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Effect of type I and type II collagen sponges as 3D scaffolds for hyaline cartilage-like tissue regeneration on phenotypic control of seeded chondrocytes in vitro

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Abstract

Biodegradable polymers have been adopted as materials of three-dimensional (3D) scaffolds for regenerating cartilage-like tissues by using tissue engineering. On the other hand, it is well recognized that phenotypic control of seeded chondrocytes is indispensable for constructing not fibrous but hyaline cartilage-like tissue. According to the context, type II collagen is focused as a candidate of scaffold materials for articular cartilage regeneration. This study compared viability and gene expressions of seeded chondrocytes in type I and type II collagen sponges. The results show that the seeded chondrocytes were well distributed and alive in the both sponges in 20 days of culture, and no significant difference in type I, type II and aggrecan gene expressions was found between both sponges. The effect of type II collagen as a scaffold material having outside in-signals on regeneration of hyaline cartilage-like tissue was not detected at least at the initial stage of tissue regeneration.
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Keywords: Type II collagen; Cartilage regeneration; Phenotype; Chondrocyte; Gene expression

1. Introduction

Articular cartilage is well known to be hardly healed in the case of defects because of its avascular nature and low mitotic activity of parenchymal cells [1]. For the purpose to treat clinically the defects of articular cartilage, many trials have been done by using tissue engineering approaches. As cell sources, allogenic and autologous cells isolated from articular cartilage [2,3], pericondrium [4] and mesenchymal stem cells [5] from bone marrow, have been adopted for that purpose. It is also well recognized that three-dimensional (3D) scaffolds are useful for regenerating cartilage-like tissue at high efficiency whether it is to be regenerated in vivo or in vitro.

Naturally derived biodegradable collagen and synthetic biodegradable poly(alpha ester)s, such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer of poly(D,L-lactic-co-glycolic acid) (PLGA), have been widely used to construct temporary scaffolds for tissue engineering [6–10]. PLA, PGA and PLGA are biocompatible and are among few synthetic polymers approved by the Food and Drug Administration (FDA) for certain human clinical application such as surgical sutures and some implant devices. In our previous study, we have construct a biodegradable hybrid sponge nested with collagen microsponges, so that the hybrid sponge could have enough initial mechanical strength at the same time with having cell recognition signal from collagen [11].

Articular chondrocytes are known to loose their phenotype according to subculturing and proliferation in vitro, loosing type II collagen expression and starting

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producing type I collagen. For constructing articular cartilage-like tissue, it is indispensable that chondrocytes could produce not type I collagen, but type II collagen, avoiding to construct fibrous cartilage. As an approach to controlling phenotype of cultured chondrocytes in scaffolds, Nehrer et al. [12,13] have tried to construct a sponge of type II collagen with glycosaminoglycan, and compared the morphology of seeded chondrocytes and DNA and glycosamionoglycan contents in the scaffold with those in type I collagen sponge. However, phenotypic expression of cultured chondrocytes is still unknown at the molecular level in the sponge composed of only type II collagen.

The objective of this study was to construct both type I and type II collagen sponges, and evaluate viabilities and type I, type II collagen gene expressions of chondrocytes cultured in type I and type II collagen sponges, respectively.

2. Materials and methods

2.1. Preparation of collagen sponges

Acid collagen solution (pH 2.8–3.0, 10 mg/ml) was prepared by treating bovine skin for type I collagen, or bovine nose for type II collagen with pepsin after delipidation, decalcification, and removal of mucopolysaccharides. Each solution, which was dispensed in 24-well plate (0.6 ml/well) was frozen at -20 °C for 5 days, and freeze-dried for 3–5days. The freeze-dried samples were cross-linked by using a UV cross-linker (SPECTROLINKER XL-1500, 254 nm, 15 w $\times$ 6) at 9000×100 mJ/cm^2, followed by thermal dehydration cross-linking (110 °C, 120 min).

2.2. Cell culture

Chondrocytes were prepared from elbow articular cartilage of 4-week-old calves obtained from a local abattoir within 3 days after slaughter. The joints were exposed under aseptic conditions, and the cartilage was collected in slices with a surgical blade. The slices were rinsed with cold phosphate-buffered saline (PBS) two times, and minced into flakes of about 1 mm^3 and digested with a solution of 0.2% collagenase (CLS 1, Worthington Biochemical, USA) in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, NY, USA) with gentle shaking overnight at 37 °C. The cells were rinsed in PBS two times with centrifugation. The cells were suspended in DMEM containing 10% fetal bovine serum and 2% antibiotic. Then the sponges were put into the silicone tubes the inner diameter of which was almost the same with outer diameters of the sponges sterilized, and 200 ml of the cell suspension (5×10^6 cells/unit) was poured into each tube. The medium was exchanged every 3 days.

