Mineral Trioxide Aggregate Induces Bone Morphogenetic Protein-2 Expression and Calcification in Human Periodontal Ligament Cells

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Abstract

Introduction: Mineral trioxide aggregate (MTA) is a therapeutic, endodontic repair material that is reported to exhibit calcified tissue-conductive activity although the mechanisms remain unclear. We hypothesize that the dissolution of calcium from MTA into the surrounding environment may play an important role in the osteoblastic/cementoblastic differentiation of human periodontal ligament cells (HPLCs). **Methods:** Two populations of HPLCs were obtained from two patients, respectively, and were cultured in the presence or absence of MTA discs and/or $CaCl_2$ in order to investigate calcium release, calcification activity, calcium-sensing receptor (CaSR) gene expression and bone morphogenetic protein-2 (BMP-2), and BMP-2 receptor protein and gene expression. **Results:** MTA released a substantial accumulation of calcium (4 mmol/L) within 14 days into culture media. After 4 weeks, the two populations of HPLCs independently exhibited calcification as well as BMP-2 distribution in the vicinity of MTA. HPLCs inherently expressed genes encoding for the CaSR and BMP-2 receptors. Exogenous $CaCl_2$ media supplementation induced CaSR gene expression in HPLCs and calcification and BMP-2 synthesis throughout the entire HPLC cultures, whereas $MgCl_2$ had no effect. Both MTA and $CaCl_2$ stimulated BMP-2 gene expression above that of baseline levels. **Conclusion:** Here we show the first report showing that HPLCs cocultured directly with MTA up-regulated BMP2 expression and calcification. These results may be through CaSR interactions that were potentially activated by the release of calcium from MTA into the culture environment. *(J Endod 2010;36:647–652)*

Key Words

Bone morphogenetic protein 2, calcification, calcium, mineral trioxide aggregate, periodontal ligament cells

The periodontal ligament (PDL) attachment apparatus consists of the PDL, the cementum, and the bone. The PDL is a highly specialized connective tissue anchoring the tooth root to the bone of the tooth socket (1). The PDL is composed of a heterogeneous cell population including fibroblasts, cementoblasts, osteoblasts, and undifferentiated mesenchymal stem/progenitor cells (2, 3). The undifferentiated mesenchymal cells included in the PDL probably exhibit a degree of plasticity because they are also capable of undergoing osteoblastic or cementoblastic differentiation *in vitro* (4) and can express osteopontin (OPN) and osteocalcin (OCN) (5) to generate calcified deposits (6). Recently, we have developed and characterized the immortalized human PDL cells (HPLCs) comparing with normal human HPLCs that showed the spindle-shaped features and calcification in the osteogenic induction media and expressed Runx2, Osterix, alkaline phosphatase, OPN, OCN, periostin, alpha-smooth muscle actin, type XII collagen, Epidermal Growth Factor (EGF)-receptor, RANKL, and osteoprotegerin (7). Furthermore, we have established and characterized two clonal cell lines from PDL stem/progenitor cells (8, 9).

Mineral trioxide aggregate (MTA) primarily consists of tricalcium silicate, dicalcium silicate, tricalcium aluminate, bismuth oxide, and gypsum. This material is reportedly biocompatible with HPLCs (10) and has been shown to induce new cementum tissue on its surface when applied to tooth root defects in monkeys (11) and canines (12, 13). A recent report suggested that when MTA was immersed in phosphate buffered saline, the carbonated apatite precipitates were formed on its surface; this could be involved in cementogenesis (14). However, the calcified tissue-conductive mechanism of MTA is not entirely clear.

Human pulp cells cultured indirectly with MTA were indicated to express bone morphogenetic protein-2 (BMP-2) (15). BMPs are known to prompt the commitment of mesenchymal stem cells into specific lineages (eg, BMP-2 supports osteoblastogenesis) (16). Additionally, BMP-2 can induce the differentiation of a subpopulation of PDL fibroblastic progenitor cells into cementoblasts (17). Mechanistically, BMPs bind to the type I and type II serine/threonine kinase receptors (18), which subsequently activate a signaling cascade to direct cell functions, including osteogenesis. However, there have been few reports showing the up-regulation of calcification and BMP2 synthesis in HPLCs cocultured directly with MTA.

