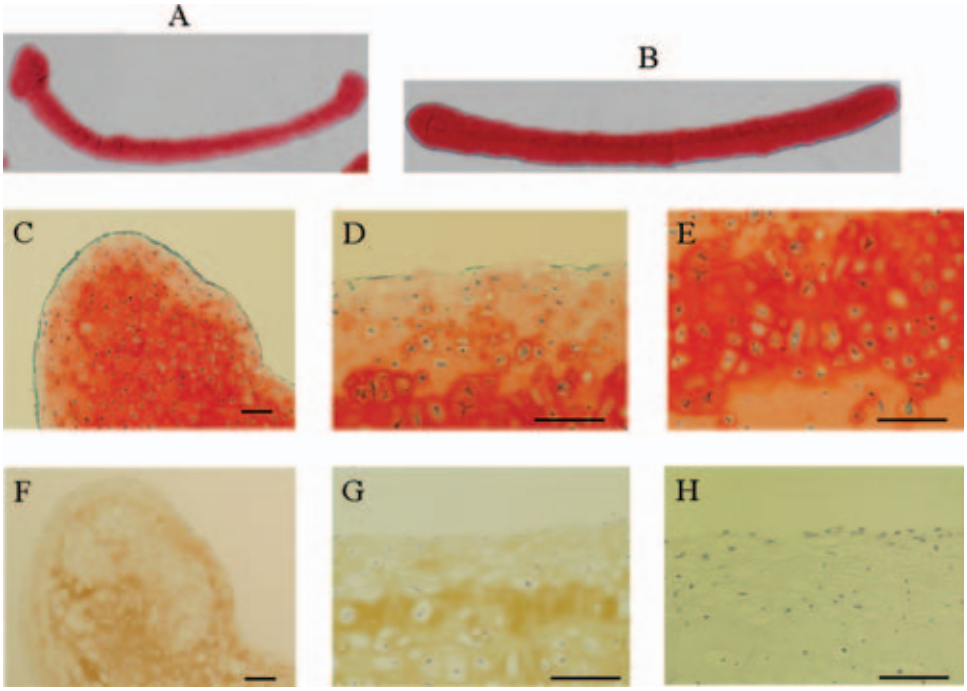

FIG. 6. Safranin O–stained paraffin sections of total images of the chondrocyte plate formed after 3 weeks of static culture (**A**) and 3 weeks of rotational culture (**B**). Safranin O–stained paraffin sections of the chondrocyte plate formed after 3 weeks of static culture. The construct reveals the accumulation of an irregular matrix in the central regions with one fibroblastic-like cell layer in the peripheral regions (**C–E**). Immunohistochemistry of paraffin sections of a chondrocyte plate that formed after 3 weeks of static culture (**F–H**). The construct was irregularly positive for type II collagen (**F, G**), but was not positive for type I collagen (**H**). Scale bar: 100 μm. Color images available online at www.liebertpub.com/ten.

