Phosphate Stimulates Differentiation and Mineralization of the Chondroprogenitor Clone ATDC5

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ABSTRACT—ATDC5 cells were employed to examine how inorganic phosphate (Pi) influences chondrocytic bone formation. 1) Pi (3 – 30 mM) plus ascorbic acid (50 μg/ml) dose-dependently accelerated proliferative differentiation and mineralization of ATDC5. 2) Northern blot analysis revealed that 10 mM Pi suppressed expression of type II collagen and PTH (parathyroid hormone) / PTH-related peptide (PTHrP) receptor, while it accelerated type X collagen expression. 3) Pi (3 – 30 mM) dose-dependently increased luciferase activity in the cells transfected with 3000 bp type X collagen promoter fused to the luciferase gene. The results suggest a regulatory role of Pi in endochondral osteogenesis.

Keywords: Phosphate, ATDC5, Endochondral osteogenesis

During embryonic long bone formation, chondroprogenitor cells proliferatively differentiate to ossifying hypertrophic chondrocytes, sequentially exhibiting the following stages of collagen production: 1) type I collagen-expressing cells to 2) type II-expressing chondrocytes that proliferate by doubling and condensation, and 3) type X-expressing hypertrophic chondrocytes that then functionally mineralize (1). This sequence of events is known to be monitored using ATDC5 cells in vitro, a clonal cell line that Atsumi et al. (2) established from mouse AT805 teratocarcinoma cells. The cells, on culture up to 25 days, differentiate from proliferating chondrocytes, which express type II collagen, aggrecans and parathyroid hormone (PTH) / PTH-related peptide (PTHrP) receptor (3), to hypertrophic chondrocytes, which express type X collagen to replace type II collagen and alkaline phosphatase, preparing for endochondral ossification (4 – 6). These cells have been utilized to analyze the molecular events that induce this specific series of phenotypic expression.

Recently, we noticed that inorganic phosphate ion (Pi) does proliferatively differentiate and mineralize as programmed normally. In this study, it was examined how Pi influences the sequential expression pattern of collagens, as the stage-specific marker proteins.

MATERIALS AND METHODS

ATDC5 from Riken Cell Bank (Ibaraki) was grown to confluence in 5% FCS/α-minimum essential medium (MEM) (Gibco BRL, Rockville, NY, USA) and then pre-cultured in 10% FCS/α-MEM, which contained basically 1 mM Pi and additionally 50 μg/ml AA, for 8 days before use by alternate day renewal of the medium. Ca deposition was monitored by o-cresolphthalein method (Calcium C-test Wako; Wako Chemical Co., Ltd., Osaka). In brief, extracellular deposits in wells were decalcified with 0.6 N HCl for 24 h and Ca contents estimated colorimetrically.

BrdU incorporation assay was performed using a colorimetric BrdU Cell proliferation Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s protocol. Cells were placed at a density of 3 × 10^4 per well in 96 well plates for culture. Absorbance at 450 nm was measured on a BioRad Model 550 microplate reader.

Northern blot analysis was done as described (7). In brief, 20 μg as total RNA from cellular extracts was separated in 1.2% agarose-formaldehyde gel, transferred onto a nylon membrane filter (Hybond N⁺; Amersham, Little Chalfont, UK) and hybridized with ^32P-labeled cDNA probes. Probes were amplified by PCR using the following...
nucleotides for the PTH/PTHrP receptor: PTH/PTHrP receptor-F 5'-CCG TGA CCT TCT TCC TCT AC-3' and PTH/PTHrP receptor-R 5'-CAC AGT GTC CCT GAG ACC TC-3'. Rat type II and mouse type X collagen cDNAs (Hind III-XbaI 550-bp fragment for type II collagen and XbaI-XhoI 1200-bp fragment for type X collagen) were kindly provided by Dr. Yasato Komatsu (Kyoto University, Kyoto).

Promoter analysis was carried out using the cells transfected with luciferase vector fused to human type X collagen promoter (3000 bp), a gift by Dr. Mikko J. Lammi (University of Kuopio, Finland) (8). Cells were transiently transfected by FuGENE6 according to the manufacturer’s protocol, with 10 µg of reporter plasmid construct and 0.1 µg of pRL-TK vector that was used to normalize the transfection efficiency between different experiments, and then cultured in the medium containing varying concentrations of Pi for 60 h. Luciferase activity of the cell lysate was assayed using the Luciferase Reporter Assay System (Promega, Madison, WI, USA) and a model TD20/20 luminometer (Promega), as described previously (9).

Statistical analyses were performed by Student’s t-test and the difference at P<0.05 was considered to be significant.

