An Inverse Agonist for Retinoic Acid Receptors Boosts Mesenchymal Stem Cell Chondrogenesis and Functional Properties of Tissue Engineered Cartilage

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Introduction

Members of the transforming growth factor family are widely used for chondrogenic induction of musculoskeletal progenitor cells. However, given the incompleteness of differentiation mediated by this morphogen on its own, interest has focused on the use of pharmacological agents that can induce chondrogenesis through alternative pathways.[1] Retinoids play a key role in skeletogenesis, with retinoic acid directly interacting with nuclear receptors to modulate transcription. Inhibition of retinoic acid receptors (RARs) positively regulates chondrogenesis through increases in SOX9 expression.[2] Few studies exist examining the potential of RAR antagonists or inverse agonists for cartilage tissue engineering,[3,4] and none have assessed the functional properties that arise from the addition of these molecules. The objective of this study was to assess the molecular and functional effects of supplementation with the pan-RAR inverse agonist BMS 493 on mesenchymal stem cells (MSCs) cultured in three-dimensional pellet and agarose culture systems. In additional to examining target pathways through the use of 96-well gene arrays, we assessed the functional effects this molecule has on matrix content and construct mechanical properties.

Methods

Juvenile bovine MSCs were expanded through passage 1 or 2. For pellet culture (P), 250,000 cells were pelleted and cultured in a non-adherent conical 96-well plate and cultured for 21 days. For hydrogel culture (H), cells (20 M/mL) were encapsulated in 2% agarose constructs (4 mm Ø, 2.25 mm thick). A 7 day hydrogel study (D7) was conducted independently from a 21 day study (D21). Constructs and pellets were fed twice weekly with chemically defined media (CM) with (+) or without (−) 10 ng/mL TGF-β3. The pan-retinoic acid receptor inverse agonist, BMS 493 (Tocris Bioscience), was added at concentrations of 0.5 μM - 2μM (P / H 7D) or 0.1 μM - 1 μM (H 21D). Cell viability was assessed with the Live/Dead Cell Viability Kit as in[5] (H D7, n=3). Constructs were paraffin processed, sectioned, and stained for proteoglycans (Alcian Blue; H D7, n=3). RNA of D7 constructs was extracted using TRIZOL-chloroform and real-time PCR of 96 genes run using a Signal Transduction PathwayFinder™ PCR Array plate (human; SABiosciences). Samples included CM−, CM+, and CM+ 2uM BMS (n=3 combined). ΔΔCt analysis was performed to assess relative expression across all samples with values normalized to GAPDH and monolayer cells taken as the control group. Additional assays (n=3), including assessment of glycosaminoglycan (GAG) content (DMMB assay as in[5] (P [2 pellets combined per n]/H 21D)) and compressive equilibrium modulus (10% stress relaxation as in,[5] H 21D), were carried out to determine the contribution of BMS to functional properties of engineered constructs. Significance ($p<0.05$) was established with 1-way ANOVA and Tukey's post-hoc correction with media as the independent variable for GAG content, eq. modulus, and viability (comparisons made within time point for pellet culture).

Results

Addition of 2 μM BMS 493 to chondrogenic media resulted in an increase in GAG in pellet cultures after 21 days in both CM− (~5.4 fold) and CM+ (~1.4 fold) conditions, with CM+2μM BMS resulting in the highest GAG content of 104 μg/pellet (Fig 1). Similarly, Alcian blue staining of hydrogels on day 7 showed an increase in the number of cells with intense pericellular staining of proteoglycans in both 2μM BMS conditions compared to controls (Fig 2). Incorporation of BMS had no effect on cell viability at day 7. Viability ranged from 78.2-82.5% in CM- conditions and 85.1-87.3 in CM+ conditions. In hydrogel cultures (21 days), incorporation of BMS at the highest concentration assessed (1 μM) in CM+ media had a striking effect on both GAG and eq. modulus (Fig 3). Interestingly, the increase in these functional outcomes was not proportional (a 59% increase in GAG with an 87% increase in eq. modulus). PCR arrays revealed the down regulation of several genes as a result of CM+2μM BMS treatment compared to CM+, including those related to metabolism

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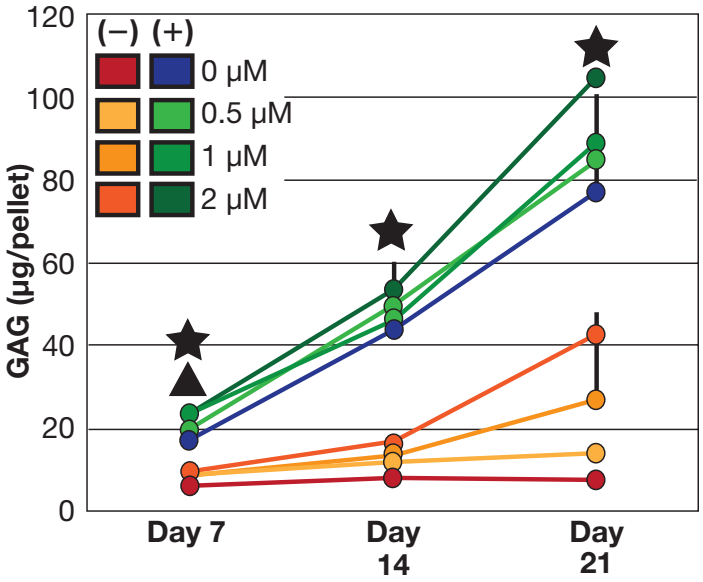

Figure 1. Increase in GAG in pellet culture with the incorporation of BMS. ($p<0.05$, star = −/+ 2μM vs. respective control; triangle = −/+ 1μM vs. respective control).

