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AGS3 is involved in TNF-α medicated osteogenic differentiation of human dental pulp stem cells

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ABSTRACT

Dental pulp stem cells (DPSCs) are multipotent adult stem cells capable of differentiating along the osteoblast, adipocyte, and chondrocyte lineages. Regulating differentiation of DPSCs may be a useful tool for regenerative medicine and cell-based therapy in oral diseases. Multisignaling pathways are involved in osteogenic differentiation of DPSCs. Recent studies show that cAMP/PKA/CREB signaling could stimulate the expression of genes such as bone morphogenic proteins 2 (BMP2), inhibitor of DNA binding 2 (ID2), bone sialoprotein, osteocalcin, and type XXIV collagen, which have been implicated in osteogenesis and bone formation. Activator of G-protein signaling 3 (AGS3, gene name G-protein signaling modulator-1, Gpsm1), an accessory protein for G-protein signaling, plays an important role in regulating the phosphorylation of cyclic AMP response element-binding protein (p-CREB). However, the involvement of AGS3 in osteogenic differentiation of DPSCs has not been explored. Our data indicated that increased expression of AGS3 would inhibit osteogenic differentiation of DPSCs exposed to inflammatory cytokine tumor necrosis factor α (TNF-α) via cAMP/PKA/CREB signaling. The negative role of AGS3 in osteogenic differentiation was further confirmed by knocking down and over expression of AGS3. Our findings may provide clinical implications for osteoporosis.

1. Introduction

Bone loss through oral diseases such as periodontitis or periapical infections is highly prevalent among adults, however, there is no effective treatment to restore lost bone (Jansson et al., 1995; Xu et al., 2014). Transplantation of mesenchymal stem cells (MSCs) has been proposed as a strategy with enormous potential because of their great availability, vigorous self-renewal, and multipotency without serious ethical or technical problems (Mareddy et al., 2009; Venkataramana et al., 2012). Numerous researches have shown that MSCs can differentiate into osteoblasts, chondrocytes and adipocytes (Purpura et al., 2004; Jaiswal et al., 1997). DPSCs have been identified as a new branch of MSCs that may provide the ability to aid in tissue engineering (Gronthos et al., 2002). Furthermore, it was demonstrated that DPSCs have higher ability of proliferation and osteogenic differentiation than that of bone marrow derived MSCs (Mori et al., 2010; Alge et al., 2010). Moreover, DPSCs have great potential to allogenic transplantation to restore tissue damages without the use of immunosuppressive agents (Pierdomenico et al., 2005; Zheng et al., 2009). The more important is that extracted teeth as a waste product of medical procedures can be easily obtained without posing serious ethical and technical problems (Rodriguez-Lozano et al., 2012). Therefore, DPSCs possess a great promise for banking and long-term cryopreservation to that can further extend their therapeutic

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application in bone loss (Mori et al., 2011; D'Alimonte et al., 2013). However, when DPSCs are transplanted for cell replacement therapy, transplanted stem cells mostly are exposed to unfavorable conditions such as hypoxia or cytokines release from inflammatory cells (Yang et al., 2012). Then, inflammatory cytokines could change their characteristics.

The pathogenesis of alveolar bone loss surrounding the tooth involves a multitude of bacterial- and host-related factors (Rettori et al., 2012). TNF-α, for instance, has an extremely broad spectrum of biological activity and plays a central role in many inflammatory diseases (Choy and Panayi, 2001). Researches have implied that the concentrations of TNF-α apparently contribute to the pathogenesis of periodontitis (Gorska et al., 2003) and higher serum concentrations of TNF-α are detected in periodontitis patients (Engebretson et al., 2007; Havemose-Poulsen et al., 2005). Furthermore, it has been indicated that TNF-α, a modulator of immune responses, can induce bone destruction by promoting osteoclast differentiation and inhibiting osteoblast differentiation (Lisignoli et al., 2004; Zhao et al., 2012). So we used TNF-α treatment to mimic the inflammatory microenvironments and investigate the mechanisms of osteogenesis in DPSCs treated with TNF-α. Early researches have demonstrated that TNF-α down regulates expression of BMP2 and alkaline phosphatase (ALP) in DPSCs (Kong et al., 2013). However, different concentrations of TNF-α treatment have lead to distinct results. We have confirmed that TNF-α (10 ng/mL) promoted osteogenic differentiation of DPSCs by activating the NF-κB pathway (Feng et al., 2013a). Other scholars have also found that TNF-α at lower concentrations (0.01 and 0.1 ng/mL) enhances ALP activity in MSCs when compared with the control cells (treated with 0 ng/mL TNF-α), while cells treated with TNF-α at higher concentrations (100 ng/mL) show decreased osteogenic differentiation capability (Huang et al., 2011). Similarly, TNF-α (50 ng/mL) suppressed alkaline phosphatase activity and stimulated mineralized matrix deposition in vitro osteogenic differentiation of MSCs (Mountziaris et al., 2013). However, the mechanism of TNF-α on osteogenic differentiation of DPSCs remains to be unclear.

