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Expression of odontogenic ameloblast-associated protein in the dental follicle and its role in osteogenic differentiation of dental follicle stem cells

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ABSTRACT

Objective: Odontogenic Ameloblast-Associated Protein (ODAM) is encoded by a secretory calcium-binding phosphoprotein cluster gene, which generally plays an important role for mineralization. Dental follicle (DF) is essential in regulating bone formation for tooth eruption. This study aims to reveal ODAM expression in the DFs of developing and erupting molars, and to determine the possible role of ODAM.
Design: DFs were collected from human third molars and rat mandibular molars for gene expression assessment and for establishment of cell cultures. RT-PCR and western blot were conducted to determine ODAM expression. Over- or silencing expression of ODAM in the dental follicle stem cells (DFSCs) was done by transfecting the cells with ODAM plasmid or siRNA to evaluate ODAM effects on osteogenesis.
Results: Rat DFs weakly expressed ODAM at early-postnatal days, but a chronological increment of ODAM expression from days 1 to 11 was observed. Differences in expression of ODAM were seen in the human DFs of different individuals. In vitro, ODAM was expressed in DFSCs, but almost no expression in DF-derived fibroblast-like cells. Forcing the DFSCs to overexpress ODAM accelerated osteogenesis, whereas continuously silencing the ODAM in the DFSCs reduced osteogenesis only at 2 weeks of osteogenic induction.
Conclusions: ODAM is differentially expressed in the DFs of different age molars. Its expression is coincident with the increased bone formation of tooth crypt during tooth eruption in rat DFs. Increase of ODAM expression may accelerate osteogenic differentiation of DFSCs. Thus, ODAM expression in the DF may regulate bone formation for timely tooth eruption.

1. Introduction

Tooth development involves a series of complicated mineralization processes. Teeth and other hard tissues can be viewed as the results of mineralization in the extracellular matrix. Secretory calcium-binding phosphoproteins (SCPPs) are macromolecules involved in mineralization in bone development. Odontogenic Ameloblast-Associated Protein (ODAM, also known as Apin) is encoded by a gene in the SCPP cluster (Kawasaki & Weiss, 2003), and ODAM has been reported to be primarily expressed in the cells and tissues related to mineralization, such as in ameloblasts (Moffatt, Smith, St-Arnaud, & Nanci, 2008) and in the enamel organ (Moffatt et al., 2008; Park et al., 2007). It is believed that ODAM plays an essential role in tooth maturation (Kestler et al., 2008). In previous studies, we observed a rapid bone growth in the bony crypt of rat erupting molars, and we proposed that the bone growth in the tooth crypt is essential for the tooth to erupt (Wise, Yao, & Henk, 2007; Wise, He, Gutierrez, Ring, & Yao, 2011).

The dental follicle (DF) is a loose connective tissue sac surrounding the unerupted tooth. A major function of DF is to regulate tooth eruption via timely and spatially controlling osteogenesis and osteoclastogenesis (Wise and King, 2008). In a preliminary study, we found that the rat DF expresses ODAM. It is logical to ask if expression of the ODAM in the DF varies during tooth eruption, and if the human DF also expresses ODAM. Given that the function of ODAM is related to mineralization, it was the objective of this study to determine if the ODAM produced by the DF participates in osteogenesis for the alveolar bone growth seen in the erupting teeth. To address these questions, we collected human DFs from impacted third molars of human patients to determine ODAM expression and rat DFs from pups to determine

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the chronological expression of ODAM in the first mandibular molar at different postnatal days prior to the onset of the tooth eruption. Because studies have proved the existence of stem cells in the DF (Honda et al., 2011; Yao, Pan, Prpic, & Wise, 2008), we have proposed that the dental follicle stem cells (DFSCs) may contribute to bone formation needed for tooth eruption. Thus, another objective of this study was to explore ODAM expression in the DFSCs and to determine if ODAM can promote osteogenic differentiation of DFSCs.

2. Materials and methods

2.1. Dental follicle and cell cultures

Human DFs were collected from the impacted wisdom teeth removed at the Pacific Oral and Facial Surgery Center (Livermore, CA, USA). Human tissue collection has been approved by Institutional Review Board (IRB) of Louisiana State University. DFs of Sprague Dawley rats were surgically collected from the first mandibular molars of rat pups at postnatal days 1, 3, 5, 7, 9 and 11 in 3 independent litters for RNA extraction to determine chronological gene expression. The protocol for using rats has been approved by Institutional Animal Care and Use Committee (IACUC) of Louisiana State University. For establishment of cell cultures, the DFs collected from rat pups or from the human patients were minced into small pieces and then digested with trypsin to obtain cell suspension. The cell suspension was centrifuged at 3000 RPM to collect cell pellets. For DFSC cultures, cells were cultured in the medium consisting of α-Minimum Essential Medium plus 20% fetal bovine serum (Atlanta Biologicals, Inc., Flowery Branch, GA, USA) and proper antibiotics. Our previous studies showed that cell populations derived from dental tissue under this culture condition contain stem cells (Yao et al., 2008). For non-stem dental follicle cell (DFC) cultures, cells were cultured in Minimum Essential Medium and 10% Gibco newborn calf serum (Thermo Fisher Scientific Inc., USA) with antibiotics. Studies indicated that DFC cultures derived from this condition are fibroblast-like cells (Wise, Lin, & Fan, 1992), and do not possess capability of differentiation (Yao et al., 2008). For establishing both DFSC and DFC cultures, the primary cells were cultured in T-25 polystyrene flasks at 37 °C, 5% CO_2. Non-adherent cells were removed by changing the medium after overnight incubation. The adherent cells remaining in the flasks were grown to 80–90% confluency before passaging into a T-75 flask. For subsequent passages, each T-75 flask was passaged into 3 flasks. The established cultures were subjected to osteogenic differentiation assay to evaluate their osteogenesis capability at passage 3, as described below in the section of "Induction of osteogenic differentiation". The cells of passages 3 to 7 were used for the experiments in this study.

