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Human urine-derived stem cells can be induced into osteogenic lineage by silicate bioceramics via activation of the Wnt/β-catenin signaling pathway

Junjie Guan [a, 1], Jieyuan Zhang [a, 1], Shangchun Guo [c], Hongyi Zhu [a], Zhenzhong Zhu [a], Haiyan Li [b, **], Yang Wang [a, c, *], Changqing Zhang [a, c, *], Jiang Chang [b, d]

[a] Department of Orthopedic Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, China
[b] Med-X Research Institute, School of Biomedical Engineering, Shanghai Jiao Tong University, 1954 Huashan Road, Shanghai 200030, China
[c] Institute of Microsurgery on Extremities, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, China
[d] Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, China

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ABSTRACT

Human urine-derived stem cells (USCs) have great application potential for cytotherapy as they can be obtained by non-invasive and simple methods. Silicate bioceramics, including calcium silicate (CS), can stimulate osteogenic differentiation of stem cells. However, the effects of silicate bioceramics on osteogenic differentiation of USCs have not been reported. In this study, at first, we investigated the effects of CS ion extracts on proliferation and osteogenic differentiation of USCs, as well as the related mechanism. CS particles were incorporated into poly (lactic-co-glycolic acid) (PLGA) to obtain PLGA/CS composite scaffolds. USCs were then seeded onto these scaffolds, which were subsequently transplanted into nude mice to analyze the osteogenic differentiation of USCs and mineralization of extracellular matrix formed by USCs *in vivo*. The results showed that CS ion extracts significantly enhanced cell proliferation, alkaline phosphatase (ALP) activity, calcium deposition, and expression of certain osteoblast-related genes and proteins. In addition, cardamonin, a Wnt/β-catenin signaling inhibitor, reduced the stimulatory effects of CS ion extracts on osteogenic differentiation of USCs, indicating that the observed osteogenic differentiation of USCs induced by CS ion extracts involves Wnt/β-catenin signaling pathway. Furthermore, histological analysis showed that PLGA/CS composite scaffolds significantly enhanced the osteogenic differentiation of USCs *in vivo*. Taken together, these results suggest the therapeutic potential of combining USCs and PLGA/CS scaffolds in bone tissue regeneration.

1. Introduction

Recent studies have demonstrated that urine-derived stem cells (USCs) possess self-renewal and multilineage differentiation potential [1–3]. Several investigations used USCs as cell source to form engineered urethral tissue [4,5]. For example, USCs seeded in bacterial cellulose scaffolds can form multilayered urothelium and cell matrix [4]. Cells differentiated from USCs were seeded on a modified 3D porous small intestinal submucosa in order to form engineered urethral tissue. Results showed that the cells formed multiple uniform layers on the scaffolds, which was similar to that of native urinary tract tissue [5]. As USCs can be obtained using non-invasive and simple methods, they represent a promising alternative stem cell population for tissue regeneration. However, whether USCs could serve as cell source for bone tissue regeneration is still unknown. In our previous study, we have demonstrated that USCs share similar biological characteristics with adipose stem cells (ASCs) and could further differentiate into an osteogenic lineage *in vitro* [2]. These evidences suggested that USCs might be a good cell source for bone tissue engineering. In addition, to better

* Corresponding authors. Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, China. Tel.: +86 21 64369181; fax: +86 21 64701361.
** Corresponding author. Tel.: +86 21 62933243.
E-mail addresses: haiyan.li@sjtu.edu.cn (H. Li), wangy63cn@126.com (Y. Wang), zhangcq@sjtu.edu.cn (C. Zhang).
[1] These authors contributed equally to this work.

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apply USCs in bone tissue engineering, it is critical to develop bioactive material that could facilitate the proliferation and osteogenic differentiation of USCs.

