Redifferentiation of dedifferentiated bovine chondrocytes when cultured in vitro in a PLGA–collagen hybrid mesh

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Abstract **Bovine articular chondrocytes dedifferentiated and lost their ability to express articular cartilage-specific extracellular matrices such as type II collagen and aggrecan when cultured in a culture flask during in vitro multiplication. A poly(DL-lactic-co-glycolic acid) (PLGA)–collagen hybrid mesh was prepared and used to redifferentiate the dedifferentiated cells. The two passaged dedifferentiated chondrocytes were seeded in a PLGA–collagen hybrid mesh and cultured in vitro in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The cells adhered to the hybrid mesh, distributed evenly, and proliferated to fill the spaces in the scaffold. The gene expression of type I collagen, type II collagen, and aggrecan was analyzed after the cells were cultured in the hybrid mesh for 2–12 weeks. The expression of the gene encoding type I collagen was downregulated, whereas those of type II collagen and aggrecan were upregulated. Histological examination by hematoxylin–eosin and safranin O/fast green staining indicates that the cells regained their original round morphology. In addition, a homogeneous distribution of articular cartilage extracellular matrices was detected around the cells. These results suggest redifferentiation of the differentiated chondrocytes in the hybrid mesh. The hybrid mesh, which facilitated the redifferentiation of the dedifferentiated multiplied chondrocytes, would be an effective scaffold for the assembly of cells to regenerate three-dimensional cartilaginous tissue.**

Key words: Redifferentiation; Chondrocyte; Cartilage; Tissue engineering; Hybrid mesh

1. Introduction

Autologous transplantation of articular chondrocytes using tissue engineering has undergone rapid development for treating articular cartilage defects [1–3]. Cells must be multiplied in vitro by a monolayer culture because of the very limited amount of donor biopsy material available. However, monolayer culture multiplication leads to dedifferentiation. This is a process during which chondrocytes change from their original round morphology to a spindle fibroblast-like shape, lose their ability to express articular cartilage-specific extracellular matrices (ECM) such as type II collagen and aggrecan, and produce fibroblast-specific ECM, type I collagen [4–6]. To be used for clinical applications, dedifferentiated cells must be redifferentiated. Different methods have been tried to induce redifferentiation of dedifferentiated articular chondrocytes to regain their cartilaginous features. Application of growth factors and creation of an environment supporting a spherical morphology such as in pellets or polymer gels have often been used to fulfill this purpose [7–11].

A three-dimensional biodegradable porous scaffold plays a vital role in a tissue engineering approach to accommodate enough cells and assemble them into three-dimensional tissue [12]. An optimal scaffold should be able to facilitate redifferentiation of the multiplied dedifferentiated chondrocytes into cartilaginous phenotypes while assembling the cells into three-dimensional tissue. In the present study, a scaffold of this type was developed by hybridizing collagen microsponges within a synthetic knitted mesh made of poly(DL-lactic-co-glycolic acid) (PLGA) and used for in vitro redifferentiation of dedifferentiated bovine chondrocytes. Two passaged dedifferentiated chondrocytes were seeded in the hybrid mesh and cultured in vitro in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The gene expression of type I collagen, type II collagen, and aggrecan was examined by Northern blot analysis; the morphological change and cartilaginous ECM deposition were evaluated by histological and immunohistological staining.

2. Materials and methods

2.1. Cell cultures

Bovine chondrocytes were isolated from articular cartilage derived from the shoulder of a 4-week-old calf by digestion with 0.2% collagenase type II (Worthington Biochemical, Lakewood, NJ, USA). The cells were seeded in T-75 culture flasks and subcultured in DMEM supplemented with 10% fetal bovine serum, 4500 mg/l glucose, 584 mg/l glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 0.1 mM non-essential amino acids, 0.4 mM proline, and 50 mg/l ascorbic acid (culture medium) under an atmosphere of 5% CO_2 at 37°C. The cells were passage cultured for up to four passages as they became confluent. Meanwhile, after two passages, the cells were cultured in a biodegradable three-dimensional porous scaffold, a PLGA–collagen hybrid mesh. To accomplish this, after two passages, the chondrocytes were collected and suspended in culture medium at a density of 5×10^6 cells/ml. The chondrocyte suspension was dropped onto the PLGA–collagen hybrid mesh and cultured in culture medium under an atmosphere of 5% CO_2 at 37°C.

