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Original article

Purmorphamine increased adhesion, proliferation and expression of osteoblast phenotype markers of human dental pulp stem cells cultured on beta-tricalcium phosphate

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ABSTRACT

Objectives: Growth factors play a significant role in cell proliferation and differentiation during different stages of the bone repair. However, several limitations have been brought researchers attention to an osteoinductive small molecule including Purmorphamine. In this study, we aimed to evaluate the effect of Purmorphamine on adhesion, proliferation and differentiation of human dental pulp stem cells (hDPSCs) seaded on beta-tricalcium phosphate (β-TCP) granules.
Methods: hDPSCs were established from extracted wisdom teeth of healthy volenteers. Cells at passage 3 were seeded on β-TCP in the presence or absence of Purmorphamine. Cell adhesion and proliferation were assessed using scanning electeron microscopy (SEM) and DNA counting assay, respectively, after 1, 3 and 5 days. Then, hDPSCs seeded on β-TCP were subjected to osteogenic medium with or without Purmorphamine. After 7 and 14 days osteogenic diffrentiation capability of hDPSCs were determined using real-time RT-PCR and alkaline phosphatase (ALP) activity assay.
Results: The significant increase in amount of DNA was observed at day 3 and 5 in the presence of Purmorphamine. SEM imaging also was confirmed the DNA counting assay; in all given time points, hDPSC attachment and growth was significantly higher in the presence of Purmorphamine. ALP activity was increased by Purmorphamine at both 7 and 14 days of induction. Purmorphamine showed to effect on osteopontin expression at earlier stage of osteogenic differentiation, whereas for osteocalcin expression, this effect was more evident at later stage of differentiation.
Conclusion: Purmorphamine had a promotive effect on adhesion, proliferation and osteogenic differentiation of hDPSCs cultured on β-TCP. The outcome of the current study would help in development of in vitro culture conditions for better osteogenic differentiation of hDPSCs prior to transplantation.

1. Introduction

Tissue engineering has emerged as a promising treatment approach for regeneration of skeletal defects. Combination of progenitor/stem cells, a scaffold and an appropriate growth factor have been extensively used for bone regeneration [1–3]. It been demonstrated that poly peptides such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF) can regulate cell proliferation, differentiation and extracellular matrix synthesis during different stages of the bone repair [4–6]. Despite their pivotal role in treatment of skeletal defect, their clinical applications faced some challenges [7]. Using DNA recombinant technology and prokaryotic systems, growth factors have been produced that did not undergo post-translational modifications that routinely occur in human body, so their activity

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and stability may be compromised [8]. Moreover, to be clinically effective, the dosages far more than physiological levels should be administrated. These has been raised some concerns about pathological side effects and toxicity [9]. The route of administration and the cost of production are also another drawbacks in administration of growth factors in clinics [10].

Due to these limitations, specific small molecules that have modulatory roles in osteoblastic differentiation, named osteoinductive small molecules, have been synthesized [10]. Several studies showed the small molecules can promote cellular responses such as differentiation and matrix mineralization and have beneficial effects on healing process of bone deficiencies [11–13]. In compared to growth factors, small molecules are more stable and affordable and also they show reduced risk of host immune reaction [14]. Moreover, immediate responses can be achieved due to their rapid effect and can be easily metabolized in the cell culture [15].

Purmorphamine is a small molecule first discovered by Wu et al. Their study on mouse mesenchymal progenitor cells revealed the ability of Purmorphamine to up-regulate the genes involved in proliferation and osteogenic differentiation [12].Later studies on a rodent model showed that Purmorphamine up-regulate the expression of genes required for osteogenic differentiation through activation of the Hedgehog (Hh) signaling pathway [12,16]. However, studies on human bone marrow mesenchymal stem cells (hBMMSCs) showed controversial results. Some studies have shown the up- regulatory effect of Purmorphamine on osteogenesis of hBMMSCs [17–20]. In contrast, others showed an inhibitory effect of this small molecule [21].