2.3. RNA extraction

Cells were isolated from cultured sponges by collagenase for 1 h at 37 °C. Then, the cells were solubilized with 1 ml Trizol reagent (Invitrogen Life Technologies, Rockville, MD). Each lysate was mixed with 200 μl of chloroform and was centrifuged at $12{,}000 \times g$ for 15 min at 4 °C. The clear supernatant was transferred to a clean tube and was mixed with 0.5 ml isopropanol. The RNA was precipitated with 70% ethanol and was dissolved in RNAse-free water.

2.4. Reverse transcriptase/polymerase chain reaction (RT-PCR)

cDNA synthesis was performed with reverse transcriptase (SuperScript II, Invitrogen Life Technologies). The reaction was performed in a 20-μl volume containing 1 μg of total RNA, 10 mM dNTPs and 500 μg/ml oligo (dT). The tubes were incubated at 65 °C for 5 min and were cooled quickly on ice for 2 min. Then 5 $\times$ First-Strand Buffer (Invitrogen) and 0.1 M DTT (Invitrogen) were added. The tubes were incubated at 42 °C for 5 min and then 1 μl of SuperScript II was added. Following 15 min heat inactivation at 70 °C, the cDNAs were amplified in a 20-μl reaction volume containing 1 μl of cDNA, 0.1 μl of TaKaRa Ex Taq HS (TaKaRa, Shiga, Japan), 2 μl of 10 $\times$ Ex Taq Buffer (TaKaRa), 1.6 μl of dNTP Mixture (TaKaRa), 1 μl of forward and 1 μl of reverse primers. Polymerase chain reaction (PCR) primer sequences are presented in Table 1. The PCR profile included initial denaturation at 95 °C for 15 min, followed by 25 cycles (type I collagen and type II collagen) or 28 cycles (GAPDH and aggrecan) of denaturation at 94 °C for 30 s, subsequent annealing at 67 °C (GAPDH and aggrecan) or 55 °C (type I collagen and type II collagen) for 30 s, and extension at 72 °C (for 30 s). The final cycle (at 72 °C) included 7 min for extension. The PCR products were visualized on ethidium-stained 2.0% agarose gel. The band images were semi-quantified using NIH image software.

2.5. SEM observation

The structures of both type I and II collagen sponges were observed by scanning electron microscope (SEM). The cross-sections were made by cutting the sponges with a

Table 1
Primer sequences used in RT-PCR

Gene	Primer	Sequence
GAPDH	Forward	5′ -ACCACAGTCCATGCCATCAC-3′
	Reverse	5′ -TCCACCACCCTGTTGCTGTA-3′
Type I collagen	Forward	5′ -ATGCTCAGCTTTGTGGATACGCGG-3′
	Reverse	5′ -AGGAAAGCCACGAGCACCCTGTGG-3′
Type II collagen	Forward	5′ -AGCGTCCCCAAGAAGAACTGGTGG-3′
	Reverse	5′ -GTCCACACCGAATTCCTGCTCGGG-3′
Aggrecan	Forward	5′ -CACTGTTACCGCCACTTCCC-3′
	Reverse	5′ -GACATCGTTCCACTCGCCCT-5′