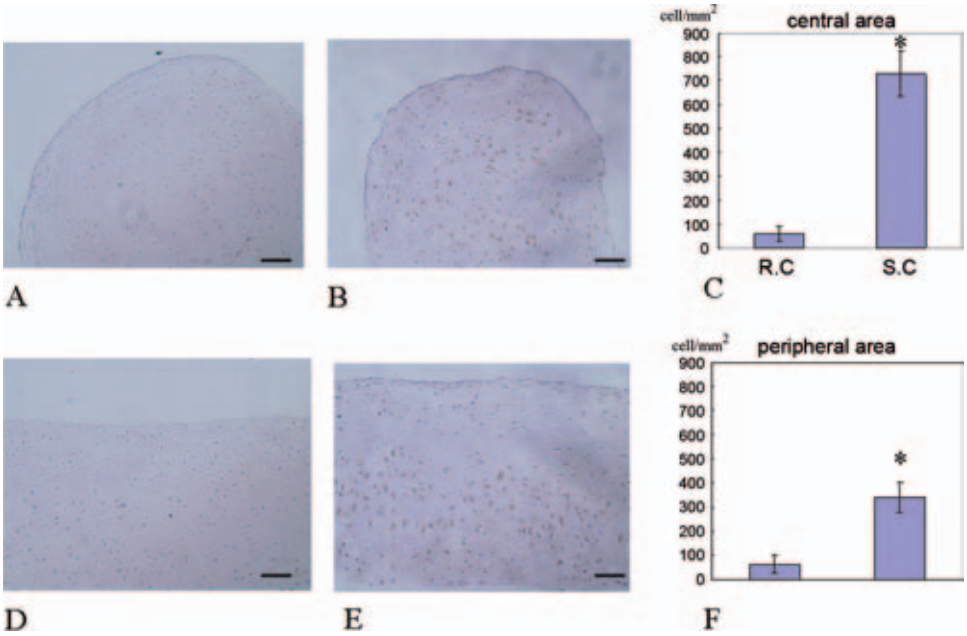

FIG. 7. TUNEL-positive cells were mainly observed in the central region of the chondrocyte plates after 3 weeks of static culture (**B, E**); on the other hand, they were almost absent in the plates after 3 weeks of rotational culture (**A, D**). Scale bar: 100 μm. A significant difference was seen between the number of TUNEL-positive cells located in the central area in comparison to those located in the peripheral area ($*p < 0.001$) (**C, F**). Color images available online at www.liebertpub.com/ten.

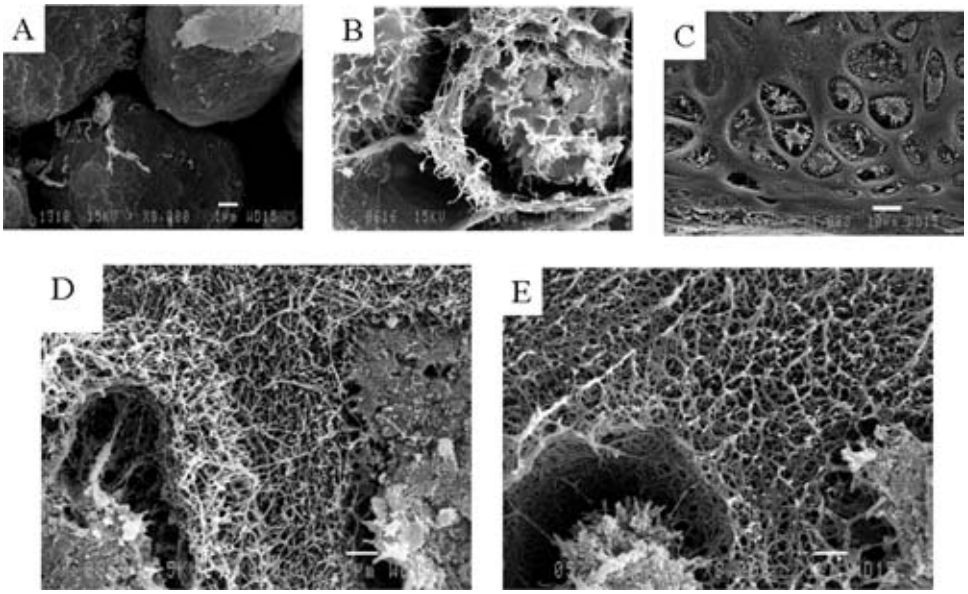

FIG. 8. The scanning electron microscopic appearance of the chondrocytes plate that formed after 8 h of inoculation in the mold (**A**). Subsequently, the chondrocyte plate formed after 7 days of primary static culture (**B**). Sequentially, the appearance of the chondron units in the chondrocyte plate formed after 3 weeks of rotational culture (**C, D**). Similarly, the chondron units were appeared in the 3 weeks of static culture (**E**). Scale bars: (**A, B, D, E**), 1 μm; (**C**), 10 μm.

primary static culture, so that the chondrocyte plate will have a constant shape in the rotational culture system. After primary static culture, the chondrocyte plate formed an extracellular matrix that was similar to an artificial scaffold, and that also had a honeycomb form. The chondrocytes were also distributed uniformly in the plate.