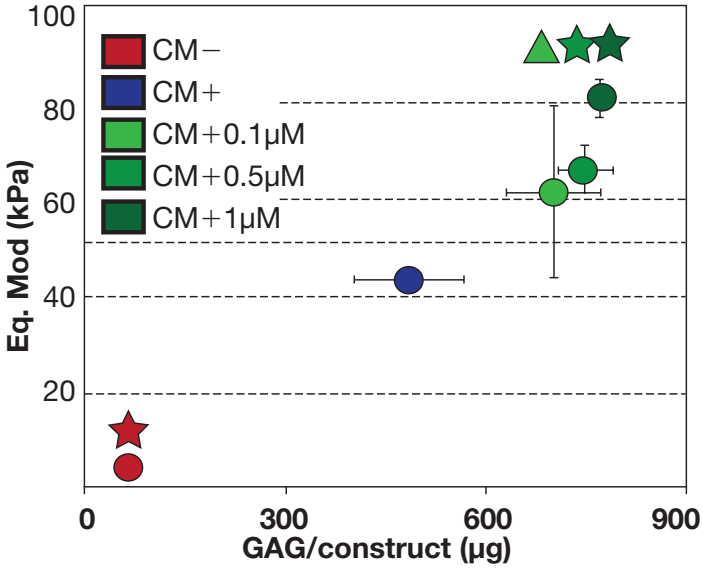

Figure 2. Increase in GAG is disproportionate to increase in eq. modulus in hydrogels after 21 days. ($p<0.05$, star = GAG and eq. mod vs. CM+; triangle = GAG only vs. CM+).

(NQO1, LDHA), lipid biosynthesis (ASCL4), anti-apoptotic function (BCL2, BIRC3), and a retinol binding protein (RBP1) (Fig 2). Upregulation of a gene whose product binds fatty acids (PPARD), and a gene involved in chondrogenesis (WNT5A) also occurred in CM+BMS treated versus CM+ cells.

Discussion

Here we show that antagonism of RARs elicited via an inverse agonist is highly beneficial for early maturation of stem cell based cartilage constructs. Incorporation of the inverse agonist BMS not only resulted in an increase in GAG in pellet and hydrogel cultures, but also an ~2 fold increase in eq. modulus by 21 days. We also found positive regulation of

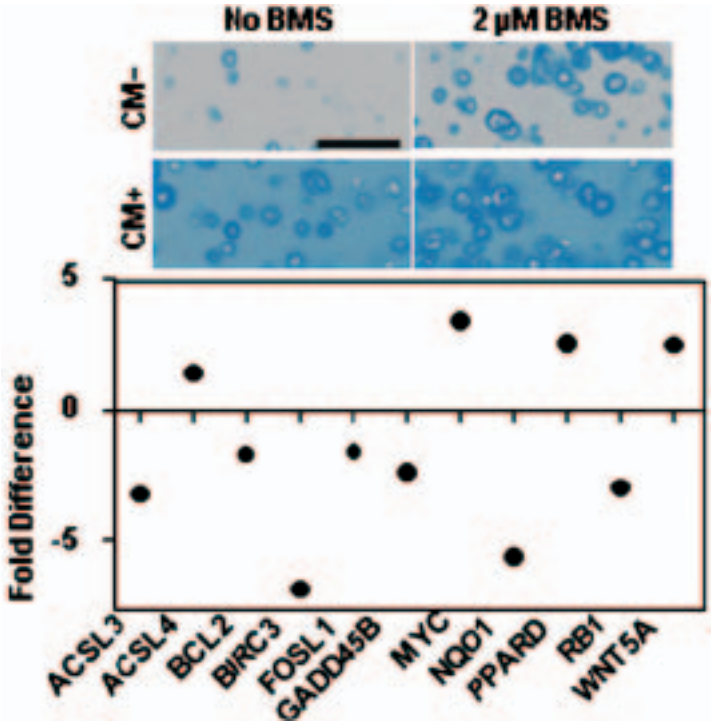

Figure 3. Top) Increase in cells with intense pericellular GAG in both CM− and CM+conditions with addition of BMS. (Scale = 100 μm) Bottom) Highest fold changes of CM+2μM BMS to CM+ after 7 days of hydrogel culture.

genes that could prove to be useful in an *in vivo* setting of an osteoarthritic joint, such as WNT5A, which inhibits canonical WNT signaling and thereby promotes chondrogenesis. In addition, we observed down-regulation of metabolic (NQ01, LDHA) and anti-apoptotic genes occurred (BLC2 and BIRC3). Down-regulation of these latter two genes indicates that the cells exposed to BMS were under less stress than those in CM+, and so less in need of activation of anti-apoptotic pathways. While we observed no significant differences in viability of these constructs at the early time point at which gene expression was assessed, longer term evaluation in the context of BMS may reveal differential viability relative to CM+ cultures. Alternatively, if CM+ cells are under stress, while BMS cells are less so, more efficient utilization of cellular resources (and production of extracellular matrix) may occur, leading to the improved functional outcomes we observed.

Significance

In this study, the use of an RAR inverse agonist significantly enhanced the functional properties of stem cell based cartilage constructs, potentially reducing the *in vitro* culture time necessary prior to implantation. Our findings also suggest that additional activated pathways, including those involved in cell stress and apoptosis, may warrant further investigation as markers of efficient chondrogenic activity in 3D culture.

Acknowledgements

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