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Original Article

Combined effects of fibroblast growth factor 2 and dexamethasone on differentiation of human cementoblasts

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Abstract: Fibroblast growth factor 2 (FGF-2) is known to play a pivotal role in bone remodeling and osteogenesis. Dexamethasone is a potent modulator of osteogenic differentiation and can increase the bone formation capacity of cementoblasts. However, the combined effects of FGF-2 and dexamethasone on cementoblasts and the way they regulate the activities of cementoblasts have not been fully understood. The aim of this study was to clarify and investigate the effects of the combination of FGF-2 and dexamethasone on growth and differentiation in human cementoblasts and to determine the underlying mechanism. A series of experiments including MTT, alkaline phosphatase (ALP) activity, alizarin red staining, RT-PCR, and Western blot were carried out to evaluate the proliferation and differentiation of cementoblasts. In addition, the changes of Wnt signaling pathway molecules were checked to analyze the possible mechanism. Compared to the treatment of cementoblasts with either FGF 2 or dexamethasone alone, the combination of FGF-2 and dexamethasone synergistically increased cell proliferation, ALP activity, nodule formation, and expression of differentiation markers. In addition, FGF 2 and dexamethasone enhanced expression of Wnt3a, Runx2, β-catenin, and p-GSK-3β, all of which were inhibited by DKK1. Taken together, FGF-2 and dexamethasone act synergistically, enhancing each other's ability to stimulate cementoblast growth and differentiation via Wnt signaling pathways. These results support the therapeutic potential of a combination strategy for facilitating cementogenesis and aiding periodontal regeneration.

Keywords: Cementoblasts, fibroblast growth factor 2, dexamethasone, differentiation, Wnt signaling pathway

Introduction

Cementum, which is formed by cementoblasts, is a mineralized tissue that covers the tooth root surface and plays an essential role in anchoring teeth to surrounding alveolar bone [1]. Cementogenesis is one of the most important processes in periodontal development and regeneration [2, 3]. Cementoblasts share the ability to form a mineralized matrix and express genes associated with osteogenic differentiation in a similar manner to osteoblasts [4], including cementum protein 1 (CEMP1), alkaline phosphatase (ALP), cementum-derived attachment protein (CAP), runt-related transcription factor 2 (Runx2), type I collagen, and noncollagenous proteins such as bone sialoprotein (BSP) and osteocalcin (OCN) [5-7]. However, the molecular and cellular regulators that promote cementoblast differentiation are not fully understood, hampering the establishment of targeted periodontal regeneration.

Fibroblast growth factor 2 (FGF-2), a signaling peptide that binds heparin and heparan sulfate, has been reported to have numerous biological activities including stimulation of cell growth, survival, morphogenesis, migration, differentiation, chemotaxis, angiogenesis, and wound healing and tissue repair [8-13]. In addition, FGF-2 is known to play a pivotal role in bone remodeling and osteogenesis [14, 15]. Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid hormones [16]. It has major effects on a number of organ systems including endocrine, cardiovascular, gastrointestinal, ophthalmic, and musculoskeletal systems [17]. Dexamethasone is a potent

Table 1. Oligonucleotide primer sequences utilized in RT-PCR

Gene	Primer sequences
RUNX2	F: 5'-CCTTGGGAAAAATTCAAGCA-3'
	R: 5'-AACACATGACCCAGTGCAAA-3'
Osteocalcin	F: 5'-CCCCGCTTCCTCTTTAGACT-3'
	R: 5'-GCCAACCCCAAAGGATATT-3'
CAP	F: 5'-GCGGAACTCTGAGGTGGTCCATT-3'
	R: 5'-CAGGCCAGTCCGGTGGTATGG-3'
BSP	F: 5'-GAACCACTTCCCCACCTTTT-3'
	R: 5'-TCTGACCATCATAGCCATCG-3'
GAPDH	F: 5'-GAGTCAACGGATTTGGTCGT-3'
	R: 5'-TTGATTTTGGAGGGATCTCG-3'

modulator of osteogenic differentiation and can increase the bone formation capacity of cementoblasts *in vitro* [18, 19].

Since FGF-2 and dexamethasone affect mineralization, we hypothesized that combined stimulation by FGF-2 and dexamethasone would augment cementoblast differentiation to a greater degree than treatment with either stimulus alone. However, controversial opinions exist with regard to the combined effects of FGF-2 and dexamethasone in proliferation, differentiation, and mineralization [18, 20]. Thus, the aim of this study was to examine the impact of FGF-2 and dexamethasone, alone or in combination, on the proliferation and differentiation of human cementoblasts. In addition, we investigated related signaling pathways.

