Octacalcium phosphate bone substitute materials: Comparison between properties of biomaterials and other calcium phosphate materials

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Octacalcium phosphate (OCP) is a material that can be converted to hydroxyapatite (HA) under physiological environments and is considered a mineral precursor to bone apatite crystals. The structure of OCP consists of apatite layers stacked alternately with hydrated layers, and closely resembles the structure of HA. The performance of OCP as a bone substitute differs from that of HA materials in terms of their osteoconductivity and biodegradability. OCP manifests a cellular phagocytic response through osteoclast-like cells similar to that exhibited by the biodegradable material β-tricalcium phosphate (β-TCP). The use of OCP for human cranial bone defects involves using its granule or composite form with one of the natural polymers, viz., the reconstituted collagen. This review article discusses the differences and similarities in these calcium phosphate (Ca-P)-based materials from the viewpoint of the structure and their material chemistry, and attempts to elucidate why Ca-P materials, particularly OCP, display unique osteoconductive property.

Keywords: Octacalcium phosphate, Hydroxyapatite, β-tricalcium phosphate, Biomaterials property, Bone substitute materials

INTRODUCTION

Octacalcium phosphate (OCP) is a calcium phosphate (Ca-P) material that serves as a precursor phase for the formation of hydroxyapatite (HA) from supersaturated solutions with respect to HA. The structure of OCP is composed of apatite layers stacked alternately with a hydrated layer, and therefore, is closely associated with HA structure. OCP is a possible precursor of bone and tooth apatite crystals and likely forms clusters. Bioactive properties of OCP as a bone substitute material have attracted the attention of not only biomaterials scientists but also oral and orthopedic surgeons specialized in repairing bone defects. A higher osteoconductivity was first observed in the bone tissue response of mouse cortical bone where OCP was placed onto the calvaria in its granule form, as compared to other Ca-P materials, including the anhydrous form of dicalcium phosphate (DCP), amorphous calcium phosphate (ACP), calcium-deficient HA (CDHA), and stoichiometric HA (both HAs were prepared without sintering). Our subsequent studies further provided evidence that OCP tends to biodegrade in the implanted sites in the bone.

A review paper classified OCP as an osteoconductive and biodegradable material like HA and β-tricalcium phosphate (β-TCP), respectively. However, a series of studies, including this first experiment, further revealed that OCP is capable of stimulating bone formation through osteoblast differentiation and osteoclast formation, which might have properties between osteoconductivity and osteoinductivity. HA and β-TCP have been widely used clinically in oral and orthopedic surgeries. We have also found that such cellular stimulatory capacity of OCP can be acquired during thermodynamic conversion to HA, via its hydrolysis under physiological environments. Based on these unique bioactive properties, we have been developing bone substitutes utilizing OCP and natural polymers, such as collagen, gelatin, alginate, and hyaluronic acid, by forming composite materials with the desired shape for the treatment of bone defects. One of the composite materials with collagen was recently approved as an implant for oral surgery after a university clinical trial, followed by a company-initiated clinical trial. Other investigators have reported the possible application of different forms of OCP, such as coating, 3D printing, blocks, and others. Thus, the intrinsic property of OCP is continuously arousing the interest of biomaterial scientists.

In order to understand and describe the unique property of OCP as a bone substitute material, it may be essential to examine the differences and similarities of OCP in comparison with an osteoconductive HA and a biodegradable β-TCP from the materials science viewpoint.

BIOMINERALIZATION INVOLVING PRECURSOR OCP AND CONVERSION OF SYNTHETIC OCP IN VIVO

It has been reported that the preferable formation of OCP in physiological solution conditions could be predicted from the solution saturation level with respect to the specific Ca-P phases, which define the degree of supersaturation (DS). An essential finding is that human serum is approximately saturated with respect to OCP, whereas it is highly supersaturated with respect

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to HA. When OCP was incubated in a physiological solution without adding calcium and phosphate ions, the solution composition became saturated with respect to OCP after the partial dissolution of OCP. However, upon incubating OCP in a simulated body fluid (SBF) containing inorganic ions corresponding to the concentration of human physiological plasma, it became only slightly supersaturated with respect to OCP under a highly supersaturated condition with respect to HA, suggesting that hydrolysis from OCP to HA is still possible. An interesting finding of this equilibrium condition is that the solution is also slightly supersaturated with respect to β-TCP, although the appearance of this phase has never been reported in bone mineralization. Because OCP can be converted to an apatitic structure when implanted in various animal models, it is reasonable to assume that the conversion of the synthetic OCP implanted in vivo through hydrolysis simulates the formation of OCP and its maturation to bone-apatite crystals, as expected from the chemical composition analysis of the human serum.