RESULTS

Under our preculture condition of 1 mM Pi plus AA, ATDC5 cells failed to mineralize within 30 days culture (Fig. 1: A, baseline, and C), although proliferation proceeded time-dependently at least for 10 days (Fig. 1E). Elevated Pi content was found to initiate Ca deposition within a few days and the effect developed in a culture time- and dose-dependent manner (Fig. 1: A and B), producing cartilaginous nodules (Fig. 1D), which appears to be related to accelerated proliferation (Fig. 1E). β-Glycerophosphate (β-GP) potentiated the Pi effect, possibly as a

![Fig. 1. Pi accelerates nodule formation and proliferation of ATDC5 cells. In panel A, the culture medium contained 1 mM Pi (baseline), 10 mM Pi (○) or 10 mM Pi plus 100 µM β-GP (●), in the presence of AA (△), n = 8. In panel B, cells were cultured for 4 days in the media containing 1 to 30 mM Pi in addition to AA. n = 8. Panels C and D show typical microscopic outlooks of cells after 4 days culture in the presence of 1 and 10 mM of Pi, respectively. In panel E, time courses of proliferation at 1 mM Pi alone (○) and 1 mM before 10 mM Pi from day 4 (●), were compared. ***significantly different from the control at P<0.001. n = 8.](image-url)
supplier of Pi (Fig. 1A). Elevating Ca content alone up to 30 mM failed to induce mineralization (data not shown).

Next, it was examined by Northern blot analysis how 10 mM Pi affects the sequential expression pattern of mRNAs of chondrocyte marker proteins, which would be related with accelerated mineralization. The results in Fig. 2 revealed that 10 mM Pi markedly decreased the expressions of mRNAs of PTH/PTHRP receptor and type II collagen in a mineralization-related manner, which otherwise appeared to occur constantly, while it significantly increased the expression of mRNA of type X collagen, a marker protein of mineralizing hypertrophic chondrocytes. A similar expression pattern of the marker proteins has been reported to develop when ATDC5 cells slowly reached to its pre-mineralization stage of differentiation during 21 days of culture in the presence of insulin, selenite and transferrin (2–4). Although the data are not presented here, mRNA of type III Pi carrier Glvr-1 (10) was constantly expressed and not affected by Pi stimulus.

Finally, we performed promoter analysis using the cells transfected with luciferase vector fused to type X collagen promoter. The obtained results in Fig. 3 clearly demonstrated that increased Pi promoted luciferase gene expression in a Pi concentration-dependent manner, which appears to be similar to the dependency in Fig. 1B. In the case of MC3T3-E1 cells transfected with the same vector, we have observed that the osteoblastic cells failed to acquire the reactivity toward Pi, possibly because a silencer element in the −3000 to −2400 region of type X collagen promoter is active for this cell line (data not shown).

**DISCUSSION**

The accelerating effect of Pi on in vitro bone formation was first noted by Bellows et al. (11) with rat calvaria osteoblastic cells and characterized as a signal to initiate mineralization. In addition, β-GP was active. Lately, the classic effectiveness of β-GP was identified to be due to liberated Pi by Beck et al. (12), and they reported that Pi acts as a novel extracellular signal for induction of osteopontin gene expression in osteoblastic MC3T3-E1 cells. However, it is unknown if Pi exerts such effect on chondrocytic mineralization.

According to Shukunami et al. (3), ATDC5 cultured in the maintenance medium containing transferrin and selenite stopped growing at confluence and thereafter remained
undifferentiated without forming nodules, and only in the differentiation medium supplemented with insulin, the cells proliferatively differentiated to the nodule forming stage after 21 days, thereafter entering a long-lasting mineralization stage. Our study indicated that even in the absence of such additives including insulin, elevating Pi alone was able to largely condense the culture time needed for mineralization, from 21 to a few days. Expression studies revealed that, during 8 days culture with elevated Pi, the signal gradually suppressed the expressions of collagen II and PTH/PTHrP receptor on one hand and enhanced that of type X collagen on the other, indicating that initially expressed and accumulated type II collagen would be degraded and replaced by type X collagen, just as observed originally (3). 

In addition, reporter assay confirmed that Pi can dose-dependently accelerate the promoter-mediated expression of type X collagen, a marker protein of the pre-mineralization stage.

Previously, Beier et al. (8) who compared a variety of bovine cells transfected with different type X collagen promoter fragments, have reported that the enhancer effect developed only in the presence of Ca phosphate used as a transfection reagent or as an additive after Lipofectin transfection. In this study, we selected FuGENE6 (non-liposomal cationic reagent) for fusion and the cells were never exposed to a high Ca phosphate environment. From our results, it is highly possible that in the experiment by Beier et al., Ca phosphate activated the expression of type X collagen.

In conclusion, simply elevating Pi content (not Ca) of the culture medium, which contains AA but not other special supplements, was found to dose-dependently accelerate proliferation, differentiation and mineralization of ATDC5 cells, which developed in a well-known but timewise condensed schedule; and this accelerated culture appears to provide a usable model for studying the mechanisms of both Pi action and chondrocytic mineralization.

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