Journal of Dental Sciences (2016) 11, 463–467

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ORIGINAL ARTICLE

Effects of fibroblast growth factor-2 on cell proliferation of cementoblasts

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Received 6 June 2016; Final revision received 26 June 2016
Available online 5 November 2016

KEYWORDS
cell proliferation;
cementoblast;
fibroblast growth factor-2;
regeneration;
Stro-1

Abstract *Background/purpose:* Fibroblast growth factor (FGF)-2 is known as a signaling molecule that induces tissue regeneration. Little is known about the effect of FGF-2 on cementoblasts for periodontal and periapical regeneration. The aim of this study was to investigate the effects of FGF-2 on murine immortalized cementoblast cell line (OCCM.30).
Materials and methods: Cell growth and proliferation was judged by using alamar blue reduction assay. Flow cytometry analysis was used to evaluate Stro-1 positive cells expression with or without FGF-2. Western blot was used to evaluate the expression of phosphorylated serine–threonine kinase Akt (p-Akt) and extracellular signal-regulated protein kinase (p-ERK) in cementoblasts.
Results: FGF-2 was found to increase cell growth in a dose-dependent manner ($P < 0.05$). The concentration of 10 ng/mL FGF-2 enhanced cell proliferation in a time-dependent manner ($P < 0.05$). In addition, 10 ng/mL FGF-2 significantly increased the number of Stro-1 positive cells in the first 24 hours ($P < 0.05$). Moreover, 10 ng/mL FGF-2 was found to upregulate p-Akt and p-ERK in a time-dependent manner ($P < 0.05$).
Conclusion: Taken together, FGF-2 could increase cementoblast growth, proliferation, and Stro-1 positive cells. These enhancements are associated with the upregulation of p-Akt and p-ERK expression. The application of FGF-2 may provide benefit for periodontal and periapical regeneration during the early phase of wound healing.

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http://dx.doi.org/10.1016/j.jds.2016.06.007

Introduction

Traditional periodontal and endodontic therapy can be predictably used to arrest mild to moderate periodontal and periapical bony defects; however, it may be inadequate for the treatment of disease characterized by wide circumferential defects. Surgical procedures could provide better access in these situations. Recently, many techniques and materials have become available to promote periodontal and periapical tissue regeneration.[1] Growth factors are responsible for the wound healing process by stimulating and regulating numerous cell activities such as mitogenesis, chemotaxis, metabolism, and differentiation.[2] These may play a pivotal role in bone remodeling, osteogenesis, and cementogenesis in periodontal and periapical tissue regeneration.

Fibroblast growth factor-2 (FGF-2), a signaling peptide that binds heparin and heparan sulfate, can modulate the expression of the main components of connective tissue including glycosaminoglycans, proteoglycans, collagen, and noncollagenous protein.[3] FGF-2 is considered to be involved in the early stage of the wound healing process by the stimulation of growth, migration, and proliferation on the cells derived from pulp[4] and periodontal ligament.[5–7] In addition, FGF-2 has been reported to be effective in human periodontal regeneration procedure.[8,9]

Cementum, which has similar composition and properties to bone, is a mineralized tissue that is synthesized by cementoblasts during tooth root formation and plays an essential role in anchoring teeth to surrounding alveolar bone. Cementoblasts play a critical role in the healing of periodontal ligament and cementum in periapical portions.[10] However, little is known about the precise mechanisms of FGF-2 on cementoblasts, hampering the establishment of more effective therapies for periodontal and periapical regeneration. The aim of this study was to investigate FGF-2 on murine immortalized cementoblast cell line (OCCM.30) by measuring cell growth, proliferation, Stro-1 positive cells, and the phosphorylated proteins Akt and ERK expression.

