The Role of Integrin αv in Proliferation and Differentiation of Human Dental Pulp Cell Response to Calcium Silicate Cement

Chi-Jr Hung, DDS, MD, PhD,[*†] *Hsin-I. Hsu, MS,*[‡] *Chi-Chang Lin, PhD,*[§] *Tsui-Hsien Huang, DDS, MD, PhD,*[*†] *Buor-Chang Wu, DDS, MD, PhD,*[*†] *Chia-Tze Kao, DDS, MD, PhD,*[*†] *and Ming-You Shie, PhD*[*]

Abstract

Introduction: It has been proved that integrin αv activity is related to cell proliferation, differentiation, migration, and organ development. However, the biological functions of integrin αv in human dental pulp cells (hDPCs) cultured on silicate-based materials have not been explored. The aim of this study was to investigate the role of integrin αv in the proliferation and odontogenic differentiation of hDPCs cultured with the effect of calcium silicate (CS) cement and β-tricalcium phosphate (TCP) cement. **Methods:** In this study, hDPCs were cultured on CS and TCP materials, and we evaluated fibronectin (FN) secretion and integrin αv expression during the cell attachment stage. After small interfering RNA transfection targeting integrin αv, the proliferation and odontogenesis differentiation behavior of hDPCs were analyzed. **Results:** The results indicate that CS releases Si ion–increased FN secretion and adsorption, which promote cell attachment more effectively than TCP. The CS cement facilitates FN and αv subintegrin expression. However, the FN adsorption and integrin expression of TCP are similar to that observed in the control dish. Integrin αv small interfering RNA inhibited odontogenic differentiation of hDPCs with the decreased formation of mineralized nodules on CS. It also down-regulated the protein expression of multiple markers of odontogenesis and the expression of dentin sialophosphoprotein protein. **Conclusions:** These results establish composition-dependent differences in integrin binding and its effectiveness as a mechanism regulating cellular responses to biomaterial surface. *(J Endod 2014;40:1802–1809)*

Key Words

Calcium silicate cement, fibronectin, human dental pulp cell, integrin αv, odontogenesis

Mineral trioxide aggregate (MTA) has long been used as a root-end dental filling material and is widely used in several clinical applications of endodontics (1). MTA received approval for general medical use in 1998 and is comprised of a mixture of 75% Portland cement (CaO and SiO_2), 20% Bi_2O_3, and 5% gypsum (1). Not only does MTA have good biocompatibility (2), it also has been proven to promote osteogenesis in human dental pulp cells (hDPCs) (3, 4). In dentistry, calcium silicate (CS)-based cements have been formulated into dentin replacement restorative materials (5), but there is reason to believe its performance can be made more effective by decreasing the setting time and improving the handling properties in the clinical setting (6). In our previous study, we produced a fast-setting CS cement that contains CaO, SiO_2, and Al_2O_3, which were shown to have a reduced setting time (7). In addition, CS cement not only exhibits good osteoconduction effects (8, 9) but also reduces inflammation markers in primary hDPCs (10, 11) and *in vivo* (12). The release of Si concentrations from silicate-based materials influences the behavior of different cell types, such as inhibiting osteoclastogenesis in macrophages (13) and angiogenesis in hDPCs (14, 15). In addition, the amount of Si ions in CS-based materials can affect the adsorption of various types of extracellular matrices (ECMs) such as collagen I, fibronectin (FN), and vitronectin and promote the up-regulation of mitogen-activated protein kinase/extracellular signal-regulated protein kinase 1/2 (MAPK/ERK 1/2) and MAPK/p38, signaling the pathway more effectively than Ca components (11, 16).

