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Influence of three-dimensional culture in a type II collagen sponge on primary cultured and dedifferentiated chondrocytes

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Abstract

Background. Once articular cartilage is destroyed, the intrinsic reparative ability is poor. Therefore, various techniques have been developed to repair articular defects. Many kinds of scaffolds have been used for cultured chondrocyte transplantation. In this study, we developed a sponge consisting of type II collagen. We investigated the influence of three-dimensional culture on the maintenance of the chondrocyte phenotype and on the redifferentiation of dedifferentiated chondrocytes.

Methods. Chondrocytes were isolated from the rib cartilage of rats and were cultured in plastic dishes for a week (P0). The cells were then dissociated with trypsin and subcultured for another 2 weeks (P1). Primary isolated chondrocytes were cultured in the type II collagen sponges for 3 weeks (S1). We compared the gene expression of S1 for chondrogenic markers with the expression of P0 and P1 by reverse transcription-polymerase chain reaction (RT-PCR). The cells were then dissociated with trypsin and subcultured for another 2 weeks (P1) and then another 6 weeks (P3). Cells of P1 were subsequently cultured in type II collagen sponges for 4 weeks (P1r). At each time point, gene expression of chondrogenic markers was examined by RT-PCR.

Results. Gene expression of COL2A1, COL10A1, and aggrecan in S1 was the same as in P0. Gene expression of COL10A1 and aggrecan in P1r was higher than in P1 and P3. Gene expression of COL1A1, COL2A1, and SOX9 in P1r was lower than in P1 and P3. Gene expression of ALP and osteocalcin in P1r was detected.

Conclusions. These results show that culture in type II collagen sponges could maintain the chondrocyte phenotype; however, dedifferentiated chondrocytes differentiated to hypertrophic chondrocytes. These finding suggest that the complex of cells and scaffolds with primary cells was more useful than that with dedifferentiated chondrocytes in laboratory and clinical application.

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Introduction

It is known that once articular cartilage tissue is destroyed the intrinsic ability of the remaining tissue to repair is poor. Therefore, treatment of damaged articular cartilage is difficult. Various techniques, such as subchondral drilling, abrasion arthroplasty,[1] microfracture methods,[2] and autologous osteochondral grafts,[3] have been developed to repair articular defects. However, no method has yet been established as the premier standard. Transplantations using autologous cultured chondrocytes were reported in 1994.[4,5] More recently, matrix-based autologous cultured chondrocyte transplantation has been developed. There have been many studies on transplanting cells to repair cartilage defects using various artificial matrices as carriers for cultivating chondrocytes. However, the transplanted tissue using these scaffolds takes a long time to restore biological and mechanical characteristics similar to those of articular cartilage because the scaffold itself has no cartilaginous matrix component. It therefore seems more useful to use a major structural component of cartilage, such as type II collagen and proteoglycan, as a scaffold. We developed a sponge consisting of type II collagen to bring biochemical characteristics of hyaline cartilage to the scaffolds.

Chondrocytes cultured in monolayer can proliferate more than when in three-dimensional (3-D) culture, but their chondrocytic characteristics gradually disappear.[6,7] The 3-D environment is thought to be beneficial for restoring the lost chondrocytic characteristics; therefore, several studies have applied the monolayer culture for cell proliferation and 3-D culture for redifferentiation of the dedifferentiated chondrocytes.[7] It is still unclear, however, whether 3-D culture can restore the chondrocytic phenotype sufficiently.

The purpose of this study was to investigate the potential of type II collagen sponges as scaffolds as well as the influence of a 3-D culture in combination with the

presence of type II collagen on the maintenance of the chondrocyte phenotype and the redifferentiation of dedifferentiated chondrocytes.

Materials and methods

Production of the type II collagen sponge

Type II collagen (Nitta Gelatin, Osaka, Japan) was extracted from bovine nasal cartilage with pepsin after delipidation, decalcification, and removal of mucopolysaccharides. Collagen was dissolved in dilute hydrochloric acid at concentration of 20 mg/ml (pH 2.8–3.0), dispensed in a 24-well plate (0.6 ml/well), frozen at –20°C for 5 days, and freeze-dried for 3–5 days. The freeze-dried collagen was crosslinked using an ultraviolet (UV) crosslinker (Spectrolinker XL-1500; Spectronics, Westbury, NY, USA) (254 nm, 15 W × 6) at 9000 × 150 mJ/cm^2 followed by dehydrothermal crosslinking (110°C, 180 min).

