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RESEARCH ARTICLE

Studies of the humoral factors produced by layered chondrocyte sheets

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Abstract

The authors aimed to repair and regenerate articular cartilage with layered chondrocyte sheets, produced using temperature-responsive culture dishes. The purpose of this study was to investigate the humoral factors produced by layered chondrocyte sheets. Articular chondrocytes and synovial cells were harvested during total knee arthroplasty. After co-culture, the samples were divided into three groups: a monolayer, 7 day culture sheet group (group M); a triple-layered, 7 day culture sheet group (group L); and a monolayer culture group with a cell count identical to that of group L (group C). The secretion of collagen type 1 (COL1), collagen type 2 (COL2), matrix metalloproteinase-13 (MMP13), transforming growth factor-β (TGFβ), melanoma inhibitory activity (MIA) and prostaglandin E2 (PGE2) were measured by enzyme-linked immunosorbent assay (ELISA). Layered chondrocyte sheets produced the most humoral factors. PGE2 expression declined over time in group C but was significantly higher in groups M and L. TGFβ expression was low in group C but was significantly higher in groups M and L ($p < 0.05$). Our results suggest that the humoral factors produced by layered chondrocyte sheets may contribute to cartilaginous tissue repair and regeneration. Copyright © 2012 John Wiley & Sons, Ltd.

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Keywords **articular cartilage; chondrocyte sheet; co-culture; enzyme-linked immunosorbent assay; humoral factor; scanning electron microscopy; temperature-responsive culture dish**

1. Introduction

Osteoarthritis (OA) is a common joint disease that is characterized by the gradual degeneration of articular cartilage over a long period. This disease develops mainly in weight-bearing joints of the lower limbs, such as the knee and hip joints, and causes pain and joint dysfunction. OA eventually limits the ability to perform activities of daily living and compromises quality of life. Articular cartilage defects are classified as either full- or partial-thickness defects, according to whether or not they penetrate the marrow spaces of subchondral bone. The treatments currently used for full-thickness defects of articular cartilage include microfracture surgery (Mithoefer *et al*., 2006; Steadman *et al*., 2001, 2002), mosaicplasty (Hangody *et al*., 1997, 2001; Szerb *et al*., 2005) and autologous chondrocyte implantation (Brittberg *et al*., 1994; Nagai *et al*., 2008a, 2008b; Peterson *et al*., 2003). These types of surgery can only be applied to a small area of articular cartilage defect, such as traumatic osteochondral lesions or osteochondritis dissecans. Microfracture surgery and drilling are techniques that encourage the regeneration of damaged cartilage by filling osteochondral defects with mesenchymal stem cells derived from the bone marrow. It is believed that the regenerated cartilage was not hyaline cartilage, but fibrocartilage (Peterson *et al*., 2003). The functions and properties of fibrocartilage are inferior to those of hyaline cartilage, and the outcomes at long-term follow-up tend to be poor. Mosaicplasty can be used to transplant hyaline cartilage to damaged areas, although this is associated with donor site morbidity. In animal experiments, Nagai *et al*. (2008a, 2008b) reported good outcomes of allograft implantation using a scaffold-free chondrocyte plate to resolve this malfunction.

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Meanwhile, full-thickness defects of articular cartilage are observed during the end stages of osteoarthritis, and these frequently spread across a wide area. Because such a cartilage condition is not an indication for these types of surgery, arthroplasty must be employed for treating severe OA as a basic remedy. Although the clinical results of arthroplasty have improved with the development of new surgical techniques and with the selection of appropriate medical devices, the stress on the patient's body from the invasive procedure is of concern.

During the initial stages of osteoarthritis, partial-thickness defects of articular cartilage are observed and they develop into full-thickness defects with progression of stages (Redman *et al*., 2005). One key issue is how to start treatment at an early stage of the disease, but only a limited number of repairing cells can enter the injured sites (Paget, 1969). For this reason, it is important to develop new protocols for cartilage repair and regeneration using tissue engineering.

