Vitamin D Promotes Odontogenic Differentiation of Human Dental Pulp Cells via ERK Activation

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The active metabolite of vitamin D such as 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) is a well-known key regulatory factor in bone metabolism. However, little is known about the potential of vitamin D as an odontogenic inducer in human dental pulp cells (HDPCs) in vitro. The purpose of this study was to evaluate the effect of vitamin D3 metabolite, 1α,25(OH)2D3, on odontoblastic differentiation in HDPCs. HDPCs extracted from maxillary supernumerary incisors and third molars were directly cultured with 1α,25(OH)2D3 in the absence of differentiation-inducing factors. Treatment of HDPCs with 1α,25(OH)2D3, at a concentration of 10 nM or 100 nM significantly upregulated the expression of dentin sialophosphoprotein (DSPP) and dentin matrix protein1 (DMP1), the odontogenesis-related genes. Also, 1α,25(OH)2D3 enhanced the alkaline phosphatase (ALP) activity and mineralization in HDPCs. In addition, 1α,25(OH)2D3 induced activation of extracellular signal-regulated kinases (ERKs), whereas the ERK inhibitor U0126 ameliorated the upregulation of DSPP and DMP1 and reduced the mineralization enhanced by 1α,25(OH)2D3. These results demonstrated that 1α,25(OH)2D3 promoted odontoblastic differentiation of HDPCs via modulating ERK activation.

INTRODUCTION

Dental pulp tissues contain dental pulp stem cells (DPSCs) that are thought to be progenitor/stem cells which have the capacity to proliferate and differentiate into odontoblasts and odontoblastoids under high serum conditions (Laino et al., 2005). DPSCs have the potential to regenerate dentin-pulp-like complexes and express dentin-specific markers such as alkaline phosphatase (ALP), type I collagen (COLI), osteocalcin (OCN), dentin matrix protein 1 (DMP1), and dentin sialophosphoprotein (DSPPP), like odontoblasts (Gronthos et al., 2002). Vitamin D controls mineral homeostasis by influencing intestinal absorption, renal reabsorption of calcium and phosphorus, and deposition and mobilization of calcium in mineralized tissues. Vitamin D deficiency causes hypocalcified dentin and delayed tooth eruption; thus, demonstrating that vitamin D has an essential role in dentin formation (Kim et al., 1982; Pitaru et al., 1982). The active form of vitamin D, 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3), plays a crucial role in regulating bone metabolism and mineralization of skeletal and dental tissues (Barron et al., 2008; van Driel et al., 2004).

Mitogen-activated protein kinases (MAPKs) are second messengers that play a critical role in cellular responses including growth, proliferation, differentiation, and apoptosis in mammalian cells (Yang et al., 2013). MAPKs are composed of three well-characterized families, including extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK (Johnson and Lapadat, 2002). Several studies have reported that p38 MAPK activation plays an important role during osteoblastic differentiation. Reports demonstrated that ERK signaling is necessary for the differentiation of mesenchymal stem cells and skeletal development (Ge et al., 2007; Greenblatt et al., 2010; Wu et al., 2012). Furthermore, MAPKs, particularly ERKs, are associated with mineral trioxide aggregate-induced odontoblastic differentiation of human DPSCs (Zhao et al., 2012).

A previous study demonstrated that vitamin D3 metabolites such as 1α,25(OH)2D3 and 25-hydroxycholecalciferol (25OHD3) could induce osteogenic differentiation in human dental pulp and human dental follicle cells (Khanna-Jain et al., 2010). However, the effect of 1α,25(OH)2D3 on odontoblastic differentiation of HDPCs and the mechanism underlying the effect have not been fully investigated. Understanding the mechanisms that regulate odontoblastic differentiation in HDPCs will have important implications for developing new therapeutic strategies to prevent dental pulp injury. The present study aimed to investigate whether 1α,25(OH)2D3 could promote odontoblastic differentiation of HDPCs and whether 1α,25(OH)2D3 could affect the activation of MAPKs during the odontoblastic differentiation process.