Cell culture method of study 1 (maintenance of chondrocytic character)

All of the experimental protocols were approved by the Kitasato University School of Medicine Animal Care Committee. Chondrocytes were enzymatically isolated from rib cartilage from 6-week-old Wistar rats. Growth plate cartilage was dissected out from rib cartilage. The portion of permanent cartilage was cut into small pieces with a scalpel. Cartilage pieces were digested with trypsin [2.5 mg/ml in phosphate-buffered saline (PBS); Wako, Osaka, Japan] for 1 h at 37°C, and the digested solution was discarded to exclude superficial fibrous tissue. The remaining tissue was then incubated in Dulbecco's modified Eagle's medium (DMEM) containing collagenase 2.5 mg/ml (Worthington type II) at 37°C overnight. The isolated cells were centrifuged and resuspended at a concentration of 1×10^7 cells/ml. The cell suspension of 100 µl was seeded in the type II collagen sponge described above. This sponge can hold 100–150 µl of fluid. After preculture for 1 h at 37°C, culture medium was added. Primary chondrocytes were cultured in type II collagen sponges for 3 weeks (S1). Cultures were maintained in a humidified atmosphere consisting of 95% air/5% CO_2 at 37°C. The culture medium, containing 10% fetal bovine serum (FBS) (GIBCO product; Invitrogen, Carlsbad, CA, USA), antibiotic-antimycotic liquid (GIBCO), and L-ascorbic acid (50 µg/ml), was changed twice a week.

Cell culture method of study 2 (redifferentiation of dedifferentiated chondrocytes)

Chondrocyte isolation was performed as described for study 1. The isolated cells were cultured in 25-cm^2 flasks (Falcon) for a week [passage (P0)] in the DMEM culture medium described above for study 1. The cells were then dissociated with trypsin and subcultured in 75-cm^2 flasks (Corning) for more 2 weeks (P1). These cells were subcultured in new 75-cm^2 flasks for more 4 weeks (P3). The cells of P1 were seeded in the type II collagen sponge at a density of 1×10^6 cells/ml as described above in study 1 and were cultured for 4 weeks (P1r).

Cell proliferation

At the 5th and 10th days of culture, at a density of 1×10^5 cells/ml, the proliferation of primary chondrocytes in collagen sponges was analyzed by the WST-8 assay (Cell Counting Kit-SF; Tesque Nacalai, Kyoto, Japan).

Histological evaluation

In S1 and P1r, collagen sponges that had been cultured with chondrocytes were washed with PBS, fixed with 10% neutral buffered formalin, dehydrated in alcohol, embedded in paraffin, and cut into sections 2 µm thick. Each specimen was stained with safranin O.

Conventional electron microscopy

Small blocks obtained from the collagen sponge in S1 were further fixed with 2% glutaraldehyde in phosphate buffer for 2 h, washed with 0.1 M cacodylate buffer for 2 h, and postfixed with 2% osmium tetroxide in phosphate buffer for 1 h at 4°C. After dehydration in a graded series of ethanol, the sponges were embedded in Quetol-812 (Nissin EM, Tokyo, Japan). Ultrathin sections were cut with an ultramicrotome, double-stained with uranyl acetate and lead citrate, and examined with an HU-12A transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV in the Kitasato Electron Microscopy Center.

