



Dental mesenchymal stem cells encapsulated in an alginate hydrogel co-delivery microencapsulation system for cartilage regeneration



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ABSTRACT

Dental-derived mesenchymal stem cells (MSCs) are promising candidates for cartilage regeneration, with a high capacity for chondrogenic differentiation. This property helps make dental MSCs an advantageous therapeutic option compared to current treatment modalities. The MSC delivery vehicle is the principal determinant for the success of MSC-mediated cartilage regeneration therapies. The objectives of this study were to: (1) develop a novel co-delivery system based on TGF- β 1 loaded RGD-coupled alginate microspheres encapsulating periodontal ligament stem cells (PDLSCs) or gingival mesenchymal stem cells (GMSCs); and (2) investigate dental MSC viability and chondrogenic differentiation in alginate microspheres. The results revealed the sustained release of TGF- β 1 from the alginate microspheres. After 4 weeks of chondrogenic differentiation in vitro, PDLSCs and GMSCs as well as human bone marrow mesenchymal stem cells (hBMMSCs) (as positive control) revealed chondrogenic gene expression markers (Col II and Sox-9) via qPCR, as well as matrix positively stained by Toluidine Blue and Safranin-O. In animal studies, ectopic cartilage tissue regeneration was observed inside and around the transplanted microspheres, confirmed by histochemical and immunofluorescent staining. Interestingly, PDLSCs showed more chondrogenesis than GMSCs and hBMMSCs ($p < 0.05$). Taken together, these results suggest that RGD-modified alginate microencapsulating dental MSCs make a promising candidate for cartilage regeneration. Our results highlight the vital role played by the microenvironment, as well as value of presenting inductive signals for viability and differentiation of MSCs.

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1. Introduction

Several treatment modalities have been introduced to regenerate or enhance the repair of articular cartilage, such as the grafting of autologous osteochondral tissue or the transplantation of autologous chondrocyte suspensions [1,2]. However, for each of these strategies, the biological and mechanical properties of the formed tissue are inferior to those of native articular cartilage [3]. An advantageous alternative therapeutic option is the regeneration of cartilage tissue using mesenchymal stem cells (MSCs). MSCs are multipotent cells that can differentiate into multiple lineages depending on the nature of the environmental signals which they receive. Specifically, MSCs undergo chondrogenesis and deposit a cartilage-specific matrix in pellet cultures and in a variety of biomaterials in the presence of appropriate growth factors. Most of the studies on chondrogenic differentiation have focused on applications using bone marrow MSCs (BMMSCs). However, it is well known that MSCs reside in a wide spectrum of post-natal tissue types including the orofacial tissues [4–6], while neural crest origin are attractive for craniofacial regenerative strategies as they might

be more plastic to differentiate into craniofacial tissues [5–7]. Among the dental-derived MSCs, periodontal ligament stem cells (PDLSCs) and gingival mesenchymal stem cells (GMSCs) are of particular interest as they can be harvested easily, accessible through the oral cavity and they can often be obtained as discarded biological samples in dental clinics [8,9]. Moreover, both in vitro and in vivo studies have confirmed the multilineage differentiation capabilities of these dental-derived MSCs [10,11].

However, an appropriate microenvironment and signaling molecules are required in order to effectively differentiate MSCs into chondrocytes [12]. It has been reported that growth factors such as TGF- β 1, BMP-4 and FGF-2 are often required in the process of chondrogenesis [13]. Particularly, studies have reported that transforming growth factor-beta (TGF- β) plays an important role in chondrogenesis of MSCs [14] by stimulating chondrocyte proliferation while preventing cartilage hypertrophy [15]. In addition, it is well known that the cell delivery vehicle has an important role in the in vivo performance of MSCs and the success of the regenerative therapy. Therefore, we sought to design an appropriate microenvironment by engineering the physiochemical properties of the extracellular MSC microenvironment in order to tailor the niche characteristics and direct cell phenotype through differentiation [16,17]. Hydrogel biomaterials have been widely used for cartilage

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tissue engineering. Among the hydrogel biomaterials, alginates, which are natural hetero-polysaccharides isolated from brown sea algae, are of particular interest due to their unique properties, including injectability and biodegradability [18,19]. Alginate can provide a three-dimensional (3-D) scaffold that facilitates the spatial distribution of MSCs, thus resulting in a structural organization that resembles the native *in vivo* microenvironments. Moreover, alginate microspheres have been used extensively for controlled delivery of growth factors (e.g. TGF- β), making them desirable biomaterials for chondrogenesis [20,21]. In the present study, we developed a novel co-delivery system that provides a 3-D architecture of RGD-coupled alginate hydrogel loaded with a TGF- β 1 ligand for microencapsulation of dental MSCs. This approach ensures optimized cartilage regeneration and provides a potential application for reconstruction of the temporomandibular joint disk and for applications in the appendicular skeleton.

2. Materials and methods

All the animal experiments in the current study were performed in accordance with the guidelines published by the Institutional Animal Care and Use Committee at the University of Southern California, and the American Association for Accreditation of Laboratory Animal Care.