MATERIALS AND METHODS

Cell cultures and vitamin D preparation
Maxillary supernumerary incisors and third molars were extracted after obtaining informed consent from three healthy...
adults who presented to Chonnam National University Dental Hospital (CNUDH). Informed consent was obtained from the donors and the protocol was approved by the Institutional Ethics Committee (CNUDH, Korea). Immediately after extraction, molars were kept in phosphate-buffered saline. The extracted maxillary supernumerary incisors and third molars were split open. Pulp tissues were removed under sterile conditions, minced with a surgical knife, and placed into 6-well cell culture plates containing minimum essential medium (α-MEM, Gibco Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Primary cultured cells obtained from three donors were pooled prior to perform the experiments.

1α,25(OH)2D3 (Sigma-Aldrich, USA) was maintained in 10 μM stock solutions in dimethyl sulfoxide (DMSO) and treated in culture medium with 10 nM and 100 nM concentration.

**Cell viability assay**

Cell viability was measured using MTT assay. For viability experiments, suspensions of HDPCs at a concentration of 2 × 10^5 cells/well were seeded into 96-well plates in α-MEM supplemented with 10% FBS. Concentrations of 1α,25(OH)2D3 were set at 10 nM and 100 nM. After one or two days, MTT was added to each well and were incubated for four hours. Dimethyl sulfoxide (DMSO) was added to each well, and the optical density was measured at 570 nm on a multwell plate reader. Background absorbance of the medium in the absence of cells was subtracted.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA from the HDPCs was extracted with the Trizol reagent (Life Technologies, USA) according to the manufacturer’s instructions. A total of 1 μg RNA was reverse transcribed using the first-strand complementary DNA (cDNA) synthesis kit (Gibco BRL, USA). The cDNA was amplified in a final volume of 20 μl containing 2.5 mmol/L magnesium dichloride, 1.25 U Ex Taq polymerase (Bioneer, Korea), and 1 mmol/L of specific primers. Amplification was performed for 30 cycles in a DNA thermal cycler. Primer sequences for differentiation markers are listed in Table 1. PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

**Alkaline phosphatase (ALP) activity**

HDPCs (1 × 10^5 cells/well) in 12 well plates were exposed to 10 nM or 100 nM of 1α,25(OH)2D3 for seven days. The ALP activity was evaluated by determining the amount of p-nitrophenol produced using an ALP activity assay kit (Bio Vision, USA) according to the manufacturer’s instructions. For measuring the intracellular ALP, washed cells were lysed in assay buffer and centrifuged at 13,000 × g for three minutes to remove the insoluble material. Protein levels in the supernatant were measured by using the bicinchoninic acid method (Thermo Scientific, USA) by adding equal amount of samples into a 96 well plate and 5 mM pNPP solution. After the reaction was incubated for 60 minutes at room temperature and protected from light, stop solution was added and the absorbance was measured at 405 nm with an ELx800uv ELISA reader (Bio-Tek Instruments, USA).

**ALP staining**

HDPCs were cultured with 1α,25(OH)2D3 for 7 days. For ALP staining, media were removed and the cells were fixed with 70% ethanol for one hour. After rinsing with deionized water three times, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro-blue tetrazolium (NBT) solution (Sigma-Aldrich) was added to each well. The reaction was stopped by the addition of water. The stain was photographed under a HP Officejet Pro L7580 scanner. For quantitative analysis, the stain was extracted with 10% (w/v) cetylpyridinium chloride (Sigma) for 15 min. The stain intensity was quantified by measuring the absorbance at 570 nm on an absorbance microplate reader (Bio-Tek Instruments).

**Alizarin red S staining**

For the mineralized nodule formation assay, HDPCs were cultured with 1α,25(OH)2D3 for seven days. The culture medium was replaced with fresh culture medium every 2 days, and cells were rinsed with phosphate buffered saline and fixed in ice-cold 70% ethanol for one hour at room temperature. Subsequently, the cells and the matrix were stained with 40 mmol/L Alizarin red S (pH 4.2) for 15 min at room temperature under conditions of gentle agitation, and then washed extensively five times with deionized water and once with PBS for 15 min at ambient temperature to remove nonspecifically bound stain. The sample was photographed using a HP Officejet Pro L7580 scanner. For quantitative analysis, the stain was extracted with 10% (w/v) cetylpyridinium chloride (Sigma) for 15 min. The stain intensity was quantified by measuring the absorbance at 570 nm on an absorbance microplate reader (Bio-Tek Instruments).