2.2. Determination of ODAM expression

DFs collected from first mandibular molars of rat pups of different postnatal days and from different human impacted molars were used for RNA isolation to determine ODAM expression. Total RNA was also extracted from the established human and rat dental follicle cell cultures. Total RNA were isolated using RNeasy kit (Promega, Madison, WI. USA) or Direct-zol™ RNA Kit (Zymo Research, Irvine, CA, USA) according to the manufacturers' instructions. The total RNA was further treated with Ambion Turbo DNase I kit (Thermo Fisher Scientific Inc. USA) to remove possible contamination of DNA. Next, the RNA was quantitated and analyzed with a Nanodrop spectrophotometer. RNA samples with OD260/OD280 greater than 1.8 were processed for RT-PCR to acquire gene expression data. Briefly, equal amounts of the RNA were reverse-transcribed into cDNA with MLV reverse transcriptase. Conventional PCR or real-time PCR was conducted using the cDNA and ODAM specific primers, and actin was used as the internal control in all RT-PCR analyses. For conventional PCR, the PCR products were electrophoresed in an agarose gel to determine the gene expression. For real-time PCR, threshold cycle (C_T) values were obtained by running the reactions in ABI 7300 real-time PCR system and relative gene expression (RGE) was calculated using the delta C_T method.

To determine if ODAM protein was indeed exist in the DF, rat pups of postnatal day 8 was sacrificed and DFs of 1st and 2nd molars were isolated and lysed in CytoBuster™ Protein Extraction Reagent (EMD Millipore Corporation) containing protease inhibitors. 20 μg of the protein from each sample was used for western blotting analysis for hybridization with anti-ODAM antibody (Biorbyt, Cambridge, UK). HRP conjugated secondary antibody was used for detection ODAM signal with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher, Rockford, IL, USA).

2.3. Induction of osteogenic differentiation

Cells (DFSCs or DFC) were seeded in the flasks or plates at a cell density around 8.0k-10.0k cells/cm^2 in α-MEM plus 20% FBS (V/V). After the cells reached about 90% confluency, the cell growth medium was replaced with osteogenic induction medium consisting of DMEM-low glucose (1.0 g/L), 10% FBS, 50 μg/mL ascorbate-2 phosphate, 10^{-8} M dexamethasone and 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA) with medium changes every 4 days for designated times. The cells then were fixed with 10% Neutral Buffered Formalin for 5 min and then stained with 1% Alizarin red solution for 5 min. The Alizarin red solution was removed and the cells were rinsed with deionized water. For further assessment of osteogenesis, alkaline phosphatase (ALP) assay was conducted in some experiments as detailed below.

2.4. Alkaline phosphatase (ALP) assay

ALP activity was measured with the QuantiFluo™ Alkaline Phosphatase Assay Kit (BioAssay Systems, Hayward, CA, USA). Specifically, 150 μl CytoBuster Protein Extraction Reagent (Millipore Novagen) containing protease inhibitor cocktail was added to each well and the cells were scraped off from the culture surface. The cell suspension was collected into a 1.5ml- microtube and placed in ice for 30 min to allow lysis of the cells. Next, the lysate was centrifuged at 15,000 rpm for 5 min. Then, 10 μl of cell lysate supernatant of each sample was pipetted into each well of black 96-well plate, and 90 μl of assay buffer was added to each well. ALP standard curve was established in the same manner by mixing 10 μl of ALP standard with 90 μl of assay buffer in each well. After 15 min of incubation at room temperature, the plate was read at excitation of 360 nm and emission of 450 nm with a SpectraMax-M2 plate reader (Molecular Devices, LLC, Sunnyvale, CA). The ALP activity was calculated using the equation given in the manufacturer's protocol (BioAssay Systems).

2.5. Effect of ODAM overexpression and knockdown on osteogenesis of DFSCs

To study the effect of ODAM on osteogenesis, ODAM overexpression experiments were conducted by transfecting plasmid vector, namely, pCMVrODAM containing rat ODAM (rODAM) coding sequence driven by a CMV promoter into human and rat DFSCs. The plasmid, pCMV3tag3 (Agilent Technologies, Santa Clara, CA, USA) without insertion of the ODAM coding sequence, was also transfected into the cells to serve as the control. Briefly,