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Phosphate regulates chondrogenesis in a biphasic and maturation-dependent manner

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ABSTRACT

Inorganic phosphate (Pi) has been recognized as an important signaling molecule that modulates chondrocyte maturation and cartilage mineralization. However, conclusive experimental evidence for its involvement in early chondrogenesis is still lacking. Here, using high-density monolayer (2D) and pellet (3D) culture models of chondrogenic ATDC5 cells, we demonstrate that the cell response to Pi does not correlate with the Pi concentration in the culture medium but is better predicted by the availability of Pi on a per cell basis (Pi abundance). Both culture models were treated with ITS+, 10mM β-glycerophosphate (βGP), or ITS+/10mM βGP, which resulted in three levels of Pi abundance in cultures: basal (Pi/DNA < 10 ng/μg), moderate (Pi/DNA=25.3 – 32.3 ng/μg), and high abundance (Pi/DNA > 60 ng/μg). In chondrogenic medium alone, the abundance levels were at the basal level in 2D culture and moderate in 3D cultures. The addition of 10 mM βGP resulted in moderate abundance in 2D and high abundance in 3D cultures. Moderate Pi abundance enhanced early chondrogenesis and production of aggrecan and type II collagen whereas high Pi abundance inhibited chondrogenic differentiation and induced rapid mineralization. Inhibition of sodium phosphate transporters reduced phosphate-induced expression of chondrogenic markers. When 3D ITS+/βGP cultures were treated with levamisole to reduce ALP activity, Pi abundance was decreased to moderate levels, which resulted in significant upregulation of chondrogenic markers, similar to the response in 2D cultures. Delay of phosphate delivery until after early chondrogenesis occurs (7 days) no longer enhanced chondrogenesis, but instead accelerated hypertrophy and mineralization. Together, our data highlights the dependence of chondroprogenitor cell response to Pi on its availability to individual cells and the chondrogenic maturation stage of these cells and suggest that appropriate temporal delivery of phosphate to ATDC5 cells in 3D cultures represents a rapid model for mechanistic studies into the effects of exogenous cues on chondrogenic differentiation, chondrocyte maturation, and matrix mineralization.

1. Introduction

Understanding the factors regulating chondrogenesis and endochondral ossification is important to treating developmental disorders and the cartilaginous diseases, as well as improving stem cell-based cartilage tissue regeneration. Dysfunction in chondrocyte proliferation can lead to developmental issues, such as achondroplasia (Pannier et al., 2010) and hypochondroplasia (Krejci et al., 2008). Hypertrophic differentiation of chondrocytes, a process that occurs normally during endochondral ossification, is associated with pathogenic changes in osteoarthritic articular cartilage (Von Der Mark et al., 1992; Johnson et al., 2003; Thouverey et al., 2009). Mesenchymal stem cells (MSCs) also express hypertrophic markers after chondrogenic induction in vitro (Farrell et al., 2014; Mauck et al., 2006) and in vivo (Pelttari et al., 2006), hindering their clinical application for cartilage tissue regeneration.

One potent factor that mediates cell fate in chondrocytic cells is inorganic phosphate (Pi). Studies in ATDC5 (Magne et al., 2003; Altaf et al., 2006; Newton et al., 2012), primary growth plate (Kim et al., 2010), and limb bud cells (Boskey et al., 1997) have established that Pi

Abbreviations: βGP, β-glycerophosphate; Pi, inorganic phosphate; 2D, two-dimensional; 3D, three-dimensional; qPCR, quantitative polymerase chain reaction; sGAG, sulfated glycosaminoglycan; ALP, alkaline phosphatase; PiT1, sodium-dependent phosphate cotransporter 1, pyrophosphate-PPi

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in the range of 2–4 mM upregulates collagen type X expression and apoptosis in mature chondrocytes, thus, inducing terminal differentiation. The role of Pi as a signaling molecule has also been demonstrated in several other cell types (Shih et al., 2014a; Beck, 2003; Rangrez et al., 2012; Chang et al., 2006; Spina et al., 2013), including osteochondral progenitor cells in which it induces osteogenic differentiation (Shih et al., 2014a; Beck, 2003). Furthermore, many of these cell-mediated activities are regulated by other exogenous cues, such as growth factors (Cecil et al., 2005; Watanabe et al., 2001a; Palmer et al., 2000) and matrix composition (Boskey et al., 1997).

