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Chondrogenically primed tonsil-derived mesenchymal stem cells encapsulated in riboflavin-induced photocrosslinking collagen-hyaluronic acid hydrogel for meniscus tissue repairs

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ABSTRACT

Current meniscus tissue repairing strategies involve partial or total meniscectomy, followed by allograft transplantation or synthetic material implantation. However, allografts and synthetic implants have major drawbacks such as the limited supply of grafts and lack of integration into host tissue, respectively. In this study, we investigated the effects of conditioned medium (CM) from meniscal fibrochondrocytes and TGF-β3 on tonsil-derived mesenchymal stem cells (T-MSCs) for meniscus tissue engineering. CM-expanded T-MSCs were encapsulated in riboflavin-induced photocrosslinked collagen-hyaluronic acid (COL-RF-HA) hydrogels and cultured in chondrogenic medium containing TGF-β3. *In vitro* results indicate that CM-expanded cells followed by TGF-β3 exposure stimulated the expression of fibrocartilage-related genes (*COL2, SOX9, ACAN, COL1*) and production of extracellular matrix components. Histological assessment of *in vitro* and subcutaneously implanted *in vivo* constructs demonstrated that CM-expanded cells followed by TGF-β3 exposure resulted in highest cell proliferation, GAG accumulation, and collagen deposition. Furthermore, when implanted into meniscus defect model, CM treatment amplified the potential of TGF-β3 and induced complete regeneration.

Statement of Significance

Conditioned medium derived from chondrocytes have been reported to effectively prime mesenchymal stem cells toward chondrogenic lineage. Type I collagen is the main component of meniscus extracellular matrix and hyaluronic acid is known to promote meniscus regeneration. In this manuscript, we investigated the effects of conditioned medium (CM) and transforming growth factor-β3 (TGF-β3) on tonsil-derived mesenchymal stem cells (T-MSCs) encapsulated in riboflavin-induced photocrosslinked collagen-hyaluronic acid (COL-RF-HA) hydrogel. We employed a novel source of conditioned medium, derived from meniscal fibrochondrocytes. Our *in vitro* and *in vivo* results collectively illustrate that CM-expanded cells followed by TGF-β3 exposure have the best potential for meniscus regeneration. This manuscript highlights a novel stem cell commitment strategy combined with biomaterials designs for meniscus regeneration.

1. Introduction

The meniscus is a fibrocartilaginous tissue found in the knee that plays a critical role in joint function and health. It contributes to load transmission and distribution [1,2], shock absorption [3] and joint stability during flexion, extension, and rotation [4]. Located between the femoral condyle and tibial plateau, the meniscus protects the tibial and femoral cartilage and subchondral bone from biomechanical stress and wear. Meniscal tears are one of the most prevalent knee injuries, resulting in 60–70 meniscectomies per 100,000 population in the United States, annually [5]. If left untreated, meniscal injuries impair knee function and

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predispose patients to osteoarthritis [6]. Treatment of meniscal injuries is necessary to prevent joint problems that severely compromise the patient's quality of life.

Surgical meniscal repair is dependent on the location of the injury due to spatial variations in vascularity and self-repair capacity. The meniscus is divided into outer one-third, vascularized zone and inner two-third, avascular zone [7]. Only minimal surgical intervention, if any, is necessary to assist self-repair in the vascularized zone. The avascular zone, however, lacks intrinsic repair capacity, and thus, requires more invasive surgical measures, i.e. partial or total meniscectomy. Currently, allografts or synthetic scaffolds are available to substitute for removed meniscus tissue. However, problems related to graft availability, disease transmission, integration into host tissue, and tissue ingrowth and differentiation into fibrocartilage render allografts and synthetic implants as inapt for meniscus replacement. Recently, several studies have combined meniscal fibrochondrocytes and ECM-mimetic scaffolds for meniscus tissue engineering [8–10]. We have demonstrated that collagen-riboflavin-hyaluronic acid (COL-RF-HA) hydrogel stimulated fibrocartilage-related gene expressions and GAG accumulation of encapsulated fibrochondrocytes [9]. Riboflavin-induced photocrosslinking was employed as a non-cytotoxic crosslinking method to mechanically strengthen the collagen hydrogel. Moreover, crosslinked hyaluronic acid was incorporated into collagen-riboflavin hydrogel for chondrogenic stimulation and mechanical support.The frequency and severity of the symptoms varies according to the size and mobility of the meniscal tear Here we have utilized mesenchymal stem cells (MSCs) isolated from human tonsillar tissues (T-MSCs) in conjunction with COL-RF-HA hydrogel for meniscus tissue engineering. Previous studies have extensively characterized and confirmed that T-MSCs are: (1) positive for MSC-specific markers and negative for hematopoietic cell markers [11,12], and (2) potential cell sources for bone [13–15] and cartilage [16,17] tissue engineering applications. In order to effectively differentiate T-MSCs to meniscus tissue, we have employed conditioned medium from fibrochondrocytes for an efficient fibrochondrogenic commitment of T-MSCs. We have previously reported that morphogenetic factors secreted from chondrocyte can effectively promote chondrogenic commitment of a variety of stem cells, including human embryonic germ cells, human embryonic stem cells, and human mesenchymal stem cells in conjunction with transforming growth factor beta 1 (TGF-β1) [18–20]. TGF-β family of growth factor activates TGF-β receptors, followed by a signaling cascade of SMAD protein complexation and transcription of mRNAs involved in chondrogenesis [21]. Even though TGF-β family has been shown to induce effective chondrogenic commitment of stem cells [22], we hypothesize that T-MSCs priming with fibrochondrocyte-derived conditioned medium may potentiate TGF-β-induced chondrogenesis and meniscus tissue formation.

