Macromolecular Crowding Enhances Osteogenic Potential in Cell-Secreted Matrix-Loaded Spheroids

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INTRODUCTION: Bone deficits due to fracture, trauma, tumor resection, and congenital diseases represent a significant clinical problem to all patients across the lifespan. Tissue engineering is an exciting alternative to deliver bone-forming cells to the damaged tissue site, but these cells must be adequately instructed to achieve the desired goal upon transplantation. Mesenchymal stromal cells (MSCs) are used in tissue engineering due to their multilineage potential and safety profile. In addition, MSCs secrete a complex extracellular matrix (ECM), which serves as a storehouse of adhesive cues that naturally sequesters and presents instructive growth factors. Previously, we described a method to modulate the function of MSC-secreted ECM by tuning the culture conditions and recovering the ECM following decellularization of the cultures. Our published data demonstrate improved MSC survival and osteogenic differentiation from a cell-secreted ECM. Furthermore, we demonstrated that the response of MSCs to soluble osteoinductive cues is increased in the presence of this ECM, offering a biomimetic tactic to potentiate growth factor signaling at lower dosages than are frequently administered. However, the capacity of the ECM to serve as an effective platform for presenting inductive cues is unknown, especially in spheroids, dense cell aggregates that outperform monodisperse cells through increased cell-cell interactions. Herein, we aimed to **1)** use macromolecular crowding (MMC) to increase production or retention of endogenous GAG content within the ECM and **2)** characterize MMC ECM-loaded spheroid osteogenic differentiation. If successful, this approach will provide an exciting method to entrap endogenous and exogenous heparin-binding growth factors such as BMP-2 for local presentation to cells at lower dosages, thereby reducing unwanted side effects.

METHODS: Human bone marrow-derived MSCs (RoosterBio) were preconditioned for 7 days with 50 µg/mL L-ascorbic acid 2-phosphate (A2P) to promote ECM production. MSCs were seeded at 50,000 cells/cm^2 in growth media supplemented with A2P for ECM deposition. λ-carrageenan (75 µg/mL) was used as a macromolecular crowding agent by supplementing A2P-containing growth media. Cells were cultured for 9 days to deposit ECM. In order to study the influence of decellularization on retention of GAG content, we evaluated two methods: *i)* our conventional decellularization method using a mixture of Triton X-100, and NH_4OH followed by treatment with Deoxyribonuclease I (DNase); or *ii)* a solution of DNase only. We included a non-decellularized group rinsed twice with PBS and without other treatment as a control.

We tested removal of DNA following decellularization with a DNA assay, total protein with a bicinchoninic (BCA) assay, and GAG content with a dimethyl methylene blue (DMMB) assay. Following deposition of ECM (30 µg/cm^2) on tissue culture plastic (TCP), we seeded BMP Responsive calvarial osteoblasts (BRITER) at 50,000 cells/cm^2 to observe stimulation by endogenous BMP-signaling after 24 hr using bioluminescence imaging. Collagen-coated TCP and uncoated TCP served as negative controls. We imaged BRITER cells in an IVIS machine.

To produce spheroids, we dispensed cell suspensions with or without ECM onto agarose microwells. All spheroids were made with 15,000 cells/spheroid and ECM-loaded spheroids with 5 µg ECM/spheroid. We centrifuged the microwells with cell suspensions and ECM at 900 rpm for 8 min and incubated them for 48 hours until formation. Spheroids were cultured in standard osteogenic media (OM) on agarose microwells for 21 days, stained with 0.5 µM Calcein AM and 1.25 µM Propidium Iodide, and imaged on a confocal microscope. We used a Calcium CPC Liquicolor StanBIO kit for calcium quantification.

All experiments were performed with at least three independent replicates. Statistical analysis was performed using a one-way ANOVA with multiple comparisons test. Groups with statistically significant differences do not share the same letters.

