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Insulin-like growth factor 1 promotes the proliferation and committed differentiation of human dental pulp stem cells through MAPK pathways

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ABSTRACT

Objectives: Insulin-like growth factor 1 (IGF-1) is a broad-spectrum growth-promoting factor that plays a key role in natural tooth development. Human dental pulp stem cells (hDPSCs) are multipotent and can influence the reparative regeneration of dental pulp and dentin. This study was designed to evaluate the effects of IGF-1 on the proliferation and differentiation of human dental pulp stem cells.
Methods: HDPSCs were isolated and purified from human dental pulps. The proliferation and osteo/odontogenic differentiation of hDPSCs treated with 100 ng/ml exogenous IGF-1 were subsequently investigated.
Results: MTT assays revealed that IGF-1 enhanced the proliferation of hDPSCs. ALP activity in IGF-1-treated group was obviously enhanced compared to the control group from days 3 to 9. Alizarin red staining revealed that the IGF-1-treated cells contained a greater number of mineralization nodules and had higher calcium concentrations. Moreover, western blot and qRT-PCR analyses demonstrated that the expression levels of several osteogenic genes (e.g., RUNX2, OSX, and OCN) and an odontoblast-specific marker (DSPP) were significantly up-regulated in IGF-1-treated hDPSCs as compared with untreated cells ($P < 0.01$). Interestingly, the expression of phospho-ERK and phospho-p38 were also up-regulated, indicating that the MAPK signaling pathway is activated during the differentiation of hDPSCs.
Conclusions: IGF-1 can promote the proliferation and osteo/odontogenic differentiation of hDPSCs by activating MAPK pathways.

1. Introduction

Dental pulp is a soft connective tissue present within the core of human teeth, whose primary functions include the production of dentin and maintenance of the biological and physiological vitality of the dentin (Shi & Gronthos, 2003). In response to specific stimuli, such as injury or decay, dental pulp will produce odontoblast-like cells along with reparative dentin structures.

Abbreviations: ALP, alkaline phosphatase; a-MEM, a-minimum essential medium; BMPs, bone morphogenetic proteins; CPC, cetyl pyridinium chloride; DSP, dentin sialoprotein; DSPP, dentin sialophosphoprotein; ERK, extracellular regulated protein kinases; FBS, fetal bovine serum; FGFs, fibroblast growth factors; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDPSCs, human dental pulp stem cells; IGF-1, insulin-like growth factor 1; IGFs, insulin-like growth factors; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OCN, osteocalcin; OD, optical density; OSX, osterix; PBS, phosphate buffered saline; PDGFs, platelet-derived growth factors; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RUNX2, runt-related transcription factor2; TGFs, transforming growth factors.

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Reparative regeneration is facilitated by the stem cells that reside within the dental pulp. Most studies have shown that human dental pulp stem cells (hDPSCs) are highly accessible and multipotent and can participate in osteo/dentinogenesis, adipogenesis, chondrogenesis, myogenesis, and neurogenesis (Al-Habib, Yu, & Huang, 2013; Arminan et al., 2009; Stevens et al., 2008). The ability of dental pulp stem cells to differentiate into osteo/odontoblast lineages is of great interest to dental researchers, due to their capacity to build and restore dental pulp and dentin.

Conditioned media collected from developing tooth germ cells has been shown to enhance the expression of mineralization-related genes and the formation of mineralized DPSC nodules *in vitro* (Yu et al., 2006). Induced DPSC pellets can produce regular dentin-pulp complexes containing dentinal tubules and predentin *in vivo* (Yu et al., 2006). Moreover, DPSCs are capable of differentiating into osteoblasts both *in vitro* and *in vivo* and can produce bone-like tissues (Carinci et al., 2008). When seeded onto three-dimensional biomaterial scaffolds within osteogenic medium containing vitamin D_3, the mRNA levels of osteogenic markers in DPSCs were significantly up-regulated (Khanna-Jain et al., 2012). To date, various growth factors and signaling molecules have been shown to participate in the regulation of pulp and dentin formation, including transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), and fibroblast growth factors (FGFs) (Kim et al., 2012). These molecules can affect the metabolic process of dental pulp tissues and stem cells, ultimately leading to hard tissue formation.

