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MAPK Signal Pathways in Regulation of Odontoblastic Differentiation by Induction of HO-1 in Human Dental Pulp Cells

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MAPK 경로를 통한 HO-1과 분화 표지자 발현

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Abstract The purpose of this study was to examine the MAPK signaling pathways involved in regulation of HO-1 and the odontoblast differentiation markers during the odontoblastic differentiation for HDPCs. We evaluated cell growth by MTT assay and differentiation marker mRNA expression by RT-PCR. When the cells were treated with p38 inhibitor (SB203580, 10 μM), JNK inhibitor (SP600125, 10 μM), and ERK inhibitor (PD98059, 20 μM) for 7 days, cell growth and expression of HO-1 and differentiation makers were significantly decreased in HDPCs. Our results suggest that odontoblastic differentiation is positively regulated by HO-1 induction in HDPCs via ERK, JNK, and p38 signaling pathways. Thus, pharmacological HO-1 induction might represent a potent therapeutic approach for pulp capping and the regeneration of HDPCs.

Key words Human dental pulp cells, Mitogen-activated protein kinase, Heme oxygenase-1, Differentiation

Introduction

Human dental pulp cells (HDPCs) have characteristics of mesenchymal stem cells (MSCs) and can differentiate into odontoblasts[1]. Dentinal repair in the postnatal organism occurs through the activity of odontoblasts[2], which are thought to arise from the proliferation and differentiation of a precursor population, residing within the pulp tissue[3]. This mechanism means that differentiated and undifferentiated cells within dental pulp may contribute to the regeneration process[4]. Odontoblasts secrete type I collagen and other noncollagenous proteins such as osteonectin (ON), osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein-1 (DMP-1), and dentin sialophosphoprotein (DSPP)[5,6], which has been used as mineralization markers for odontoblast/osteoblast-like differentiation of HDPCs[2,7]. Thus, we used RT-PCR to investigate the expression of extracellular matrix proteins such as OPN, DMP-1, and DSPP in HDPCs as an indicator of odontoblastic differentiation.

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism. Heme is degraded to free iron, carbon monoxide (CO) and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. Of the three known heme oxygenases, HO-1(heat shock protein 32) is the only inducible isoform[8]. HO-1 and its metabolic products play important regulatory roles in both physiological and pathological status[9]. Data from current studies suggest that HO-1 plays a vital role in control of cell growth and differentiation[10]. In addition, several studies demonstrated that expression of HO-1 is related to osteoblastic differentiation in periodontal ligament cells (PDLCs)[11], and neuronal differentiation in MSCs[12]. The cobaltic protoporphyrin IX (CoPP) has been shown to strongly induce HO-1 expression both *in vivo and in vitro*[13,14]. Recently, we reported that inducing of HO-1 expression by CoPP, HO-1 inducer, enhance the cell viability and expression of the odontoblastic differentiation markers such as OPN, DMP-1, and DSPP on HDPCs[15].

A number of intra-cellular signaling molecules and their

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downstream transcription factors have been identified to involve HO-1 expression[16]. Among them, the mitogen-activated protein kinase (MAPK), as one of upstream pathways are well described. In mammalian cells, three major subgroups of MAPKs families have been clearly characterized: namely classical MAPK (ERK), C-Jun N-terminal kinase/ stress-activated protein kinase (JNK/ SAPK) and p38 kinase (p38)[17]. The MAPK pathways are most important signaling pathways controlling the cell proliferation and differentiation in response to the exogenous growth factors[18]. Recently, we reported that nicotine-induced HO-1 expression is mediated by the p38 and ERK-1/2 MAP kinases in IHOK and HN12 cells[19]. Moreover, induction of HO-1 is known to be regulated by MAPK such as p38, JNK, and ERK in macrophage[20]. However, various signaling pathways involved in regulation of HO-1 during odontoblastic differentiation in HDPCs have not yet completely understood.

Although previous studies have proved that HO-1 induction by CoPP upregulates the expression of osteoblastic differentiation marker in HDPCs, molecular control mechanism underlying inductive signal on growth and differentiative functions in odontoblastic differentiation remains to be elucidated.

Thus, the purpose of this study was to examine the MAPK signaling pathways involved in regulation of HO-1 and the odontoblast differentiation markers, including OPN, DMP-1, and DSPP during the odontoblastic differentiation for HDPCs.

