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Comparative study on bone regeneration by synthetic octacalcium phosphate with various granule sizes

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ABSTRACT

The present study was designed to investigate whether the granule size of synthetic octacalcium phosphate (OCP) and the resultant intergranular spaces between the granules formed by the filling affect its osteoconductive and biodegradable characteristics in a mouse calvaria critical-sized defect up to 10 weeks after implantation. Mercury intrusion porosimetry showed that OCP granules having distinct diameter sizes ranging from 53 to 300 (S-OCP), 300 to 500 (I-OCP) and 500 to 1000 μm (L-OCP) produced distinct intergranular spaces between OCP granules ranging from 28.8 to 176.6 μm. The dissolution rate of OCP, estimated by the phosphate concentration in the culture medium, was the highest in S-OCP, followed by I-OCP and L-OCP, while the specific surface area of OCP decreased. Histological and histomorphometric analyses showed that bone formation around the implanted granules increased significantly with increasing granule size coupled with activating the appearance of TRAP- and cathepsin K-positive osteoclastic cells. The rate of new bone formation formed with L-OCP was two times higher than that formed with S-OCP at 10 weeks after implantation. The results indicated that the osteoconductive and biodegradable properties of OCP can be augmented by increasing the granule size, most probably by thus providing enough spaces between the granules, suggesting that the intergranular spaces formed by the granules may work similarly to pores, as reported in porous ceramic materials. It seems likely that the enhancement of bone formation by OCP is accompanied by simultaneous activation of osteoclastic resorption of OCP.

1. Introduction

Biodegradable calcium phosphate compounds, such as β-tricalcium phosphate (β-TCP) [1] and octacalcium phosphate (OCP) [2] biomaterials, have been extensively investigated as bone substitute materials in the repair of various bone defects. This is because these calcium phosphate materials are anticipated to be replaced with higher volume of new bone than non-biodegradable calcium phosphate, such as sintered hydroxyapatite (HA), which has a stoichiometric Ca/P molar ratio 1.67 if implanted in bone defects, even critical-sized ones [3]. OCP has been proposed as a precursor of biological apatite crystals in bone and tooth [4], although the chemical nature of the first mineral formed in vertebrate biomineralization remains controversial [5,6]. Apart from what kind of mineral is formed first at the onset in osteogenesis, recent intensive studies on the experimental application of synthetic OCP have shown that it has the potential to enhance new bone formation [7–12].

Previous studies reported that OCP is more capable of facilitating the differentiation of mouse bone marrow stromal ST-2 cells into osteoblastic cells than non-sintered Ca-deficient HA formed via OCP origin [13] or sintered HA ceramics [14] if incubated on calcium phosphate coatings in vitro even in the absence of osteogenic supplements, such as dexamethazone and β-glycerophosphate. The OCP crystal assembly also has the potential to support the differentiation of rat bone marrow stromal cells into osteoblasts [15,16]. Although the solution-mediated and osteoclastic cell-mediated biodegradable properties of OCP could be one of the factors leading to higher osteoconduction in vivo [7,17–21], it has been suggested that the transitory nature of OCP into HA in a physiological environment also plays a role in stimulating osteoblastic cells to differentiate both in vitro and in vivo during OCP–HA conversion [13,22–25]. The osteoconductive characteristics of OCP were first demonstrated in granular form in the subperiosteal region of mouse calvaria [22,23], showing the rapid appearance of new bone more clearly than HA or Ca-deficient HA [22]. Furthermore, it was confirmed that OCP was unique in that fine filaments and granular materials were formed around the OCP particles, the structure of which was very similar to that of the starting locus of intramembranous bone mineralization or so-called bone nodules

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[22]. Thus, intensive interest in this material has been given to pursue its possible clinical application and its efficacy for use in various forms, including coating on metal implants [7–10] and composites with polymers, such as collagen [11,26], gelatin [27] and alginate [12].

It is becoming clear that the microstructure of calcium phosphate biomaterials affects their osteoconductive characteristics [28–30]. Osteoblastic differentiation in vitro and bone regeneration in critical-sized defects are controlled by the crystal size and the surface area of OCP crystals [30]. Subtle changes in stoichiometry caused by the partial hydrolysis of OCP significantly affects the osteoconductive and biodegradable characteristics if the material is implanted in the rat tibia intramedullar canal [31]. Ca-deficient HA and β-TCP with rod-shaped crystals, prepared by hydrothermal treatment, enhanced bone formation and biodegraded more than those with a sphere-like morphology, prepared by conventional sintering processes, when implanted in the rabbit femur intramedullar canal [28,29]. Thus, the microstructure of the calcium phosphate crystals has been considered to have an effect on the biological response within bone tissue [28–30].

It is widely accepted that another critical factor in enhancing bone regeneration is the pore distribution within the materials [32,33]. Pore sizes in the range of 150–500 μm should provide a better condition for the growth of new bone within the sintered bulk materials [34]. On the other hand, it has been reported that the bone formation in rabbit femur intramedullar defects is regulated by the granule size of calcium phosphate ceramics [35]. It seems likely that the distinct granule size may provide distinct intergranular spaces that allow the involvement of mechanisms similar to the porous structure encouraging cell migration in a three-dimensional scaffold.

