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Ectopic bone formation cannot occur by hydroxyapatite/β-tricalcium phosphate bioceramics in green fluorescent protein chimeric mice

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ABSTRACT

Many studies have shown that calcium phosphate ceramics (CP) have osteoconductive and osteoinductive properties; however, the exact mechanism of bone induction has not yet been reported. This study was performed to investigate if destroying immunological function will influence osteogenesis, to explain the mechanism which is unclear. In this study, twenty C57BL/6 mice were divided into two groups ($n = 10$), in group 1, a hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) ceramic was implanted into both the left and right leg muscles of each mouse; in group 2, ten mice experienced lethal irradiation, then were injected bone marrow (BM) cells from green fluorescent protein (GFP) transgenic mice by tail veil, after bone marrow transplantation (BMT), heart, liver, spleen, lung, kidney, and muscle were harvested for biological analysis, after the GFP chimera model was established successfully, the same HA/β-TCP ceramic was implanted into both leg muscles of each mouse immediately after irradiation. 45 and 90 days after implantation, the ceramics of the two groups were harvested to perform with hematoxylin and eosin (HE) and immunohistochemistry (IHC) staining; the results showed that there was no bone formation in group 2, while new bone tissues were detected in group 1. Our findings suggest that the BM cell from GFP transgenic mice is a good biomarker and it could set a good platform for chimera model; it also shows that BM cell is one of cell resources of bone induction, and destruction of immune function will impede osteoinduction by CP. Overall, our results may shed light on clear mechanism study of bone induction in the future.

1. Introduction

Bone autograft is the gold standard in some clinical applications [1]. However, it is associated with some limitations: pain, infection, loss of sensibility and hematoma in donor site as well as limited availability [2]. Synthetic materials have been extensively studied as substitutes for bone autografts [3]. Calcium phosphate ceramics (CP) have been widely used in bone reconstructive surgery due to their osteoconductive and biocompatibility properties [4,5]. In addition, these biomaterials have been demonstrated to be osteoinductive in muscular sites without the addition of seed cells or osteogenic growth factors after 6–12 weeks [6–13]. This phenomenon reflects the intrinsic osteoinductivity of CP. Osteoinductivity is considered to be a clinical advantage for biomaterials used in the bone repair and bone tissue engineering. Although osteoinduction by biomaterials has been widely observed, its mechanism is still largely unknown. A proposed mechanism suggests that osteoinductive biomolecules, such as bone morphogenetic protein 2 (BMP-2), are adsorbed on the surface of CP after implantation, and then these adsorbed biomolecules involve in bone formation, which appears as osteoinduction [10]. Green fluorescent protein (GFP) is a 27-kD protein, originally discovered in jellyfish *Aequorea Victoria*. Because the fluorescence activity of GFP requires no substrates or cofactors, cells expressing the intracellular GFP marker can be observed directly by fluorescent microscopy without the requirement for antibody staining. Therefore, bone marrow cell of GFP transgenic mice would be helpful and used as a biomarker in bone marrow transplantation.

The objective of this study was to investigate the mechanisms of intrinsic osteoinduction of CP. In this study, we compared osteoinduction by the same calcium phosphate material in the same genotype mice, but implanted in two different models. The results show that the difference models resulted in significant differences in osteoinduction, and the mechanism of intrinsic osteoinduction by CP will be analyzed and discussed.

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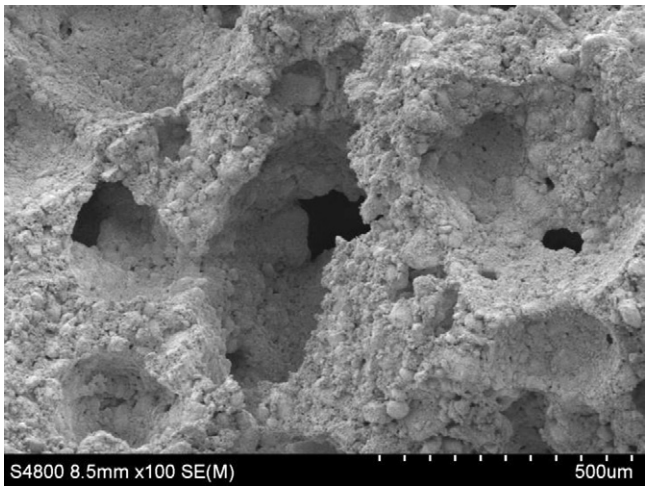

Fig. 1. A SEM micrograph showing the microstructure of porous HA/β-TCP implants.