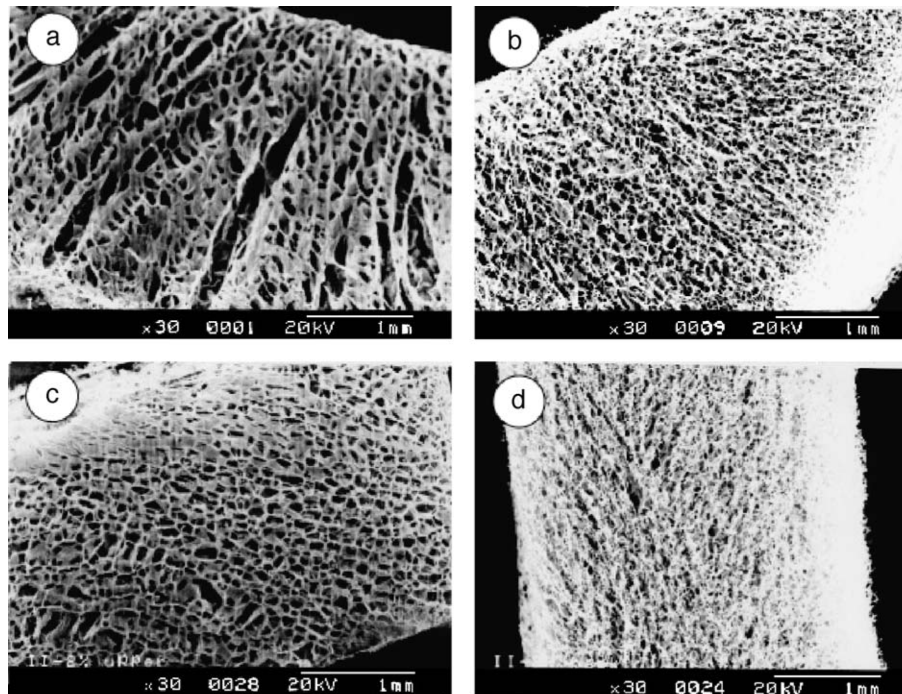

Fig. 1. SEM photographs of type I and type II collagen sponges. (a) Upper side of type I collagen sponge (× 30), (b) bottom side of type I collagen sponge (× 30), (c) upper side of type II collagen sponge (× 30), (d) bottom side of type II collagen sponge (× 30).

razor blade. The samples were coated with gold using a spatter coater (E-101, HITACHI). Then, the samples were observed with a scanning electron microscope (S-21 00A, HITACHI).

2.6. Histological staining

The samples were fixed in neutral buffered formalin, and embedded in paraffin. The embedded samples were sec-

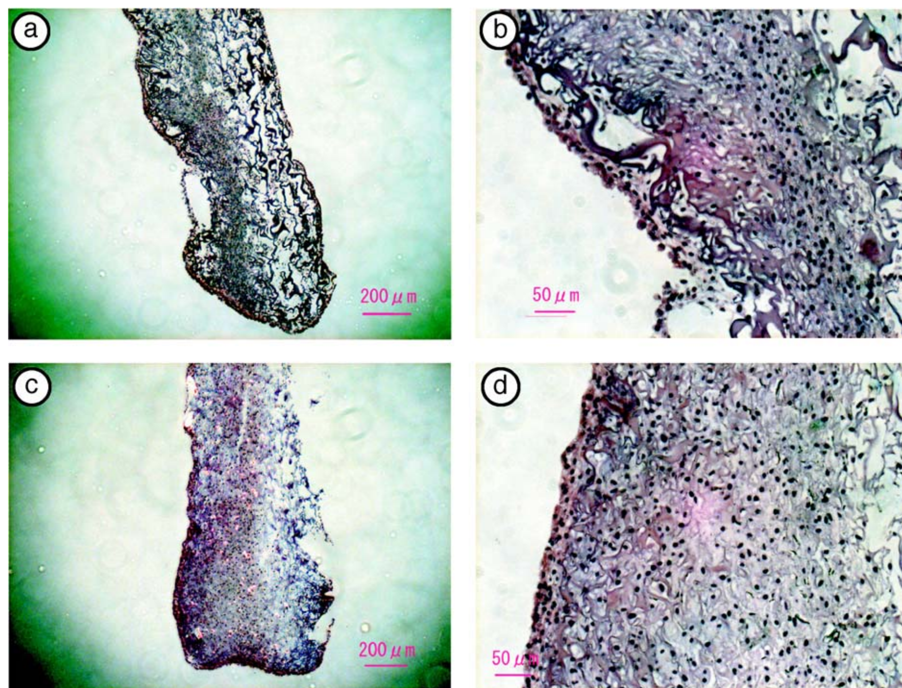

Fig. 2. HE stain of sectioned collagen sponges with seeded bovine chondrocytes. (a) Cross-section of type I collagen sponge (low magnification), (b) cross-section of type I collagen sponge (high magnification), (c) cross-section of type II collagen sponge (low magnification), (d) cross-section of type II collagen sponge (high magnification). Each sponge was cultured with seeded bovine articular chondrocytes during 20 days.

tioned with a microtome at a thickness of 5 μm. The cross-sections were pretreated with ethanol and hexane, and stained with hematoxylin and eosin.

3. Results and discussion

The SEM photographs show the microstructures of the sponges as shown in Fig. 1. Both the sponges had interpores so that the seeded cells could be penetrated into the center of the sponges. The size of pores in the type II collagen sponge was similar with that in the type I collagen sponge. However, the structure and the size at the upper side of the sponges were different from those at the reverse side of the sponges in the cases of both type I and type II collagen sponges. This difference in structure and size was thought to be caused by the fabrication method of the sponges, where the sponges were fabricated with a freeze-dried method in the 24-well plate, and the freeze-dried condition at the upper side was not identical with that at the bottom side. The similar phenomenon was described also by Nehrer et al. [12] As far as freeze-dry method is adopted for generating sponge-like structure, this difference in pore size might be inevitable. The novel method where collagen gels can be uniformly frozen should be developed for creating collagen sponges with homogeneous structure.

Fig. 2 shows the distribution of seeded chondrocytes at low magnification after 5 days of culture. The seeded chondrocytes were well distributed in any portions of both sponges, although the cell density was not uniform due to the difference in microstructure between upper and reverse sides. The seeded chondrocytes were found to be alive and neither in necrosis nor apoptosis because of sane morphology of their nuclei even after 20 days of culture as shown Fig. 2.

