Role of the Extracellular Signal-regulated Kinase 1/2 Pathway in Driving Tricalcium Silicate–induced Proliferation and Biomineralization of Human Dental Pulp Cells *In Vitro*

Rong Du, DDS, PhD, Tiantian Wu, DDS, MDS,* Wenjuan Liu, PhD,† Lifen Li, DDS, PhD,* Long Jiang, DDS, PhD,* Weiwei Peng, DDS, MDS,* Jiang Chang, PhD,† and Yaqin Zhu, DDS, PhD**

Abstract

Introduction: The aim of this study was to investigate the role of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in regulating tricalcium silicate (C3S)–driven proliferation and biomineralization of human dental pulp cells (hDPCs) *in vitro*. **Methods:** Human DPCs were cultured in C3S-containing medium and compared with untreated controls. Cell viability was measured by the methyl-thiazol-tetrazolium assay. Biomineralization was assessed by staining calcium deposits on the extracellular matrix with von Kossa and alizarin red S stains. Phosphorylated ERK1/2 was evaluated by immunoblotting. The ERK1/2 inhibitor U0126 was used to assess the role of this pathway on stage of the cell cycle and mineralization-dependent gene expressions of hDPCs by using flow cytometry and real-time polymerase chain reaction, respectively. Data were analyzed by analysis of variance followed by the Student–Newman–Keuls post hoc test, with significance set at $P < .05$. **Results:** The viability and biomineralization of hDPCs were promoted by C3S extracts ($P < .05$). Phosphorylated ERK1/2 strongly appeared after hDPCs were cultured in the C3S extracts for 30 minutes. Moreover, inhibition of the ERK1/2 pathway in C3S-treated hDPCs decreased proliferation and the expression of mineralization-dependent genes, including collagen type I, dentin sialophosphoprotein, osteopontin, and osteocalcin ($P < .05$). **Conclusions:** C3S stimulated the proliferation and biomineralization of hDPCs *in vitro*, with the ERK1/2 pathway playing a key role in the regulation of these effects. *(J Endod 2013;39:1023–1029)*

Key Words

Biomineralization, extracellular signal-regulated kinase 1/2 pathway, human dental pulp cells, proliferation, tricalcium silicate

Mineral trioxide aggregate (MTA) has been widely and successfully used as a capping agent to preserve dental pulp viability in clinical procedures. Several studies have shown that MTA can promote the differentiation of human dental pulp cells (hDPCs) and hence induce biomineralization and the formation of reparative dentin (1–6). Tricalcium silicate (C3S) has been found to be a major component of MTA (7, 8) as well as in some other pulp-capping agents (9–12). Data are scarce about the mechanism of hDPC changes after their contact with these C3S-based pulp-capping agents, but it can be supposed that C3S plays an important role. We have recently shown that C3S stimulated the proliferation and differentiation of hDPCs *in vitro* (13). However, the underlying molecular mechanisms for these effects remain unclear.

The mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved in all eukaryotes and play a key role in the regulation of gene expression and cytoplasmic signaling (14). The mammalian MAPK comprises 3 major subfamilies, namely extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), and p38, each with specific substrates and functions (15, 16). For instance, ERK1/2 preferentially regulates cell growth and differentiation (17, 18). In this regard, MTA enhanced proliferation of a human osteosarcoma cell line via ERK1/2 activation (19). Moreover, both MAPK (20–23) and ERK1/2 (22) played a role in the proliferation and differentiation of hDPCs. Taking these factors altogether, we hypothesized that C3S, the major component of MTA, may impact the behavior of hDPCs via an MAPK pathway, in particular ERK1/2.

Therefore, the aim of this study was to investigate the specific role of the ERK1/2 pathway on C3S-induced proliferation and mineralization of hDPCs.

