Odontogenic Potential of Parathyroid Hormone–related Protein (107-111) Alone or in Combination with Mineral Trioxide Aggregate in Human Dental Pulp Cells

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Abstract

Introduction: Parathyroid hormone–related protein plays an important role in bone remodeling. Its N-terminal domain parathyroid hormone–related protein (107-111) is called osteostatin (OST). OST has demonstrated osteogenic potential when combined with biomaterials such as hydroxyapatite or bioceramics. However, the odontogenic potential of OST has not yet been reported. Therefore, the aim of this study was to determine whether OST has an odontogenic effect or a synergistic effect with mineral trioxide aggregate (MTA) in human dental pulp cells (hDPCs) and to examine the underlying signaling mechanisms involved in OST-mediated odontogenic differentiation. **Methods:** Viability of hDPCs on stimulation with OST or MTA was measured. Real-time polymerase chain reaction and Western blot analyses were performed to evaluate the expression levels of odontogenic markers and the activation of extracellular signal-regulated kinase (ERK). To evaluate mineralized nodule formation, alkaline phosphatase (ALP) staining and alizarin red S staining were performed. Combined effects of OST and MTA were evaluated. **Results:** OST promoted odontogenic differentiation, as evidenced by the formation of mineralized nodules, induction of ALP activity, and upregulation of odontogenic markers (dentin sialophosphoprotein, dentin matrix protein-1, and ALP). Phosphorylation of ERK was increased by OST. However, ERK inhibitor (U0126) inhibited the increase in dentin sialophosphoprotein and dentin matrix protein-1 expression and mineralization induced by OST. A combination of MTA and OST upregulated odontogenic differentiation-associated gene expression and calcium nodule mineralization in hDPCs compared with MTA alone. **Conclusions:** The present study revealed that OST can promote odontogenic differentiation and mineralization through activating the ERK signaling pathway. A combination of MTA and OST showed a synergistic effect compared with MTA alone in hDPCs. *(J Endod 2017;■:1–7)*

Key Words

Dental pulp cells, MTA, odontogenic effect, osteostatin, parathyroid hormone-related protein

Significance

OST could promote odontogenic differentiation and mineralization, and combination of MTA and OST has synergistic effect in hDPCs compared with MTA alone. OST can be used alone or in combination with MTA for regeneration of pulp dentin complex.

Parathyroid hormone–related protein (PTHrP) is a member of the parathyroid hormone family. It was originally identified as a factor involved in humoral hypercalcemia of malignancy (1–3). PTHrP has been shown to be widely expressed in many normal tissues including kidney and bone. It seems to play an important role in bone development and bone remodeling (4). Recent studies have suggested that PTHrP can induce osteogenic features through its specific C-terminal domain PTHrP (107-139). The activity of this fragment in bone appears to reside in its N-terminal domain PTHrP (107-111), also known as osteostatin (OST) (5, 6).

To date, it is widely known that PTHrP is involved in bone formation and resorption. PTHrP (107-139) not only has a strong inhibitory effect on bone resorption in healthy mice, it also has anabolic effects that promote bone formation in diabetic mice (7, 8). Meanwhile, another study reported that PTHrP (107-139) has an inhibitory effect on proliferation and differentiation of human osteoblast-like cells (9). Recently, it has been shown that OST in combination with bioceramic materials such as Si-doped hydroxyapatite and gelatin-glutaraldehyde biopolymer-coated hydroxyapatite can increase the osteogenic potential of osteoblastic cells (10–12). However, the odontogenic potential of OST has not yet been reported.

Mineral trioxide aggregate (MTA) is a type of calcium silicate cement. It has been used for root-end filling, perforation repair, pulp capping, apexification, and apexogenesis

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(13, 14). To date, several studies have revealed that MTA has the capacity to induce odontogenic/osteogenic differentiation (15, 16). Moreover, a combination of MTA and a potent odontogenic protein can promote rapid differentiation of human dental pulp cells (hDPCs) than MTA alone (17–20).

Therefore, the purpose of this study was to determine whether OST has odontogenic potential that is due to a synergistic effect when it is used in combination with MTA in hDPCs. Moreover, the underlying signaling mechanisms of OST-mediated odontogenic differentiation were examined in this study.

Materials and Methods

Cell Culture

This study was approved by the Institutional Review Board of Chonnam National University Dental Hospital, Gwangju, Korea (IRB no. CNUDH-2016-009). Written consent was obtained from each patient included in this study. Extracted human third molars with pulp tissues were obtained from the Department of Oral Maxillofacial Surgery, Chonnam National University Dental Hospital. Tooth samples were removed aseptically, rinsed with Dulbecco phosphate-buffered saline solution (PBS) (Welgene, Daegu, South Korea), and placed in 60-mm dishes. Cells were cultured in α-minimum essential medium (α-MEM) (Gibco Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (Gibco Invitrogen), 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; Gibco Invitrogen) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Dental pulp cells between the third and fifth passages were used in this study.

