Characterization Of A 3d Model Of Mineralization In Atdc5 Cells

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Introduction: Mineralization of cartilage tissue occurs naturally in the growth plate and aberrantly in articular cartilage. The physiological mechanism of this process has been the subject of numerous studies using the chondroprogenitor mouse cell line ATDC5 detailing the role of calcium, organic and inorganic phosphates, and various hormones on the calcification process (1, 2). However, this analysis has always been performed in monolayer (2D) cultures with a variety of calcium and phosphate concentrations, many of which are supraphysiologic and supersaturating (3). With the increasing interest in modification of the matrix during this process, it is essential to develop physiologically relevant models to understand the impact of the calcium/phosphate combination on matrix accumulation and mineral formation. Herein a pellet culture model of cartilage mineralization using ATDC5 cells is presented and compared to monolayer culture. The effects of phosphate type and calcium/phosphate ratio on the progression of chondrocyte maturation, matrix production, gene expression, and mineral accumulation were examined.

Methods: Monolayer Culture (2D): ATDC5 Cells were seeded in 24 plates and cultured in DMEM/F12 containing 5% FBS (basal media). At confluence (~3 days), the media was supplemented with 1% ITS and 50μg/ml ascorbic acid-2-phosphate (AA-2P) to induce chondrogenesis (control). Pellet Culture (3D): Pellets were formed with 1x10^6 ATDC5 cells in 96 well polypropylene plates and culture in basal media for 2 days to allow pellet condensation and then switched to the control medium. All experiments went for 21 days and sGAG and Ca2+ levels were measured with the DMMB dye and Arsenazo assays, respectively. Exp #1: CaCl2 was added to control medium 2D and 3D cultures bring to Ca2+ levels to 1.3mM and treated cultures were given 10mM beta glycerophosphate (βGP). A 2nd set of 3D samples were also treated with 3 and 5 mM βGP to determine the effect of organic phosphate dose on sGAG production and mineral formation. Additionally, to determine the time-dependent effect of βGP addition on matrix deposition and quality in 3D cultures, 10mM was added at D0, D7, or D14. Quality was examined using infrared techniques. Exp #2: Ca2+ accretion was noted in 3D cultures prior to the sGAG deposition. Therefore, to examine calcification in chondrogenic and non-chondrogenic ATDC5 cultures, both 2D and 3D cultures in basal and chondrogenic media were treated with 10mM βGP for 21 days. Additional samples were treated with levamisole to inhibit alkaline phosphatase activity and ensure that the mineralization was cell mediated. Exp #3: To examine the effect of Ca2+ and inorganic phosphate (Pi) ratios on sGAG production and mineral accretion, 3D cultures were treated with either 1mM Ca2+ and 4mM Pi (Ca/Pi=0.25; standard concentrations for limb-bud mineralization studies) or 2.4 mM Ca2+ (serum Ca2+ levels) and 1, 1.5, or 4mM Pi (Ca/Pi=2.4, 1.6, and 0.6, respectively). The optimal combination of Ca2+ and Pi was also delivered after D7 and compared to the effects of delayed delivery of βGP.

Results: Organic phosphate: Preliminary studies found that mineralization only occurred with 10mM βGP (not shown), therefore Exp #1 was conducted at this concentration. As shown in Fig 1a & b, the rate of sGAG production and Ca2+ accretion per unit DNA was higher in 3D cultures. βGP also decreased
sGAG deposition in 3D cultures in a dose-dependent manner (Fig 1c & d). PCR analysis (Fig 2) indicates that this may be due to decreased levels of aggrecan gene expression with 5 and 10mM βGP. Interestingly, the timecourse of aggrecan expression was not altered whereas it was altered for collagen II expression with all concentrations of βGP. IR of pellet cultures revealed vast changes in peaks indicative of collagen and sGAG accumulation with increasing βGP dose, further supporting the biochemistry data (Fig 2). Delaying addition of βGP for 7 days or longer removed sGAG deposition inhibition and the rate of mineral deposition changes when BGP added after day 7 (Fig 3a & b). Inhibition of ALP abolished mineralization, however, mineral accrued in both 2D and 3D exposed to chondrogenic and basal media (Fig 3c). This indicates that the mineral deposited with βGP treatment includes dystrophic stoichiometric precipitates. This occurs despite the fact that conversion to free Pi is cell-mediated as evidenced by levamisole experiments. This is consistent with previous experiments demonstrating that cells that do not naturally produce mineralizable matrices can similarly be made to mineralize by the addition of βGP and is suggests that βGP supplementation of such cultures may not be appropriate for mechanistic studies of mineralization using ATDC5 cells (4).

**Discussion:**

**Significance:** An in vitro 3D model in which the role of phosphates on chondrogenic matrix deposition, cell maturation, and subsequent calcification may be studied will be useful in cartilage tissue engineering and mechanistic studies of osteophyte formation during arthritis. Previously published protocols designed to accelerate mineralization in ATDC5 cells in monolayer may lead to appreciable changes in the matrix composition and gene expression of ATDC5s in 3D culture and disrupt the normal progress of chondrogenesis.
Fig 1. ATDC5 cultures treated with 10mM BGP (open square or circle): SGAG production (a) and Ca^{2+} levels (b) in 3D (squares) and 2D (circles). BGP dose response in 3D (0, 3, 5, or 10mM): SGAG production (c) and Ca^{2+} levels (d).
Fig 2. qPCR analysis of Col2 and Aggrecan expression (Top) and IR analysis (bottom) in 3D cultures exposed to 3, 5, & 10 mM βGP.
Fig 3. Delayed administration of βGP restores normal sGAG production (a) and alters the rate of mineral accrual (b). ALP is necessary for mineral accrual but chondrogenesis is not (c).

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