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Synergistic effect of strontium and silicon in strontium-substituted sub-micron bioactive glass for enhanced osteogenesis

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ABSTRACT

Strontium-substituted sub-micron bioactive glasses (Sr-SBG) have been reported to have enhanced osteogenic differentiation capacity compared to sub-micron bioactive glasses (SBG) in our previous study. However, the underlying molecular mechanisms of such beneficial effect of Sr-SBG are still not fully understood. In this study, we synthesized Sr-SBG, studied the effects of Sr-SBG on proliferation and osteogenic differentiation of mouse mesenchymal stem cells (mMSCs), and identified the molecular mechanisms of the enhancement effect of Sr-SBG on mMSCs. The results demonstrated that Sr-SBG had more profound promotion effect on proliferation and osteogenic differentiation of mMSCs than SBG and $SrCl_2$ group which containing identical Sr concentration with Sr-SBG group. RT-qPCR and western blot analysis showed that the mRNA expressions and protein expressions involved in NFATc and Wnt/β-catenin signaling pathways were all upregulated mediated by Sr-SBG, while only Wnt/β-catenin signaling pathway related genes upregulated in SBG group and only NFATc signaling pathway activated in $SrCl_2$ group, suggesting that NFATc and Wnt/β-catenin signaling pathways played important roles in osteogenesis enhancement induced by Sr-SBG. To conform the above conclusion, cyclosporin A (CSA) was applied to inhibit NFATc signaling pathway. It was found that the enhanced osteogenic differentiation of mMSCs induced by Sr-SBG was partially abrogated and the activated Wnt/β-catenin signaling pathway was also inhibited in part. However, the effects of SBG on proliferation and osteogenesis of mMSCs were unimpaired, yet the effects of $SrCl_2$ were greatly suppressed. Taken together, these results indicated that strontium activated NFATc signaling pathway and silicate activated Wnt/β-catenin signaling pathway might synergistically mediated the enhanced osteogenesis induced by Sr-SBG.

1. Introduction

Multifunctional bioactive materials for the repair of bone defects caused by trauma, tumors, infections or genetic malformations, have attracted much attention in the past several years. In 1970s, Carlisle and Schwarz suggested that Silicon (Si) element might play an important role in skeletal development and bone repair [1,2]. Since then, many studies on silicate bioactive materials, including Si-substituted calcium phosphates [3–5], silicate-based bioceramics [6–9] and bioactive glasses [10–13], have been employed for bone regeneration. Bioactive glass is one of the typical bioactive materials possessing osteoinductive properties for its function of activating osteogenesis-related gene expression of bone marrow stromal cells (BMSCs) [14–16]. Recently, micro-nano bioactive glass (MNBG) with controlled size and morphology was developed through the combination of sol-gel technique and template self-assembly method. Compared with conventional bioactive glasses, MNBG has superior apatite formation ability and biological activity due to their significantly increased surface area [17,18]. To achieve improved osteogenesis of bioactive glasses for bone repair, it was proved that the substitution of Sr ions into bioactive glasses could enhance the osteogenic differentiation of BMSCs [19–24]. Our previous work also demonstrated that Sr-substituted sub-micron bioactive glass (Sr-SBG) could improve osteogenesis than SBG [25]. Nevertheless, the underlying molecular mechanisms are still under investigation. To develop an ideal bioactive glass-based bone repair biomaterial, it is important to clarify the molecular mechanism through

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which the biomaterial regulates osteogenic differentiation of BMSCs.

During embryonic development, Wnt signaling plays a critical role in bone formation through regulating the determination of cell fate, and influencing the proliferation and differentiation of stem cells [26–28]. Previous researches have demonstrated that the bioactive Si ions released from BG and other silicate bioceramics could promote proliferation of BMSCs and induce osteogenic differentiation of BMSCs. The underlying mechanism of the enhanced proliferation and differentiation of BMSCs mediated by silicate was supposed to be related to the Wnt signaling pathway activation [7,29,30].

Sr is a trace element which could not only stimulate bone formation but also inhibit bone resorption during bone metabolism [31–33]. The mechanism was supposed to be related to the ability of Sr ions to promote the activity of alkaline phosphatase (ALP) and mRNA expression of osteogenesis-related gene of MSCs [34,35]. It was found that systemic administration of strontium ranelate could lead to increased bone mineral density (BMD) and decreased probability of bone fractures occurrence in osteoporotic patients [36–38]. Consequently, plenty of Sr-containing bone materials were developed due to their enhanced osteogenic capacity for rapid osseointegration [3,39–42]. Recently, Fromigue et al. showed that the Nuclear factor of activated T-cells (NFATc) signaling pathway and downstream canonical and non-canonical Wnt signaling pathways were involved in strontium ranelate induced proliferation and differentiation of murine osteoblasts [43]. NFATc are transcription factors that are highly phosphorylated and remain in the cytoplasm in unstimulated cells, and are involved in the differentiation of various cell types. When intracellular calcium level increases, calcineurin will be activated followed by dephosphorylation of NFATc1, leading to the translocation of NFATc1 to nucleus and binding to the promoter of target genes [44]. It was demonstrated that NFATc1 played an important role in both osteoblasts and osteoclasts, which is expressed during osteogenic differentiation and osteoclastogenesis.