Preparation of MTA Conditioned Medium

MTA (ProRoot MTA; Dentsply, Tulsa, OK) was mixed with sterile water according to the manufacturer's instructions under aseptic conditions. MTA samples were then fabricated in the form of disks with use of a sterile cylindrical polyethylene tube (diameter 8 mm, height 3 mm) and incubated in culture medium containing 50 mL fresh α-MEM and antibiotics. After incubation for 7 days in a humidified atmosphere at 37°C and 5% CO_2, the supernatant of this preparation was filtered by using 0.20-μm filters (Minisart; Sartorius Stedim Biotech, Goettingen, Germany).

Cell Viability

HDPCs were seeded at a density of 1×10^4 cells per well on 96-well cell culture plates. After 24 hours of culture, cells were treated with different concentrations (10, 50, 100, 500, and 1000 nmol/L) of OST (Bachem, Bubendorf, Switzerland) or diluted MTA solution (1, 1/2, 1/4, and 1/16) for 24 hours. After 24 hours of culture, cell viability was analyzed by the WST-1 assay by using the EZ-Cytox enhanced cell viability assay kit (Daeil Lab Service, Seoul, Korea). Briefly, 10 μL EZ-Cytox reagent was added to each well in the 96-well plate and incubated at 37°C for 4 hours. The absorbance value was measured at a wavelength of 420 nm by using a spectrophotometer (VERSAmax multiplate reader; Molecular Devices, Sunnyvale, CA).

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

HDPCs were seeded at a density of 2×10^5 cells per well on 6-well cell culture plates with a growth medium. After 24 hours of culture, cells were treated with or without 100 nmol/L OST for 3 and 5 days. Total RNA was then extracted from cells by using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol. cDNA was synthesized by using a random primer (Promega Biotech, Piscataway, NJ) and AccessQuick real-time polymerase chain reaction (RT-PCR) system (Promega, Madison, WI). Quantitative RT-PCR was performed by using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) on a 72-well Rotor-Gene 6000 (Corbett Research, Sydney, Australia). Primer sequences used for PCR are listed in Table 1.

Relative gene expression values were analyzed by using the $2^{-\Delta\Delta Ct}$ method. To examine the combined effect of OST and MTA, the same assay described above was performed for cells cultured in diluted MTA solution (1/4) with or without 100 nmol/L OST.

Western Blot

OST-induced odontogenic protein expression and activation of extracellular signal-regulated kinase (ERK) were analyzed by Western blot. HDPCs were treated with or without 100 nmol/L OST. Cells were washed twice with PBS and extracted with cell lysis buffer (Cell Signaling Technology, Beverly, MA). Cell lysates were then centrifuged at 13,000 rpm for 10 minutes. Supernatants were collected, and protein concentrations were determined by using the Lowry protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA). Proteins were subjected to 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis at 80 V for 2 hours, followed by transfer from the gel to a polyvinylidene difluoride membrane at 10 V overnight. After blocking with 5% non-fat dried skim milk in PBS, 0.1% Tween 20 (PBST) at room temperature for 1 hour, the membranes were incubated with anti–dentin sialophosphoprotein (DSPP) (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA), anti–dentin matrix protein (DMP)-1 (1:2000; Abcam, Cambridge, UK), anti-ERK (1:3000; Santa Cruz Biotechnology), and anti-phospho-ERK (1:3000; Santa Cruz Biotechnology) at 4°C overnight. After washing 3 times with PBST, the membranes were then incubated with horseradish peroxidase (HRP)–conjugated anti-mouse immunoglobulin (Ig) G or anti-rabbit IgG secondary antibodies (1:10,000; Sigma-Aldrich, St Louis, MO) at room temperature for 1 hour. After washing with PBS 5 times, chemiluminescent HRP substrate (Millipore, Billerica, MA) was used to visualize protein signals with a Chemiluminescence Imaging System (Ez-capture; Atto, Tokyo, Japan).

Alkaline Phosphatase Staining

HDPCs were seeded at a density of 2×10^4 cells per well on 24-well cell culture plates and cultured in growth medium with or without 100 nmol/L OST for 7 days. Fresh growth medium was replaced every 2 days. After 7-day exposure, the medium was removed, and cells were washed with PBS and fixed in 70% ethanol for 1 hour, followed by rinsing with distilled water 3 times. Fixed cells were treated with 300 μL alkaline phosphatase (ALP) staining reagent (1-step NBT/BCIP solution; Thermo Fisher Scientific Inc, Rockford, IL) per well. After removing the staining reagent, a photograph was taken by using an Officejet Pro L7580 scanner (Hewlett-Packard, Palo Alto, CA). To quantitatively evaluate the staining results, the stain was treated with 10%

TABLE 1. List of Primers Used for RT-PCR

Gene	Sequences (5′–3′)
DSPP	Forward: GGG AAT ATT GAG GGC TGG AA
	Reverse: TCA TTG TGA CCT GCA TCG CC
DMP-1	Forward: TGG TCC CAG CAG TGA GTC CA
	Reverse: TGT GTG CGA GCT GTC CTC CT
ALP	Forward: GGA CCA TTC CCA CGT CTT CAC
	Reverse: CCT TGT AGC CAG GCC CAT TG
β-actin	Forward: CTC CTT AAT GTC ACG CAC GAT
	Reverse: CCT TGT AGC CAG GCC CAT TG