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Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage

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Abstract

Some treatments for full thickness defects of articular cartilage, such as cultured chondrocyte transplantation, have already been done. However, to overcome osteoarthritis, we must further study the partial thickness defect of articular cartilage. It is much more difficult to repair a partial thickness defect because few repairing cells can address such injured sites. We herein show that bioengineered layered chondrocyte sheets using temperature-responsive culture dishes may be a potentially useful treatment for partial thickness defects. We evaluated the property of these sheets using real-time PCR and histological findings, and allografted these sheets to evaluate the effect of treatment using a rabbit partial model. In conclusion, layered chondrocyte sheets were able to maintain the cartilageous phenotype, and could be attached to the sites of cartilage damage which acted as a barrier to prevent a loss of proteoglycan from these sites and to protect them from catabolic factors in the joint.

Keywords: Temperature-responsive culture dish; Cell sheet; Tissue engineering; Chondrocyte; Partial thickness defect; Articular cartilage

Articular cartilage is an avascular tissue that is nourished by the synovial fluid. Adult articular cartilage shows a poor self-repair after degeneration or injury occurs and it is therefore unlikely to be restored to normal once it has been damaged. Currently available treatments for cartilage defects include application of a periosteal patch to cover the defect [1] and mosaicplasty, in which an osteochondral pillar is grafted from a non-weight-bearing site [2]. However, the use of periosteal patches has limitations due to problems with ossification and the limited area that can be treated. Although the microfracture technique, in which drilling is employed to induce bone marrow cells to differentiate into chondrocytes, is widely used, the cartilage obtained by this technique is fibrocartilage with different characteristics to those of hyaline cartilage. Since promising results for the transplantation of cultured autologous chondrocytes have been reported [3], various articular cartilage regeneration techniques have been applied clinically, including the use of scaffolds such as atelocollagen [4] and cell transplantation therapy with bone marrow-derived mesenchymal stem cells [5]. However, the current cartilage regeneration techniques are intended for treating full thickness defects, and there have so far been no reports on the clinical application of a technique for partial thickness defects in patients with early osteoarthritis as far as we could determine by a literature search. Defects in articular cartilage are classified as either full or partial thickness defects, according to whether or not they penetrate the marrow spaces of subchondral bone. Partial thickness defects are analogous to the clefts and fissures that are seen in the early stages of osteoarthritis in humans; these fibrillated lesions grow larger and deeper during the course of

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the disease but never repair themselves spontaneously. It has also been suggested that partial thickness defects do not heal because they are walled off from marrow and thus have no access to the macrophages, endotherial cells, and mesenchymal cells that reside therein [6].

Articular cartilage is composed of scattered chondrocytes embedded in an abundant extracellular matrix (ECM). The matrix is mainly composed of type II collagen and proteoglycans, and is responsible for specific joint functions, including smooth movement and shock absorption. When cultured chondrocytes are employed in vitro, it is important to harvest cells without damaging the ECM. However, the cultured cells are damaged and the ECM is disrupted by current methods because proteolytic enzymes are used when harvesting cultured cells. To achieve the repair and regeneration of partial thickness articular cartilage defects, cultured chondrocytes can be harvested without ECM damage by using the temperature-responsive culture dish developed by Okano et al. [7].

Temperature-responsive culture dishes have already been applied to research in various fields of regenerative medicine, including the regeneration of the myocardium [8–11], vascular epithelium [12], cornea [13,14], hepatocytes [15], and renal cells [16]. This method has also been clinically applied for the corneal reconstruction [14]. The surface of a temperature-responsive culture dish is grafted with a polymer (poly-*N*-isopropylacrylamide) that becomes either hydrophilic or hydrophobic in a reversible manner, depending on the temperature [17]. The polymer has a low critical solution temperature of 32 °C, below which it becomes soluble in water. Based on this characteristic, the temperature-responsive culture dish has a weakly hydrophilic surface like that of commercially available dishes and it can be used to culture cells in a conventional manner when the temperature is 37 °C or higher. However, the surface of the dish becomes hydrophobic when the temperature falls below the critical solution temperature of 32 °C. Therefore, confluent sheets of cultured cells can be spontaneously released from the hydrophobic dish surface by reducing the temperature to below 32 °C. Using this method, cultured cells can thus be collected as a sheet without damaging cell–cell junctions and the ECM because it eliminates the need for conventional enzymatic harvesting with trypsin [11]. Such cell sheets have been reported to have various advantages, including the preservation of the early phenotype and the expression of adhesion proteins on the base [7]. Furthermore, these cell sheets can be placed on each other to prepare a layered ''tissue'' because ECM is preserved on the base, and such three-dimensional manufactured tissue has already been used for transplantation [8].