Materials and Method

1. Reagents

Minimum Essential Medium Alpha (MEM-α), fetal bovine serum (FBS) and other tissue culture reagents were obtained from Gibco BRL (Grand Island, NY). Cobalt protoporphyrin IX (CoPP) was obtained from Porphyrin Products (Logan, UT). The MAPK inhibitors such as SB203580, PD98059 and SP600125 were purchased from Calbiochem (La Jolla, CA).

2. Cell culture

We used the HDPCs lines immortalized by transfection with the telomerase catalytic subunit hTERT gene[21]. Cells were cultured in MEM-α supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Osteogenic media (OM), based on 10% FBS, MEM-α supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, 10^{-7} M dexamethasone (basic differentiation medium), were employed in order to induce differentiation[22].

3. Cell viability assay

Viable cells were detected using 3-[4,5-dimethylthiazol-2-yl]-2.5 diphenyltetrazolin bromide (MTT) dye, which forms blue formazan crystals that are able to be reduced by mitochondrial dehydrogenase present in living cells. MTT solution was prepared as a 5 mg/ml stock solution in distilled water. After treatment, 50 μL of MTT solution (2 mg/ml in PBS) were added to each samples and incubated for 4 h. The plates were then centrifuged at 200 g for 10 min and the supernatant was discarded. To each well, 50 μL of DMSO were added. The plates were then shaken until the crystals had dissolved. Reduced MTT was then measured spectrophotometrically in a dual beam microtiter plate reader at 540 nm.

4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Then 1 μg RNA was reverse-transcribed for first strand cDNA synthesis (Gibco BRL, Rockville, MD). The cDNA was amplified in a final volume of 20 μL containing 2.5 mM magnesium dicholoride, 1.25 units Ex Taq polymerase (Bioneer, Daejeon, Korea)

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) primers sequence.

Gene	Sequence (5'-3')	Size (bp)
Heme oxygenase-1 (HO-1)	Forward: AAGATTGCCCAGAAAGCCCTGG Reverse: AACTGTCGCCACCAGAAAGCTGAG	399
Osteopontin (OPN)	Forward: CCAAGTAAGTCCAACGAAAG Reverse: GGTGATGTCCTCGTCTGTA	347
Dentin-matrix protein-1 (DMP-1)	Forward: CAGGAGCACAGGAAAAGGAG Reverse: CTGGTGGTATCTTCCCCCAGGAG	213
Dentin sialophosphoprotein (DSPP)	Forward: CAGGAGCACAGGAAAAGGAG Reverse: CTGATTTGCTGCTGTCTGAC	488
GAPDH	Forward: CGGAGTCAACGGATTTGGTCGTAT Reverse: AGCCTTCTCCA TGGTGGTGAAGAC	306

and 1 μM specific primers. The sequences of the specific primers used in this study are detailed in Table 1. RT-PCR products were electrophoresed on 1.5% agarose gel with 0.5 mg/mL ethidium bromide. Bands were detected by UV illumination of ethidium bromide-stained gels.

5. Statistical analysis

Values were calculated as the mean and standard deviation. Statistical significance was evaluated by one way analysis of variance (ANOVA) using the SPSS (Version 11.0; SPSS, Chicago, IL) computer program.

Results

1. Effects of signal pathway inhibitors on cell viability during odontoblastic differentiation by inducing of HO-1 in HDPCs.

Cells were cultured with CoPP (20 mM) for 7 days in OM with 10^{-7} M dexamethasone, 10 mM-glycerophosphate and 50 μg/ml L-ascorbic Acid. To examine the effect of pharmacological inhibitors of MAPK on cell viability of HDPCs, we used MTT assay. When the cells were treated with p38 inhibitor (SB203580, 10 μM), JNK inhibitor (SP600125, 10 μM), and ERK inhibitor (PD98059, 20 μM) for 7 days, cell viability was significantly decreased compared with the control group (Fig. 1).