Cell sheet technology using temperature-responsive culture dishes was first reported by Okano *et al*. (1993) and is now used widely in corneal, myocardial, hepatic and other regenerative medicine fields (Harimoto *et al*., 2002; Kushida *et al*., 2000; Nishida *et al*., 2004; Shimizu *et al*., 2002, 2006). Nishida *et al*. (2004) reported that corneal cell sheets cultured in temperature-responsive culture dishes could adhere strongly to the cornea without scaffolding or suturing. Kushida *et al*. (2000) reported that fibronectin expression was conserved on the basal aspect of cell sheets cultured in temperature-responsive culture dishes. Cell sheets can be harvested with intact ECM and adhesion factors because the cell sheets are simply layered on top of each other, using the natural adhesiveness of the basal side. Repetition of this procedure facilitates the construction of multilayered, three-dimensional (3D) tissue structures without the need for scaffolds. Shimizu *et al*. (2006) reported that the maximum thickness of the fabricated rabbit myocardial cell sheet is three layers *in vitro*, because thicker sheets receive inadequate nutrition. They also demonstrated that repetitive allografts of cell sheets *in vivo* could not increase the thickness by more than 1 mm in myocardial tissues.

We have confirmed the ability of layered chondrocyte sheets, cultured in temperature-responsive culture dishes, to repair and regenerate partial-thickness defects of articular cartilage in animal experiments (Ebihara *et al*., 2012; Ito *et al*., 2012; Kaneshiro *et al*., 2006, 2007; Mitani *et al*., 2009). Chondrocyte sheets are obtained by simply reducing the temperature, with no need for enzymatic digestion, and multilayered chondrocyte sheets can be produced easily by placing one cell sheet over others. We developed a method for preparing chondrocyte sheets that requires less time by co-culture with synovial cells. We have shown that implanted chondrocyte sheets can survive for at least 8 weeks in the recipient joint (unpublished data), and the articular cartilage defects, separated from the implanted site of chondrocyte sheets, are well repaired, along with the implanted site. The chondrocyte sheets have excellent adhesive properties and barrier functions, but this may not be the only mechanism responsible for cartilage repair and regeneration. The purpose of this study was to identify and measure the amount of humoral factors produced by layered chondrocyte sheets, and to elucidate whether another mechanism contributes to the ability of chondrocyte sheets to repair cartilage.

2. Materials and methods

2.1. Human chondrocytes and human synovial cells

This study was performed in compliance with the Helsinki Declaration and was approved by the Institutional Review Board for Clinical Research of Tokai University School of Medicine. Human cartilage tissues and synovial tissues were obtained from six patients who underwent joint replacement surgery at Tokai University Hospital, after giving consent. The patients were four women and two men with an average age of 66 (range 37–81) years.

The tissues were sliced finely with scissors in Petri dishes. The synovial tissues and cartilage tissues were incubated for 2 and 4 h, respectively, for proteolytic digestion in 5 g/ml collagenase type 1 (Worthington Biochemical Corp., Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco Invitrogen Corp., Carlsbad, CA, USA) at 37 °C in 5% CO_2 and 95% air. After digestion, the cell suspension was passed through a cell strainer (BD Falcon™, BD Bioscience, Bedford, MA, USA) with a pore size of 100 μm and retrieved by centrifugation. Chondrocytes were maintained in a culture medium of DMEM/F12 (Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% antibiotic–antimycotic mixture (10 000 U/ml penicillin G, 10 000 μg/ml streptomycin sulphate and 25 μg/ml amphotericin B as Fungizone; Gibco), to which 50 μg/ml ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan) was added from day 4 onward. Synovial cells were maintained in a culture medium of DMEM/F12 supplemented with 10% FBS and 1% antibiotic–antimycotic mixture (all from Gibco). All experiments were performed at 37 °C in 5% CO_2 and 95% air. The cells were seeded at a density of 10 000 cells/cm^2 for both the primary culture and subcultures. The culture period was 7 days from P0 to P1, and 5 days from P1 to P2.

2.2. Temperature-responsive culture dishes and inserts

The specific method used to fabricate temperature-responsive culture dishes and inserts (CellSeed, Tokyo, Japan) has been reported (Okano *et al*., 1995). Briefly, *N*-isopropylacrylamide (IPAAm) monomer solution was spread onto commercial culture dishes, and the dishes

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were subjected to electron beam irradiation, which resulted in polymerization and covalent bonding of the IPAAm to the dish surface. The grafted dishes were then rinsed with distilled water to remove any ungrafted IPAAm. The culture dishes were sterilized using ethylene oxide gas (Sekiya *et al*., 2006).

