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Odontogenic differentiation of human dental pulp cells by calcium silicate materials stimulating via FGFR/ERK signaling pathway

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ABSTRACT

Bone healing needs a complex interaction of growth factors that establishes an environment for efficient bone formation. We examine how calcium silicate (CS) and tricalcium phosphate (β-TCP) cements influence the behavior of human dental pulp cells (hDPCs) through fibroblast growth factor receptor (FGFR) and active MAPK pathways, in particular ERK. The hDPCs are cultured with β-TCP and CS, after which the cells' viability and odontogenic differentiation markers are determined by using PrestoBlue® assay and western blot, respectively. The effect of small interfering RNA (siRNA) transfection targeting FGFR was also evaluated. The results showed that CS promoted cell proliferation and enhances FGFR expression. It was also found that CS increases ERK and p38 activity in hDPCs, and furthermore, raises the expression and secretion of DSP, and DMP-1. Additionally, statistically significant differences ($p < 0.05$) have been found in the calcium deposition in si-FGFR transfection and ERK inhibitor between CS and β-TCP; these variations indicated that ERK/MAPK signaling is involved in the silicon-induced odontogenic differentiation of hDPCs. The current study shows that CS substrates play a key role in odontoblastic differentiation of hDPCs through FGFR and modulate ERK/MAPK activation.

1. Introduction

For the past several years, significant attention has been paid to silicon (Si), due to the researches of Carlisle that showed that Si plays an important role in bone formation and repair [1]. Si has been found to be an important trace element in human body [2]. Si is located at active calcification sites and is directly included in the mineralization process of bone growth. Silicate-based ceramics have received a considerable amount of positive attention in recent years as these materials have better bioactivity than calcium phosphate-based materials [3,4]. Mineral trioxide aggregate (MTA) is a biomaterial with several clinical applications in endodontics and has beenapproved for sale in 1998 [5]. Several clinical usage studies have shown that MTA has results comparable with or better than calcium hydroxide-based materials in decreasing pulp inflammation [6]. This commercial product contains 75% Portland cement, 20% Bi_2O_3, and 5% gypsum. Not only does MTA have good biocompatibility, but also it has been proven to enhance hard-tissue formation [7]. Several studies have been suggested for the process leading to hard tissue regeneration over the MTA: the cells migrated to the interface between MTA and fibrous connective tissue, and finally, the activation of mineralization [8]. In these processes, mesenchymal stem cells are usually required and play a critical role in hard tissue formation. In our previous study, we produced a calcium silicate (CS) cement that contained CaO, SiO_2, Al_2O_3, and ZnO, which had a reduced setting time [5,9,10]. In dentistry, calcium silicate-based cements have been formulated into dentin replacement restorative materials [6,11]. In addition, the viability of human dental pulp cells (hDPCs) cultured on CSC was higher than those cultured on MTA for all tested time lengths [6,7], suggesting that the cement can be applied in endodontic treatments. Moreover, CS cement not only showed good osteoconduction effects [8,12], but also reduces inflammation of hDPCs [6,13]. In addition, the suitable concentration of silicon can inhibit osteoclastogenesis in osteoclast cells [14], and promote angiogenesis in hDPCs [15,16]. However, as described by Bohner in his leading opinion paper, until now there has been no obvious and direct evidence linking the promoted biological performance of Si-based materials to Si release [17].

Several growth and differentiation factors have been demonstrated as potential therapeutic agents for hard-tissue regeneration [18,19]. Fibroblast growth factors (FGFs) play an important role in the control of cell survival, proliferation, and differentiation in several tissues including bone and tooth [19,20]. Notably, basic FGF (FGF-2) was found to enhance cell proliferation and osteogenic differentiation in bone marrow mesenchymal cells [21] and dental pulp cells [22,23]. FGF binding to FGF receptor (FGFR) leads to receptor phosphorylation of intrinsic tyrosine residues, which activates several signal transduction

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pathways including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K) [24,25]. Some studies in animal models have shown that high expression levels of BMP2 cause premature suture fusion and occur downstream of FGFR signaling in skull growth [26,27]. FGFR was also found to activate the ERK-specific mitogen-activated protein kinase pathway, leading to increased BMP production in osteoblasts [28]. In bone tissue, phosphorylation of extracellular-regulated kinase (ERK1/2) MAPK and protein kinase C (PKC) was found to promote osteogenesis gene expression [29,30].

This study investigated the genetic changes related to osteogenic and odontogenic differentiation in hDPCs when cultured with silicate-based and calcium-based materials. The silicon ion is a major component of silicate-based materials, which played an important role in the differentiation process of hDPCs. Here we hypothesized that CS might induce hDPCs to differentiate into odontoblasts with expression of dentin matrix protein-1 (DMP-1) and dentin sialophosphoprotein (DSP) via FGFR and ERK/MAPK pathways. Therefore, this study aimedto examine the effect of CS on the terminal differentiation pathway of hDPCs, by assessing the expression levels of specific proteins and calcium deposition.

