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Capacity of octacalcium phosphate to promote osteoblastic differentiation toward osteocytes in vitro

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ABSTRACT

Octacalcium phosphate (OCP) has been shown to act as a nucleus for initial bone deposition and enhancing the early stages of osteoblastic differentiation. However, the effect on differentiation at the late stage into osteocytes has not been elucidated. The present study was designed to investigate whether OCP can promote the differentiation lineage from osteoblasts to late osteocytes using a clonal cell line IDG-SW3 compared to commercially available sintered β-tricalcium phosphate (β-TCP) and hydroxyapatite (HA) in a transwell cell culture. Special attention was paid to detect the progress of OCP hydrolysis associated with ionic dissolution products from this material. OCP induced the appearance of an alkaline phosphatase (ALP) peak in the IDG-SW3 cells compared to β-TCP and HA and increased SOST/sclerostin and FGF23 gene expression after 35 days of incubation. Analyses by X-ray diffraction, curve fitting of Fourier transform infrared spectra, and acid phosphate inclusion of the materials showed that OCP tended to hydrolyze to an apatitic structure during the incubation. Since the hydrolysis enhanced inorganic phosphate ion (Pi) release from OCP in the media, IDG-SW3 cells were further incubated in the conditioned media with an increased concentration of Pi in the presence or absence of phosphonoformic acid (PFA), which is an inhibitor of Pi transport within the cells. An increase in Pi concentration up to 1.5 mM raised ALP activity, while its positive effect was eliminated in the presence of 0.1 to 0.5 mM PFA. Calcium ions did not show such an effect. These results indicate the stimulatory capacity of OCP on osteoblastic differentiation toward osteocytes.

Statement of Significance

Octacalcium phosphate (OCP) has been shown to have a superior osteoconductivity due to its capacity to enhance initial stage of osteoblast differentiation. However, the effect of OCP on the late osteoblastic differentiation into osteocyte is unknown. This study showed the capacity associated with the structural change of OCP. The data show that OCP released inorganic phosphate (Pi) ions while the hydrolysis advanced if soaked in the media, determined by chemical and physical analyses, and enhanced osteocytes differentiation of IDG-SW3 cells more than hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). Conditioned elevated Pi-containing media in the absence of OCP enhanced the osteocyte differentiation in the range of the concentration induced by OCP, the effect of which was cancelled by the inhibitor of Pi-transporters.

1. Introduction

It has been proposed that bone formation first advances from mineral precursor deposition through an association with non-collagenous extracellular matrix proteins [1,2]. This evolves to bone apatite crystals within collagen fibers under the control of an osteoblastic cell lineage [3] and subsequent differentiation of osteoblasts into osteocytes [4]. During these biomineralization events, it is reasonable to hypothesize that osteoblastic cells undergo physicochemical effects induced by the mineral hydrolysis process concomitant with interactions between the calcium phosphate surfaces and proteins or various inorganic ions, including calcium and phosphate ions [5–8]. Amorphous calcium

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phosphate (ACP) [1,9,10] and octacalcium phosphate (OCP) [11] have been postulated as precursor phases in bone apatite crystal formation based on the formation of hydroxyapatite (HA) via these precursors from supersaturated calcium and phosphate solutions with respect to the HA phase [12].

Previous and recent intensive studies of synthetic OCP have revealed that this material displays an osteoconductive property [5,13–16] and can serve as a nucleus for initial bone deposition if implanted in various bone defects [13,17]. Therefore, synthetic OCP appears to have an advantage for promoting bone formation over other calcium phosphate bone substitutes, such as HA and β-tricalcium phosphate (β-TCP) [18,19]. The first evidence that new bone matrix formation by osteoblasts begins at the OCP surface was demonstrated through an onlay graft in granule form onto mouse calvaria [13]. In this model, the ultrastructure of the implanted OCP crystals and the initial matrix proteins that accumulated around them was very similar to the ultrastructure of bone nodules [13], which likely served as the initial locus of intramembranous bone development [20]. The initial bone nodule-like structure that formed after OCP implantation was then replaced with non-mineralized osteoid tissue embedded with osteocytes that had differentiated from osteoblasts [3,20], which was previously aligned on the OCP surface [13,18]. The osteoid tissue was then immediately calcified to convert to mineralized bone tissue [3,21].

We previously found that the stimulatory capacity of OCP to enhance new bone formation stems from its positive effects on the activity of the cells associated with bone tissue [5,22,23]. OCP enhances the differentiation of mouse bone marrow-derived stromal cells to osteoblastic cells through an increase in differentiation marker genes, such as osterix and alkaline phosphatase (ALP) [22]. OCP also enhances the formation of osteoclastic cells from bone marrow cells in the presence of osteoblasts with increased receptor activator of NF-kappaB ligand (RANKL) expression even in the absence of active vitamin D_3 [23], which is an enhancer of RANKL expression in osteoblasts. OCP has also been shown to be a metastable phase at physiological pH [24] and therefore thermodynamically favored to convert to HA [12]. However, the hydrolysis of OCP in vitro does not begin even in supersaturated simulated body fluid (SBF) in the absence of a hydrolysis enhancer [25], such as fluoride ions [7,26]. We have found that OCP tends to progressively hydrolyze into apatitic phase if implanted in a bone defect based on changes in X-ray diffraction patterns and Fourier transform infrared spectra toward an HA structure [5,13,27]. Our laboratory as well as others have observed that OCP can be hydrolyzed into HA in subcutaneous tissues [17,28,29] with an increasing Ca/P molar ratio toward Ca-deficient HA (from 1.30 to 1.49) [5,13,17,29].

