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Chondrogenic differentiation of human mesenchymal stem cell aggregates via controlled release of TGF- β 1 from incorporated polymer microspheres

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Abstract

Aggregate culture is a useful method for inducing chondrogenic differentiation of human mesenchymal stem cells (hMSC) in a three-dimensional *in vitro* culture environment. Conventional aggregate culture, however, typically requires repeated growth factor supplementation during media changes, which is both expensive and time-intensive. In addition, homogenous cell differentiation is limited by the diffusion of chondrogenic growth factor from the culture medium into the aggregate and peripheral cell consumption of the growth factor. We have engineered a technology to incorporate growth factor-loaded polymer microspheres within hMSC aggregates themselves. Here, we report on the system's capacity to induce chondrogenesis via sustained delivery of transforming growth factor- β 1 (TGF- β 1). Cartilage formation after 3 weeks in the absence of externally supplied growth factor approached that of aggregates cultured by conventional methods. Chondrogenesis in the central region of the aggregates is enabled at TGF- β 1 levels much lower than those required by conventional culture using exogenously supplied TGF- β 1, which is likely a result of the system's ability to overcome limitations of growth factor diffusion from cell culture media surrounding the exterior of the aggregates. Importantly, the inclusion of growth factor-releasing polymer microspheres in hMSC aggregates could enable *in vivo* chondrogenesis for cartilage tissue engineering applications without extensive *in vitro* culture.

Keywords

MSC; cartilage; biomaterial; tissue engineering; controlled delivery

INTRODUCTION

The tissue engineering approach to repair or regenerate damaged cartilage is often based on providing transplanted or host cells with signals to guide their behavior and induce the creation of new replacement tissue through manipulation of the cellular milieu. Human mesenchymal stem cells (hMSCs) are a promising cell source for use in cartilage tissue engineering applications.¹ These cells are pluripotent mesenchymal progenitor cells that can be readily obtained from donor bone marrow.^{2,3} They can be expanded in culture for several passages without losing their pluripotent capabilities, which permits the generation of clinically relevant numbers of undifferentiated cells. In addition, hMSCs show great

utility for cartilage tissue engineering because they have the capacity to undergo chondrogenic differentiation under specific culture conditions.^{2, 3}

Aggregate culture is a commonly used method of inducing *in vitro* chondrogenesis of hMSCs in a three-dimensional culture environment. This method has been used to study the *in vitro* chondrogenesis of rabbit⁴ and human bone marrow MSCs,⁵ and it was recently modified for high-throughput aggregate formation in a 96-well format.⁶ In this modified format, aliquots of an MSC suspension are centrifuged in polypropylene wells, allowing the cells to form free-floating condensations through cell–cell mediated interactions. In the presence of soluble signaling factors including dexamethasone and transforming growth factor- β 1 (TGF- β 1), the cells in these aggregates are capable of undergoing chondrogenic differentiation and produce cartilage-specific extracellular molecules such as glycosaminoglycans (GAG) and type II collagen.^{4–6}

Using these culture methodologies, however, the MSC aggregate is subject to limitations of the diffusion of chondrogenic factors from the surrounding medium. The outer layer of cells in an aggregate is exposed to the highest concentrations of soluble chondrogenic factors, whereas cells closer to the center of the aggregate are exposed to lower concentrations. The factors must penetrate the cell layers according to Fick's Law of Diffusion, and the inner regions are exposed to lower levels of growth factors because of the increased time required for the factors to reach the central regions of the aggregate as well as consumption by the peripheral cells as the factor diffuses through the cell layers. This could result in a nonuniform spatial pattern of aggregate chondrogenesis. Because of these limitations, a higher concentration of TGF- β 1 in the culture medium is required to achieve chondrogenic differentiation in the central region of the aggregate.⁴ Traditional chondrogenic aggregate culture requires repeated dosing of TGF- β 1 during media changes, and aggregates are typically supplemented with 10 ng/mL of TGF- β 1 every other day for a period of up to 3 weeks.^{4, 6} This repeated growth factor supplementation during extended *in vitro* culture is both costly, because of the high concentrations of TGF- β 1 required, and time-intensive.

