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CULTURED ARTICULAR CHONDROCYTES SHEETS FOR PARTIAL THICKNESS CARTILAGE DEFECTS UTILIZING TEMPERATURE-RESPONSIVE CULTURE DISHES

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Abstract

The extracellular matrix (ECM) of articular cartilage has several functions that are unique to joints. Although a technique for transplanting cultured chondrocytes has already been introduced, it is difficult to collect intact ECM when using enzymes to harvest samples. Temperature-responsive culture dishes have already been clinically applied in the fields of myocardial and corneal transplantation. Earlier studies have shown that a sheet of cultured cells with intact ECM and adhesive factors can be harvested using such culture dishes, which allow the surface properties of the dish to be reversibly altered by changing the temperature. Human chondrocytes were subjected to enzymatic digestion and then were seeded in temperature-responsive culture dishes. A sheet of chondrocytes was harvested by only reducing the temperature after the cultured cells reached confluency. A real-time PCR analysis of the chondrocyte sheets confirmed that type II collagen, aggrecan, and fibronectin were present. These results suggested that, although chondrocytes undergo dedifferentiation in a monolayer culture, multilayer chondrocyte sheets grown in a similar environment to that of three-dimensional culture may be able to maintain a normal phenotype. A histological examination suggested that multilayer chondrocyte sheets could thus prevent the loss of proteoglycans because the area covered by the sheets was well stained by safranin-O. The present experiments suggested that temperature-responsive culture dishes are useful for obtaining cultured chondrocytes, which may then be clinically employed as a substitute for periosteal patches because such sheets can be applied without a scaffold.

Key words: cell sheet, cultured cell, articular cartilage, partial thickness defect, chondrocyte, temperature-responsive culture dish

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Introduction

Temperature-responsive culture dishes have already been applied to research in various fields of regenerative medicine, including the regeneration of the myocardium (Shimizu *et al.*, 2001; 2002a; 2002b) vascular epithelium (Hirose *et al.*, 2000), cornea (Nishida *et al.*, 2004a; 2004b) hepatocytes (Harimoto *et al.*, 2002) and renal cells (Kushida *et al.*, 2000). This method has also been clinically applied clinically for the myocardium and cornea (Nishida *et al.*, 2004). The surface of a temperature-responsive culture dish is coated with a polymer (poly-N-isopropyl acrylamide) that becomes hydrophilic or hydrophobic in a reversible manner, depending on the temperature (Okano *et al.*, 1993). 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 hydrophobic surface, similar to 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 hydrophilic when the temperature falls below the critical solution temperature of 32°C. Therefore, confluent sheets of cultured cells can be spontaneously released from the hydrophilic dish surface by reducing the temperature below 32°C (temperature recovery system) (Kikuchi *et al.*, 1998). Using this method, cultured cells can thus be harvested as a sheet without damaging the cell-cell junctions and the ECM because it eliminates the need for conventional enzymatic harvesting with trypsin. Such cell sheets have been reported to have various advantages, including the preservation of the normal phenotype and the expression of adhesion proteins on the base (Okano *et al.*, 1993). Furthermore, these cell sheets can also be placed on each other to prepare layered “tissue” because ECM is preserved on the base and such three-dimensional manufactured tissue has already been used for transplantations (Shimizu *et al.*, 2002a; 2002b).

Adult articular cartilage shows a poor self repair after either degeneration or injury occurs and is therefore unlikely to be restored to normal once it has been damaged. The current available treatments for cartilage defects include the application of a periosteal patch to cover the defect (O’Driscoll *et al.*, 1986) and mosaicplasty, in which an osteochondral pillar is grafted from a non-weight-bearing site (Matsusue *et al.*, 1993). 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 from those observed in hyaline cartilage. Since promising results for the transplantation of cultured autologous cartilage cells have been reported (Brittberg *et al.*, 1994) various articular cartilage regeneration techniques have thus been clinically applied, including the use of scaffolds such as atelocollagen (Ochi *et al.*, 2002) and cell transplantation therapy with bone marrow-derived mesenchymal stem cells (MSC) (Wakitani *et al.*, 2002). 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.

In this study, human chondrocyte sheets were obtained using the temperature-responsive culture dish method and then they were combined in layers. The obtained "tissue" specimens were then compared with those produced by single sheets and the adhesion of the sheets was examined *in vitro*.

