Materials Science and Engineering C 34 (2014) 168–175

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Effect of microcavitary alginate hydrogel with different pore sizes on chondrocyte culture for cartilage tissue engineering

Lei Zeng [a,b,1], Yongchang Yao [a,b,1], Dong-an Wang [b,c,*], Xiaofeng Chen [a,b,**]

[a] School of Materials Science and Engineering, South China University of Technology, Guangzhou 510641, China
[b] National Engineering Research Center for Tissue Restoration and Reconstruction, Guangzhou 510006, China
[c] Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637457, Republic of Singapore

ARTICLE INFO

Article history:
Received 5 June 2013
Received in revised form 31 July 2013
Accepted 6 September 2013
Available online 13 September 2013

Keywords:
Chondrocyte
Hydrogel
Microcavitary
Signal pathway

ABSTRACT

In our previous work, a novel microcavitary hydrogel was proven to be effective for proliferation of chondrocytes and maintenance of chondrocytic phenotype. In present work, we further investigated whether the size of microcavity would affect the growth and the function of chondrocytes. By changing the stirring rate, gelatin microspheres in different sizes including small size (80–120 μm), middle size (150–200 μm) and large size (250–300 μm) were prepared. And then porcine chondrocytes were encapsulated into alginate hydrogel with various sizes of gelatin microspheres. Cell Counting Kit-8 (CCK-8), Live/dead staining and real-time PCR were used to analyze the effect of the pore size on cell proliferation and expression of specific chondrocytic genes. According to all the data, cells cultivated in microcavitary hydrogel, especially in small size, had preferable abilities of proliferation and higher expression of cartilaginous markers including type II collagen, aggrecan and cartilage oligomeric matrix protein (COMP). Furthermore, it was shown by western blot assay that the culture of chondrocytes in microcavitary hydrogel could improve the proliferation of cells potentially by inducing the Erk1/2-MAPK pathway. Taken together, this study demonstrated that chondrocytes favored microcavitary alginate hydrogel with pore size within the range of 80–120 μm for better growth and ECM synthesis, in which Erk1/2 pathway was involved. This culture system would be promising for cartilage tissue engineering.

1. Introduction

Articular cartilage is a firm and highly elastic structure that covers the end of bones that is readily yielding to pressure, and recovering its shape when the force is removed. However, articular cartilage is a particular tissue that lacks a blood supply to support repair and remodeling. It has limited capacity to self-regenerate after damaged by injuries or degenerative diseases[1]. At present, lots of attempts have been made to repair cartilage defects through a variety of methods such as cartilage gouging, microfracture, bone-chondral autologous and allogous grafting and autologous chondrocyte transplantation[2]. But these methods have such disadvantages as expensive cost, inflammation or lacking of donors. Recent researches in biology and material science have pushed cartilage tissue engineering to the forefront of new cartilage repair techniques. Since native cartilage has an extracellular protein matrix which is strengthened by a three-dimensional (3D) network of collagen fibrils, 3D construct can be a good mimic of the morphology of the in vivo microenvironment[1]. In a 3D construct, most of chondrocytes showed a spherical morphology, which has been demonstrated to be associated with the production of cartilage-related ECM proteins[3–5]. Among various existing biomaterials, hydrogels such as alginate[6], agarose[7] and collagen[8] appear to be more advantageous: they have injectable and biodegradable properties for convenient clinical use, and provide an aqueous environment to maintain cells [9,10].

Nevertheless, one primary bottleneck of hydrogel-based tissue engineering applications is the limited proliferation and phenotypic preservation of committed chondrocytes [11,12]. To address this challenge, we have developed a novel phase transfer cell culture strategy, which was inspired by edge flourish (EF) phenomenon. This phenomenon is observed through a spontaneous dynamic outgrowth of chondrocytes along the edge of a cell-laden hydrogel construct [13]. The chondrocytes therein exhibit higher capability of growth and extracellular matrix (ECM) secretion than those in the hydrogel bulk.

In this study, we set up a microcavitary hydrogel model, aiming to evaluate whether the size of microcavity would influence the growth and phenotype of chondrocytes. Gelatin microspheres were prepared in three kinds of size, including Small size (80–120 μm), Middle size (150–200 μm), and Large size (250–300 μm). By encapsulating these microspheres into sodium alginate with chondrocytes, we cultured the cell-laden constructs for 21 days and investigated the effect of

* Correspondence to: D. Wang, Division of BioEngineering, School of Chemical & Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, N1.3-B2-13, Singapore 637457, Republic of Singapore. Tel.: +65 6316 8890; fax: +65 6791 1761.
** Correspondence to: X. Chen, School of Materials Science and Engineering, South China University of Technology, Guangzhou 510641, China. Tel.: +86 20 39380256; fax: +86 20 22236088.
E-mail addresses: DAWang@ntu.edu.sg (D. Wang), chenxf@scut.edu.cn (X. Chen).
[1] Yongchang Yao and Lei Zeng contributed equally to this work.

0928-4931/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.msec.2013.09.003

pore size on the cell proliferation and expression of cartilage-specific genes by CCK-8, Live/dead staining and real-time PCR. Meanwhile, we sought to explore the mechanisms of cell growth within the microcavitary hydrogel for a better understanding of interaction between cells and materials.

2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Chemie GmbH, USA). All cell cultures were maintained at 37 °C, 5% CO_2 and 95% humidity.

