EphB–EphrinB Interaction Controls Odontogenic/Osteogenic Differentiation with Calcium Hydroxide

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Abstract

Introduction: Calcium hydroxide is used in direct pulp capping of uncontaminated exposed vital pulps caused by mechanical or traumatic injury. Calcium hydroxide creates a high alkaline pH environment and initiates a mineralized tissue formation in the pulp. The exact mechanism by which calcium hydroxide induces the reparative dentin formation is unknown. Because Eph receptors and ephrin ligands play a role in pulp stem cell migration and proliferation, our hypothesis is that calcium hydroxide–related odontogenic/osteogenic differentiation may be associated with Eph-ephrin interaction. The aim of this study was to investigate whether Eph-ephrin interaction regulates odontogenic/osteogenic differentiation with calcium hydroxide. **Methods:** Primary pulp cells were harvested from the molars of C57BL/6 mice. The cells were treated with calcium hydroxide. Immunofluorescence was used to detect protein expression. A knockout of the *ephrinB1* or *EphB2* gene was performed with short hairpin RNAs. Cell migration, proliferation, and gene expression were then analyzed. **Results:** Calcium hydroxide stimulated *EphB2* gene expression but suppressed *ephrinB1* gene expression at the proliferation stage. However, calcium hydroxide stimulated both *ephrinB1* and *EphB2* gene expression at the differentiation stage. In addition, EphB2 localized at ephrinB1–positive cells at the area of Dentin sialoprotein (DSP) staining, which increased with calcium hydroxide treatment. Knockdown of ephrinB1–EphB2 significantly suppressed cell proliferation. Additionally, knockdown of the *ephrinB1* gene caused cell migration, whereas a lack of the *EphB2* gene suppressed calcium hydroxide–induced mineralization from primary pulp cells. **Conclusions:** EphrinB1–EphB2 interaction contributes to calcium hydroxide–induced odontogenic/osteogenic differentiation. This observation is the first finding of the mechanism of calcium hydroxide–induced odontogenic/osteogenic differentiation. *(J Endod 2013;39:1256–1260)*

Key Words

Calcium hydroxide, Eph, ephrin, primary pulp cells, pulp capping

Calcium hydroxide has been extensively used for direct pulp capping in clinical dentistry. It is known that calcium hydroxide initiates the formation of reparative dentin at the site of pulp exposure because of its high alkaline pH. The reparative process that follows involves cell migration and the proliferation of pulp mesenchymal stem cells (1, 2). However, the exact mechanism by which calcium hydroxide induces the reparative dentin formation remains unclear.

The Eph/ephrin family is well known for its role in mediating inhibitory or repulsive cellular responses and has been shown to be expressed during tooth development (3). Eph receptors are the largest subgroup of the receptor tyrosine kinase family. Their cell surface ligands, known as ephrins, are involved in a variety of cell communications that affect processes such as cell proliferation, chemotaxis, angiogenesis, extracellular matrix remodeling, and cell differentiation (4–8). It is known that forward signaling of ephrinB1 inhibits the migration of dental pulp stem cells, while permitting mobilization of dental pulp stem cells after injury through the down-regulation of *ephrinB1* gene expression (7). In addition, forward signaling of ephrinB1 increases the number of bromodeoxyuridine-positive pulp stem cells and CD146 expression in the proliferating cells (9). Thus, a forward signal of ephrinB1 inhibits the migration of dental pulp stem cells while stimulating the proliferation of pulp stem cells. With respect to the Eph receptors, a reverse signal of EphB2 in pulp stem cells has been shown to stimulate dentin marker genes (9).

Our hypothesis is that calcium hydroxide–induced odontogenic/osteogenic differentiation may be associated with Eph-ephrin interaction. The aim of this study was to investigate how Eph-ephrin interaction regulates odontogenic/osteogenic differentiation with calcium hydroxide.