Notably, regarding the presence of OCP as a biomineral component from the material point of view, its involvement in the biomineralization process is still under debate, although there are reports based on crystallographic or spectroscopic evidence suggesting OCP is involved in the biomineralization of tissues. The OCP-like crystalline phase lacked the characteristics of OCP in the X-ray diffraction (XRD) primary peak, and therefore resembled the apatitic phase. Such OCP-like materials were found in the hydrolyzed product obtained in the presence of fluoride ions (F⁻) in the wet condition, and still possessed a solubility close to that of OCP with an apatitic crystallographic characteristic. It may be reasonable to consider that although well-crystallized OCP has not been identified in the bone during mineralization, a non-stoichiometric OCP-like phase chemically compatible with the well-crystallized OCP could be involved in bone mineralization based on the observation of the behavior of synthetic OCP in physiological conditions.

MATERIALS AND PHYSIOCHEMICAL PROPERTIES OF OCP, β-TCP, AND HA

Crystal structure of OCP, β-TCP, and HA

OCP, HA, and TCP are Ca-P compounds which are defined based on their crystal structure and chemical composition, including the molar ratio of calcium to phosphorus (Ca/P). Figure 1 shows the unit cells of OCP, HA, and β-TCP drawn by VESTA.

Matthew et al. determined the crystallographic structure of OCP (Ca₈H₂(PO₄)₆•5H₂O) to be triclinic with the space group P. The lattice parameters of the OCP unit cell are a=19.692 Å, b=9.523 Å, c=6.835 Å, α=90.15°, β=92.54°, γ=108.65°, and Z=2. The crystal structure of OCP consists of a layer of a Ca-deficient apatite-

Fig. 1  Schematic images showing the crystal structure of OCP (A), HA (B), and β-TCP (C) on the (001) plane.
like structure (4[Ca₆(PO₄)₂•0.5H₂O]) and a hydrated layer (4[CaHPO₄•2H₂O]) similar to the structure of brushite (dicalcium phosphate dihydrate; DCPD). The apatite-like structure and hydrated layer are laminated alternately toward the a-axis of the OCP unit cell. The OCP has a stoichiometric composition of 33.3 mol% acidic phosphate (HPO₄²⁻) to total phosphate present either in the hydrated layer (HP(5)O₄²⁻) or at the junction of the apatite-like structure and hydrated layer (HP(6)O₄²⁻).

HA (Ca₁₉(PO₄)₁₂(OH)₂) belongs to the hexagonal system with space group P6₃/mmm. The lattice parameters of the HA unit cells are a=9.4214 Å, c=6.883 Å, and Z=1. The structure of HA consists of two types of Ca: Ca(1), which is positioned at four sites in the unit cell, and forms a line toward the c axis, is termed as “columnar Ca”; and Ca(2), which has six sites surrounding the hydroxide ion positioned at four corners of the unit cells. The domain of the Ca₆(PO₄)₆ cluster has an ACP structure as reported by Posner and Betts⁶⁸, and is shown in the HA unit cells (Fig. 1). A crystal growth model has been proposed for aggregation, and/or stacking of the Ca₆(PO₄)₆ cluster toward the c axis of the HA unit cell during the formation of HA in solution⁴. ACP, as well as OCP, are considered as the precursors of HA. The Ca₆(PO₄)₆ cluster is also present in the apatite-like layer of OCP, which suggests that the cluster is involved in biomineralization⁴. Recently, Habraken et al. proposed that Ca₆(HPO₄)₆²⁻ complexes aggregate to form a spherical ACP cluster, which converts into HA through the Ca-deficient OCP phase⁶⁹.

Tricalcium phosphate (Ca₆(PO₄)₃) has three kinds of crystal structure, i.e., α (monoclinic), α’ (hexagonal), and β (rhombohedral) phases. HA and OCP are prepared through solution synthesis, whereas β-TCP can be prepared by solid synthesis. The lattice parameters of β-TCP are a=10.435 Å, c=37.403 Å, and Z=21⁻⁶⁹,⁷⁰. The unit cell of β-TCP has A and B columns consisting of P(1)O₆, Ca(4)O₆, Ca(5)O₆, P(1)O₆, and P(3)O₆, Ca(1)O₆, Ca(3)O₆, Ca(2)O₆, P(2)O₆, P(3)O₆, respectively. The Ca and PO₄ in each column are oriented toward the c-axis, and six B columns surround each A column.

