Journal of Inorganic Biochemistry 171 (2017) 100–107

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Characteristics of minerals in vesicles produced by human osteoblasts hFOB 1.19 and osteosarcoma Saos-2 cells stimulated for mineralization

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ARTICLE INFO

Article history:
Received 1 December 2016
Received in revised form 8 February 2017
Accepted 19 March 2017
Available online 29 March 2017

Keywords:
Mineralization
Osteoblasts
Osteosarcoma
Apatites
Vesicles

ABSTRACT

Bone cells control initial steps of mineralization by producing extracellular matrix (ECM) proteins and releasing vesicles that trigger apatite nucleation. Using transmission electron microscopy with energy dispersive X-ray microanalysis (TEM-EDX) we compared the quality of minerals in vesicles produced by two distinct human cell lines: fetal osteoblastic hFOB 1.19 and osteosarcoma Saos-2. Both cell lines, subjected to osteogenic medium with ascorbic acid (AA) and β-glycerophosphate (β-GP), undergo the entire osteoblastic differentiation program from proliferation to mineralization, produce the ECM and spontaneously release vesicles. We observed that Saos-2 cells mineralized better than hFOB 1.19, as probed by Alizarin Red-S (AR-S) staining, tissue nonspecific alkaline phosphatase (TNAP) activity and by analyzing the composition of minerals in vesicles. Vesicles released from Saos-2 cells contained and were surrounded by more minerals than vesicles released from hFOB 1.19. In addition, there were more F and Cl substituted apatites in vesicles from hFOB 1.19 than in those from Saos-2 cells as determined by ion ratios. Saos-2 and h-FOB 1.19 cells revealed distinct mineralization profiles, indicating that the process of mineralization may proceed differently in various types of cells. Our findings suggest that TNAP activity is correlated with the relative proportions of mineral-filled vesicles and mineral-surrounded vesicles. The origin of vesicles and their properties predetermine the onset of mineralization at the cellular level.

1. Introduction

Bone is a dynamic form of connective tissue composed of three cell types (osteoblasts, osteocytes and osteoclasts), extracellular matrix (ECM), which provides tensile strength, and hydroxyapatite (HA), which grants mechanical resistance [1]. Osteoblasts, as well as chondrocytes in cartilage and odontoblasts in teeth, control initial steps of mineralization by producing ECM proteins and releasing matrix vesicles (MVs), which trigger apatite nucleation [2,3]. Soft tissues do not mineralize under physiological conditions, however ectopic calcification may occur under pathological conditions. This is initiated by cells transdifferentiation toward bone formative cells due to activation of genes associated with osteochondrogenesis [4]. The transdifferentiated mineral-competent cells are able to produce collagen matrix and to release vesicles, which leads to the formation of calcium phosphate deposits (apatites) in soft tissues [3]. Therefore, understanding the molecular mechanisms of calcification of osseous cells and chondrocytes shall provide some clues on how cells in soft tissues can trigger ectopic calcification.

Skeletal tissues are regulated by various enzymatic activities, growth factors, as well as by promoters or inhibitors of mineralization. Of crucial importance are the antagonistic actions of tissue-nonspecific alkaline phosphatase (TNAP) and ectonucleotide pyrophosphatase/phosphodiesterase I (NPP1), which control HA formation [5]. Inorganic pyrophosphate (PP_i), a potent inhibitor of HA formation, is hydrolyzed by TNAP, while NPP1 hydrolyzes nucleotide triphosphates to form PP_i. Indeed, the P_i/PP_i ratio regulates HA formation [6,7] and deregulates cellular PP_i production, degradation and transport, with possible pathological consequences [8]. Ion replacement in apatite crystals is a known biomedical procedure [9]. Thus, identification of minerals produced by cells is necessary to determine how the chemistry of calcium phosphate complexes may alter physiological mineralization and ectopic calcification. Apatites are calcium and phosphate containing minerals with the general crystal unit cell formula $Ca_{10}(PO_4)_6X_2$ where X = Cl, F, OH. This group of minerals is classified as follows [10]: fluorapatite (FA) $Ca_{10}(PO_4)_6F_2$, chlorapatite (CA) $Ca_{10}(PO_4)_6Cl_2$ and hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$. Bone apatites can be more accurately described