2.3. Conventional culture group and monolayer chondrocyte sheets

In the conventional culture group, chondrocytes were seeded in culture dishes (150 cm^2; BD Falcon™) at a density of 10 000 cells/cm^2. To prepare the monolayer chondrocyte sheets, the synovial cells were seeded in companion plates, which were notched for use with cell culture inserts (9.6 cm^2; BD Falcon™), at a density of 10 000 cells/cm^2. The chondrocytes seeded on temperature-responsive inserts (4.2 cm^2; CellSeed) were seeded at a density of 50 000 cells/cm^2. The culture period of the monolayer cell sheets fabricated by co-culture was 14 days with the inserts. The conventional culture group cells were retrieved using a sterile cell scraper. After removal from the incubator, temperature-responsive culture inserts were allowed to stand at 25 °C for 30 min to release confluent cells as a monolayer chondrocyte sheet from the inserts, and the culture medium was removed. Polyvinylidene fluoride (PVDF) membranes were used as supporting membranes to retrieve the sheets according to the method of Yamato *et al*. (2001). The lifted chondrocyte sheet edges promptly attached to the overlaid supporting membrane, and the cell sheet and PVDF membrane film were detached gently from the temperature-responsive culture inserts.

2.4. Fabrication of layered chondrocyte sheets

It was possible to retrieve the chondrocyte sheets by simply reducing the temperature, removing the need for enzymatic digestion. The retrieved chondrocytes in sheet form retained their contiguity with adjacent extracellular structures, which implies that these cell sheets contain extracellular proteins, including cell–cell junctions, extracellular matrix (ECM) and adhesion proteins.

Multilayered chondrocyte sheets could be produced easily by placing one cell sheet over another, using a supporting PVDF membrane. To fabricate triple-layered sheets, it was necessary to repeat this procedure twice. Because the layered chondrocyte sheets floated in the culture medium, a cell strainer (BD Falcon™) was placed on top. When cultured for 1 week, the triple-layered chondrocyte sheets were extendable and were not damaged by mild external force (Figure 1A). Because each sheet adhered firmly and tightly to the others, the multilayered structure was retained even when it was extended. Due to the supporting membrane, the cell sheet–PVDF film exhibited good stability and handling the chondrocyte sheet was easy (Figure 1B).

2.5. Scanning electron microscopy (SEM) evaluation

The layered chondrocyte sheets were soaked in 0.1 M phosphate buffer and 2% glutaraldehyde for 2 h. They were then fixed in 1% osmium solution for 1 h and dehydrated in gradually increasing ethanol concentrations (50%, 70%, 80%, 90%, 95%, and 100%). The samples were dried using the critical point drying method, sputter-coated with gold and affixed to an adhesive interface for observation of both the top and bottom surfaces of the cell sheets by SEM (JSM-840; Jeol, Tokyo, Japan).

2.6. Measurement of humoral factors

Our studies included a monolayer, 7 day culture sheet group (group M); a triple-layered, 7 day culture sheet group (group L); and a conventional culture group with a cell count identical to that of group L (group C). For each group, $n = 6$.

After changing the culture medium to DMEM/F12 (Gibco) supplemented with 1% heat-inactivated FBS

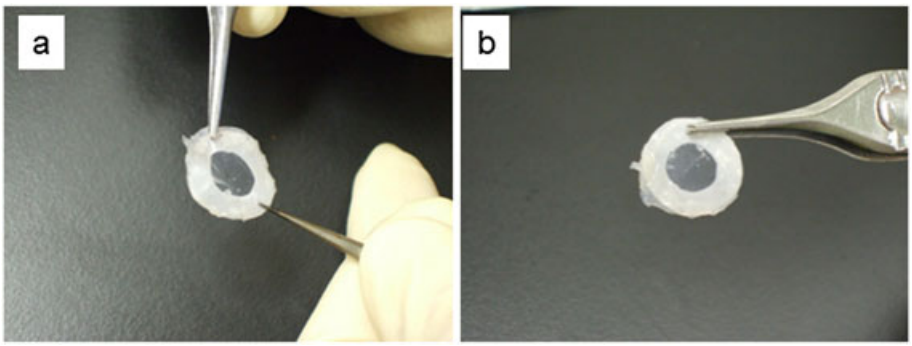

Figure 1. Manipulation of chondrocyte sheets. We could easily fabricate multilayered chondrocyte sheets by placing one cell sheet over another, using a supporting PVDF membrane. (A) Triple-layered sheets were fabricated by repeating this procedure. When cultured for 1 week, the triple-layered chondrocyte sheets were extendable and were not damaged by mild external force. (B) The supporting membrane provided good stability for the cell sheet–PVDF film, and the chondrocyte sheet could be handled easily