RESULTS SECTION: Both methods of decellularization effectively removed more than 98% of cellular DNA from the ECM as measured with a DNA assay. Compared to the conventional method (0.07±0.02 µg), the addition of carrageenan to the culture media resulted in more GAG retention (0.64±0.6 µg). GAG content was greatest when treating cells with carrageenan and decellularizing only with DNase (2.0±0.4 µg; n=4, $p<0.05$) (**Fig. 1A**). We assessed whether GAG content, increased by culture with carrageenan, would increase the retention of endogenous BMP-2 secreted by MSCs during ECM deposition. We observed nearly a 250% increase in BRITER cell activity measured by bioluminescence when cells were cultured on carrageenan-treated ECMs decellularized with only DNase compared to our conventional method of decellularization or collagen-coated and uncoated controls (**Fig. 1B**). These data suggest that endogenous retained growth factors may contribute to the efficient binding of growth factors to the ECM.

Next, we incorporated MMC dECM into spheroids and cultured them for 21 days. MMC dECM-loaded spheroids had larger diameters than the controls without ECM, and all spheroids remained alive throughout the culture time (**Fig. 1C**). The addition of MMC dECM onto spheroids significantly increased calcium deposition at Day 21 (**Fig. 1D**). These data demonstrate a beneficial effect of MMC dECM in osteogenic differentiation and calcium deposition.

DISCUSSION: This study provides evidence for retaining endogenous growth factors in decellularized matrices by increasing or retaining GAG content. At this time, it is unknown whether carrageenan increased the amount of ECM deposited by cells or preserved its presence during decellularization. This ECM platform can be easily harvested and used as a coating on a number of implantable substrates to potentially sequester endogenous growth factors from the local environment. This merits further investigation.

SIGNIFICANCE: These data demonstrate a simple approach to retain GAG and endogenous growth factors within decellularized matrices. Future work will examine cell osteogenic and chondrogenic differentiation when cultured on carrageenan modified ECMs modified with inductive factors. This approach represents a facile method to improve upon the nature of endogenous cell-secreted matrices for use as instructive biomaterials.

IMAGES AND TABLES:

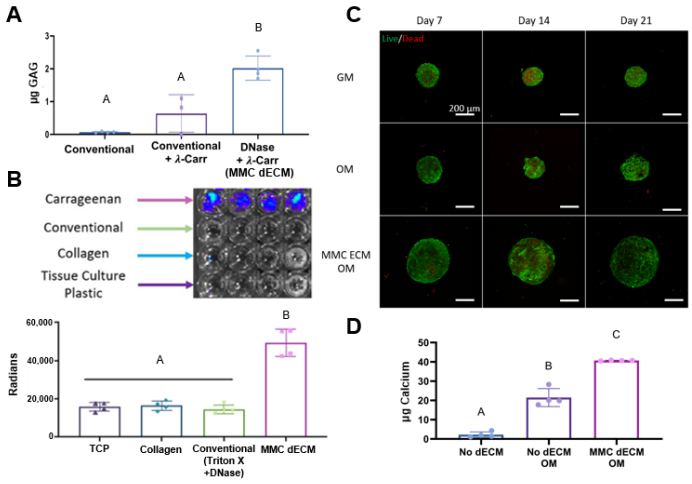

Figure 1. Decellularization and addition of carrageenan increases retention of endogenous GAGs and growth factors and increases calcium deposition. (A) Inclusion of carrageenan and decellularization with only DNase increased GAG content compared to the conventional decellularization method with Triton-X (n=3 per group). **(B)** Decellularized ECM coating with carrageenan and DNase treatment retained endogenous BMP-2, evidenced by increased radians from the bioluminescent imaging of BRITER cells (top) and signal quantification (bottom) (n=4). **(C)** Live/Dead staining of spheroids in growth media (GM), osteogenic media (OM), and MMC ECM-loaded spheroids. **(D)** MMC ECM-loaded spheroids had increased calcium content compared to spheroids without ECM. A one-way ANOVA with multiple comparisons was performed. Groups with statistically significant differences do not share the same letters.