2.1. Isolation of chondrocytes

Porcine articular cartilage was harvested from the knee of edible swine, which was bought from local butchery. Chondrocytes were enzymatically isolated from cartilage tissues of porcine. In brief, cartilage tissues were removed and cut into small pieces by scalpels. Then these cartilage pieces were incubated in high glucose Dulbecco's modified Eagle's medium (H-DMEM) (Gibco, life of technology) culture medium with 10% (v/v) fetal bovine serum (Gibco, FBS, North America) containing 1 mg/mL type II collagenase (Gibco) at 37 °C under continuous shaking overnight. In the next day, chondrocytes were filtered with a sterile 70 μm mesh and centrifuged. Then cells were resuspended in chondrocytes culture medium, which was comprised of H-DMEM culture medium supplemented with 20% (v/v) fetal bovine serum, 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid, 0.1 mM nonessential amino acids (Invitrogen), 0.4 mM proline, 0.05% vitamin C, 10000 Units/mL penicillin and 10000 μg/mL streptomycin. The cells were seeded into a 150 mL tissue culture flask (Falcon; seeding density: 4×10^4 cells/cm^2) and incubated in humidified air with 5% CO_2/95% air atmosphere at 37 °C. The culture medium was changed every 2–3 days during routine culture. At about 90% confluence, chondrocytes were collected and used in our studies.

2.2. Preparation of gelatin microspheres

Different sizes of Gelatin microspheres were prepared through a double emulsion method. Briefly, 30 mL of gelatin solution (0.1 g/mL in water) was prepared under 70 °C and then 10 mL of ethyl acetate was added into gelatin solution by stirring at room temperature for several minutes. The emulsion was further added into 50 mL of tea oil and stirred for 15 min under an ice bath. Then the gelatin microspheres were formed spontaneously in 300 mL of precooled (−20 °C) ethanol and rinsed with 1,4-dioxane/acetone in turn. After being in fuming cupboard for 1 h and then in 60 °C for about 5 h, the microspheres were collected via standard sieves. By changing the stirring rate, gelatin microspheres in different sizes including small size (80–120 μm), middle size (150–200 μm) and large size (250–300 μm) can be harvested.

For the test to trace the dissolution of gelatin microspheres, gelatin microspheres were pre-labeled with Rhodamine B. During the fabrication of gelatin microspheres, Rhodamine B was added into gelatin solution and mixed sufficiently. The gelatin microspheres appeared red when excited by green light under fluorescence microscope (NIKON, TE 2000).

2.3. Cell seeding and in vitro culture of microcavitary alginate hydrogel

The gelatin microspheres were pre-equilibrated in 10 × PBS with 10% penicillin streptomycin at 4 °C overnight to lessen the possibility of contamination. And then kept in H-DMEM with 20% fetal bovine serum for 1 h. 1.6 wt% sodium alginate solution in 0.15 M NaCl was prepared and sterilized under high temperature and high pressure. Then chondrocytes were suspended in 1.6 wt% sodium alginate solution and mixed with gelatin microspheres homogeneously. The final cell density in sodium alginate mixture was about 80 million cells per mL. The sodium alginate hydrogel beads were formed by dispensing the alginate-cell suspension dropwise (60 μL/drop) into a 102 mM $CaCl_2$ solution. The beads were gelled in about 5 min. Then the constructs were cultured in the chondrocytes culture medium and the medium was changed every 2 days. According to the size of gelatin microspheres, there were four groups in our study as follows: small group (80–120 μm), middle group (150–200 μm), large group (250–300 μm) and control group (plain hydrogel).

2.4. Scanning electronic scanning

The morphology of sodium alginate scaffolds with different pore sizes was observed by scanning electronic microscopy (SEM). After dried by critical point dryer, the samples were carefully sectioned with a razor blade and mounted onto aluminum stubs. Prior to examination, platinum was sputtered for scanning electron microscopy contrast. A 30XLFEG SEM microscope (Philips, The Netherlands) was used to perform image analysis.

2.5. Cell proliferation and viability

The proliferation of chondrocytes was determined by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). CCK-8 allows sensitive colorimetric assays with the use of WST-8{[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]} for the determination of cell viability. WST-8 is reduced by dehydrogenase activities in living cells to produce a water-soluble yellow formazan dye. The amount of the formazan dye, is directly in proportion to the number of living cells. Briefly, samples from the four groups were collected on days 1, 3, 7, 14 and 21, respectively. Each construct was incubated in 500 μL of DMEM medium containing 50 μL of cck-8 under normal culture condition for 2 h. Then the medium was removed and added into a new culture plate and the absorbance was then measured at a wavelength of 450 nm using Thermo Scientific Microplate Reader.

"Live/Dead" dye staining (Biotium) was used to detect the viability of chondrocytes in the microcavitary hydrogels following manufacturer's instruction. The viable cells were indicated with green fluorescent calcein while dead cells were indicated with red fluorescent ethidium homodimer II.

2.6. Real time quantitative PCR analysis

Hydrogel constructs from the four groups were collected at 3, 7, 14, 21 days, respectively followed by RNA extraction using RNA extraction kit (Takara). Afterwards, cDNA was synthesized from 500 ng of RNA using Reverse Transcription Reagents Kit (Takara) according to the manufacturer's instructions. Then the real-time PCR was performed in a real-time PCR machine (Chromo4 real-time PCR detection system, Bio-rad) using the real-time PCR kit (SYBR Premix II, TaKaRa). The C_T value for each gene of interest was normalized against the corresponding C_T value of housekeeping gene GAPDH. Then the relative fold change of each gene was calculated with the $\triangle\triangle C_T$ method against control group on day 3.

The primer sequences of selected genes for real-time PCR were listed in Table 1.

2.7. Western blot analysis

The constructs from each group were collected on day 14 and day 21 respectively and dissolved with sodium citrate buffer to release the cells. Total proteins were extracted from the cell pellets using the RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 μg/mL leupeptin) and quantified using BCA Protein Assay kit (Thermo scientific, Pierce) followed by SDS-PAGE electrophoresis. The proteins were transferred