Materials and Methods

Human articular chondrocytes

The cells used for the *in vitro* experiment included human articular chondrocytes obtained from patients who underwent anterior cruciate ligament reconstruction and gave their informed consent at Tokai University Oiso Hospital from December 2004 to September 2005. Chondrocytes were obtained while forming the interfoveolar ligament. Twenty-five knees from 21 patients aged 14 to 49 years (13 males and 8 females, Average 23.9 years old) were used as the source of these cells. Next, the chondrocytes were subjected to enzymatic treatment, seeded, and cultured according to the method of Sato *et al.* (2003).

Cell proliferations on a Temperature-Responsive surface

The chodrocytes were digested for one 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 hours in DMEM/F12 containing 0.016% Collagenase P (Roche, Mannhein Germany). The digested tissue was passed through a cell strainer (BD Falcon™) with a pore size of 100μm. The chondrocytes were then seeded at high density (10000cells/cm^2) into temperature-responsive surface dishes (diameter:35mm; CellSeed, Tokyo, JAPAN) and then were cultured in DMEM/F12 supplemented with 20% Fetal Bovine Serum (FBS; GIBCO, NY USA) and 50μg/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.

Staining with PKH-26

Lipophilic dye PKH-26 (Red Fluorescent Cell Linker Kit, Sigma, Japan) is a nonradioactive substance with no known cellular toxicity, having a fluorescent half-life of >100 days in erythrocytes (manufacturer's package insert). The fluorescence of PKH-26 is not transferred to other cells but it 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 minutes.

RNA isolation and cDNA synthesis

Total RNA extraction was carried out using the RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to

Table 1. List of primers used in real time PCR

Primer ID	Accession No	Sequence	Expect Size(bp)
Collagen Type I-F	NM_000088	AAG GGT GAG ACA GGC GAA CAA	170
Collagen Type I-R		TTG CCA GGA GAA CCA GCA AGA	
Collagen Type II-F	NM_033150	GGA CTT TTC TTC CCT CTC T	113
Collagen Type II-R		GAC CCG AAG GGT CTT ACA GGA	
Aggrecan1-F	NM_001135	TCG AGG ACA GCG AGG CC	94
Aggrecan1-R		TCG AGG GTG TAG GCG TGT AGAGA	
Fibronectin1-F	NM_001030524	GCA CAG GGG AAG AAA AGG AG	189
Fibronectin1-R		TTG AGT GGA TGG GAG GAG AG	
GAPDH-F	NM_002046	GCA CCG TCA AGG CTG AGA AC	142
GAPGH-R		ATG GTG GTG AAG ACG CCA GT	

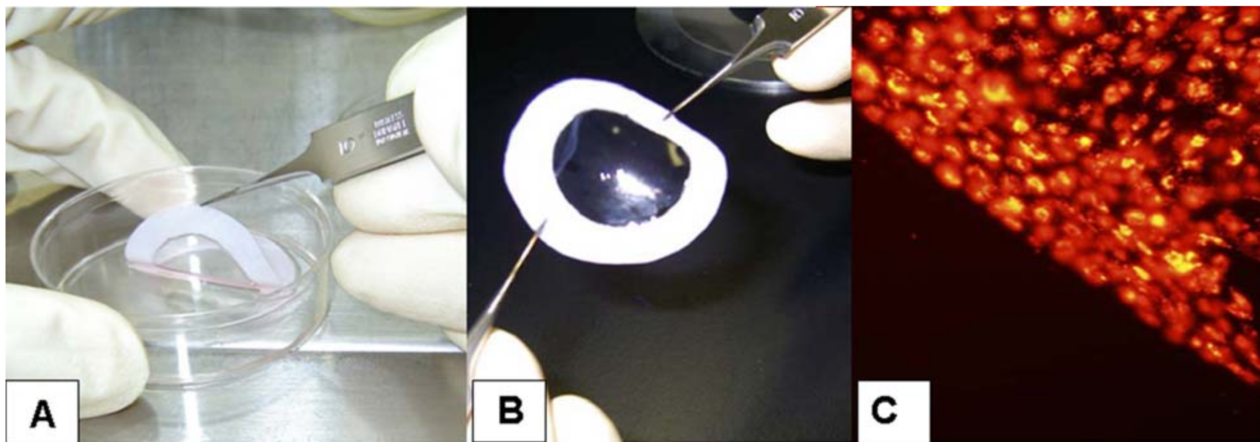

Figure 1. Cultured chondrocytes on a temperature-responsive surface could be released from the dish surface by only reducing the temperature, without the use of proteolytic enzymes (A). Confluent cultured human articular chondrocytes (B) were harvested as a single contiguous cell sheet, thus retaining the cell-cell junctions as well as the deposited ECM on the basal side. Cultured chondrocytes stained with PKH26 were observed with a fluorescence microscope, the cells did not remain after harvesting (C).

