Gene Expression and Cytokine Release during Odontogenic Differentiation of Human Dental Pulp Stem Cells Induced by 2 Endodontic Biomaterials

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Abstract

Introduction: Mineral trioxide aggregate (MTA) and calcium-enriched mixture (CEM) have shown osteogenic/cementogenic/dentinogenic activities; however, their mechanism of action is not fully understood. We aimed to evaluate the effect of these biomaterials on odontogenic differentiation of human dental pulp stem cells (DPSCs). **Methods:** Flow cytometry with stem cell markers for the confirmation of stemness and homogeneity was first performed. Then isolated DPSCs were seeded on prepared discs of MTA, CEM, differentiation medium (DM), and growth medium (GM) and incubated up to 14 days. Concentrations of transforming growth factor-β1, bone morphogenetic protein (BMP)2, BMP4, and fibroblast growth factor 4 were measured at each interval using an enzyme-linked immunosorbent assay reader. Gene expression of *dentin sialophosphoprotein, dentin matrix protein 1,* and the cytokines were evaluated by reverse-transcription polymerase chain reaction. To evaluate the cell morphology, scanning electron micrographs were taken; mineralization potential was evaluated using alizarin red S staining. **Results:** Scanning electron micrographs showed that DPSCs spread/adhered/proliferated similarly on MTA and CEM. On day 14, alizarin red S staining confirmed that mineralization occurred in all groups except GM. Expressions of *dentin matrix protein 1* and *dentin sialophosphoprotein* genes were similar in the CEM, MTA, and DM groups; they were significantly higher compared with the GM group ($P < .05$). A greater amount of *transforming growth factor-β1* gene was expressed in MTA compared with the other groups ($P < .05$). However, the expression *of fibroblast growth factor 4* and *BMP2* genes was significantly greater in the CEM group ($P < .05$). In all the tested groups, the expression of *BMP4* was less than GM ($P < .01$); however, CEM and DM were similar but more than MTA ($P < .05$). Concentrations of protein product detected using an enzyme-linked immunosorbent assay reader confirmed these gene expressions. **Conclusions:** MTA and CEM can induce osteo-/odontogenic-like phenotype differentiation of human DPSCs; however, they stimulate different gene expressions and growth factor release. *(J Endod 2014;40:387–392)*

Key Words

Biomaterials, calcium-enriched mixture, CEM cement, dental pulp capping, differentiation, human, mineral trioxide aggregate, pulp regeneration, stem cell

Pulp exposure is the reason for innumerable pulpectomy procedures of vital teeth each year worldwide. In 1999 alone, endodontists performed 4 million primary root canal therapies in private practices in the United States (1). Recently, vital pulp therapy (VPT) has become a major center of attention (2), and apart from its economic advantage, VPT allows normal tooth function, sensation, and proprioception as well as long-term survival (3). The rigid dentin periphery of the pulp chamber provides a powerful support for coronal dental pulp. Untreated dental caries may break down the integrity of the normal dentin-safe shield, leading to pulp exposure and subsequent inflammation/infection. Various researches have addressed tissue engineering strategies for reconstitution of a normal dentin-pulp complex based on the differentiation potential of dental pulp stem cells (DPSCs) (4). Theoretically, an ideal regenerative process in VPT teeth should induce dentinogenesis in new odontoblast-like cells originating from DPSCs. In turn, the dentinogenesis reintegrates the dentin shield and reprotects the threatened exposed pulp from further irritation.

