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Magnesium modification of a calcium phosphate cement alters bone marrow stromal cell behavior via an integrin-mediated mechanism

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ABSTRACT

The chemical composition, structure and surface characteristics of biomaterials/scaffold can affect the adsorption of proteins, and this in turn influences the subsequent cellular response and tissue regeneration. With magnesium/calcium phosphate cements (MCPC) as model, the effects of magnesium (Mg) on the initial adhesion and osteogenic differentiation of bone marrow stromal cells (BMSCs) as well as the underlying mechanism were investigated. A series of MCPCs with different magnesium phosphate cement (MPC) content (0~20%) in calcium phosphate cement (CPC) were synthesized. MCPCs with moderate proportion of MPC (5% and 10%, referred to as 5MCPC and 10MCPC) were found to effectively modulate the orientation of the adsorbed fibronectin (Fn) to exhibit enhanced receptor binding affinity, and to up-regulate integrin α5β1 expression of BMSCs, especially for 5MCPC. As a result, the attachment, morphology, focal adhesion formation, actin filaments assembly and osteogenic differentiation of BMSCs on 5MCPC were strongly enhanced. Further *in vivo* experiments confirmed that 5MCPC induced promoted osteogenesis in comparison to ot her CPC/MCPCs. Our results also suggested that the Mg on the underlying substrates but not the dissolved Mg ions was the main contributor to the above positive effects. Based on these results, it can be inferred that the specific interaction of Fn and integrin α5β1 had predominant effect on the MCPC-induced enhanced cellular response of BMSCs. These results provide a new strategy to regulate BMSCs adhesion and osteogenic differentiation by adjusting the Mg/Ca content and distribution in CPC, guiding the development of osteoinductive scaffolds for bone tissue regeneration.

1. Introduction

In the field of bone regeneration, adhesion, proliferation and ensuing osteogenic differentiation of stem cells or multipotent cells on the biomaterial surface are crucial for the new bone formation and the subsequent osseointegration of the implant [1]. Substantial researches have revealed that the surface topographical and biochemical properties of material had great effect on the cell responses. For example, Ding et al. found that the Si/Ca ratio of calcium silicate cements could modulate attachment and proliferation of cells [2]. Ayala R and his group demonstrated that the surface wettability of hydrogels regulated the adhesion and ensuing differentiation of marrow stromal cells (MSCs) [3]. Dalby et al. also reported using nanoscale disorder to stimulate the osteogenic differentiation of human MSCs to produce bone mineral *in vitro* [4]. Overall, tailoring the physicochemical properties of biomaterials to control cell response and execute specific cell functions is therefore becoming more attractive for the development of novel bone implant.

Currently, the mechanism involved into the surface-mediated cellular response is one of the hottest areas in the field of regenerative medicine [5]. The cell–material interaction is a very complex process which involves many cytokines and extracellular matrix (ECM) proteins. But first of all, it is primarily mediated by specific binding of cellular receptors to proteins adsorbed on the

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material surfaces [6]. Among all the cellular receptors, integrins, a widely expressed family of heterodimeric trans-membrane receptors [7], have turned out to exert principal role in anchoring cells to extracellular matrices. By binding to its extracellular ligand, a RGD peptide found in several ECM proteins such as fibronectin (Fn), integrins can activate the intracellular signaling pathways in the focal adhesion (FA) complex and further alter cell function [8,9]. Additionally, previous studies revealed the quantity and structure of Fn adsorbed on the materials were dynamically influenced by the substrate properties, which in turn affected the Fn-integrin binding and the subsequent cell adhesion [10–12]. Garcia et al. [12] found that the surface chemistry (presence of functional groups like CH_3, OH, COOH, NH_2) could modulate Fn adsorption and conformation to direct the specific integrin binding affinity, which further regulate cell adhesion and FA assembly. Foss et al. [10] also reported that nanostructured surfaces with different roughness were able to influence the adsorption of Fn and direct cell adhesion, FA assembly and actin expression. Besides, recent reports demonstrated that the substrate-induced intracellular FA formation and actin polymerization could stimulate osteogenic differentiation [13–18]. Specifically, in the pioneering works, Seo et al. reported that micropit surfaces could enhance FA formation and intracellular actin polymerization, and thereafter stimulate osteogenic differentiation through the RhoA-ROCK-MLCK (myosin II) pathway [17]. Khang et al. also demonstrated that a hybrid surface structure with both nano- and submicron-scaled roughness of titanium could promote osteoblast differentiation by initiating integrin activation and accelerating cyclins expressions [18]. Inspired by the above, incorporating metal cations into bone repair materials, which often exert effect on the physicochemical properties and ensuing proteins adsorption onto the substrates, may modulate the cell response, including cell adhesion and differentiation, and the ensuring bone regeneration process.

Magnesium is a vital and widely used component for the bone substitutes. In this study, we endeavored to systematically study the effects of the magnesium element on the initial responses and the ultimate differentiation of cells, as well as the mechanism involved. Calcium phosphate cements (CPC), a promising candidate for the treatment of bone defects in clinic [19,20], was used as the matrix model. The magnesium precursor (MPC) consisted of $Ca(H_2PO_4)_2 \cdot H_2O$ and MgO in a molar ratio of 1:2 was incorporated into CPC to obtain the MPC-modified CPC (MCPC). MCPC was chosen because the addition of MPC could quicken the hydration process and improve the mechanical strength without any obvious effect on the self-setting property [21–23]. The adsorption dynamics and conformation of Fn on the CPC/MCPC was assayed. The rat bone marrow stromal cells (BMSCs) were used to study the cell adhesion and viability, the formation of focal adhesion and the osteogenic differentiation on MCPCs substrates in detail. Finally, in order to verify the cell-instructive and the osteoinductive effect of MCPCs *in vivo*, the bone repair ability of macroporous MCPCs scaffolds were evaluated in a rat critical-sized calvarial defect model.