In this study, human chondrocyte sheets with ECM were obtained using the temperature-responsive culture dish method and then were combined in layers. Next, the ''tissue'' was compared with that of a single sheet and the adhesion of the sheets was examined both in vivo and ex vivo. We therefore herein demonstrated the first step toward bioengineering cartilageous tissues for the treatment of partial thickness cartilage defects using cell sheet technology.

Materials and methods

All procedures using animals in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) published by the National Institutes of Health, USA, and the Guidelines of Tokai University on Animal Use.

Temperature-responsive culture dishes. Specific procedures for the preparation of temperature-responsive culture dishes (provided by CellSeed, Tokyo, Japan) have all been previously described [17]. Briefly, *N*-isopropylacrylamide (IPAAm) monomer solution was spread onto commercial tissue culture polystyrene dishes. These dishes were then subjected to electron beam irradiation, thus resulting in polymerization and covalent bonding of IPAAm to the dish surface. Poly-IPAAm (PIPAAm)-grafted dishes were rinsed with cold distilled water to remove ungrafted IPAAm. The culture dishes were finally sterilized by ethylene oxide gas [8].

Human articular chondrocytes. The cells used for the in vitro experiment included human articular chondrocytes derived from patients who had undergone anterior cruciate ligament reconstruction and gave their informed consent at the Tokai University Oiso hospital from December 2004 to August 2005. Chondrocytes were obtained while forming the interfoveolar ligament and then they were isolated by enzymatic treatment. Twenty-five knees of 25 patients aged 14–49 years old (Average 23 y/o, 19 males and 6 females) were used as the source of these cells. Next, the chondrocytes were subjected to enzymatic treatment, and then were seeded, and cultured according to the method of Sato et al. [18].

Articular chondrocytes from Japanese white rabbits. Twelve Japanese white rabbits aged 3–4 weeks old and weighing about 800–1000 g were used as the source of articular cartilage cells. Cartilage samples were collected from the femoral compartment of the knee joint, and were subjected to the same enzymatic treatment process as that used for human articular cartilage cells. Thereafter, the isolated cells were seeded and cultured in temperature-responsive culture dishes.

Cell proliferations in temperature-responsive surface. Chondrocytes were digested for a hour in Dulbecco's modified Eagle's medium/F12 (D-MEM/F12; Gibco, NY, USA) containing 0.4% Pronase E (Kakenseiyaku Inc.) and subsequently for 4 h in DMEM/F12 containing 0.016% Collagenase P (Roche, Mannheim, Germany). The digested tissue was passed through a cell strainer (BD Falcon™) with a pore size of 100 μm. The cells were then seeded at high density (10,000 cells/cm^2) into temperature-responsive surface dishes (diameter: 35 mm provided by CellSeed, Tokyo, Japan) and cultured in DMEM/F12 supplemented with 20% fetal bovine serum (FBS; GIBCO, NY, USA), 50 μg/ml ascorbic acid (Wakojunyakukougyou Corp., Japan), and 1% antibiotics–antimycotic (GIBCO, NY, USA) at 37 °C in an atmosphere of 5% CO_2 and 95% air for a week. Human articular cartilage cells were also seeded into commercially available culture dishes (diameter: 35 mm, Iwaki, Japan) at a concentration of 1000 cells/cm^2 and cultured under the same conditions.

Staining with PKH-26. To evaluate the chondrocyte morphology and the status of chondrocyte sheet, isolated chondrocytes were labeled with PKH-26. The lipophilic dye PKH-26 is a nonradioactive substance with no known cellular toxicity, having a fluorescent half-life of >100 days in erythrocytes (according to the manufacturer's package insert) [19]. The fluorescence of PKH-26 is not transferred to other cells but does transfer to daughter cells. Labeling was performed according to the manufacturer's instructions, i.e., reacting 2×10^7 cells with PKH-26 in F12/DMEM without serum at 25 °C for 3 min.