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(Gibco) and 1% antibiotic–antimycotic mixture (Gibco), the specimen was extracted from the culture supernatant in equal volumes over 5 days. The amounts of collagen type 1 (COL1), collagen type 2 (COL2), matrix metalloproteinase-13 (MMP13), melanoma inhibitory activity (MIA), transforming growth factor-β (TGFβ) and prostaglandin E2 (PGE2) secreted into culture supermatant were measured using enzyme-linked immunosorbent assay (ELISA). The assays were COL1 ELISA (ACBio, Kanagawa, Japan), COL2 ELISA (MDB, Zurich, Switzerland), activated MMP13 ELISA (MDB), MIA ELISA kit 96-well plate (Roche, Mannheim, Germany), human TGFβ ELISA Kit (R&D Systems, Minneapolis, MN, USA) and PGE2 (R&D Systems). The biochemical data were analysed using one-factor analysis of variance (ANOVA) followed by individual *post hoc* comparisons (Tukey–Kramer). $p < 0.05$ was considered significant.

3. Results

3.1. SEM

SEM analysis revealed that the top and basal aspects of the chondrocyte sheets had completely different structures. On the top aspect of the sheet, a network of laminated ECM structures had a 'piled-up' appearance, comprising sheet-like configurations and amorphous shapes. These edges were occasionally dog-eared on the culture medium side (Figure 2A).

By contrast, the basal aspect of the sheet that bonded to the bottom of the culture dish expressed a smooth ECM pattern and numerous mound-like elevations. Compared with the top aspect of the sheet, the arrangement of the accumulated ECM surface on the basal aspect was smoother and had a parallel pattern (Figure 2B).

3.2. Analysis of humoral factors by ELISA

COL1 secretion tended to increase with each subculture. COL1 secretion did not differ significantly between the three groups from P0 (group M, 98.10 ± 3.46 ng/ml; group L, 94.41 ± 0.92 ng/ml; and group C, 92.89 ± 5.25 ng/ml on day 5) through P2 (Figure 3A).

COL2 secretion increased progressively in P0 (group M, 113.39 ± 49.26 ng/ml; group L, 189.22 ± 125.47 ng/ml; and group C, 163.49 ± 19.36 ng/ml on day 5) but very little in P1–P2. COL2 secretion did not differ significantly between the three groups (Figure 3B).

MMP13 secretion was suppressed consistently in P0–P2 and did not differ significantly between the three groups (Figure 3C).

MIA secretion was significantly higher for group L in P0 (group M, 7.32 ± 0.82 ng/ml; group L, 9.87 ± 1.50 ng/ml; and group C, 4.78 ± 0.39 ng/ml on day 1. group M, 18.38 ± 7.98 ng/ml; group L, 30.67 ± 20.34 ng/ml; and group C, 26.50 ± 3.14 ng/ml on day 5). The same pattern was seen in P1 (group M, 15.58 ± 7.42 ng/ml; group L, 21.99 ± 7.61 ng/ml; and group C, 8.89 ± 2.59 ng/ml on day 1). In P2, MIA secretion was significantly higher in groups M and L for days 1–5, but almost no MIA secretion was observed in group C (group M, 7.42 ± 3.38 ng/ml; group L, 16.05 ± 4.44 ng/ml; and group C, 0.26 ± 0.08 ng/ml on day 5) (Figure 4A).

Almost no TGFβ secretion was observed in any of the passages for group C. In P0 and P1, TGFβ secretion was significantly higher for groups M and L (group M, 2.61 ± 1.42 ng/ml; group L, 2.66 ± 1.05 ng/ml; and group C, 0.18 ± 0.02 ng/ml on day 5 in P0) (Figure 4B).