RNA extraction

The RNA extraction analysis was performed as described previously.[8] For each time point (P0, P1, P3, S1, P1r), total RNA was extracted from collagen sponges that had been cultured with chondrocytes at an initial density of 1×10^6 cells/ml (S1, P1r) and from monolayer cultures (P0, P1, P3). Sponges were homogenized in 1 ml SET buffer [1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 10 mM Tris-HCl, pH 7.5] containing proteinase K 65 mg/ml (Merck, Darmstadt, Germany)

using scissors. The homogenate was then incubated for 1 h at 45°C and mixed with 0.5 vol. phenol saturated with 0.5 M Tris-HCl (pH 7.5) followed by mixing with 0.5 vol. chloroform. Total nucleic acids were extracted with phenol/chloroform, precipitated overnight with 2 vol. ethanol at –4°C, pelleted, and dissolved in water treated with diethylpyrocarbonate (DEPC; Sigma, St. Louis, MO, USA). Total RNA was dissolved in DEPC-treated water, and the concentration of total RNA was determined by measuring the absorbance at 260 nm. Total RNA from monolayer culture was extracted using Isogen (Nippon Gene, Tokyo, Japan).

Reverse transcription polymerase chain reaction

The reverse transcription-ploymerase chain reaction (RT-PCR) analysis was performed as described previously.[9] The RT reaction mixture contained 1 µg total RNA, 50 pmol random 9-mers, and 200 U Superscript II reverse transcriptase (GIBCO BRL, Tokyo, Japan) in a total volume of 20 µl. After denaturation of mRNA at 70°C for 10 min, the reactions were preincubated for 10 min at 25°C and incubated at 42°C for 50 min. After denaturation at 70°C for 15 min, 0.2–1.0 µl portions; of the RT product cDNA were amplified using Ready To Go PCR Beads (Pharmacia Biotech, Uppsala, Sweden) containing ~1.5 U Taq DNA polymerase. Unless otherwise noted, 12.5 pmol primers, sense and antisense, were added; and the following profile was used: 1 cycle at 94°C for 3 min, followed by a set cycle of the following: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. All primers used are listed in Table 1. Primers for aggrecan, SOX9, and alkaline phosphatase (ALP) were designed from GeneBank sequences (accession nos. J03485, AF421878, and J03572). Cycle titrations with individual primer sets were simultaneously performed on aliquots containing the same cDNA reverse-transcribed from a single RNA preparation. The sequence of amplified products by RT-PCR were confirmed by direct sequence analysis.

Results

The type II collagen sponge used in these experiments was 3 mm thick and round with a diameter of 14 mm (Fig. 1A). A scanning electron micrograph of this sponge shows its porous structure. Average size of the pores in the sponge was 150 µm in diameter (Fig. 1B).

Results of study 1

Histology

Light microscopy showed that the primary cultured chondrocytes in S1 had entered the sponge pores, but the chondrocytes were not distributed uniformly in the sponge. The condensed chondrocytes synthesized more aggrecan stained by safranin O. High magnification showed that the cells in S1 seemed to be round (Fig. 2). Electron microscopy demonstrated that the cells in S1 seemed to be inactive in the sponge pores because the appearance of chondrocytes in S1 may be closer to the appearance of chondrocytes in articular cartilage (Fig. 3).

Table 1. Oligodeoxynucleotide primers used for the polymerase chain reaction

Target cDNA	Primer sequence (5′-3′)	Product size (bp)	PCR cycles
GAPDH[a]	5′:CACCATGGAGAAGGCCGGGG 3′:GACGGACACATTGGGGGTAG	418	20
COL1A1[b]	5′:CCTACCACTGCAAGAACAGC 3′:AACAGACAGGAGTACCACCG	375	30
COL2A1[a]	5′:AGGAGGCTGGCAGCTG 3′:CACTGGCAGTGGCGAG	204	30
COL10A1[a]	5′:ACAAAGAGCGGACAGAGACC 3′:AGAAGGACGAGTGGACATAC	442	24
COL11A1[c]	5′:CAGACTCAGAAGCCTCACAG 3′:TCCCTCTACAAACATACCAG	488	30
Aggrecan[d]	5′:TAGAGAAGAAGAGGGGTTAGG 3′:AGCAGTAGGAGCCAGGGTTAT	322	23
SOX9[d]	5′:CGGAACAGACTCACATCTCTCCTAATGC 3′:CGAAGGTCTCAATGTTGGAGATGACGTC	292	30
ALP[d]	5′:CGACACGGACAAGAAGCCCTT 3′:ACTTCTGTTCCTGCTCGAGGTTG	485	28
Osteocalcin[a]	5′:TCTGACAAACCTTCATGTCC 3′:AAATAGTGATACCGTAGATGCG	198	28