Harvesting of cell sheets. Each culture dish was removed from the incubator when the cells reached confluence and then was let stand at about 25 °C for 30 min. After the culture medium was removed, the cell sheet was harvested using a polyvinylidene difluoride (PVDF) membrane

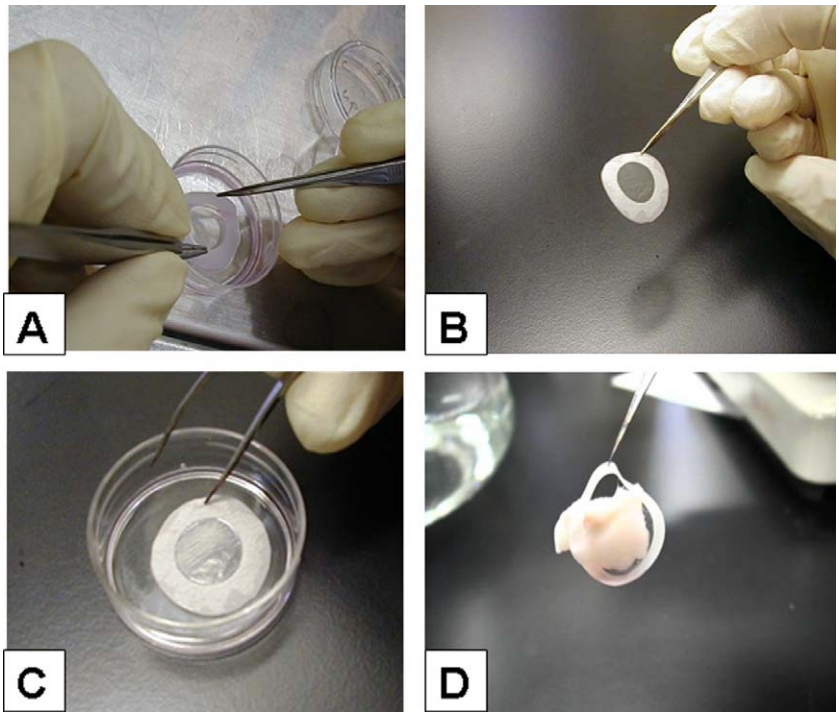

Fig. 1. Harvest of the cell sheets. (A) Cultured chondrocytes by using a temperature-responsive surface could be released from the dish surface only by reducing the temperature without proteolytic enzyme. (B) Confluent cultured chondrocytes were harvested as a single contiguous cell sheet retaining cell–cell junction as well as deposited ECM on the basal side. (C) The chondrocyte sheets could be layered and thereafter adhere to other cell sheets. The culture of the three-layered chondrocyte sheets could be continued for 1 week. (D) After the chondrocyte sheets and porcine cartilage tissue were organ-cultured together for 1 week, they demonstrated a good adhesive ability when the chondrocyte sheets were lifted and not separated by any mild external force.

according to the method reported by Yamato et al. [20]. In brief, the PVDF membrane was placed on the cell sheet and then the sheet was rolled up with the membrane from one corner (Fig. 1A). Cultured human chondrocytes could be successfully harvested as a single contiguous cell sheet by this method (Fig. 1B). Then each cell sheet was placed on top of another confluent cell sheet to prepare multilayered sheets. Since the multilayered sheets floated in the culture medium, a 0.4-μm cell culture insert (Falcon, USA) was placed on top to prevent this and then the culture of the sheets was continued for 1 week.

Three-layer sheets of cartilage cells from Japanese White rabbits were also prepared. A 0.4-μm filter was also used to compress these sheets onto the culture dish and incubation was done for 3 weeks to prepare the multilayered sheets for transplantation.

RNA isolation and cDNA synthesis. Total RNA extraction was carried out using the RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. RNA quality from each sample was assured by the A260/280 absorbance ratio and by electrophoresis of 1.2% agarose formaldehyde gel. 1.0–2.0 μg of total RNA was reverse transcribed into single strand cDNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The RT reaction was carried out for 60 min at 42 °C and then for 5 min at 95 °C in a thermocycler.

Primer design and real time PCR. All oligonucleotide primer sets were designed based upon the published mRNA sequence. The expected amplicon lengths ranged from 70 to 200 bp. Oligonucleotide primers used in this study are listed in Table 1. The real time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA) by using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Two to 2.5 μl of cDNA template was used for real time PCR in a final volume of 25 μl. cDNA was amplified according to the following condition: 95 °C for 15 s and 60 °C for 60 s from 35 to 45 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A melting curve analysis was performed (0.5 °C/s increase from 55 to 95 °C with continuous fluorescence readings) at the end of cycles to ensure that single PCR products were obtained. The amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. All reactions were repeated in 6 separate PCR runs using RNA isolated from 4 sets of human samples. The results were evaluated using the SmartCycler II software program. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize samples. To monitor crossover contaminations of PCR, RNase-free water (Qiagen Inc., Valencia, CA) was included in the RNA extraction and it was used as a negative control. To ensure quality of data, a negative control was always applied in each run.

