Mesenchymal Stem Cell-Based Cartilage is Unstable in Very Long Term In-Vitro Culture

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Introduction

Adult derived progenitor cells, including mesenchymal stem cells (MSCs), are a promising cell source for the treatment of non-healing musculoskeletal disorders. Soluble factors combined with appropriate 3D culture materials support the chondrogenic differentiation of these cells,[1,2] and in some instances, a mechanically viable cartilage-like tissue can be produced.[3,4] However, recent studies have also demonstrated that this chondrogenic phenotype is unstable, producing mineralized matrix akin to bone when implanted subcutaneously[5] or challenged with hypertrophic differentiation media *in vitro*.[6] While the focus of the cartilage tissue engineering community has largely been skewed towards the anabolic functionality of these cells, stability must be achieved if constructs are to function *in vivo*. Previously, we have shown that MSC viability in agarose hydrogels is markedly lower than chondrocytes (CH) cultured identically.[7] Because agarose is an unsupportive 3D material (does not provide biologic cues or attachment), alternative hydrogels may yield different outcomes. We have recently shown that hyaluronic acid (HA)-based materials can support the development of tissues that match some native properties.[3] The objective of this study was to evaluate MSC instability in long term agarose and HA culture by delineating the time course of decline in MSC viability in comparison to chondrocytes and by assessing the peaks and declines in matrix accumulation and mechanical properties across gel types.

Methods

Juvenile bovine bone marrow MSCs (jbMSCs, P2) and primary CH were encapsulated in 2% agarose (Ag) cylinders (4 mm ø x 2.5 mm) at a density of 20M cells/mL. Constructs were fed twice weekly with 1 mL of chemically defined media (CM) with (+) or without (−) 10 ng/mL TGF-β3 as in[7] through 168 days. In a separate study, jbMSCs (P2) were encapsulated at a density of 60M cells/mL in 1% (w/v) methacrylated HA crosslinked via a UV initiated addition reaction.[3] Constructs were fed thrice weekly with CM+ through 126 days. Compressive equilibrium (eq.) modulus was evaluated via unconfined compression (10% strain) (n=3 Ag, 4 HA) and samples were papain digested for biochemical assessment of glycosaminoglycans (GAG) via the dimethylmethylene blue assay. Percent viability in the center of the constructs (n=3, Ag) was assessed in the center of the constructs as in[7] with the Live/Dead Cell Viability Kit. Constructs were paraffin processed and stained for proteoglycans (PGs, Alcian Blue) and calcium deposits (Alizarin Red). Significance ($p<0.05$) was established with 2-way ANOVA (Ag; day and media type as independent variables) and 1-way ANOVA (HA; day as independent variable) and Tukey's post-hoc.

Results

In the center of Ag constructs, CHs retained stable viability in CM(−); however, the population was only stable through day 56 in CM+ (Fig 1). MSCs in CM(−) and CM(+) showed marked declines in viability as early as day 7, retaining only 50% in CM(−) and 62% in CM(+) (compared to ~90% at day 1). Although MSC CM(−) continued to decline from day 28 to day 56, viability stabilized for CM(+) between days 28 and 112 before declining once again. In terms of eq. modulus, values for CH CM(+) constructs increased from day 56 to day 168, whereas those for MSC CM(+) constructs did not (Fig 1). Interestingly, there was a spike in eq. modulus of MSC CM(−) at the last time point due to mineralization as this tissue stained heavily for calcium deposits (not shown). In MSC-seeded HA constructs, similar instability was observed at later time points. GAG content peaked at ~1.6 mg/construct at day 63, before declining to ~1.2 mg by day 84 and ~0.27 mg by day 126 (Fig 2). Similarly, compressive properties peaked at 218kPa at day 49, declining to 110kPa by day 84 before plummeting to almost zero at day 126 ($p<0.001$). Staining of proteoglycans in day 168 (Ag) and day 126 (HA) MSC CM+ constructs showed a marked decrease in intensity compared to day 56 constructs (Fig. 3). These data confirm the degradation of the previously established matrix.

Discussion

Recent advances in stem cell based tissue engineered cartilage (particularly with regard to