Gravity caused the cell suspension to settle, producing cell condensation in the mold. The process of cell aggregation is significant in chondrogenesis. The earliest morphological events associated with limb skeleton differentiation are the reduction of the intercellular space and the consequent increase in cell condensation and cell-to-cell contact in the areas of prospective cartilage formation.[13] Moreover, a high-density culture of chondrocytes, which promotes cell-to-cell contact, has been significantly associated with extracellular matrix biosynthesis.[14,15] In the first step, 8 h later, the cell suspension in the mold showed three-dimensional cell aggregation, forming a chondrocyte plate without any scattering. In the second step, after primary static culture for 7 days, the chondrocytes in the chondrocyte plate secreted specific extracellular matrices, which stained with safranin O and showed abundant amounts of type II collagen.

However, a high-density culture may limit the amount of mass transfer. Therefore, numerous bioreactors have been developed for the high-density, three-dimensional *in vitro* culture of chondrocytes.[16] These systems provide oxygen and allow nutrient diffusion, as well as an adequate mechanical load. However, undesirable side effects have been reported in bioreactors associated with high-shear systems. The constructs are grown in spinner flasks, which constitute high-shear systems, and form fibrous outer capsules made of multiple cell layers within 1 week of tissue cultivation.[17]

In the chondrocyte plate constructed with the rotational culture system, the peripheral region that was not stained with safranin O had only 2–3 layers after 3 weeks of cultivation. Although no hydromechanical analysis was conducted in this study, the authors speculate that a rotational culture system is not a high-shear bioreactor.

In addition to rotational flow at a speed of 70 rpm, negligible chondrocytic cytotoxicity was detectable in the peripheral region with TUNEL staining. Few TUNEL-positive

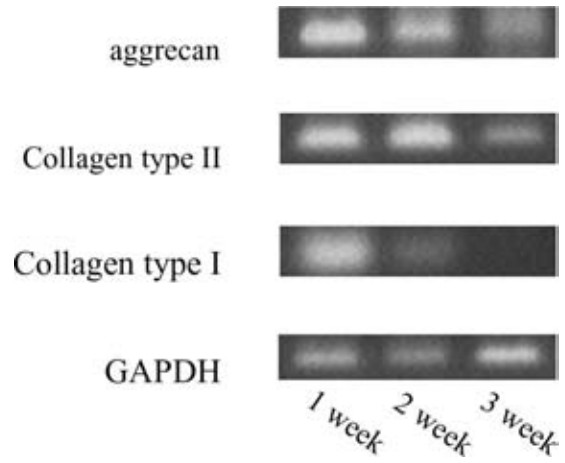

FIG. 9. RT-PCR showing the expression of aggrecan, type II collagen, and type I collagen genes at 1, 2, and 3 weeks after rotational culture.

cells were found in the plate after rotational culture for 3 weeks, although TUNEL-positive cells did appear in the static culture during the same period. The chondrocyte plate grown in static culture for 3 weeks had a thin and irregular shape macroscopically, and a histologically irregular accumulation of extracellular matrix and apoptotic cells apparent on TUNEL staining. These findings suggest that the advantages of a rotational culture system include oxygen and nutrient diffusion and an adequate dynamic load.

Actually, no major apparent differences were observed by SEM or RT-PCR in the chondrocyte plate after 3 weeks in rotational culture or static culture. Basically, the chondrocyte plate was a three-dimensional aggregate. Therefore, the chondrocyte plates in static culture prevent and reverse the phenotypic modulations of chondrogenesis, as in the pellet culture system.