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COL1A1, pro-α1(I)collagen; COL2A1, pro-α1(II)collagen; COL10A1, pro-α1(X)collagen; COL11A2, pro-α1(XI)collagen; ALP, alkaline phosphatase; PCR, polymerase chain reaction

[a,b,c] Primer sequences reported in references 10, 11, and 12, respectively, were used

[d] Primers were constructed according to reported sequences as described in the text

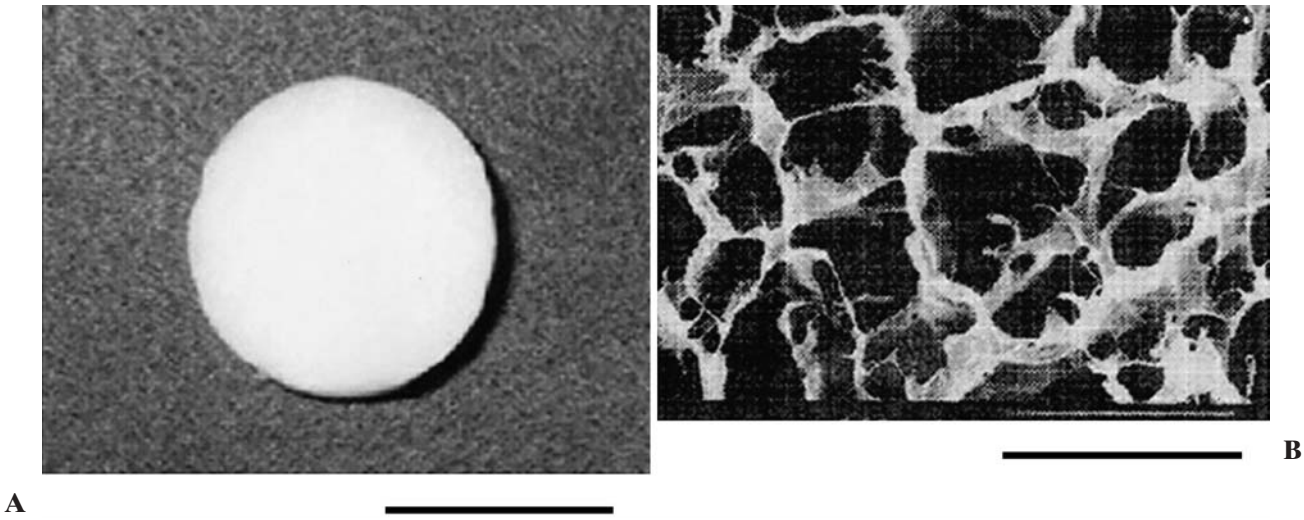

Fig. 1. **A** Macroscopic appearance of a type II collagen sponge. *Bar* 10 mm. **B** Scanning electron micrograph of a type II collagen sponge. *Bar* 200 μm