**Chemical composition of OCP, β-TCP, and HA**

The Ca/P molar ratios of stoichiometric OCP, HA, and β-TCP are 1.33, 1.67, and 1.50, respectively. Various non-stoichiometric compositions related to HA (Ca/P molar ratio below 1.67), such as the Ca-deficient HA (Ca₁₉₋ₓ(HPO₄)ₓ(PO₄)₂₋ₓ(OH)₂₋ₓ), carbonate-containing HA, are well known.

Wet chemical synthesis is used to obtain non-stoichiometric OCP with Ca/P=1.27, which contains excess acidic phosphate (approximately 40 mol%). The acidic phosphate contains 50–60% stable HP(6)O₄²⁻, exchangeable HP(5)O₄²⁻, and 15–20% unstable HPO₄²⁻. The unstable HPO₄²⁻ exists on the surface of the OCP crystal. The composition of the non-stoichiometric OCP is Ca₁₉₋ₓ(HPO₄)ₓ(PO₄)₂₋ₓ(OH)₂₋ₓ(10-x)H₂O. During HA formation through the aggregation of the calcium phosphate complexes, the formation of Ca-deficient OCP (Ca₆(HPO₄)₅(PO₄)₂⁻) was identified by wide-angle synchrotron X-ray scattering⁸⁰. The ratio of HPO₄ to the total phosphorus and Ca/P molar ratio in OCP gradually changes during hydrolysis⁷¹,⁷².

**Thermal stability of OCP, β-TCP, and HA**

The crystal structure of Ca-P is sensitive to the surrounding temperature. The stoichiometric HA transforms into oxyapatite through the loss of water molecules at higher temperatures (1,200–1,400°C)⁷³, although β-TCP and HA form from CDHA above 700–800°C⁷⁴,⁷⁵. β-TCP is a low-temperature phase, which transforms into α-TCP above 1,125°C.

The thermal stability of OCP is lower than that of HA or β-TCP because OCP has a large amount of water in its structure. The complete decomposition of OCP to HA occurs above approximately 300°C⁷⁴,⁷⁵. The onset of dehydration in the hydrated layer starts at approximately 170°C during heat treatment with increasing temperature at 10°C/s⁷⁵. During heating, the hydrated layer contracts due to dehydration, and “collapsed OCP” is formed below the temperature required for the transformation into HA. The lattice parameters of the “collapsed OCP”, which coexists with the OCP structure, are a=18.86 Å and γ=114.65°⁷⁵. In the crystal structure of OCP, Ca²⁺ can be substituted with other cations such as magnesium (Mg²⁺), strontium (Sr²⁺), and zinc (Zn²⁺)⁷⁶. The substituted ions such as Sr²⁺ and Mg²⁺ can reduce the thermal stability of OCP and increase the content of the “collapsed OCP” at high temperatures⁷⁵.

**Solubility of OCP, β-TCP, and HA**

The solubility of Ca-P is related to the thermodynamic stability of its structures, which in turn depends on the concentration of the surrounding ions, pH, and temperature. The solubility product constant (Kₛ) is an equilibrium constant for a solid dissolving in an aqueous solution. The values of Kₛ for Ca-P phases are 37°C⁷⁸-⁸². Based on the Kₛ values of Ca-Ps, the phase diagram consisting of solubility isotherms in the Ca(OH)₂-H₃PO₄-H₂O system at 37°C shows that the Ca-Ps are soluble at pH 7.4, and that the solubility increases as HA<β-TCP<OCP<DCPD⁴⁰. This also implies that the thermodynamic stability of OCP is lower than that of β-TCP as well as HA under the physiological pH condition.

The DS with respect to Ca-P is an indicator of the solubilities in arbitrary solutions, and is calculated using Eqn. (1):

\[
DS=(\frac{IP}{Kₛ})^{1/ν}
\]

where IP and ν are the ionic activity products and the number of ions in Ca-P, respectively. The analytical results for the Ca²⁺ and inorganic phosphate (Pi) ion concentrations and pH of the solution can be used to calculate the IP values based on the three mass balance values for the ions, assuming the formation of ion complexes and generation of ion strength³¹,⁸³,⁸⁴. In a physiological environment, the ionic strength is ~150–
On the other hand, ion diffusion-crystallization in the hydrated layer is specific to OCP hydrolysis. In this reaction, the protons and HP(5)O_4^2- in the hydrated layer diffuse to outside the OCP lattice. Subsequently, the crystal structure is reconstructed and crystallized to form HA. The surface hydrolysis of OCP through ion diffusion has been observed during alkaline extraction. In hot water, HA is epitaxially formed on OCP through the diffusion of ions and water molecules; therefore, the obtained HA (CDHA) maintains the morphology of the original OCP crystals.