Histological examination. A cartilage tissue sample measuring about 3×3-cm was collected from the femoral condyle of an adult pig. The surface of the sample was polished with coarse sandpaper to a depth of about 1 mm to prepare a partial-thickness cartilage defect model. Thereafter, a three-layer human articular chondrocyte sheet was placed over the damaged site (with the basal surface downwards). To prevent the sheet from floating in the culture medium, filter paper immersed in medium was placed over the cell sheet until its initial fixation was confirmed. Another cartilage tissue sample with a partial-thickness defect model without cell sheets was cultured as a control. After 1 week, the samples were cut into sections and stained with safranin-O to assess the effect of the cell sheet.

Transplantation of chondrocyte sheets. The articular cartilage of the medial femoral condyle of Japanese White rabbits weighing about 3000 g was removed to a depth of less than 1 mm using a file to prepare a model of partial thickness cartilage damage. The damaged cartilage was covered with a three-layered chondrocyte sheet, which was stabilized with a nylon suture until the initial fixation was achieved. This was done in four knees of two rabbits as the transplantation group. At the same time, the articular cartilage of the medial femoral condyle was similarly filed, but not covered with a cell sheet, in four knees of two rabbits (the control group). The cartilage was harvested after 4 weeks, fixed in 4% PFA for 1 week, and decalcified with K-CX Decalcifying Solution (Fujisawa Pharmaceutical,

Table 1
List of primers used in real time PCR

Primer ID	Accession No.	Sequence	Expect size (bp)
MMP3-F MMP3-R	NM_002422	ATT CCA TGG AGC CAG GCT TTC CAT TTG GGT CAA ACT CCA ACT GTG	138
MMP13-F MMP13-R	NM_002427	TCA CGA TGG CAT TGC TGA CA AGG GCC CAT CAA ATG GGT AGA	77
TIMP1-F TIMP1-R	NM_003254	CAG CGT TAT GAG ATC AAG ATG GAC CA AGT GAT GTG CAA GAG TCC ATC CTG	186
ADAMTS5-F ADAMTS5-R	NM_007038	GAG CCA AGG GCA CTG GCT ACT A CGT CAC AGC CAG TTC TCA CACA	120
Collagen Type I-F Collagen Type I-R	NM_000088	AAG GGT GAG ACA GGC GAA CAA TTG CCA GGA GAA CCA GCA AGA	170
Collagen Type II-F Collagen Type II-R	NM_033150	GGA CTT TTC TTC CCT CTC T GAC CCG AAG GGT CTT ACA GGA	113
Aggrecan1-F Aggrecan1-R	NM_001135	TCG AGG ACA GCG AGG CC TCG AGG GTG TAG GCG TGT AGAGA	94
Fibronectin1-F Fibronectin1-R	NM_001030524	GCA CAG GGG AAG AAA AGG AG TTG AGT GGA TGG GAG GAG AG	189
GAPDH-F GAPGH-R	NM_002046	GCA CCG TCA AGG CTG AGA AC ATG GTG GTG AAG ACG CCA GT	142

Japan) for 1 week. Next, the specimens were embedded in paraffin, cut into sections, and stained with safranin-O and toluidine blue for evaluation.

Statistical analysis. The Dunn test was used as a post hoc test using the one-way analysis of variance as a statistical analysis of the results using real time PCR.

Results

Multilayered cell sheets

The chondrocyte sheets could be easily prepared by placing one sheet on another and then culturing them together (Fig. 1C). It was possible to continue the culture of the three-layered sheets. Three-layer human articular chondrocyte sheets cultured for 1 week were extendable and were not damaged by any mild external force (Fig. 1D).

Three-layer rabbit articular chondrocyte sheets cultured for 3 weeks maintained their original shape without shrinking when PVDF was removed. As a result, it was thus possible to create round grafts.