2. Materials and methods

2.1. Preparation of MCPC scaffolds

MCPC consisted of CPC powder, MPC powder and cement liquid (ultrapure water). CPC was prepared with equimolar tetracalcium phosphate (TTCP, $Ca_4(PO_4)_2O$) and dicalcium phosphate anhydrous (DCPA, $CaHPO_4$) by the same procedure as our previous study [24]. MPC was formed with calcium dihydrogen phosphate (CDP, $Ca(H_2PO_4)_2 \cdot H_2O$) and magnesium oxide (MgO) in a molar ratio of 1:2 [23]. In the experiment, TTCP/DCPA powders (component 1 CPC, prepared according to the previous reference) were mixed with CDP/MgO mixture (component 2 MPC, prepared according to the previous reference) to form MCPCs with MPC content in the range from 5% to 20% by weight of component (listed and named in Table 1). After adding water at a liquid to powder ratio of 0.3 (mL/g), the mixture was stirred to form homogeneous pastes and then transferred to a stainless steel mold with a diameter of 12 mm. After that, the resulting pastes were uniaxially pressed at 2 MPa for 1 min. Finally, MCPC disc-shaped pellets with 12 mm in diameter and 1.5 mm in thickness (0.2 g/pellet) were put into a constant temperature oven at 37 °C and 100% relative humidity. After 72 h, the hardened MCPC samples with different MPC content were obtained. The porous scaffolds for *in vivo* experiment were prepared through the NaCl particle leaching procedure described in our previous study [24]. All samples were sterilized by damp-heat sterilization at 121 °C, 1.21 MPa for 30 min. The HA pellets and beta-tricalcium phosphate (beta-TCP)/HA pellets with 5% beta-TCP in HA (named as 5% CaP/HA) were also prepared by the procedures as described in Supplementary Files. All the raw chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China).

Table 1
The compositions, setting time and compressive strength of cement samples.

Samples	CPC (wt%)	MPC (wt%)	Setting time (min)	Compressive strength (Mpa)
CPC	100	0	15 ± 0.5	38.2 ± 5.8
5MCPC	95	5	13 ± 0.4	52.7 ± 4.5
10MCPC	90	10	11 ± 0.3	55.9 ± 5.2
20MCPC	80	20	9 ± 0.5	63.5 ± 6.1

2.2. Physicochemical characterization

2.2.1. Determination of setting time and compressive strength

One gram of powder for each kind of cement was mixed with deionized water at the designated liquid to powder ratio to form a paste, which was then placed in a glass tube (Ø 6 × 10 mm). The top and bottom surfaces of the tube were tightly covered with two sheets of plastic film held by a "C" clamp and then stored at 37 °C in a 100% humidity box for setting. The samples were tested at various intervals using a Vicat apparatus, which consists of a frame bearing a movable rod, weighing 300 g, with a 1 mm stainless-steel needle at the end. The setting time was determined to be the time taken before the needle was unable to penetrate more than 1 mm into the sample. This is in accordance with the ASTM Test Method C 187-98. Each experiment was performed in triplicate and the average value was calculated. After hardening for 72 h, the hardened cement sample (Ø 6 × 10 mm) was uniformly polished on both sides. The compressive strength of the sample was measured at a loading rate of 1 mm/min using a universal testing machine (AG-2000A, Shimadzu Autograph, Shimadzu Co., Ltd., Japan). Three replicates were carried out for each group, and the results were expressed as means ± standard deviation (means ± SD).

2.2.2. Chemical phase composition and surface morphology

The MCPC samples were characterized by X-ray diffraction (XRD, Rigaku D/Max 2550, Cu K α radiation, Japan). Surface morphology of cement samples, the energy dispersive spectrometer (EDS) elements analysis and mapping of calcium and magnesium on cement surfaces were examined with scanning electron microscope (SEM, S-4800, Hitachi, Japan).

2.2.3. Surface roughness and contact angle

Surface roughness of the cements and the corresponding sterilized samples was determined using the atomic force microscopy (AFM). The AFM images (20 × 20 μm) were acquired in tapping mode using a commercial Nanoscope IIIA Multimode SPM (Veeco Instruments, Santa Barbara, USA). Advancing sessile drop water contact angles were measured on the surface of MCPCs using JJC-1 static contact angle equipment (Changchun No.5 Optical Instrument Co., Ltd., China). Milli-Q water was used with a drop volume of approximately 0.02 mL. Results are presented as an average of eight measurements on at least three different surfaces.

2.2.4. Ion release of cements in cell culture medium

The variation of the concentration of Ca^{2+} and Mg^{2+} from MCPC was studied. In brief, the MCPCs were immersed in cell culture medium (α-MEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin sulfate at a solid/liquid mass ratio of 0.2 g/g at 37 °C with 5% CO_2. The medium was changed every 48 h. After 4, 24, 72, 168 h, the cement samples were removed and the culture medium was collected. The concentration of Ca^{2+} and Mg^{2+} in medium was analyzed with an inductively coupled plasma atomic emission spectrometer (ICP-AES, Perkin-Elmer Optima 2000).

2.2.5. pH value of cements modified cell culture medium

The variation of pH value after cements soaking was studied. In brief, the cements were immersed in cell culture medium (α-MEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin sulfate at a solid/liquid mass ratio of 0.2 g/g at 37 °C with 5% CO_2. The medium was changed every 48 h. After 1, 2, 4, 8, 12 days, the cement samples were removed and the pH value of culture medium was assayed by pH meter (FE20, Mettler Toledo, Switzerland).