**Western blot analysis**

Cell lysates (50-100 μg) were placed in the NP-40 lysis buffer. Protein concentrations were detected using the BCA protein assay kit (Thermo Scientific). Proteins were separated by 12% polyacylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, UK) according to standard procedures. The membranes were blocked in 5% non-fat dry milk for one hour and incubated with anti-DSP, anti-DMP1, anti-phospho-ERK, anti-ERK, anti-phospho-p38, anti-p38, anti-phospho-JNK, or anti-JNK antibodies (Cell Signaling, USA) for 4 h at room temperature. After incubation with specific peroxidase-conjugated secondary antibodies (Sigma) for one hour, blot bands were detected using an enhanced chemiluminescence

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**Table 1. Sequences of PCR Primers used RT-PCR**

| Genes | GenBank number | Sequences (5’-3’) | Length of product |
|-------|----------------|-------------------|------------------|
| DSP   | NM_0147208     | Forward: CAGTGATGAACTCATAAGG | 488 bp |
|       |                | Reverse: CTGATTGTGCTGCTGTCGAC |       |
| DMP1  | NM_001079911   | Forward: CAGGAGACAGGAAAAAGGAG | 213 bp |
|       |                | Reverse: CTGGTTGATCTTGGGCCACT |       |
| GAPDH | NM_001256799   | Forward: AGTCACGGGATTGGTCTGCTG | 185 bp |
|       |                | Reverse: ACAAGCTTCGGTTCTCGAG |       |
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Fig. 1. Effect of 1α,25(OH)2D3 on cell viability of HDPCs. The viability of HDPCs treated with different concentrations (10 and 100 nM) of 1α,25(OH)2D3 for 1, 2, and 7 days was investigated by MTT assay and expressed as the percentage of control (0 day). Values are expressed as the mean ± SD of three replicates of one representative experiment.

Statistical analysis
All experiments were performed in triplicate using three independent cell cultures. Each value represented the mean ± SD. Statistical significance was determined using the Student’s t-test compared to the control. Differences with \( p < 0.05 \) were considered statistically significant.

RESULTS

Effect of 1α,25(OH)2D3 on cell viability of HDPCs
The effect of 1α,25(OH)2D3 on cell growth of HDPCs was evaluated by MTT assay. As shown in Fig. 1, cell viability of HDPCs was slightly increased by incubation with 10 nM or 100 nM 1α,25(OH)2D3 for 1, 2, and 7 days. However, cell viability of HDPCs was unaffected by incubation with 10 nM or 100 nM 1α,25(OH)2D3 for 7 days compared to that of the untreated cells.

Effect of 1α,25(OH)2D3 on odontoblastic differentiation of HDPCs
1α,25(OH)2D3 has been shown to induce the expression of osteoblast differentiation marker genes in various cells including HDPCs and human dental follicle cells (Khanna-Jain, 2010). To investigate the potency of HDPCs for odontoblast-like differentiation after 1α,25(OH)2D3 treatment, the mRNA expression and protein levels of odontoblastic differentiation markers (DSP and DMP1) were detected in HDPCs treated with 1α,25(OH)2D3 at a concentration of 10 nM for up to 1 day by RT-PCR and Western blot. DSP and DMP1 mRNAs were markedly upregulated by 1α,25(OH)2D3 at concentrations of 10 nM and 100 nM (Fig. 2A). In addition, Western blot findings further confirmed the upregulation of DSPP (DSP) and DMP1 protein in the 1α,25(OH)2D3-treated HDPCs (Fig. 2B).

