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Effect of nanostructure on osteoinduction of porous biphasic calcium phosphate ceramics

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ABSTRACT

In order to evaluate the effect of the nanostructure of calcium phosphate ceramics on osteoinductive potential, porous biphasic calcium phosphate (BCP) ceramics with a nano- or submicron structure were prepared via microwave sintering and compared to conventional BCP ceramics. The selective protein adsorption of bovine serum albumin and lysozyme (LSZ) and the osteogenic differentiation of human mesenchymal stem cells in vitro was investigated. Porous BCP nanoceramics showed higher ability to adsorb proteins, especially low molecular weight protein of LSZ, than conventional BCP ceramics, and the BCP nanoceramics promoted bone sialoprotein expression more than conventional BCP did. Further in vivo study to investigate ectopic bone formation and bone repair efficiency proved the highly osteoinductive potential of nanostructured BCP ceramics. The results suggest that nanostructured BCP ceramics have the potential to become a new generation of bioceramics for bone tissue engineering grafts.

1. Introduction

Due to their good biocompatibility and bioactivity, calcium phosphate (CaP) ceramics have long been widely used clinically as a hard tissue repair or substitute [1–3]. Although the bone implants are osteoconductive, they often lack the osteogenicity needed to support bone healing in large defects and are slowly degraded in vivo [4,5]. Furthermore, it has been recognized since 1991 that CaP ceramics with specific composition and structure can induce ectopic bone formation in the body [6,7]. Owing to having higher osteoinductive potential than pure hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP, $Ca_3(PO_4)_2$), biphasic calcium phosphate (BCP) ceramics containing HA and TCP phases have recently attracted much attention as an ideal bone graft substitute [8,9]. BCP ceramics can be obtained by controlling the Ca/P ratio in the course of CaP synthesis. However, the phenomenon of bone induction by CaP ceramics in ectopic sites is so weak (taking months for a small amount of bone) that many approaches have been explored such as the addition of osteogenic cells [10,11] or bone morphogenetic proteins (BMPs) to enhance early bone formation [12,13].

With the development of nanoscience and nanotechnology, bone apatite has been proved to consist of nanosized carbonated CaP crystals [14,15]. Most in vitro studies have showed that bone-forming cells tend to interact with the nanoscale surface of biomaterials, and the nanoscale feature of implanted materials is critical to preventing the body from rejecting artificial parts [16,17]. It has been widely reported that a nanoscale surface can promote the adhesion, proliferation and other functions of bone-related cells [18,19]. In addition, the inorganic component of human bone is in fact similar in composition to carbonate-substituted CaP ceramics, and the structure of bone is an interconnective porous compact [20,21]. Therefore, simulating the chemistry, microstructure and porous structure of mineralized tissue in bioceramic materials may be a means of improving the level of stimulation of CaP ceramics in osseous tissue [22,23]. We hypothesized that porous carbonated biphasic CaP nanoceramics would not only aid in osteogenesis but may facilitate and accelerate bone healing in skeletal defects by enhanced osteoinduction.

Previous work has shown that the nanocharacteristics of implant surfaces have a direct influence on tissue response by affecting protein adsorption and by modulating the expression level of cellular genes, leading not only to enhanced osteoblast differentiation and local factor production in vitro but also to increased bone–implant contact in vivo [24,25]; thus, improved clinical rates of wound healing can be achieved easily [26,27]. In previous work, we reported the preparation of porous carbonated BCP ceramics with a nanostructure by microwave sintering with activated

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carbon as embedding material, and its cellular response suggests that these nanoceramics promote osteoblast adhesion, proliferation and differentiation [28]. The present objective was to determine the selective protein adsorption of bovine serum albumin/lysozyme (BSA/LSZ) and the osteogenic differentiation of human mesenchymal stem cells (hMSCs) in vitro, and the tissue response in vivo of porous ceramics with nano, submicron or micron structure. To the knowledge of the authors, this is the first experiment to evaluate in vivo osteoinduction of porous nanoceramics and its relationship to protein adsorption and gene expression in vitro.

2. Materials and methods

2.1. Materials preparation

Porous ceramics implants were produced by a slurry foaming method as described previously [28]. In short, Ca-deficient HA was synthesized as the precursor by the wet precipitation method. Analytically pure $(NH_4)_2HPO_4$ solution were dropped into $Ca(NO_3)_2{\cdot}4H_2O$ solution. The white precipitate was centrifuged and washed, followed by adding methylcellulose (MC) and polyethylene glycol as dispersant and viscous agent. With analytically pure H_2O_2 as a vesicant, the slurry was heated in a domestic microwave oven (Galanz, PR China) for 30–60 s and the foaming slurry was poured immediately into the molds with good permeability. After drying and removing the organic addition, the as-prepared porous green bodies were transferred into a microwave furnace for sintering. The microwave sintering furnace was replaced in a domestic microwave oven (2.45 GHz/750 W, Sanyo, Japan). Samples were embedded by activated carbon filled in a hollow porous mullite cube. The temperature of specimens in the microwave furnace was measured by an optical fiber pyrometer. The BCP green bodies were sintered by microwave at 950 and 1050 °C for 5 min to obtain nanosized BCP ceramics (nBCP) and submicron-sized BCP ceramics (mBCP), respectively. By comparison, conventional sintering with the same green bodies was conducted at 1100 °C with a heating rate of 5 °C min^{-1} and a holding time of 2 h to obtain micron-sized BCP ceramics (cBCP).

