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A scaffold-filter model for studying the chondrogenic differentiation of stem cells *in vitro*

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ABSTRACT

This study was undertaken to explore the synergistic effect of scaffold materials and a cartilage-like environment on the chondrogenic differentiation of stem cells. Because stem cells encapsulated in a cartilage scaffold will be induced by scaffold molecules as well as permeable molecules from the surroundings, it is impossible to optimize a chondro-inducible scaffold without considering environmental sensitivity. How do we know if a designed scaffold will be sufficient prior to implantation? In this study, bone marrow mesenchymal stem cells (bMSCs) were seeded in various scaffolds, including collagen hydrogel, collage/sodium alginate hydrogel, collagen sponge and silk fibroin sponge. The cell-scaffold complex was encapsulated in a filter pocket to avoid direct contact with co-cultured chondrocytes. Scaffolds differed in the ability to adsorb inducible molecules expressed by chondrocytes, as evidenced by various expressions of cartilage specific proteins and genes. Collagen hydrogel unexpectedly supported chondrogenic differentiation in an environment filled with chondrocytes secretion better than other reinforced scaffolds, which is consistent with the previous experiment *in vivo*. This result indicated that the environmental sensitivity of a scaffold is important for *in vivo* chondro-induction. This *in vitro* scaffold-filter model may be useful as a precursor to investigate the chondro-inducing potential of various scaffolds for cartilage repair.

1. Introduction

Cartilage is incapable of rejuvenating itself and often degenerates after injury. Thus, the implantation of chondroblasts and scaffold materials appears to be a promising strategy for cartilage repair [1]. However, there is a limited source of autologous chondrocytes, and seed cells obtained from ectopic cartilage often require additional surgery [2]. As bone marrow mesenchymal stem cells (bMSCs) can differentiate into chondroblasts under defined conditions [3] and can be rather easily isolated and expanded, bMSCs have become a prime cell source for cartilage regeneration.

According to a tissue-engineering concept, cartilage scaffolds can be designed to induce bMSC differentiation to chondroblasts at the implantation site. Several biodegradable and biocompatible materials have been experimentally and/or clinically studied [4,5]. Polymers such as polyglycolic acid (PGA) enhance proteoglycan synthesis, whereas collagen matrices stimulate the synthesis of collagen. For cartilage repair, ideal scaffolds should mimic the natural extracellular matrix (ECM). Collagen can be extracted and purified from a variety of sources and offers low immunogenicity and good formability. Collagen sponges promote cell adhesion, whereas collagen hydrogels protect cell leakage and are conducive to 3D cultured chondrocytes. However, the poor mechanical property of collagen scaffolds limits their applications. To overcome this shortcoming, collagen scaffolds can be cross-linked by chemical or physical methods or modified with natural/synthetic polymers or inorganic materials [6]. The exploration of new cartilage scaffolds is continuing. Wang et al. suggested promising prospects for a porous silk sponge in cartilage repair [7]. An alginate gel with good mechanical properties has applications for cell encapsulation. Three-dimensional polycaprolactone (PCL), poly (glycerol sebacate) (PGS), and poly (1,8 octanediol-co-citrate) (POC) scaffolds have been studied to determine the materials' effects on cartilage regeneration [8–10]. Novel designed cartilage scaffolds have always exhibited good bioactivities *in vitro*, but their applied effects *in vivo* are not as satisfactory. Collagen-based materials are still the most commonly used cartilage scaffolds [11].

Exogenous growth factors such as TGF-β1, dexamethasone, vitamin C and insulin-transferrin-selenium (ITS) are widely used in the chondrogenic differentiation of bMSCs *in vitro* [12–15]. BMP-2,4, or 6 are even added as enhancers [16]. However, stem cell exposure to massive inducible molecules may produce hypertrophic chondrocytes to facilitate endochondral bone formation [17]. As Macdonald et al.

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suggested [18], the gradual delivery of inducible molecules to target cells more closely recapitulates the native scenario. Furthermore, the cartilage environment may play a role in stabilizing the bMSC phenotype. The ossification of implants and adjacent tissues is reportedly inhibited when bMSCs are directly transplanted into cartilage defects without inducing differentiation *in vitro* [19].

In general, primary chondrocytes constantly secrete specific inducible factors, such as TGF-β1 [20], IGF-1 [21], BMP-2 [22] and FGF-2 [23]. The bMSC-scaffold implants should be able to interact gradually with secreted molecules from the surrounding chondrocytes. Thus, the adsorption and permeation of secreted inducible molecules into a bMSC-scaffold may contribute to chondrogenesis induction *in vivo* [24,25]. However, the adsorbability and infiltrability of a scaffold are affected by its composition, structure, elasticity, and other factors. How do we know if the prepared scaffold is feasible for interaction with secreted inducible molecules and further chondroinduction of bMSCs before implantation?

In this study, we developed a system for bMSC based cartilage engineering (with inducible molecules secreted by the chondrocyte to interact with bMSC), which was supported by scaffolds and protected by a semipermeable membrane to inhibit direct contact with chondrocytes. This *in vitro* model helps recapitulate the native interaction between the cell-scaffold implant and chondrocytic secretory factors. Thus the biological activity of bMSCs within various scaffolds after implantation may be predictable, providing references for the screening of scaffolds in cartilage tissue engineering.

