



Osteoinduction of hydroxyapatite/ β -tricalcium phosphate bioceramics in mice with a fractured fibula

Lijia Cheng^a, Feng Ye^a, Ruina Yang^a, Xiaofeng Lu^a, Yujun Shi^a, Li Li^a, Hongsong Fan^b, Hong Bu^{a,c,*}

^a Key Laboratory of Transplant Engineering and Immunology, Ministry of Health, West China Hospital, Sichuan University, Chengdu, People's Republic of China

^b National Engineering Research Center for Biomaterials, Sichuan, People's Republic of China

^c Department of Pathology, West China Hospital, Sichuan University, Chengdu, People's Republic of China

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ABSTRACT

Many studies have shown that calcium phosphate ceramics can induce bone formation in non-osseous sites without the application of any osteoinductive biomolecules, but the mechanisms of this phenomenon (intrinsic osteoinduction of bioceramics) remain unclear. In this study, we compared the intrinsic osteoinduction of porous hydroxyapatite/ β -tricalcium phosphate (HA/ β -TCP) implanted in mice at different sites. In 30 mice the left fibula was fractured and the right fibula was kept intact. A porous HA/ β -TCP cylinder was implanted into both the left (group 1) and right (group 2) leg muscles of each animal. In addition, two HA/ β -TCP cylinders were bilaterally implanted into leg subcutaneous pockets (group 3) in each of the remaining 15 mice. New bone formation was studied in the three groups by histology, histomorphometry and immunostaining. In group 1 new bone was observed at week 6 and bone marrow appeared at week 12. In group 2 new bone was observed at week 8 and bone marrow appeared at week 12. The new bone area percentage in group 1 was significantly higher than in group 2 at both weeks 8 and 12. In contrast, group 3 did not show any new bone within the period studied. These differences were explained based on the location of the implants and thus their proximity to the osteogenic environment of fracture healing. The results support the hypothesis that intrinsic osteoinduction by calcium phosphate ceramics is the result of adsorption of osteoinductive substances on the surface.

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1. Introduction

Bone grafts are in great demand. Although autografts remain the gold standard in clinical applications, they are of limited availability and are associated with donor site morbidity as well as pain, infection, loss of sensibility and hematoma [1]. Therefore, synthetic materials have been extensively studied as substitutes for autografts [2,3].

Calcium phosphate ceramics are promising synthetic bone replacement materials. Because they share chemical similarities with human bone mineral, they show excellent biocompatibility with bone [4]. In addition, calcium phosphate ceramics have been observed to induce bone formation at non-osseous sites in animals without the addition of any osteogenic biomolecules [5–8]. This phenomenon reflects the intrinsic osteoinductivity of calcium phosphate bioceramics [9–19]. Osteoinductivity is considered to be a clinical advantage for biomaterials used in the repair and engineer-

ing of bone [20–22], but the mechanisms of this intrinsic osteoinduction remain unclear. A proposed mechanism suggests that osteoinductive biomolecules such as bone morphogenetic protein 2 (BMP-2) are adsorbed onto the surface of calcium phosphate bioceramics after their implantation and these adsorbed osteoinductive biomolecules then initiate bone formation, which appears as osteoinduction. Some findings support this mechanism. For example, porous calcium phosphate implants prepared by sintering at a lower temperature showed a higher osteoinductivity than those sintered at a higher temperature [15]. It was suggested that the lower temperature sintering produced a higher specific surface area and thus allowed adsorption of more bone morphogenetic proteins. However, sintering temperature may also have affected additional material properties, such as crystallinity, dissolution rate and phase structure (i.e. by thermal decomposition). These additional properties may be confounding factors in any analysis of the mechanism of osteoinduction [15]. In addition, osteoinduction by calcium phosphates occurred often in larger animals (e.g. goats and pigs) [11,14,15,18,19], but infrequently in smaller animals (e.g. mice and rats) [17]. This species-dependent difference was suggested to be due to the different levels of bone morphogenetic proteins in body fluid among species, but quantitative data on these levels are unavailable [17].

* Corresponding author. Address: Key Laboratory of Transplant Engineering and Immunology, Ministry of Health, West China Hospital, Sichuan University, Chengdu, People's Republic of China. Tel.: +86 028 85164030.

E-mail address: hongbu@scu.edu.cn (H. Bu).

The objective of this study was to investigate the mechanisms of intrinsic osteoinduction of calcium phosphate ceramics. In this study, we compared osteoinduction by the same calcium phosphate material in the same species, but implanted at different sites. We will show that this difference resulted in significant differences in osteoinduction and will relate it to the mechanism of intrinsic osteoinduction by calcium phosphate bioceramics.

2. Materials and methods

2.1. Calcium phosphate samples

Cylindrical hydroxyapatite/ β -tricalcium phosphate (HA/ β -TCP) samples (3×5 mm) (Fig. 1A) were provided by the National Engineering Research Center for Biomaterials (Chengdu, Sichuan, China). The samples contained 60 wt.% HA and 40 wt.% β -TCP and were prepared by sintering at 1100 °C for 2 h. The volumetric porosity was approximately 50% and the pore size ranged from 300 to 500 μ m.