The response to Pi is orchestrated by the activity of phosphatases, such as alkaline phosphatase (ALP) (Bellows et al., 1991; Mikami et al., 2016; An et al., 2017), the availability of pyrophosphate (PPi) (Tenenbaum, 1987; Addison et al., 2007), and activity of sodium-dependent phosphate transporters in skeletal (Suzuki et al., 2006; Cecil et al., 2005; Kimata et al., 2010) and non-skeletal systems (Li et al., 2006; Yang et al., 2004). ALP cleaves PPi to release Pi (Murshed et al., 2005), and ALP expression in the growth plate progressively increases from being hardly detectable in the proliferative zone to higher in the maturing zone, and highest in the hypertrophic zones (Miao and Scutt, 2002). Deficiency of ALP activity causes hypophosphatasia, which is a skeletal disease associated with diminished or absent hypertrophic zones in the growth plate (Fedde et al., 1999). The expression profile of sodium-dependent phosphate cotransporter 1 (PiT-1) has been found to be variable during endochondral ossification. *In vitro*, expression levels were found to be highest during the early stages of chondrogenesis in the CFK2 chondroprogenitor cell line (Wang et al., 2001) and during the proliferative and the early phase of hypertrophy in ATDC5 cells (Kimata et al., 2010; Guicheux et al., 2000). *In vivo*, the expression profile of PiT-1 has been variable depending on species (Palmer et al., 1999). The effects of Pi during terminal differentiation require the activity of PiT-1 (Suzuki et al., 2006; Cecil et al., 2005; Kimata et al., 2010; Mansfield et al., 2001), as inhibition of its activity negates Pi induced apoptosis during terminal differentiation (Mansfield et al., 2001).

Although ALP activity is highly upregulated in hypertrophic and terminally differentiated chondrocytes during endochondral ossification (Miao and Scutt, 2002), Pi is present in the resting and proliferative zones of the growth plate (Kakuta et al., 1985). However, few studies have investigated whether chondrogenic differentiation or early events in the endochondral ossification pathway are regulated by Pi. Kimata et al. demonstrated that Pi treatment upregulated cell proliferation through ERK1/2 mediated cyclin D1 expression in the ATDC5 cell line and primary chondrocytes (Kimata et al., 2010). Wang et al. also showed that transient Pi treatment upregulated collagen type II gene expression (Wang et al., 2001). Taken together with the previously reported expression profile of PiT-1 and activity of ALP, these studies suggest that Pi plays a direct regulatory role in early chondrogenic differentiation and proliferation during endochondral ossification.