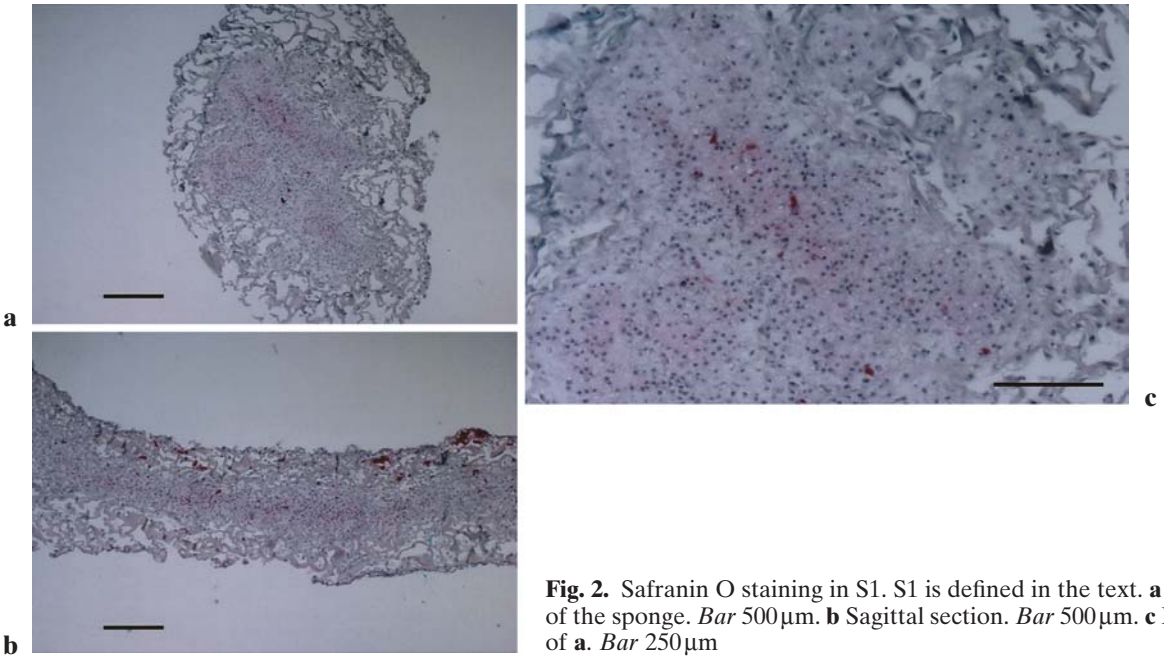

Fig. 2. Safranin O staining in S1. S1 is defined in the text. **a** Horizontal section of the sponge. *Bar* 500 μm. **b** Sagittal section. *Bar* 500 μm. **c** High magnification of **a**. *Bar* 250 μm

WST-8 assay

At the 5th and 10th days of culture, the proliferation of primary chondrocyte cultured in both monolayer and in the type II collagen sponge was significantly increased. At the 10th day of culture, the increase in the proliferation of cells in the type II collagen sponge was significantly less than those in monolayer culture (Fig. 4) ($P < 0.001$).

Gene expression

The expression of chondrogenic marker genes such as COL2A1, COL10A1, COL11A2, and aggrecan in P1 was lower than in P0. Also, expression of these genes in S1 was higher than in P1. Gene expression of COL2A1, COL10A1, and aggrecan in S1 was the same as in P0. The gene expression of COL11A2 in S1 was higher than in P0. The expression of osteogenic marker genes such as COL1A1 in P1 was higher than in P0. Also, expression of COL1A1 in S1 was lower than in P1 but was higher than in P0 (Fig. 5).

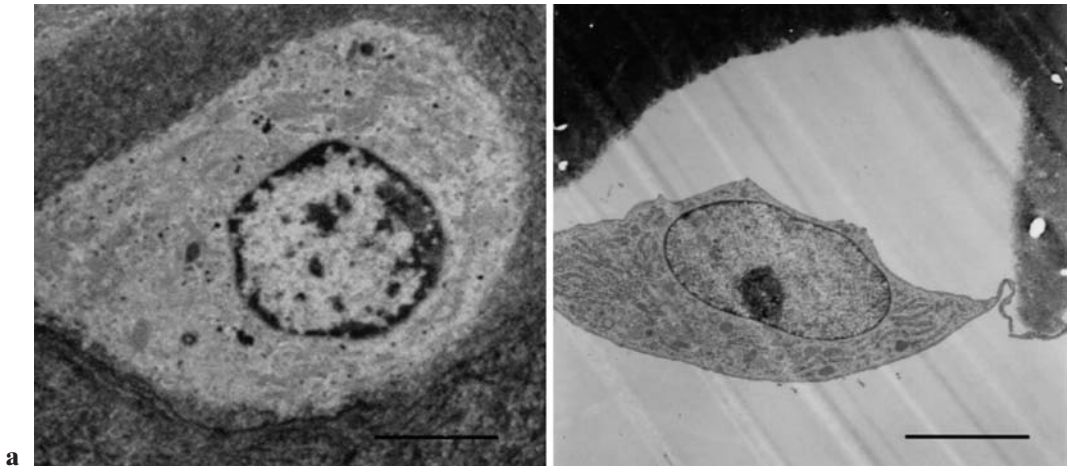

Fig. 3. Electron micrograph of S1 and normal articular cartilage. **a** Normal articular cartilage. *Bar* 5 μm. **b** S1 is defined in the text. **a**,**b**, *Bars* 5 μm