Materials and methods

Cell culture

An immortalized human cementoblast cell line (HCEM) was kindly provided by Professor Liu (Fudan University, Shanghai, China). Cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate (All reagents were from Gibco/Sigma-Aldrich, CA). Cells were stimulated with a final concentration of 2 ng/mL or 20 ng/mL for FGF-2 and 10 nM or 100 nM for dexamethasone. The cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Cell proliferation

Cell proliferation was evaluated using 3-(4, 5-methyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assays. 5×10^3 cells were seeded into each well of a 96-well plate, grown overnight, and exposed to FGF-2 and dexamethasone. The control group was incubated in media supplemented with ethanol vehicle. The MTT (0.5 mg/mL) assays were performed at 3 days and 7 days later. After removal of the medium, absorbance was then measured spectrophotometrically at 570 nm (Thermo Scientific, USA).

ALP activity assay

ALP activity was measured by using a substrate assay as described previously [21]. Cells were seeded in 6-well plates at a density of 10^5 cells per well and cultured with or without FGF-2 and dexamethasone for 7 or 14 days. Cells were rinsed with ice-cold PBS, scraped into 1 mL lysis buffer (10 mM Tris-HCl, and 0.2% Triton X-100), sonicated on ice for 20 seconds, and then centrifuged at 4,000 g for 10 minutes at 4°C. ALP activity in the supernatant was measured using p-nitrophenyl phosphate as the substrate in 2-amino-methyl-1-propanol containing $MgCl_2$ (Sigma). After incubation at 37°C for 15 minutes, the reaction was stopped with 0.5 N NaOH and the absorbance at 405 nm was measured using a microplate reader (Thermo Scientific, USA). All ALP activity results were normalized to the total protein in the cell lysate.

Alizarin red staining

Cells were seeded in 6-well plates at a density of 10^5 cells per well and cultured with or without dexamethasone and FGF-2 for 21 days. Cells were fixed with 4% paraformaldehyde and stained with 2% Alizarin red for 30 minutes, washed with PBS, and observed under a microscope.

RT-PCR

The total cellular RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and quantified by spectrometry at 260 and 280 nm. cDNA was synthesized and amplified using the Transcript First-strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) in accordance with the manufacturer's protocol. RT-PCR was performed using a Super Real PreMixPlus (SYBR Green) kit (Tiangen, Beijing, China) with candidate gene sets or GAPDH primers (**Table 1**). The reactions were performed on an ABI 7500 instrument (Applied Biosystems, Carlsbad, CA, USA). All data from

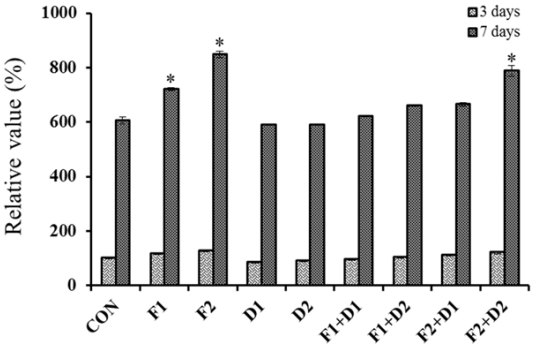

Figure 1. Effect of FGF-2 and/or dexamethasone on the proliferation of cementoblasts. *P<0.05.

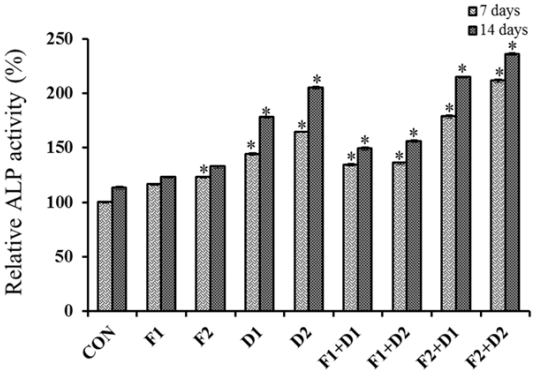

Figure 2. Effect of FGF-2 and/or dexamethasone on alkaline phosphatase activity in cementoblasts. *P<0.05.

each sample were assessed by 2-ΔΔCt analysis. The measured genes that displayed a significant difference (P<0.01) were considered differentially expressed genes.

