The effects of calcium silicate cement/fibroblast growth factor-2 composite on osteogenesis accelerator in human dental pulp cells

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Abstract  Background/purpose: To examine the effects of fibroblast growth factor-2 (FGF-2)/calcium silicate (CS) cement on material characters and in vitro primary human dental pulp cell (hDPC) behavior.

Materials and methods: Setting time and diametral tensile strength (DTS) of CS and CS/FGF-2 composite were measured. PrestoBlue assay was used for evaluating primary hDPC proliferation. Alkaline phosphatase and osteocalcin expression in HDPCs cultured on the specimens were determined by enzyme-linked immunosorbent assay. One-way analysis of variance was used to evaluate the significance of the differences between the mean values.

Results: Setting time and DTS of CS were not significantly different (P > 0.05) between CS hydration with H2O or FGF-2. Cell proliferation and osteogenic properties increased significantly (P < 0.05) with FGF-2 mixed CS. The CS/FGF-2 composite enhanced hDPC proliferation and osteogenic differentiation as compared to pure CS cement.

Conclusion: CS combined with FGF-2 is biocompatible with hDPCs. It not only promotes hDPC proliferation but also helps in differentiating reparative hard tissue. Thus, we suggest that the CS/FGF-2 composite has the potential for hard tissue defect repair.

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Introduction

Mineral trioxide aggregate (MTA) is a bio compatible material and developed by Torabinejad’s team in 1993, which has several clinical applications in endodontic treatment, such as root-end fillings, pulp capping, pulpotomy, perforation repairs, and apexification treatment.1-3 It is a complex mixture of 20% bismuth oxide for radiopacity and similar to Portland cement, which consists of tricalcium silicate (Ca3SiO5), dicalcium silicate (Ca2SiO4), tricalcium aluminate (Ca3Al2O6), tetracalcium aluminoferrite, and 5% gypsum.4 In previous studies, we found that the newly developed calcium silicate cement (CS) showed an advantageously shortened setting time,1 excellent bioactivity, and good biocompatibility.5 Moreover, we recently showed that CS not only stimulates the proliferation and differentiation of human dental pulp cells (hDPCs) in vitro,7-9 but also reduced inflammation in vivo.10 Several growth factors have been verified as potential therapeutic agents for hard-tissue formation.11,12 Fibroblast growth factors (FGFs) play an important role in the control of cell adhesion, proliferation, and differentiation in several tissues including bone.13,14 Notably, FGF-2 has been found to enhance cell proliferation and osteogenic differentiation in bone marrow mesenchymal cells.15,16 FGF-2 signaling played an important role of in the control of osteoprogenitor cells, and knockout of FGF-2 gene in mice results in decreased bone marrow stromal cell osteogenic differentiation and changed bone formation.17 FGF binding to FGF receptor (FGFR) leads to receptor phosphorylation of intrinsic tyrosine residues, including mitogen-activated protein kinase and phosphatidylinositol 3-kinase.18,19 In hard tissue, phosphorylation of extracellular-related kinase, mitogen-activated protein kinases, and protein kinase C have been proved to enhance osteogenic gene expression.20,21 In a clinical case, tricalcium phosphate ceramic combined with growth factor and placed in a bone defect after periapical surgery was shown to enhance bone regeneration.22 It can help regeneration of vital tissue with necrotic pulp and periapical lesions.22 Growth factors/cytokines are key factors in tissue wound healing and this combination reduced immune function and promoted proliferation and differentiation of cells participating in wound healing.23 FGF-2 is expressed during tooth development, associated with tooth morphogenesis, and promotes the differentiation of dental pulp cells into odontoblasts in culture.24 A search of the literature reveals an absence of any publication discussing the cellular effects of the growth factor combined with CS. The aim of the present study was to evaluate CS/FGF-2 composite properties and pulp cell biological effects. It is hypothesized that CS combined with FGF-2 can promote the osteogenesis protein markers expression and help in mineralization.

