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Original Article

Inorganic phosphate induces cementogenic/osteogenic gene expression and mineralization in dental follicle cells in vitro

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ABSTRACT

Introduction: Phosphate is an essential factor in skeletal mineralization. Inorganic phosphate (Pi) is an important regulator of various genes in osteoblasts, cementoblasts, and dental pulp cells. However, the effects of Pi on the precursor cells of periodontal cells remain unknown. The present study aimed to assess the effects of Pi on dental follicle cells that contribute to periodontium formation as precursor cells.
Material and methods: Murine dental follicle cells (SVF4) were treated with Pi, and the corresponding effects on mineralization and gene expression were observed. Mineralized nodule formation during SVF4 differentiation was assayed by alizarin red S staining. Expression of genes associated with differentiation and mineralization was analyzed by quantitative real-time reverse-transcriptase polymerase chain reaction.
Results: Treatment of SVF4 cells with 3 mM Pi induced mineral nodule formation after 2 weeks. In addition, treatment with 3 mM Pi upregulated the mRNA expression of bone morphogenetic protein 2 and osteopontin and downregulated the mRNA expression of noggin. The effects of Pi on SVF4 were blocked by treatment with phosphonoformic acid, also known as foscarnet, which is the competitive inhibitor of Na-Pi transport.
Conclusions: Our findings demonstrated that Pi enhanced dental follicle cell differentiation by upregulating cementogenic/osteogenic gene expression.

1. Introduction

Phosphate is an essential factor in skeletal mineralization and is present complexed with calcium in hydroxyapatite crystals deposited onto collagen matrix. In addition, phosphate is required for various cellular functions, including deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA) synthesis, membrane phospholipid synthesis, energy metabolism, and intracellular signaling. Previous studies have shown that inorganic phosphate (Pi) is an important regulator of various genes in osteoblasts [1], cementoblasts [2–4], and dental pulp cells [5].

Cells within the follicle region, a loose connective tissue surrounding the developing tooth, play critical roles in the process of tooth eruption [6–10]. In addition, follicle cells are also believed to be capable of differentiating into periodontal cells (cementoblasts, periodontal ligament fibroblasts, and osteoblasts) as required for periodontium development. In a previous study, we used cementoblasts to examine the role of Pi and identify Pi-regulated genes that are associated with cementoblast activity and function [3]. However, the effects of Pi on the precursor cells of cementoblasts remain unknown. Therefore, in this study, we aimed to further understand the effects of Pi on dental follicle cells that contribute to the periodontium.

2. Materials and methods

2.1. Cell culture

The murine dental follicle cell line SVF4 was kindly provided by Dr. Martha J. Somerman (National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institute of Health, Bethesda, MD, USA). Isolation, immortalization, and characterization of SVF4 cells were performed as previously described [11–13]. Immortalized follicle cells, designated as SVF4 cells, were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Carlsbad, CA, USA) containing 10% FBS (GIBCO), 100 U/ml penicillin, and

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100 μg/ml streptomycin (GIBCO) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Mineralized nodule formation assay

SVF4 cells were seeded in 24-well tissue culture plates at 2×10^4 cells/well in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. One day after seeding, the medium was replaced with medium containing mineralized nodule induction supplements [DMEM with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid (Sigma–Aldrich St. Louis, MO, USA), and 10 mM β-glycerophosphate (Sigma–Aldrich)] and 0, 1, 2, and 3 mM Pi. Cells were then cultured for 1, 2, and 3 weeks. A stock solution of 100 mM Pi was prepared by combining one part of sodium phosphate monobasic monohydrate (Sigma–Aldrich) with four parts of sodium phosphate dibasic heptahydrate (Sigma–Aldrich) in DMEM and filter sterilizing the solution. The formation of mineralized nodules was evaluated by alizarin red S staining.

2.3. RNA extraction

SVF4 were plated in 60-mm dishes at a concentration of 2×10^4 cells/well in DMEM containing 10% FBS. One day after seeding, media were replaced with DMEM containing 5% FBS, and 0.1–5 mM Pi was added. Total RNA was isolated using the Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Real-time reverse-transcriptase polymerase chain reaction