Silicates, including silicate bioglass and silicate bioceramics, have been reported to be able to stimulate osteoblastic differentiation and bone regeneration in many studies [6–8]. Previous studies have shown that Si plays an important role during skeletal development and regeneration [9,10]. Si has been widely incorporated into bioceramics to enhance their bioactivity [7,11–14]. Calcium silicate (CS), one of the silicate bioceramics with simple components, has shown a great osteostimulatory effect without the need of additional exogenous growth factors [15,16]. It has been demonstrated that CS can stimulate osteogenic differentiation of several kind of adult stem cells including human bone marrow mesenchymal stem cells (BMSCs), orofacial mesenchymal stem cells as well as tooth germ stem cells [17–19]. In addition, several studies have shown that CS ion extracts or composite scaffolds containing CS possess pro-angiogenic properties [13,20], which may further stimulate bone regeneration through enhancing vascularization and angiogenesis to deliver oxygen and nutrients to seeded cells in bone tissue engineering. Therefore, scaffolds containing CS show great potential for bone tissue regeneration.

To develop an ideal silicate-based biomaterial, it is important to clarify the molecular mechanism through which the biomaterial regulates stem cell differentiation. The canonical Wnt/β-catenin signaling cascade plays a critical role in cell proliferation, differentiation and apoptosis [21]. It has been demonstrated that the Wnt/β-catenin signaling pathway is involved in bone formation [22]. Recently, Zhou et al. reported that the Wnt/β-catenin signaling pathway was involved in the regulation of bredigite bioceramic-induced osteogenic differentiation of periodontal ligament cells [23]. Han et al. demonstrated that Si ions stimulated the osteogenic differentiation of BMSCs and that the Wnt/β-catenin signaling pathway played an important role in this process [24]. Therefore, we hypothesize that Wnt/β-catenin signaling pathway may also play an important role on proliferation and differentiation of USCs with the presence of CS. However, little is known about the involvement of Wnt/β-catenin signaling in the USCs osteogenic differentiation upon CS interaction.

This study was conducted to investigate the application potential of USCs as cell source for bone tissue engineering by studying the *in vitro* and *in vivo* osteogenic differentiation of USCs when they were cultured with CS ion extracts or on scaffolds containing CS and to explore the role of the Wnt/β-catenin signaling pathway during this process.

2. Materials and methods

2.1. Synthesis of CS powders

Chemical co-precipitation method was used to obtain CS powders [15,16]. Briefly, chemical co-precipitation happened during continuous mixing of an aqueous solution of Na_2SiO_3 (1 mol/L) and aqueous solution of $Ca(NO_3)_2$ (1 mol/L) at ambient temperature overnight (molar ratio: Na_2SiO_3:$Ca(NO_3)_2$ = 1:1). Then, CS suspension was obtained after the reacted mixture were filtered and washed with deionized water and ethanol. After that, CS suspension was dried at 80 °C overnight and calcined at 800 °C for 2 h to obtain CS powder. The CS particles were sieved to obtain CS powder with size between 100 and 150 μm.

2.2. Ion extract preparation and ion concentration determination

Ion extracts of the CS were prepared according procedures reported in the literature [13,20]. Briefly, 1 g of CS powder was soaked in 5 ml of serum-free USC growth medium and incubated at 37 °C for 24 h. The mixture was centrifuged to collect supernatant. After sterilization with a filter (Millipore, 0.22 μm), serial dilutions of extracts (1/64, 1/128 and 1/256) were prepared using USC growth medium and stored at 4 °C for future use. The ion concentrations of the Ca, P and Si in the extracts dilutions were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). USCs growth medium was also analyzed by ICP-AES as the control medium.

2.3. Cell isolation and culture

USCs were isolated from human urine according to procedures described our previous studies [2]. Urine was obtained from healthy volunteers (22–28 years old). Informed consent was obtained from all donors, and the research plan was approved by the Ethics Committees of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital. For the isolation and proliferation of USCs, penicillin and streptomycin were added to the fresh urine samples (200 ml) at standard concentrations to minimize contamination. After the urine samples were centrifuged at 400 × g for 10 min at room temperature, the supernatant was discarded, and the sediment was washed with phosphate buffered saline (PBS). The cells were washed and centrifuged again. Then, the sediment was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% (vol/vol) fetal bovine serum (FBS) (Gibco), 10 ng/ml human epidermal growth factor (hEGF), 2 ng/ml platelet-derived growth factor (PDGF), 1 ng/ml transforming growth factor-β (TGF-β), 2 ng/ml basic fibroblast growth factor (bFGF), 0.5 μM cortisol (hydrocortisone), 25 μg/ml insulin, 20 μg/ml transferrin, 549 ng/ml adrenaline (epinephrine), 50 ng/ml triiodothyronine (triiodothyronine, T3), L-glu and antibiotics. The cell suspension was plated onto gelatin-coated 24-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed after 7 days of culture. The non-adherent cells were removed by thoroughly washing the plates with PBS. The colonies derived from single cells were marked. The culture medium was refreshed twice a week thereafter. When the cells became nearly 80% confluent, they were passaged by digestion with 0.25% trypsin containing 1 mM EDTA. USCs at passages P4–P5 were used for the experiments.