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http://dx.doi.org/10.1016/j.jinorgbio.2017.03.006
0162-0134/

as CO_3-substituted apatites (CSA). Among the substituting ions in apatites that are reported in bone and tooth mineral and/or used in biomedical applications are F^-, Cl^-, B^+, Cu^{2+}, Na^+, K^+, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Si^{2+} or Sr^{2+} chloride, citrate, carbonate [9,11–17]. The choice of osteoblast cell lines to induce mineral formation is crucial since each cell line exhibits distinct characteristics of the extracellular matrix and mineralization.

Osteosarcoma-derived cells are commonly used as osteoblastic models [18]. Among the three osteosarcoma cell lines tested: MG-63, Saos-2 and U-2 OS, Saos-2 cells revealed the most mature osteoblastic labeling profile [18]. They are able to secrete MVs [19] and are good *in vitro* models of human osteoblast-like cell implant materials [20]. Human bone marrow mesenchymal stromal cells (BMMSCs) are excellent model systems, however their scarcity, heterogeneity and limited lifespan restricted their use [20]. Undifferentiated human fetus hFOB cells possess similar markers as BMMSCs and are widely used as a model of normal osteoblastic differentiation [21]. In this report, we compared the formation of minerals by two selected cell models: osteosarcoma Saos-2 cells and human fetus hFOB 1.19 cells. Our aim was to determine the amount and composition of minerals, the activity of TNAP and the number of vesicles in these two different cell lines, to better understand mechanisms of mineralization during distinct stages of differentiation.

2. Materials and methods

2.1. Cell culture and treatment

Human fetus hFOB 1.19 SV40 large T antigen transfected osteoblasts (ATCC CRL-11372) were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium with 2.5 mM L-glutamine (ATCC) supplemented with 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin (Sigma), 0.3 mg/ml G418 (Sigma) and 10% FBS (v/v, Gibco). The cells were grown at 34 °C in atmosphere of 5% CO_2.

Human osteosarcoma Saos-2 cells (ATCC HTB-85) were cultured in McCoy's 5A medium with 1.5 mM L-glutamine (ATCC) supplemented with 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin (Sigma) and 15% FBS (v/v, Gibco). The cells were grown at 37 °C in atmosphere of 5% CO_2.

Cells were stimulated for mineralization by treatment with 50 μg ml^{-1} ascorbic acid (AA, Sigma) and 7.5 mM β-glycerophosphate (β-GP, Sigma) for 7 days [22].

2.2. Synthesis of apatites

2.2.1. Hydroxyapatite (HA)

Hydroxyapatite was obtained from commercially available powder $Ca_{10}(PO_4)_6(OH)_2$ (Bio-Gel HTP Gel, Bio-Rad Lab.). Ca to P ratio, calculated from the atomic numbers, is 1.67.

2.2.2. Fluorapatite (FA)

Fluorapatite was synthesized according to Yao et al., with modifications [23]. A mixture of 75 ml of 0.01 M phosphate buffer (Na_2HPO_4 and NaH_2PO_4 (Sigma) in a 1:1 ratio, pH 6.7) with 0.003 M NaF (Sigma) was prepared in a flask into which 25 ml of 0.05 M solution of $Ca(C_2H_3O_2)_2$ 1 × H_2O (POCh) was added under constant mixing at 1000 rpm. The reaction mixture was placed in an oven at 100 °C for 3 h with occasional stirring. The precipitate was left to cool and the fluorapatite crystals formed were then filtered through filter paper (Whatman No 5) and placed in glass beakers to dry at 100 °C for 24 h.

2.2.3. Chlorapatite (CA)

Chlorapatite was synthesized according to Kannan et al., with modifications [24]. A 52 ml solution of 1 M $Ca(NO_3)_2$ 4 × H_2O (Sigma) was prepared in a flask, into which 30.64 ml of 0.6 M solution of $(NH_4)_2HPO_4$ (Sigma) was added under constant mixing at 1000 rpm. Then, 10.21 ml of 0.2 M NH_4Cl (Sigma), pH 9.0, earlier adjusted with 1.37 ml NH_4OH (POCh), was slowly added. The reaction mixture was placed in an oven at 90 °C for 2 h with occasional stirring. The resulting precipitate was then left for 24 h at room temperature to mature. The chlorapatite crystals formed were filtered through filter paper (Whatman No. 3) and placed in glass beakers to dry at 80 °C for 48 h.