To elucidate the role of phosphate in early chondrogenic events, we used ATDC5 cells in 2D and 3D cultures. This cell line, established by Atsumi et al. from the mouse teratocarcinoma cells AT805, recapitulates the phases of endochondral ossification, from chondrogenic commitment to hypertrophic differentiation, with the addition of insulin (Atsumi et al., 1990; Shukunami et al., 1996, 1997). In these cells, chondrogenic maturation and matrix mineralization occur on an accelerated timescale if given an exogenous source of phosphate (Altaf et al., 2006; Newton et al., 2012). Since ALP activity has also been shown to change as chondrocytes differentiate and mature (Miao and Scutt, 2002; Sakano et al., 1993), we used β-glycerophosphate (βGP) as our phosphate source to ensure cells control the release of phosphate. Although 10 mM βGP is considered a supraphysiologic concentration of organic phosphate for in vitro studies (Bonewald et al., 2003), it was found in this study that the maximum Pi concentration in the media using this level of βGP is 4 mM, the concentration employed in many previous mechanistic studies (Kimata et al., 2010; Magne et al., 2003; Wang et al., 2001). So that the Ca × Pi never reaches levels that lead to dystrophic precipitation of $CaPO_4$, calcium concentration was maintained at 1.3 mM (Yang et al., 2004; Hunter and Bader, 1989). Using this media formulation, we found that the response of chondroprogenitor cells was regulated by Pi availability on a per cell basis (Pi abundance). Specifically, moderate Pi abundance upregulates markers of chondrogenesis whereas high abundance levels inhibit chondrogenesis and stimulate rapid matrix mineralization. We also show that this biphasic response to Pi concentration is mediated by ALP activity and cellular uptake of Pi and is dependent on the maturation stage of chondrocytes. Finally, delaying the addition of exogenous phosphate to ATDC5 cells in 3D cultures upregulates the expression of hypertrophic markers and accelerates terminal differentiation without dystrophic mineral formation, demonstrating that this culture system may serve as a rapid and physiologic model of endochondral ossification.

2. Methods

2.1. Cell culture

ATDC5 cells (Sigma) were maintained in DMEM/F12 (Life Technologies) supplemented with 5% FBS (Life Technologies) and 1% Antibiotic-Antimycotic (Life Technologies). Chondrogenesis of ATDC5 cells was induced with chondrogenic differentiation medium (ITS+) consisting of the growth medium supplemented with 1% ITS+ Premix (Corning) and 50 μg/ml ascorbate acid-2-phosphate (Sigma). Co-treatment medium (ITS+/βGP) consists of ITS+ medium supplemented with 10 mM β-glycerophosphate (Sigma). Non-chondrogenic mineralizing medium (βGP) consists of growth medium supplemented with 10 mM βGP. Calcium concentration was maintained at 1.3 mM.

2.1.1. Monolayer (2D) culture

Four days prior to chondrogenic induction, cells were seeded at the density of 6000 cells/cm^2 in multiple well plates. When cells reached 100% confluence (D0), differentiation was initiated by replacing the growth medium with differentiation medium. Cultures were fed with 2 ml medium every other day, and maintained for 21 days.

2.1.2. Pellet (3D) culture

Four days prior to chondrogenic induction, cells were seeded at the density of 6000 cells/cm^2. When cells reached 100% confluence (D0), they were trypsinized and centrifuged into pellets containing 2.5×10^5 cells in round-bottomed polypropylene 96-well-plate. Cultures were fed with 200 μl medium every other day. Pellet cultures were maintained for 21 days with differentiation medium. In delayed treatment, pellets were switched to ITS+/βGP medium after 7 or 14 days in ITS+ medium.

2.2. Biochemical analysis

Cartilage-specific matrix production was measured by the 1,9-dimethylmethylene blue (DMMB) assay as previously described (Carrion et al., 2016). Both monolayer cell tissues and pellets were digested with 1 mg/ml proteinase K in 0.1 M ammonium acetate at 50 °C for 16 h. Digested samples were mixed with DMMB dye (pH 1.5) at a ratio of 1:20, and the GAG content of the samples were determined by comparing the ratio of 525 nm to 595 nm readings to the standard curve derived from shark chondroitin sulfate. Measured GAG content of each sample was normalized by its DNA content using Hoechst 33258 dye (Sigma) as previously described (Kim et al., 1988). To measure mineral content of each sample, insoluble residues from the proteinase K digestion were collected and hydrolyzed by with 10% (w/w) acetic acid for 48 h at 40 °C. Hydrolyzed solution was added to Arsenazo III dye (Pointe Scientific) at ratio of 1:15, and the absorbance was read at 650 and 500 nm. The mineral content was calculated by