Effect of Nifedipine on the Differentiation of Human Dental Pulp Cells Cultured with Mineral Trioxide Aggregate

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Abstract

Introduction: Mineral trioxide aggregate (MTA) can induce differentiation of the dental pulp cells into odontoblast-like cells and generate a dentin-like mineral structure. The mechanisms underlying MTA-induced odontoblastic differentiation in human dental pulp cells (HDPCs) are not completely understood. The purpose of this study was to evaluate the effect of nifedipine as calcium channel blocker on MTA-induced odontoblastic differentiation in HDPCs. **Methods:** HDPCs extracted from maxillary supernumerary incisors and third molars were directly cultured on MTA with or without nifedipine in the culture medium. Cell growth and expression of odontoblastic differentiation markers were determined by using methyl-thiazol-diphenyl-tetrazolium assay and reverse transcription–polymerase chain reaction analysis, respectively. Phosphorylation of mitogen-activated protein kinase was measured by Western blotting, and calcium deposition was assessed by using alizarin red S staining. **Results:** MTA at a concentration of 1 mg/mL significantly up-regulated the expression of dentin sialophosphoprotein and dentin matrix protein-1 and enhanced mineralized nodule formation. However, nifedipine attenuated the MTA-induced odontoblastic differentiation in HDPCs. In addition, MTA-induced mineralization was blocked by inhibition of extracellular signal-regulated kinase (ERK), p38, and Jun N-terminal kinase (JNK) by using U0126, SB203580, and SP600125, respectively. Furthermore, phosphorylation of ERK and JNK in response to MTA was inhibited when the medium was supplemented with nifedipine. **Conclusions:** This study showed that calcium ions released from MTA play an important role in odontoblastic differentiation of HDPCs via modulation of ERK and JNK activation. *(J Endod 2013;39:801–805)*

Key Words

Differentiation, human dental pulp cells, mineral trioxide aggregate, mitogen-activated protein kinase, nifedipine

Mineral trioxide aggregate (MTA) is a biocompatible material with many clinical applications in endodontic treatment as a root-end filling material, root or furcal perforation repair material, and for apexification and obturation of the root canal system (1, 2). Especially, MTA is used as an effective pulp-capping material because it is able to stimulate pulp tissue repair and dentin bridge formation by the defensive mechanism of early pulpal wound healing (3). Many recent clinical usage studies with histologic evaluation have shown that MTA showed results comparable with or better than traditional calcium hydroxide–based materials with regard to the frequency of dentin bridge formation and degree of pulp inflammation (4, 5).

During the reparative process of exposed pulps, primary odontoblasts lost as a result of extensive damage are replaced with newly differentiated odontoblast-like cells from the dental pulp. Differentiated and undifferentiated human dental pulp cells (HDPCs) within the dental pulp may contribute to the dentinal regeneration process. Odontoblasts secrete several collagenous and noncollagenous proteins, including osteonectin (OC), osteopontin (OPN), bone sialoprotein, dentin matrix protein-1 (DMP-1), and dentin sialophosphoprotein (DSPP), which have been used as mineralization markers for the odontoblast-like/osteoblast-like differentiation of HDPCs (6, 7). However, the intracellular signaling mechanism responsible for the regulation of odontoblastic differentiation induced by MTA in HPDCs is still poorly understood.

Schroder (8) demonstrated that calcium ions are necessary for differentiation and mineralization of pulp cells. Many studies have shown that the presence of large quantities of calcium ions played a role in the mineralization process and specifically modulated OPN and bone morphogenetic protein-2 levels during pulp calcification (9, 10). Fridland and Rosado (11) reported that calcium was the main component of MTA, which was based on a chemical analysis of the salts dissolved from MTA in water. In

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Supported by a grant (CNHRICM-2010) from Hospital Research Institute of Clinical Medicine and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0075373 and 2010-0006901). Also supported by the National Research Foundation of Korea (NRF) grants funded by the Korean government (MEST) (2011-0030761).

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0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2012.12.037

TABLE 1. RT-PCR Primers Sequence

Gene	Sequence (5′-3′)	Size (base pairs)
DSPP	Forward: CAGTGATGAATCTAATGG Reverse: CTGATTTGCTGCTGTCTGAC	488
DMP-1	Forward: CAGGAGCACAGGAAAAGGAG Reverse: CTGGTGGTATCTTGGGCACT	213
OCN	Forward: CCCAGGCGCTACCTGTATCAA Reverse: GGTCAGCCAACTCGTCACAGTC	112
ALP	Forward: GGACCATTCCCACGTCTTCAC Reverse: CCTTGTAGCCAGGCCCATTG	137
GAPDH	Forward: GAGTCAACGGATTTGGTCGT Reverse: GACAAGCTTCCCGTTCTCAG	185

GAPDH, gylceraldehyde-3-phosphate dehydrogenase.

addition, Takita et al (12) reported that MTA-induced proliferation of pulp cells is related to the release of calcium ions from MTA. However, the effect of calcium ions released from MTA on the differentiation and mineralization of HDPCs has not been investigated.