2.3. Calcium mineral detection

The presence of calcium deposits in the cells was confirmed by staining with Alizarin Red-S (AR-S, Sigma) [25]. Cell cultures were washed with 5 ml of phosphate buffer saline (PBS, 125 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.0) and incubated with 5 ml of 0.5 g 100 ml^{-1} AR-S in PBS pH 5.0 for 30 min at room temperature. Then, after washing 3 times with 5 ml of PBS to remove free calcium ions, the stained cultures were photographed under an inverted AxioObserver Z1 microscope (Zeiss) using transmitted light and Red/Green/Blue filters. The quantitative analysis of calcium salts levels in the cells was assessed by destaining with cetylpyridinium chloride (CPC, Sigma) [26]. Cell cultures were incubated with 5 ml of 10 g 100 ml^{-1} CPC in PBS, pH 7.0, for 30 min at room temperature. The solution was transferred to centrifuge tubes and centrifuged at 132 × *g* for 1 min at room temperature (MPW-350R, MPW Medical Instrument). 1 ml of supernatant was used for absorbance measurements at 562 nm using a BioMate3 spectrophotometer (Thermo Electronics Co.). $[Ca^{2+}]$ was calculated as described [27]. The obtained results were referenced to the standard curve of AR-S prepared earlier.

2.4. TNAP activity assay

Cell cultures, either resting or stimulated for 7 days, were treated according to a collagenase digestion protocol [28]. Medium from cell cultures was collected, whereas cells were washed with PBS and incubated with crude collagenase (500 U ml^{-1}, type IA; Sigma) in a solution of 0.25 M sucrose, 0.12 M NaCl, 0.01 M KCl, and 0.02 M Tris-HCl buffer, pH 7.45, at 37 °C for 3 h. Then, cells were mechanically scraped, passed 10 times through a 0.5 × 16 syringe followed by 2 times sonication for 10 s at 20% power of a S-250D digital sonifier (Branson Ultrasonic S.A.) and centrifugation at 500 × *g* for 5 min (5417C, Eppendorf). The pellet was suspended in 500 μl of Hank's balanced salt solution (HBSS, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.6 mM KH_2PO_4, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose, 2.38 mM $NaHCO_3$, pH 7.4). The collected supernatant was analyzed for protein concentration, using the Micro BCA Reagent (Pierce), and TNAP activity, using ALP Yellow pNPP Liquid Substrate System for ELISA (Sigma). The reaction was initiated with the addition of 10 μl (0.5 μg of protein) aliquots of the enzyme to 96-well plates containing 200 μl of p-NPP as substrate, preincubated at 37 °C for 5 min, and the absorbance was recorded at 405 nm for 1 h with 15 s intervals using a Spectra Max M5e multi-detection reader (Molecular Devices). As a control, 10 μl of 100 μM TNAP inhibitor, levamisole (tetramisole hydrochloride, Sigma), was added immediately prior to the measurement. Reaction was stopped using 50 μl of 3 M NaOH. Experiments were repeated at least 3 times. TNAP activity was calculated as U/mg protein, where 1 U = 1 μmol pNPP hydrolyzed per min.

2.5. Preparation of minerals and cells for transmission electron microscopy and X-ray microanalysis

2.5 mg of synthetically produced minerals (HA, CA, FA) were suspended in 0.5 ml of deionized water and incubated at 37 °C in atmosphere of 5% CO_2 for 1 h. 10 μl of the suspension was dropped on Formvar/Carbon 300 Mesh Ni grids (Agar Scientific Ltd.) placed in a porcelain multi-well plate. Then the samples were dried for 30 min at room temperature. The sample-covered grids were counterstained with 2.5 g 100 ml^{-1} uranyl acetate in ethanol for 20 min at room temperature