The calcium-sensing receptor (CaSR) is a class 3 G-protein-coupled protein and plays a pivotal role in the regulation of extracellular calcium homeostasis by modulating the rate of parathyroid hormone secretion (19). It is also expressed in many cell types,

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including bone cells, which are not directly involved in systemic calcium homeostasis (20). The CaSR was shown to be involved in the differentiation of immature osteoblasts using a CaSR agonist or a dominant-negative variant of the CaSR (21, 22).

In this study, we aimed to analyze the bioactive effects of MTA on the cementoblastic/osteoblastic differentiation of HPLC. Here we report that MTA is a calcium-releasing material that can induce the osteoblastic/cementoblastic differentiation of HPLCs, which express the CaSR and BMP-2 receptors that are potentially involved in their stimulated osteogenesis.

Materials and Methods

Materials

ProRoot MTA was purchased from Dentsply Tulsa Dental (Tulsa, OK). Calcium chloride ($CaCl_2$) and magnesium chloride ($MaCl_2$) were purchased from WAKO Pure Chemical Industries Ltd (Osaka, Japan). Tissue culture wares were purchased from Becton Dickinson Labware (Lincoln Park, NJ).

Distilled water was mixed with MTA powder at ratio of 0.33 (w/p), and MTA discs were then prepared according to a modification of Al-Rabeah's method (23). Briefly, MTA mixtures were dispensed into plastic lids of Eppendorf microcentrifuge tubes. The specimens remained in a CO_2 incubator with a humidified atmosphere at 37°C for 12 hours. The set MTA discs (9 mm in diameter and 1 mm in thickness) were removed from the lids and rinsed with alpha-minimum essential medium (α-MEM; Gibco-BRL, Grand Island, NY), supplemented with 50 μg/mL of streptomycin and 50 U/mL of penicillin (Gibco-BRL), and were further immersed for 6 h in the same media. The discs were then placed in a 48-well or a 24-well tissue culture plate (1 disc/well) (Becton Dickinson Labware) for cell assays.

Cell Culture

Two HPLC populations were isolated as described previously (7, 24) from the healthy premolars of a 22-year-old woman (HPLC-2H) and a 14-year-old male (HPLC-2I), respectively, who visited the Dental Hospital of Kyushu University for orthodontic extractions. Briefly, the middle third region of the PDL was stripped from the root surface of the extracted tooth with a scalpel. The tissues were washed with α-MEM and incubated at 37°C for 20 minutes in α-MEM containing 0.2% collagenase and 0.25% trypsin. The dispersed cells were centrifuged, and the pellet was resuspended in α-MEM containing 10% fetal bovine serum (FBS; SAFC Bioscience, Lenexa, KS) and seeded onto 35-mm Primaria dishes (Becton Dickinson Labware). Cells from passages five through six were used in this study. HPLCs (1×10^5 cells/well) were cultured in 10% FBS/α-MEM through all the present culture systems and cultured on tissue culture plastic in the absence or presence of MTA discs, $CaCl_2$, or $MgCl_2$ for 2 to 4 weeks. All cultures were maintained in a CO_2 incubator. The procedures in this study were performed in compliance with the regulations of Kyushu University.

Human mesenchymal stem cells (hMSCs) were purchased from Cambrex (East Rutherford, NJ) and maintained in α-MEM with Single-Quots (Cambrex). Normal human skin fibroblasts (NFSFs) were purchased from Riken Cell Bank (Tsukuba, Japan) and maintained in 10% FBS/ α-MEM.