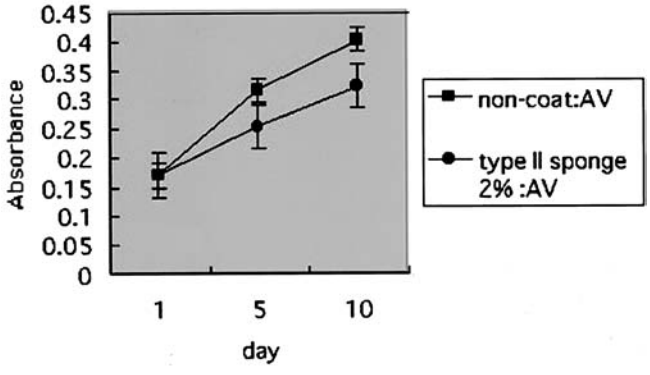

Fig. 4. WST-8 assay of cultured chondrocytes from monolayer culture and type II collagen sponge culture. Measurements were taken on days 1, 5, and 10 of culture. *Squares*, monolayer culture; *circles*, type II collagen sponge culture. *AV*, average

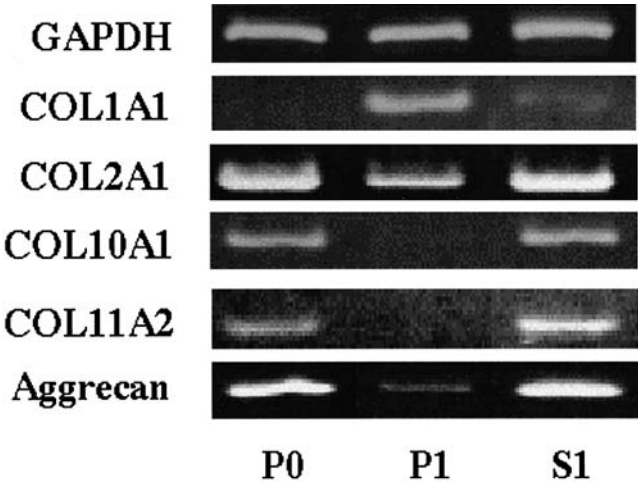

Fig. 5. Gene expression of primary cultured chondrocytes in type II collagen sponges. *P0*, *P1*, and *S1* are defined in the text. *COL1A1*, pro-α1(I)collagen; *COL2A1*, pro-α1(II) collagen; *COL10A1*, pro-α1(X)collagen; *COL11A2*, pro-α1(XI)collagen

Results of study 2

Histology

Light microscopy showed that the chondrocytes in P1r were distributed unevenly in the sponge pores, similar to the result for primary chondrocytes cultured in sponges. The chondrocytes in P1r synthesized an extracellular matrix, but the matrix was not stained by safranin O. The cells in P1r seemed to be round, similar to the cells in S1 (Fig. 6).

Gene expression

The expression of chondrogenic marker genes such as COL10A1 and aggrecan in P1r were higher than in P1 and P3. The expression of COL11A2 in P1r was higher than in P3 and was the same as in P1. The expression of COL10A1 in P1r was the same as in P0. The expression of COL11A2 and aggrecan in P1r was lower than in P0. The gene expression of COL2A1 and SOX9 decreased gradually with every passage (P0–P3). Expression of these genes in P1r was lower than in P0, P1, or P3. The ALP and osteocalcin genes were detected in P1r but not in P0, P1, or P3. The expression of osteogenic marker genes such as COL1A1 in P1r was lower than in P1 and P3 but higher than in P0 (Fig. 7).

