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Calcium phosphate nanoparticles are associated with inorganic phosphate-induced osteogenic differentiation of rat bone marrow stromal cells

Xiao-rong Chen [1], Jing Bai [1], Shuai-jun Yuan, Cai-xia Yu, Jian Huang *, Tian-lan Zhang, Kui Wang

Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100191, PR China

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ABSTRACT

In the present study, we demonstrated that calcium phosphate (CaP) nanoparticles formed in cell culture media were implicated in the process of high inorganic phosphate (Pi) mediated osteogenic differentiation of rat bone marrow stromal cells (BMSCs). Exposure of BMSCs in vitro to high Pi-containing media reduced alkaline phosphatase (ALP) activity and the expressions of osteoblast-specific genes. The sediments of CaP nanoparticles were observed at the cell surface and some of them were concomitantly found inside cells at high Pi concentration. In addition, treatment the cells with pyrophosphate (PPi), an inhibitor of calcium crystal formation, abrogated the ALP activity induced by high Pi, suggesting the contribution of CaP nanoparticles. Moreover, for isolated CaP nanoparticles, there was a trend of conversion from amorphous calcium phosphate to hydroxyapatite with elevated Pi. The particle size of CaP increased and the surface morphology changed from spherical to irregular due to increased concentrations of serum proteins incorporated into CaP nanoparticles. The study demonstrated that those physicochemical properties of CaP nanoparticles played an important role in modulating BMSCs differentiation. Furthermore, the addition of Pi in the osteogenic media resulted in a dose-dependent increase in matrix mineralization, while treatment of the cells with PPi suppressed Pi-induced calcium deposition. The findings indicated that calcium deposition in the matrix partly came from the spontaneous precipitation of CaP nanoparticles.

1. Introduction

Inorganic phosphate (Pi) plays an essential role in diverse biological processes, including energy metabolism, cell signaling, nucleic acid synthesis, membrane function and bone mineralization [1]. As the second most abundant mineral ion found in the human body, Pi is not only a prerequisite for normal hydroxyapatite formation, it is also integral to osteoblast function during the differentiation process in vitro [2]. Increasing evidences indicate that adequate control of Pi homeostasis is crucial for bone mineralization [3,4]. A number of disease states, such as end-stage renal disease, hyperparathyroidism, multiple myeloma, oncogenic osteomalacia, cause changes in serum and cellular phosphate levels, which result in bone impairment and influence the aging process and lifespan [5,6]. Despite the considerable progress that have been made to understand the responses to the elevated Pi in skeletal biology, but the mechanisms governing this need for further investigation.

It is often stated that the ionic product of calcium and phosphate in many biological fluids including blood has always exceeded the solubility product for hydroxyapatite or other calcium phosphates, but precipitates do not take place spontaneously under physiologic conditions. Several in vitro and in vivo studies proved the existence of different types of calcification inhibitor in serum, which prevent the formation and buildup of insoluble minerals [7,8]. Given these considerations, serum must still be regarded as a 'metastable' calcium and Pi solution, and an imbalance between calcification inhibitors and promoters could be a key mechanism toward ongoing unwanted precipitation [9]. Recent in vivo and in vitro investigations support the formation of mineral-organic nanoparticles in pathologic situations like chronic kidney disease [10,11]. It is possible that calcium phosphate (CaP) particles will generate once the concentration of the ions exceed the solubility product of the relevant phase in the extracellular milieu, and that these particles then influence cell function.

* Corresponding author.
E-mail address: jhuang@bjmu.edu.cn (J. Huang).
[1] Contributed equally to this article.

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Bone marrow stromal cells (BMSCs) are the most common source of osteoprogenitor cells and play an essential role in bone formation and remodeling. Therefore, investigating the physicochemical properties of CaP nanoparticles and their possible interactions with BMSCs will likely broaden our understanding of high Pi-related bone impairment in vivo. In this study, the concentration of Pi was based on that used in the literature [5,12,13]. Our results indicated that CaP nanoparticles formed in culture media were associated with the process of high Pi mediated osteogenic differentiation BMSCs and the physicochemical properties of CaP nanoparticles played an important role in modulating the biological response of BMSCs.

2. Materials and methods

2.1. BMSCs cell isolation and culturing

BMSCs were isolated and expanded according to the methods reported by Barbash [14]. All experimental protocols involving bone marrow collection were approved by the ethical guidelines of Peking University. Bone marrow was flushed from the tibias and femurs with DMEM. The single-cell suspension was then centrifuged, and the supernatant was suspended in DMEM which contains 0.916 mM of Pi, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells between passages three and seven were used for all experiments.

For the induction of osteogenic differentiation, BMSCs were cultured in differentiation media containing 5% FBS, 50 μg/ml ascorbic acid, and 100 nM dexamethasone. Appropriate amounts of sodium phosphate buffer (1 M Na_2HPO_4 adjusted to pH 7.4 with 1 M NaH_2PO_4) were added into the media to achieve final Pi concentrations of 2.0, 4.0, 6.0, 8.0 and 10.0 mM, respectively. For the experimental groups involving the inhibitor pyrophosphate (PPi), osteogenic media containing different concentration of PPi were added. On each media change, fresh inhibitor was added into the fresh media.