2.4. Cell proliferation assay

The proliferation of USCs in the CS ion extracts was examined using the Cyquant assay (Cyquant Cell Proliferation Assay Kit, C7026; Invitrogen). Briefly, 0.5 × 10^5 USCs were seeded in six-well plates in CS extracts diluted in USC growth medium at 1/64, 1/128 and 1/256. After being cultured for 1, 3, 5 and 7 days, cell number was quantified as the manufacturer's instructions.

2.5. ALP staining and activity of USCs cultured in CS ion extracts

USCs were seeded at 5 × 10^4 cells/well in a 24-well plate and cultured with control medium and CS extracts diluted with control medium at 1/64, 1/128 and 1/256. ALP staining was performed according to the manufacturer's instructions (Beyotime, C3206, China). Briefly, USCs were first washed with PBS and then rinsed with deionized water. Thereafter, USCs were incubated in a mixture of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. After the incubation, cells were observed under an inverted bright field microscope (TY9648; Leica). Images were acquired with a CCD camera. ALP activity was detected at days 7 and 14 after addition of CS ion extracts to the USCs cultures as previously described [25]. In brief, the cells were washed with PBS and then submerged in 0.2% Triton X-100. Lysates were transferred to a 1.5-ml tube and centrifuged at 20,817 g for 5 min at 4 °C. The supernatant was mixed with ALP assay working solution according to the manufacturer's instruction (Jiancheng, China). Absorbance was measured at 405 nm on a plate reader. The ALP activity was normalized to the total protein content as measured by the BCA protein assay kit (Thermo Scientific, 23228, Rockford, IL) [2].

2.6. Calcium deposition assay

USCs were seeded at 5 × 10^5 cells/well in a six-well plate cultured with control medium and CS extracts diluted with control medium at 1/64, 1/128 and 1/256. Accumulated calcium was measured at day 14 according to QuantiChrom Calcium Assay Kit (BioAssay Systems Hayward, CA). In brief, the cells were washed with deionized water and lysed in 0.1 N HCl. Cell extracted were prepared in 0.1 N HCl at 4 °C for a minimum of 4 h and centrifuged at 15,249 rpm for 5 min. Total calcium in the supernatant was determined according to the manufacturer's instructions. Total calcium was calculated from standard solutions prepared in parallel and normalized to the total DNA content, which was detected using a commercially available kit (DNeasyTM, Qiagen, Valencia, CA). The values of blank samples were subtracted from the calcium-containing test samples.

2.7. RNA extraction and real-time quantitative PCR analysis

RT-PCR was performed to measure the gene expression of ALP, Runx2 and OCN from USCs cultured in different medium. The involvement of the Wnt/β-catenin signaling pathway was analyzed by measuring the expression of axis inhibition protein 2 (Axin2), beta-catenin (β-catenin), ahr and nkd through RT-PCR since ahr and nkd have been demonstrated to serve as Wnt target genes in many literatures [26–28]. Total RNA was isolated from USCs cultured in different concentrations of the CS powder using TRIzol Reagent (Life Technologies). We used TakaRa PrimeScript 1st strand cDNA Synthesis Kit (6110A) for cDNA synthesis. Complementary DNA was synthesized using 1 μg RNA and following the manufacturer's instructions. RT-PCR was performed using SYBR Green detection reagent (SYBR Premix, Roche). The housekeeping gene β-actin was used as an internal control. The primer sequences for the target genes are listed in Table 2. All reactions were run in triplicate in three independent experiments.