Staining with PKH-26

Chondrocytes stained with PKH-26 showed a relatively round shape. No residual cells could be detected at sites from which the cell sheets were collected (Fig. 2A). During the continuous culture, the layered areas showed marked

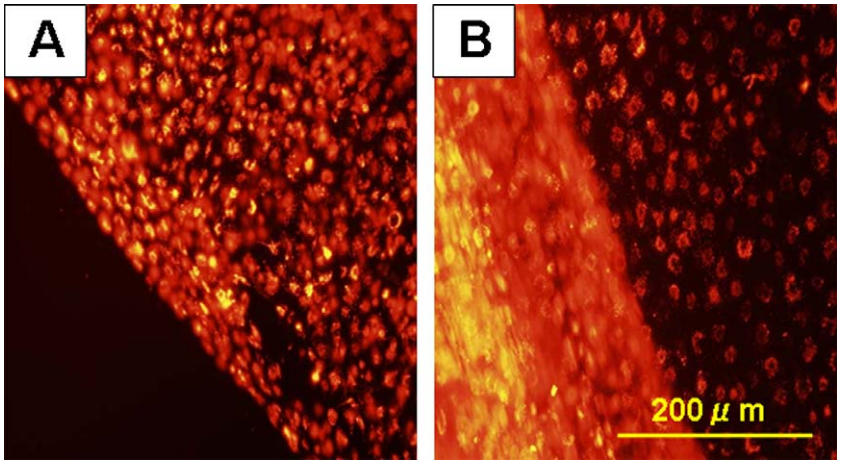

Fig. 2. Staining with PKH26. The cultured chondrocytes stained with PKH26 were observed with a fluorescence microscope. (A) No fluorescence remained at all in the dish after harvesting, thus, indicating that the chondrocyte sheet was completely harvested as continuous tissue, while the chondrocytes maintained their spherical shape, and they did not clearly change into fibroblast-like cells. (B) The fluorescence level at the layered cell sheets was remarkably higher than that at the monolayer cell sheet because the fluorescence level depends on the accumulation of cells in the culture dish.

accumulation of cells in comparison to the single-layered areas (Fig. 2B).

Analysis of gene expression

The mRNA expressions of FN1, COL2, AGC1, and TIMP1 were observed at significantly high levels, while those of COL1, MMP3, and ADAMTS5 were significantly seen at low levels in the layered chondrocyte sheets in comparison to the monolayer culture (Fig. 3).

Ex vivo histological examination

After the chondrocyte sheets and porcine cartilage tissue were organ-cultured together for 1 week, they remained well attached to each other when lifted and thus were not separated by any mild external force (Fig. 1D). A histological examination after safranin-O staining confirmed that the cell sheet adhered directly to the porcine cartilage partial damage model without a scaffold (Fig. 4A). The area not covered by the cell sheet was poorly stained with safra-