For real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis, the extracted RNA was DNAse-treated (DNA-free™; Ambion, Austin, TX), and cDNA was synthesized from 1.0 μg of total RNA using a cDNA synthesis kit for RT-PCR (Transcriptor First Strand cDNA Synthesis Kit; Roche, Basel Schweiz). The generated cDNA products (2 μL) were used as templates for PCR amplification in 20-μL reaction volumes using the DNA Master SYBR Green I kit (Roche) on a LightCycler system (Roche). Primers were designed using LightCycler probe design software (Roche) for amplification of the following target mouse genes: osteopontin (OPN), bone morphogenetic protein 2 (BMP-2), NF-κB, early growth response 2 (Egr2), bone morphogenetic protein receptor type-1A (BMPR1A), bone morphogenetic protein receptor type-2 (BMPR2), noggin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The designed primers were as follows (forward/reverse): OPN (5′-TTTACAGCCTGCACCC-3′/5′-CTAGCAGTGACGGTCT-3′); BMP-2 (5′-CCCGATCACCTCTCTT-3′/5′-ACCGCAGTCCGTCTAA-3′); NF-κB (5′-AGTGAACCGAAAC-CCT-3′/5′-TCCGTAGTTCGAGTAGC-3′); Erg2 (5′-CGAGTAG-CTTCGCTCC-3′/5′-TGGCGGCGATAAGAAT-3′); BMPR1A (5′-GAGGAGCCAACTACCC-3′/5′-GACGGCTTCCTGGATTA-3′); BMPR2 (5′-AATGGAACGTACCGCT-3′/5′-CTGCTCCGTATCGACC-3′); noggin (5′-CACGCTACGTGAAGGT-3′/5′-GGAACACTTACACTCGG-3′); GAPDH (5′-ACCACAGTCCATGCCATCAC-3′/5′-TCCACCA-CCCTGTTGCTGTA-3′). Gene expression analysis of OPN, BMP-2, NF-κB, BMPR1A, BMPR2, Egr2, and noggin was performed using GAPDH as the housekeeping/reference gene. Amplification was run using the following profile: 40 cycles of 95/5, 55/7, and 72/20 [temperature (°C)/time (seconds)].

For the relative quantification of expression, LightCycler Relative Quantification Software, version 1.0 (Roche Diagnostics, Germany) was used to compare the expression of target genes to that of GAPDH (reference gene) with calibrator normalization and amplification efficiency correction.

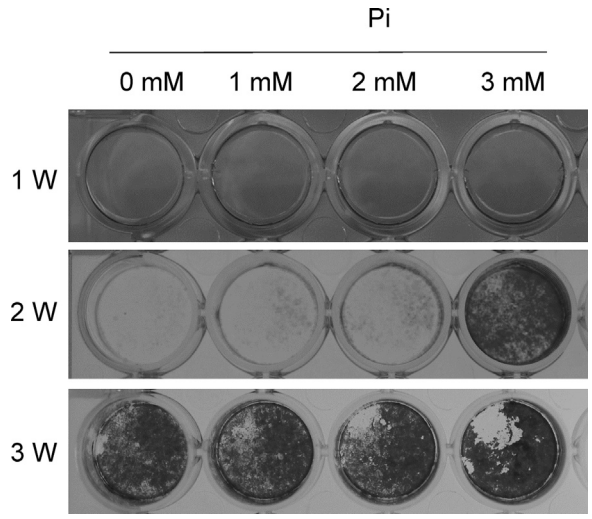

Fig. 1. Effect of phosphate on mineralized nodule formation by SVF4 cells. Image shows mineralized nodule formation with or without Pi treatment as evaluated by alizarin red S staining. Mineralized nodule formation increased 2 weeks after treatment with 3 mM Pi.

2.5. Effect of phosphate transport inhibition

To determine whether Pi entry into cells is required for the expression of target genes, culture media were supplemented with 1 mM phosphonoformic acid (PFA) (Sigma–Aldrich) in addition to 3 mM Pi treatment and incubated for 72 h.

2.6. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Inorganic phosphate induces mineral nodule formation in dental follicle cells in vitro

Mineralized nodule formation was not observed under all Pi conditions tested after 1 week of treatment (Fig. 1). Increased nodule formation was observed in cells treated with 3 mM Pi after 2 weeks. Cells treated with 3 mM Pi showed significantly enhanced nodule formation compared to those treated with 0, 1, and 2 mM Pi.

Pi enhanced cell-mediated mineral nodule formation in a dose-dependent manner after 3 weeks of treatment.

3.2. Inorganic phosphate induces cementogenic/osteogenic gene expression in dental follicle cells in vitro

Treatment with Pi for 48 h affected the expression of genes associated with follicle cell differentiation and mineral regulation, namely OPN and BMP-2, respectively (Fig. 2a and b). Treatment with 3 mM Pi was observed to elicit the maximum increase in mRNA levels for both genes, while the effects of 5 mM Pi were less pronounced than those of 3 mM Pi.

Next, we performed a time-course experiment. A dose of 3 mM Pi was selected for these experiments because this dose induced the most robust responses in gene expression. The results revealed that changes in the expression of OPN and BMP-2 were observed after 24 and 48 h, respectively (Fig. 2c and d).