It has been shown that when OCP is hydrolyzed to HA through acceleration in hot water [7] or in a solution with low fluoride concentration at physiological temperature and pH [6,7], OCP tends to hydrolyze to Ca-deficient HA, which is accompanied by an uptake of calcium ions (Ca^{2+}) from the surrounding solution and the release of inorganic phosphate (Pi) ions [6]. OCP hydrolysis can induce progressive changes in chemical composition, including the acid phosphate content of the total phosphorus in the crystals [7,30], binding affinity of serum protein adsorption onto the surfaces [7], and a subtle decrease in pH around the crystals [31] as hydrolysis advances. Therefore, it is highly probable that OCP hydrolysis is involved in acquiring the stimulatory capacity to activate bone tissue-related cells [5]. However, the effect of the physicochemical environment induced by OCP on osteoblastic cell lineage at the later differentiation stages into osteocytes has not been examined.

It is becoming clear that osteocytes embedded in bone matrix can control mineralization of collagen [32], respond to mechanical stimuli [33], and regulate phosphate homeostasis locally and systemically through factors such as fibroblast growth factor 23 (FGF23) [34]. In addition, osteocytes are actively involved in the regulation of bone formation by producing sclerostin, which is encoded by the SOST gene and associated with osteoclast activity [35,36], and in the maintenance of bone quality [34]. Since pre-osteoblastic cells are likely exposed to ions during OCP hydrolysis [5,6] and may be activated down an osteoblastic differentiation lineage [22,37], it is of interest to understand whether OCP can affect the subsequent differentiation of osteoblasts into osteocytes in a similar fashion during the crystal maturation process of OCP, which may simulate the mechanism of natural bone mineralization and late osteoblastic cell differentiation into osteocytes. The present study was designed to investigate whether OCP can stimulate the later stages of osteoblast differentiation using a clonal cell line immortomouse/Dmp1-GFP-SW3 (IDG-SW3) [38] and determine how the ionic exposure derived from OCP controls its differentiation.

2. Materials and methods

2.1. Preparation of calcium phosphates

OCP was synthesized by mixing calcium acetate and sodium hydrogen phosphate solutions under a constant pH 5–6 and temperature at 65 °C according to a well-established wet synthesis method previously reported [13]. The precipitate was recovered from the reacting solution, washed several times with water, and dried at 105 °C for subsequent use. Commercially available HA (Apaceram-G®, HOYA, Tokyo, Japan) and β-TCP (OSferion®, Olympus Terumo Biomaterials Corp., Tokyo, Japan) were used. These calcium phosphates were sieved between 32 and 48 meshes to obtain granules with a particle size of 300–500 μm. The sieved powders were sterilized at 120 °C for 2 h before being used in cell culture. The morphology of the calcium phosphate materials was observed using a JEOL analytical scanning electron microscope (SEM) JSM-6390LA (Tokyo, Japan) with an accelerating voltage of 10 kV. Au-Pd was sputtered before observation. OCP exhibited an aggregate form of plate-like crystals (Fig. S1A). HA and β-TCP (Fig. S1B and 1C) were composed of sintered grains, but included some pores within them.

2.2. Osteocyte cell culture

The mouse osteocyte-like cell line (IDG-SW3) was purchased from Kerafast, Inc. (Boston, MA, USA). The cells were expanded on plates coated with 1% porcine skin type I collagen (Nippon Meat Packers, Tsukuba, Ibaraki, Japan) in αMEM (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco by life technologies, Carlsbad, USA), 1% penicillin streptomycin (Nacalai tesque, Kyoto, Japan), and 50 U/ml mouse IFN-γ (R&D systems, Minneapolis, USA) at 33 °C in a 5% CO_2 incubator. To induce osteocyte differentiation, cells were seeded on the type I collagen coating at a concentration of 2×10^6 cell/ml on 24-well plates in osteogenic media with 50 μg/ml ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan) and 4 mM β-glycerophosphate (Tokyo Chemical Industry, Tokyo, Japan) in the absence of IFN-γ at 37 °C. A transwell (Cell Culture Insert, 8.0 μm pore size, FALCON) containing 4 mg of calcium phosphate particles (HA, β-TCP, or OCP) was applied to each well. The culture medium was replaced every two or three days.

2.3. Measurement of DNA content and activity of alkaline phosphatase (ALP) of IDG-SW3 cells

Cells were collected at day 7, 14, 21, 28, and 35 after seeding. After rinsing with phosphate buffered saline (PBS), the cells were

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