Integrins are the major transmembrane receptors for cell adhesion to the ECM (17). All integrins are heterodimers composed of noncovalently linked alpha and beta subunits. Among the receptor systems, integrins bind to specific ECM components, such as collagen, vitronectin, and FN, influencing cell adhesion behavior (17, 18). Integrin-mediated interactions are vital to the maintenance of normal cell functions because they mediate the interaction between individual cells and the ECM and therefore have important implications for cell adhesion (19), proliferation (20), and differentiation (11). In addition, integrin protein expression changed accordingly, with higher levels of $\alpha 2\beta 1$ and $\alpha v\beta 3$ subintegrins in the cells on the Si- and Ca-rich substrates, respectively, which were ascribed to the collagen-binding and FN-binding subintegrin on primary cells, respectively (11). Integrin αv is essential for various biological functions, and it binds to and competes for the arginine-glycine-aspartic site on FN (21, 22). Recent studies have shown that integrin αv is not only required for osteoblast proliferation (23) but also for activation of the focal adhesion kinase/ERK/MAPK and phosphoinositide 3-kinase signaling pathways (24). These findings indicate that integrin αv might be affected by osteogenic differentiation of cells. The odontogenic differentiation of hDPCs is a biological process similar to osteogenic differentiation. There are several proteins involved in bone and dentin mineralization, and they are similar but distinct. In comparison with bone, dentin has been shown to contain less osteopontin, bone sialoprotein, and osteocalcin but more dentin sialoprotein (DSP) and dentin matrix protein 1 (DMP-1) (25). Thus, we suggest integrin αv may be included in the odontogenic differentiation of hDPCs.

From the *School of Dentistry and ‡Institute of Oral Science, Chung Shan Medical University; †Department of Stomatology, Chung Shan University Hospital; and §Department of Chemical and Materials Engineering, Tunghai University, Taichung City, Taiwan.

Address requests for reprints to Dr Chia-Tze Kao, School of Dentistry, Chung Shan Medical University, Taichung City, Taiwan or Dr Ming-You Shie, Department of Chemical and Materials Engineering, Tunghai University, Taichung City, Taiwan. E-mail addresses: ctk@csmu.edu.tw or eviltacasi@gmail.com

0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2014.07.016

Based on these factors, we hypothesize that CS cement may influence FN adsorption and accelerate adhesion protein formation, in particular, integrin αv. Thus, the roles of integrin αv involved in the regulation of odontogenic differentiation of hDPCs are also investigated in the present study, which investigates the specific role of DMP-1 and DSP protein expression on CS-induced odontogenesis. Additionally, small interfering RNA (siRNA) transfection is used to explore the integrin αv level in hDPCs during the proliferation and differentiation stages.

Materials and Methods

Specimen Preparation

The CS cement used in this research was made according to our previously reported laboratory procedures (13). Appropriate amounts of CaO (65%; Sigma-Aldrich, St Louis, MO), SiO_2 (25%; High Pure Chemicals, Saitama, Japan), and Al_2O_3 (5%, Sigma-Aldrich) powders were mixed. After sintering at 1400°C for 2 hours, the granules were ball milled in 99.5% EtOH using a centrifugal ball mill (Retsch S 100, Hann, Germany) for 12 hours and then dried in an oven. In addition, beta-tricalcium phosphate (TCP, Sigma-Aldrich) was compared with CS in this study. Before the preparation of the cement specimens, 50 mg powdered zeta potential was placed in 1 mL phosphate-buffered saline (PBS; Gibco, Langley, OK) and after soaking for 3 hours was measured by particle electrophoresis using the Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Three samples were measured for each powder condition, and the average is reported in this analysis. The CS and TCP powders were mixed according to the liquid/powder ratio of 0.35 mL/g and placed in a 24-well plate (GeneDireX, Las Vegas, NV) to a thickness of 2 mm fully covering each well; the samples were then stored in an incubator at 100% relative humidity and 37°C for 1 day. Before performing the cell experiments, all the specimens were sterilized by immersion in 75% ethanol followed by exposure to ultraviolet light for 1 hour.