Western blot

Cells were harvested and homogenized on ice in RIPA lysis buffer. Lysates were centrifuged at 12,000× g for 20 minutes at 4°C. The protein concentrations were measured using a BCA kit (Pierce Chemical, Rockford, IL, USA). Equivalent amounts of protein extracts (50 μg) were separated by 10% SDS-polyacrylamide gels electrophoresis and transferred onto PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked for 1 hour with 5% non-fat dry milk in Tris-buffered saline/Tween-20 (TBST) buffer and incubated with primary antibodies against Wnt3a, Runx2, β-catenin, and p-GSK-3β (1:1000 dilution) (Cell Signaling Technology, Inc., Danvers, MA) at 4°C overnight. Blots were washed three times and incubated with secondary antibodies for 1 hour at room temperature. The signals were developed using enhanced chemiluminescence (ECL) reagent (Tiangen Biotech Co., Ltd., Beijing, China). Western blot bands were visualized and quantified with Quantity One software.

Statistical analysis

Statistical analysis was performed with SPSS software version 13.0. Results were expressed as the mean ± standard deviation (SD). The significance of differences between the two groups was tested by Student's t-test or ANOVA. A *P* value of <0.05 was considered statistically significant.

Results

Effect of FGF-2 and dexamethasone on cell proliferation

The MTT assays were performed at 3 and 7 days to examine the effects of FGF-2 and dexamethasone, either alone or in combination, on cell growth of human cementoblasts. For these studies, an FGF-2 concentration of 2 ng/mL or 20 ng/mL and a dexamethasone concentration of 10 nM or 100 nM were chosen, based on previous studies. Cell growth was increased by the addition of FGF-2 alone relative to controls. The combination of FGF-2 at 20 ng/mL and dexamethasone at 100 nM resulted in a statistically significant increase in cell proliferation compared with either factor alone (**Figure 1**).

Effect of FGF-2 and dexamethasone on ALP activity

The effects of FGF-2 and dexamethasone on ALP activity were determined by substrate assay. As shown in **Figure 2**, ALP activity in cementoblasts was significantly increased after being treated with either FGF-2 or dexamethasone over 7 or 14 days culture (P<0.05). FGF-2 at 20 ng/mL and dexamethasone at 100 nM resulted in a maximum increase in ALP activity on days 7 and 14. Based on these preliminary data, we chose to use these concentrations in subsequent experiments.

Effect of FGF-2 and dexamethasone on calcium deposition and RUNX2, OCN, CAP, and BSP expression of cementoblasts

To determine the combined effects of FGF-2 and dexamethasone on the differentiation of cementoblasts, alizarin red staining and osteo-

