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TISSUE ENGINEERING

Co-culture of infrapatellar fat pad–derived mesenchymal stromal cells and articular chondrocytes in plasma clot for cartilage tissue engineering

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Abstract

Background. Cell source plays a deterministic role in defining the outcome of a cell-based cartilage regenerative therapy and its clinical translational ability. Recent efforts in the direction of co-culture of two or more cell types attempt to combine the advantages of constituent cell types and negate their demerits. *Methods.* We examined the potential of co-culture of infrapatellar fat pad–derived mesenchymal stromal cells (IFP MSCs) and articular chondrocytes (ACs) in plasma clots in terms of their ratios and culture formats for cartilage tissue engineering. *Results and discussion.* It was observed that IFP MSCs and ACs interact positively to produce a better quality hyaline cartilage-like matrix. While a supra-additive deposition of sulfated Glycosaminoglycans (sGAG), collagen type II, aggrecan and link protein was observed, deposition of collagen type I and X was sub-additive. (Immuno)-histologically similar cartilage was generated *in vitro* in IFP MSC:AC ratio of 50:50 and pure AC groups thus yielding a hyaline cartilage with 50% reduced requirement of ACs. Subsequently, we investigated if this response could be improved further by enabling better cell-cell interactions using scaffold-free systems such as self-assembled cartilage or by encapsulating cellular micro-aggregates in plasma clot. However, it was inferred that while self-assembly may have enabled better cell-cell interaction, poor cell survival negated its overall beneficial role, whereas the micro-aggregate group demonstrated highly heterogeneous matrix deposition within the construct, thus diminishing its translational utility. Overall, it was concluded that co-culture of IFP MSCs and ACs at a ratio of 50:50 within plasma clots demonstrated potential for cell-based cartilage regenerative therapy.

Key Words: *articular chondrocytes, cartilage tissue engineering, co-culture, infrapatellar fat pad–derived mesenchymal stromal cells, plasma clot*

Introduction

Articular cartilage repair is clinically challenging because of the hypo-cellular and avascular nature of cartilage tissue. In recent years, cartilage tissue engineering (CTE) has gained significant attention as an alternate strategy toward cartilage repair and regeneration. CTE uses cells, growth factors and biomaterials either alone or in combination to repair/regenerate articular cartilage tissue and its therapeutic outcome is strongly linked to these factors. Therefore, much effort has been devoted toward studying these individual factors and their combinations to effectively repair cartilage tissue [1]. Of these factors, the contribution of cells to cartilage repair is central because they are the functional units that synthesize, deposit and remodel extracellular matrix (ECM), which ultimately forms functional cartilage tissue. Therefore, it is essential to identify optimal cell sources that can be exploited for CTE.

The ideal cell source for CTE should be the one that can be easily isolated without significant donor site morbidity, can be easily cultured *in vitro* and secretes cartilage-specific ECM, which would lead to good quality hyaline cartilage [2]. In this regard, the two most widely studied cell populations for CTE are articular chondrocytes (ACs) and mesenchymal stromal cells (MSCs) [3]. ACs are the residing cell population of articular cartilage and are known to regulate various anabolic and catabolic pathways to attain cartilage tissue homeostasis. It is because of these reasons

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that chondrocytes were the foremost cell population used for cartilage tissue engineering [4,5]. Although chondrocytes enable good quality cartilage formation *in vitro* and *in vivo*, they have certain disadvantages like poor expansion capacity and high tendency to dedifferentiate upon *in vitro* expansion [6]. Moreover, large-sized cartilage biopsy specimens harvested for isolating chondrocytes have been shown to be associated with high risk of developing donor site morbidity [7]. Overall, although chondrocytes have good cartilage-forming capacity, because of the disadvantages associated with them, they have limited translational utility. MSCs, on the other hand, are a relatively abundant cell source in the human body and have a higher expansion capacity *in vitro* [8]. Further, MSCs are capable of differentiating into multiple cell types including chondrocytes. Although MSCs possess these advantages, it has been shown that MSCs form cartilage tissue rich in collagen type-I (fibrocartilaginous), which is mechanically inferior to the hyaline cartilage of articular joints and, at times, MSCs also show poor matrix deposition [9]. Further, hypertrophic differentiation has been observed when only MSCs were used to generate cartilage, which eventually leads to calcification of cartilage [10].