ALP activity was measured in HDPCs treated with 1α,25(OH)2D3 at a concentration of 10 nM or 100 nM for 7 days. 1α,25(OH)2D3 enhanced the ALP activity in a dose-dependent manner (Fig. 3A). Increased ALP activity by 1,25(OH)2D3 was clearly confirmed by ALP staining in the 1α,25(OH)2D3-treated cells for 7 days. The ALP-positive cells were increased in the 1α,25(OH)2D3-treated group compared to the untreated control group (Fig. 3B). To determine the effect of 1α,25(OH)2D3 on mineralization in HDPCs, mineralized nodule formation in HDPCs was examined using Alizarin Red S staining after 7 days of culture with 1α,25(OH)2D3 at concentrations of 10 nM and 100 nM. 1α,25(OH)2D3 clearly increased the area of calcified nodules in a dose-dependent manner. The area of calcified nodules was increased up to three-fold by 1α,25(OH)2D3 at a concentration of 100 nM compared to that in the control cells (Fig. 3C).

1α,25(OH)2D3-induced odontoblastic differentiation of HDPCs via ERK activation
To determine whether 1α,25(OH)2D3 stimulation of HDPCs could trigger the activation of MAPK cascades, phosphorylation of MAPKs was investigated by Western blot analysis. As shown in Fig. 4, treatment with 1α,25(OH)2D3 increased the phosphorylation of ERK and the total level of ERK. However, treatment with 1α,25(OH)2D3 failed to activate p38 and JNK phosphorylation. In addition, after incubation with 100 nM 1α,25(OH)2D3 for 2 hours, phosphorylated ERK, p38 and JNK were observed at different time points (0, 30, 60, 90, and 120 min) in HDPCs. Phosphorylated ERK level was significantly increased, and peaked at 90 minutes after incubation with 1α,25(OH)2D3, whereas phosphorylated JNK was diminished in a time-dependent manner (Fig. 4B).

To further determine the role of ERK signaling in 1α,25(OH)2D3-induced odontoblastic differentiation of HDPCs, cells were pretreated with U0126 (an ERK inhibitor) for 1 h followed by treatment with 100 nM 1α,25(OH)2D3 for 2 days. Inhibition of ERK activation using U0126 decreased the 1α,25(OH)2D3 upregulated mRNA expression and protein level of differentiation markers (DSP and DMP1) in HDPCs (Fig. 4C). Also, it had no influence in changes of cell morphology among three groups, untreated group, 100 nM 1α,25(OH)2D3-treated group with or without U0126 at 7 days (data not shown).

In addition, after incubation for 7 days in the presence of U0126 with 100 nM 1α,25(OH)2D3, pretreatment with U0126 reduced the 1α,25(OH)2D3-stimulated mineralization detected by Alizarin Red S staining, indicating that ERK MAPK signaling was involved in 1α,25(OH)2D3-induced odontoblastic differen-
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**Fig. 3.** Effects of 1α,25(OH)2D3 on activity and expression of ALP and the formation of calcified nodules in HDPCs. Cells were cultured with 1α,25(OH)2D3 (10 nM and 100 nM) for 7 days. (A) ALP activity was detected in 1α,25(OH)2D3-treated HDPCs. Values are expressed as the mean ± SD of three independent experiments. *p < 0.05 compared to the control. (B) ALP staining was performed and the quantification of ALP staining was graphed. (C) Cells were stained with Alizarin red S as described in the ‘Materials and Methods’ section, and the representative photograph of calcified nodules stained with Alizarin red S staining is shown. The quantification of Alizarin red S was graphed. Values are expressed as the mean ± SD of three replicates of one representative experiment. *p < 0.05 or **p < 0.01 compared to the control. The data shown are the representatives of three independent experiments with triplicates for each experiment.