Signaling molecules (ie, growth and differentiation factors) and responding DPSCs are the 2 main control keys of dentin regeneration/dentinogenesis. The transforming growth factor (TGF) super family, including TGF-β, activins/inhibins, and bone morphogenetic protein (BMP), regulate cell proliferation/differentiation, epithelial-to-mesenchymal change, and embryonic maturity. TGF-β1 is a potent inducer for normal odontogenic differentiation and dentinogenesis (5). BMP2, BMP4, and fibroblast growth factor (FGF)-4 signalings are also important participants in embryonic tooth development, regulating early tooth morphogenesis and subsequent odontogenic differentiation (6, 7). In addition, one of the most highly expressed odontoblast-specific terminal differentiation genes is *dentin sialophosphoprotein (DSPP)* (8). Dentin matrix protein 1 (DMP1) also plays a regulatory

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role in collagen matrix organization and dentin mineralization and can also function as a signaling molecule (9).

Previous researches have shown that mineral trioxide aggregate (MTA) and calcium-enriched mixture (CEM) cement are promising endodontic biomaterials for capping primary/permanent human exposed pulps (10, 11) and that they are nontoxic and biocompatible (12, 13). They have shown that MTA and CEM have similar effects in terms of hard-tissue formation. Moreover, when they were applied in bone defects, they showed osteogenesis (14); after perforation repair and root-end filling, they induce cementogenesis (15, 16); and after pulp capping or pulpotomy of vital dental pulp, MTA and CEM stimulate dentinogenesis (17, 18). There have been a few studies regarding the effects of MTA on DPSCs (19–21); however, the exact mechanism of dentinogenesis after dental pulp exposure and the DPSCs' response to MTA and CEM cement have not yet been completely explicated. The aim of this study was to evaluate the biologic effects of the 2 biomaterials on odontogenic differentiation of DPSCs.

Materials and Methods

Preparation of MTA and CEM Samples

MTA (ProRoot; Dentsply, Tulsa, OK) and CEM (BioniqueDent, Tehran, Iran) were prepared according to manufacturer instructions, were casted in disk shape with a 5-mm diameter aseptically, and were incubated at 37°C and humidity of 95% for 24 hours for complete setting. After this period, all samples were seeded by cells at the same time.

Cell Culture

An intact human third molar tooth was disinfected by 70% ethanol and dissected at the crown-root border. Pulpal tissue was digested enzymatically using 0.1% collagenase, 0.05% trypsin, and 0.5 mmol/L EDTA in 0.1 mol/L phosphate buffer saline solution (PBS) for 20 minutes at 37°C. Suspended cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 100 IU/mL penicillin-streptomycin, and 0.25 g/mL Fungizone (Invitrogen, Carlsbad, CA) and incubated at 37°C, 5% CO_2_ condition, and a humidity of 85%. Cells were subcultured at 80% confluency 3 times. Third passage cells were evaluated for surface markers using flow cytometry using antibodies against CD29, CD44, CD49b, CD90, and STRO1 (Invitrogen).

Cells were seeded on prepared MTA and CEM disks at 5×10^5 cells/cm^2 in 24-well culture plates containing DMEM and supplemented with 15% FBS and 100 IU/mL penicillin-streptomycin. The same number of cells were cultured in similar culture plates with DMEM (growth medium [GM]) and DMEM supplemented with 10 mmol/L glycerol 2-phosphate (Fluka, St Louis, MO), 0.2 mmol/L L-ascorbic acid 2-phosphate (L-ascorbic acid 2-phosphate hydrate sesquimagnesium salt; Sigma-Aldrich, St Louis, MO), 0.1 μmol/L dexamethasone (Sigma-Aldrich), and 0.1 mmol/L 1, 25-dihydroxy vitamin D3 (Sigma-Aldrich) as differentiation medium (DM). All samples were incubated for 1, 3, 7, and 14 days at 37°C, 5% CO_2_, and humidity of 85%.

Enzyme-linked Immunosorbent Assay Reader

Supernatant of samples were saved at the aforementioned intervals, and concentrations of FGF4, BMP2, BMP4, and TGF-β1 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, MA) according to manufacturer instructions. Briefly, 100 μL cell supernatants were entered into the test in triplicate incubations. Washings and adding antibodies, enzymes, substrate, and chromogenic solutions were performed, and the reactions were stopped according to a timetable. Optical densities of samples were measured at 450 nm using the Biotek ELX800 microplate reader (Biotek, Winooski, VT). Standard curves were plotted using known concentrations of proteins included in the kits, and concentrations of mentioned growth factors were calculated against optical density and stated in ng/μL.