In group C, PGE2 secretion declined with time. By contrast, PGE2 secretion was significantly higher in groups M and L in P0 (group M, 1.32 ± 0.19 ng/ml; group L, 1.51 ± 0.29 ng/ml; and group C; 0.81 ± 0.39 ng/ml on day 5) and in P2 (group M, 0.62 ± 0.04 ng/ml; group L, 1.88 ± 0.15 ng/ml; and group C, 1.06 ± 0.15 ng/ml on day 1) (Figure 4C).

4. Discussion

We confirmed the availability of layered chondrocyte sheets cultured in temperature-responsive culture dishes for repair and regeneration of partial-thickness defects of articular cartilage in animal experiments. SEM examination indicated

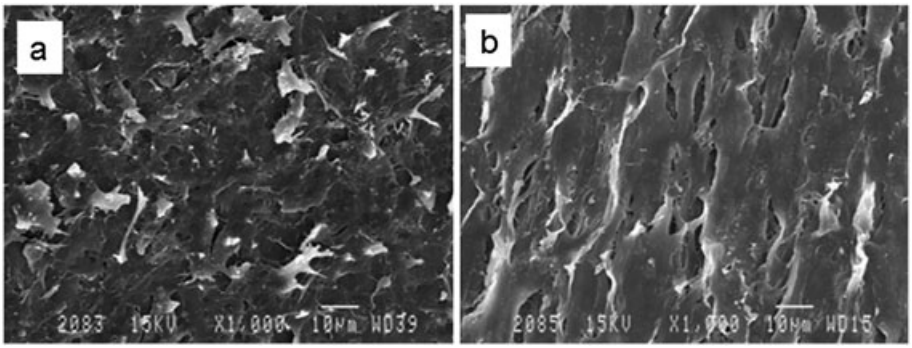

Figure 2. SEM analysis showed that the top and basal aspects of the chondrocyte sheets had completely different structures. (A) A network of laminated ECM structures was observed on the top aspect of the sheet, and the ECM sheet edges were occasionally dog-eared on the culture medium side. (B) The basal aspect of the cell sheet, which bonded to the bottom of the culture dish, was covered with a smooth ECM pattern, and numerous mound-like elevations were observed. Compared with the top aspect of the chondrocyte sheet, the arrangement of the accumulated ECM surface on the basal aspect was smoother and had a parallel pattern. Scale bar = 10 μm

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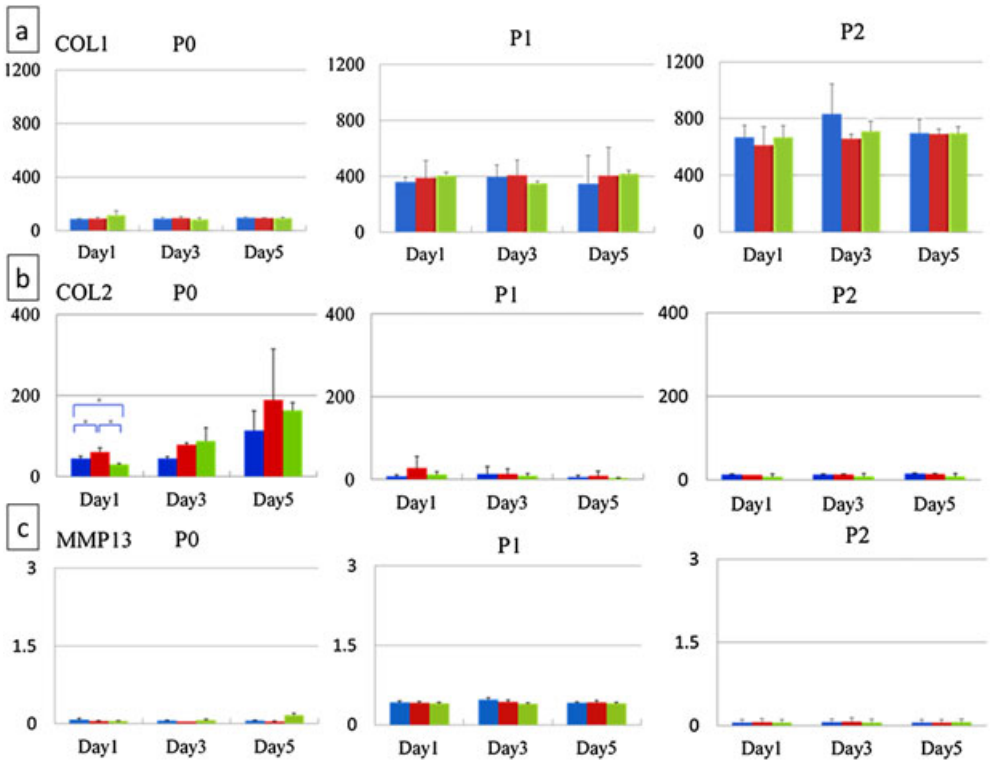