The higher DS with respect to HA acts as a driving force to promote the conversion of metastable Ca-P, including OCP, into HA in physiological environments. SBF was maintained in a supersaturated condition with respect to HA after the incubation of OCP granules, which allows the formation of HA (Table 1). XRD also indicated that the hydrolysis of OCP occurs, although it was not easy to detect apatite formation from XRD even after its immersion in SBF. Analysis of OCP using the curve-fitted spectra of Fourier transform infrared (FT-IR) shows that the OH^- content in the apatitic structure slightly increased and HPO_4^2- in the non-apatitic structure modestly decreased after incubation in SBF for two weeks. This also supports the hydrolysis of OCP in SBF. Such analysis also demonstrated that the hydrolysis of OCP for the conversion into HA had been induced in a cell culture medium supersaturated with respect to OCP and HA.

On the other hand, the transformation of OCP into HA in vivo has been confirmed by ultrastructural observation and crystallographic analysis. Notably, fine de novo HA crystals were formed on plate-like OCP crystals placed onto mouse calvaria at three weeks post-implantation. It has been reported that several ion species regulate the conversion of OCP into HA. Serum and SBF contain Mg^{2+}, which serve as an inhibitor, whereas serum also contains F-, which serve as a promoter to induce the hydrolysis of OCP through the dissolution-reprecipitation reaction. We also confirmed the progress of the conversion of OCP into HA at a lower fluoride ion concentration at 37°C. Tung et al. reported that Mg^{2+} delays the nucleation of HA during the hydrolysis of OCP, although F- accelerates the nucleation regardless of the presence of Mg^{2+}. Ito et al. reported that the

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Table 1 DS of calcium phosphates in SBF incubated with Ca-P granules for seven days.

|          | HA       | OCP      | β-TCP    |
|----------|----------|----------|----------|
| SBF original | 1.00×10^{12} | 2.37×10^{3} | 1.98×10^{3} |
| HA       | 4.00×10^{6}  | 1.85×10^{6} | 1.34×10^{4} |
| OCP       | 2.35×10^{6}  | 1.22×10^{6} | 9.79×10^{4} |
| β-TCP     | 1.29×10^{9}  | 6.30×10^{1}  | 6.43×10^{9} |

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nucleation of HA could be the rate-determining process for the conversion of OCP into HA\(^{40}\). Thus, it is assumed that both the thermodynamic stability and kinetics of HA formation govern the conversion of OCP into HA in the physiological condition both in vitro and in vivo.

Recently, via spectroscopic analysis with curve fitting, we reported that OCP, when mechanically mixed with ACP, promoted its conversion into an apatitic structure compared to the use of OCP alone in SBF\(^{33}\). After two weeks of incubation of the mixture, SBF approached close to a saturation with respect to OCP, and maintained a higher supersaturation with respect to HA. Thus, ACP has a role in providing a favorable ion environment for conversion in terms of the thermodynamic stability of HA and OCP due to its higher solubility.

The DS values for SBF incubated with \(\beta\)-TCP also showed that it is possible to induce HA precipitation, although HA could not be formed on \(\beta\)-TCP\(^{53}\). Other research groups also demonstrated that the ability of \(\beta\)-TCP to form HA is poor in SBF\(^{26,59}\). Different results have been reported regarding the in vitro environment for HA formation on the surface of \(\beta\)-TCP. Earlier literature works indicated that the apatite layer was not observed between the surface of \(\beta\)-TCP and newly formed bone\(^{46,97}\), although it was suggested that carbonate-substituted apatite formed on \(\beta\)-TCP\(^{98}\). In contrast, it has been demonstrated that \(\alpha\)-TCP forms HA through a hydrolysis reaction in SBF\(^{26,59}\). The SBF and serum are undersaturated with respect to \(\alpha\)-TCP. Thus, the difference in the thermodynamic stability of TCP regulates the HA formation in the physiological environment.