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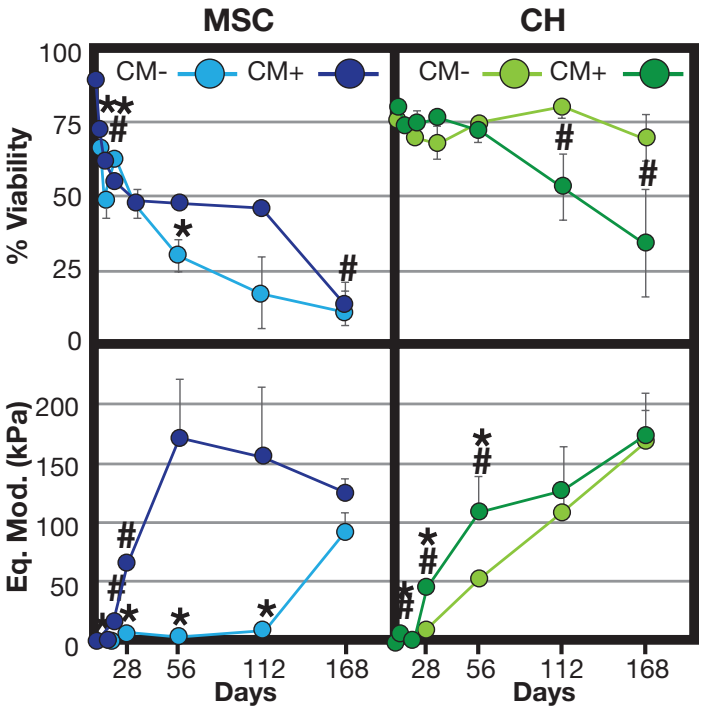

Figure 1. (Top) Viability in the center of constructs is less stable for MSCs, particularly at early time points ($p<0.05$, * vs. previous time point CM−, # vs. previous time point CM+). (Bottom) Eq. modulus of CH and MSC-laden constructs. ($p<0.05$, * vs. peak CM−, # vs. peak CM+).

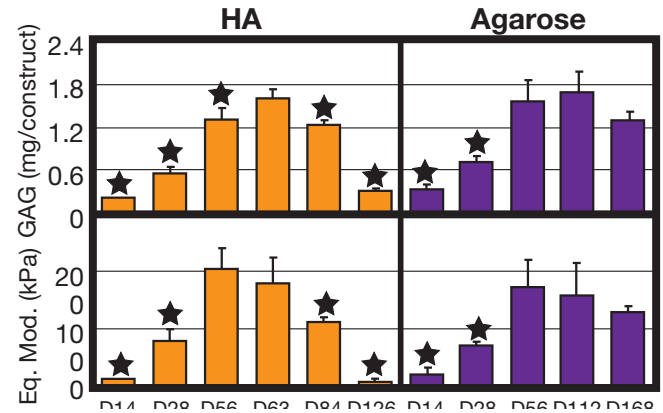

Figure 2. GAG content (top) and eq. modulus (bottom) decline significantly from peak values at later time points in HA hydrogels. ($p<0.05$, star vs. peak).

functional properties) support the promise of MSCs for use in cartilage repair; however, the lack of long term stability of these constructs both *in vitro* and *in vivo* raises important new considerations. In this study, we identified a rapid, followed by a progressive, decline in MSC viability in 3D Ag constructs. Furthermore, we showed that with extended time in culture, the instability in MSC phenotype and/or viability resulted in the destruction of previously established matrix and mechanical properties. Perhaps most interestingly, we observed spontaneous calcification of matrix in conditions where TGF-beta was not present after very long culture durations. This instability is not simply a function of Ag culture, as a very similar (and perhaps even advanced) time course of

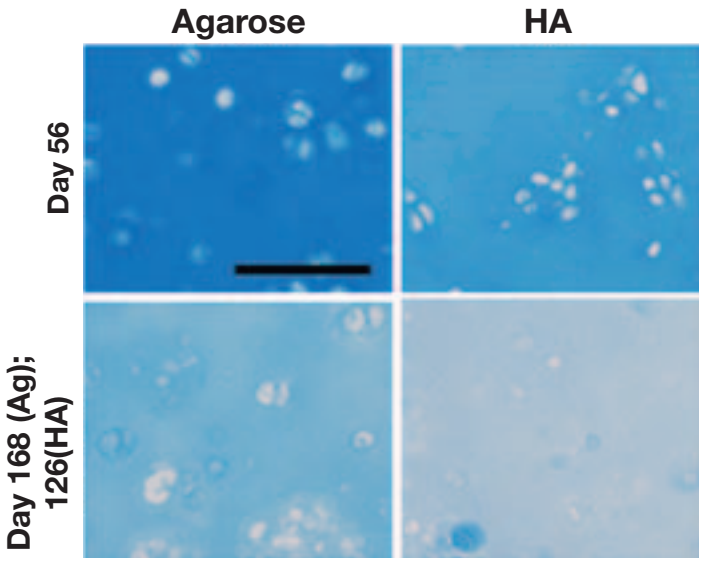

Figure 3. Decreased proteoglycan staining intensity at later time points suggests breakdown and/or loss of matrix. Scale = 100 μm.

self-destruction was observed in HA gels. These data suggest that even in a controlled culture environment, removed from any perturbations arising from *in vivo* soluble factors, chondrogenic instability may be inherent to MSCs and will need to be addressed to further the clinical applicability of this cell type.

Significance

This work demonstrates a pronounced instability in MSC-based cartilage cultured for extended durations, independent of biomaterial scaffold, and despite achievement of near native mechanical properties. If MSC-based cartilage constructs are to be used clinically, concerns related to whether this instability would be progressive and unpreventable after implantation will need to be addressed.

Acknowledgements

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