Combined Effects of Growth Hormone and Mineral Trioxide Aggregate on Growth, Differentiation, and Angiogenesis in Human Dental Pulp Cells

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Abstract

Introduction: The aim of this study was to evaluate the effects of growth hormone (GH) on mineral trioxide aggregate (MTA) with regard to cell adhesion, growth, odontoblastic differentiation, and angiogenesis in human dental pulp cells and the underlying signal pathway mechanisms. **Methods:** Cell adhesion and proliferation were assessed by adhesion analysis and cell counting. Differentiation was examined by alkaline phosphatase activity, alizarin red staining, and reverse transcriptase polymerase chain reaction for marker genes. Angiogenesis was evaluated by human umbilical vein endothelial cell migration and capillary tube formation assays. Signaling pathways were analyzed by Western blotting and confocal microscopy. **Results:** Combined treatment with GH and MTA enhanced cell adhesion, growth, alkaline phosphatase activity, calcified nodules, expression of marker mRNAs, migration, and capillary tube formation, compared with treatment with MTA or GH alone. In addition, GH plus MTA increased expression of bone morphogenetic protein-2 mRNA, phosphorylation of Smad 1/5/8, extracellular signal-regulated kinase, JNK, and p38 MAPK, and increased the levels of the transcription factors Runx2 and Osterix, compared with MTA alone. **Conclusions:** Collectively, our results demonstrate that a combination of MTA and GH promotes cell adhesion, growth, differentiation, and angiogenesis of MTA in human dental pulp cells via the activation of bone morphogenetic protein and MAPK pathway. *(J Endod 2016;42:269–275)*

Key Words

Angiogenesis, growth, growth hormone, human dental pulp cells, MTA, odontogenic differentiation

Mineral trioxide aggregate (MTA) is a modified preparation of Portland cement with radiopacifier (bismuth oxide, Bi_2O_3) that has been used for more than a decade as a dental material in root-end fillings, perforation repairs, pulp capping, and apexification (1, 2). MTA has the capacity to induce odontogenic/osteogenic differentiation (3–5). Our previous direct pulp-capping experiment demonstrated that MTA induced the formation of dentin bridges with less inflammation than calcium hydroxide (6). In addition, MTA has been reported to be less cytotoxic than other root-end filling or endodontic materials both *in vivo* and *in vitro* (7, 8). However, MTA has several disadvantages, including discoloration potential, difficult handling characteristics, the presence of toxic elements in the material composition, a long setting time, and high material costs (9, 10).

The processes of osteogenesis and bone remodeling are orchestrated by a constellation of local growth factors, cytokines, and systemic hormones. To improve or accelerate osteogenic potential, growth and differentiation factors have been used in tissue regeneration (11). Moreover, a combination of growth factor and tricalcium phosphate has been shown to enhance bone regeneration in a bony defect after periapical surgery (12). Recently, it was reported that MTA combined with fibroblast growth factor-2 promoted greater proliferation and differentiation in human dental pulp cells (HDPCs) than MTA alone (13). Moreover, we demonstrated that a combination of MTA and enamel matrix derivative promoted more rapid differentiation in HDPCs, as compared with pure MTA (14). In addition, the MTA/bone morphogenetic protein-2 (BMP-2) combination promoted more rapid differentiation in MC3T3-E1 cells than did MTA or enamel matrix derivative alone (15). MTA mixed with hydration accelerators such as calcium chloride and low-dose citric acid more upregulated osteogenic genes than that of MTA alone in mouse osteoblasts (5).

Growth hormone (GH), a single-chain peptide hormone, is produced and stored by somatotroph cells located in the anterior pituitary gland (16). Osteoblasts and chondrocytes have receptors for GH (17). Consequently, addition of the hormone to these cells *in vitro* increases cell proliferation, maturation, and differentiation (18, 19). GH levels decrease with age, and GH deficiency may contribute to metabolic bone diseases, including postmenopausal and senile osteoporosis (20). Moreover, GH has been shown to be an important physiologic regulator of bone metabolism; this can be mediated directly or indirectly through insulin-like growth factor-I (18). Although GH upregulated the expression of BMP-2 and BMP-4 mRNAs in HDPCs (21), the combined effects of GH and MTA in HDPCs have not yet been reported.

Because *in vivo* GH treatment stimulated dentinogenesis in rat molars (22), we hypothesized that combined stimulation by MTA and GH would augment odontoblastic

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differentiation and angiogenesis to a greater degree than treatment with MTA alone. Therefore, the purpose of the present study was to investigate whether MTA and GH would have synergistic effects on adhesion, growth, migration, and odontogenic differentiation in HDPCs, compared with treatments with MTA or GH alone. In addition, human umbilical vein endothelial cells (HUVECs) under the conditioned medium from HDPCs were used to investigate the effect of MTA and/or GH on angiogenesis. Moreover, the underlying signaling mechanisms of this combined treatment were examined.