To address issues of sustained local protein delivery, growth factor-encapsulating polymer microspheres are widely used as delivery vehicles in tissue engineering technologies. One polymer commonly utilized for microsphere-mediated protein delivery is poly(lactide-co-glycolide) (PLGA), a biocompatible, biodegradable polymer that breaks down hydrolytically into lactic acid and glycolic acid, which are natural metabolic by-products.⁷ Growth factor release from PLGA microspheres occurs via diffusion out of the microspheres and polymer degradation, and it can be controlled by varying polymer properties such as molecular weight, copolymer ratio, and degree of crystallinity.^{7, 8} Through adjusting polymer formulation parameters, the protein release kinetics can be tailored to fit the requirements of a specific system.

To overcome the TGF- β 1 diffusion limitations associated with conventional aggregate culture and potentially enable *in vivo* aggregate chondrogenesis without extensive *in vitro* culture, we report here on a new engineered technology in which growth factor-encapsulating polymer microspheres are incorporated within hMSC aggregates.⁹ In this system, chondrogenic growth factor is released from PLGA microspheres uniformly dispersed within the cell aggregates, which bypasses the problems of TGF- β 1 diffusion from the culture medium. This allows for a more homogenous spatial pattern of chondrogenic differentiation within the aggregates and permits a much lower amount of growth factor to be delivered to achieve a similar chondrogenic response compared with exogenous TGF- β 1 supplementation. As the polymer microspheres degrade and the growth factor diffuses out, TGF- β 1 is released throughout the interior of the aggregates in a controlled manner, eliminating the need for repeated TGF- β 1 supplementation *in vitro* during media changes.

MATERIALS AND METHODS

Materials

Poly(lactic-*co*-glycolic acid) 5050 2A (50:50, 0.18 dL/g inherent viscosity) was purchased from Lakeshore Biomaterials. Poly(vinyl alcohol) (PVA), papain, and chondroitin 6-sulfate were purchased from Sigma-Aldrich. Calf thymus DNA standard was obtained from Rockland Immunochemicals. TGF- β 1 was from PeproTech, and Safranin O, Hoechst 33258, and Tween-20 were from Acros Organics. ITS β Premix was from BD Biosciences, and dexamethasone was obtained from MP Biomedicals. Ascorbate 2-phosphate was purchased from Wako Chemicals, USA. Sodium pyruvate and nonessential amino acids were obtained from Lonza. Ethyl acetate and other standard chemicals were obtained from Fisher Chemical. Polypropylene 96-well plates were purchased from Phenix Research Products. Statistical analysis software used InStat V. 3.06 software for Windows from GraphPad.

Polymer microsphere synthesis

Polymer microspheres were synthesized by a double emulsion technique as previously described.¹⁰ Briefly, 250 mg of PLGA dissolved in ethyl acetate was combined with 100 μ L of diH₂O containing 5 μ g TGF- β 1 (or diH₂O only for empty control microspheres) and sonicated to form the primary emulsion. The primary emulsion was combined with 1 mL of 5% (w/v) poly(vinyl) alcohol (PVA) and then vortexed to form the secondary emulsion. The mixture was immediately poured into a stirring extraction solution containing 0.3% PVA (w/v) in diH₂O. The microspheres were stirred for 3 h at room temperature to allow for solvent evaporation, collected by filtration, flash-frozen in liquid nitrogen, and lyophilized until dry. Microspheres were sterilized via a 10 min exposure to ultraviolet (UV) light (TUV 30W/G30T8; Philips) at a distance of 30 cm.

Polymer microsphere characterization

The PLGA microspheres were imaged using scanning electron microscopy (SEM; XL30 ESEM; Philips, Eindhoven, The Netherlands) to examine the surface morphology before and after UV sterilization. In addition, the mean microsphere diameter was determined using ImageJ (NIH) image analysis software (*N*5 1296 microspheres). Protein release profiles from the growth factor-loaded microspheres were determined by placing 5 mg of microspheres into 1 mL of 0.02% Tween-20 in PBS at 37°C (*N*5 4). Every 1 or 2 days, samples were centrifuged, the supernatant was removed, and 1 mL of fresh release medium was added to the samples. Supernatants from the release samples were stored at -80°C until analyzed. TGF- β 1 release was quantified using an ELISA kit (R&D Systems).

hMSC isolation and preparation

A human bone marrow aspirate was harvested from the posterior iliac crest of a healthy human volunteer donor after informed consent under a protocol approved by the University Hospitals of Cleveland Institutional Review Board. hMSCs were isolated from the bone marrow aspirate and cultured in the Skeletal Research Center Mesenchymal Stem Cell Facility as previously described.¹¹