Figure 3. Secretion of COL1, COL2 and MMP13. (A) COL1 secretion tended to increase with each subculture passage. (B) COL2 secretion increased progressively in P0. (C) MMP13 secretion was suppressed consistently from P0 through P2. In the graph, group M is shown in blue, group L in red and group C in green; vertical axis units, ng/ml; $*p < 0.05$

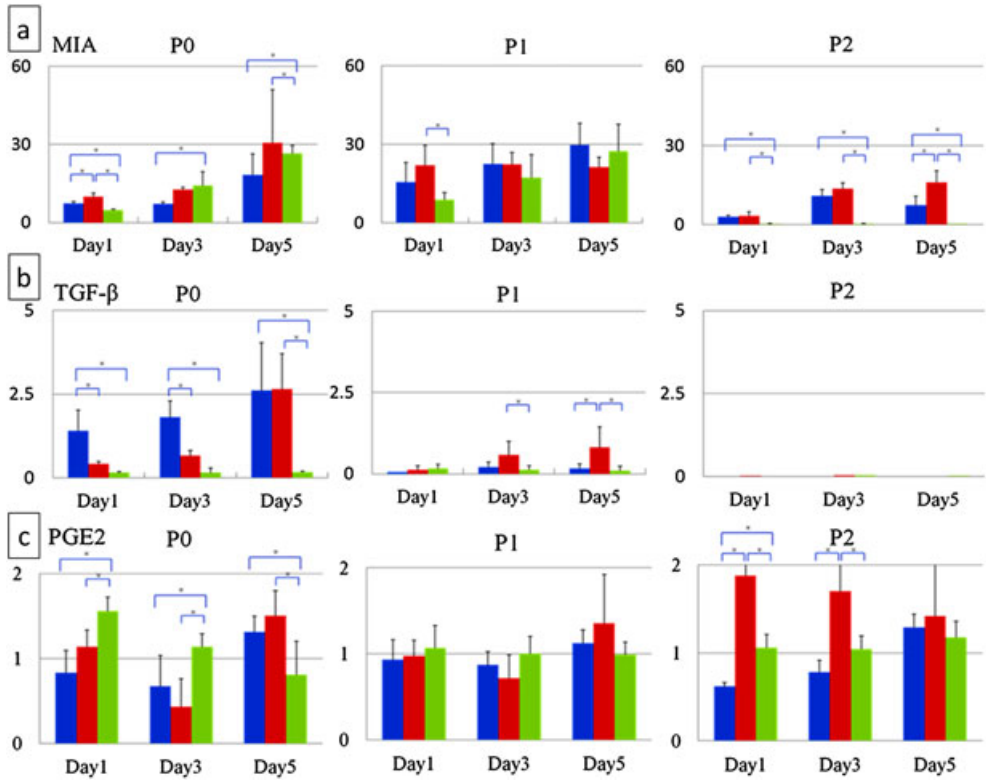

Figure 4. Secretion of MIA, TGFβ and PGE2. (A) MIA secretion was significantly higher for group L than group C in P0 and P2. (B) TGFβ secretion was significantly higher for groups M and L than group C in P0 and P1. (C) PGE2 secretion was significantly higher for groups M and L than group C in P0 and P2. In the graph, group M is shown in blue, group L in red and group C in green; vertical axis units, ng/ml; $*p < 0.05$

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that the top and bottom of the cell sheets had completely different structures. A network of laminated ECM structures on the top aspects of the sheets resembled the normal superficial cartilage zone, the lamina splendens initially proposed by MacConaill (1951) and later identified by Ckark (1990). However, SEM showed that the sheets did not have a smoother surface than normal articular cartilage. In this study, it was not possible to observe a distinct fibrous collagen structure, which may exist beneath the lamination. SEM of the superficial zone in ordinary cartilage shows several layers of collagen fibrils lying immediately beneath the lamina splendens and forming a mesh of interwoven fibrils that run parallel to the articular surface. In the superficial zone, the chondrocytes appear to be located beneath the layers of collagen fibrils. By contrast, on the basal aspect, numerous mound-like elevations were observed, which resembled an aggregation of chondron-like shapes. This smooth surface was more similar to the surface of normal cartilage than was the top side of the cell sheets. The smooth, flat surface of the basal side implies abundant accumulation of extracellular proteins, which is reflected in their characteristic adhesiveness. The effects of gravity during the culture period and the flat bottoms of the culture dishes may also have contributed to this even surface structure.