Materials and Methods

Isolation and Culture of hDPCs

Human dental pulp tissues were obtained from periodontal healthy and noncarious third molars or premolars extracted because of orthodontic treatment from patients aged 15–20 years. Informed consent was acquired before extractions, and the study was approved by the Ethics Committee of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

From the *Department of General Dentistry, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai; and †Biomaterial and Tissue Engineering Research Center, Shanghai Institute of Ceramics, Chinese Academy of Science, Shanghai, People's Republic of China.

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Address requests for reprints to Dr Yaqin Zhu, Department of General Dentistry, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, 639 Zhi Zao Ju Road, Shanghai 200011, PR China. E-mail address: zyq1590@163.com

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Briefly, the teeth were washed 3 times in sterile phosphate-buffered saline (PBS) solution (0.01 mol/L, pH 7.4) immediately after extraction. Then, a tooth was cut around the cementoenamel junction with sterilized dental fissure burs and split with a gypsum cutter to reveal the pulp chamber. The dental pulp was carefully removed from the pulp chamber. Isolated pulp tissues were minced into 1- to 3-mm^3 pieces and rinsed in PBS buffer supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin. Pieces were then adhered to the bottom of a culture dish by using sterile petroleum jelly (Mingshi, Shanghai, China), covered with Thermanox coverslip (NUNC, Naperville, IL), and incubated in Dulbecco modified Eagle medium (DMEM) (Gibco, Grand Island, NY) supplemented with 2 mmol/L glutamine (Sigma, St Louis, MO), 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.25 mg/mL amphotericin B (Fungizone; Gibco), and 20% fetal bovine serum (FBS) (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed at 3-day intervals. Cells were collected by trypsinization (0.2% trypsin and 0.02% ethylenediaminetetraacetic acid) (Gibco) and subcultured with DMEM containing 10% FBS (24). For this study we used hDPCs obtained on the third to fifth cell culture passages. Data presented herein were representative of experiments with pulp from 11 different donors.

Preparation of RD Medium and C3S Extracts

To simulate the physiologic calcium concentrations, RD medium (25–27) was prepared by mixing RPMI 1640 (Gibco, Guangzhou, China) and DMEM (Gibco, Grand Island, NY) media at a 1:1 (vol:vol) ratio. Because RPMI and DMEM contain 0.4 mmol/L and 1.8 mmol/L ionized calcium, respectively, RD medium should contain approximately 1.1 mmol/L ionized calcium ion. The serum concentration of ionized calcium ranges from ~1.1–1.35 mmol/L, so RD medium would provide cells a nearly physiologic calcium condition. This medium was then used to prepare the C3S extracts and also as the control condition (10% FBS) during the experiments.

The C3S powder was prepared by the sol-gel method (28). Following the International Standard Organization (ISO10993-5) protocol, C3S powder was dissolved in serum-free RD medium at a concentration of 200 mg/mL and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 24 hours without agitation. Then, the mixture was centrifuged for 10 minutes; the supernatant was sterilized through a 0.22-mm filter (Millipore, Billerica, MA) and stored at 4°C until further use for a maximum of 30 days. Then, C3S extracts at different concentrations were obtained by further diluting the 200 mg/mL stock solution with RD medium supplemented with 10% FBS.

Cell Viability Assay

The influence of C3S on hDPCs' viability was determined by using the methyl-thiazol-tetrazolium (MTT) (Amresco, Solon, OH) assay following the manufacturer's directions. For this purpose, hDPCs were seeded at a density of 2×10^3 cells per well into 96-well plates and incubated in a humidified atmosphere of 5% CO_2 and 95% air for 24 hours. Then, the culture medium was removed and replaced by 200 μL C3S extracts at different concentrations (50, 5, 0.5, 0.05, and 0.005 mg/mL) to define the optimal extract concentration supporting cell viability. The RD medium alone was used as a negative control. Media were replaced every 3 days. At different time points (1, 3, 6, and 9 days) after initiation of culture, 20 μL MTT solution was added into each well to a final concentration of 5 mg/mL. After an additional 4-hour incubation period, the supernatant was discarded, and 200 μL dimethyl sulfoxide (Sigma) was added to solubilize the formazan crystals. Then, the optical density of each preparation was determined at a wavelength of 490 nm by using an enzyme-linked immunosorbent assay plate reader (ELx 800; BIO-TEK, Burlington, VT). Increased purple coloring correlated with increasing numbers of viable cells.