2. Materials and methods

2.1. Specimen preparation

In this study, CS cement (composition: 65% CaO, 25% SiO_2, 5% ZnO, and 5% Al_2O_3) and β-tricalcium phosphate (β-TCP) were used. The CS cement was made according to our previously reported laboratory procedures [9,15]. Appropriate amounts of as-received powders were sintered at 1400 °C for 2 h, and the granules were ball milled in EtOH for 6 h using a centrifugal ball mill (Retsch S 100, Hann, Germany) and then dried in an oven at 120 °C. CS and commercial β-TCP (Sigma-Aldrich) powders were mixed according to the liquid/powder ratio of 0.3 mL/g. After mixing with ddH_2O, the cement plaster fully covered 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 24 h. Before cell experiments, all specimens were sterilized by immersion in 75% ethanol followed by exposure to ultraviolet (UV) light for 1 h.

2.2. Phase composition

To investigate the crystalline phase hydration of CS and β-TCP cements was performed by X-ray diffraction (XRD; Shimadzu XD-D1, Kyoto, Japan) over the 2-theta range from 20° to 50° with a scanning speed of 1°/min.

2.3. Cell isolation and culture

Human dental pulp cells (hDPCs) were isolatedat an established laboratory. The hDPCs were freshly derived from different caries-free donors (aged 18–26 years) with intact premolars that were extracted for orthodontic treatment purposes, as described previously. The patients 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 fresh tissue was then immersed in cold phosphate-buffered saline (PBS; Caisson, North Logan, UT) solution and digested in 0.1% type I collagenase (Sigma-Aldrich) at 37 °C for 30 min. After being transferred to a new 10 cm-dish (GeneDireX), the cell suspension was cultured in Dulbecco's modified Eagle medium (DMEM; Caisson), supplemented with 20% fetal bovine serum (FBS; GeneDireX), and penicillin G/streptomycin (PS, Caisson) in a humidified atmosphere with 5% CO_2 at 37 °C and changed medium every 3 days. Cell cultures between the third and sixth passages were used.

2.4. Cell viability assays

The hDPC suspensions at a density of 10^4 cells/mL were directly seeded over each specimen at 37 °C in a 5% CO_2 atmosphere. To evaluate the ions the concentration for cell viability was cultured with calcium-free DMEM (No. 21068028, Gibco, Langley, OK). After different culturing times (0.5, 1, 2, and 3 days), cell viability was evaluated using the PrestoBlue® assay (Invitrogen, Grand Island, NY). The reagent PrestoBlue® was used for real-time and repeated monitoring of cell cytotoxicity, which is based on the detection of mitochondrial activity. Briefly, at the end of the culture period, the medium was discarded and the wells were washed twice with PBS. Each well was filled with a solution that contained PrestoBlue® and fresh DMEM (1:9) at 37 °C for 30 min. The solution in each well was transferred to a new 96-well plate. Plates were read using a multi-well spectrophotometer (Hitachi, Tokyo, Japan) at 570 nm with a reference wavelength of 600 nm. Cells cultured on a tissue culture plate were used as control (Ctl). The results were obtained in triplicate from six separate experiments for each test.

2.5. Ion concentration

After culturing for 3 days, the calcium and silicon ion concentration released from cements on calcium-free DMEM was analyzed using an inductively coupled plasma-atomic emission spectrometer (ICP-AES; Perkin-Elmer OPT 1MA 3000DV, Shelton, CT, USA). Three samples were measured for each data point. The results were obtained in triplicate from three separate samples for each test.

2.6. Western blot

Western blot analysis was carried out using cell lysates and a culture medium prepared with cultured hDPCs for 3 days. Cells were lysed in NP-40 lysis buffer (Invitrogen) at 4 °C for 30 min and the lysates were centrifuged at 13,000 *g*. The cell lysates (30 μg protein) were separated using SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes. The membrane was blocked in 5% bovine serum albumin (BSA, Gibco) for 1 h and then immunoblotted with the primary anti-DMP-1, anti-DSP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERK1/2, anti-phosphor-ERK1/2, anti-p38, anti-phosphor-p38, anti-JNK, anti-phosphor-JNK (Abcam Inc., Cambridge, MA), anti-FGFR and β-actin (GeneTex, San Antonio, TX) antibodies for 2 h, then washed three times in a tris-buffer saline containing 0.05% Tween-20 (Sigma-Aldrich). A horseradish peroxidase (HRP)-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 bioimaging system; Frederick, MD) and ImageJ (National Institutes of Health, Bethesda, MD). Protein expression levels were normalized to the β-actin band for each sample. The results were obtained in triplicate from three separate samples for each test.

2.7. Alizarin Red S stain

Accumulated calcium deposition was observed using Alizarin Red S staining according to a previous study [31]. In brief, the specimens were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min and then incubated in 0.5% Alizarin Red S (Sigma-Aldrich) at a pH of 4.0 for 15 min at room temperature. After the cells were washed with PBS, photographs were taken using an optical microscope (BH2-UMA, Olympus, Tokyo, Japan) equipped with a digital camera (Nikon, Tokyo, Japan) at 200× magnification. In addition, Alizarin Red was quantified using a solution of 20% methanol and 10% acetic acid in water. After 15 min, the liquid was transferred to a 96-well plate, and the quantity of Alizarin Red was read on a spectrophotometer at 450 nm. To further clarify the MAPK inhibitor effect on cell mineralization, the culture medium was mixed with three types of MAPK