manufacturer's instructions. The RNA quality from each sample was assured by the A260/280 absorbance ratio and by electrophoresis of 1.2% agarose formaldehyde gel. 1.0-2.0mg 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 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 70bp-200bp. The oligonucleotide primers used in this study are listed in Table 1. Real time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA) using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA).02-2.5ml of cDNA template was used for real time PCR in a final volume of 25ml. cDNA was amplified according to the following conditions: 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 55–95°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 the samples. To monitor any crossover contaminations of PCR, RNase-free water (Qiagen Inc., Valencia, CA) was included in the RNA extraction and then used as a negative control. To ensure the quality of the data, a negative control was always applied in each run.

Statistical analysis

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

Histological examination

A cartilage tissue sample measuring about 3×3 cm^2 was collected from the femoral condyle of an adult pig. The surface of the sample was polished with coarse sandpaper (#40) to a depth of about 1 mm to prepare a partial-thickness cartilage defect model. Next, a three-layer human articular cartilage cell 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 of a partial thickness defect without covered by any cell sheet was cultured as a control. After one week, the samples were cut into sections, and then were stained with safranin-O to assess the effect of the cell sheet.

Results

Harvesting of cell sheets

Each culture dish was removed from the incubator when the cells reached confluence and then was allowed to stand at about 25°C for 30 minutes. After the culture medium was removed, the cell sheet was collected using a polyvinylidene difluoride (PVDF) membrane according to the method reported by Yamato *et al*. (2001). 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 collected as cell sheets by this method (Fig. 1B). Next, each cell sheet was placed on top of another confluent cell sheet to prepare multilayered sheets.

Staining with PKH-26

Chondrocytes stained with PKH-26 showed a relatively round shape. No residual cells could be detected at the sites from which the cell sheets were harvested (Fig. 1C).