Materials and methods

Cell culture

Immortalized murine cementoblasts (OCCM.30) was a generous gift from Dr Somerman's laboratory (University of Washington, Seattle, WA, USA).[11] The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% fetal calf serum (FCS) and antibiotics (Gibco BRL, Gaithersburg, MD, USA). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell proliferation assay

Cells were seeded at a 2×10^4 cell density per well into 96-well culture plates for 24 hours. The culture medium was replaced with fresh DMEM with 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL FGF-2 for 24 hours. In addition, cells were incubated with 10 ng/mL FGF-2 for 0 hours, 24 hours, 48 hours, and 72 hours, respectively. Cell proliferation was tested using the alamar blue dye (BUF012A/B alamarBlue; AbD serotec, Oxford, UK) as described previously.[12]

Stro-1 staining by flow cytometry

Cells were stained with anti-Stro-1 antibody (R&D Systems, Minneapolis, MN, USA) and phycoerythrin-conjugated goat antimouse immunoglobulin G antibody (Miltenyi Biotech., Auburn, CA, USA), with labeling according to the manufacturer's instructions. Red (> 650 nm) fluorescence emission from 10,000 cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Western blot

Cells arrested in G_0 by serum deprivation (0.5% FCS; 48 hours) were used in the experiments.[13] Nearly confluent monolayers of cells were washed with serum-free DMEM and immediately thereafter exposed at the indicated incubation times to 10 ng/mL FGF-2. Cultures without FCS were used as negative control. Cell lysates were collected at 1 day, 2 days, and 3 days. The extraction of proteins from cells and immunoblotting analysis were performed as described previously.[14,15] p-Akt (phosphorylated serine—threonine kinase Akt) and p-ERK (extracellular signal-regulated protein kinase) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

Triplicate experiments were performed throughout this study. All assays were repeated three times to ensure reproducibility. The significance of the results obtained

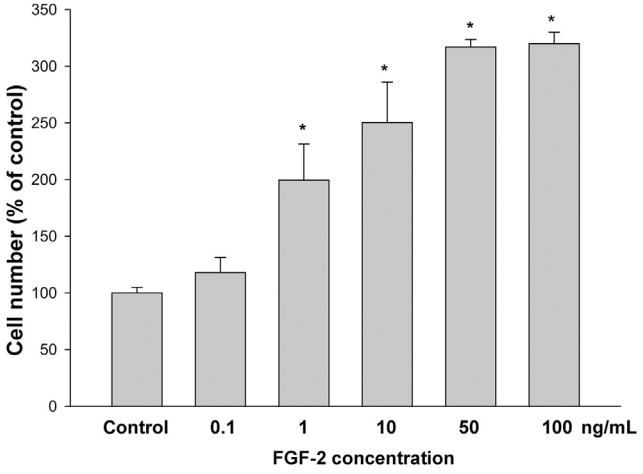

Figure 1 Effects of various concentrations (0.1 ng/mL, 1 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL) of FGF-2 on cementoblasts as measured by alamar blue assay. Each point and bar represent mean ± SD. * Significant differences from control values with $P < 0.05$.

from control and treated groups was statistically analyzed by paired Student *t* test. A P value of < 0.05 was considered statistically significant.

Results

Figure 1 shows the effects of FGF-2 for cell proliferation on cementoblasts. FGF-2 increased the alamar blue absorbance of cementoblasts in a dose-dependent manner ($P < 0.05$). The cell numbers of FGF-2 at 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL was 1.2-, 2.0-, 2.5-, 3.2-, and 3.2-fold, respectively, as compared with untreated control. In addition, 10 ng/mL FGF-2 was found to increase cementoblasts proliferation in a time-dependent manner compared with untreated controls ($P < 0.05$). The cell numbers increased by about 2.5-, 3.9-, and 7.0-fold at Day 1, Day 2, and Day 3, respectively (Figure 2).