Material and Methods

Animals and Cell Culture of Pulp Stem Cells

Primary pulp cells were harvested from 1- to 2-month old male C57BL/6 mice (a total of 6 mice), minced in phosphate-buffered saline (PBS), and incubated in PBS containing 3 mg/mL collagenase type I (Sigma-Aldrich, St Louis, MO) and 4 mg/mL dispase (Sigma-Aldrich) for 30 minutes at 37°C (10, 11). The cells were cultured on plates with proliferation condition media (alpha-Minimum Essential Medium [alpha-MEM] supplemented with 20% fetal bovine serum, 2 mmol/L L-glutamine, and 100 U/mL penicillin-streptomycin; Life Technologies, Grand Island, NY). Fifty micrograms per milliliter of L-ascorbic acid (Sigma-Aldrich) was added to the media on day 7 and every 2–3 days afterward. For the last 4 days of the culture period, 5 mmol/L beta-glycerol phosphate (EMD Millipore, Billerica, MA) was added to the media for mineralization. All primary cells were used in their second or third

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passage. Cells were treated with calcium hydroxide as described by Ji et al (2). The final concentration of 1 μg/mL was made by adding 1 μL of 1 mg/mL calcium hydroxide stock into the 12-well plate with 1 mL media. Primary pulp cells were proliferating at 7 days (proliferation stage) until they became confluent at 8–9 days. After confluence, ascorbic acid was added, and cells began differentiating in the plates. At 14 days (differentiation stage), cells were accumulated to create nodule formation.

Migration Assay

Mouse primary pulp cells were placed in an upper chamber of QCM Chemotaxis Cell Migration Assay (EMD Millipore). Twenty-four-well flat-bottom plates were incubated with or without calcium hydroxide for 72 hours at 37°C in 5% CO_2. The interior of the transwell was cleaned to remove cells that did not migrate through the membrane. Migrated cells were quantitated using a cell-permeable fluorescent dye (excitation maximum: ~485 nm; emission maximum: ~520 nm).

Proliferation Assay

Mouse primary pulp cells were placed in 96-well or 12-well plates and then incubated with or without calcium hydroxide for at 37°C in 5% CO_2. Cells in 12-well plates were corrected on day 7 and counted using a hemocytometer. Cells in 96-well plates were incubated for 72 hours with or without calcium hydroxide. tetrazolium dye (MTT) solution (Biotium Inc, Hayward, CA) was added to the 100 μL medium in each well according to the manufacturer's instructions. Cells were incubated at 37°C for 4 hours. Dimethyl sulfoxide (DMSO) was directly added into the medium in each well and pipetted in succession to dissolve the formazan salt. The absorbance signal was measured on a spectrophotometer at 570 nm. The background absorbance was measured at 630 nm. Fold changes in the normalized absorbance values (background absorbance subtracted from signal absorbance) relative to the negative control were calculated.

Quantitative Real-time Reverse-transcription Polymerase Chain Reaction

Total RNA from primary pulp cells was isolated with TRIzol reagent (Sigma-Aldrich) and reverse transcribed to complementary DNA with TaqMan Reverse Transcription Reagents (Life Technologies) according to the manufacturer's instructions. The sequences were amplified by adding 2.5 μL complementary DNA to the polymerase chain reaction (PCR) mixture (22.5 μL) containing each primer (0.2 μm) and 12.5 μL Platinum SYBR Green qPCR SuperMix uracil-DNA glycosylase (UDG) (Life Technologies). The reactions were preincubated at 50°C for 2 minutes for decontamination of deoxyuridine (dU)-containing DNA by UDG and then incubated at 95°C for 2 minutes to inactivate UDG and activate Taq. The PCR program continued 49 cycles of denaturation at 95°C for 15 seconds with the annealing and elongation of the primers at 60°C for 30 seconds. Fold changes in gene expression relative to control samples were calculated using the following formula: $2^{(\Delta Ct \text{ control or negative control} - \Delta Ct \text{ calcium hydroxide or shRNA})}$. All of the samples were normalized to beta-actin. The primer sequences were as follows: alkaline phosphatase (ALP), forward: 5′-tcccacgttttcacattcgg-3′, reverse: 5′-cccgttaccatataggatggcc-3′; osterix (Osx), forward: 5′-agaggttcactcgctctgacga-3′, reverse: 5′-ttgctcaagtggtcgcttctg-3′; bone sialoprotein (BSP), forward: 5′-acaccccaagcacagacttttg-3′, reverse: 5′-tcctcgtcgctttccttcact-3′; dentin matrix protein-1 (DMP-1), forward:5′- ctcaggacagtagccgatcc-3′, reverse: 5′ tgggtttgttgtggtaagca-3′; dentin sialophosphoprotein (DSPP), forward 5′-ctgggaagagccaagat cag-3′, reverse: 5′-ctccactccttggtgtccat-3′; and beta-actin, forward: 5′-tcctcctgagcgcaagtactct-3′, reverse: 5′-cggactcatcgtactcctgctt-3′. The PCR reactions and program are described in a previous publication (12).

