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# Bone formation enhanced by implanted octacalcium phosphate involving conversion into Ca-deficient hydroxyapatite

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**Abstract**

The present study was designed to investigate whether hydrolysis of synthetic octacalcium phosphate (OCP) into hydroxyapatite affects bone formation. Mouse bone marrow stromal ST-2 cells and primary calvarial osteoblastic cells were cultured on the dishes pre-coated with OCP or its hydrolyzed Ca-deficient hydroxyapatite (OCP hydrolyzate; HL). The capacity of proliferation and differentiation was determined up to day 20. Granules of OCP and HL were implanted into critical-size rat calvaria defects for 4 and 12 weeks, and then bone formation was measured by histomorphometry. Structural changes of incubated and implanted OCP were determined by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The proliferation of both ST-2 and primary osteoblasts cultured on OCP or HL was initially inhibited, whereas their differentiation to osteoblasts was promoted at last. Implantation of OCP in bone defect more significantly enhanced bone formation than that of HL until 12 weeks. OCP tended to convert to apatite in vitro and in vivo. The conversion of the implanted OCP was ascertained to advance gradually with implantation periods. Taken together, these results suggest that OCP supports appositional bone formation and OCP-apatite conversion may be involved in this stimulatory capacity of OCP.


*Keywords:* Bone formation; Octacalcium phosphate (OCP); Hydroxyapatite (HA); Conversion; Biomineralization

## 1. Introduction

Synthetic octacalcium phosphate ($Ca_8H_2(PO_4)_6 \cdot 5H_2O$; OCP) has been investigated as a bone substitute material in various forms, such as coatings on metallic implants [1–4] and granules [5,6]. These studies estimated the longevity of the OCP coatings [1], osteoblastic cell proliferation on OCP [2] and the ability to form bone by implantation in subcutaneous tissue or bone defects [3–6]. However, the mechanism of the induction of osteoconductive characteristics by OCP remains to be resolved. OCP has been suggested to be a precursor of biological apatite crystals in bones and teeth [7]. There is a general consensus that the OCP structure stacks apatitic layers alternatively with hydrated layers, and that the transition of OCP to hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HA) is thermodynamically favored [8,9]. Because of the transitory characteristics of OCP in the physiological condition [7–9], the detection of OCP in these calcified tissues is not easy. The direct evidence of the presence of OCP, however, was obtained as the inclusion in the central part of a dentin crystal and apatite in the outer most layers of the same crystal [10].

The mineral prototype of bones and teeth is usually considered to be basic calcium phosphate HA [11–13]. The biomineral displays better crystallinity and a higher molar ratio with mineral development [11–13]. The final biological apatite crystals are constituted of poorly crystalline HA

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with a low Ca/P molar ratio, i.e. Ca-deficient HA, containing foreign ions, such as carbonate and fluoride. Conversion of synthetic OCP into HA has been investigated in various physiological media, such as simulated body fluid [14] and ultrafiltered human serum [15], and by murine tissue implantation [5,16,17]. It has been shown that the apatite converted from OCP in in vitro physiological conditions was a Ca-deficient HA, which has a chemical composition with a lower Ca/P molar ratio and a higher acid phosphate content [18], as observed in biological crystals [19]. The Ca-deficient HA formed via OCP contained a small amount of OCP as a residual inclusion within the formed apatite [20,21] and further retained the original plate-like morphology of OCP [18,20].

Ca-deficient HA can also be prepared by direct precipitation from calcium and phosphate solutions [22]. We previously reported that OCP enhanced bone formation more than stoichiometric HA or Ca-deficient HA, when they were implanted in murine bone [5,16,23]. The implanted OCP was shown to convert to an apatite structure, determined by X-ray diffraction (XRD) [16,17]. The Ca-deficient HA used was obtained not via OCP, but from direct precipitation [16]. It is reasonable to assume that apatite converted from OCP by its being implanted in the bone could be Ca-deficient HA, and maintains the original OCP characteristics, as observed in in vitro OCP conversion studies [18,20,21]. Since OCP is a potential precursor of biological apatite crystals in the bones and teeth [7–9], the Ca-deficient HA formed via OCP, rather than the directly precipitated Ca-deficient HA, would probably be analogous to biological apatite. It is becoming clear that the major incorporation of foreign ions (e.g. carbonate ions) into biological apatite takes place during the hydrolysis of OCP [24]. Moreover, it has been indicated that the hydrolysis of OCP affects the interaction with biomolecules, such as serum constituents [17,18]. However, the biological significance of OCP and its hydrolysis, if implanted in bone, is still unclear.