Quantification of Calcium Ion Release

Sixteen MTA discs were set on the bottom of a 24-well plate (1 disc/well), immersed in 500 μL of 10% FBS/α-MEM, and maintained at 37°C in a CO_2 incubator. Media was collected on days 1, 3, 7, and 14 (n = 4 discs per time point). The conditioned media were exposed to a QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA), and the calcium content was calculated according to the manufacturer's instructions using a microplate reader (ImmunoMini NJ-2300; System Instruments, Tokyo, Japan) at an absorbance of 590 nm. After subtraction of the blank (normal medium) optical density values from the sample optical density values, the corresponding value (mg/dL) was divided by the slope of the calcium standard curve. Because 1 mg/dL of calcium equals a concentration of 250 μmol/L, the obtained values were converted into molar concentrations.

Detection of Calcified Deposits

Cells were cultured with MTA discs on tissue culture plastic for 4 weeks under normal culture conditions. The formations of calcified deposits were visualized by von Kossa staining. Briefly, cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in deionized water and stained with 2% silver nitrate (Nacalai Tesque Inc, Kyoto, Japan) as previously described (25, 26).

Immunocytochemistry

HPLCs cultured for 4 to 9 days were fixed with 4% parafornaldehyde and 0.5% dimethyl sulfoxide (WAKO Pure Chemical Industries Ltd) in phosphate buffered saline. A rabbit polyclonal antihuman BMP-2 antibody (LifeSpan Biosciences, Inc, Seattle, WA) or a rabbit polyclonal antihuman BMP receptor-IA (BMPR-IA) antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) was suspended in 1% horse serum in phosphate buffered saline at a concentration of 10 μg/mL and applied to the fixed cells. Staining was performed with Nichirei-Histofine (Nichirei Biosciences, Inc, Tokyo, Japan) according to the manufacturer's instructions. The color reaction was developed with a new fuchsin chromogen (Nichirei Biosciences, Inc), and cells were counterstained with methyl green (Vector Laboratories, Inc, Burlingame, CA). All procedures were performed at room temperature.

Real-time Reverse Transcription-Polymerase Chain Reaction Analysis

First-strand complementary DNA was synthesized from 1 μg of total cellular RNA using an ExScript RT Reagent kit (Takara Bio Inc, Shiga, Japan), and PCR was performed using SYBR Green I (Takara Bio Inc.), in a Thermal Cycler Dice Real Time System (Takara Bio Inc) according to our previous description (9). After initial denaturation at 95°C for 10 seconds, subsequent amplification was followed by 40 cycles of 95°C for 5 seconds, the appropriate annealing temperature for 30 seconds, 72°C for 30 seconds (amplification), and a dissociation program at 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. Specific primer sequences were as follows: CaSR (128 bp), forward 5'-TCCACGGTCAGATTTGCTGTTC-3', reverse 5'-TTGATGTCCCATCAGTCTGCAC-3'; BMP2 (74 bp), forward 5'-TCCACTAATCATGCCATTGTTCAGA-3', reverse 5'-GGGACACAGCATGCCTTAGGA-3'; BMPR-IA (97bp), forward 5'-AGTGTCTCCAGTCAAGCTCTGGGTA-3', reverse 5'-CCATCTCTGCTGCGCTCATTTA-3'; BMPR-II (199bp), forward 5'-ACGGAGTATTCACTTCTGAGGATG-3', reverse 5'-GCAAGGCTTCAGACAGCTTATACA-3'; and β-Actin (β-Act) (89bp), forward 5'-ATTGCCGACAGGATGCAGA-3', reverse 5'-GAGTACTTGCGCTCAGGAGGA-3'. A β-act primer was used as an internal control. Expressions of the target genes were calculated from the delta–delta Ct ($\Delta\Delta$Ct) values. Experiments were performed in duplicate. Representative data are shown.

Results

Calcium Release From MTA

MTA is rich in calcium oxide, which is converted to calcium hydroxide on contact with tissue fluid. The calcium hydroxide further dissociates into calcium and hydroxide ions. The calcium ions released