Materials and Methods

Sample Preparation

White ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK) was mixed according to the manufacturer's instructions. Each sample (1 cm in diameter, 10 mm; thickness, 2 mm) was allowed to set for 24 hours at 37°C in 100% humidity. MTA was sterilized by gamma-radiation with 37.2 gray before being used to culture cells. Commercially available GH was obtained from LG Life Sciences (Seoul, Korea).

Cell Culture

Immortalized HDPCs transfected with the human telomerase catalytic component were provided by Professor Takashi Takata (Hiroshima University, Japan) (23). The cells were grown in α-minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Odontogenic differentiation was induced by changing to osteogenic supplement (OS) medium containing 50 μg/mL L-ascorbic acid and 10 mmol/L β-glycerophosphate. The medium was replaced every 2 days during the incubation period.

Cell Proliferation and Alkaline Phosphatase Activity

The proliferation rates of HDPCs were assessed by cell counting by using trypan blue exclusion. Alkaline phosphatase (ALP) activity was measured in 0.7 mol/L 2-aminomethyl-1-propanol (pH 10.3) and 6.7 mmol/L $MgCl_2$ by using p-nitrophenyl phosphate (3 mmol/L final concentration) as the substrate. The absorbance was measured at a wavelength of 405 nm by using an enzyme-linked immunosorbent assay (ELISA) reader.

Alizarin Red S Staining

Cells were stained with 40 mmol/L alizarin red S (pH 4.2) for 10 minutes with gentle agitation. The level of alizarin red S staining was observed under light microscopy.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from cells with Trizol reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. One microgram RNA was reverse-transcribed with oligo $(dT)_{15}$ primers by using AccuPower RT PreMix (iNtRON Biotechnology, Gyeonggi-do, South Korea). Next, the generated cDNAs were amplified with AccuPower PCR PreMix (Bioneer Corp, Daejeon, South Korea). Polymerase chain reaction (PCR) products were subjected to electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Cell Adhesion Assay

Endothelial cell medium (ECM) Gel solution (50 μL; Cell Biolabs, San Diego, CA) was poured onto a 96-well culture plate and allowed to solidify (37°C, 1 hour). HDPCs were seeded in a 96-well plate and allowed to attach for the indicated times at 37°C. Adherent cells were fixed with 10% formalin for 30 minutes at room temperature and stained with 0.5% crystal violet for 10 minutes. The cell-bound stain was dissolved by incubating the wells with 1% sodium dodecylsulfate (SDS) overnight in the dark. The absorbance was measured at a wavelength of 595 nm by using an ELISA reader (Beckman Coulter).

In Vitro Angiogenesis and Migration

HUVECs were cultured in ECM (ScienCell Research Laboratories, Carlsbad, CA) at 37°C under a 5% CO_2 atmosphere. ECM Gel solution (50 mL; Cell Biolabs) was poured onto a 96-well culture plate and allowed to solidify (37°C, 1 hour). HUVECs (1.5×10^4 cells/well) were seeded on the ECM gel and were cultured with conditioned medium obtained from HDPCs treated with MTA and GH for 3 days. After 12 hours, tube formation was observed and quantified under a light microscope.

Cell migration was assessed by *in vitro* scratch assay. HUVECs were wounded by using a 200-μL pipette tip and washed 3 times with 1× phosphate-buffered saline (PBS) to remove cell debris. After HUVECs were incubated with conditioned medium for 24 hours, the migration of the cells was quantified. The cell migration rate was expressed as the relative fold of the initial area.

Western Blot Analysis

Samples were lysed in RIPA lysis buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% SDS, 1% Nonidet P-40, 1:100 proteinase inhibitor cocktail, 50 mmol/L β-glycerophosphate, 50 mmol/L sodium fluoride). Protein concentration was determined by using Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were resolved by SDS–polyacrylamide gel electrophoresis and were then transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Western blot analysis was performed as described previously (24). The proteins were visualized by using an enhanced chemiluminescence system (Amersham, Piscataway, NJ).

Immunofluorescence

Briefly, treated cells were fixed with 4% paraformaldehyde for 30 minutes after permeabilization with 0.1% Triton X-100. After washing in PBS buffer, slides were blocked with 1% normal goat serum for 1 hour and then incubated with mouse monoclonal anti-human p-Smad 1/5/8 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours by using a 1:100 dilution. The slides were washed with PBS, incubated with fluorescein isothiocyanate–conjugated goat anti-mouse immunoglobulin G (Invitrogen Life Technologies, Grand Island, NY) at a 1:100 dilution for 1 hour, and nuclei were counterstained with 10 μg/mL propidium iodide. Slides were imaged at ×200 magnification on a confocal microscope (Cell Voyager, Yokogawa, Japan).

Statistical Analysis

The results were expressed as the mean ± standard deviation, and an analysis of variance with Bonferroni test was used for multiple comparisons (SPSS v22.0, Chicago, IL).