Various strategies have been proposed to circumvent the problems associated with both ACs and MSCs. Co-culture of these cell populations is one of such strategies and it has shown better neo-cartilage formation compared with monocultures [2,11]. It has been observed that in co-culture settings, chondrocytes enhance chondrogenic differentiation of MSCs while various factors secreted by MSCs facilitate proliferation of chondrocytes enabling a synergistic response in cartilage formation [12]. Previous studies have shown that the expression of cartilage-specific ECM markers increased significantly in a co-culture than in MSC monocultures [13]. Most importantly, using MSCs along with chondrocytes lowers the amount of chondrocytes required for the treatment, thus reducing the need of large-size cartilage biopsy specimens making the therapy translationally more viable. Recently, MSCs derived from infra-patellar fat pad (IFP MSCs) are gaining attention because they were shown to possess chondrogenic potential and be effective in treating osteoarthritis in pre-clinical studies [14,15]. Therefore, we hypothesized that IFP MSCs may demonstrate good neo-cartilage formation when co-cultured with ACs. In addition, IFP MSCs also hold the advantage of easy isolation along with chondrocytes as both infra-patellar fat pad and cartilage can be resected through the same surgical procedure, thereby avoiding multiple invasive procedures. Since it has been observed previously that the ratio of ACs to MSCs used in the co-culture influences chondrogenesis, optimizing this ratio in co-culture systems is essential [16,17]. The first part of this study examines co-culture of IFP MSCs and ACs at different ratios in plasma clots. Plasma clots were chosen as the scaffold material because it is composed of fibrin network, which has been shown to improve chondrocyte viability and induce chondrogenesis [18]. The efficacy of fibrin gels in treating cartilage defects has been demonstrated in both pre-clinical and clinical studies [19,20].

In addition to the ratio of these two cell types, it is also critical to understand the extent of cell-cell contact and paracrine signaling required for effective cellular communication in co-cultures. While direct co-culture systems like monolayer culture and 3-D pellet cultures facilitate both direct cell-cell interaction and paracrine signaling, hydrogel-based cultures while facilitating paracrine signaling may hinder direct cell-cell interaction to some extent [21]. Therefore, the second part of this study examined the influence of varying cell-cell interactions in this co-culture system on cartilage formation. For this, three different culture formats, namely, IFP MSCs/ACs homogeneously dispersed in plasma clot (PC; hindered cell-cell interaction due to intervening plasma clot between adjacent cells), IFP MSC/AC microaggregates encapsulated in plasma clot (MA; combination of cell-cell interactions within matrix and good diffusion between aggregates) and self-assembled IFP MSCs/ACs (SA) without PCs (good cell-cell interactions but poor nutrient diffusion), were examined for *in vitro* cartilage formation.

Materials and methods

Isolation and culture of cells

Goat ACs were isolated from stifle joints of goats (collected from local slaughterhouse) and cultured in Complete Dulbecco's modified Eagle's medium (DMEM) – high glucose (Sigma-Aldrich) (DMEM supplemented with 1 mmol/L sodium pyruvate, 2.5 µg/mL amphotericin B, 100 U/mL penicillin–streptomycin [Himedia Labs], 50 µg/mL ascorbic acid phosphate and 10% heat inactivated fetal bovine serum [FBS; Gibco]) as reported previously [22]. To isolate IFP MSCs, infrapatellar fat pad was harvested from the same stifle joint. The tissue was minced and washed with phosphate-buffered saline (PBS) containing 5 µg/mL amphotericin B, 200 U/mL penicillin–streptomycin and 2.5 µg/mL ciprofloxacin for 20 min. The minced tissue was digested in incomplete medium containing 1.5 mg/mL collagenase and 2% FBS for 12–16 h. Digested tissue was filtered through a cell strainer (70 µm), centrifuged, washed and resuspended in complete cell culture medium. The cells were then seeded onto tissue culture dishes and cultured in complete medium with 5 ng/mL basic fibroblast growth factor (bFGF; Sino Biologicals). Cells between passage numbers P2 to P4 were used for all experiments.

Characterization of MSCs isolated from goat IFP

Morphology of the isolated IFP MSCs was visualized and imaged using an inverted phase contrast microscope (TS100, Nikon). To assess the self-renewal potential of IFP MSCs, colony forming unit–fibroblast (CFU-F) assay was performed. For CFU-F assay, 100 cells were seeded onto a 100-mm cell culture dish followed by culture in routine conditions for 10 days. For visualization of the colonies, cells were stained with crystal violet (3% in methanol) for 10 min and imaged.