IGF-1 is a major regulator of growth, differentiation, and apoptosis in numerous cell types and tissues whose biological functions are mediated by the IGF-1 receptor (IGF-1R) (Reboucas et al., 2013). Many studies have demonstrated that IGF-1 plays an important role in both bone and tooth development. IGF-1 can increase the proliferation and osteogenic gene expression of mesenchymal stromal cells and enhance bone morphogenic protein-2 (BMP-2)-induced mineralization (Doorn et al., 2013). IGF-1 augments bone formation in a sheep model when defects are created in humeral and femoral condyles, which indicates that IGF-1 has the osteoinductive potential for bone healing (Luginbuehl et al., 2013). When IGF-1 is released from the bone matrix during bone remodeling, this action will stimulate the osteoblastic differentiation of MSCs. Further investigation demonstrated that IGF-1 can stimulate osteoblast differentiation due to the activation of the mammalian target of rapamycin (mTOR) through the PI3 K/Akt pathway (Xian et al., 2012). Moreover, IGF-1 can promote the proliferation and osteogenic differentiation of hDPSCs *via* PI3 K/Akt pathway (Feng et al., 2014). In addition, IGF-1 can stimulate the osteoblast differentiation and mineralization in stem cells *in vitro*, while proliferation can be blocked by an ERK/MAPK inhibitor or PI3-kinase inhibitor, suggesting that the effects of IGF-1 on osteoblast differentiation may be mediated *via* MAPK and Akt pathways (Zhang et al., 2012). Therefore, IGF-1 is an essential growth factor that regulates the processes necessary for the proliferation and differentiation of mesenchymal stem cells.

Our previous studies have revealed that exogenous IGF-1 can stimulate the proliferation and expression of several osteoblast-related markers *in vitro* in stem cells harvested from apical papillae (SCAPs), while it increases the osteogenic abilities of SCAPs *in vivo* (S. Wang et al., 2012). Moreover, IGF-1 can modify the ultrastructure and enhance the proliferation and osteogenic differentiation of periodontal ligament stem cells (PDLSCs) *via* the ERK and JNK MAPK pathways (Yu et al., 2012). In this study, multi-colony derived hDPSCs were isolated from healthy third molars and purified as described previously (Yu et al., 2010). The effects of IGF-1 on the proliferation and committed differentiation of hDPSCs was then examined *in vitro*, and the potential mechanisms by which IGF-1 mediates the differentiation of hDPSCs were explored.

2. Materials and methods

2.1. IGF-1 preparation and isolation of hDPSCs

IGF-1 powder (Peprotech, Offenbach, Germany) was dissolved in distilled water according to the manufacturer's instructions. Then the IGF-1 solution was aliquoted and stored at −70 °C. To isolate the stem cells from dental pulp, dental pulp tissues were obtained from healthy patients undergoing third molar extractions (age range: 20–28 years) at Jiangsu Provincial Stomatological Hospital. All subjects provided informed consent prior to sample collection. And all experimental protocols were approved by the Ethics Committee of the Stomatological School of Nanjing Medical University. Dental pulp stem cells were isolated and cultured as previously described (Yu et al., 2010). Briefly, the dental pulp was gently removed from the tooth, then minced and digested in a solution containing 3 mg/mL collagenase type I (Sigma) and 4 mg/mL dispase (Sigma) at 37 °C for 30 min. Then, these cells were purified by using rabbit anti-STRO-1 antibody (Santa Cruz) and sheep anti-rabbit IgG Dynabeads (Dynal Biotech) according to the standard procedures for magnetic activated cell sorting (MACS). The purified stem cells were cultured in α-minimum essential medium (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). The medium was changed every 3 days, and the cells were passaged by trypsinization (Gibco) once they reached 80% confluency. Cells between passages 2–4 were used for experimentation.

2.2. MTT assay

The proliferation of hDPSCs was measured by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl nyltetrazolium bromide, Sigma) assays. Briefly, hDPSCs were seeded in 96-well plates (Costar) at an initial density of 3×10^3 cells/well until they reached 60% confluence, at which point they were serum-starved for 24 h. IGF-1, at a concentration of 100 ng/mL, was added to the cells in the experimental groups. Cells cultured in α-MEM medium (supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) without IGF-1 served as the control group. After 1, 3, 5, 7, and 9 days of culture, the cells were treated with the MTT reagent followed by incubation at 37 °C for 4 h. Then, the medium was removed, and the cells were rinsed twice with 0.01 M PBS. Dimethyl sulfoxide (Sigma) was added to each well to dissolve the formazan crystals. The optical density (OD) values were read by using an automatic enzyme-linked immunosorbent assay reader (ELx800, BioTek Instruments Inc., USA) at 490 nm. The experiment was repeated at least three times and the data were described as mean ± SD.

2.3. Alkaline phosphatase activity and alizarin red staining

HDPSCs were seeded in 96-well plates (Costar) at an initial density of 3×10^3 cells/well until they reached 60% confluence, at which point they were serum-starved for 24 h. The α-MEM medium, containing 100 ng/mL IGF-1 was added to the cells in the experimental groups,while cells cultured without IGF-1 served as the control group. Each sample was assessed at days 3, 5, 7, and 9 with an ALP kit (Sigma). Briefly, cells in 96-well plates were washed with 0.01 M PBS three times, then lysed in 50 μL Lysis Buffer, incubated at 4 °C overnight, then 50 μL alkaline buffer solution and 50 μL stock substrate solution were added and mixed well. Fifteen minutes later, 110 μL 0.5 N NaOH was added to stop the reaction.