2. Materials and methods

2.1. Hydrogels and sponges

Type I collagen was extracted from calf skin and suspended in a 0.5 mol/L acetic acid solution at a concentration of 7 mg/mL as previously described [26]. The obtained neutral collagen solution was partly lyophilized to prepare a collagen sponge (CS), which was later crosslinked by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). Subsequently 20 mg/mL sodium alginate solutions were sterilized by saturated steam in an autoclave at 120 °C for 20 min, cooled to 4 °C and diluted to a concentration of 5 mg/mL with neutral collagen to form a collagen/sodium alginate mixture. Both the pure collagen solution and the collagen/sodium alginate mixture were kept at 4 °C for hydrogel preparation and cell encapsulation, which are described in Section 2.4. Silk fibroin was a generous gift from Suzhou University. The lyophilized silk sponge was named SK.

2.2. BMSC isolation and culture

The bMSCs were harvested from the femur and tibia of newborn rabbits. Each bone was cut on both ends under sterile conditions, and the marrow space of each bone was washed with a culture medium of alpha-modified Eagle's medium (a-MEM) (Gibco, Gaithersburg, MD) containing 10% FCS and antibiotics (penicillin 100 U/mL, streptomycin 100 U/mL). The bMSCs were collected after centrifugation, then suspended and cultured on uncoated dishes. Non-adherent cells were removed by changing the culture medium after 3 days of culture. After two weeks of primary culture, cells of each dish were passaged to two 10 cm culture dishes. The medium was changed every 3 days. Only the second passages were harvested for seeding in scaffolds.

2.3. Chondrocyte isolation and culture

Hyaline cartilages were obtained from the joints of newborn rabbits and digested with 2 mg/mL collagenase type II in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) for 16 h at 37 °C. Isolated chondrocytes filtered through a 60 μm aperture nylon cloth (Cadisch and Sons, London) to remove the undigested matrix, washed with PBS and then re-suspended in α-MEM supplemented with 10% fetal calf serum and antibiotics (penicillin 100 U/mL, streptomycin 100 U/mL). Chondrocytes were seeded into culture flasks at an initial density of 3×10^5 cells/mL and cultured until the 2nd passage at 5% CO_2, 37 °C.

2.4. Cell encapsulation and seeding

The bMSCs were trypsinized, counted and mixed gently with the pure collagen solution or the collagen/sodium alginate mixture to reach a concentration of 2×10^7 cells/mL. Subsequently, cell-material mixtures were cultured in a 96-well plate at 37 °C for solidification. The obtained collagen hydrogel and collagen/sodium alginate hydrogel were called CH and CAH, respectively. Ten minutes later, the cell-CH composites were transferred into culture media while the cell-CAH composites were immersed in a solution of 75 mmol/L $CaCl_2$, 30 mmol/L HEPES, 150 mmol/L NaCl and 10 mmol/L KCl for crosslinking. Fifteen minutes later, the cell-CAH composites were washed with PBS and then placed in culture media.

For porous sponges, the cell suspension was seeded onto pre-wetted scaffolds including the collagen sponge (CS) and silk sponge (SK) at a concentration of 2×10^7 cells/cm^3. After 4 h, the cell-seeded sponges were immersed into fresh culture media. The cells encapsulated into the hydrogel or sponge are illustrated in Fig. 1.

2.5. Cell-scaffold culture in vitro

The cell-scaffold complexes cultured with different methods were divided into three groups: control group, inductive group and coculture group. All samples were cultured in culture media at 5% CO_2, 37 °C. A summary of the sample information is presented in Table 1. An illustration of the experimental methods is displayed in Fig. 1.

2.6. MTT assay

After 21 days of *in vitro* culture, cell-scaffold complexes were transferred into a 24-well plate and cultured with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)(Sigma-Aldrich) for 4 h, then washed with Hanks solution two times and gently mixed with 1 mL dimethyl sulfoxide (DMSO, Wako). After being crushed and centrifuged at 11,000 rpm to guarantee complete extraction of the formazan pigment by DMSO, the samples were detected by a microplate reader (Bio-Rad 550) at 490 nm.

2.7. Cell morphology and viability

After 21 days of *in vitro* culture, cell viability was measured by fluorescein diacetate (FDA, Ex/Em = 488 nm/540 nm) and propidium iodide (PI, Ex/Em = 535 nm/617 nm) staining. Briefly, the cell-scaffold complexes were washed with PBS and stained with FDA (5 μg/mL) and PI (10 μg/mL) for 10 min. Subsequently, the samples were washed with PBS 3 times and viewed with a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) to visualize FDA stained live cells and PI stained dead cells. Multi-channel overlays were assembled by Leica LAS software. The cell morphology, attachment and distribution in the scaffolds were imaged.

2.8. Histological and immune-histochemical staining

After 21 days of *in vitro* culture, cell-scaffold complexes were fixed overnight in 10% neutral buffered formalin, dehydrated in graded alcohols, embedded in paraffin and then sectioned (5 μm). Using standard immune-histochemical techniques, serial sections were stained with hematoxylin-eosin and safranin O, respectively. For immune-histochemical staining, sections were treated with pepsin for 30 min, washed with Tris-buffer, and treated with mouse anti-collagen type II