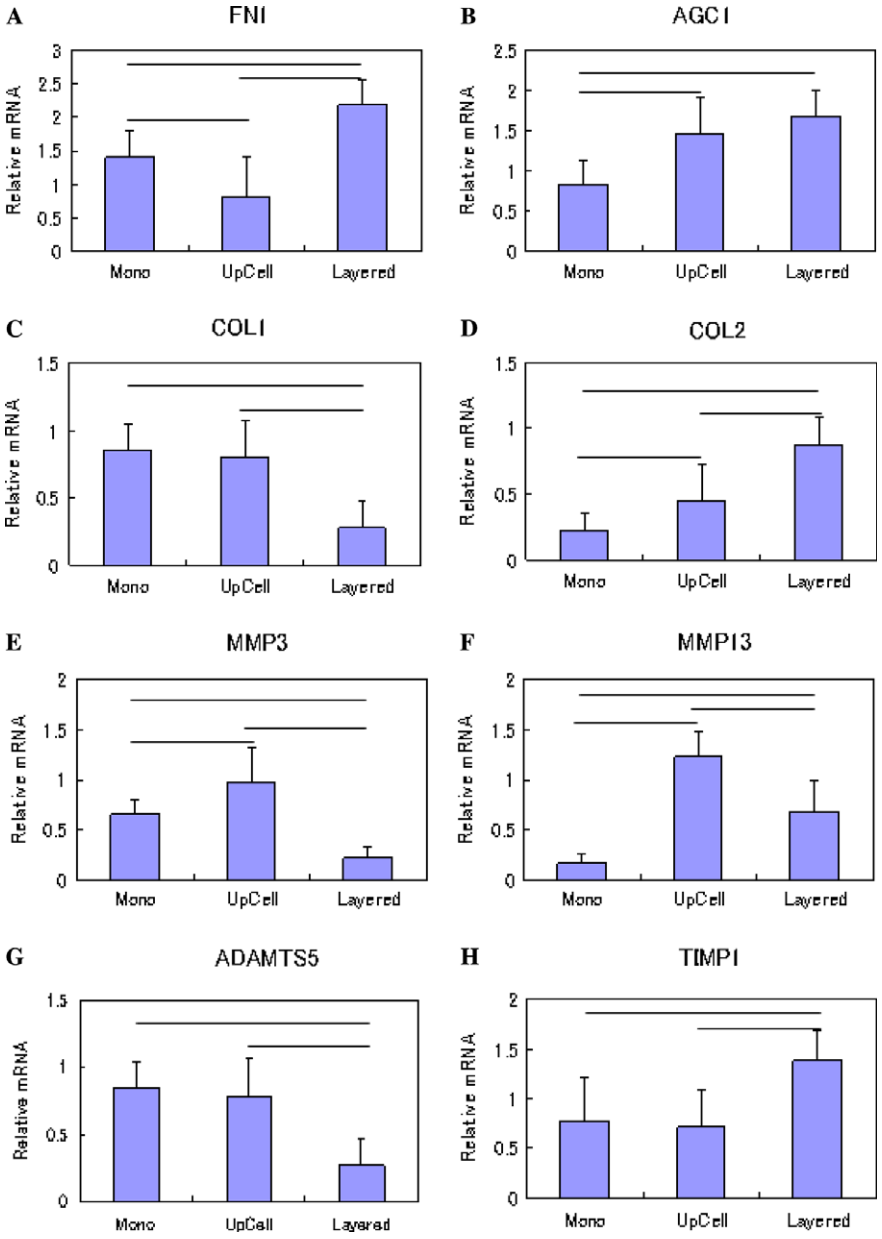

Fig. 3. Relative expression of mRNA. The mRNA expression of fibronectin one of three-layered chondrocyte sheets was higher than monolayer culture (A). The mRNA expressions of aggrecan and type II collagen of chondrocyte sheets were higher than that of monolayer culture (B,D). The mRNA expression of type I collagen demonstrated a low level in the three-layered chondrocyte sheets (C). The expressions of MMP3 and ADAMTS5, which promote cartilage degeneration, were low in the three-layered chondrocyte sheets (E,G) while the mRNA expression of TIMP1, which is antagonistic factor of MMP3, showed a significantly high level in the three-layered chondrocyte sheets (E,H). Mono: conventional monolayer culture, UpCell: monolayer culture using temperature-responsive culture dish, Layered: layered chondrocyte sheets (—, $P < 0.05$).

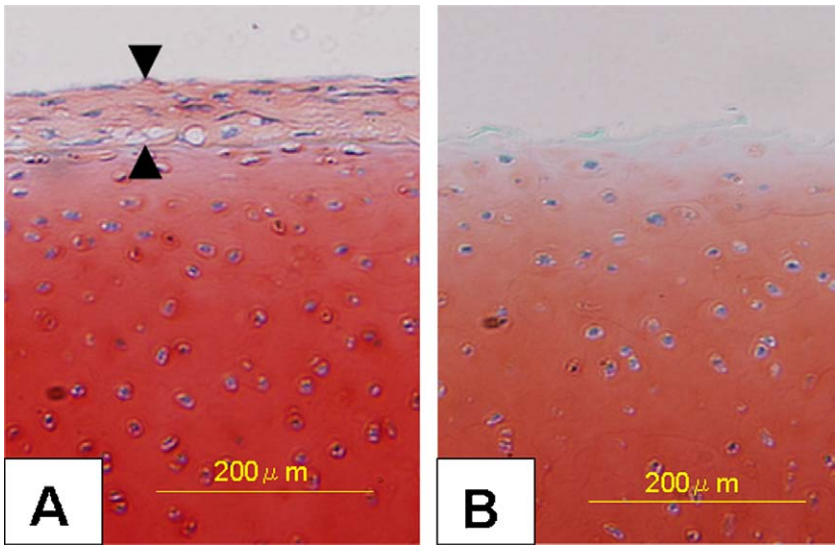

Fig. 4. A histological analysis of an ex vivo study. (A) By organ culture, chondrocyte sheets could adhere well to the partial-thickness defect model of porcine articular cartilage. The partial-thickness defect model with culturing chondrocyte sheets on it maintained a good stainability with safranin-O staining. The arrows demonstrate the layered chondrocyte sheets on the partial-thickness model. (B) The partial-thickness defect model without covering the chondrocyte sheet on it showed a poor stainability with safranin-O staining.