Discussion

Primary chondrocytes

It is well known that chondrocytes in a monolayer culture easily lose their typical phenotype and tend to be-

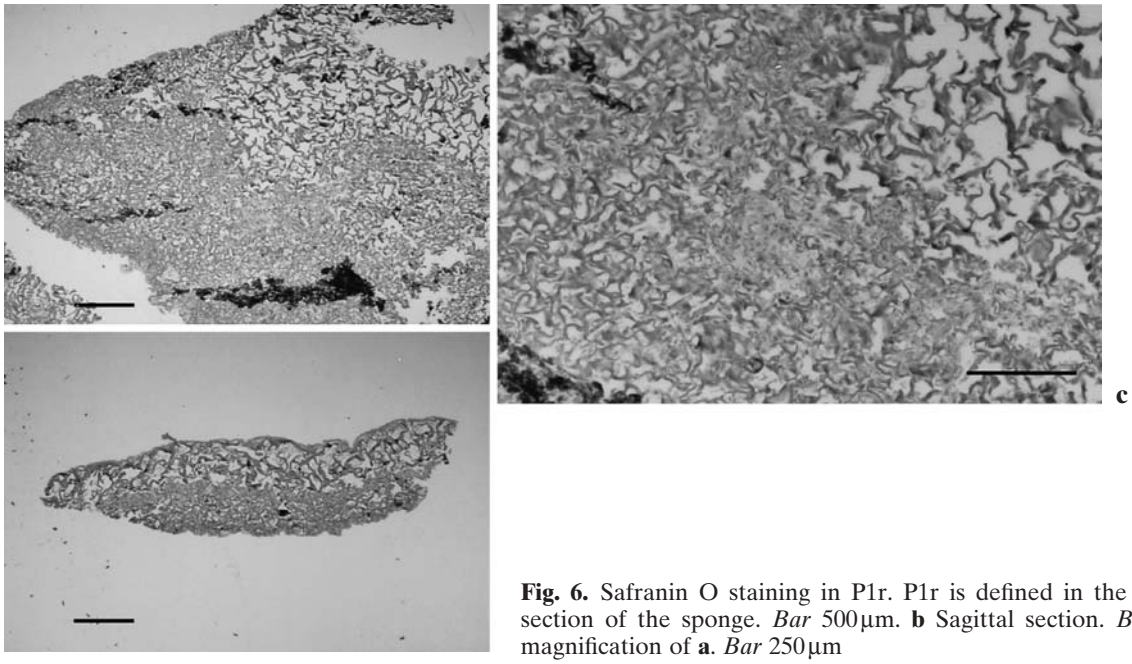

Fig. 6. Safranin O staining in P1r. P1r is defined in the text. **a** Horizontal section of the sponge. *Bar* 500 μm. **b** Sagittal section. *Bar* 250 μm. **c** High magnification of **a**. *Bar* 250 μm

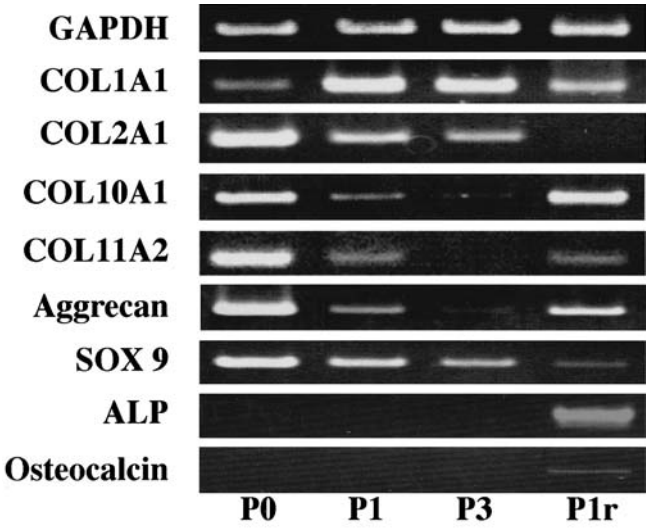

Fig. 7. Gene expression of dedifferentiated chondrocytes cultured for 4 weeks in type II collagen sponges. *P0*, *P1*, *P3*, and *P1r* are defined in the text. *ALP*, alkaline phosphatase