Plasmids, Short Hairpin shRNA Transfection

Vectors that express *ephrinB1* and *EphB2* shRNAs under the control of the U6 promoter were constructed by inserting pairs of annealed DNA oligonucleotides into the lentivirus-shRNA expression vector (GeneCopeia, Rockville, MD). Supernatants containing infectious lentivirus were amplified by HEK293 cells. Mouse primary pulp cells were plated into 24- or 96-well plates and infected with 20 multiplicity of infection (MOI) of viruses for 6 hours. Fresh medium was used after the incubation period, and the cells were cultured for an additional 3 days to examine silencing effects.

Immunofluorescence Assay

Mouse primary pulp cells were grown in 24-well plates with or without calcium hydroxide and then fixed in 4% paraformaldehyde in PBS. Primary antibodies for anti–ephrinB1, anti- EphB2 (R&D Systems Inc, Minneapolis, MN), anti-Oct3/4, and anti-DSP (Santa Cruz Biotechnology, Dallas, TX) were used. Secondary antibodies conjugated to green-fluorescent Alexa Fluor 488 dye (Life Technologies) or red-fluorescent Texas Red dye (Santa Cruz Biotechnology) were used at a dilution of 1:300. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich).

Statistical Analysis

All results are expressed as mean ± standard deviation of triplicate measurements with all experiments being repeated a minimum of 3 times. Statistical analyses were performed using the Student *t* test.

Results

Calcium Hydroxide Affected EphrinB1 and EphB2 Interaction

To investigate the effect of calcium hydroxide on ephrinBs and EphBs, we examined the gene expression of ephrinBs and EphBs with or without calcium hydroxide treatment in primary pulp cells. Calcium hydroxide stimulated EphB2 gene expression at the proliferation stage while inhibiting *ephrinB1* gene expression (Fig. 1*A*). However, calcium hydroxide stimulated the *ephrinB1* gene and the EphB2 gene at the differentiation stage (Fig. 1*B*). Calcium hydroxide had no effect on *ephrinB2*, *EphB1*, *EphB4*, and *EphB6* (Fig. 1*A* and *B*).

Next, we performed an immunofluorescence assay to examine where ephrinB1 and EphB2 were expressed. Calcium hydroxide caused cell aggregation and nodule formation during high levels of ephrinB1 and EphB2 expression. EphB2 localized ephrinB1–positive cells with or without calcium hydroxide (Fig. 1*C*). To examine if ephrinB1 is associated with stem cells, we used stem cell markers such as Oct3/4, which partially localized to ephrinB1–positive cells (Fig. 1*D*). DSP expression, which increased with calcium hydroxide, was shown to localize surrounding or overlapping ephrinB1–positive cells (Fig. 1*E*). Calcium hydroxide significantly stimulated cell numbers on day 7 (Fig. 1*F*).

Calcium Hydroxide Stimulated Cell Migration through EphrinB1

To assess if *ephrinB1* and *EphB2* genes were associated with calcium hydroxide–induced cell migration, we performed knockdown of *ephrinB1* and *EphB2* genes. As shown in Figure 2*A*, shRNA of *ephrinB1* and *EphB2* caused a 50%–60% reduction of genes in primary pulp cells. A high concentration (1 μg/mL) of calcium hydroxide significantly increased cell migration in the negative control. Knockdown of ephrinB1 induced cell migration with calcium hydroxide compared