Previous studies evaluate the effect of Purmorphamine on proliferation and osteogenic capability of different types of cells. Up to our knowledge, the promotive effect of Purmorphamine on cells cultured on 3-D scaffold has not been evaluated before. The main focus of the current study is to evaluate the effect of Purmorphamine on adhesion, proliferation and differentiation of cells cultured on beta-tricalcium phosphate (β-TCP) granules. In particular, we have used human dental pulp stem cells (hDPSCs) for investigation of the Purmorphamine effects, since their clinical application seems feasible. Harvesting and isolating these progenitor population are relatively easy and their high osteogenic potential in treatment of skeletal defects has been shown before [22]

2. Material and methods

2.1. Culturing human DPSCs (hDPSCs)

hDPSCs were obtained from pulp tissues of extracted wisdom teeth of three healthy volunteer donors refereed to Department of Oral and Maxillofacial surgery at Shahid Beheshti University of Medical Sciences. Briefly, the surface of extracted teeth were cleaned by several washes in sterile Phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, United States) followed by immersion in 1% providone-iodine solution (Iran Najo, Tehran, Iran) for 2 min. Then, they were washed again in sterile PBS. The root of cleaned teeth were separated from crown. Then, the pulp tissues were isolated from the pulp chamber with sterile forceps and digested in a solution of 3 mg/ml collagenase type I (Sigma-Aldrich, St. Louis, MO, United States) for 1 h at 37 °C. Digested cells were centrifuged to collect cell pellets. Cells were re-suspend in medium containing DMEM (Life Technologies, Carlsbad, CA, United States), 20% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, United States) + 1% Penicillin-Streptomycin 10,000 u/ml (Life Technologies, Carlsbad, CA, United States). This medium has been used previously as a growth medium for dental stem cells [23]. Cell suspension was immediately plated in a T-25 flask and placed at 37 °C and 5% CO_2, and non-adherent cells were removed by medium change a day after. At this stage cells were called passage 0 (P0) .The remaining cells were cultured until they reached 80–90% confluency. Medium were changed every 4 days. Cells were passed until P3 using 0.25% trypsin-EDTA (Life Technologies, Carlsbad, CA, United States).

2.2. Characterization of hDPSCs

In order to confirm whether the isolated/cultured hDPSCs display the mesenchymal stem cell (MSC) phenotype, the cells were subjected to flow cytometry analysis for expression of positive (CD44, CD90, CD73 and CD105) and negative (CD45 and CD34) MSC markers. Briefly, cells at P3 were treated with 0.05% trypsin-EDTA. The harvested cells were centrifuged and then, cell pellets were re-suspended at concentration of 10^5 per sample in PBS and incubated for 30 min at 4 °C in the dark room with antibodies. After incubation time, cells were washed with phosphate-buffered saline (PBS). Analysis was next performed using flow cytometer. The positive control expression was defined as the level of florescence greater than 99% of the corresponding unstained cells.

Also, hDPSCs were subjected to osteogenic induction medium in order to evaluate their differentiation capability towards the osteoblast lineage. Osteogenic medium consists of DMEM-LG (Invitrogen, Grand Island, NY, USA) 10% FBS, 1% Penicillin-Streptomycin 10,000 u/ml, 50 μg/ml ascorbate-2 phosphate, 10–5 mM dexamethasone and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA). After 14 days of incubation, cells were washed with PBS and fixed with 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO, USA). Next, cells were stained with 1% Alizarin Red solution (GFS Chemicals, Inc., Columbus, OH, USA) for 5 min.

2.3. Preparation of purmorphamine

Purmorphamine [2-(1-naphthoxy)-6-(4-morpholinoanilino)-9- cyclohexylpurin] (Sigma-Aldrich, St. Louis, MO, United States) was re-constituted in dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, United States) to make 1 mM stock solution according to the manufacturer' protocol and aliquots were stored at −20 °C.

2.4. Cell seeding on scaffold

In this study, β-TCP granules (Lasak, Praha, Czech Republic) with pore size of 100–200 μm (macro pores) and 1–5 μm (micro pores) were used as a 3D-scaffold. About 200 mg of β-TCP granules were placed in the bottom of an individual well in 24-well plates under sterile conditions. DPSCs P3 at a cell density of 10^4/well were seeded on β-TCP granules. Cell-scaffolds were cultured either in stem cell growth medium or osteogenic medium according to different experiment purposes. Experiments were performed in triplicates, i.e., three independent hDPSC primary cultures. Purmorphamine at concentration of 2 μM was also added to the culture medium. Schematic of experimental design is illustrated in Fig. 1.

2.5. Evaluation of cell proliferation using DNA counting assay

Cell-scaffolds were cultured in stem cell growth medium with 2 μM Purmorphamine for 24 h, 3 days and 5 days. Cell-scaffolds cultured in the medium without Purmorphamine were served as the control. Proliferation of hDPSCs were evaluated using DNA counting assay. Briefly, cell-scaffolds were lyzed in Trizol solution (Life Technologies, Carlsbad, CA, United States) and incubated at