**Ion environment in physiological condition regulated by Ca-Ps**

The dissolution, conversion, and crystal growth of Ca-Ps regulate the ion environment in physiological conditions. We demonstrated that the Ca\(^{2+}\) concentration increases and Pi ion concentration decreases in physiological conditions such as a cell culture medium incubated with OCP. This is because OCP incorporates Ca\(^{2+}\) and releases Pi ions during the hydrolysis reaction\(^{39}\). The decrement or increment of these ions in the medium depends on the amount of OCP incubated\(^{28,30,108}\). Furthermore, the pH locally decreased at the area adjacent to the OCP surface of its granules in a Tris-HCl buffer containing F\(_{-}\), although the buffering effect occurs to maintain the pH under the physiological condition\(^{101}\). When \(\beta\)-TCP is incubated, the concentrations of Ca\(^{2+}\) and pH increase, whereas the concentration of the Pi ions decreases in the medium\(^{30}\). Such behavioral change in the ion concentration could be attributed to HA formation on the \(\beta\)-TCP surface\(^{102}\). To summarize, the ion environment around the materials may regulate the mechanisms of apatite formation on biodegradable Ca-Ps, which could actively be involved in cellular response both in vitro and in vivo.

**Adsorption of protein onto OCP and HA**

The adsorption of serum-derived proteins has been shown to regulate cell adhesion, proliferation, and differentiation onto bioceramics, and is involved in bone formation\(^{109}\). Results of an in vivo study indicated that new bone formation by osteoblasts could be initiated from the surface of OCP granules accumulating on non-collagenous serum-derived proteins containing a lectin-binding glycoprotein\(^{15,54}\). Our group has compared the behavior of serum protein adsorption onto OCP and HA in vitro to investigate why these materials exhibit different cellular responses. In a saturated solution with respect to OCP, the adsorption isotherm of bovine serum albumin (BSA) onto OCP in comparison with that of HA (CDHA) prepared through the hydrolysis of OCP in hot water can be described using a Langmuir model\(^{91}\). This indicates that OCP had a higher adsorption affinity and a more significant number of adsorption sites for BSA relative to HA\(^{91}\). We also recently reported that the adsorption of BSA onto OCP was enhanced by increasing the DS with respect to OCP and HA in the solution simulating the ion environments induced by the dissolution of OCP\(^{94,95}\). These results indicate that solution supersaturation could regulate the adsorption affinity of serum proteins.

Furthermore, proteome analysis was performed to identify the adsorbed proteins onto OCP and HA in rat serum in vitro\(^{103}\). The adsorption of α2HS-glycoprotein was detected at the site of the OCP granules implanted in the defect\(^{54}\). Interestingly, the adsorption amount of apolipoprotein E, known as a regulator of osteoblastic differentiation\(^{106}\) and complement component 3, which is reported to promote the migration of osteoclast precursor cells\(^{107}\), was more abundant on the surface of OCP relative to HA\(^{105}\). Taken together, serum protein adsorption onto OCP could also contribute to the higher capacity of OCP to enhance the osteoblast and osteoclast activities in vivo.

**Effect of macromolecules around OCP on its conversion and apatite formation**

The formation and maturation of Ca-P phases in the presence of the matrix molecules around the crystals are other factors when the bioactivity of OCP, \(\beta\)-TCP and HA is to be considered. The physicochemical properties of composite materials consisting of OCP and natural polymers, such as collagen, gelatin, and alginate, were also evaluated in vitro. These composites were prepared by mixing OCP granules with a natural polymer solution\(^{20,22,108}\) or by the co-precipitation of OCP and the polymer in the solution\(^{24,35,36,109}\). The cell culture medium of α-MEM became supersaturated with respect to HA and slightly supersaturated with respect to OCP after incubation with OCP/collagen, which was prepared by the mixing method\(^{29}\). FT-IR spectra and XRD patterns showed that apatite formation could have occurred on the OCP/natural polymer composites after incubation in SBF. These reports indicate that OCP favors hydrolysis and forms HA even if OCP exists in the matrix molecules in a physiological environment. From the XRD and FT-IR analyses, it was found that apatite formation tends to be accelerated on OCP-based composites\(^{24,35,108,110}\) compared to OCP alone\(^{35}\). Furthermore, the fabrication
process of the composites regulates the apatite-forming ability of the OCP-based composites. For OCP/alginate incubated in SBF, the FT-IR peaks attributed to OCP in the composites prepared by the co-precipitation method seemed to reduce rapidly compared to the peaks for the composites prepared by the mixing method. OCP prepared through co-precipitation with collagen also formed apatite in SBF after incubation for a day. Recently, it was reported that OCP crystals co-precipitated with gelatin released a more substantial amount of Pi ions compared to OCP crystals without gelatin in the solution saturated with respect to OCP. OCP prepared through co-precipitation with collagen also formed apatite in SBF after incubation for a day. Recently, it was reported that OCP crystals co-precipitated with gelatin released a more substantial amount of Pi ions compared to OCP crystals without gelatin in the solution saturated with respect to OCP. The solution incubated with the co-precipitated OCP became slightly undersaturated with respect to OCP, relative to that of the OCP without the gelatin, suggesting that the solubility of the co-precipitated OCP crystal could be higher in physiological environments. In vivo experiments also showed that OCP/gelatin composites prepared by co-precipitation exhibited higher degradability in a rat calvaria defect model. On the other hand, XRD confirmed that the crystallinity of the co-precipitated OCP with gelatin, collagen, or alginate tended to be lower than that of OCP prepared without the polymers. These results suggest that the use of macromolecules with OCP could support apatite formation induced by the hydrolysis of OCP in physiological conditions regardless of the preparation process of the composites. The macromolecules co-precipitated with OCP could also regulate the crystalline characteristics of OCP, which in turn contribute to dissolution and apatite formation. The effect of the presence of macromolecules on the physiological properties of β-TCP has not been examined yet. Although a macromolecule effect could be expected in DS when Ca-P in the solution is incubated with β-TCP alone, further studies are required to confirm the same.

