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Identification of dicalcium phosphate dihydrate deposited during osteoblast mineralization in vitro

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ABSTRACT

The hydroxyapatite (HAP) with variable chemical substitutions has been considered as the major component in the mineralized part of bones. Various metastable crystalline phases have been suggested as transitory precursors of HAP in bone, but there are no consensuses as to the nature of these phases and their temporal evolution. In the present study, we cultured rat calvarial osteoblasts with ascorbate and β-glycerophosphate to explore which calcium phosphate precursor phases comprise the initial mineral in the process of osteoblast mineralization in vitro. At the indicated time points, the deposited calcium phosphate was analyzed after removing organic substances from the extracellular matrix with hydrazine. The features comparable to dicalcium phosphate dihydrate (DCPD) and octacalcium phosphate (OCP), in addition to HAP, were detected in the mineral phases by high resolution transmission electron microscopy. And there was a trend of conversion from DCPD- and OCP-like phases to HAP in the course of mineralization, as indicated by Fourier-transform infrared microspectroscopy, energy-dispersive X-ray spectroscopy and synchrotron X-ray powder diffraction analyses. Besides, biochemical assay showed a progressive decrease in the ratio of mineral-associated proteins to calcium with time. These findings suggest that DCPD- and OCP-like phases are likely to occur on the course of osteoblast mineralization, and the mineral-associated proteins might be involved in modulating the mineral phase transformation.

1. Introduction

Bone biomineralization is a complex process modulated by organic macromolecules under cellular control. In the form of non-stoichiometric calcium phosphate, carbonated hydroxyapatite (HAP) has been described as the most thermodynamically stable mineral phase in bone [1]. The crystals of HAP are initially observed in the gap regions of aligned type I collagen fibrils and grow in the shape of nanometer-size irregular platelets with their c-axes oriented along the long axis of the collagen fibril [2]. Mineral formation of bone is understood to be a sequential process, but a clear understanding of the mechanism(s) in the early stages of mineralization has been elusive [3–5]. The proposed precursors of bone mineral are amorphous calcium phosphate (ACP) [6,7], dicalcium phosphate dihydrate (DCPD, $CaHPO_4 \cdot 2H_2O$) [8], and octacalcium phosphate (OCP, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$) [9]. However, the identification of a precursor phase in bone mineralization is a challenging task, because these minerals may transform during specimen preparation [10–13]. A recent study with cryogenic transmission electron microscopy assay demonstrated that apatite formation in zebra fish bone did not occur directly by the association of ions from solution but proceeded through an ACP precursor phase [14,15]. Evidence from Raman microspectroscopy further pointed out that an OCP-like phase presented prior to the formation of carbonated HAP at the suture boundaries of mice calvaria [16]. The identification of mineral precursors has provided valuable clues to a full understanding of the process of bone mineralization.

Osteoblasts are the primary cells contributing to bone formation. Osteoblasts can be obtained by enzymatic digestion of fetal rat calvaria and cultured in the presence of ascorbic acid (AA) and β-glycerophosphate (β-Gp). These cells can undergo a sequence of differentiating events restricted in time, such as the expression of alkaline phosphatase, osteopontin, and osteocalcin, as well as nodule formation and mineral deposition [17,18]. Therefore, a timing study of osteoblast culture would be useful to the identification of calcium phosphate precursors deposited at the different stages of mineralization. One of the principal issues hindering the study of mineral phase is that the high levels of matrix obscure the signals arising from small amounts of mineral. To eliminate the interference effects of proteins, hydrazine has commonly been used in bone mineral research to isolate calcium phosphate crystals from the extracellular matrix [19–21]. As claimed by previous investigators, hydrazine treatment either caused only insignificant alterations to bone mineral or did not alter bone mineral in any way [22–27]. In the present investigation we exploited the mineralizing capabilities of

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osteoblast cultures to monitor what mineral was formed at the different stages of mineralization. At the indicated time points, the organic matrix was removed by hydrazine and the deposited calcium phosphate was analyzed. By combined analyses of the high resolution transmission electron microscopy (HRTEM), energy-dispersive X-ray spectroscopy (EDX), Fourier transform infrared microspectroscopy (FT-IRM) and synchrotron X-ray powder diffraction (XRD), we have demonstrated that HAP yields, at least in part, from the DCPD- and OCP-like phases in the mineral deposition by osteoblast cultures.

2. Materials and methods

2.1. Cell culture

Osteoblasts were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague–Dawley rats (1% trypsin in phosphate buffered saline for 30 min; 0.2% collagenase type II in Dulbecco's modified Eagle medium (DMEM) for 30 min; 0.2% collagenase type II in DMEM for 90 min), as previously described [28]. The first two digests were discarded. Cells from the third digest were collected by centrifugation and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (termed complete media) at 37 °C in 95% humidified air containing 5% CO_2.

All procedures were performed in accordance with the regulations laid down by the ethical guidelines of Peking University.

2.2. Mineralization assay

For the mineralization experiments,osteoblasts were plated at 50,000 cells/cm^2 in 100 mm tissue culture dishes and allowed to adhere for 24 h at 37 °C/5% CO_2 in a humidified incubator before treatment with osteogenic media (complete media supplemented with 50 μg/ml AA and 10 mM β-GP (Sigma, St. Louis, MO)). The cell cultures were maintained for up to 7, 14, 21 and 28 days respectively and the culture medium was replaced every 3 days.