2.2. Differentiation of BMSCs

Alkaline phosphatase (ALP) activity was quantified as the trait of osteoblastic development of the BMSCs. After culturing in the differentiation media for up to 7 days, the cells were washed three times with PBS, lysed with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for ALP activity. ALP values were normalized to protein content that was measured with BCA protein assay.

BMSCs in 24-well plates were cultured in differentiation media for 14 and 21 days, respectively. Then, matrix mineralization was assessed by a modified Wada procedure [15]. Alternatively, matrix mineralization was detected by Alizarin Red S staining. The stained matrix was observed and recorded with a Nikon Diaphot inverted microscope.

2.3. Real-time quantitative polymerase chain reaction

Total RNA was extracted using Trizol reagents (Invitrogen) as described by the manufacturer's protocol. The concentration of extracted RNA was determined with an ultraviolet spectrophotometer. 300 ng total RNA was then reverse-transcribed into cDNA by PrimeScript™ RT reagent (TaKaRa). The primers of core-binding factor α1 (Runx2), bone sialoprotein (BSP), osteopontin (OPN), collagen I alpha I telopeptide (COL1A1) were listed in Table 1. qRT-PCR analysis was performed with an ABI7500 Sequence Detection System (Applied Biosystems) using the SYBR Prime-Script™ PCR (TaKaRa). Each experiment was performed for three biological replicates. Each gene expression level was normalized to 18S mRNA content. Relative quantification was calculated with the $2^{-\Delta\Delta Ct}$ method [16].

Table 1
Primers for real time PCR analysis.

Target	Forward	Reverse
COL1A1	CCAGCTGACCTTCCTGCGCC	CGGTGTGACTCGTGCAGCCA
BSP	CCGGCCACGCTACTTTCTT	TGGACTGGAAACCGTTTCAGA
OPN	GACTGGCAGTGGTTTGCTTTTGCC	TGGGTCAGGCTTCAGCCAAGTG
Runx2	AGATGGGACTGTGGTTACCG	GGACCGTCCACTGTCACTTT
18S	GACCGGCGCAAGACGAACCA	GCATCGCCAGTCGGCATCGT

2.4. The deposition and cellular uptake of CaP nanoparticles

The depositions of the particles formed in DMEM in the presence of cells were observed by scanning electron microscopy (SEM). Cells were seeded on cover slips for 24 h, then exposed to Pi-containing DMEM supplemented with 5% FBS for another 72 h. The cells were fixed with 3% glutaraldehyde in PBS followed by 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4). After dehydration in a series of graded ethanol, the samples were sputtered with gold and viewed with a SEM (JEOL, JEM-5600LV).

The cellular uptakes of the particles were observed by transmission electron microscopy (TEM). The samples were fixed, postfixed, and dehydrated as described for SEM. Afterward, cultures were

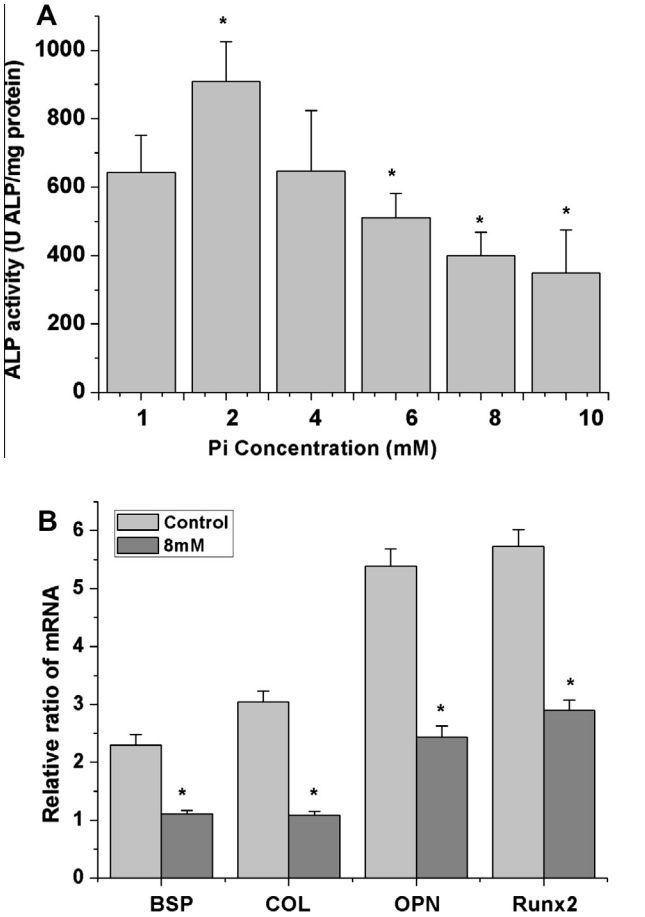

Fig. 1. Effect of Pi on BMSCs differentiation. (A) ALP activity of rat BMSCs. Data are presented as the quantities of the total ALP activity normalized to total protein; (B) The mRNA expression in 8 mM Pi treated BMSCs cultures as determined by Real-time q-PCR analyses. Values are means ± SD. $^{*}P < 0.05$ versus the corresponding control group.