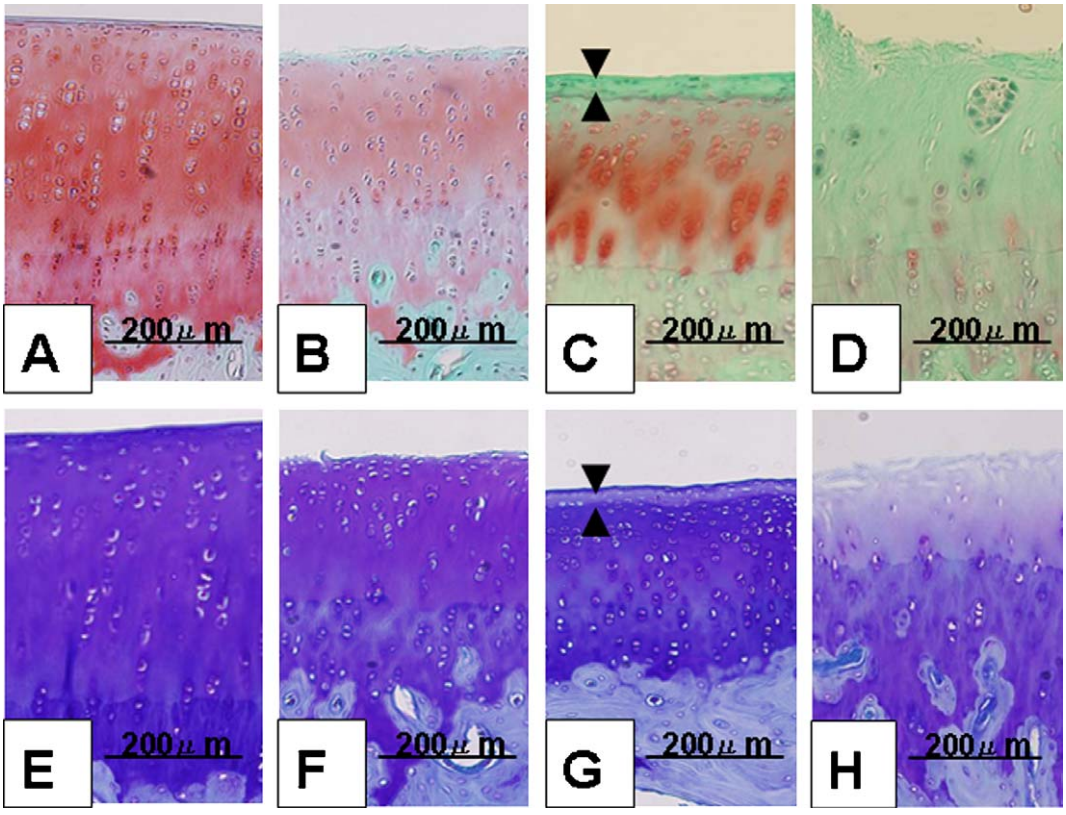

Fig. 5. A histological analysis of an in vivo study [(A–D) Safranin-O staining, (E–H) Toluidine blue staining]. The normal articular cartilage of the Japanese white rabbit femoral chondrocyte is shown in (A,E). The partial-thickness defect model is shown in (B,F). The partial-thickness defect models which covered the three-layered chondrocyte sheets (C,G) showed a better stainability than those not covered by the chondrocyte sheets (D,H). The partial-thickness defect models themselves showed progressive cartilage degeneration with fibrillation (D,H). The arrows demonstrate the layered chondrocyte sheets (C,G).

nin-O in comparison to that covered by the cell sheet in the porcine cartilage partial-thickness defect model (Fig. 4B).

Histological findings of the allografted chondrocyte sheet and injured sites

The three-layered cell sheet remained well attached to tissue sections at 4 weeks after transplantation. The area covered by the sheet was better stained than that not covered with it, as also observed in the above-mentioned ex vivo experiment (Fig. 5C, D, G, and H). In the cartilage partial damage model, the area not covered with the multilayered sheets showed progressive cartilage degeneration with fibrillation and poor staining of the matrix at 4 weeks (Fig. 5D and H). In contrast, the area covered with the three-layered sheets showed only relatively mild degeneration and a well-stained matrix (Fig. 5C and G).

Discussion

It has been reported that cell sheets cultured in temperature-responsive culture dishes and harvested by using the temperature recovery system preserve the ECM and adhesive factors on the base and thus readily adhere to other cell sheets [7]. The present study confirmed that chondrocytes could be harvested as sheets and thus be made into multilayered "tissue" by culturing in temperature-responsive dishes and then be collected using a temperature recovery system.

The importance of the treatments and prophylaxes for osteoarthritis is increasing due to the progressively aging society. However, we only have a few conservative therapies at this time, such as NSAID administration and the injection of hyaluronic acid. Namely, there is still no means to prevent future exacerbations of cartilage degeneration.