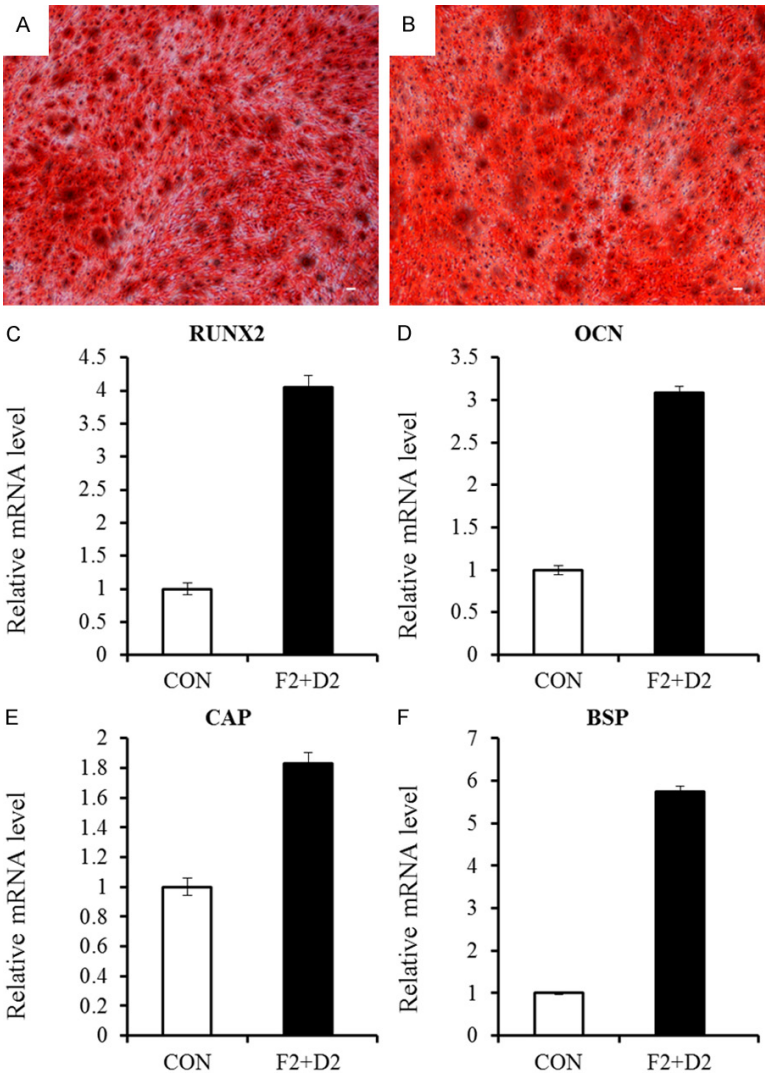

Figure 3. Combined effect of FGF-2 and dexamethasone on calcium deposition and mRNA expression of cementoblastic markers. Alizarin red S staining of calcium deposition at 21 d in CON group (A) and F2+D2 group (B). *P<0.05. RT-PCR analysis of RUNX2 (C), OCN (D), CAP (E) and BSP (F) mRNA expression at 14 d. *P<0.05.

blast/cementoblast-related gene expression were performed.

Treatment with FGF-2 and dexamethasone for 21 days enhanced mineralized nodule formation (**Figure 3A**, **3B**). Cementoblasts treated with FGF-2 and dexamethasone exhibited increased mRNA expression of osteoblastic/cementoblastic markers including RUNX2, osteocalcin (OCN), CAP, and BSP (**Figure 3C-F**).

Effect of FGF-2 and dexamethasone on Wnt signaling in cementoblasts

Since Wnt signaling could mediate bone formation and remodeling, we evaluated the combined effects of FGF-2 and dexamethasone on Wnt signaling pathways. As shown in **Figure 4A**, the combination of FGF-2 and dexamethasone increased the protein level of Wnt3a, Runx2, β-catenin, and phosphorylated GSK-3β in cementoblasts. To further investigate whether the combination of FGF-2 and dexamethasone induced cementoblast differentiation through activation of the Wnt signaling pathway, cells were pretreated with DKK-1 (Dickkopf related protein 1) and a secreted antagonist of Wnt signaling for 1 hour before treatment with FGF-2 and dexamethasone. The increase in expression of Wnt3a, Runx2, β-catenin, and phosphorylated GSK-3β protein expression induced by FGF-2 and dexamethasone was significantly inhibited by DKK-1 (**Figure 4B**).

Discussion

Cementoblasts are responsible for cementum formation and root resorption repair, a process essential to establishing the attachment of periodontal ligament to roots. Cementoblasts differentiate from either dental follicle cells during root development or progenitors in the periodontal ligament and share many similar characteristics with osteoblasts *in vivo* and *in vitro* [22-24]. As an important growth factor or steroid hormone, FGF-2 and dexamethasone have been shown to promote osteoblastic differentiation of osteoprecursor cells [25]. In this research, we investigated the effects of FGF-2 and dexamethasone on proliferation, differentiation, and protein expression of cementoblasts under predetermined concentrations (2 or 20 ng/mL FGF-2, 10 or 100 nM dexamethasone) and investigated whether the effects of the combination of FGF-2 and dexamethasone were comparable to those of FGF-2 and dexamethasone alone in terms of growth and differentiation in

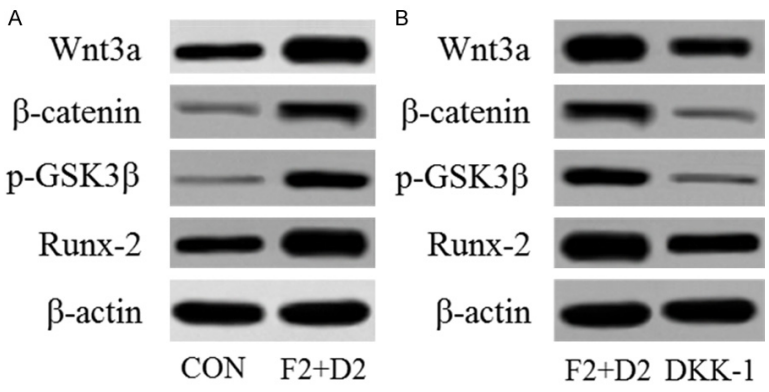

Figure 4. Combined effects of FGF-2 and dexamethasone on Wnt pathway in cementoblasts and the effects of DKK1. A: Protein expression of Wnt3a, Runx2, β-catenin, and p-GSK-3β were determined by Western blot. B: Western blot analysis of the DKK1 effect on the expression of Wnt pathway molecules. *P<0.05.

human cementoblasts. In addition, we focused on how cementoblasts responded to FGF-2 and dexamethasone.