Three groups of materials were produced for the subsequent in vitro and in vivo evaluation. First, to decrease the effect of different porous structures, nBCP and cBCP granules were prepared for protein adsorption after ceramics blocks were crushed and sieved. Second, nBCP and cBCP disk-like ceramics ($\phi 14 \times 1$ mm) were obtained after cylinder-like ceramics had been cut and carefully ground for study of osteogenic gene expression. Third, the three kinds of cylinder-like samples (ϕ × 12 mm) were fabricated for implantation. After porous ceramics blocks were cut and carefully ground. Subsequently, the disk-like and cylinder-like samples were thoroughly washed with pure water several times. Finally, the samples were dried and autoclaved prior to use. The flowchart of sample preparation and subsequent in vitro and in vivo evaluation is shown in Fig. 1.

2.2. Materials characterization

The phase composition of all BCP samples was analyzed using X-ray diffraction (XRD; X'pert Pro MPD, Philips) and Fourier transform infrared spectroscopy (FTIR; FTIR1750, PerkinElmer, USA). The porosity and the microstructure of the sintered ceramics were examined by the Archimedes method and by scanning electron microscopy (SEM; JSM-5900 LV, JEOL, Japan), respectively. Specific surface area was determined by the Brunauer–Emmett–Teller method (BET; ASAP-2010, USA). Zeta potential was determined using a Nano ZS90 Zetasizer (Malvern, UK). The compressive

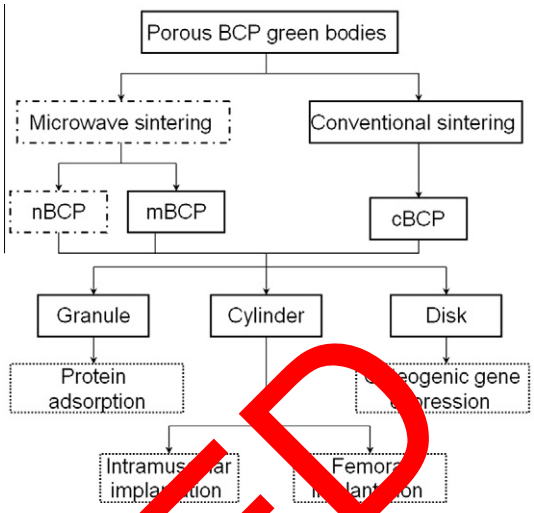

Fig. 1. A flowchart of ceramics sample preparation.

strengths were measured with a WDW-5 Biomechanical Testing Machine (Changchun Instrument, Changchun, PR China).

2.3. Protein adsorption

The protein adsorption experiments were conducted following Zhu et al.'s method [29]. Briefly, mixtures of nBCP or cBCP granules (0.1 g) and a binary BSA/LSZ system (1 ml) were incubated at room temperature for 1 h with continuous agitation of the suspended particles. After centrifuging for 5 min at 10,000 rpm, the granules were washed three times with 1 ml of deionized water. After the washing operation, 200 ml of 2% (w/v) sodium dodecyl sulfate (SDS) solution was added to the tubes with rapid stirring for 1 min. Then the suspensions were centrifuged for 7 min and the supernatants containing the desorbed proteins were collected. The samples with SDS-desorbed proteins were separated by polyacrylamide gel electrophoresis (PAGE), which was performed on a Mini-PROTEAN 3 system (Bio-Rad) according to the method of Laemmli [30]. The gels were stained with Coomassie Brilliant Blue R-250 and scanned with a Chemi-Doc™ XRS system (Bio-Rad). The protein bands present in the gels were analyzed by Quantity One® 1-D Analysis Software, and the amounts of adsorbed BSA and LSZ were calculated according to a standard curve, which was obtained from a series of optical densities of BSA and LSZ bands with known concentrations.

2.4. Osteogenic gene expression

hMSCs were seeded on nBCP and cBCP ceramics in 24-well plates at a concentration of 2×10^4 cells ml^{-1}. As a control, we seeded 5000 cells cm^{-2} on tissue culture flasks in osteogenic medium [31]. At day 7 after MSC differentiation, osteocalcin (OCN), runt-related transcription factor 2 (Runx2) and bone sialoprotein (BSP) gene expression were examined by quantitative polymerase chain reaction (qPCR) analysis.

Briefly, total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. The primers used in this study are listed in Table 1. The RNA extracts were analyzed by conventional reverse transcription (RT)-PCR (FTC2000, Canada). Cycle conditions were set to 2 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 1 min at 60 °C, and ending in a default dissociation step. Primers for the experiment were designed using Primer Express 3.0 (Applied

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