2.2. Scaffold fabrication

The hybrid mesh was prepared by forming collagen microsponges in the openings of a knitted mesh of PLGA [13]. Briefly, a Vicryl knitted mesh made of polylactin 910 (a 90:10 copolymer of glycolic acid and lactic acid) was immersed in an aqueous solution of collagen

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in acetic acid (bovine, type I, pH 3.2, 0.5 wt%, Koken, Tokyo, Japan) and frozen at −80°C for 12 h. The mesh was then freeze-dried under a vacuum of 0.2 Torr for 24 h and further cross-linked by treatment with glutaraldehyde vapor saturated with a 25% glutaraldehyde aqueous solution by placing the mesh and the glutaraldehyde aqueous solution in a closed box at 37°C for 4 h. Subsequently, the mesh was treated with a 0.1 M glycine aqueous solution to block unreacted aldehyde groups and freeze-dried after complete washing with deionized water. The microstructure of the hybrid mesh was observed by scanning electron microscopy (SEM). The hybrid mesh was 200 μm thick. The meshes were cut into 1.0 cm diameter disks and were then sterilized with ethylene oxide for cell culture.

2.3. Northern blot analysis

In up to four passages, the cells after each passage in the monolayer culture were directly dissolved in Isogen reagent (Nippongene, Toyama, Japan). Cells cultured in the hybrid mesh for 0, 2, 4, or 12 weeks were frozen in liquid nitrogen and powdered by an electric crusher. The sample powder was dissolved in Isogen reagent. The total RNA was extracted with chloroform, precipitated with isopropanol, and washed with 70% ethanol. After recovery in DEPC-treated water, the total RNA was determined and stored at −80°C. The total RNA (0.2 μg) was electrophoresed through a 1.2% agarose gel containing formaldehyde, blotted onto Hybond N+ membrane (Amersham Pharmacia, Amersham, UK), and cross-linked by exposure to UV light for 2 min. After prehybridization with hybridization buffer (5×SSC, 0.1% (w/v) SDS, 5% (w/v) dextran sulfate sodium salt, and 5% (v/v) blocking reagent (Amersham Pharmacia)) for 1 h, the membrane was incubated overnight with cDNA probes for type IA2 bovine collagen, type IIA bovine collagen, and bovine aggrecan, which were labeled with fluorescein-11-dUTP at 65°C in hybridization buffer. After hybridization, the membrane was washed once with 1×SSC/0.1% (w/v) SDS at 65°C for 15 min and once in 0.1×SSC/0.1% (w/v) SDS at 65°C for 15 min. After being incubated with blocking reagent in buffer solution (100 mM Tris–HCl, 300 mM NaCl, pH9.5) at room temperature for 1 h, the membrane was incubated with alkaline phosphatase-labeled anti-fluorescein antibody in buffer solution containing 0.5% bovine serum albumin at room temperature for 1 h. After three 10 min washes, with buffer solution containing 0.3% Tween-20, the bands were reacted with detecting reagent (CDP-Star, Amersham Pharmacia) for 5 min and exposed to Kodak X-OMAT X-ray film (Eastman Kodak, Rochester, NY, USA). The experiment was conducted with two samples, each of which showed a similar trend.

2.4. Histological and immunohistological staining

The cells cultured in the hybrid mesh were fixed in neutral buffered formalin, embedded in paraffin, and sectioned (10 μm thick). The cross-sections were stained with hematoxylin–eosin and safranin O/fast green. The type II collagen was immunohistologically stained using rabbit anti-bovine type II collagen polyclonal antibody (Chemicon International, Temecula, CA, USA) and DAKO LSAB Kit Peroxidase (Dako, Carpinteria, CA, USA) according to the instructions accompanying the kit.