come dedifferentiated.[6,13] In this process, chondrocytes change from their original round morphology to a spindle fibroblast-like shape, lose their ability to express the genes of articular cartilage-specific extracellular matrices (ECM) such as type II collagen and aggrecan, and start to express the type I collagen gene. Previous studies have shown that 3-D cultures using agarose,[6,14] alginate,[15,16] and type I collagen gels[17,18] maintain the phenotype of articular chondrocytes in vitro. In this study, the primary cultured chondrocytes seeded in type II collagen sponges synthesized a rich ECM that stained with safranin O. The cultured chondrocytes in sponges were round. Electron microscopy demonstrated that chondrocytes in sponges seemed to be as inactive as articular chondrocytes in situ. PCR analysis showed that chondrocytes seeded in type II collagen sponges maintained COL2A1, COL10A1, COL11A2, and aggrecan gene expression. These results indicate that 3-D culture of primary chondrocyte cells in the type II collagen sponge maintained the chondrocyte phenotype similar to the 3-D culture using alginate,[15,16] agarose,[13,14] or type I collagen scaffolds.[17,18]

Previous studies have reported favorable effects when using type II collagen for cartilage engineering.[19] It is reported that type II collagen scaffolds increase proteoglycan synthesis.[19] Our result showed that the ability of chondrocytes to proliferate in the type II collagen sponge was much less than in monolayer culture, but the type II collagen sponge could maintain the chondrocyte phenotype. These results suggest that the type II collagen sponge is a good scaffold for producing cartilage-like tissue in vitro when using sufficient number of primary chondrocytes.

Electron microscopy shows that chondrocytes in the type II collagen sponges seemed to be inactive in the sponge pores. In general, it is known that articular cartilage has poor development of subcellular organelles such as the Golgi apparatus and endoplasmic reticulum. Histologically, in articular cartilage chondrocytes do not show mitotic activity and seem to be somewhat inactive. On the other hand, growth plate cartilage develops subcellular organelles and ECM, and

chondrocytes show mitotic activity. In this study, it seems that the appearance of chondrocytes in type II collagen sponge may be closer to the appearance of chondrocytes in articular cartilage because in the type II collagen sponges they have poor development of subcellular organelles.

Redifferentiation

Previous reports have described various methods for cartilage tissue generation using chondrocyte transplantation. Recently several matrix-based autologous chondrocyte transplantation procedures have been developed, and clinical results have been improved.[20] However, the volume harvested from articular cartilage is limited. It is difficult to maintain the chondrocyte phenotype and increase the cell number rapidly. Therefore, monolayer culture is used to increase the cell number for clinical application, even though chondrocytes must be dedifferentiated.

Several studies have suggested that dedifferentiated chondrocytes can restore the chondrocyte phenotype when they are seeded at high density in a 3-D matrix or cultured at high density as pellet cultures[21,22] and suspension cultures. Some studies have investigated the morphological change, GAG production, and biosynthetic activity in 3-D cultures using a type II GAG matrix or type I/III collagen matrix; and they have shown that these cultures induced redifferentiation of dedifferentiated chondrocytes.[23,24] However, the expression and synthesis of type II collagen in redifferentiated chondrocytes was not evaluated. Other studies showed that redifferentiated chondrocytes cultured in hyaluronan-based biomaterials and a PLGA-collagen matrix reexpressed type II collagen[6,25]; however, the expression and synthesis level of type II collagen was not compared to that in primary cultured chondrocytes. It was still unclear whether redifferentiated chondrocytes sufficiently restored the phenotype of articular cartilage.

Our results demonstrated that the COL II, XI, and SOX9 gene expression in dedifferented chondrocytes cultured in the type II collagen sponge decreased when compared to the expression in primary cultured chondrocytes. These cells in type II collgen sponge also expressed ALP and osteocalcin genes. These results suggest that the dedifferentiated chondrocytes differentiated to hypertrophic chondrocytes. The environment of collagen sponges may not be proper for making the dedifferentiated chondrocytes restore the phenotype of permanent cartilage. It is known that the chondrocytes isolated from rib cartilage can differentiate into hypertrophic chondrocytes and synthesize ALP and COL X collagen in a certain environment.[26] The characteristics of these chondrocytes from rib cartilage are not always identical to those of chondrocytes from articular cartilage, such as distribution of insulin-like growth factor-I mRNA.[27] Further investigation of the redefferentiation of dedifferentiated chondrocytes from articular cartilage is required.

Conclusions

Our findings suggest that we should use primary chondrocytes instead of the cells amplified in monolayer culture to create hyaline cartilage-like tissue.

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