**Fig. 4.** 1α,25(OH)2D3-induced odontogenic differentiation of HDPCs via ERK MAPK in HDPCs. (A) HDPCs were cultured with 1α,25(OH)2D3 (10 nM and 100 nM) for 2 days. The cellular expression and phosphorylation of ERK (p-ERK), p38 (p-p38), and JNK (p-JNK) were detected by Western blot analysis. (B) After incubation for the time points (0, 30, 60, and 120 min) with 100 nM 1α,25(OH)2D3, the expression of ERK, p38 or JNK and their phosphorylation were analyzed by Western blotting analysis. (C) HDPCs were pretreated with the ERK MAPK inhibitor (U0126, 10 μM) for one hour and then cultured with 1α,25(OH)2D3 (100 nM) for 2 days. Expression levels of DMP1 and DSPP (DSP) as the odontoblastic differentiation markers were determined using RT-PCR analysis or Western blotting. (D) After 7 days in the presence of U0126 with 100 nM 1α,25(OH)2D3, calcified nodule stained with Alizarin red S staining is shown, and the quantification of Alizarin red S was graphed. Results were representative of three independent experiments. **p < 0.01 compared to the control, #p < 0.05 compared to the 1α,25(OH)2D3-treated group. The data shown are the representatives of three independent experiments with triplicates for each experiment.

**DISCUSSION**

Proliferation, chemotaxis, and differentiation of dental pulp cells into odontoblasts are involved in the dentinal regeneration process, including reparative dentin formation and dental pulp healing when there is damage to the dental pulp tissue (Yamamura, 1985). Progenitor/stem cells within the dental pulp have the capacity to proliferate and differentiate into dentin-forming odontoblasts (Gronthos et al., 2000). The role of 1α,25(OH)2D3 in tooth formation is well known based on the in vivo and clinical studies.

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Deficiency of 1α,25(OH)2D3 would result in hypocalcification of the dentin and enamel, leading to unmineralized dental structure (Barron et al., 2008).

The present study investigated the effect of 1α,25(OH)2D3 on odontoblastic differentiation and mineralization of HDPCs. A previous study reported that vitamin D3 metabolites (1α,25(OH)2D3 and 25OHD3) could stimulate osteogenic differentiation in HDPCs (Khanha-Jain et al., 2010). The differentiation of dental pulp cells into odontoblastic cells was evaluated by the expression of odontoblastic differentiation-associated genes (DSPP, ALP, and DMP1), ALP activity, and mineralization. To examine the effect of 1α,25(OH)2D3 on odontoblastic differentiation in HDPCs, cells were cultured in a medium containing 1α,25(OH)2D3 without the differentiation-inducing factors (dexamethasone and β-glycerophosphate). The present results showed that 1α,25(OH)2D3 stimulated the mRNA and protein expression of genes associated with odontoblastic differentiation, such as DSPP (DSP) and DMP1. The expression of DSP and phosphorin (pp), two dentin non-collagenous proteins, is associated with odontoblast-mediated biomineralization (Feng et al., 1998; Ritchie et al., 1997). The result of upregulation of DSPP and DMP1 in HDPCs cultured with 1α,25(OH)2D3 was similar to that in a previous study by Tonomura (2007), which reported that the level of DSP was increased in human dental pulp-derived cells cultured with 1α,25(OH)2D3 in differentiation-inducing media (dexamethasone and β-glycerophosphate). However, it was different from the result in the study by Ritchie (2004), which reported that vitamin D3 upregulated osteopontin but had no effect on DSP-PP mRNA in rat tooth organ culture. Moreover, the present result of DMP1 upregulation by 1α,25(OH)2D3 treatment was inconsistent with that in a previous report which showed that murine cementoblasts and osteocyte-like cells exhibited decreased DMP1 mRNA level in the presence of 1α,25(OH)2D3 (Nociti et al., 2014).