2. Effects of signal pathway inhibitors during odontoblastic differentiation by HO-1 inducing in HDPCs.

Cells were cultured with CoPP (20 mM) for 7 days in OM same procedure as described in the legend to Fig. 1. To determine whether activation of the MAPK pathways are involved in the expression of HO-1 and odontoblastic differentiation markers, we examined the effects of MAPK inhibitors during odontoblastic differentiation in HDPCs. Cells were treated with p38 inhibitor (SB203580, 10 μM), JNK inhibitor (SP600125, 10 μM) and ERK inhibitor (PD98059, 20 μM) for 7 days. Treatment with SB203580, SP600125 and PD98059 significantly decreased the mRNA expression of HO-1 and odontobalstic differentiation markers such as OPN, DMP-1 and DSPP in HDPCs (Fig. 2).

Discussion

HDPCs are able to produce a reparative dentin matrix secreted by a second generation of odontoblast- like cells after dental injury and irreversible odontoblast damage[23]. Thus, activation of HDPCs represents a potential cellular approach to pulp capping or dentinal regenerative treatment[24]. During dentin formation, odontoblasts synthesize several noncollagenous proteins and secrete these into the dentin extracellular matrix[25]. One of these proteins is dentinsialophosphoprotein (DSPP), which is believed to play a regulatory role in the mineralization of reparative dentin; it serves as a specific marker for the odontoblastic phenotype[26]. Osteopontin (OPN) is a major noncollagenous protein synthesized by differentiated osteoblasts and deposited into the mineralizing matrix[27]. In addition, expression of dentin matrix protein 1 (DMP-1) is also an important characteristic of odontoblast[28].

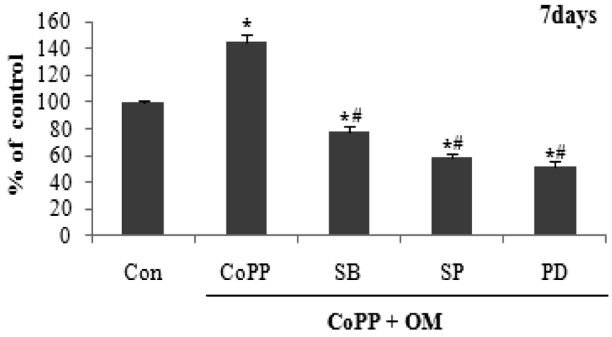

Fig. 1. Effects of signal pathway inhibitors of MAPKs during odontoblastic differentiation by induction of HO-1 in HDPCs. Cells were cultured with CoPP (20 mM) for 7 days in OM and co-treated with p38 inhibitor (SB203580, 10 μM), JNK inhibitor (SP600125, 10 μM), and ERK inhibitor (PD98059, 20 μM) for 7 days. Cell viability was evaluated using MTT assay. These data are representative of three independent experiments.

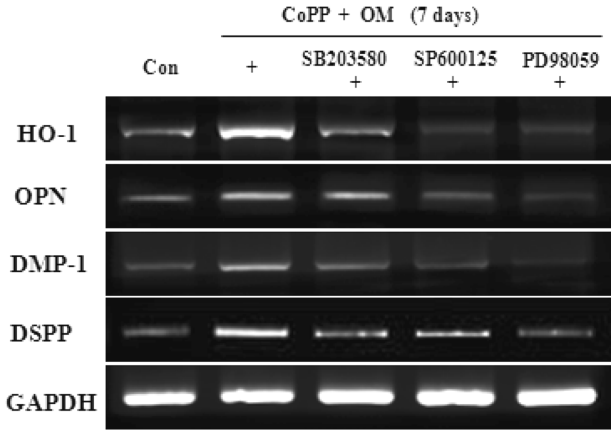

Fig. 2. Effects of signal pathway inhibitors of MAPK during odontoblastic differentiation by induction of HO-1 in HDPCs. Cells were treated with p38 inhibitor (SB203580, 10 μM), JNK inhibitor (SP600125, 10 μM), and ERK inhibitor (PD98059, 20 μM) for 7 days in OM with CoPP (20 mM). mRNA expression of HO-1 and odontoblastic differentiation markers such as OPN, DMP-1 and DSPP were assessed by RT-PCR analysis. Similar data were obtained from three independent experiments.