3. Results

Chondrocytes isolated from bovine articular cartilage were subcultured in a monolayer in DMEM supplemented with 10% fetal bovine serum. The chondrocytes after two passages were seeded in the PLGA–collagen hybrid mesh and cultured in vitro. The hybrid mesh was prepared by forming cobweb-like collagen microsponges in the openings of a knitted mesh made of PLGA (Fig. 1a). Observation by a phase contrast microscope indicates that the cells were entrapped by the collagen microsponges in the hybrid mesh immediately after cell seeding (Fig. 1b). Chondrocytes adhered to the cobweb-like hybrid mesh and showed uniform distribution on the mesh. They proliferated and regenerated cartilaginous matrices filling the void spaces in the hybrid mesh (Fig. 1c,d).

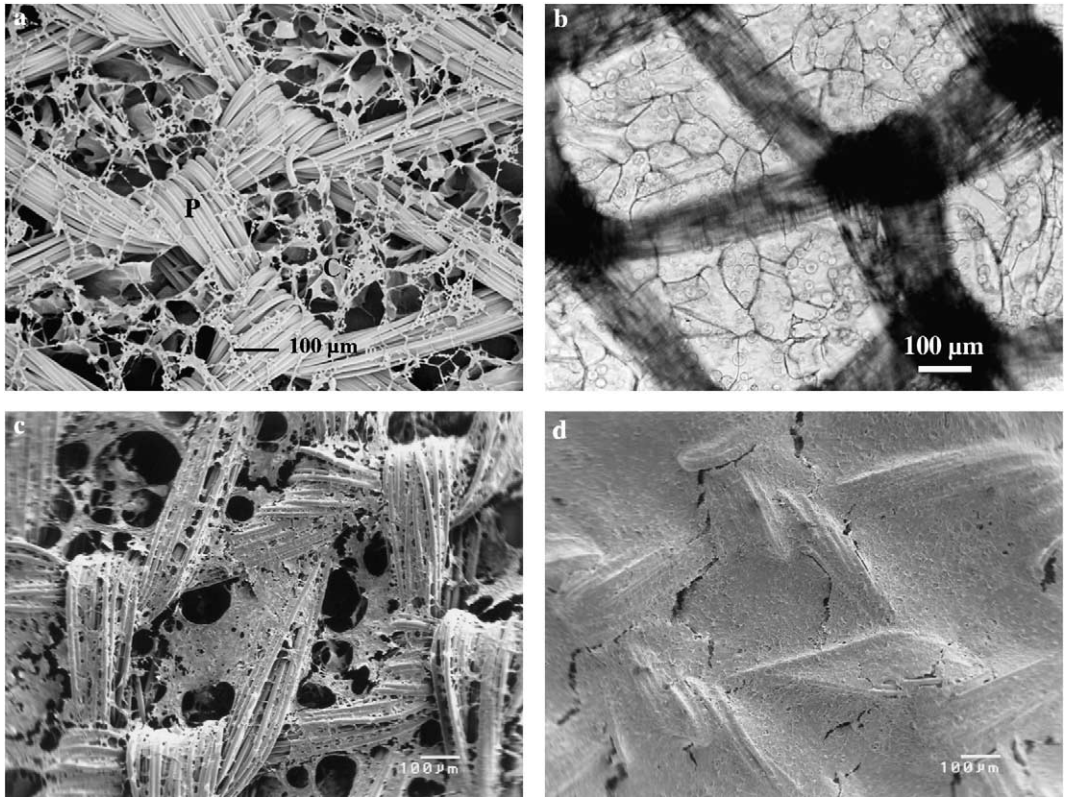

Fig. 1. SEM micrograph of PLGA–collagen hybrid mesh (a), phase contrast micrograph of two passaged cells immediately after seeding in the hybrid mesh (b), and SEM micrographs of chondrocytes cultured in the hybrid mesh for 1 week (c) and 4 weeks (d). P indicates PLGA knitted mesh and C indicates collagen microsponges.