hDPC Isolation and Culture

The hDPCs were freshly derived from caries-free, intact premolars that had been extracted for orthodontic treatment purposes as described previously (11). The patient gave informed consent, and approval from the Ethics Committee of the Chung Shan Medicine University Hospital was obtained (CSMUH No. CS11187). The tooth was split sagittally with a chisel. The pulp tissue was immersed in PBS (Caisson, North Logan, UT) solution and digested in 0.1% collagenase type I (Sigma-Aldrich) for 30 minutes. After being transferred to a new cultured dish, the cell suspension was cultured in Dulbecco modified Eagle medium (DMEM, Caisson) containing 20% fetal bovine serum (GeneDireX) and 1% penicillin (10,000 U/mL)/streptomycin (10,000 mg/mL) (Caisson) and was kept in a humidified atmosphere with 5% CO_2 at 37°C. The medium was changed every 3 days. The hDPCs were subcultured through successive passaging at a 1:3 ratio until they were used for experiments (passages 3–8).

Cell Adhesion Assays

The suspended cells were kept at a density of 1.5×10^4/specimen and directly seeded over each sample. Cell cultures were incubated at 37°C in a 5% CO_2 atmosphere. After being cultured for different lengths of time (ie, 1, 3, and 6 hours), cell adhesion ability was evaluated using the PrestoBlue assay (Invitrogen, Grand Island, NY). Briefly, each specimen was filled with medium with a 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 and 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. Cells cultured on tissue culture plates without cement were used as a control (Ctl).

FN Secretion

Cells were cultured on different substrates for 1, 3, and 6 hours, and the cell culture media were then collected and stored at room temperature. The enzyme-linked immunosorbent assay kits of human FN were obtained from Abcam (Cambridge, MA). Following the manufacturer's instructions, we used a 3-hour assay, which has a high sensitivity. The reaction was terminated by the addition of stop solution and read at 450 nm using a multiwell spectrophotometer.

FN Adsorption on Substrates

After being cultured for different periods of time, the amount of FN secreted from cells onto the cement's surface was analyzed using the enzyme-linked immunosorbent assay. The cells were detached using a trypsin-EDTA solution (Cassion) after being washed 3 times with cold PBS. Specimens were then washed 3 times with PBS containing 0.1% Tween 20 (PBS-T; Sigma-Aldrich, St Louis, MO) followed by blocking with 5% bovine serum albumin (Gibco) in PBS-T for 1 hour. Dilutions of primary antibodies were set at 1:500. After this procedure, samples were incubated with antihuman beta-actin or antihuman FN antibody (GeneTex, San Antonio, TX) for 3 hours at room temperature. Afterward, samples were washed 3 times with PBS-T for 5 minutes and incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature with shaking. The samples were then washed 3 times with PBS-T for 10 minutes each, and then One-Step Ultra TMB substrate (Invitrogen) was added to the wells and developed for 30 minutes at room temperature in the dark; after this, an equal volume of 2M H_2SO_4 was added to stop and stabilize the oxidation reaction. The colored products were then transferred to new 96-well plates and read using a multiwell spectrophotometer at 450 nm with reference at 620 nm according to the manufacturer's recommendations. All experiments were performed in triplicate. Additionally, beta-actin antibodies were used as a control.

Western Blot

Western blotting was performed on cells cultured on different cement specimens for a predetermined time to evaluate the differences in their protein levels. Cells were lysed in NP-40 lysis buffer (Invitrogen) at 4°C for 30 minutes, and the lysates were centrifuged at 13,000*g*. The protein concentrations of the lysates were measured using a Bio-Rad DC Protein Assay kit (Richmond, CA), and the proteins (30 μg) were then resolved using standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 5% bovine serum albumin (Sigma-Aldrich) in Tris-buffer saline containing 0.05% Tween 20 for 1 hour, the membranes were incubated with primary antibodies against beta-actin, integrin αv (GeneTex), DMP-1, and DSP (Santa Cruz Biotechnology, Santa Cruz, CA). A horseradish peroxidase–conjugated secondary antibody was subsequently added, and the proteins were visualized with enhancement using enhanced chemiluminescent detection kits (Invitrogen). The stained bands were scanned and quantified using a densitometer (Syngene, Frederick, MD) and Scion Image software (Scion Corporation, Frederick, MD). Protein expression levels were normalized to the beta-actin band for each sample.