Activator of G-protein signaling 3 (AGS3) has been first identified during a functional screen for mammalian proteins that activates heterotrimeric G-protein signaling in a receptor-independent manner in *Saccharomyces cerevisiae*. It has been shown that AGS3 could regulate the phosphorylation of cyclic AMP response element-binding protein (p-CREB) (Shao et al., 2014). Thus, cAMP/PKA/CREB pathway could regulate osteoclast-inducing potential of MSCs, PKA stimulators increased phosphorylation of CREB to regulate expression of Runx2 and the ratio of RANKL/OPG (Yang et al., 2008), treatment of hMSCs with db-cAMP lead to PKA activation, which stimulates in vitro osteogenic differentiation of hMSCs (Siddappa et al., 2008). And it has been reported that cAMP/PKA signaling participates in osteogenic differentiation and bone formation in osteoblasts (Wang et al., 2010). However, how does AGS3 regulate osteogenic differentiation of DPSCs still remained controversial.

In this study, we found TNF-α at high concentration (100 ng/mL) decreased osteogenesis and analyzed the function of AGS3 on osteogenic differentiation of DPSCs for the first time. Additionally, we studied the role of the cAMP/PKA/CREB pathway in this process. Our data revealed that AGS3 inhibits osteogenic differentiation of DPSCs treated with TNF-α via the cAMP/PKA/CREB signaling pathway. Our findings may provide clinical implications for osteoporosis.

2. Materials and methods

2.1. Cell cultures

Bone marrow aspirates were obtained aseptically from three donors (male, 40–65 years old) with informed consent. Five milliliters of heparinized BM were mixed with an equal volume of phosphate-buffered saline (PBS). Then, the resuspended cells were layered over Ficoll solution (1.077 /mL) and centrifuged at $2000 \times g$ for 25 min at room temperature. The mononuclear cells were collected at the interface and resuspended in low-glucose Dulbecco Modified Eagle Medium (L-DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS). Then, the cells were plated at a density of 2×10^7 cells per 25 cm^2 dish and cultured at 37 °C in a 5% CO_2 incubator, and the medium was replaced and non-adherent cells were removed after 5 days and every three days thereafter. When the MSCs became nearly confluent, the adherent cells were released from the dishes with 0.25% trypsin-EDTA (Gibco, USA), and then replanted at a density of 1×10^6 cells per 25 cm^2 dish.

Human dental pulp was obtained from freshly extracted mature human third molars from healthy subjects recruited at the Ethics Committee of the Affiliated Hospital of Nantong University. Informed consent was obtained from each subject, and the study was approved by the institutional Medical Ethics. Freshly extracted teeth were stored in serum-free alpha minimum essential medium (a-MEM; Hyclone, Logan, UT, USA) and transported to the laboratory within 60 min of extraction.

Pulp tissue was isolated from the crown and root and digested in PBS containing 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 h at 37 °C. Single-cell suspensions were obtained by passing the digested tissues through a 70-μm cell strainer (BD Falcon). Cell suspensions of dental pulp were seeded into 10-cm culture dishes and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2. The culture medium was changed every 3 days. Cells from the third to fifth passages were used in the follow-up experiment.

DPSCs exhibited a fibroblast-like morphology (Supplement Fig. 1A). DPSCs obtained from the cell clones that were grown for 14 days were characterized by immunofluorescence staining and flow cytometric analysis. Immunofluorescence staining of DPSCs revealed that the cells positively expressed STRO-1 and Vimentin and were negative for Cytokeratin expression (Supplement Fig. 1B–D). For flow cytometric analysis, we have already revealed that these cells were highly positive for CD29 and CD105, but negative for CD31 and CD34 (Feng et al., 2013b). The multipotency of DPSCs was identified by chondrogenic, adipogenic, osteogenic and neurogenic. Chondrogenic differentiation of DPSCs was demonstrated by positive staining with toluidine blue (Supplement Fig. 1E). After 3 weeks in adipogenic medium, DPSCs developed into Oil Red O-positive lipid-laden fat cells (Supplement Fig. 1F). After 2 weeks of osteogenic induction, the cells stained positive for mineral nodules with Alizarin red S staining (Supplement Fig. 1G). The intensity of neurogenic differentiation was examined by immunofluorescence, DPSCs expressed Nestin marker (Supplement Fig. 1H).

2.2. Osteogenic differentiation

DPSCs/MSCs were plated at a density of 2×10^4 cells/cm^2 and incubated in osteogenic differentiation medium containing 0.1 μM dexamethasone, 10 mM β-glycerophosphate (Sigma), 50 μg/mL ascorbic acid (Sigma) and 10% FBS . DPSCs were differentiated for 3, 5, 7, and 14 days in the absence or presence of TNF-α. TNF-α was added every 3 days. The degree of extracellular matrix calcification was estimated using an Alizarin red S (Sigma) and Alkaline phosphatase (JianCheng, Nanjing, China) staining.

DPSCs were plated at a density of 2×10^4 cells/cm^2 and cultured in growth medium supplemented with osteogenic medium for 14 days. 100 ng/mL TNF-α was added or not. For pharmacological activation of cAMP/PKA/CREB signaling pathway, DPSCs were supplemented with 1mM dibutyryl-cAMP (db-cAMP, PKA activity