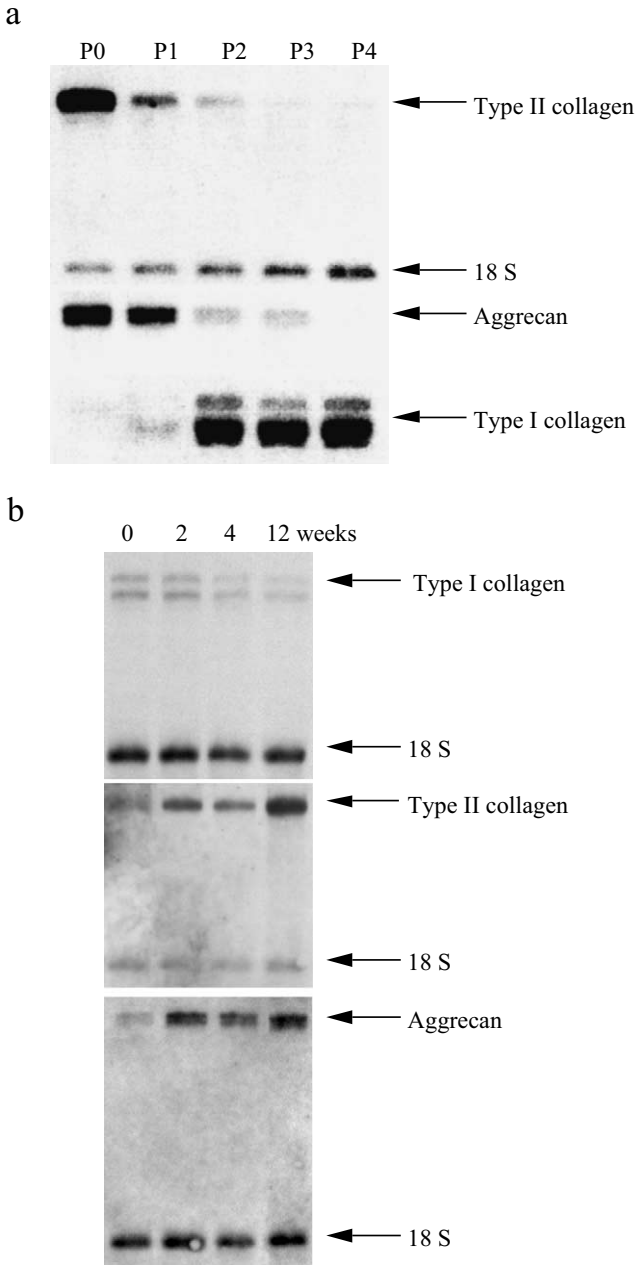

Fig. 2. Northern blot analysis of the genes encoding type I collagen, type II collagen, and aggrecan of bovine chondrocytes cultured in monolayer for 0, 1, 2, 3, and 4 passages (a), and two passaged chondrocytes cultured in the hybrid mesh for 0, 2, 4, and 12 weeks (b).

The gene expression of type I collagen, type II collagen, and aggrecan of chondrocytes cultured both in monolayer and in the hybrid mesh was examined by Northern blot analysis. When cultured in monolayer, the expression of the genes encoding type II collagen and aggrecan was weakened with the increase of passage and dramatically decreased after two passages. Meanwhile, the gene encoding type I collagen became detectable and was strongly expressed after two passages (Fig. 2a). These results indicated the chondrocytes dedifferentiated after two passages. When the dedifferentiated chondrocytes were seeded and cultured in the hybrid mesh, the expression of mRNAs for type II collagen and aggrecan was upregulated and that of type I collagen mRNA was downregulated. After culture in the hybrid mesh for 12 weeks, the gene expression of type I collagen was very weakly detectable, but those of type II collagen and aggrecan reached their highest levels (Fig. 2b).