In the present study, we hypothesized that the effect of enhancing bone formation may be associated with a process of conversion from OCP to Ca-deficient HA. The goal of this study was to examine the effect of OCP and its hydrolysis on proliferation and differentiation in osteoblastic cells in vitro and bone formation in vivo. OCP was compared with its hydrolyzed Ca-deficient HA (referred to as OCP hydrolyzate; HL, hereafter). A better understanding of bone formation by OCP and the effect on hydrolysis to apatite may lead not only to the development of bone substitute materials, but also to the elucidation of the biomineralization mechanism of bone formation.

## 2. Materials and methods

### 2.1. Preparation of synthetic OCP and HLs

Reagent-grade chemicals were used in this study. A batch of OCP was prepared by direct precipitation at pH 4.7–4.8 and 70 °C according to a previously reported method [25]. Two-thirds of the synthesized OCP slurry was filtered and washed four times with deionized water. Furthermore, half of the washed precipitate was re-dispersed in deionized water for OCP slurry stock and the remnant was dried in an oven at 105 °C as implant material stock. The remaining first OCP slurry was subsequently maintained at 70 °C with constant stirring for 48 h. The process has been confirmed to produce a hydrolyzed apatitic product from OCP (HL) with various degrees of conversion, depending on the hydrolysis periods [18]. At 6 h, a portion of the slurry was taken for physical and chemical analyses. At 48 h, the resulting HL slurry was filtered and washed four times with deionized water and then re-dispersed in deionized water with a solid versus solution ratio of 5 in weight per volume. The slurry was refrigerated at −4 °C. A portion of the slurry was also used to characterize the physical and chemical analyses to re-confirm the OCP–apatite conversion, and the remainder was used as a coating material for cell culture. Granules of OCP and HL were used as implants. The granules were obtained by grinding the dried slurry cake and by sieving between 32 and 48 meshes (particle sizes of 300–500 μm). The sieved granules were sterilized by heating at 120 °C for 2 h. Our previous study showed that such heating does not affect the physical properties, such as the crystalline structure or the specific surface area of OCP granules [17].

### 2.2. Characterization of synthetic OCP and HLs

The synthetic OCP and HL were characterized by XRD and Fourier transform infrared spectroscopy (FTIR). Powder XRD patterns of the synthetics were obtained by a scanning step with Cu Kα X-rays on a Rigaku Electrical Co., Ltd. (Tokyo), RAD-2B diffractometer at 40KV, 20 mA. FTIR spectra of the synthetics were obtained using a diffuse-reflectance attachment to a Jasco FT/IR 350 (Jasco, Tokyo, Japan). Approximately 500 spectral scans were usually conducted over the range of 4000–400 $cm^{-1}$ with a resolution of 4 $cm^{-1}$. The calcium and phosphorus contents of the synthetics were determined with an atomic absorption spectrophotometer and colorimetry and acid phosphate was determined using a previously reported procedure [12] after pyrolysis of the solid at 600 °C for 24 h under atmospheric conditions. Specific surface areas of the synthetics were determined by nitrogen adsorption.

### 2.3. Coating of synthetic OCP and HL slurries onto plates for cell culture

Fifty microliters of the OCP and HL slurries were dropped onto each well in 6-well plates (Corning Inc., NY, USA) using a pipette. The plates were placed in an oven at 80 °C to dry overnight. Heating allows the slurries to dry for sufficient adherence to the plate during incubation. The plates were immersed in 70% alcohol for sterilization (disinfection) and dried at room temperature on a clean bench for cell culture.

### 2.4. Cell culture

Mouse bone marrow stromal ST-2 cells were obtained from RIKEN Cell Bank (Tsukuba Science City, Ibaraki, Japan). Primary osteoblastic cells were obtained from neonatal ddy strain mice calvaria by sequential collagenase-dispase digestion [26]. Cells were maintained in α-minimal essential medium (α-MEM) containing 10% FBS (Sigma-Aldrich, St. Louis, MO), and inoculated at $3 \times 10^5$ cells/well in 6-well plates. Alkaline phosphatase (ALP) activity was stained using an azo-dye method as described previously [27] and analyzed using a Charge-Coupled Device (CCD) camera with the Compact Color Vision System (Keyence, Tokyo, Japan). Cell proliferation was estimated by counting the number of nuclei under a fluorescent microscope after 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining.

### 2.5. XRD of incubated OCP in medium

The OCP coating was examined with XRD to study the structural changes during incubation. The OCP coatings were incubated in α-MEM