Based on the important role of Wnt/β-catenin signaling pathway mediated by silicate and NFATc signaling pathway induced by Sr element in osteogenesis, this study was conducted to investigate the effect of Wnt/β-catenin and NFATc signaling pathway on Sr-SBG stimulated osteogenic differentiation of mouse bone marrow-derived mesenchymal stem cells (mMSCs) since that both Sr and Si ions are dominant components of Sr-SBG. We hypothesized that Sr-SBG could promote osteogenic differentiation of mMSCs through the synergistically effect of SBG activated Wnt/β-catenin pathway and Sr activated NFATc pathway. To test this hypothesis, we conducted three experimental groups: SBG group, Sr-SBG group and $SrCl_2$ group, and investigated their effect on proliferation and osteogenesis of mMSCs and their influence on activation of the two signaling pathways above by RT-qPCR and western blot analysis. Meanwhile, NFATc signaling pathway was blocked by using cyclosporin A (CSA) to evaluate the osteogenic differentiation capacity of mMSCs induced by Sr-SBG. The results could further verify whether NFATc and Wnt/β-catenin signaling pathways played a synergistic effect on the enhanced osteogenesis of Sr-SBG or not. This study might clarify a key scientific problem before the application of Sr-SBG in clinic.

2. Materials and methods

2.1. Materials

Ethanol (AR), tetraethyl orthosilicate (TEOS) (AR), calcium nitrate tetrahydrate (CN) (AR), and strontium nitrate (SN) (AR) were obtained from Guangzhou Chemical Reagent Factory, P.R. China. Triethylphophate (TEP) (AR) and Dodecylamine (DDA) were purchased from Aladdin (Shanghai, P.R. China). All chemical reagents were used directly without other treatment.

2.2. Synthesis of SBG and Sr-SBG and their ion dissolutions

SBG and Sr-SBG were prepared according to the previous described method by the combination of sol-gel and template self-assembly technique using DDA as both catalyst and template agent [17,18,25]. The constituents ratio of SBG was SiO_2: CaO: P_2O_5 = 60: 36: 4 (mol/mol), and the constituents ratio of Sr-SBG was SiO_2: CaO: SrO: P_2O_5 = 60: 30: 6: 4 (mol/mol). Typically, a given amount of ethanol was mixed with deionized water. Then, 4 g DDA was dissolved into the mixed solution above by stirring at 40 °C for 10 min. After that, two equal proportion of total 16 ml TEOS was added to the above mixture under vigorous stirring for 30 min interval. Finally, TEP and CN or SN was added under stirring in order with 30 min intervals. After another 3 h vigorous stirring, the resulting milky suspension was aged for 6 h and centrifuged at 4000 rpm to obtain white gel precipitate. The white precipitate was rinsed with ethanol and deionized water for three times each, and freeze-dried for 48 h. Eventually, SBG and Sr-SBG powders were obtained after sintering in air at 650 °C for 3 h. The ion dissolutions of SBG and Sr-SBG in Dulbecco modified Eagle medium (DMEM) was prepared as previously described. Briefly, SBG and Sr-SBG powders were autoclaved and then added into DMEM medium at a ratio of 1 mg/ml. The suspension was maintained at 37 °C in a shaker with the speed set as 120 rpm for 48 h. The ion dissolutions of SBG and Sr-SBG were obtained by centrifugation and filtration using a 0.22 μm syringe filter. 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin were added to the ion dissolutions for cell culture in the following experiments.

2.3. Characterization of SBG and Sr-SBG

Scanning electron microscopy (SEM) (DSM 982-Gemini, Zeiss, Germany) was conducted to characterize the morphology of SBG and Sr-SBG. The particle size distribution was assayed on Zetasizer Nano ZS (Malvern Instruments, UK). X-ray photoelectron spectroscopy (Kratos Axis UlraDLD, UK) and Energy-dispersive X-ray spectroscopy were used to test the element composition of SBG and Sr-SBG. And the concentrations of ion dissolution of SBG and Sr-SBG were tested by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (PS1000-AT, Leeman, USA).

2.4. Cell culture

mMSCs was obtained from American Type Culture Collection (ATCC® CRL-12424™) and used in the subsequent experiments. mMSCs were cultured in DMEM with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin, under a 5% CO_2 incubator at 37 °C. After the confluence reached to 90%, cells were passaged using 0.25% trypsin-EDTA. The third to sixth passages of mMSCs were used for cell proliferation, osteogenic differentiation and activated/inactivation signaling pathway studies. Four groups (a. Control group with DMEM medium; b. SBG group with ion dissolution of SBG; c. Sr-SBG group with ion dissolution of Sr-SBG; d. $SrCl_2$ group with equal Sr ion concentration with Sr-SBG group) were set up in the following experiments.

2.5. Proliferation assay

Proliferation of mMSCs was carried out by a Cell Counting Kit-8 assay kit (CCK-8, Dojindo Laboratories, Kumamoto, Japan). mMSCs were seeded at a density of 3000 cells/well in a 96-well plate. After cells attached, the culture medium was replaced by ion dissolution of each group. The culture medium of each group was refreshed every 3 days. 1, 3 and 7 days later, cells were harvested for CCK-8 assay (five samples for each group). For each well, the medium was displaced by 100 μl DMEM containing 10% CCK-8 and maintained in the incubator at 37 °C for 1 h. The absorbance of each well was measured at 450 nm by

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