The morphological changes of chondrocytes were examined by histological staining. When cultured in monolayer, the chondrocytes showed spread spindle-like morphology. Histological examination of the cells cultured in the hybrid mesh using hematoxylin–eosin staining indicates a uniform spatial cell distribution in the mesh (Fig. 3). The chondrocytes remained viable and secreting extracellular matrix components to form homogeneously compact cartilage tissues. The chondrocytes showed a round morphology. The safranin O-positive stain indicated that glycosaminoglycans were abundant and homogeneously distributed around the cells. Immunohistological staining with an antibody to type II collagen showed a homogeneous extracellular staining for type II collagen. The histological and immunohistological results coincided with the results of gene expression, which indicates the redifferentiation of the dedifferentiated cells in the hybrid mesh.

4. Discussion

Recently, much attention has been focused on cartilage tissue engineering and chondrocyte transplantation. For autologous transplantation, cells must multiply so the required cell amount is obtained. In the process of multiplication in a monolayer culture, dedifferentiation seems to be unavoidable. It remains a great challenge to be able to redifferentiate the dedifferentiated cells to regain their original phenotype.

Here, we reported on a method to redifferentiate dedifferentiated chondrocytes using a PLGA–collagen hybrid mesh as a porous scaffold. Bovine chondrocytes grown in a monolayer culture dedifferentiated and gradually lost their ability to express type II collagen and aggrecan. Instead the gene encoding type I collagen was expressed. However, when the two passage dedifferentiated chondrocytes were seeded in the PLGA–collagen hybrid mesh and cultured in vitro in DMEM containing 10% fetal bovine serum, the cells were redifferentiated into their cartilaginous characteristics. The redifferentiation of dedifferentiated chondrocytes was demonstrated by the results of both Northern blot analysis and histological examination. Northern blot analysis of total RNA indicates an increase in type II collagen and aggrecan mRNA expression. In contrast, the level of type I collagen was gradually reduced. Histological examination demonstrated the morphological change from spindle-like to round and the deposition of abundant safranin O-positive cartilaginous ECM surrounding the cells. Immunohistological staining further demonstrated the existence of type II collagen and its homogeneous distribution.

The effects of the hybrid mesh on the redifferentiation of dedifferentiated expanded chondrocytes may be explained by the even distribution of a sufficient number of cells in the hybrid mesh. Such a situation is similar to that of a high-density cell culture, which has been reported to facilitate redifferentiation of dedifferentiated chondrocytes [14]. Cell–cell interactions play important roles in chondrocyte differentiation and phenotype expression [15]. The cell distribution in the hybrid mesh meets the requirements for the redifferentiation of seeded cells. As revealed by SEM observation, the collagen microsponges, which formed in the synthetic PLGA