We have reported that layered chondrocyte sheets possess excellent adhesive properties and barrier functions for defending against invasion by catabolic factors in synovial fluid and for preventing leakage of proteoglycan in ECM from damaged cartilage (Ebihara *et al*., 2012; Ito *et al*., 2012; Kaneshiro *et al*., 2006, 2007; Mitani *et al*., 2009).

We also confirmed that COL2 secretion increased and MMP13 secretion was inhibited, suggesting that layered chondrocyte sheets maintain an intact ECM and exert a chondroprotective action. The MMP13 concentration of synovial fluid is 1.928 ± 0.373 ng/ml in OA and 2.35 ng/ml in normal joints (Kim *et al*., 2010; Lehmann *et al*., 2010). Although MMP13 secretion increased slightly in P1, the level was low – 0.5 ng/ml or less. In P1 and P2, COL2 secretion was virtually non-existent. These data suggest that repeated subculture causes chondrocyte dedifferentiation (Schnabel *et al*., 2002).

The layered chondrocyte sheets secreted MIA, TGFβ and PGE2, which play an important role in chondrocyte differentiation and tissue repair. MIA is a small, soluble protein secreted by malignant melanoma cells and chondrocytes. It modulates osteogenic differentiation toward a chondrogenic direction and stabilizes cartilage differentiation (Schubert *et al*., 2010). Because MIA level in synovial fluid reflects cartilage remodelling, it is believed that this factor may provide a marker for objectively estimating the restorative reaction of chondrocytes (Saito *et al*., 2002). In this study, MIA secretion was maintained better in groups M and L than in group C, even in P2.

In OA, interleukin-1 (IL-1) stimulates the expression of MMPs and aggrecanases, which induce cartilage degeneration. IL-1 can reduce the production of cartilage-specific macromolecules, including COL2 (Pujol *et al*., 2008). On the other hand, TGFβ stimulates production of ECM components by chondrocytes and can counteract the effects of catabolic cytokines such as IL-1 (Blaney Davidson *et al*., 2006). The TGFβ signalling pathway is crucial for the maintenance of articular cartilage homeostasis and for preventing its disruption (Roman-Blas *et al*., 2007). In this study, TGFβ secretion levels were high in group L in P0 and P1 but very low in P2, a pattern similar to that for COL2.

PGE2 exerts pleiotropic effects in various tissues through EP1–4 receptors. EP2 is the main receptor expressed in articular cartilage, and the PGE2 signal through EP2 stimulates articular chondrocyte growth (Aoyama *et al*., 2005). Continuous inhibition of PGE2 accelerates the progression of OA (Nishitani *et al*., 2010). In this study, group L had a higher secretory capacity for PGE2, suggesting that the implantation of layered chondrocyte sheets may cause more efficient tissue repair processes of partial-thickness cartilage defects.

TGFβ and PGE2 also have an inhibitory effect on T cell proliferation (Aggarwal and Pittenger, 2005; Di Nicola *et al*., 2002). This inhibitory effect might be beneficial in counteracting immune rejection, such as graft-versus-host disease, suggesting one possible future clinical application.

In our experiments, some humoral factors were no longer secreted with successive subculture passages. Therefore, we believe that the use of P0 cells would be appropriate in clinical application, considering the culture period and probability of contamination.

5. Conclusions

Layered chondrocyte sheets secrete various humoral factors. We observed significantly higher secretory capacity of TGFβ and PGE2, which play an important role in cartilage repair and regeneration, in layered chondrocyte sheets fabricated using a co-culture method. In addition to the excellent adhesive properties and barrier functions of the sheets themselves, the abundant secretion of these humoral factors supports the efficacy of layered chondrocyte sheets in cartilage repair.

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