To clarify the role of FGF-2 and dexamethasone, we first examined the effect of FGF-2 and/or dexamethasone on the proliferation of cementoblasts (**Figure 1**). Dexamethasone alone exhibited no obvious effect on the proliferation of cementoblasts in our present research. Exponential growth began on day 2 in all FGF-2 and combined FGF-2 plus dexamethasone groups. Our results demonstrate that FGF-2 or dexamethasone stimulates cementoblasts growth. The growth stimulatory effects by FGF-2 and dexamethasone are consistent with the results of a previous study performed in osteoprecursor cells [25].

To illuminate the role of FGF-2 and dexamethasone on the differentiation of cementoblasts, we studied the level of ALP activity, the expression of RUNX2, OCN, CAP, and BSP, and calcium deposition in FGF-2 and/or dexamethasone treated cementoblasts. As an early marker of cementoblasts differentiation, ALP plays an important role in transferring phosphate groups from the cells to the matrix. As illustrated in **Figure 2**, ALP activity of the cementoblast also increased with the increase of FGF-2 or/and dexamethasone concentration. This is similar to previous reports which have shown that treatment with dexamethasone presented a significant induction in ALP activity, leading to osteoblastic differentiation of bone marrow cells [26, 27]. Our current study shows that the addition of 20 ng/mL FGF 2 to 100 nM dexamethasone produces a significant increase in ALP activity in comparison with that of the 100 nM dexamethasone group. From the perspective of composition, cementum consists of about 45%-50% inorganic material and 50% collagen and proteoglycans [28]. Non-collagenous proteins existing in cementum include BSP, ALP, dentin matrix protein 1, osteopontin, and several growth factors. Runx2 transactivates ALP, OCN, and BSP promoters in osteoblastic cells [29, 30]. OCN is a late marker for cementoblast differentiation and regulates the mineral deposition. BSP, which is mainly lying on the root surface while cementogenesis occurs, has been proven to trigger mineralization and enhance adhesion and differentiation of cementoblasts [18, 31]. The previous report [32] and the present findings (**Figures 2**, **3**) indicate that the expression levels of ALP, OCN, and BSP are increased during cementoblast differentiation and are accompanied by increased expression levels of Runx2. Classical Alizarin Red-S staining has been used to evaluate the level of mineralization. As shown in **Figure 3**, upon 21 days of co-incubation with FGF 2 and dexamethasone, red-stained mineralized nodules were clearly observed from the control group and treated group. However, due to the effects of FGF 2 and dexamethasone on cementoblast differentiation, the degree of mineral nodule formation increased in the FGF 2 and dexamethasone treated group. Taken together, the presence of FGF 2 and dexamethasone resulted in an increase of ALP activity and further induced the differentiation of cementoblasts. This is consistent with the effect of FGF 2 and dexamethasone on osteoprecursor cells which indicates that cementoblasts share similar characteristics with osteoblasts [25].

The previous study suggested that Wnt signaling pathway may decrease cementoblast differentiation by inhibiting Runx2 expression [33]. Wnt signaling is a key pathway for regulating

bone formation and remodeling through a number of mechanisms including renewal of stem cells, stimulation of preosteoblast replication, induction of osteogenesis, and inhibition of osteoblast and osteocyte apoptosis. This suggests that activation of the Wnt signaling pathway may assist in regeneration of bone and associated periodontal tissues [34, 35]. Some factors and proteins such as osterix, dentin sialoprotein, and bone morphogenetic protein-2 regulate the differentiation of cementoblasts by maintaining a low level of Wnt/β-catenin signaling via a positive regulation of DKK1 [36, 37]. In this study, the expression levels of Wnt3a, Runx2, β-catenin, and p-GSK-3β were enhanced in the combined presence of FGF 2 and dexamethasone. In addition, our results demonstrate that the expression of Wnt3a, Runx2, β-catenin, and GSK-3β induced by FGF 2 and dexamethasone are partially blocked by the addition of DKK1. Here, for the first time, we provide evidence that the Wnt signaling pathway may be involved in the enhancement of cementogenesis achieved by the combination of FGF 2 and dexamethasone.

Conclusions

In conclusion, our present research sheds light on the effect of FGF 2 and dexamethasone on cementoblasts. Based on our results, the combined treatment of FGF 2 and dexamethasone has additive effects on cementoblast differentiation through Wnt signaling pathways. Thereby, the combination of FGF 2 and dexamethasone may offer a new therapeutic approach for cementum and periodontal regeneration. However, due to the complex environment *in vivo*, more studies are still much needed before further application in the clinic.

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Disclosure of conflict of interest

None.

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