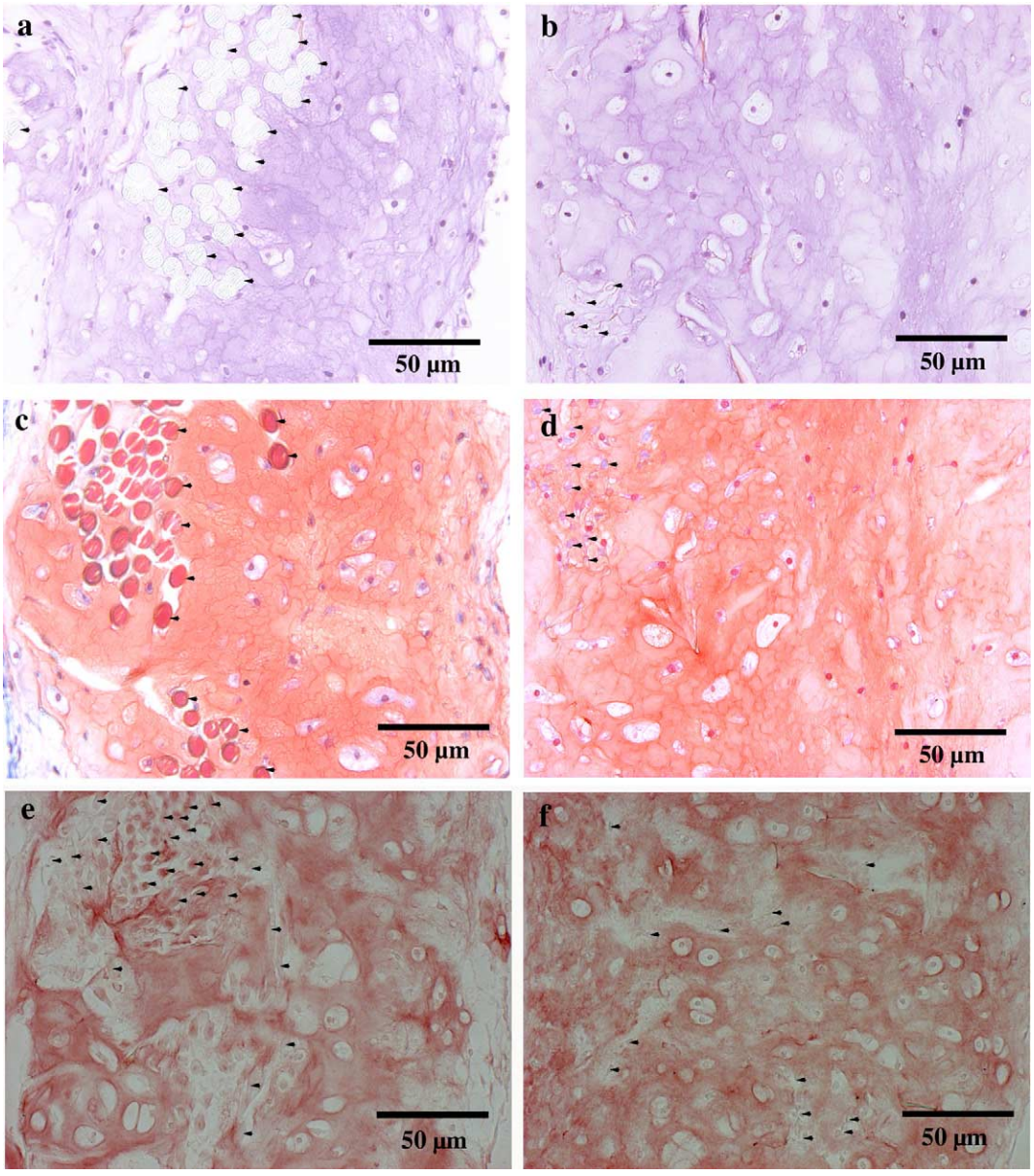

Fig. 3. Hematoxylin–eosin (a,b) and safranin O/fast green (c,d) staining. Immunohistological staining of type II collagen (e,f) of the cells cultured in the hybrid mesh for 2 (a,c,e) and 12 (b,d,f) weeks. The round objects that do not have nuclei are the cross-sections of remaining PLGA fibers (arrows).

knitted mesh, facilitated cell seeding and even cell loading in the mesh.

Many strategies have been proposed to regain differentiated cartilaginous phenotypes such as the application of growth factors and culturing the cells in pellets or polymer gels. Supplements of insulin-like growth factor 1, fibroblast growth factor 2, or transforming growth factor β2 in a culture medium have been reported to elicit some effect on the redifferentiation of articular cartilage [7,10,16–19]. However, the effect of a growth factor may depend on both the cellular phenotype and the 'culture history' of the cells before the addition of a growth factor. The optimal choice of growth factors should vary with time during the tissue engineering process and is dependent on the changing phenotype and the presence of ECM components, making the process complex. Through the use of PLGA–collagen hybrid mesh as the scaffold, dedifferentiated chondrocytes could be redifferentiated without the addition of any special growth factors, making the process easier. In addition, the hybrid mesh could hold the seeded cells together and assemble them into tissue of a designed shape. The hybrid mesh would be useful both for cartilage tissue engineering and for the redifferentiation or differentiation induction of cells.

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