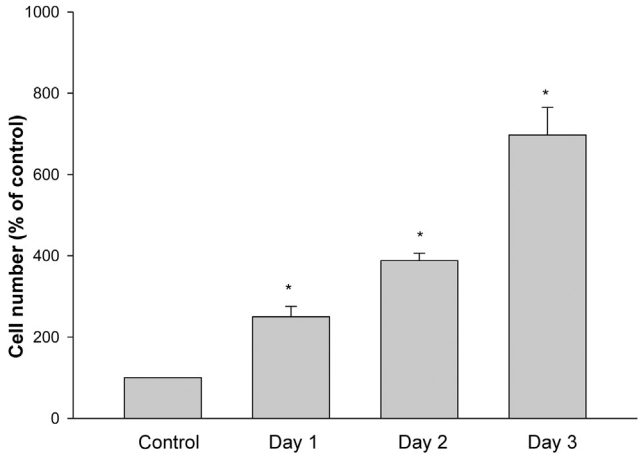

Figure 2 Effects of 10 ng/mL of FGF-2 on cell proliferation of cementoblasts were measured by alamar blue assay for 3-day incubation period. Each point and bar represent mean ± SD. * Significant differences from control values with $P < 0.05$.

Stro-1 has been used as a marker for most mesenchymal stem/progenitor cells. As shown in Figure 3, the expression of Stro-1 positive cells was about 9.65%, 17.0%, 12.0%, and 7.07% in the presence with 10 ng/mL FGF-2 at 0 hours, 24 hours, 48 hours, and 72 hours, respectively. FGF-2 was found to significantly enhance the number of Stro-1 positive cells in the first 24 hours ($P < 0.05$).

Based on the Western blot results, FGF-2 was found to upregulate Akt and ERK phosphorylation in cementoblasts (Figure 4). The quantitative measurement was made by the AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA, USA). As shown in Figure 5A, the levels of p-Akt activity increased by about 1.4-, 1.7-, and 4.5-fold after the stimulation of FGF-2 at Day 1, Day 2, and Day 3, respectively, compared with the untreated controls ($P < 0.05$). Moreover, Figure 5B demonstrates that the levels of p-ERK increased by about 2.0-, 3.9-, and 8.4-fold after the stimulation of FGF-2 at Day 1, Day 2, and Day 3, respectively, compared with the untreated controls ($P < 0.05$).

Discussion

Cementum is an important component of periodontal attachment apparatus and a key to establishing and regenerating functional periodontal and periapical tissues.[16] Rapid induction and appropriate accumulation of cementoblasts may be critical during tissue healing and regeneration for successful repair and regeneration of