BIOLOGICAL CHARACTERISTICS OF OCP COMPARED WITH β-TCP AND HA

Bone cellular response around OCP

1. Osteoclast-osteoblast crosstalk on OCP, β-TCP, and HA

Biodegradable Ca-Ps, such as OCP and β-TCP, can promote bone regeneration through bone remodeling, which involves the coupling of bone resorption by osteoclasts along with bone formation by osteoblasts. Recently, it was found that the osteoclast can coordinate osteoblast differentiation locally through several coupling factors. For example, the activation of EphrinB2 (EfnB2) ligands on osteoclasts coupled with EphB4 receptors on osteoblasts promotes osteoblast differentiation. Besides, there are several coupling factors secreted by osteoclasts. Sphingosine-1-phosphate (S1P), collagen triple helix repeat containing 1 (Cthrc1), and complement component 3a (C3a) are secreted by osteoclasts to enhance osteoblast differentiation. In contrast, Semaphorin4D (Sema4D) released by osteoclasts inhibits osteoblast differentiation. However, it is still unknown how osteoclasts crosstalk with osteoclasts on Ca-Ps.

In order to investigate the role of different kinds of Ca-Ps with varying chemical compositions on osteoclast formation and osteoclast-osteoblast crosstalk, mouse bone marrow macrophages were cultured on disks composed of OCP, HA, β-TCP, and mixtures of these compounds. Large tartrate-resistant acid phosphatase (TRAP)-positive cells, indicating multinucleated osteoclasts, were observed more frequently in the cultures with OCP or β-TCP disks compared to HA disks. The ability of osteoclast formation on OCP was almost the same as that of β-TCP, while the expression patterns of the coupling factors varied depending on the type of Ca-P. β-TCP and the HA/β-TCP mixture induced EfnB2 and Cthrc1 expression, whereas OCP and HA/OCP mixtures promoted C3a expression (Fig. 2). These results suggest that the crystal phase of Ca-P can affect the regulation of osteoblastic differentiation by coupling factors derived from osteoclasts.

XRD analysis revealed that OCP and HA/OCP mixtures tended to convert to HA during osteoclast culture. The concentration of Pi was higher in the media of OCP and HA/OCP mixtures than HA or β-TCP, suggesting that Pi was released through OCP-HA conversion. On the other hand, the crystal structures of HA, β-TCP, and the HA/β-TCP mixtures did not change before and after osteoclast culture. Taken together, the differences in the chemical environments, such as crystal structure and ion concentration, may influence the regulation of coupling factor expression in osteoclasts.

2. Osteoclasts

Osteoclasts play a vital role in determining the biodegradability of Ca-Ps. The effect of OCP on osteoclast differentiation was examined using co-cultures of...

Fig. 2  Comparison of osteoclast-osteoblast crosstalk for OCP and β-TCP.
mouse bone marrow macrophages and UAMS-32 mouse stromal cells. Cell culture plates were prepared with and without OCP coating. Compared with the un-coated culture plates, OCP-coated plates promoted the formation of TRAP-positive cells in a dose-dependent manner after 4–6 days of culture. Besides, RT-PCR analysis showed that the expression of TRAP and cathepsin K, which are typical osteoclast markers, increased in the cells cultured on OCP-coated culture plates.