In success of the transplantation, we consider that the importance of tissue-engineering cartilage lies not only in the quantity of extracellular matrix that can be produced, but also in maintaining the distribution of the cells and the extracellular matrix. Moreover, as a matter of course, the central region of the graft must maintain cartilage differentiation. The distribution of the peripheral region facing in the articular surface is particularly important, because emission of aggrecan and cellular damage are anticipated if the peripheral region carrying a direct load is not a smooth and tight surface.

In this study, the peripheral region of the chondrocyte plate in static culture was not smooth and opened in pores. On the other hand, the peripheral region of the plate in rotational culture was smooth and tight. Therefore, in the distribution of cells and extracellular matrix, it was also considered that the rotational culture system is advantageous in comparison with the static culture.

The levels of type II collagen and aggrecan mRNAs appeared to decline after 2 weeks of rotational culture. However, histological evaluation with safranin O staining and type II collagen immunohistochemical staining showed that proteoglycans and type II collagen increase after 2 weeks of rotational culture. Adherent cells exposed to external forces tend to flatten out and thus induce stress-protection mechanisms in order to remain firmly attached to the substrate. Flat chondrocytes proliferate faster than spherical chondrocytes.[18] One possibility is that in the fibroblast-like cells of the peripheral region, proliferation may be faster than in the spherical cells of the central region. The peripheral region also forms a capsule around the central region.

Therefore, the mRNAs of genes associated with the synthesis of the cartilage matrix decreased after 2 weeks of rotational culture, not only because of the limitations in mass transfer, resulting from the presence of the self-generated extracellular matrix, but also because the peripheral region forms a capsule to restrain the growth of the central region. In this study, the chondrocyte plate showed only a slight increase in thickness after 3 weeks of cultivation (data not shown).

In a clinical context, the cell number obtained by biopsy is limited, and there are also some disadvantages in performing cell proliferation in a monolayer culture. A rotational culture system can create scaffold-free artificial tissue with relatively few cells compared with other scaffold-free systems. In this study, we have shown that it is possible to make a chondrocyte plate with a cartilaginous phenotype from dedifferentiated chondrocytes.

The major disadvantage of the rotational culture system is that the extracellular matrix that is constructed in the primary static culture relies on its endogenous cellular capacity to synthesize extracellular matrix. We intend to demonstrate, in the future, that a greater endogenous capacity can be achieved with a period of primary static culture.

A successful approach to the generation of tissue-engineered cartilage must provide for the survival of the newly constructed tissues and their integration into the adjacent host tissues after implantation.[14] Until now, *in vitro* studies have shown an improved integration of native and engineered cartilage using constructs at an early stage of chondrogenesis.[19] Conversely, the acquisition of a proper level of rigidity is considered to be essential to support transplanted artificial cartilage during physiological loading.[20] As mentioned above, after only 2–3 weeks of rotational culture, the chondrocyte plate was stable enough to be handled with surgical forceps and the plate did not lose its cylindrical shape. Histologically, the central region of the chondrocyte plate consisted of an abundant extracellular matrix, whereas the peripheral region, exposed to moderate shearing force, displayed a smooth cell alignment and an extracellular matrix with a superficial zone, similar to that observed *in vivo*. These findings suggest that the chondrocyte plate is able to withstand physiological loading *in vivo*.

It is also important to create a constant form, which will be advantageous for the implantation of the chondrocyte plate in the future. We estimated when the optimum conditions for implantation can be determined from the results of quantitative analyses with biochemical, gene expression, and biomechanical assays. To evaluate the repair of articular cartilage, chondrocyte plates are now allografted into full-thickness defects in the knee joints of rabbits.

In conclusion, the rotational culture system described above is a very simple bioreactor. Our artificial cartilage consists of immature cartilaginous constructs that are viable, and maintain a constant form and a proper level of rigidity.

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Address reprint requests to:
Masato Sato, M.D., Ph.D.
143 Shimokasuya
Isehara, Kanagawa
259-1193 Japan

E-mail: sato-m@is.icc.u-tokai.ac.jp

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