Microsphere-incorporated hMSC aggregate formation

Sterilized microspheres were added at a concentration of 3.5 or 7.0 mg microspheres/mL to chemically defined medium containing DMEM-HG with 1% ITS⁺ Premix, 37.5 μ g/mL ascorbate-2-phosphate, 10⁻⁷ M dexamethasone, 1% nonessential amino acids, and 1% sodium pyruvate. First passage hMSCs were trypsinized, suspended in this solution at a concentration of 1.25 $\times$ 10⁶ cells/mL, and then dispensed into sterile V-bottom polypropylene microplates in aliquots of 200 μ L per well. The plates were centrifuged at

500g to form free-floating cell aggregates. Cell aggregates were transferred to fresh polypropylene microplates after 48 h, and the aggregate medium was replaced every other day. The mass of unincorporated microspheres was measured by collecting the remaining medium after 48 h to determine the microsphere loading efficiency into the aggregates. Media from four wells containing the unincorporated microspheres were combined into a single tube, and the microspheres were collected via centrifugation ($N = 3$). The microspheres were rinsed three times with diH_2O , flash-frozen in liquid nitrogen, lyophilized, and weighed.

Aggregate analysis

After 3 weeks of culture, at least three aggregates from each group were harvested for DNA and GAG quantification, and two from each group were processed for histologic examination. For biochemical analyses, aggregates were digested in 200 μL of a papain solution¹² for 2 h at 65°C. Four hundred microliters of 0.1N NaOH was added to the digests, which were then incubated for 30 min at room temperature and then neutralized with 400 μL of a solution containing 0.1N HCl, 5M NaCl, and 100 mM NaH_2PO_4 . A standard Hoechst 33258 dye assay was used to quantify total DNA in the aggregates.¹³ Fluorescence was measured on a Safire microplate reader ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 358/452$ nm; Tecan, Durham, NC) and compared to calf thymus DNA standards. GAG content was quantified using a previously described dot-blot assay.¹⁴ Aggregates for histologic evaluation were fixed for 20 min in 10% neutral buffered formalin, paraffin embedded, and sectioned. Adjacent sections were stained for GAG content with Safranin O and counterstained with Fast Green. For statistical analysis, two-tailed unpaired *t*-tests were performed using GraphPad InStat software, with $p < 0.05$ considered significant.

RESULTS AND DISCUSSION

Before their inclusion into cell aggregates, the polymer microspheres were characterized to determine size, surface morphology, and growth factor release kinetics. Prior to use in *in vitro* cell culture, the polymer microspheres were sterilized via exposure to UV light. Microspheres were examined using SEM before and after 10 min UV sterilization to monitor any changes in surface morphology, as changes to the surface would be one indicator of UV damage. No change was observed, and the microspheres appeared smooth both before [Fig. 1(A)] and after [Fig. 1(B)] UV exposure. As determined from the SEM images, the average diameter of the microspheres was 6.8 ± 4.1 μm , which is similar to the size of the cells.¹⁵ This aids in creating a well-mixed suspension of cells and polymer microspheres, and results in cell aggregates with microspheres dispersed throughout. Protein release kinetics from growth factor-loaded microspheres show sustained release of TGF- β 1 over the first 21 days, with a total cumulative release of 608 ± 24 pg per mg of microspheres after 3 weeks [Fig. 1(C)].

After forming and culturing microsphere-incorporated hMSC aggregates for 3 weeks, their chondrogenic differentiation was assessed through biochemical assays for DNA and GAG content as well as histological examination. Aggregate histology indicated greater GAG content in aggregates formed with the 1.4 mg/well initial microsphere loading [Fig. 2(B,D)] compared with those formed with the 0.7 mg/well initial microsphere loading [Fig. 2(A,C)], as is qualitatively apparent from the area and intensity of Safranin O stain. Biochemical assay results confirmed that aggregates formed with the 1.4 mg/well initial microsphere loading produced larger quantities of GAG [Fig. 3(A)] and GAG normalized to DNA [Fig. 3(B)] per aggregate after 3 weeks by comparison to those formed with the 0.7 mg/well initial microsphere loading, but the difference between these two groups was not significant ($p = 0.1953$). The average GAG content of aggregates formed with 0.7 and 1.4 mg/well initial microsphere loading corresponds to 27 and 55%, respectively, of the values obtained

by Penick et al. for aggregates cultured for 3 weeks in medium supplemented with 10 ng/mL TGF- β 1. Additionally, DNA assay results showed no significant difference in DNA content between aggregates formed with the 0.7 or 1.4 mg/well initial microsphere loadings [Fig. 3(C)], confirming that the polymer microsphere loadings had no differential effects on cell viability within the aggregates.