Materials and methods

Preparation of CS specimens

The CS cement was made according to our previously reported laboratory procedures.5,25 Appropriate amounts of 65% CaO (Showa, Tokyo, Japan), 25% SiO2 (High Pure Chemicals, Saitama, Japan), and 5% Al2O3 (Sigma-Aldrich, St Louis, MO, USA) powders were mixed by a conditioning mixer (ARE-250; Thinky, Tokyo, Japan). After sintering at 1400°C, the granules were ball milled in EtOH using a centrifugal ball mill (Retsch S 100; Retsch, Hann, Germany) and then dried in an oven. CS cement was mixed according to the liquid/powder ratio of 0.33 mL/g. FGF-2 (ProSpec, Rehovot, Israel) was dissolved in double distilled (dd)H2O, and the concentration was 3 μg/mL. After mixing, the cement fully covered of the 24-well plate (GeneDireX, Las Vegas, NV, USA) to a thickness of 2 mm. The samples were stored in an incubator at 100% relative humidity and 37°C for 1 day. Prior to the cell experiments, all specimens were sterilized by immersion in 75% ethanol followed by exposure to UV light for 1 hour.

Setting time and strength

After mixing, the cement was placed into a Teflon cylindrical mold (diameter = 6 mm) under a pressure of 0.7 MPa for 1 minute and tested using a 400 g Gillmore needle with a 1 mm diameter, according to International organization for standardization 9917-1 (ISO 9917-1). Six specimens were tested for each measurement.

As for mechanical performance testing, the cement was molded into a cylindrical mold to a diameter of 6 mm and thickness of 2 mm, and the specimens were stored in an incubator at 100% relative humidity and 37°C for 24 hours to set. The testing was conducted on an EZ-Test machine (Shimadzu, Kyoto, Japan) at a loading rate of 0.5 mm/minute to obtain diametral tensile strength (DTS).

In vitro release of FGF-2

As for FGF-2 release testing, the cement (0.1 g) was molded into a cylindrical mold to a diameter of 6 mm and thickness of 3 mm and the specimens were stored in an incubator at 100% relative humidity and 37°C for 24 hours to set. The release of FGF-2 was measured by soaking the specimens in 1 mL of Dulbecco’s modified Eagle medium (DMEM; Caisson Laboratories, North Logan, UT, USA) at 37°C for several time points. The amount of FGF-2 in DMEM was measured using the FGF-2 enzyme-linked immunosorbent assay kit (ELISA; Invitrogen, Grand Island, NY, USA), and the FGF-2 content following the manufacturer’s instructions. All experiments were carried out in triplicate.

Human dental pulp cell isolation and culture

Human dental pulp cells (hDPCs) were derived freshly from a caries free, intact premolar that was extracted for the purpose of orthodontic treatment. The patient provided informed consent, and approval for the study was obtained from the committee of the Chung Shan Medicine University Hospital (CSMUH No. CS111877). The tooth was split sagittally with a chisel and pulp tissue was immersed in phosphate buffered saline (PBS) contained type I collagenase. The tissue was cut into fragments and placed in plates containing DMEM, supplemented with 10% fetal bovine serum (Caisson Laboratories) for 3 days. Cell culture media were supplemented with 100 units/mL penicillin-G, 100 μg/
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mL streptomycin, and 0.25 μg/mL fungizone. Subculture was successively passed at a ratio of 1–3 until used for testing (Passage 4–8).

FGF-2 effect of cell proliferation

The proliferation of hDPCs cultured in the presence of FGF-2 was evaluated by PrestoBlue assay (Invitrogen). Briefly, 5 × 10^4 cells/mL hDPCs were seeded in a 24-well overnight with normal DMEM. Then, hDPCs were treated with DMEM containing FGF-2 at concentrations of 12.5 ng/mL, 25 ng/mL, and 50 ng/mL for different times (1, 3, 7 days). At the end of the culture period, the medium was discarded and washed with PBS three times. Each well was filled with a 1:9 solution of PrestoBlue in fresh DMEM and incubated at 37°C for 60 minutes. The solution in each well was transferred to a new 96-well plate. Plates were read using a multiwell spectrophotometer (Hitachi, Tokyo, Japan) at 570 nm with a reference wavelength of 600 nm. The results were obtained in triplicate from three separate experiments for each test.