The aim of this study was to investigate the genetic changes related to odontoblastic differentiation in HDPCs when MTA was applied to cells and the role of calcium ions, a major component of MTA, in the differentiation of HDPCs by using an L-type calcium channel blocker, nifedipine.

Materials and Methods

Preparation of MTA Materials

The method for preparation of MTA in this study was according to the previous study (13). MTA powder (ProRoot MTA; Dentsply/Tulsa, Tulsa, OK) was added to α-minimal essential medium (α-MEM) at a concentration of 20 mg/mL. The solution was vortexed until completely suspended and then incubated for 1 week in the incubator (5% CO_2, 37°C). Supernatant obtained from this preparation was filtered before use. Cells were treated every other day with freshly prepared MTA solutions. To make MTA disk, MTA was mixed according to the manufacturer's instructions. A disk (diameter, 10 mm; thickness, 3 mm) was allowed to set for 24 hours at 37°C, placed in culture medium for 1 week in the incubator, and filtered before being used to culture cells.

Cell Culture

Maxillary supernumerary incisors and third molars were extracted after obtaining informed consent from 3 healthy adults who presented to Chonnam National University Dental Hospital. Immediately after extraction, the molars were kept in phosphate-buffered saline. The extracted maxillary supernumerary incisors and third molars were split open, and the pulp tissues were removed under sterile conditions, minced with a surgical knife, and placed in 6-well cell culture plates containing α-MEM (Gibco Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For the mineralization experiments, cells were cultured in 50 mg/mL ascorbic acid and 10 mmol/L β-glycerophosphate–containing media as described previously (14).

Cell Viability Assay

The cell viability was measured by using a methyl-thiazol-diphenyl-tetrazolium (MTT) assay. Briefly, cells were seeded at a concentration of 20,000 cells/well in 96-well plates in α-MEM with 10% FBS. The concentration of MTA was set at 0.01, 0.1, 1, and 10 mg/mL, on the basis of previous reports (13). After 24 hours, the media were changed for fresh media containing the indicated concentrations of MTA with 2% FBS; the control cells were cultured in α-MEM with 2% FBS. After incubation for 1, 3, and 5 days, MTT was added to each well for the last 4 hours of the experiment, and the reaction was stopped by the addition of dimethyl sulfoxide. The optical density was determined at 570 nm on a multiwell plate reader.

Reverse Transcription–polymerase Chain Reaction

The total RNA of HDPCs was extracted by using the Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Then, 1 ug RNA was reverse transcribed for first-strand complementary DNA synthesis (Gibco BRL, Rockville, MD). The complementary DNA was amplified in a final volume of 20 uL containing 2.5 mmol/L magnesium dichloride, 1.25 U Ex Taq polymerase (Bioneer, Daejeon, Korea), and 1 mmol/L specific primers. Amplification was performed for 30 cycles in a DNA thermal cycler. Primer sequences for differentiation markers are detailed in Table 1. The polymerase chain reaction (PCR) products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

Alizarin Red S Staining

For mineralized nodule assay, HDPCs were cultured with mitogen-activated protein kinase (MAPK) inhibitors (U0126, SB203580, and SP600125) for 1 hour and then replaced with MTA extract for 7 days. The culture medium was replaced with fresh culture medium every 2 days. After 14 days of treatment, the calcium deposition of HDPCs was studied by using 0.1% alizarin red S staining solution (Sigma-Aldrich, St Louis, MO). The samples were fixed with 70% ice-cold ethanol for 1 hour, rinsed twice with phosphate-buffered saline, and stained with 40 mmol/L alizarin red solution for 10 minutes under conditions of gentle agitation. The alizarin red S staining was photographed under light microscopy.

Western Blot

HDPCs were grown in serum-free α-MEM for 12 hours and pretreated with or without nifedipine for 4 hours before MTA exposure. Cell lysates (50–100 μg) were placed in a NP-40 lysis buffer. Protein concentrations were detected by using BCA protein assay kit. Proteins were separated by 12% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the standard procedures. The membrane was blocked in 5% nonfat dry milk for 1 hour and incubated with anti-phospho–extracellular signal-regulated kinase (ERK), anti-phospho-p38, and anti-phospho–Jun N-terminal kinase (JNK) antibodies (Cell Signaling, Danvers, MA) for 1 hour at room temperature. After incubation with the specific peroxidase-coupled secondary antibodies (Sigma) for 1 hour, the blotted bands were detected by using