Most negative-control aggregates cultured with empty microspheres in the absence of TGF- β 1 disintegrated over the 3-week culture period, and those that could be harvested produced no GAG as evidenced by histology (data not shown). Lack of GAG production in the negative controls indicated that the PLGA microspheres themselves were not promoting aggregate chondrogenesis. Positive-control aggregates formed with empty microspheres and cultured in medium containing TGF- β 1 exhibited no significant change in DNA or GAG content by comparison to normal chondrogenic control aggregates containing no microspheres, as was apparent from biochemical analysis and histologic examination (data not shown). This demonstrates that the empty microspheres did not adversely affect cell viability or chondrogenic differentiation in the presence of exogenously supplemented TGF- β 1.

To determine the absolute quantity of growth factor incorporated into each hMSC aggregate, microsphere incorporation efficiencies were determined. The incorporation efficiencies for aggregates formed with initial loadings of 0.7 and 1.4 mg of polymer per well were $70.5\% \pm 9.2\%$ and $59.8\% \pm 1.9\%$, respectively, resulting in actual microsphere incorporations of 0.49 ± 0.06 mg and 0.84 ± 0.03 mg of microspheres per aggregate for the two groups. Assuming that release profiles from the microspheres within the aggregates are similar to those obtained in the release study [Fig. 1(C)], these microsphere masses correspond to 298 and 511 pg of total TGF- β 1 released per aggregate over the 3-week culture period, or an average release of 65 and 115 pg/mL per day for aggregates cultured in 200 μ L of medium. Interestingly, chondrogenesis is limited to only the very outer regions of aggregates cultured by conventional methods with comparable levels of exogenous TGF- β 1 supplementation.⁵ Johnstone et al. observed that culturing MSC aggregates in media containing TGF- β 1 in concentrations below 10 ng/mL resulted in decreased aggregate chondrogenesis. When cultured in medium containing 1 ng/mL TGF- β 1, chondrogenesis was limited to the outer third of the aggregates after 21 days of culture. When cultured in medium containing 500 pg/mL TGF- β 1, chondrogenesis was even more limited, suggesting that transport problems of growth factor from the culture medium were restricting chondrogenesis to only the outermost regions of the aggregates.

CONCLUSIONS

We have shown that TGF- β 1-loaded polymer microspheres incorporated within hMSC aggregates can induce chondrogenesis and cartilage formation after 21 days in the absence of externally supplied growth factor. Through the use of this system, it is possible to induce aggregate chondrogenesis with amounts of TGF- β 1 much lower than those required by conventional aggregate culture methods, likely because of the ability of the growth factor released from the incorporated microspheres to overcome the transport problems associated with delivering growth factor from the culture medium into the central regions of the aggregates. The use of growth factor-loaded polymer microsphere-incorporated hMSC aggregates eliminates the need for repeated TGF- β 1 dosing over a multiweek *in vitro* culture period, and *in vivo* chondrogenesis could be enabled without extended prior *in vitro* culture. Studies are currently underway to systematically investigate the effects of varying growth factor release profiles and microsphere amount on aggregate chondrogenesis.

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Figure 1.

Polymer microsphere morphology and growth factor release profile. Scanning electron microscopy (SEM) images of poly(lactide-co-glycolide) (PLGA) microspheres containing 100 ng transforming growth factor-β1 (TGF-β1) per mg microspheres (A) before and (B) after ultraviolet (UV) sterilization (scale bar = 10 μm). (C) TGF-β1 release kinetics from polymer microspheres (avg ± SD).

Figure 2. Histological evidence of chondrogenesis after 3 weeks (scale bar = 100 μ m). (A, B) Low- and (C, D) high-magnification images of aggregates containing (A and C) 0.7 mg/well or (B and D) 1.4 mg/well of microspheres encapsulating TGF- β 1. Safranin O/Fast Green stain.

Figure 3.

DNA and glycosaminoglycan (GAG) content for hMSC aggregates made with 0.7 mg/well (gray) or 1.4 mg/well (black) of growth factor-containing microspheres (avg $\pm$ SD). (A) GAG content, (B) GAG content normalized to DNA, and (C) DNA content.