Cell proliferation

Cell suspensions at a density of 10^5 cells/mL were directly seeded over each specimen. Cell cultures were incubated at 37°C in a 5% CO₂ atmosphere. After different culturing times, cell proliferation was evaluated using the PrestoBlue assay. Cells cultured on the tissue culture plate without the cement were used as a control (Ctl). The samples of CS or Ctl mixed with FGF-2 were named CSF or CtlF (DMEM containing 25 ng/mL FGF-2), respectively.

Alkaline phosphatase activity assay

Secretion of alkaline phosphatase (ALP) activity, it was determined at 3 days, 7 days, and 14 days after cell seeding. Briefly, the cells were lysed using 0.2% NP-40, and centrifuged at 3600g for 15 minutes. ALP activity was determined by p-nitrophenyl phosphate (pNPP; Sigma-Aldrich) as the substrate. Each sample was mixed with pNPP in 1 M diethanolamine buffer for 15 minutes. The reaction was stopped by the addition of 5 N NaOH and quantified by absorbance at 405 nm. All experiments were done in triplicate.

ELISA analysis for osteocalcin protein

Osteocalcin (OC) protein released from pulp cells cultured on different substrates for 7 days and 15 days after cell seeding. The OC ELISA kit (Invitrogen) was used to determine OC content following the manufacturer’s instruction. OC concentration was measured by correlation with a standard curve. Blank disks were treated as controls. All experiments were done in triplicate.

Small interfering RNA transfection

Small interfering RNA (siRNA) for FGFR was purchased from GeneDireX. In the experiments, hDPCs were harvested and seeded on a 24-well plate at a concentration of 2 × 10^5 cells per well, and transiently transfected with FGFR-specific siRNAs (FGFR-specific siRNA sense: 5'-GGAGAGAU GAUGGAAGACCCGGA-3' and antisense: 3'-AUCGGUGU CAUCCCCAUCAUGC-5') or mock siRNA (sense: 5'-UUCU CGCAGUGUGUCAGT-3' and antisense: 3'-ACUGAGAC CGUUCAGAGATT-5') using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions for 48 hours. Cellular levels of the proteins specifically silenced were assessed using a western blot, immunofluorescence staining, and cytotoxicity.

After transfection, cell pellets were lysed in a NP-40 lysis buffer (Invitrogen) at 4°C for 30 minutes, and the lysates were centrifuged at 15,000g. Total protein concentration was measured using the Bio-Rad DC Protein Assay kit. Thirty μg of protein extracts were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were blotted onto polyvinylidene difluoride membranes. After blocking in 5% bovine serum albumin (Sigma-Aldrich) for 1 hour, the membrane was immunoblotted with the primary anti-FGFR and β-actin (GeneTex, San Antonio, TX, USA) for 2 hours, then washed three 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 visualized with enhancement using enhanced chemiluminescent detection kits (Invitrogen). The stained bands were scanned and quantified using a densitometer (Syngene bioimaging system; Syngene, Frederick, MD, USA) and ImageJ (National Institutes of Health, Bethesda, MD, USA). 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.

Immunofluorescence staining

After transfection for 2 days, hDPCs were washed with cold PBS, and the adherent cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 30 minutes at room temperature and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (PBS-T). The cells were blocked with PBS supplemented with 5% bovine serum albumin prior to incubation with primary antibodies against anti-FGFR for 2 hours. To reveal the FGFR, hDPCs were washed with PBS-T, followed by incubation with goat anti-rabbit IgG antibodies conjugated to Alexa Fluor 488 (Invitrogen). Finally, the nuclei and F-actin cytoskeleton were stained with 0.05% 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and phalloidin conjugated to Alexa Fluor 546 (Invitrogen) in PBS for 60 minutes, respectively. After washing three times with PBS-T, the cells were viewed under indirect immunofluorescence using a Zeiss Axioskop2 microscope (Carl Zeiss, Thornwood, NY, USA).