The influence of the solubility of biphasic calcium phosphate (BCP) on osteoclast resorption was investigated using neonatal rabbit bone cells. BCP is a mixture of HA and β-TCP. Cells were cultured for two days on HA and β-TCP, and two types of BCP with HA/β-TCP ratios of 25/75 and 75/25. Resorption lacunae were observed on pure β-TCP and BCP 25/75, whereas osteoclasts did not resorb BCP 75/25 or pure HA. Pure β-TCP had the highest chemical solubility; it was BCP 25/75 in which the osteoclasts formed well-organized resorption lacunae. These results suggest that the solubility of Ca-Ps would affect osteoclast resorption.

3. Osteoblasts
When OCP was implanted in vivo, active cuboidal osteoblasts were observed around OCP granules. To investigate the osteoblastic differentiation induced by OCP, mouse bone marrow stromal ST-2 cells and primary calvarial osteoblastic cells were cultured on dishes pre-coated with OCP or HA. Osteoblastic differentiation was measured by alkaline phosphatase (ALP) activity, and mRNA expression levels of Runx2, osterix (Osx), collagen type I (Col-1), ALP, and osteopontin (OPN). The ALP staining of ST-2 or primary calvarial osteoblastic cells was more intense in OCP-coated wells than in HA-coated wells. The enzymatic ALP activity of ST-2 cells cultured on OCP-coated wells increased progressively with increasing OCP concentration; however, for the HA-coated group, the activity was constant regardless of HA content. Furthermore, OCP enhanced the expression of osteogenic markers, including Osx, Col-1, and ALP by day 21 of culturing. Thus, OCP has the potential to improve osteoblastic differentiation compared to HA.

4. Osteocytes
Osteocytes are terminally differentiated from osteoblasts and are embedded in the bone matrix. In order to investigate the effect of OCP on the transition of osteoblasts to late osteocytes, mouse IDG-SW3 cells were cultured in transwells with OCP, commercially available β-TCP, or HA. OCP increased the activity of ALP, an osteoblastic marker, on IDG-SW3 cells, compared to β-TCP and HA. The expression of Dmp1, a marker of mineralizing osteocytes, was also promoted in the presence of OCP relative to other groups. Furthermore, OCP enhanced the expression of mature osteocyte markers, SOST/sclerostin, and FGF23, compared to β-TCP and HA after 35 days of culture.

To clarify the role of OCP in promoting osteocyte differentiation, the IDG-SW3 cell culture was performed in various concentrations of Pi. Here, the hydrolysis of OCP to HA advanced in the culture, and was accompanied by the release of Pi and incorporation of Ca. Thus, increasing the Pi concentration in the culture media to 1.5 mM enhanced the ALP activity. This effect was eliminated by the addition of the Pi transport inhibitor phosphonoformic acid (PFA) to the media. These results suggested that the increase in Pi during OCP-HA conversion would stimulate osteocyte transition from osteoblasts.

5. Vascular endothelial cells
It is widely accepted that the induction of angiogenesis in the bone defect is one of the essential processes for the success of bone regeneration by implanted biomaterials. OCP can promote early angiogenesis and facilitate new bone formation in a rat calvarial defect model. To clarify how OCP can promote angiogenesis in vitro, human umbilical vein endothelial cells (HUVECs) were cultured in the presence or absence of OCP granules. It was found that OCP enhanced the capillary-like tube formation in HUVEC culture with a specific OCP dose (1 mg per well). This result indicates that OCP can promote direct angiogenesis by vascular endothelial cells.

It has been reported that the chemical composition of Ca-Ps influences neovascularization. In this study, HA, two types of BCPs, and β-TCP were implanted under the thigh muscle pouches of a mouse to evaluate the angiogenesis of these materials in vivo. BCP containing 70% β-TCP and β-TCP increased the vessel density (number of CD34 or CD105 positive cells) significantly at four weeks after implantation compared with HA or BCP containing 30% β-TCP. In vitro analysis also showed that the conditioned media collected from the human fibroblast culture on especially β-TCP and BCP, which had a higher content of the β-TCP phase, promoted the blanch points of blood vessels in the HUVEC culture.

The results of earlier studies and our studies suggest that biodegradable Ca-Ps, such as β-TCP and OCP, are advantageous to induce angiogenesis. The potential of angiogenesis in β-TCP and OCP might be related to the favorable ion condition around Ca-Ps. Ca signaling has been reported to be essential for the migration and proliferation of HUVECs. Further studies are necessary to understand the mechanism by which biodegradable Ca-Ps promote angiogenesis.