The results indicate that the both sponges had interpores at any parts which were necessary for 3D scaffolds in tissue engineering and made seeded cells adhering and producing matrices even in the center of the sponges.

Fig. 3a shows the time-course changes of type I collagen expressions of the seeded chondrocytes in the type I and type II collagen sponges, respectively, where the bands by RT-PCR were semiquantified using NIH-image. The type I collagen expressions were decreased according to culture time in both sponges, and no significant difference in the expressions was found between the sponges. Fig. 3b shows the time-course changes of type II collagen expressions of the seeded chondrocytes in the type I and type II collagen sponges, respectively. The type II collagen expressions were decreased in 2 weeks of culture and recovered to initial revels in 4 weeks of culture in both sponges, and no significant difference in the expressions was found between the sponges. Fig. 3c shows the time-course changes of aggrecan expressions of the seeded chondrocytes in the type I and type II collagen sponges, respectively. The aggrecan expression was slightly increased with culture

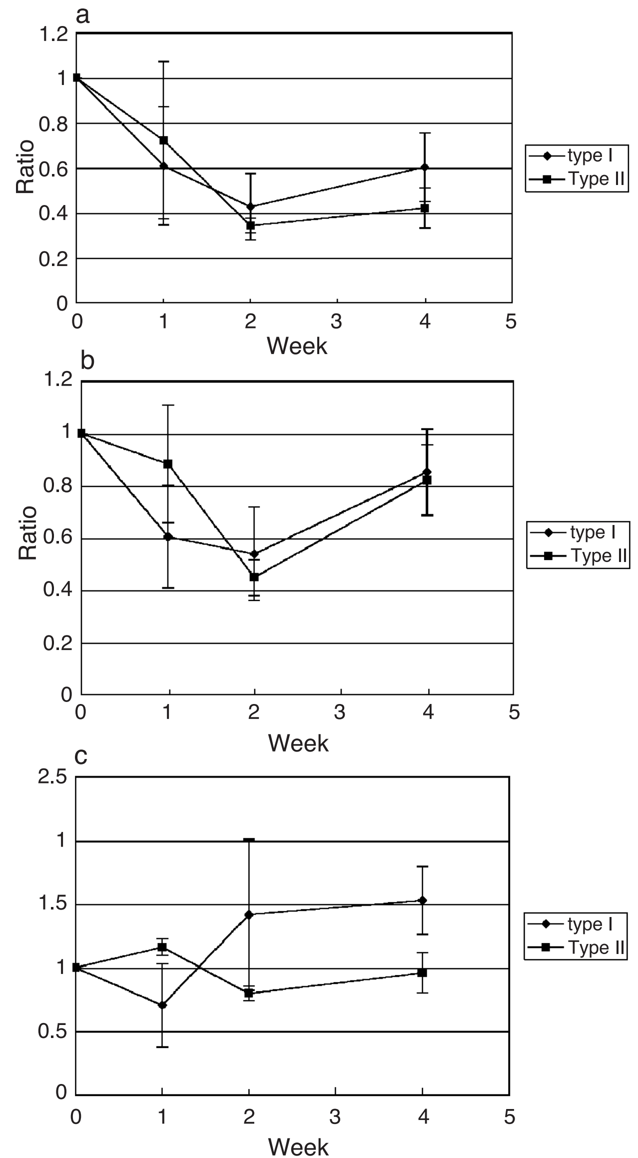

Fig. 3. Time-course changes of type I, type II collagen and aggrecan gene expressions semiquantified by using NIH-image. (a) Type I collagen gene expression, (b) type II collagen gene expression, (c) aggrecan gene expression. The RT-PCT was independently done by using three collagen sponges with seeded chondrocytes at each time point. (mean ± S.E.M., $n=3$) The vertical axis represents a ratio of semiquantified value of targeted gene expression to that of GAPDH.

time in type I collagen sponge, while the expression was not changed throughout culture time in type II collagen sponge.

Type I collagen is cytocompatible and widely used in the field of tissue engineering. However, it is not certain whether type I collagen is an adequate scaffold material or not for constructing hyaline cartilage-like tissue because an essential element of articular cartilage matrices is not type I but type II collagen. This study shows that the type I collagen sponge had equivalent effect with the type II collagen sponge in cell-seeding efficiency and type I, type II and aggrecan gene expressions. One of our studies also

shows redifferentiation of dedifferentiated bovine chondrocytes when cultured in vitro in a PLGA-type I collagen hybrid mesh [14]. Those results might indicate that the type I collagen sponge was as useful as type II collagen sponge for constructing hyaline cartilage-like tissue at least at the initial stage of tissue regeneration.

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