Biochemical Engineering Journal 103 (2015) 68–76

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

A three-dimensional dynamic coculture system enabling facile cell separation for chondrogenesis of mesenchymal stem cells

Feiyue Xu, Lei Xu, Qi Wang, Yan Zhou, Zhaoyang Ye*, Wen-Song Tan*

State Key Laboratory of Bioreactor Engineering, School of Bioengineering, East China University of Science and Technology, Shanghai 200237, China

ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form 22 May 2015
Accepted 6 July 2015
Available online 13 July 2015

Keywords:
Mesenchymal stem cells
Coculture
Bioreactors
Microcarriers
Biomedical
Tissue cell culture

ABSTRACT

Coculture has emerged as a strategy to improve chondrogenesisof mesenchymal stem cells (MSCs) for cartilage regeneration. In the present study, a new system integrating cell encapsulation, dynamic stimulation and coculture with articular chondrocytes (ACs), termed as three-dimensional (3D) dynamic coculture, was developed for chondrogenic differentiation of MSCs. In addition, a novel mechanism was exploited to enable facile separation of cocultured cells. Rabbit bone-marrow derived MSCs (rMSCs) and rabbit ACs (rACs) were encapsualted in alginate gel beads, respectively. In rACs-laden beads, magnetic Fe_3O_4 nanoparticles were incorporated. Dynamic coculture was performed in a spinner flask. Fe_3O_4 nanoparticles had no negative effects on growth of rACs, although a slight inhibition on production of glycosaminoglycans (GAG) by rACs was noticed. An alginate concentration of above 1.8 w/v% was favorable for dynamic culture of rMSCs and rACs. Coculturing rMSCs with rACs in chondrogenic medium in this system demonstrated an improved chondrogenesis than monocultured rMSCs according to GAG quantification, histological staining, and gene expression analysis of collagen I, II and X. However, supplementation of 2% FBS did not show posive effects on chondrogenesis of cocultured rMSCs. This proof-of-concept study demonstrates that such a separable 3D dynamic coculture system holds great potential in cartilage tissue regeneration.

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1. Introduction

Damaged articular cartilage has limited self-repair capacity due to its avascular nature. Albeit clinical practices such as microfracture and autologous tissue grafting are established, an effective long-term regeneration remains elusive [1]. Hence, a cell-based therapy, "autologous chondrocyte implantation" (ACI), is developed by applying autologous chondrocytes in collagen matrix with the potential of regenerating hyaline cartilage [2]. Alternatively, tissue engineering based on seeding cells into biomaterial scaffolds aiming at fabricating off-the-shelf tissue replacements is also promising for cartilage repair [2]. In both methodologies, chondrocytes have been exploited, which however are associated with several issues, such as limited availablity and unfavorable dedifferentiation (loss of the phenotype) during *ex vivo* expansion [3].

In contrast, mesenchymal stem cells (MSCs) are able to differentiate toward chondrocytes and proliferate *in vitro* and they can be readily acquired from diverse adult tissue origins including bone marrow and adipose tissue, representing a very attractive cell source for cartilage regeneration [4]. However, in current induction protocols, particularly with transform growth factor β (TGF-β), chondrogenesis of MSCs tends to express a hypertrophic phenotype and subsequent calcification, leading to an unstable hyaline cartilage tissue [5,6]. Nonetheless, cartilage tissue engineered from MSCs is generally inferior in mechanical properties compared to one engineered from chondrocytes as well as a native articular cartilage tissue [7,8].

Various strategies have been explored to improve *in vitro* chondrogenesis of MSCs. A three-dimensional (3D) microenviroment as well as mechanical stimulation are recognized very critical. For example, when encapsulated in 3D hydrogels, efficient chondrogenesis of MSCs can be achieved [9–11]. By designing specified bioreactors, appropriate mechanical loading has been extensively

Abbreviations: ACs, articular chondrocytes; CAM, calcein-AM; 3D, three-dimensional; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAG, glycosaminoglycans; α-MEM, α-minimum essential medium; MSCs, mesenchymal stem cells; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; OD, optical density; PBS, phosphate-buffered saline; PI, propidium iodide; rACs, rabbit articular chondrocytes; rMSCs, rabbit bone-marrow derived MSCs; TGF-β, transform growth factor β.

* Corresponding authors at: The State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Mei-Long Road, P. O. Box 309#, Shanghai 200237, China. Fax: +86 21 64252250.

E-mail addresses: zhaoyangye@ecust.edu.cn (Z. Ye), wstan@ecust.edu.cn (W.-S. Tan).

http://dx.doi.org/10.1016/j.bej.2015.07.003
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Table 1
Experimental design of cell culture studies.

