Effect of Verapamil, a Calcium Channel Blocker, on the Odontogenic Activity of Human Dental Pulp Cells Cultured with Silicate-based Materials

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Abstract

Introduction: This study examines how calcium silicate cement extracts influence the behavior of human dental pulp cells (hDPCs) through calcium channels and active mitogen-activated protein kinase pathways, in particular extracellular signal-related kinase (ERK). **Methods:** HDPCs are treated with various silicon concentrations both with and without verapamil, after which the cells' viability and odontogenic differentiation markers are determined by using PrestoBlue assay and Western blot, respectively. **Results:** The silicon promoted cell proliferation and inhibited calcium channel blockers. It was also found that silicon increased ERK and p38 activity in a dose-dependent manner. Furthermore, it raised the expression and secretion of alkaline phosphatase, osteocalcin, dentin sialophosphoprotein, and dentin matrix protein-1. In addition, statistically significant differences ($P < .05$) have been found in the secretion of osteocalcin in ERK inhibitor + verapamil between the silicon concentrations; these varations are dose-dependent and indicate that ERK signaling is involved in the silicon-induced odontogenic differentiation of hDPCs. **Conclusions:** The current study shows that silicon ions released from calcium silicate substrates play a key role in odontoblastic differentiation of hDPCs through calcium channels and modulate ERK activation. *(J Endod 2014;40:1105–1111)*

Key Words

Calcium channel blocker, calcium silicate cement, human dental pulp cells, MAPK, odontogenic differentiation, verapamil

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Mineral trioxide aggregate (MTA) is a biomaterial with several clinical applications in endodontics (1). Several clinical usage studies have shown that MTA has results comparable with or better than calcium hydroxide–based materials in decreasing pulp inflammation (2). This commercial product contains 75% Portland cement, 20% Bi_2O_3, and 5% gypsum. It was approved for sale in 1998 (1). Some *in vitro* studies showed that not only does MTA enhance hard-tissue formation (3), but it also inhibits osteoclast differentiation (3). In our study we found that radiopaque and hydraulic calcium silicate cement (CSC) require a shorter setting time (4) and have better biocompatibility than MTA and thus may have the potential to serve as a root-end filling material (5). Therefore, CS-based cements have been formulated into dentin replacement restorative materials in dentistry (6). In addition, human dental pulp cell (hDPC) viability cultured on CSC was higher than when cultured on MTA for all time lengths tested (2), suggesting that the cement can be applied in endodontic treatments. Moreover, CSC not only exhibits good osteoconduction effects (5) but also reduces inflammation of hDPCs (2, 7). In addition, the suitable concentration of silicon can inhibit the osteoclastogenesis in osteoclast cells (8) and promote angiogenesis in hDPCs (9).

Human dental pulp cells have the ability to differentiate along the osteoblast lineage and contribute to the odontogenic process (10). The most striking characteristic of hDPCs is their ability to form a dentin-pulp–like complex that is composed of mineralized nodules with tubules lined with odontoblasts as well as fibrous tissue containing vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth (10). According to the literature, CS-based materials promote the proliferation of hDPCs (11, 12). During the odontogenic process, hDPCs secrete various proteins, including osteocalcin (OC), alkaline phosphatase (ALP), bone sialoprotein, dentin matrix protein-1 (DMP-1), and dentin sialophosphoprotein (DSP), which are all used as biomineralization markers for the odontoblast-like/osteoblast-like differentiation from hDPCs (13). However, the mechanism of intracellular signaling transduction responsible for the regulation of odontoblastic differentiation induced by CS-based materials in hDPCs is still poorly understood. The ratios of silicon or Ca in CS-based materials may play a dose-dependent role in increasing cell growth (11, 14). Extracellular Ca ions have been documented as being a major proponent in transmitting messages that affect cell behavior, thus having a significant effect on the regulation of the proliferation and differentiation of osteoblasts (15). However, even though it is known that silicon promotes bone formation and the calcification process in the early stages, the mechanism by which silicon enhances cell behavior, including cell attachment, remains unclear. To understand the mechanism of silicon-induced cell attachment and proliferation enhancement is important for the future expansion of the applications of silica-based materials. On the other hand, although silicon plays important roles in the early stages of bone formation, the mechanism by which silicon ion promotes odontogenesis activity, including initial cell differentiation, remains unclear.

The aim of this study was to examine the protein changes related to odontoblastic differentiation in hDPCs when CS-based material is applied to cells and to examine the role of silicon ions in the differentiation of hDPCs by using an L-type calcium channel blocker, verapamil. This study showed how silicon manipulates the sequential

molecular events through the Ca channel and promotes extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) during odontogenic differentiation.

Materials and Methods

Specimen Preparation

In this study, MTA (white ProRoot MTA; Dentsply, Tulsa, OK) and CSC (composite: 65% CaO, 25% SiO_2, and 5% Al_2O_3) were used. The method for the preparation of CS powder has been described elsewhere (4). Appropriate amounts of CaO (Showa, Tokyo, Japan), SiO_2 (High Pure Chemicals, Saitama, Japan), and 5% Al_2O_3 (Sigma-Aldrich, St Louis, MO) powders were mixed and sintered at 1400°C for 2 hours. MTA and CSC had been mixed according to the same liquid/powder ratio of 0.3 mL/g. After mixing, the cement was used to fully cover each well of the 24-well plate (GeneDireX, Las Vegas, NV) to a thickness of 2 mm, and the samples were stored in an incubator at 100% relative humidity and 37°C for 1 day. Before cell experiments were performed, all specimens were sterilized by immersion in 75% ethanol, followed by exposure to ultraviolet light for 1 hour.