To study the multi-lineage differentiation potential of isolated IFP MSCs, the cells were cultured in adipogenic induction medium (complete medium supplemented with 1 μmol/L dexamethasone, 10 μg/mL insulin and 100 μmol/L indomethacin), osteogenic induction medium (complete medium supplemented with 10 mmol/L β-glycerophosphate, 100 nmol/L dexamethasone and 50 μg/mL ascorbic acid phosphate) [23] and chondrogenic induction medium (basal medium supplemented with 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.25 mg/mL bovine serum albumin (BSA), 1 mmol/L proline, 50 μg/mL ascorbic acid phosphate, 100 nmol/L dexamethasone, 1X insulin, transferrin, sodium selenite + linoleic-BSA (ITS+1) and 10 ng/mL transforming growth factor [TGF] β1 [Sino Biologicals Inc.]) [24] with media being changed twice a week. Cells cultured in complete DMEM cell culture medium were used as control. For chondrogenic differentiation, cells were seeded at 3×10^5 cells per 10 μL and allowed to condense for 1 h before addition of medium to enable micromass formation. After 14 days, oil red O, alkaline phosphatase and alcian blue staining were used to evaluate adipogenic, osteogenic and chondrogenic differentiation, respectively, as reported previously [25].

Chondrogenic differentiation

For co-culture studies, different IFP MSC:AC ratios (100:0, 75:25, 50:50 and 0:100) were suspended in citrated plasma at a final concentration of 2×10^6 cells per 50 μL. A drop (50 μL) of this suspension was placed on a polystyrene dish and $CaCl_2$ was added to it (at a final concentration of 0.1%). After 45 min of incubation at 37°C, the clotted plasma samples were transferred to well plates containing chondrogenic induction medium and the media was changed twice a week. The plasma clots with cells were harvested for morphological study, histology, biochemical analysis and immunohistochemistry at predetermined time points (14 and 28 days). The IFP-MSCs and ACs were co-cultured in an animal matched manner (i.e. both the cell types in a single co-culture experiment were from the same animal).

Histological analysis and immunohistochemistry

For histology and immunohistochemistry, the plasma clots were fixed in neutral buffered formalin for 15 min, embedded in optimum cutting temperature (OCT) compound (Tissue-Tek) and sectioned at a thickness of 10 μm (Leica CM1900). Standard histology protocols were followed for staining the cryo-sections with hematoxylin and eosin for morphological analysis, safranin O for sulfated Glycosaminoglycans (sGAG), picro-sirius red for total collagen and alcian blue for sGAG [24]. Stained slides were serially dehydrated in ethanol followed by xylene and finally mounted in DPX medium before imaging (Leica DM2500). Immunohistochemical analysis was performed for positive cartilage markers (collagen type II, chondroitin sulfate, aggrecan and link protein), fibrocartilage marker (collagen type I) and hypertrophy marker (collagen type X) as reported previously [24]. The immunohistochemistry data was quantified using image analysis (Image J software). Integrated density was calculated using the formula given as follows. Mean value was obtained by quantifying integrated densities from at least eight images (two images each from four different samples). .

$$\text{Integrated density} = \log_{10}\left[\frac{\text{Area of interest} \times \text{mean pixel intensity}}{\text{Area of interest} \times 255}\right]$$

Biochemical analysis

The samples were harvested for biochemical analysis after 14 and 28 days of culture. For this, the samples were digested in 0.2 mg/mL papain at 65°C for 3 h, followed by quantification of sGAG and DNA using dimethylmethylene blue assay and Hoechst assay, respectively [24].

Chondrogenic differentiation of cells in different 3-D formats

In the second part of the study, 50:50 co-culture group was cultured in three different culture formats, namely, PC, MA and SA, to vary the extent of cell-cell and cell-matrix interactions (Figure 1B). In the PC group, cells were encapsulated in PC as described in the Chondrogenic Differentiation section. For the SA group, 2×10^6 cells were suspended in 50 μL complete cell culture medium and were allowed to condense at 37°C in 24-well plates for 2 h. Finally, to generate cellular MAs, an agarose microwell-based approach was used as reported previously [26]. Briefly, a mold that consisted of an array of pillars (300 × 300 × 300 μm) separated by a distance of 150 μm was fabricated using a laser engraving machine on acrylic glass. This was then placed over a layer of