FGFR knockdown inhibitors on proliferation and OC level

After transfection, cells were cultured on different substrates for 1, 3, and 7 days. The cell proliferation and OC protein secretion were analyzed by PrestoBlue assay and ELISA, respectively.
Statistical analysis

A one-way variance statistical analysis was used to evaluate the significance of the differences between the groups in each experiment. A Scheffe's multiple comparison test was used to determine the significance of the deviations in the data for each specimen. In all cases, the results were considered statistically significant with $P < 0.05$.

Results

Setting time and DTS

The setting time is one of the most clinically relevant factors. CS set within $15.3 \pm 1.0$ minutes, and $15.6 \pm 1.3$ minutes when mixed with ddH$_2$O and FGF-2, respectively ($P > 0.05$; Fig. 1A). Fig. 1B shows the DTS values of cement specimens. One-way analysis of variance of the DTS data shows that the variations in strength between specimens are not significant ($P > 0.05$). CS had a strength value of $3.2 \pm 0.2$ MPa and $3.3 \pm 0.1$ MPa when mixed with ddH$_2$O and FGF-2, respectively.

In vitro release of FGF-2

The amount of FGF-2 loaded in CS was about 100 ng. The in vitro release profiles of FGF-2 from CS are plotted in Fig. 2. The initial burst release was observed in the first 12 hours, with 20.67 ng FGF released from CS. Approximately 27.33 ng FGF-2 was released over 3 days.

hDPC proliferation

The proliferation of hDPCs cultured in DMEM with FGF-2 concentrations of $0$-50 ng/mL is shown in Fig. 3. There was no significance between $0$ ng/mL and $12.5$ ng/mL ($P > 0.05$) at all times. As the concentrations of FGF-2 were increased to $25$ ng/mL, hDPCs were significantly increased at Day 1 ($P < 0.05$). In addition, we evaluated the cell proliferation on CS without and with FGF-2 (Fig. 4). Cell viability steadily increased in Ctl on Days 1-7, indicating increasing numbers of viable cells. The optical density values of the two FGF-2-containing groups were significantly higher ($P < 0.05$) than those obtained without FGF-2 after 1 day. Moreover, the CSF showed highest hDPCs growth ($P < 0.05$) at all groups for all culture time periods.

Osteogenesis

ALP expression of hDPCs cultured on different substrates was examined. As shown in Fig. 5, analysis of quantitative examination data shows that the ALP activity of cells cultured on the composites increased over time throughout the assay.
period with a higher value in the substrates containing FGF-2. The ALP activity of the hDPCs seeded on CSF was higher than that of the cells on other groups with a statistically significant difference (P < 0.05) throughout the culture period.

OC released from hDPCs was measured 7 days and 14 days after seeding (Fig. 6). OC secretion on all groups increased significantly from Day 7 to Day 14. At Day 7, there was a significant increase of OC in FGF-2-containing groups. Interestingly, the OC secretion by cells cultured on CtlF and CS was similar (P > 0.05), and significantly lower (P < 0.05) than that secreted by cells on CSF.

**FGFR knockdown**

Western blots showed effective FGFR knockdown by pairs of FGFR-specific siRNA in the cells compared to Ctl and the control with mock siRNA transfection, respectively (Fig. 7A). Transfection with FGFR siRNA optimally reduced FGFR protein levels by 56% in hDPCs after 48 hours. To further investigate the effects of siRNA on hDPCs functions, FGFR was analyzed using immunofluorescence microscopy (Fig. 7B). The assembly of the F-actin cytoskeleton was subsequently examined with rhodamine-labeled (red). It can be clearly seen that cells expressed lower levels of FGFR (green) after siRNA transfection. There was no cytotoxicity of hDPCs after the FGFR knockdown procedures (Fig. 7C).
FGFR knockdown inhibitors on proliferation and OC level

As shown in Fig. 8, the proliferation (Fig. 8A) and OC (Fig. 8B) levels were analyzed after treatment of FGFR siRNA on hDPCs. For all time-points, FGFR knockdown by the use of FGFR siRNA decreased hDPCs proliferation and OC secretion on CS/FGF-2 composite, and there was no significance (P > 0.05) between samples with and without FGF-2.

