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# Effect of fibroblast growth factor-1 on the expression of early growth response-1 in human periodontal ligament cells

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*Background and Objectives:* Early growth response-1 is a nuclear transcription factor implicated in regulating cell proliferation. Fibroblast growth factor-1 is the prototypic fibroblast growth factor involved in the proliferation and differentiation of various cell types. Expression of early growth response-1 induced by fibroblast growth factor-1 thus may be very important for cell growth, during both development and wound healing in oral tissue. However, little is known about the expression and kinetics of early growth response-1 in fibroblast growth factor-1-stimulated oral cells. The aim of this study was to investigate the effects of fibroblast growth factor-1 on the expression of early growth response-1 in human periodontal ligament cells.

*Material and Methods:* Periodontal ligament cells were cultured in medium containing 1, 10 or 100 ng/mL of fibroblast growth factor-1 for 45 min or with 10 ng/mL of fibroblast growth factor-1 for 15, 30, 45, 60 or 120 min. The proliferation of periodontal ligament cells was evaluated by measuring 5-bromo-2′-deoxyuridine incorporation. The expression of early growth response-1 mRNA and protein, and the localization of early growth response-1 protein, were examined by western blotting, northern blotting and immunocytostaining.

*Results:* 5-Bromo-2′-deoxyuridine incorporation correlated directly with increases in fibroblast growth factor-1 concentration, and 5-bromo-2′-deoxyuridine incorporation peaked 45 min after starting treatment. Early growth response-1 protein was expressed in response to a concentration of fibroblast growth factor-1 as low as 1 ng. Peak expression of early growth response-1 mRNA was observed at 15 min and that of early growth response-1 protein at 60 min. The 140-kDa early growth response-1 protein was not detected in the nuclear fraction, and the peak expression of the 80-kDa early growth response-1 protein occurred at 60 min. Early growth response-1 localized in or around the nucleus at 30 min.

*Conclusion:* These results show that a concentration of fibroblast growth factor-1 as low as 1 ng induces the expression of early growth response-1 protein, and that the 80-kDa early growth response-1 protein functions in the nucleus of periodontal ligament cells treated with fibroblast growth factor-1.

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The early growth response gene family includes early growth response-1, -2, -3 and -4 (1–3). This gene family encodes nuclear transcription factors that are implicated in the regulation of cell proliferation (3,4), as well as zinc finger DNA-binding proteins that target DNA regulatory sequences in specific genes. Expression of this family has been demonstrated in human periodontal ligament fibroblasts (5), NIH 3T3 cells (6,7) and several tumor cell lines (7–10) cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Expression of early growth response protein may be induced during the G0 and G1 phases of the cell cycle. Early growth response protein is also thought to be an immediate-early gene product because it is rapidly and transiently induced by numerous stimuli, including growth factors, mechanical injury or inflammation (11,12). Interestingly, some studies have shown that early growth response-1 protein binds to the promoter region of growth factor genes (13–16). Accordingly, these data suggest that early growth response-1 protein may play a role in the regulation of cell proliferation as well as in differentiation and signaling. When culturing human periodontal ligament cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, we previously observed both 82-kDa and 130-kDa early growth response-1 protein bands (5). Although the molecular mass of native early growth response-1 protein is reported to be 82 kDa (1), we speculate that, like the other transcription factors, early growth response-1 protein is phosphorylated or glycosylated after translation, which would account for the larger apparent size of one of the bands (17–19).

Fibroblast growth factor is a pleiotropic growth factor that regulates proliferation, migration and differentiation of target cells and enhances wound healing or regeneration of oral tissues (20–22). In addition, the expression of early growth response-1 is induced by mitogenic, hypertrophic and differentiation signals in various cells. Because early growth response-1 is also strongly expressed during wound healing (2,23), it appears to have a specific role in the regulation of cell growth. Although multiple changes in early gene expression thus occur during the growth of oral cells, little is known about the regulatory role of early growth response-1.

In this study, we investigated the effects of fibroblast growth factor-1 on the expression of early growth response-1 protein and mRNA in human periodontal ligament cells using molecular biological and immunocytochemical techniques.