2.1. Progenitor cell isolation and culture

Human PDLSCs and GMSCs were isolated and cultured according to previously published protocols by Seo et al. and Zhang et al. [10,11]. The teeth and gingival tissues were obtained from healthy male patients (18–25 years old) undergoing third molar extractions according to IRB approval from the University of Southern California. Only subjects without any history of periodontal disease were included in this study.

For assay of colony forming units-fibroblastic (CFU-F), 0.1×10^6 cells were seeded in a culture dish and cultured for 14 days. Subsequently, the cells were stained with Toluidine Blue. A cell count of more than 50 in one colony was counted as a positive CFU-F. Passage 4 cells were used in the experiments and hBMMSCs were used as the positive control group.

2.2. Flow cytometric analysis

Approximately 5×10^5 cells at passage four were incubated with specific PE- or FITC-conjugated mouse monoclonal antibodies for human CD34 and CD45 (as negative: markers for hematopoietic stem cells); and CD73, CD105, CD 146, and CD 166 (as positive: markers for mesenchymal stem cells) (BD Biosciences, San Jose, CA), or isotype-matched control IgGs (Southern Biotechnology Associates, Birmingham, Alabama) and subjected to flow cytometric analysis using a Beckman Coulter flow cytometer (Beckman Coulter, Brea, CA) and analyzed using FACScan software (BD Biosciences).

2.3. Biomaterial fabrication and cell encapsulation

Custom-made RGD-coupled alginate with high guluronic acid content (NovaMatrix FMC Biopolymer, Norway) was utilized in this study. Alginate was purified and partially oxidized (2%) to increase its degradability according to the methods in the literature [22–24]. Subsequently, the alginate was mixed with TGF- β 1 (Abcam, Cambridge, MA) ($50 \mu\text{g ml}^{-1}$) under rigorous stirring, concentrated and freeze-dried under reduced pressure.

PDLSC and GMSC, as well as hBMMSC (as a positive control), were encapsulated in TGF- β 1 ligand ($50 \mu\text{g ml}^{-1}$) loaded alginate

at a density of 2×10^6 cells ml^{-1} of alginate solution [25]. Micro-bead formation was accomplished using a microfluidic device with a two-channel fluid jacket microencapsulator equipped with a micropipette. Alginate ($200 \mu\text{l h}^{-1}$) and soybean oil (10 ml h^{-1}) (Sigma) were injected into the device using syringe pumps. Alginate droplets were sheared off by the soybean oil, flowed out of the device and were added dropwise into a petri dish containing 100 mM CaCl_2 solution to form microspheres. The formed constructs were incubated at 37 °C for 45 min to complete cross-linking and then washed three times in non-supplemented Dulbecco's modified Eagle's medium (DMEM). Alginate hydrogel without cells was used as the negative control in this study.

2.4. *In vitro* release study

TGF- β 1 ($50 \mu\text{g ml}^{-1}$) loaded alginate microspheres were incubated in 500 ml of high-glucose DMEM supplemented with 100 U ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin in 48-well plates on a rotational shaker at 37 °C for 1 week. At each selected time interval, the medium was collected and analyzed for released TGF- β 1 using an anti-human recombinant TGF- β 1 ELISA kit (Abcam). At the end of release study, the residual entrapped TGF- β 1 was extracted by dissolving the scaffolds in 10% sodium citrate solution in distilled water and the percentage of cumulative released TGF- β 1 was measured.

2.5. Cell survival assay

Survival of the encapsulated MSCs was measured as described previously [22–24] using calcein AM to stain live cells and ethidium bromide homodimer-1 to stain dead cells (Invitrogen, Carlsbad, CA). The percentage of live cells was measured using ImageJ software. Additionally, an MTT assay (Invitrogen) was utilized to further evaluate the metabolic activity of encapsulated MSCs according to methods previously published [26].

2.6. *In vitro* chondrogenic assay

Encapsulated PDLSCs and GMSCs as well as hBMMSCs (2×10^6 in 1 ml of alginate) were cultured with a chondrogenic medium containing DMEM with 15% FBS, 2 mM L-glutamine, 1% ITS (BD Bioscience), 100 nM Dex, 100 μM ascorbic acid, 2 mM sodium pyruvate (R&D Systems, Minneapolis, MN), 100 U ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin.

4 weeks after the induction, the samples were fixed with 4% PFA, and paraffin sections were made. Chondrogenic differentiation, as indicated by matrix production, was determined by staining with 0.1% Safranin-O (Sigma-Aldrich, St Louis, MO) and 0.1% Toluidine Blue (Sigma-Aldrich) solution. Because the polyanions of alginate stained intensely by Safranin-O and Toluidine Blue resulted in a strong background, the sections were first washed with Ca^{2+} -free PBS to remove the cross-linker Ca^{2+} and dissociate the alginate prior to staining.

Sections were immunolabeled using primary Sox 9 and collagen II antibodies (Abcam) at 4 °C overnight, detected using Alexa fluor conjugated secondary antibody (1:200 dilution; Invitrogen), and counterstained with DAPI. The chondrogenic assays were determined from three independent samples for each experimental group. Five areas were randomly selected from each sample, then the positive area in the field was calculated with NIH Image-J software (NIH, Bethesda, MD) and shown as a percentage of the area over total field area.

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