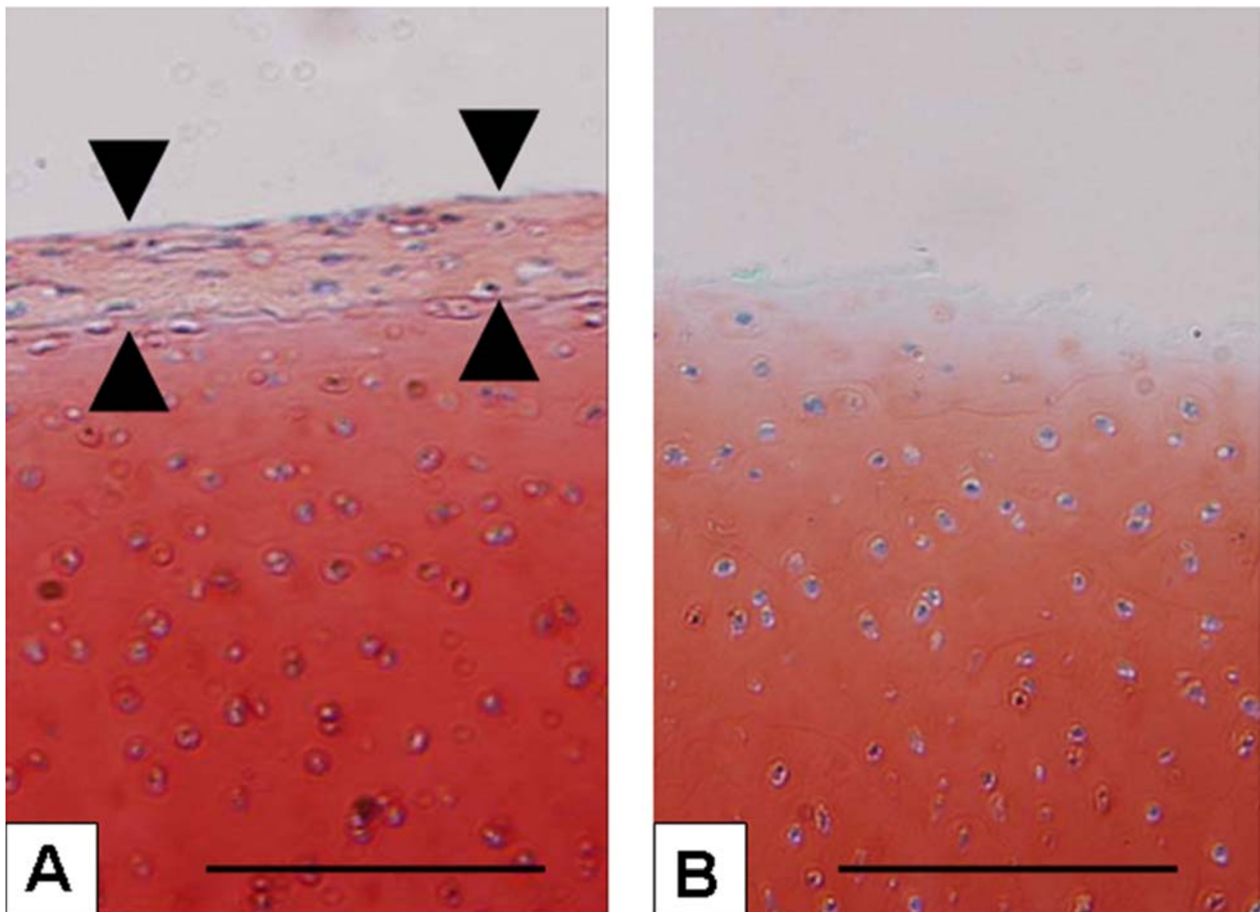

Figure 2. In an organ culture, chondrocyte sheets were adhesive to the partial thickness defect model of porcine articular cartilage. The partial thickness defect model, to which the chondrocyte sheets adhered, maintained a good stainability with safranin-O staining (A); the partial thickness defect model, without their surface being covered by chondrocyte sheets, showed poor stainability with safranin-O staining (B) (Scale line, 200µm).

Multilayered cell sheets

Multilayered chondrocyte sheets could be easily prepared by placing one sheet on another and then culturing them together. It was possible to either continue the culture of the multilayered sheets or to combine a synovial cell sheet as the superficial layer with a chondrocyte sheet as the underlying layer. Three-layered human articular chondrocyte sheets cultured for one week were thus extendable and they were not damaged by any mild external force.

***Ex vivo* histological examination**

After three-layered chondrocyte sheets and porcine cartilage tissue were organ-cultured together for one day, they remained well attached to each other. A histological examination after safranin-O staining confirmed that the cell sheet adhered directly to the porcine cartilage partial damage model and not to the scaffold (Fig. 2A). The area not covered by the cell sheet was poorly stained with safranin-O in comparison to that covered by the cell sheet in the porcine cartilage partial damage model (Fig. 2B).

Analysis of gene expression

The mRNA expressions of fibronectin 1, the expression of three-layered chondrocyte sheets was higher than that of the monolayer culture (Fig. 3A). By the mRNA expressions of aggrecan (Fig. 3B) and type II collagen (Fig. 3D), the expressions of chondrocyte sheets under temperature recovery conditions were higher than the expressions of cultured by conventional dishes, the expressions of three-layered chondrocyte sheets were higher than monolayer culture. The mRNA expressions of type I collagen was low in three-layered chondrocyte sheets (Fig. 3C).

Discussion

The cell sheets cultured in temperature-responsive culture dishes and collected using the temperature recovery system have been reported to preserve the ECM and adhesive factors on the base and thus readily adhere to other cell sheets (Shimizu *et al*., 2002b). The present study confirmed that chondrocytes could be harvested as sheets and thus made into multilayered “tissue” by culturing in temperature-responsive dishes, followed by collection using the temperature recovery system.