Real-time Reverse-transcription Polymerase Chain Reaction

A quantitative analysis of the expression of *FGF4, BMP2, BMP4, TGF-β1, alkaline phosphatase (ALP), collagen type I, DSPP,* and *DMP1* genes relevant to odontogenic differentiation of cells was performed. Samples with loaded cells were crashed by sonication followed by the removal of cement residuals using a cell strainer with 40-μmol/L pores. Pass-throw suspension composed of both intact and lysed cells was used for RNA isolation procedure. Briefly, the total RNA was collected from cells using the RNeasy Mini Kit (Qiagen, Seoul, Korea). The standard reverse-transcription reaction was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo, Waltham, MA) according to the manufacturer's instructions. Subsequent real-time polymerase chain reaction (RT-PCR) was performed with Power SYBR Green Real-Time Master Mix and Stepone Real-Time PCR (Applied Biosystems, Carlsbad, CA). The mentioned primers (Table 1) were used, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was used as the housekeeping gene. Each reaction was triplicated.

Scanning Electron Microscopy

For scanning electron microscopic analysis, culture medium was removed, the cell loaded specimens were rinsed in PBS twice, and the cells were then fixed with 2.5% glutaraldehyde solution (Sigma-Aldrich). After 90 minutes, they were rinsed with PBS and fixed with 1% osmium tetroxide (Sigma-Aldrich). Specimens were dehydrated in ethanol solutions of ascending concentrations (ie, 30%, 50%, 70%, 90%, and 100%) for ~20 minutes at each concentration. The specimens were then left to dry in air. After being completely desiccated, the specimens were mounted on copper stubs, coated with gold, and observed by scanning electron microscopy.

Alizarin Red Staining

At the end of the second week, wells were washed with PBS, and cells attached onto bottom of wells were fixed with methanol (Merck, Whitehouse Station, NJ) for 10 minutes. Fixed cells were exposed to dye solution (1% alizarin red S in ammonia water 25%) for a period

TABLE 1. Specific Primer Sets Target for Differentiation Markers and *GAPDH* Used as the Control

Gene name	Direction	Sequence
DMP1	Forward	5′-CAACTGGCTTTTCTGTGGCAA-3′
	Reverse	5′-TGGGTGCGCTGATGTTTGCT-3′
DSPP	Forward	5′-CAACCATAGAGAAAGCAAACGCG-3′
	Reverse	5′-TTTCTGTTGCCACTGCTGGGAC-3′
TGF-β1	Forward	5′-CCCAGCATCTGCAAAGCTC-3′
	Reverse	5′-GTCAATGTACAGCTGCCGCA-3′
Collagen type I	Forward	5′-GCCCTGTCTGCTTCCTGTA-3′
	Reverse	5′-TTTGGGTTGGTTGTCTGTTT-3′
BMP2	Forward	5′-GCTAGTAACTTTTGGCCATGATG-3′
	Reverse	5′-GCGTTTCCGCTGTTTGTGTT-3′
BMP4	Forward	5′-TCCACAGCACTGGTCTTGAG-3′
	Reverse	5′-GGGATGTTCTCCAGATGTTCTT-3′
FGF4	Forward	5′-CCAACAACTACAACGCCTACGA-3′
	Reverse	5′-CCCTTCTTGGTCTTCCCATTCT-3′
ALP	Forward	5′-CCACAAGCCCGTGACAGA-3′
	Reverse	5′-GCGGCAGACTTTGGTTTC-3′
GAPDH	Forward	5′-ACAGTCAGCCGCATCTTCTT-3′
	Reverse	5′-ACGACCAAATCCGTTGACTC-3