The present study was conducted to investigate the effect of OCP granule size ranging from about 50 to 1000 μm on osteoconductive characteristics in mouse calvaria critical-sized defects. It is still not known how the size of an OCP granule and the resultant intergranular spaces affect the osteoconductive and biodegradable properties of this material. We hypothesized that the distinct granule size of OCP may provide a certain condition for new bone formation and biodegradation of OCP by providing spatial and chemical stimulation/signals in activating osteoblasts, thereby augmenting its osteoconductive properties in mouse calvaria critical-sized bone defect.

2. Materials and methods

2.1. Synthesis and characterization of OCP and preparation of the granules

OCP was synthesized according to a method reported previously [22]. The granules, consisting of an OCP crystal aggregate, were prepared by lightly grinding the dried OCP cake using a pestle and mortar and then passing through a standard testing sieve. Granules with diameters ranging from 53 to 300, 300 to 500 and 500 to 1000 μm were used for in vivo implantation and in vitro dissolution experiments. The granules are referred to hereafter as small-OCP (S-OCP) for 53–300 μm, intermediate-OCP (I-OCP) for 300–500 μm and large-OCP (L-OCP) for 500–1000 μm. OCP granules were characterized by powder X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). XRD patterns were recorded by a step scanning at 0.05° intervals from 3.0 to 60.0°, with Cu K_α X-rays on a diffractometer (Mini Flex; Rigaku Electrical Co., Ltd., Tokyo, Japan) at 30 kV, 15 mA. The 2θ range measurement included the primary peak (1 0 0) of OCP at 4.9°. Joint Committee for Powder Diffraction Standard (JCPDS) numbers 26-1056A9 for OCP and 9-432 for HA were utilized to identify their crystalline phases. The FTIR spectrum of OCP was obtained by FTIR spectroscopy (FREEXACT-2; HORIBA, Kyoto, Japan), with the sample diluted with KBr over a range of 4000–400 cm^{-1} with 4 cm^{-1} resolution. The porosity, intergranule size and surface area of granules were determined by mercury intrusion porosimetry using a PoreMaster (Sysmex Co., Kobe, Japan). The morphology of the OCP crystals and the granules was examined using a JEOL analytical scanning electron microscope JSM-6390LA (Tokyo, Japan) operating at an accelerating voltage of 10 kV. Gold sputtering was performed using the powder samples before the observation. The sieved granules were sterilized by heating at 120 °C for 2 h in an oven before the implantation. Our previous study confirmed that heating does not affect the physical properties of OCP, such as the crystal structure determined by XRD or the specific surface area [23].

2.2. Solubility estimation of OCP granules immersed in a culture medium

The concentration of inorganic phosphate (Pi) ions in alpha minimal essential medium (αMEM) was determined quantitatively by the Phosphor C tests (Wako Pure Chemical Industries, Osaka, Japan). Five milligrams of OCP granules was immersed in 250 μl of αMEM for 3 days at 37 °C under a 5% carbon dioxide environment. The supernatants after the immersion were collected for Pi quantitative analyses.

2.3. Experimental animal model

All animal handling and surgical procedures were approved by the Animal Research Committee of Tohoku University. Granules (4 mg) were implanted into critical-sized calvaria defects (4.2 mm in diameter) [30] in 9- to 10-week-old ICR mice weighing 35–39 g. A control experiment without implantation was also conducted. Five mice were used for histological observation in each group. The OCP-implanted animals for histological observations were dissected at 2, 6 and 10 weeks, and fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer. The samples were decalcified, dehydrated and embedded in paraffin. Thin sections (5 μm in thickness) were prepared and stained with hematoxylin–eosin (H-E) and tartrate-resistant acid phosphatase (TRAP). Cathepsin K was immunohistochemically stained with mouse anti-human cathepsin K monoclonal antibody (F-92; Fuji, Takaoka, Japan) then chromogen diaminobenzidine, followed by counterstaining with hematoxylin to confirm osteoclast-like cells [36]. Before immunostaining, the specimens were pretreated with a 3% solution of hydrogen peroxide in absolute methanol to remove the endogenous peroxidase. The immunoreaction was visualized with a histofine mouse stain kit (Nichirei Biosciences, Inc., Tokyo, Japan) according to the manufacturer's instructions.

2.4. Observation of filling of OCP granules using paraffin-embedded sections

To evaluate the packing condition of OCP granules, 4 mg of OCP granules was mixed with the melt paraffin and filled into a disk mold with a 4 mm diameter. After the paraffin was hardened, the molded disks were sectioned at 10 μm and observed through a light microscope.

2.5. Quantitative micrograph analysis

The procedure used in the histomorphometrical analysis has been described previously [30]. Briefly, light micrographs of sections stained with H-E (at least 15 sections) were used, and photographs that showed the overall defect were taken and scanned using Leica DMI 4000B (Leica Microsystems CMS GmbH, Wetzlar,