Based on the results of this study, we suggest that the use of bioengineered chondrocyte sheets may thus be potentially useful in the treatment of partial thickness defects of articular cartilage. The advantages of such cell sheets are that they are easy to culture and proliferate, and most importantly, they have a good adhesion and barrier function. This means that they can protect against intra-articular catabolic factors while also preventing proteoglycan escape from the injured site. They have a promising growth factor supply. Furthermore, such cell sheets could be useful as an alternative to periosteum itself. Although focal gene delivery using a cell sheet was not mentioned in this paper, it may also have a good clinical potential for such treatment as well. We think that the development of therapeutic apparatus less invasively delivering the cell sheet to injured site may therefore be fundamental to expand its use for the treatment of patients demonstrating the early stages of osteoarthritis.

Shimizu et al. [10] reported that three-dimensional layers of myocardial tissue conducted electrical stimuli and maintained the phenotype of cardiac muscle. Articular chondrocytes are known to dedifferentiate into fibroblast-like cells when cultured as a single layer. Real-time PCR showed that three-layered chondrocyte sheets had an increased expression of type II collagen and aggrecan, as well as a reduced expression of type I collagen (Fig. 3B–D). This indicates that multilayered chondrocyte sheets maintained a normal phenotype because these sheets provided a three-dimensional structure that formed an environment close to that of three-dimensional culture. The chondrocytes stained with PKH-26 showed a relatively round shape (Fig. 2). Although articular chondrocytes cultured in temperature-responsive culture dishes showed a delayed adhesion and were slow to reach confluence despite a high density compared with cells cultured in commercially available culture dishes, the surface coating of the new dishes was useful for maintaining the phenotype of cultured chondrocytes. Nishida et al. [13,14] reported that corneal cell sheets cultured in temperature-responsive dishes could strongly adhere to the cornea without either scaffolding or suturing. Kushida et al. [16] reported that the fibronectin expression was preserved on the basal side of the cell sheets cultured in temperature-responsive culture dishes. In the present study, layered chondrocyte sheets were shown to adhere to porcine cartilage after a 1-day organ culture (Fig. 4). It is possible that an increase of fibronectin in the multilayered chondrocyte sheets may have been involved (Fig. 3A). Good adhesion could thus be obtained because harvesting without enzymatic treatment makes it possible to better preserve the expression of both fibronectin and adhesion proteins such as integrin. We think matrix–matrix interaction therefore plays an important role in the adhesion between chondrocyte sheet and injured cartilage, and speculate that some types of enzymes may modify the surface of each matrix and thereafter integrin families and fibronectin exert their actions. We are now studying this adhesive phenomenon using a cDNA microarray.

Another interesting aspect about these chondrocyte sheets is the fact that catabolic factors, such as MMP3 [21–24], MMP13 [23–26], and ADAMTS5 [27,28], are observed to decrease at the time of layering, while the expression of TIMP1 with antagonistic actions against MMP3 is increasing (Fig. 3E–H). Namely, the layered chondrocyte sheets, which are going to be transplanted, have few destructive factors that degenerate cartilage, and they also have good adhesion properties that help to both protect and repair the cartilage surface.

Although an experimental study for the allograft of layered chondrocyte sheet was established up to 2 months, we also confirmed the good adhesion and the inhibiting effect of cartilage degeneration at the injured sites. The sites where the cell sheets showed adhesion were well stained with safranin-O. Therefore, it is suggested that multilayered chondrocyte sheets may serve as a barrier that prevents proteoglycan loss from damaged cartilage, while also protecting the injured site from the catabolic factors in synovial fluid. In the future, we would like to elucidate, how long such chondrocyte

sheets can adhere and live at the grafted sites, while also clarifying the optimum conditions for the adhesion to injured sites for other cell sheets, such as synovial sheets and therefore the combination of both chondrocyte sheets and synovial sheets may thus also be possible.

Although we have to carry out further research to perform more clinical studies, we believe that the use of chondrocyte sheets is useful for the treatment of partial thickness defects of articular cartilage for the reasons stated above.

Conclusions

Human articular chondrocytes could be obtained as sheets without damaging the extracellular matrix by using the newly described temperature-responsive culture dish. Layered chondrocyte sheets were able to maintain the normal phenotype of chondrocytes, and they could be attached to sites of injured cartilage and act as a barrier to prevent the loss of proteoglycan from these sites, while also protecting them from catabolic factors in the knee joints of the rabbits.

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