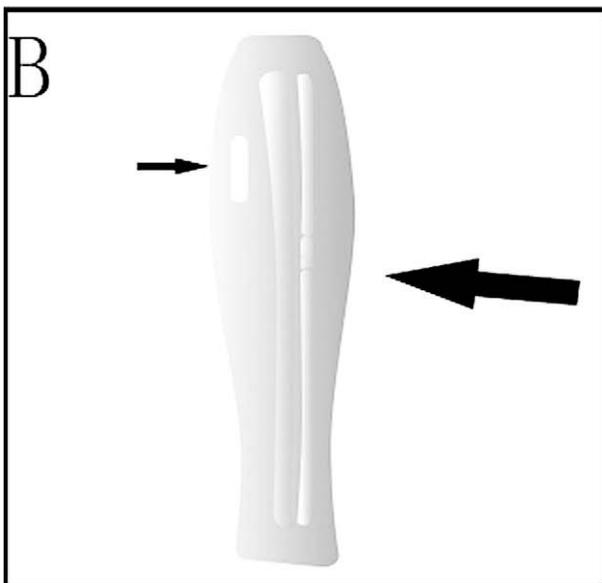
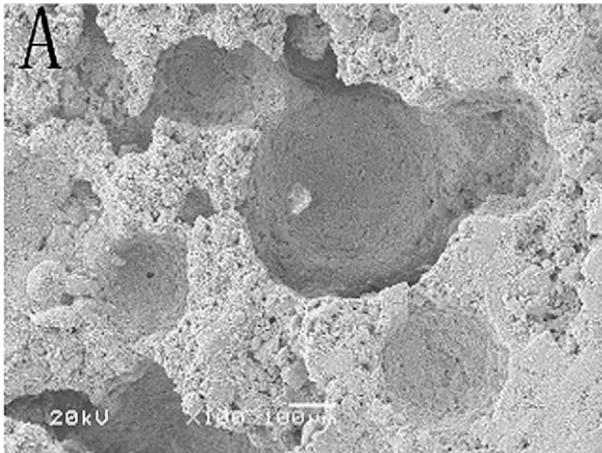


Fig. 1. (A) An SEM micrograph showing the microstructure of porous HA/ β -TCP implants. (B) A schematic showing the fibular fracture and the location of implantation in the left leg muscle. Small arrow, the implanted HA/ β -TCP; large arrow, fibular fractures.

2.2. Implantation

Forty-five 8-week-old male C57BL/6 mice were obtained from the Laboratory Animal Center of Sichuan University (Chengdu, Sichuan, China) and were maintained in a temperature and light controlled environment ventilated with filtered air. They were intraperitoneally anesthetized and their legs were disinfected. In 30 mice the left fibula was fractured at two locations (approximately 1 mm apart) with ophthalmic forceps (Fig. 1B). An HA/ β -TCP sample was implanted into both the left (group 1) and right (group 2) leg muscles of each animal. In the other 15 mice bilateral subcutaneous pockets were created in each mouse and an HA/ β -TCP sample was implanted into each pocket (group 3). All procedures were performed under general anesthesia. The wounds were closed by single interrupted suturing.

The study was approved by the Animal Care and Use Committee of Sichuan University. The operative procedures and animal care were performed in compliance with NIH guidelines on the care and use of laboratory animals, under the supervision of a licensed veterinarian.

2.3. Histology

The samples were harvested at post-operative weeks 2, 4, 6, 8 and 12. They were immediately fixed in 10% neutral formalin buffer solution for approximately 24 h at room temperature, decalcified in 10% ethylenediaminetetraacetic acid (EDTA), pH 7.0, for about 20 days at room temperature, washed with diethyl pyrocarbonate (DEPC), dehydrated and embedded in paraffin (melting point 56–58 °C). The embedded samples were cut into 5 μ m thick histological sections and transferred onto 3-aminopropyltriethoxy silane-coated glass slides. The sections were stained with hematoxylin and eosin (HE) and the 12 week sections were stained with Masson-trichrome.

2.4. Immunostaining

We analyzed the expression of BMP-2 in all three groups at week 12 by immunostaining. BMP-2 is a member of the transforming growth factor β superfamily and is involved in the induction of cartilage and bone. The 12 week slides were deparaffinized, rehydrated and rinsed with double-distilled water. They were treated with 3% H_2O_2 for 15 min in the dark to block endogenous peroxidase, rinsed three times with double-distilled water, immersed in a Tris-EDTA buffer (pH 9.0) and kept in a 95 °C water bath for 45 min. After cooling to room temperature they were rinsed three times (5 min each) with phosphate-buffered saline (PBS) and incubated in mouse monoclonal antibody against BMP-2 (1:1000) (Abcam) at 4 °C overnight. Then the slides were rinsed three times with PBS and incubated in a horse-radish peroxidase (HRP)-labeled secondary antibody at room temperature for 30 min. Finally, they were developed with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin.

2.5. Bone histomorphometry

New bone tissue was studied by optical microscopy and measured with Image-Pro Plus (Media Cybernetics, Bethesda, MD). Five image fields (100 \times) were captured on each slide; the areas of new bone growth and total tissues were measured and the area percentage of new bone was calculated as the ratio between the new bone area and the total tissue area.

2.6. Statistical analyses

Data are expressed as means \pm standard deviation and were analyzed by paired ANOVA (SPSS 10.0, SPSS, Chicago, IL). A $P < 0.05$ was considered statistically significant.

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