Expression and activity of ALP, which is regarded as an early marker of hard tissue formation or odontogenic differentiation, were investigated to confirm the odontogenic capacity of HDPCs. In the present study, 1α,25(OH)2D3 at concentrations of 10 nM and 100 nM increased the expression and activity of ALP in HDPCs. In correlation with the increment of ALP expression and activity, the initiation of odontogenesis resulted in progression of mineralized matrix formation when HDPCs were cultured with 1α,25(OH)2D3, as assessed by Alizarin red staining. These results suggested that odontoblastic differentiation in HDPCs was stimulated by 1α,25(OH)2D3. This finding was consistent with that in previous report, which suggested that the addition of 1α,25(OH)2D3 significantly increased ALP activity in cultured dental pulp cells in the presence of dexamethasone and β-glycerophosphate (Kido et al., 1991; Tonomura et al., 2007). However, the present study showed that ALP activity and mineralization were induced by 1α,25(OH)2D3 in the basal medium (alpha-minimum essential medium, 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin) in the absence of additional osteo/odontogenic factors. Generally, additional osteo/odontogenic factors such as ascorbic acid, β-glycerophosphate, dexamethasone, and bone morphogenetic protein-2 (BMP-2) are added to the culture medium to stimulate mineralization. There are reports suggesting that vitamin D3 induced ALP activity and mineralization in basal cell culture medium without osteogenic factors, although it showed high effects in osteogenic medium (Khanha-Jain et al., 2010; Mason et al., 2014). The components including calcium, phosphate, and vitamins of the alpha-minimum essential medium may be used for induction of ALP activity and mineralization in HDPCs during a short period.

Several studies indicated that MAPKs are involved in the regulation of osteoblastic and odontoblastic differentiation. Mineral trioxide aggregate used as an endodontic material for pulp capping and regeneration promotes odontoblastic differentiation in human dental pulp stem cells via the MAPK pathway (Zha et al., 2012). JNK activity is required for late-stage odontoblastic differentiation induced by BMP-2 (Qin et al., 2014). LPS promotes odontoblastic differentiation of HDPCs via the ERK and p38 MAPK signaling pathways (He et al., 2013). ERK and p38 MAPK significantly enhanced odontogenic differentiation (Zhang et al., 2012). p38 MAPK is involved in BMP-2-induced odontoblastic differentiation of HDPCs (Qin et al., 2012). The p38 protein is activated in response to growth factors to mediate ALP expression in dental pulp cells (Wang et al., 2006). However, the role of ERK in odontoblastic differentiation is controversial. Calcium silicate (CS) plays a key role in the odontoblastic differentiation of HDPCs via the activation of ERK MAPK (Liu et al., 2014). Intermittent traction stretch promotes the osteoblastic differentiation of bone mesenchymal stem cells by the ERK1/2-activated Cbfa1 pathway (Wu et al., 2012). In contrast, ERK pathways negatively regulate osteoblastic differentiation and mineralization in C2C12 cells and MC3T3-E1 cells (Kono et al., 2007; Nakayama et al., 2003). The present study explored whether 1α,25(OH)2D3 could affect MAPK activation in the process of odontoblastic differentiation in HDPCs. 1α,25(OH)2D3 increased ERK phosphorylation, while could not increase p38 and JNK phosphorylation in HDPCs. Inhibition of ERK activation by the ERK inhibitor (U0126) reduced 1α,25(OH)2D3-induced upregulation of DSPP (DSP) and DMP1 and mineralization in HDPCs. These results suggested that activation of the ERK1/2 signaling pathway might upregulate the expression of odontoblastic differentiation-associated genes and mineralization of nodules under 1α,25(OH)2D3 treatment of HDPCs. However, DSPP (DSP) and DMP1 expression and mineralization in the 1α,25(OH)2D3 and U0126-treated group was similar and not low compared with those in the control group, indicating that odontoblastic gene regulation and mineralization of these cells does not seem to be inhibited by the ERK inhibitor to the level of control. These results suggest the involvement of other mechanisms besides ERK MAPK pathway in 1α,25(OH)2D3-induced odontoblastic differentiation of HDPCs. A further study is needed to assess whether 1α,25(OH)2D3- induced expression of genes associated with odontoblastic differentiation is mediated by transcriptional regulation of vitamin D receptor with MAPK pathway.

Taken together, this study revealed that 1α,25(OH)2D3 promoted the odontoblast-like cell properties, including odontoblast-related DSPP and DMP1 upregulation, high ALP activity, and calcification of HDPCs through the activation of ERK MAPK. These findings suggest that vitamin D may be useful in inducing odontogenesis and facilitating dentin regeneration.

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