Shimizu *et al*. (2002a) 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

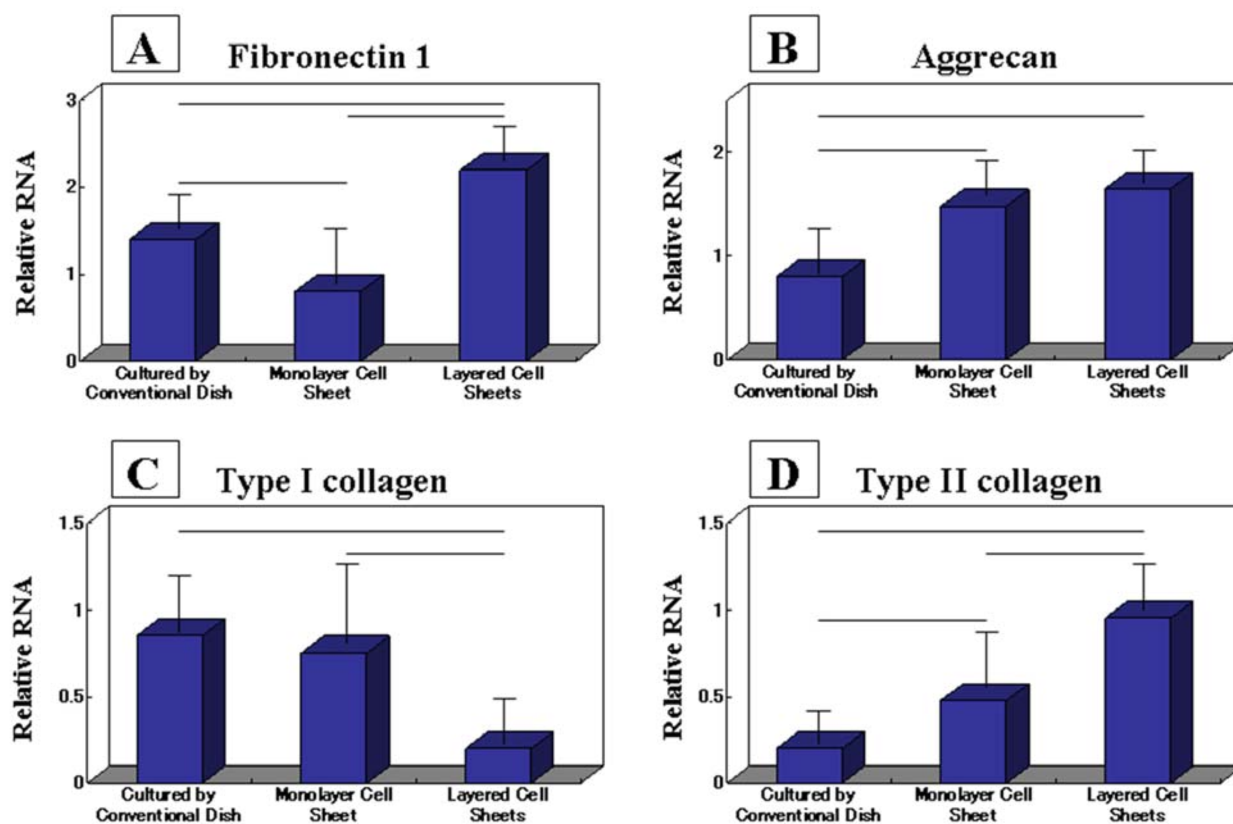

Figure 3. The mRNA expressions of fibronectin 1, type II collagen and aggrecan of three-layered chondrocyte sheets were significantly higher than monolayer culture, and that of type I collagen was significantly lower than monolayer culture (——— : $p<0.05$).

showed multilayer chondrocyte sheets to increase the expression of type II collagen (Fig. 3D) and aggrecan (Fig.3B), as well as to reduce the expression of type I collagen (Fig. 3C), thus indicating that multilayered chondrocyte sheets maintained a normal phenotype because these sheets provided a three-dimensional structure that formed an environment closely similar to that of a three-dimensional culture. Although the 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 nevertheless useful for maintaining the phenotype of cultured chondrocytes. Nishida *et al*. (2004a; 2004b) reported that corneal cell sheets cultured in temperature-sensitive dishes could strongly adhere to the cornea without either scaffolding or suturing. Kushida *et al*. (1999) reported the fibronectin expression to be preserved on the base of cell sheets grown in temperature-responsive culture dishes. In the present study, a chondrocyte sheet was shown to adhere to porcine cartilage after organ culture for one day. We detected a large increase of fibronectin in the multilayered chondrocyte sheets (Fig. 3A). Good adhesion could be obtained because harvesting without enzymatic treatment resulted in the preservation of the expression of both fibronectin and adhesion proteins such as integrin.

Many cells have to be collected to cover a large area of damaged cartilage, but the number of chondrocytes that can be collected from non-weight-bearing sites is limited. Repeated subculturing may lead to problems such as dedifferentiation while also increasing the risk of infection due to long *ex vivo* exposure. Cell source candidates include mesenchymal stem cells that show excellent growth. Previous studies have shown that differentiation from bone marrow mesenchymal stem cells to cartilage cells can be induced *in vitro*. Sekiya *et al*. (2005) reported that when bone marrow, periosteum, or synovial membrane-derived mesenchymal stem cells were pellet-cultured in a differentiation-inducing culture medium, then the synovial membrane-derived mesenchymal stem cells produced the largest cartilage mass with an abundant cartilage matrix, thus resulting in the greatest differentiation into cartilage cells. Synovial cells are considered to be a useful cell source because it is easier to collect them than chondrocytes which have to be obtained from non-weight-bearing sites and because they also show good growth (Data not shown).