Biomineralization Assay

The hDPCs were seeded into 6-well plates and cultured with C3S extracts (5 mg/mL) or RD medium alone for 21 days in humidified atmosphere (5% CO_2, 95% air). The von Kossa and alizarin red S (AR) stains were used to assess for mineralized deposit formation. Briefly, cell layers were rinsed with PBS and either fixed with 4% paraformaldehyde solution, stained with 1% silver nitrate ($AgNO_3$), and exposed to ultraviolet light for 30 minutes (von Kossa staining) or fixed with 95% alcohol and stained with 0.5% AR (pH 4.2) for 40 minutes at 37°C (AR staining). Six-well plates were then scanned (Canon, Tokyo, Japan), and the images were captured and saved with PhotoStudio 2000 software.

Effect of C3S Extracts on ERK1/2 Phosphorylation

The hDPCs cultured in C3S extracts (5 mg/mL) were harvested at selected time points (0, 15, 30, 60, 90, and 120 minutes), and each sample (cells collected from 1 dish) was treated with 100 μL modified lysis buffer (RIPA lysis buffer: protease inhibitor: phosphatase inhibitor = 100:1:1) for 15 minutes on ice following the manufacturer's instructions. Cell lysates were then cleared at 12,000 rpm for 15 minutes at 4°C. The protein concentrations were determined with bicinchoninic acid method according to the manufacturer's instructions (Beyotime, HaiMen, Jiangsu). Forty micrograms of protein from each sample was boiled in 5× sodium dodecylsulfate gel-loading buffer for 10 minutes and subjected to 12% gel electrophoresis. After sodium dodecylsulfate–polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour with 5% nonfat dried milk in Tris-buffer saline containing 0.1% Tween-20 and incubated with primary antibodies against ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) (rabbit immunoglobulin G, 1:1000; Cell Signaling, Danvers, MA) overnight at 4°C. After being washed 3 times in Tris-buffer saline containing 0.1% Tween-20, membranes were incubated for 1 hour with horseradish peroxidase–labeled secondary antibodies (anti-rabbit immunoglobulin G, 1:2000; Cell Signaling) and were resolved by using an ECL kit (Thermo, Waltham, MA). Images were captured by Alliance 4.7 software (UVITec Ltd, Cambridge, UK) and analyzed with ImageJ software. For quantification of p-ERK, each p-ERK band was normalized to the corresponding total (t)-ERK1/2 band. Therefore, ERK1/2 activity is presented as the ratio of p-ERK1/2 normalized to t-ERK1/2.

Effects of ERK1/2 Pathway on the Proliferation and Differentiation of hDPCs Cultured in C3S Extracts

Effects of ERK1/2 Inhibitor on ERK1/2 Phosphorylation. The U0126 (Cell Signaling) ERK1/2 inhibitor was used for these experiments. The hDPCs were therefore cultured in C3S extracts (5 mg/mL) with 3 test conditions: (1) no additions (control); (2) +U0126 (10 μmol/L): hDPCs were pretreated with U0126 (10 μmol/L) for 2 hours before addition of C3S extracts to the culture; and (3) +U0126 (20 μmol/L): hDPCs were pretreated with U0126 (20 μmol/L) for 2 hours before addition of C3S extracts to the culture. After a 30-minute incubation period in a humidified atmosphere of 5% CO_2 and 95% air, protein was obtained for Western blotting as detailed above. The optimal inhibitory concentration of U0126 was selected in the following experiments.