Experiment	Culture description	Culture medium	Assay
Effects of Fe_3O_4 on rACs	• Monoculture of rACs-laden beads • 12-Well culture plate • Static • 14 d	• Chondrocyte growth medium	• MTT • GAG quantification
Effects of alginate concentration	• Coculture of rACs- and rMSCs-laden beads (equal amounts) • 125-mL spinner flask • 50 rpm • 28 d	Chondrocyte growth medium	• Live/dead staining • MTT • GAG quantification • Histological staining
Chondrogenesis of rMSCs	• Coculture of rACs- and rMSCs-laden beads (equal amounts) • Monoculture of rMSCs-laden beads as control • 125-mL spinner flask • 50 rpm • 28 d	• Chondrogenic medium • Chondrogenic medium + 2% FBS	• GAG quantification • Histological staining • Immunofluorescence staining • RT-PCR

explored to improve chondrogenesis [12–14]. Recently, coculturing MSCs and chondrocytes has emerged as an additional strategy to promote chondrogenesis by permitting cell–cell interactions between the two different cell types [15,16]. Many studies have demonstrated that expression of cartilage-related markers including collagen II and aggrecan can be stimulated by coculturing MSCs with chondrocytes [17–21]. Moreover, collagen X, a hypertrophic marker of chondrocytes, can be downregulated by coculture, which may eventually lead to generation of a hyaline cartilage phenotype [22–24]. It is believed that the paracrine secretion by chondrocytes (*e.g.*, parathyroid hormone-related protein) plays a major role to instruct the chondrogenesis of MSCs at the presence of TGF-β, although some indicate that a direct cell–cell contact might also be essential [19,20,22,24].

Current set-ups to enable coculture include formation of mixed cell pellet permtting direct cell–cell contacts, applying transwell insert to separate cocultured cells in culture plates, or seeding cell mixture into a same scaffold (either a hydrogel or porous scaffold) [17,21,24,25]. Firstly, all these reported coculture studies are performed in a static culture condition; secondly, these coculture modes are impractical for scaling-up to meet the needs in clinic, wherein a large volume of cellular materials is generally required. In the present study, in order to integrate all potential instructive cues for achieving improved *in vitro* chondrogensis of MSCs, a coculture system combining 3D encapsulation in microcarriers and dynamic culture with chondrocytes in a spinner flask was developed. Such a microcarrier-based suspension culture in spinner flasks is expected to be amenable to future scaling-up. Meanwhile, in mixed coculture, it is generally challenging to analyze individual cell types, requiring tedious molecular technologies as reported in literature [26,27]. Herein, a mechanism of exploiting magnetic Fe_3O_4 nanoparticles was applied to enable facile separation of cocultured cells for convenient analysis.

2. Materials and methods

2.1. Cell isolation and culture

All animal experiments were performed at Shanghai Laboratory Animal Center (Shanghai) in accordance with the institutional guidelines of animal care and use committee. Rabbit MSCs (rMSCs) were isolated from bone marrow of 2-month-old New Zealand white rabbits following a density-gradient method using Ficoll-Paque Plus solution (density: 1.077 g/mL; GE Healthcare) [22,28]. Collected bone marrow was resuspended in phosphate-buffered saline (PBS; pH 7.2), gently layered on an equal volume of Ficoll-Paque Plus solution in a 50-mL centrifuge tube and centrifuged at 400 g for 30 min. The second layer from top (low-density mononuclear cells) was harvested and washed with growth medium consisting of α-minimum essential medium (α-MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 units/mL of penicillin and 100 units/mL of streptomycin. Cells were initially seeded at 1×10^5 cells/cm^2 in growth medium and subcultured at 5×10^3 cells/cm^2 in a humidified atmosphere of 5% CO_2 at 37 °C. Cells at passage 3 were used. The identity of rMSCs was confirmed with surface antigen analysis and tri-lineage differentiation assay (data not shown).

Rabbit ACs (rACs) were isolated from 2-month-old New Zealand white rabbits as described [29]. Small fragments of articular cartilage tissues were treated with trypsin briefly and then digested with collagenase II (0.1%, 200 units/mg; Invitrogen) in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) to release cells. Chondrocyte growth medium consisted of high-glucose DMEM, 10 mM *N*-(2-Hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 10% FBS, 0.1 mM nonessential amino acids, 0.4 mM proline, 0.05 mg/mL vitamin C, 100 units/mL of penicillin and 100 units/mL of streptomycin. Cells were passaged once at 1×10^4 cells/cm^2 for use.

2.2. Cell encapsulation and culture of cell-laden beads

Sodium alginate (low viscosity, Sigma) dissolved in 0.9% saline was sterilized at 105 °C for 20 min. In general, for cell encapsulation, at 25 °C, cells (rMSCs or rACs) were resuspended in an alginate solution and then adjusted to obtain a determined alginate concentration using 0.9% saline. The final cell densities were set at 1×10^6 cells/mL and 5×10^5 cells/mL for rMSCs and rACs, respectively. For gelation, the suspension was delivered through a syringe with a 25-gauged needle as droplets into $CaCl_2$ solution (102 mM) and incubated for 10 min at 25 °C. To incorporate Fe_3O_4 nanoparticles (20 nm; Sigma) in rACs-laden alginate beads, nanoparticles were supplemented at 0.2 w/v% in the cell-alginate suspension and following the same gelation procedure as above.

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