HDPC Isolation and Culture

The hDPCs were obtained from an established laboratory. They were freshly derived from a caries-free intact premolar that was extracted for orthodontic treatment. The patient gave informed consent, and this process received approval from the Ethics Committee of the Chung Shan Medicine University Hospital (CSMUH no. CS11187). The tooth was split sagittally with a chisel. The pulp tissue was then immersed in phosphate-buffered saline (PBS) (Caisson Laboratories, North Logan, UT) solution and broken down in 0.1% collagenase type I (Sigma-Aldrich) for 30 minutes. After being transferred to a new plate, the cell suspension was cultured in Dulbecco modified Eagle medium (DMEM) (Caisson Laboratories), supplemented with 20% fetal bovine serum (GeneDireX), 10 units/mL penicillin G solution, and 10 mg/mL streptomycin (PS; Caisson Laboratories) in a humidified atmosphere measuring 5% CO_2 at 37°C. The cells were subcultured through successive passaging at a 1:3 ratio until being used for experiments (passages 3–8).

Ion Concentration

The silicon ion concentration released from cement on DMEM was analyzed by using an inductively coupled plasma-atomic emission spectrometer (Perkin-Elmer OPT 1MA 3000DV, Shelton, CT) after being cultured for 1, 3, and 5 days. Three samples were measured for each data point. The results were obtained in triplicate from 3 separate samples for each test.

Preparation of Test Medium Containing Different Silicon Concentration

Cements (1 g) were immersed in 10 mL DMEM for 1 day, and the supernatants were passed through a 0.22-μm filter (Millipore, Billerica, MA) to obtain the extract. The silicon ion concentration thus extracted was analyzed by using an inductively coupled plasma-atomic emission spectrometer. A detailed description of the dilution of the test medium with various silicon ion concentrations is given elsewhere (14). The cement extract and DMEM were used to prepare 4 different media with various silicon ion concentrations (1, 2, and 4 mmol/L).

Cytotoxicity

HDPC suspensions with a density of 5×10^3 cells/mL were directly seeded onto substrates. The cell cultures were incubated at 37°C in a 5% CO_2 atmosphere. After different culture times (1, 3, and 5 days), cell viability was evaluated by using the PrestoBlue assay (Invitrogen, Grand Island, NY). Briefly, at the end of the culture period, the medium was discarded, and the wells were washed with cold PBS twice. Each well was then filled with a medium composed of 1:9 ratio of PrestoBlue in fresh DMEM and incubated at 37°C for 30 minutes. The solution in each well was then transferred to a new 96-well plate. Plates were read in a multiwell spectrophotometer (Hitachi, Tokyo, Japan) at 570 nm with a reference wavelength of 600 nm. The results were obtained in triplicate from 3 separate experiments for each test. Furthermore, the influence of the silicon concentration on cell viability for hDPCs without and with the pretreatment of verapamil (5 μmol/L; Sigma-Aldrich) and EGTA (5 mmol/L; Sigma-Aldrich) was evaluated after treatment with extract on 24-well plate. Cells cultured on tissue culture plate without cement were used as a control.

Western Blot

Western blot analysis was carried out by using cell lysates and a culture medium prepared with cultured hDPCs for 5 days, because hDPCs can start differentiating and expressing odontogenic markers after 3 days in culture (12). Cells were lysed in NP-40 lysis buffer (Invitrogen) at 4°C for 20 minutes, and the lysates were centrifuged at 15,000*g*. Protein concentrations were detected by using a BCA protein assay kit. Cell lysates (30 μg protein) were separated by using sodium dodecylsulfate–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After blocking in 5% bovine serum albumin (Sigma-Aldrich) for 1 hour, the membrane was immunoblotted with the primary anti-phospho-ERK1/2, anti-phospho-p38, anti-phospho–c-Jun N-terminal kinases (JNK), anti-ALP, anti-OC, anti-DSP, anti-DMP-1, and anti-β-actin (GeneTex, San Antonio, TX) for 2 hours and then washed 3 times in a Tris-buffer saline containing 0.05% Tween-20 (Sigma-Aldrich). A horseradish peroxidase–conjugated secondary antibody was subsequently added, and the proteins were visually enhanced by using enhanced chemiluminescent detection kits (Invitrogen). The stained bands have been scanned and quantified by using a densitometer (Syngene Bioimaging System, Frederick, MD) and ImageJ software (National Institutes of Health, Bethesda, MD), after which the protein expression levels were normalized to the β-actin band for each sample. The results were obtained in triplicate from 3 separate samples for each test.

Effects of ERK1/2 Inhibitor and Verapamil on Cell Differentiation

To further clarify the ERK1/2 inhibitor and verapamil effects on cell differentiation, ERK1/2 inhibitors (PD98059, 50 μm) were added to the culture medium. After being cultured for 5 days, the hDPC differentiation was evaluated by using a commercially available OC enzyme-linked immunosorbent assay kit (Abcam, Cambridge, MA). Following the manufacturer's instructions, we used the 2-hour assay, which has a higher sensitivity. The reaction was terminated by adding a stop solution and read at 450 nm by using a multiwell spectrophotometer. This experiment was repeated independently 3 times.

Alizarin Red S Stain

The accumulated calcium deposition after 5 days was analyzed by using alizarin red S staining, as in a previous study (14). In brief, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes and then incubated in 0.5% alizarin red S (Sigma-Aldrich) at pH 4.0 for 15 minutes at room temperature in an orbital shaker (25 rpm). To quantify the stained calcified nodules, samples were immersed in 1.5 mL 5% sodium dodecylsulfate in 0.5N HCl for 30 minutes at