For future clinical application, we will evaluate the combination of cell sheets using a synovial membrane-derived cell sheet as the superficial layer and a chondrocyte sheet for adhesiveness as the base layer.

Conclusions

Human articular chondrocytes could be obtained as sheets without damaging the extracellular matrix using the

temperature-responsive culture dish method. Multilayered cell sheets could be created for continuous culture in an environment similar to a three-dimensional culture and they were able to maintain the normal phenotype of chondrocytes. Multilayered chondrocyte sheets could then be attached to sites of cartilage damage to act as a barrier to prevent the loss of proteoglycan from the damaged area.

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References

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med **331**: 889-895.

Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A, Okano T, (2002) Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. J Biomed Mater Res **62**: 464-470.

Hirose M, Hyeong Kwon O, Yamato M, Kikuchi A, Okano T (2000) Creation of designed shape cell sheets that are noninvasively harvested and moved onto another surface. Biomacromolecules **1**: 377-381.

Kikuchi A, Okuhara M, Karikusa F, Sakurai Y, Okano T (1998) Two-dimensional manipulation of confluently cultured vascular endothelial cells using temperature-responsive poly (N-isopropylacrylamide)-grafted surfaces. J Biomater Sci Polym Ed **9**: 1331-1348.

Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T (1999) Decrease in culture temperature releases monolayer endothelial cell sheets together with deposited fibronectin matrix from temperature-responsive culture surfaces. J Biomed Mater Res **45**: 355-362.

Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T (2000) Temperature-responsive culture dishes allow nonenzymatic harvest of differentiated Madin-Darby canine kidney (MDCK) cell sheets. J Biomed Mater Res **51**: 216-223.

Matsusue Y, Yamamoto T, Hama H (1993) Arthroscopic multiple osteochondral transplantation to the chondral defect in the knee associated with anterior cruciate ligament disruption. Arthroscopy **9**:318-321.

Nishida K, Yamato M, Hatashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, Okano T (2004a) Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded *ex vivo* on a temperature-responsive cell culture surface. Transplantation **15**: 379-385.

Nishida K, Yamato M, Hatashida Y, Watanabe K, Yamamoto K, E Adachi, Nagai S, Kikuchi A, Maeda N, Watanabe H, Okano T, Tano Y (2004b) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N Engl J Med **16**: 1187-1196.

O'Driscoll SW, Salter RB (1986) The repair of major osteochondral defects in joint surfaces by neochondrogenesis with autogenous osteoperiosteal grafts stimulated by continuous passive motion. An experimental investigation in the rabbit. Clin Orthop Relat Res **208**: 131-140.

Ochi M, Uchio Y, Kawasaki K, Wakitani S, Iwasa J (2002) Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J Bone Joint Surg Br **84**: 571-578.

Okano T, Yamada N, Sakai H, Sakurai Y (1993) A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). J Biomed Mater Res **27**: 1243-1251.

Sato M, Asazuma T, Ishihara M, Kikuchi T, Masuoka K, Ichimura S, Kikuchi M, Kurita A, Fujikawa K (2003) An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc. J Biomed Mater Res A **64**: 248-256.

Sekiya I, Sakaguchi Y, Yanagishita K, Shinomiya K, Muneta H, (2005) Synovium-derived cells have superior proliferation and chondrogenic potential: Comparison of human stem cells derived from various mesenchymal tissue. J Jpn Orthop. Assoc **79**: 940-945.

Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T (2002a) Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res **90**: e40-e48.

Shimizu T, Yamato M, Isoi Y, Akutsu T, Shibata T, Abe K, Kikuchi A, Umezu M, Okano T (2002b) Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. J Biomed Mater Res **60**: 110-117.

Shimizu T, Yamato M, Kikuchi A, Okano T (2001) Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. Tissue Eng **7**: 141-151.

Wakitani S, Imoto K, Saito M, Murata N, Yoneda M (2002) Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage **10**: 199-206.

Yamato M, Ushida M, Kushida A, Konno C, Kikuchi A, Okano T (2001) Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. Tissue Eng **7**: 473-480.