Porous Microcarrier-Enabled Three-Dimensional Culture of Chondrocytes for Cartilage Engineering: A Feasibility Study

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INTRODUCTION

Once articular cartilage is damaged in adult, it is almost intractable to regenerate due to the limited capacity for self-repair [1]. The long-term outcome of current clinical treatments has often been unsatisfactory. On the other hands, tissue engineering approach, i.e., utilizing cells and matrices together to regain the biological functions of damaged tissues, has shown some promise for the cartilage repair [2]. Among the cell sources introduced for cartilage engineering, chondrocytes, mesenchymal stem cells, and pluripotent stem cells have been used [3]; on the while, as for the biomatrices, synthetic and natural polymers, including poly(lactic acid), poly(glycolic acid), their copolymer, alginate, chitosan, and hyaluronan have often been used [4-7].

Scaffolds provide the three-dimensional (3D) environment onto which cells can grow and produce extracellular matrix of cartilage. While different types have been developed, including hydrogels [8], sponges [9], and fibrous meshes [10], injectable form has some merits of minimal incision during the transplantation [11,12]. One typical form of injectable scaffold is the porous microparticles that can be used to expand and scale up cells through 3D culture methods and then fill large defect spaces [12,13].

Previously, we showed that blended poly(D, L-lactide) (PLDLA)/poly(caprolactone) (PCL) porous microspheres significantly accelerated osteoblasts expansion and differentiation under proper dynamic culture conditions [13]. Based on this, we seek to utilize the blended microspheres for cartilage engineering. As a first step toward this, here we evaluate the chondrocyte growth and maintenance of chondrogenic phenotypes upon the porous microspheres under dynamic culture conditions, which can provide information on the feasible usefulness of the porous microspheres for future cartilage tissue engineering.

MATERIALS AND METHODS

Microsphere preparation

PCL (80 kDa, Sigma-Aldrich, USA) blended with PLDLA (L-lactide: D, L-lactide=70:30, Sigma-Aldrich, USA) was fabricated into porous microspheres as described in our previous report [13]. In brief, the PCL and PLDLA were dissolved separately in chloroform at 10%. To generate pores, camphene was

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added at 60% in the solvent. The PLDLA and PCL solutions were mixed at 1:3 by volume with the camphene solution. The solution was dropped into ice-cooled 2% poly(vinyl alcohol) solution, while gentle stirring at 450 rpm. After the pore generation, the microspheres were filtered through Millipore filter paper, washed with ice-cooled distilled water, and freeze-dried for further uses. The microsphere morphology was observed by scanning electron microscopy (SEM, Hitachi S-3000H, Japan) and optical microscopy. The size distribution of microspheres was determined based on the images.

Cell seeding and culture
Rat primary articular chondrocytes used in this study were harvested from articular cartilage of the knees of Sprague Dawley rats. Briefly, the collected cartilage slices were minced into about 1 mm² pieces and incubated in 0.2% type II collagenase solution for 18 h. The cell suspension was filtered through a 40 μm cell strainer and centrifuged at 300×g at room temperature for 10 min. The cell pellets were washed twice with phosphate buffer saline (PBS). The cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM, 4.5 g/L glucose; Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator containing 5% CO₂ at 37°C. The cells were used after four passages for the following experiments.

Before seeding cells, microspheres were sterilized with 70% ethanol for 2 h and washed with PBS solution three times. One milliliter of 1×10⁶ suspended cells was added to the pre-wetted microspheres of 30 mg with the culture medium for 12 h, and cell-microsphere constructs were incubated under shaking with a sway of 45° at 3 rpm for 6 h using MyLab SLRM-3 Intelli-Mixer (SLRM-3, SeoulLin Bioscience, Korea).

Thereafter, the cell-microsphere constructs were divided into experimental group and control group, and then a spinner flask culture system (S-flask 4500-1L, TAITEC, Japan) was employed. The control group was cultured in normal medium (DMEM consisting of the normal medium supplemented with 10% FBS and 1% penicillin/streptomycin); whereas, the experimental group was cultured in chondrogenic medium consisting of the normal medium supplemented with 10% FBS and 1% penicillin/streptomycin, 50 μg/mL ascorbic acid, 1% ITS premix (Sigma, USA), 100 nM dexamethasone (Sigma, USA), and 10 ng/mL transforming growth factor-β1 (PeproTech, USA). The stirring speed of the spinner flask was set at 30 rpm. The experiments were carried out up to 14 days. The media were changed twice each week.

Cell behavior observation in 2D and 3D environment
After 14 days, the cell-microsphere constructs were moved onto a 35-mm culture dish or embedded into 3D type I collagen hydrogels. The collagen hydrogels were prepared as described in our previous study [14]. The final concentration of collagen was 2 mg/mL. The constructs of both groups were cultured using the same normal medium at each time point. An inverted optical microscope (Olympus, Japan) was used to observe the cell behaviors.

Quantitative real-time polymerase chain reaction
Quantitative analysis of the chondrocyte-related genes expressed at day 14 was conducted using a Rotor-Gene RG-3000A qPCR machine (Australia). The first strand complementary DNA was synthesized from the total RNA (1 μg) using a SuperScript first strand synthesis system for real-time PCR (Invitrogen, USA) according to the manufacturer’s instructions. The reaction mixture was made up to 50 μL. Real-time PCR was conducted using SYBR GreenER qPCR SuperMix reagents (Invitrogen, USA). The relative transcript quantities were calculated using the ΔΔCt method with β-Actin as the endogenous reference gene amplified from the samples. The primer sequences of the genes are summarized in Table 1.

Immunofluorescence staining
Immunostaining of the harvested samples at day 14 was performed to detect the expression of type II collagen. The samples were incubated with 5% normal goat serum (Vector Laboratories, USA) in PBS for 30 min to suppress nonspecific staining, and then incubated with a primary antibody, anti-type II collagen (1:150 dilution, sc-52658; Santa Cruz Biotechnology, USA), and then incubated with a primary antibody, anti-type II collagen. The specimens were subsequently incubated with the FITC-conjugated antibody against mouse IgG (1:100 dilution, sc-52658; Santa Cruz Biotechnology, USA), and then incubated with a primary antibody, anti-type II collagen. The nuclei of the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The

Table 1. Primer sequences of chondrogenic genes for quantitative real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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<tbody>
<tr>
<td>SOX9</td>
<td>5’-CGTCACAGCTCAGACAC-3’</td>
<td>5’-TGCGCCACACAGCATGA-3’</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>5’-GAGTGGAAAGCCAGCAGTTG-3’</td>
<td>5’-CTCCATGTTCGAGACACATTTCA-3’</td>
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<tr>
<td>Aggrecan</td>
<td>5’-CTAGCTCGGTAGCTGACT-3’</td>
<td>5’-TGACCAGAGGTCACCAAG-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-ACGGTGACATCCGTAAGAC-3’</td>
<td>5’-TAATCTCCTTCTGCATCCTG-3’</td>
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