6. Macrophages
Biomaterial implantation triggers an inflammatory reaction. Therefore, the regulation of immune responses is crucial to reveal the osteogenic capacity of biomaterials. In particular, macrophages play an essential role in regulating the initial immune responses. Therefore, immune responses induced by the culture with OCP and other Ca-Ps to biodegradation and subsequent bone formation were investigated. OCP was compared to CDHA, which is an OCP hydrolyzate...
in the defect. However, defects implanted with HA showed slight newly formed bone, with the remaining being HA granules. Histomorphometric analysis revealed that the volume of the newly formed bone implanted on the three Ca-P materials decreased as OCP ≥ β-TCP > HA (Table 2). The volume of the remaining materials was highest in HA followed by OCP and β-TCP; the latter two were almost compatible (Table 2).

Picrosirius red staining is a useful tool to appraise collagen networks in bone tissue. The area of the oriented collagen matrix stained with yellowish-green was observed more in the OCP-implanted group than in the HA-implanted group or β-TCP-implanted group. These results suggest that the osteoconductivity of OCP could be higher than those of the other two Ca-P materials in terms of the collagen orientation.

2. Osteoconductivity and biodegradability of OCP/gelatin (OCP/Gel) composites compared with β-TCP in rabbit tibia

We have succeeded in developing a composite scaffold with OCP and gelatin21,22,36. Gelatin consists of biodegradable polymers denatured from collagen. Our earlier study showed that OCP/Gel exhibits rapid biodegradability and excellent bone formation capacity in rat calvarial bone defect26. However, little is known about whether OCP/Gel can induce bone regeneration in long bones. The OCP/Gel composite was implanted in a rabbit tibia defect and compared with two types of commercially available β-TCP to assess the bone formation and bioabsorbability21. In micro-CT analysis for the OCP/Gel group, the radiopaque area suggested that HA converted from OCP and newly formed mineralized bone at two weeks. The radiopacity in the intramedullary area decreased at four weeks. Bone defects were restored entirely and continued with the bone cortex at eight weeks. On the contrary, β-TCP (porosity 71–80%) and β-TCP (porosity 60%) remained almost unchanged until four weeks or eight weeks, respectively.

Histological analysis showed that active bone formation had already started in the OCP/Gel group at two weeks. The new bone was connected in the cortical area until eight weeks. Moreover, the entire OCP/Gel was almost resorbed in the intramedullary area. On the

Table 2 Quantitative analysis of the volume of newly formed bone and remaining implants in mouse calvarial defects with implantation of OCP, HA, and β-TCP at ten weeks post-implantation

|                          | the volume of newly formed bone (×10⁵ μm²±SD) | the volume of remaining implants (×10⁵ μm²±SD) |
|--------------------------|---------------------------------------------|---------------------------------------------|
| Control (untreated defect)| 0.3±0.3                                     | —                                           |
| OCP                      | 13.3±3.5                                    | 9.1±4.7                                     |
| HA                       | 5.6±4.0                                     | 13.2±4.6                                    |
| β-TCP                    | 8.7±4.3                                     | 7.2±1.5                                     |

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other hand, in the β-TCP (porosity 71–80%) and β-TCP (porosity 60%-implemented group, slight bone formation was observed at the surface of materials. The bone defects were not completely bridged by newly formed bone at eight weeks. Besides, material resorption advanced more slowly in β-TCP (porosity 71–80%) and β-TCP (porosity 60%) than in OCP/Gel.

Histomorphometric findings revealed that the percentage of newly formed bone in the OCP/Gel group was significantly higher than that in the β-TCP (71–80%) and β-TCP (60%-implemented group at eight weeks in the cortical area. The percentage of remaining materials showed that OCP/Gel was resorbed faster than β-TCP (71–80%) and β-TCP (60%). The entire OCP/Gel was almost resorbed at eight weeks. These results suggest that the OCP/Gel composite rapidly biodegraded and promoted the formation of new bone compared to commercially available β-TCP in rabbit tibia defects.

**CONCLUSION**

It has been challenging to understand the bioactivity of OCP, HA, and β-TCP materials due to the differences not only in their physicochemical properties but also their morphological characteristics. Further studies may elucidate the similarities and differences in features between these bone-substituting materials, which should lead to the development of next-generation highly functional Ca-P based-materials.

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**CONFLICTS OF INTEREST**

The authors have no conflicts of interest to declare.

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