Discussion

MTA has a wide range of applications, including pulp capping, perforation repair, apexification procedures, and root-end filling. In a previous study we confirmed that MTA and CS were calcium-silicate based materials with similar physical–chemical and biological properties. Additionally, the viability of pulp cells cultured on CS cement was greater than those cultured on MTA for all culture times, suggesting that CS cement could be used in...
endodontic materials. Growth factors are naturally occurring proteins that cause cellular adhesion, proliferation, and differentiation. Several reporters directed at clinical use have progressed, including use of enamel matrix derivative (EMD), platelet-derived growth factor-BB, bone morphogenic protein (BMP)-2, BMP-7, and transforming growth factor. The growth factors (BMP-7, epithelial growth factor, transforming growth factor, and insulin-like growth factor -1) have been used to alter the sensitivity of hDPCs in commonly used restoration materials. Previous studies have shown that FGF-2 promotes alveolar bone formation and cementum regeneration in dogs. Moreover, FGF-2 is known to be involved in all stages of osteogenesis, including osteoblast cell proliferation, differentiation, maturation, and apoptosis. By contrast, the problem with this kind of administration is that a large amount of FGF-2 protein was used because of its fast release in vivo. To overcome this disadvantage, in attempting to treat more severe bone defects with FGF-2, the concept of a substitute needs to be introduced. Many studies have developed composites for the delivery of FGF-2 based on sulfated or natural materials, showing hopeful results. Therefore, we have carried out CS mixed with FGF-2 and for local delivery in order to evaluate the capacity of these systems for inducing cell proliferation and differentiation.

Setting time is the most important factor in clinical use; a long setting duration could affect the cement’s inability to maintain dimensional stability and support stresses when placed into a hard bone defect. As for CS containing FGF-2, the setting time and DTS were similar to pure CS (P > 0.05). The rate of FGF-2 release from CS was in accordance with an earlier report of the release characteristics of porosity. We conjecture that the primary mechanism of FGF-2 release from the CS surface is prior to 12 hours. The efficacious concentrations of FGF-2 for different biological function have been determined in several previous studies. Previous studies verify that FGF-2 at a concentration of >10 ng/mL may have adverse effects on bone formation. Recently, Sogo et al. found that FGF-2 immobilized at 2.72 ng/cm² on the hydroxyapatite surface has that ability to promote new bone formation. The present study showed that CS combined with growth factors on cultured hDPCs could promote proliferation at a long time period of culture. We presume that the amount of FGF-2 released from CS was high enough to enhance hDPCs behavior. Our findings are similar to the above result that FGF-2 combined with CS showed synergistic effects on culture hDPC proliferation than CS alone. The increasing culture time and concentrations appeared better for hDPC growth.

ALP is an important biochemical marker of bone and dentin formation which is secreted by osteoblasts. ALP expression was dominantly increased in the growth factor-included group at all time points. ALP is considered to play a key role in the mineralization of reparative dentin. The high ALP expression in hDPCs culture in the CS/FGF-2 group can promote in reparative dentin formation. OC is a non-collagenous protein found in bone and dentin. It is secreted by osteoblasts and thought to play a role in mineralization and calcium ion homeostasis. In the present study, the expression of the OC marker was higher in the CS/FGF-2 groups. There is a synergistic effect to hDPCs cultured with growth factor combined CS groups. However, FGFR knockdown inhibited the proliferation and differentiation of hDPCs on CS/FGF-2, indicating that the blockade of FGF-2 signal transduction affects cell behavior.

In conclusion, the CS combined with growth factors was biocompatible with hDPCs. It not only promotes hDPCs to proliferation but also helps in differentiating reparative dentin. We suggest that this CS/FGF-2 composite has the potential for used in reparative dentin.

Conflicts of interest
The authors declare that they have no conflicts of interest.

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