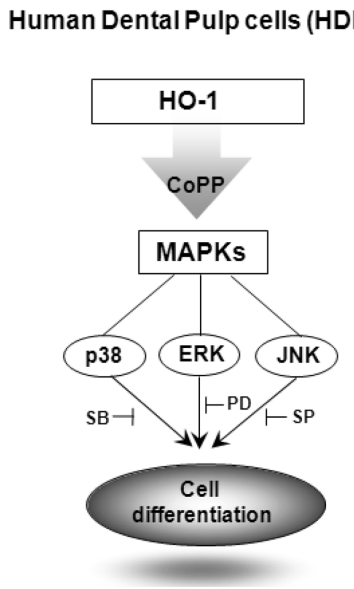

Fig. 3. MAPK pathways activated by HO-1 leading to odontoblastic differentiation in human dental pulp cell.

These proteins have been used as mineralization markers for odontoblast-like differentiation of pulp cells [5,7].

HO is a rate-limiting enzyme of heme catabolism and is widely distributed in mammalian tissues. HO-1 can be induced in a variety of tissues by its natural substrate heme and other synthetic metal porphyrins, such as cobalt-protoporphyrin IX (CoPPIX)[29]. Recent study indicated that CoPP treatment resulted in an elevated HO-1 expression and reduced inflammation in macrophages. In addition, CoPP-mediated HO-1 induction alters the inflammatory cytokine response in T-cells. Moreover, data from current studies suggest that HO-1 plays a vital role in control of cell growth and differentiation[10]. We have previously shown that *in vitro* administration of hemin to PDL cells leads to HO-1 induction, and to enhanced osteoblastic differentiation[30]. However, there are no published reports for signal pathways involved in CoPP-mediated induction of HO-1 during odontoblastic differentiation in HDPCs.

The role of MAPK has previously been demonstrated in various cell culture systems, and contradictory results were observed on the regulatory role of different MAPK pathways in HO-1 gene expression[31]. Several studies have indicated a major role for the MAPK in HO-1 activation[32]. Moreover, induction of HO-1 is known to be regulated by p38, JNK, and ERK[14]. Increasing evidence indicates that functions of MAPK pathways in the regulation of HO-1 expression and differentiation; however, the mechanism of MAPK in CoPP-induced expression of HO-1 and odontoblastic differentiation are unclear.

In our data, after pharmacological inhibition of MAPK pathways, cell viability was significantly suppressed in HDPCs. Furthermore, we were able to show that mRNA expressions of odontoblastic differentiation markers such as BSP, OPN, DSPP, and DMP-1 inducted by HO-1 were decreased when the cell were exposed the MAPK pathway inhibitors. These data provide evidence that odontoblastic differentiation induced by HO-1 may be partly mediated by an MAPKs signal pathway in HDPCs. With these results, we speculated that odontoblastic differentiation by HO-1 gene expression can be induced via signaling pathways involving MAPKs (ERK, JNK, p38).

To determine whether the proliferation and differentiation of HDPCs afforded by CoPP are associated with HO-1 expression via MAPK pathways, we used the inhibitors of ERK, JNK, and p38 during differentiation by inducing of HO-1 in HDPCs. When the cells were treated with SB203580, SP600125, and PD98059, mRNA expression of HO-1 and differentiation markers were suppressed in HDPCs. These results suggest that the ERK, JNK, p38 pathways are involved in CoPP-induced expression of HO-1 and osteoblastic differentiation.

Therefore, the targeted MAPK pathways in regulation of HO-1 during odontoblastic differentiation may represent a novel therapeutic approach to promote or to regenerate living human dental tissues for tissue engineering.

Summary

Enhanced HO-1 activity by CoPP is involved in proliferation and differentiation in HDPCs. However, MAPK signaling pathways involved in pulp cell growth and differentiation by HO-1 induction have not been well explored. The purpose of this study was to investigated the role of the MAPK signaling pathways involved in regulation of HO-1 and the odontoblast differentiation markers, including OPN, DMP-1, and DSPP during the odontoblastic differentiation for HDPCs.

The HO-1 induction by CoPP in HDPCs increased cell growth and upregulated the mRNA levels for odontoblastic markers, such as OPN, DMP-1, and DSPP. Moreover, treatment of cells with ERK, JNK, and p38 inhibitors blocked CoPP-induced HO-1 and differentiation markers.

These results suggest that odontoblastic differentiation is positively regulated by HO-1 induction in HDPCs via ERK, JNK, and p38 signaling pathways. Therefore, HO-1 induction through MAPK pathways by HO-1 inducer might represent a potent therapeutic approach for pulp capping and regeneration of HDPCs.

Acknowledgements

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