2. Materials and methods

2.1. Calcium phosphate samples

Cylindrical hydroxyapatite/β -tricalcium phosphate (HA/β-TCP) samples (Fig. 1) were provided by the National Engineering Research Center for Biomaterials (Chengdu, Sichuan, China). Briefly, the sintering was conducted at 1100 °C with a heating rate of 5 °C min^{-1} and a holding time of 2 h to obtain ceramics. The samples were cut into cylinder with a diameter of 3 mm and a height of 5 mm. The samples contained 60 wt.% HA and 40 wt.% β-TCP, and the volumetric porosity was approximately 50% and the pore size ranged from 300 to 500 μm. Subsequently, samples were thoroughly washed with ultrapure water several times. Finally, samples were dried and autoclaved prior to use.

2.2. Chimera

The outline of the whole protocol is presented in Fig. 2. 8-week-male C57BL/6 mice from genetic engineering mice center of Sichuan University (Chengdu, China) were used as recipients. GFP transgenic mice (GFP mice) of C57BL/6 background were kindly provided by Dr. Mo (Stem Cell Laboratory of National Key Laboratory of Sichuan University, Chengdu, Sichuan) and maintained in our animal facility. Male GFP mice aged 6–8 weeks were used as bone marrow (BM) donors. 10 recipient C57BL/6 mice were irradiated at dose of 13 Gy. BM cells of GFP mice were collected by flushing the bone shafts of the femora and tibia with DMEM medium. The cells were then washed once in this medium and suspended in phosphate-buffered saline (PBS) and 1×10^7 cells were injected via the tail vein.

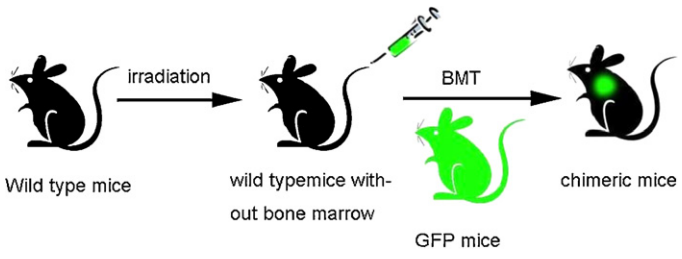

Fig. 2. Protocol of chimeric mice production.

2.3. Western blotting

One month after bone marrow transplantation (BMT), proteins were collected from fresh skeletal muscle, heart, liver, spleen, lung, kidney of wild type mice, GFP mice and chimeric mice, and separated using a 10% polyacrylamide gel. After transferring the proteins on a nitrocellulose membrane, the proteins were blocked with a 5% defatted milk solution. The membrane was then probed with mouse monoclonal antibody against GFP (1:2500, Chemicon) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, 1:5000, Chemicon), and then probed with a secondary antibody using a 1 in 5000 dilution of alkaline phosphatase conjugated anti-mouse IgG.

2.4. Fluorescence observation

Frozen sections of various tissues were cut at 6-mm thickness, and the nucleus were stained with 4′,6-diamidino-2-phenylindole (DAPI). Frozen sections were examined under a fluorescence microscope for detection of GFP-positive cells.

2.5. Implants

20 wild type mice (group 1, $n = 10$) and chimeric mice (group 2, $n = 10$) were both intraperitoneally anesthetized and their legs were disinfected. The operations were done immediately after BMT. One HA/β-TCP sample was implanted into both the left and right leg muscles of each animal. 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.

2.6. Histology

The HA/β-TCP samples were harvested at 45 and 90 post-operative days. 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 μm thick histological sections and transferred onto 3-aminopropyrytrietoxy silane-coated glass slides. The ceramic sections were stained with hematoxylin and eosin (HE).

2.7. Immunohistochemistry

The 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 PBS and incubated in mouse monoclonal antibody against GFP (1:1000, Chemicon) at 4 °C overnight. Then the slides were rinsed three times with PBS and incubated in a horseradish peroxidase (HRP)-labeled secondary antibody at room temperature for 30 min. Finally, they were developed with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin.