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Magnesium modification up-regulates the bioactivity of bone morphogenetic protein-2 upon calcium phosphate cement via enhanced BMP receptor recognition and Smad signaling pathway

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ABSTRACT

Efficient presentation of growth factors is one of the great challenges in tissue engineering. In living systems, bioactive factors exist in soluble as well as in matrix-bound forms, both of which play an integral role in regulating cell behaviors. Herein, effect of magnesium on osteogenic bioactivity of recombinant human bone morphogenetic protein-2 (rhBMP-2) was investigated systematically with a series of Mg modified calcium phosphate cements (xMCPCs, x means the content of magnesium phosphate cement wt%) as matrix model. The results indicated that the MCPC, especially 5MCPC, could promote the rhBMP-2-induced *in vitro* osteogenic differentiation via Smad signaling of C2C12 cells. Further studies demonstrated that all MCPC substrates exhibited similar rhBMP-2 release rate and preserved comparable conformation and biological activity of the released rhBMP-2. Also, the ionic extracts of MCPC made little difference to the bioactivity of rhBMP-2, either in soluble or in matrix-bound forms. However, with the quartz crystal microbalance (QCM), we observed a noticeable enhancement of rhBMP-2 mass-uptake on 5MCPC as well as a better recognition of the bound rhBMP-2 to BMPR IA and BMPR II. *In vivo* results demonstrated a better bone regeneration capacity of 5MCPC/rhBMP-2. From the above, our results demonstrated that it was the Mg anchored on the underlying substrates that tailored the way of rhBMP-2 bound on MCPC, and thus facilitated the recognition of BMPRs to stimulate osteogenic differentiation. The study will guide the development of Mg-doped bioactive bone implants for tissue regeneration.

1. Introduction

Bone morphogenetic proteins (BMPs) are multifunctional regulators in a multitude of cellular processes and elicit a crucial role in tissue engineering applications [1,2]. In particular, recombinant human bone morphogenetic protein-2 (rhBMP-2) has been cleared by the FDA for the repair of bone defects [3,4]. Unfortunately, the clinical use of rhBMP-2 is greatly limited by a short biological half-life, rapid local clearance and propensity for side effects like ectopic ossification as well as high costs of treatment. To address these problems, various delivery matrices, including cross-linked heparin [5], graphene oxide [6], biomimetic hydrogel [7], and apatite [8] have been adopted for rhBMP-2. These delivery systems can enhance the efficiency of bone regeneration to some extent by increasing local concentration and lifetime of bioactive rhBMP-2. However, the environmental factors [9] like pH, ionic strength, electrolytes, *etc.* often lead to the changes in secondary/tertiary structure and thereby the osteoactivity of rhBMP-2, which directly undermined its therapeutic efficacy. Therefore, a better understanding and control of rhBMP-2's adsorption and the osteogenic bioactivity during its application *in vitro* and *in vivo* would be of great significance to development of the rhBMP-2-based osteoinductive scaffolds for bone tissue regeneration.

Incorporation of bioactive ions like Si, Mg, Ca, Zn, and Sr has been considered as an effective strategy to improve the bioactivity of

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biomaterials [10,11]. Among them, magnesium ion (Mg^{2+}), the second most abundant intracellular divalent cation, has been known to be involved in diverse cellular functions and plays an essential positive role in bone biomineralization [12,13]. The increasing of soluble Mg^{2+} could up-regulate the gene expression of COL10A1 and VEGF in human bone marrow stromal cells (hBMSCs), consequently leading to the enhanced ECM mineralization [14]. Our previous study also demonstrated that appropriate magnesium enhanced cell adhesion and osteogenic differentiation of hBMSCs by upregulation of integrin α5β1 expression [15]. However, till now, the impact of Mg modification on the conformation and osteogenic bioactivity of rhBMP-2 loaded on the substrates has been paid little attention.

With this respect, we endeavored to study the Mg modification-induced changes of conformation and osteogenic capacity of rhBMP-2 on matrix *in vitro* and *in vivo*. Calcium phosphate cement (CPC), which has been widely applied for bone substitution [16] and drug delivery [17] in clinic, was chosen as a substrate model. In order to achieve the advantages of rapid setting, better mechanical properties, as well as significantly improved biodegradability [18,19], magnesium phosphate cement (MPC) was introduced to CPC precursor to obtain Mg-doped CPC (MCPC) with various Mg content. RhBMP-2 was physisorbed by freeze-drying process and its conformational changes were analyzed using far-UV circular dichroism (CD) spectroscopy. Pluripotent skeletal muscle myogenic progenitor C2C12 cell line, a widely used cell-model for evaluation of the osteoblast differentiation, was chosen to determine the *in vitro* bioactivity of rhBMP-2. The effects of Mg amount in CPC and different existence forms on cell adhesion, proliferation, rhBMP-2-induced osteodifferentiation activity and related Smad signaling pathway were investigated in detail. The adsorption dynamics of rhBMP-2 and BMP receptors on MCPC sensors was in real time monitored by QCM technique. A rat critical-sized calvarial defect model was used to evaluate the osteoinductive properties and bone regeneration efficacy of rhBMP-2 loaded MCPC *in vivo*.

2. Materials and methods

2.1. Fabrication and characterization of MCPC samples

The CPC powders were made up of equimolar tetracalcium phosphate (TECP, $Ca_4(PO_4)_2O$) and dicalcium phosphate anhydrous (DCPA, $CaHPO_4$). Briefly, TECP was developed by a solid-to-solid reaction between calcium carbonate and calcium phosphate at 1500 °C for 8 h. Dicalcium phosphate dehydrate (DCPD, $CaHPO_4{\cdot}2H_2O$) was prepared from ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) and calcium nitrate ($Ca(NO_3)_2$) in the acidic environment. DCPA was obtained by removing the crystallization water in DCPD at 120 °C for 5 h.

The MPC powders were formed by mixing magnesia (MgO) with calcium dihydrogen phosphate ($Ca(H_2PO_4)_2$) in a molar ratio of 2:1. MgO was prepared by heating basic magnesium carbonate pentahydrate ($(MgCO_3)_4{\cdot}Mg(OH)_2{\cdot}5H_2O$) in a furnace at 1500 °C for 6 h. The resultant powder was initially cooled to room temperature (RT), and then ground in a planetary mill for 5 min, followed by sieving through 200 meshes. The obtained MPC powders were kept in a desiccator for further experiment.

The MCPC powders consisted of CPC and MPC at a specific ratio (Table S1). The mixture was stirred to form homogeneous pastes with the addition of pure water. After that, the resulting pastes were uniaxially pressed at 2 MPa for 1 min in a stainless steel mold with a diameter of 10 mm. Finally, MCPC disc-shaped pellets (Φ10 × 2 mm) were put into a constant temperature oven at 37 °C and 100% relative humidity for 3 days until the hardened MCPC samples with different MPC content were obtained. For *in vivo* experiment the porous MCPC scaffolds (Φ5 × 2 mm) were prepared using leaching method with sodium chloride (NaCl) particles as porogens.

The phase compositions of the hardened MCPCs before and after rhBMP-2 adsorption were characterized by X-ray diffraction (XRD, Rigaku D/Max 2550, Japan). The surface morphology of cement samples were observed by scanning electron microscope (SEM, S-4800, Hitachi, Tokyo, Japan), as well as the energy dispersive spectrometer (EDS) element analysis and mapping on MCPC surfaces.

For the release of Mg^{2+}, MCPC samples were submerged in cell culture medium (DMEM supplemented with 10% fetal bovine serum) at 37 °C with 5% CO_2. After 1, 3 and 7 days, the supernatant was collected and filtered to remove released particles for the follow-up test. The concentrations of released Mg^{2+} were examined by inductively coupled plasma optical emission spectroscopy (ICP-AES).

2.2. In vitro *release kinetics of rhBMP-2*

RhBMP-2 was physisorbed onto MCPC surface and dried overnight under vacuum. To investigate the release profiles of rhBMP-2, MCPCs were incubated in media containing 1 mL PBS. Half of supernatant (500 μL) was collected and replaced with fresh media to maintain constant volume after 1, 3, 7, 12, 24, 72 and 168 h. Concentrations of rhBMP-2 in the collected media samples were determined by ELISA followed the manufacturer's instructions (PeproTech, Rocky Hill, NJ). The cumulative release of rhBMP-2 was then expressed as a percentage of the initial loading amount (2 μg).

2.3. Circular dichroism spectroscopy

The structure of rhBMP-2 protein released from MCPC was examined using a spectropolarimeter (Model J-715, Tokyo, Japan) to measure the Far-UV CD spectra (190–260 nm) at room temperature, compared to that of native rhBMP-2. The rhBMP-2 released from MCPC was purified by centrifugation (8000 rpm, 5 min) and removal of the precipitation. Then the supernatant was analyzed in a cylindrical cuvette (0.1 cm pathlength) with a time constant of 0.5 s and a scan rate of 100 nm/min. Data were collected every 0.5 nm with a bandwidth of 1 nm, and 5 scans were averaged in order to increase the signal-to-noise ratio. The final spectrum was baseline-corrected and the data presented as mean residue ellipticity (θ).

2.4. Quartz crystal microbalance (QCM) measurements

A gold-coated quartz crystal with 5 MHz fundamental resonance frequence was used in this study. All QCM chips were cleaned by immersion in hot piranha solution (3:1 concentrated H_2SO_4/30%H_2O_2) for 30 min and then rinsed in ultrapure water followed by ethanol. The MCPC substrates were developed by the electrophoretic deposition (EPD) of MCPC solution onto the gold-plated sensors.

The adsorption behavior of rhBMP-2 and BMPRs onto the MCPC sensors was monitored in real-time at 25 ± 0.05 °C using a QCM technique (Q-Sense AB, Biolin Scientific, Sweden). All QCM experiments were conducted in flow-through mode using a peristaltic pump at a rate of 20 μL/min. For the test, a flat baseline was quickly established by passing PBS for 10 min. And then rhBMP-2 solution (50 μg/mL) was pumped into the system to allow the attainment of the adsorption plateau for 60 min approximately. After adsorption was complete, the freshly prepared BMPR IA, BMPR IB, and BMPR II were introduced respectively to bind rhBMP-2 until the adsorption equilibrium was reached again. In the case of a thin non-dissipative