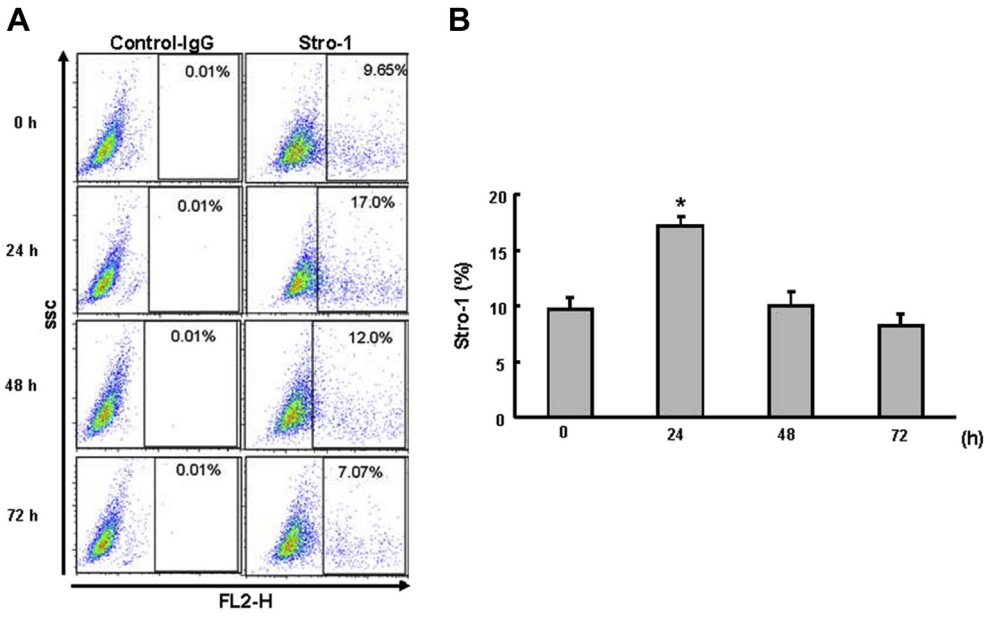

Figure 3 Effect of FGF-2 on Stro-1 positive cells in cementoblasts. The cells cultured with and without FGF-2 were stained with anti-Stro-1 and PE-conjugated antibodies. Stro-1 positive cells were further measured by flow cytometry analysis for 0 hours, 24 hours, 28 hours, and 72 hours. The percentages of positive cells (%) are displayed as mean ± SD. * Significant differences from control values with $P < 0.05$.

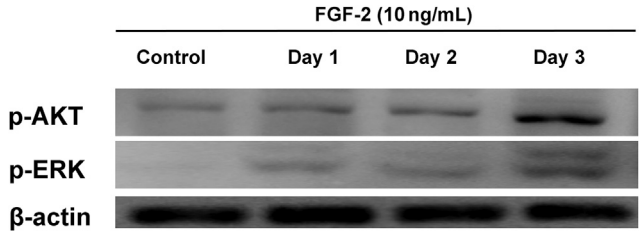

Figure 4 Kinetics of p-Akt and p-ERK expression in cementoblasts exposed to FGF-2 for 1 day, 2 days, and 3 days, respectively, using Western blot analysis. β-Actin was performed in order to monitor equal protein loading.

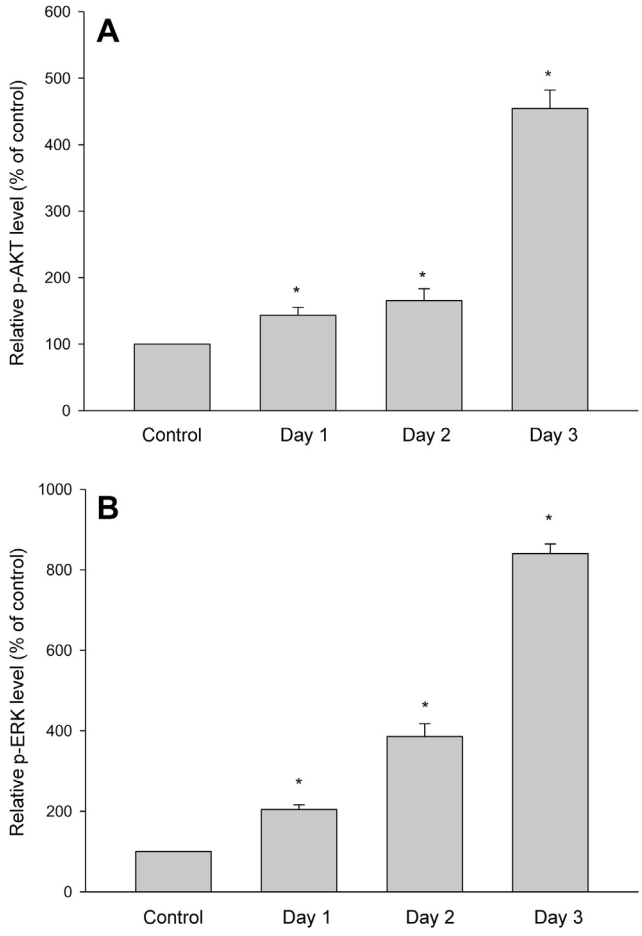

Figure 5 Levels of (A) p-Akt and (B) p-ERK protein treated with FGF-2 were measured by AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA, USA). The relative level of p-Akt (A) and p-ERK (B) protein expression was normalized against β-actin signal, and the control was set as 1.0. Optical density values represent mean ± SD. * Significant difference from without FGF-2 control values with $P < 0.05$.

periodontal and periapical tissues. In this study, FGF-2 was found to stimulate cementoblasts cell growth and proliferation. Our results are in agreement with those of Hakki et al,[17] who reported that FGF-2 could act as a mitogenic response on cementoblasts by cell morphology observation and DNA synthesis assay. In addition, similar results were found in pulp cells,[4] periodontal ligament cells,[5–7] and stem cells from the apical papilla.[18] These have clearly shown the potent mitogen of FGF-2. In addition, the mitotic activity of FGF-2 is not cell type-specific.