2. Materials and methods

2.1. T-MSC isolation

Human tonsillar tissues were obtained from 9 patients younger than 10 years old undergoing tonsillectomy with informed consent at the Department of Otorhinolaryngology- Head and Neck Surgery of Ewha Woman's University Mok-Dong Hospital. T-MSC isolation was performed as previously described [12,14]. Briefly, harvested tonsillar tissues were chopped, and digested with collagenase type I (210 U/mL; Invitrogen) and DNase I (10 μg/mL; Sigma-Aldrich) for 30 min at 37 °C with shaking. Tissue digest solution was filtered through a wire mesh and centrifuged to obtain cell pellets. T-MSCs were isolated by density gradient centrifugation using Ficoll-Paque gradient media (GE Healthcare). T-MSCs from 9 donors were pooled altogether and were maintained in growth medium (GM), containing Dulbecco's modified Eagle medium (DMEM, Hyclone), 10% (v/v) fetal bovine serum (FBS, Biowest), 1% (v/v) L-glutamine (Gibco), 1% (v/v) penicillin/streptomycin (pen/strep, Gibco) and 1% (v/v) antibiotic-antimycotic (Gibco), and sub-cultured once a week.

2.2. Chondrogenic priming of T-MSC

T-MSCs were expanded with conditioned medium (CM) for cell priming. Conditioned medium was prepared from meniscal fibrochondrocytes isolated from New Zealand white rabbits. Briefly, the inner, avascular region of the meniscus was minced and digested with 0.2% (w/v) collagenase (Worthington) in DMEM/F12 (Gibco) supplemented with 10% (v/v) FBS and 1% (v/v) pen/strep at 37 °C for 16 h with gentle shaking. Isolated fibrochondrocytes were then cultured in DMEM/F12 containing 10% (v/v) FBS, 1% (v/v) pen/strep, 50 μg/ml vitamin C (Sigma-Aldrich), 100 μM non-essential amino acid (Gibco) and incubated at 37 °C in 5% CO_2. Fibrochondrocytes (P1) were then incubated with serum-free DMEM/F12 for 48 h to obtain fibrochondrocyte-conditioned medium (CM). CM was supplemented with 10% FBS and 1% pen/strep before use. T-MSCs (P4) were expanded with either GM or CM for two weeks, encapsulated in COL-RF-HA hydrogel and cultured in chondrogenic differentiation medium with or without TGF-β3 (10 ng/mL; eBioscience). Chondrogenic medium was prepared with high glucose DMEM, 100 nM dexamethasone (Sigma-Aldrich), 50 mg/mL ascorbate-2-phosphate (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1% ITS + Premix (BD Biosciences). A total of four groups of cell-laden constructs were studied. Group names (GM, GMT, CM, CMT) were designated based on: the type of medium (GM or CM) used to expand T-MSCs in monolayer and the presence or absence of TGF-β3 (T) in culture medium of cell-laden constructs.

2.3. COL-RF-HA hydrogel fabrication

Riboflavin-induced photocrosslinked collagen hydrogel containing crosslinked hyaluronic acid (COL-RF-HA) was prepared as described previously [9]. Briefly, bovine type I collagen solution (3 mg/mL in 0.01 N acetic acid, 300 kDa; Advanced Biomatrix) was mixed with 10X PBS (Gibco) and riboflavin 5′-phosphate sodium salt hydrate (riboflavin; Sigma-Aldrich) and neutralized with 0.1 M sodium hydroxide (Sigma-Aldrich). Crosslinked hyaluronic acid (XL-HA) was synthesized by amide bond formation between hyaluronic acid (HA) and hexamethylenediamene (HMDA) to increase retention in hydrogel [23]. Briefly, HA (64 kDa; Lifecore Co.) and HMDA (Sigma-Aldrich) were mixed in MES buffer (Thermo Scientific) at a molar ratio of 1:1. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbonate (Thermo scientific) and 1-Hydroxybenzotriazole (GL Biochem Ltd.) were added as coupling reagents, followed by incubation at 37 °C for 2 h. After completing crosslinking reaction, XL-HA was dialyzed against 1× PBS for 3 days to eliminate unreacted material and freeze-dried for 3 days. Lyophilized XL-HA was pulverized and stored at −20 °C until use. For cell encapsulation, final concentrations of collagen and crosslinked HA were 2.4 mg/mL and 1% (w/v), respectively. Cell pellets were gently pipetted with COL-RF-HA precursor solution (150 μL) at a concentration of 1×10^6 cells/construct. Hydrogels (diameter = 8 mm, height = 3 mm) were incubated at 37 °C for 90 min and exposed to UV light (3.5 mW/cm^2) for 3 min.