Mineralization of the cultures was visualized directly in the culture dishes by 2.5% silver nitrate staining (von Kossa staining) which stains mineralized areas in black.

2.3. Mineral-associated protein assay

According to the procedure described by Grynpas et al. [29], the mineral-associated proteins were extracted twice with 0.50 M EDTA at 4 °C for a total of 48 h. The protein content was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Calcium concentrations were measured from mineral-dissolving, hydrochloric acid extracts of cell layers (also containing mineralized extracellular matrix) using a calcium kit (Diagnostic Chemicals). The protein expression was normalized to the total calcium content in the mineralized matrix.

2.4. Isolation and characterization of mineral phase

Calcium phosphate crystals were isolated from the organic matrix synthesized by osteoblasts by a modified methods described by Kuhn et al. [20]. Briefly, cells and matrix layers were harvested from the culture dishes after the culture medium was removed, rinsed quickly three times with neutral pH, 0.01 M Tris buffer, and lyophilized. The samples were then incubated with anhydrous hydrazine (10 mg/10 ml) at 4 °C under rotary mixing for 24 h and intermittent ultrasonication for about 5 min each hour to remove the organic matrix as much as possible. After 24 h the solvent was removed and the residue was washed twice with 100% ethanol. The residue was assayed as described below. As controls for mineralization analysis, we also cultured synthetic HAP nanopowder of <200 nm particle size (Sigma-Aldrich) with osteogenic medium and went through every procedure used for cell sample preparation.

HRTEM observations were carried out using a Hitachi H-9000 NAR electron microscope with electron acceleration energy of 300 kV. The fast Fourier transforms (FFT) of the HRTEM images were created by software named Digital Micrograph (version 3.5.2; Gatan Inc., USA).

EDX analysis was performed using a Hitachi S-5200 field emission SEM equipped with EDX spectroscopy (ISIS, Link Analytical, Oxford Instruments)

Synchrotron XRD spectra are collected using beam line BL14B1 at the Shanghai Synchrotron Radiation Facility, China. The X-ray wavelength was 1.2398 Å with the energy of 10 keV. We obtained the diffraction patterns by performing integration from the two-dimensional images with FIT2D software. The diffraction angle 2θ was converted to the condition of Cu Kα radiation wavelength ($\lambda = 1.54$ Å) through the Bragg's relation: $\lambda = 2d \cdot \sin\theta$. Phase identification was performed with the XRD analytical software Jade 5.0 (MDI, Livermore, CA) using the powder diffraction file (PDF). PDF card 09-0077 was used as the reference for DCPD, card 09-0432 for HAP, and card 26-1056 for OCP.

FT-IR spectra were recorded on a Nicolet Magna 750-II spectrometer equipped with a Nic-Plan TM IR microscope. Data were collected in transmission mode, 128 scans per point at 4 cm^{-1} resolution. The measurement was in the range of 4000–450 cm^{-1} and performed at random positions in the samples.

3. Results

3.1. Osteoblast mineralization

At the end of the experimental period, culture dishes were stained by the von Kossa reagent. As shown in Fig. 1, the calcium deposition stained in black was detected first in the cultures of osteoblasts at day 7, and the extent of mineralization increased up to the furthest time point at day 28.

3.2. HRTEM, EDX, synchrotron XRD and FT-IRM results

HRTEM was performed on deposited calcium phosphate in osteoblast cultures to observe changes in crystalline phases after removal of the organic components at different time intervals. As shown in the crystallized domains in Fig. 2, the measured interplanar spaces of 0.410, 0.522 and 0.824 nm correspond respectively to the (200), (101), and (100) planes of HAP. And those of 0.360 nm and of 0.798 nm correspond to the (220) face of OCP and the (020) plan of DCPD, respectively. From HRTEM analysis, DCPD phase was clearly identifiable at day 7, and OCP phase appeared usually at day 14 and 21, while HAP phase was the dominant phase at day 28. The probability of the three crystalline phases appeared in HRTEM images ($n = 10$) for each time point (7, 14, 21 and 28 days) implied that the thermodynamically metastable phases like DCPD and OCP appeared at the initial stage of the mineralization process of osteoblasts in vitro, and then partially transformed to HAP over time.

Further evidence for the notion is from the EDX data shown in Fig. 3. The molar ratios between calcium and phosphorus (Ca/P) of the mineral phases, as calculated from the EDX analysis ($n = 3$) at day 7, 14, 21 and 28, are 1.10 ± 0.42, 1.37 ± 0.08, 1.72 ± 0.08 and 1.53 ± 0.03, respectively. The first three Ca/P ratios were found to be in agreement with the theoretical stoichiometric molar ratio for DCPD (Ca/P = 1.00), OCP (Ca/P = 1.33) and HAP (Ca/P = 1.67), respectively. The Ca/P ratio around 1.53 at day 28 is lower than the stoichiometric value of 1.67 for HAP, but is consistent with nonstoichiometry of calcium deficient HAP. The EDX data showed a change in the Ca/P ratio from 1.10 to 1.53 reflecting the temporal transformation of the mineral phases.

The synchrotron XRD profiles of mineral phases are shown in Fig. 4. The peaks were substantially broadened because of the smaller crystal