Cell proliferation plays an important role in the wound healing process, which enhances cell population over a wounded space at early stages and is a prerequisite for tissue formation at later stages. In this study, FGF-2 was found to increase cementoblast cell proliferation. The properties of FGF-2 could offer great potential during periodontal and periapical wound healing.

Stro-1, a cell marker present on all clonogenic stromal precursors, is a reliable marker for mesenchymal stem cells over the past decade.[19] Usually, mesenchymal progenitor cells often express stem cell markers at a lower level. In this study, we first demonstrated that FGF-2 could enhance Stro-1 positive cells in cementoblast cultures. Similar results were reported in pulp cells,[20] periodontal ligament cells,[6] and stem cells from the apical papilla[18] cultured with FGF-2. Therefore, FGF-2 might function as a factor to maintain the cell behaviors that define stem cell stemness such as strengthening of self-renewal ability and regulating cell proliferation and differentiation *in vitro*. Thus, cell proliferation stimulated by FGF-2 may be attributable to the increased Stro-1 expression in this study.

To further investigate the mechanism of FGF-2-induced signaling proteins related to cell proliferation, Western blot analysis of the phosphorylated proteins was performed. Akt is known to be involved in cell proliferation. In this study, phosphorylation of Akt was increased by FGF-2 in cementoblasts. Our results are in agreement with those of Shimabukuro et al,[5] who reported that FGF-2 activated cell migration and the phosphorylation of p-AKT protein expression in mouse periodontal ligament cells MPDL22. Moreover, a recent study has shown that FGF-2 rapidly induced activation of AKT in bone marrow-derived mesenchymal stem cells.[21] These findings suggest that the Akt pathway is involved in FGF-2-stimulated cell proliferation.

ERK is also known to be involved in cell proliferation. In this study, the phosphorylation of ERK was increased by FGF-2 in cementoblasts. Previously, the activation of the p-ERK pathway by FGF2 was reported in an immortalized osteocyte cell line MLO-Y4,[22] primary calvaria osteoblasts from neonatal mouse,[22] Hertwig's epithelial root sheath cells,[23] and bone marrow-derived mesenchymal stem cells.[21] Taken together, the cell proliferation by FGF-2 may be via the p-ERK signal transduction pathway.

Signaling pathways including mitogen-activated protein kinases, transforming growth factor (TGF)-β, Notch, and phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B pathways are crucial to maintain stemness and differentiation in dental stem cells. Insulin-like growth factor 1 treatment could promote the differentiation of Stro-1 positive human periodontal ligament stem cells via ERK pathway.[24] Signaling pathways such as TGF-β, ERK, Akt, and Wnt were found to be activated in stem cells from human exfoliated deciduous teeth.[25] In the present study, we identified that FGF-2 treatment induced stemness marker Stro-1 expression in cementoblasts at 24 hours. FGF-2 treatment also activated p-Akt and p-ERK expression in cementoblasts. These results may link the possibility of Akt or ERK activation involved in the augmentation of Stro-1 in cementoblasts. Identification of the mechanisms leading to

the upregulation of Stro-1, and the activation of Akt, ERK, or other stemness-related signaling pathways in FGF-2-stimulated cementoblasts prior to 24 hours still need to be further investigated.

Within the limitations of this laboratory study, FGF-2 was found to increase cementoblast growth and proliferation during 72 hours. FGF-2 might be a powerful promoter of the proliferation of cemenotoblast in periodontal and periapical regeneration in early wound healing. This may be via the p-Akt and p-ERK signal transduction pathways.

Conflicts of interest

The authors declare that they have no conflict of interest.

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