## Material and methods

### Culture of periodontal ligament cells

All experiments were approved by the Ethics Committee of Osaka Dental University (No. 050351) and were performed in triplicate. Primary cultures of periodontal ligament cells were obtained from three patients after obtaining informed consent, as described previously (24). Periodontal tissues were scraped from the mid-root surface and transferred onto a 22.1-$cm^2$ culture dish. Outgrowing periodontal ligament cells were initially cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 μg/mL of streptomycin, 100 IU/mL of penicillin and 10% fetal calf serum in a humidified atmosphere, containing 5% $CO_2$ in air, at 37°C. Periodontal ligament cells between passages 3 and 7 (15 cultures) were cultured for 2 d in Dulbecco's modified Eagle's medium without fetal calf serum. Then these periodontal ligament cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1, 10 or 100 ng/mL of human recombinant fibroblast growth factor-1 (Wako Pure Chemicals, Osaka, Japan) and antibiotics for 45 min for dose–response experiments, or were cultured in Dulbecco's modified Eagle's medium supplemented with 10 ng/mL of fibroblast growth factor-1 for 0, 15, 30, 45, 60 or 120 min for time-course experiments. In time-course experiments, nuclear and cytoplasmic fractions were extracted from periodontal ligament cells (15) for western blot analysis. Controls were treated identically, except that no fibroblast growth factor-1 was added.

### 5-Bromo-2′-deoxyuridine incorporation

The proliferation of periodontal ligament cells was evaluated by measuring the incorporation of 5-bromo-2′-deoxyuridine, which was measured by indirect enzyme-linked immunosorbent assay. Periodontal ligament cells were seeded at a density of 3000 cells/well in a 96-well plate. After incubating with fibroblast growth factor-1 in Dulbecco's modified Eagle's medium and 10 μM 5-bromo-2′-deoxyuridine (Oncogene Research Products, Cambridge, MA, USA), 5-bromo-2′-deoxyuridine incorporation was measured using peroxidase-conjugated anti-5-bromo-2′-deoxyuridine, according to the manufacturer's instructions (Oncogene Research Products). The Student's paired-sample *t*-test was used to analyze the data, and significance was set at $p < 0.05$.

### Northern blot analysis

Total RNA was extracted from fibroblast growth factor-1-stimulated periodontal ligament cells, and a sample containing 7 μg of total RNA was fractionated and transferred onto a nylon membrane (Hybond-N+ Amersham Pharmacia Biotech, Bucks., UK) and then hybridized to a fluorescence-labeled early growth response-1 cDNA probe. Hybridization was visualized with CDP-Star reagent (Amersham Pharmacia Biotech) following exposure of the membranes to X-ray film. A fluorescence-labeled β-actin probe was also hybridized to the stripped membranes. Both probes were synthesized by reverse transcription-polymerase chain reaction and were then amplified using primers for human early growth response-1 or human β-actin. The primers used to produce the probes included early growth response-1 (sense, 5′-CACGTACTCCTCTGTT-3′; antisense, 5′-AGACACTGTACAAGGG-3′) and β-actin (sense, 5′-TTGCCGACAGGATGCAGAA-3′; antisense, 5′-GCCGATCCACACGGAGTACT-3′).

### Western blot analysis

Protein samples from fibroblast growth factor-1-stimulated periodontal ligament cells in 75-$cm^2$ tissue culture flasks were solubilized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer (25), fractionated and then transferred electrophoretically to membranes. After blocking with 5% skimmed milk at 4°C overnight, the resulting membranes were then incubated with antibody to early growth response-1 protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by labeled streptavidin-biotin (26). Expressed proteins were finally visualized by exposing the membranes to X-ray film, as described previously (5).

### Immunocytochemical analysis

Periodontal ligament cells were seeded at a density of 3000 cells/well in chamber slides, fixed with acetone-ethanol for 60 min, permeabilized with phosphate-buffered saline/0.5% Triton X-100, and nonspecific reactions were blocked with phosphate-buffered saline containing 1% bovine serum albumin. Treated cells were incubated with rabbit anti-early growth response-1, followed by fluorescein isothiocyanate-conjugated immunoglobulins (Dako A/S, Glostrup, Denmark). Finally, all images were visualized by a confocal laser-scanning microscopy system (FV300-BX51WI; Olympus, Tokyo, Japan) in order to observe the nuclear region of periodontal ligament cells. Sections incubated with phosphate-buffered saline instead of primary antibody were used as negative controls.

## Results

### 5-Bromo-2′-deoxyuridine incorporation

Measurements of 5-bromo-2′-deoxyuridine incorporation (Fig. 1) correlated directly with increased fibroblast growth factor-1 concentration. In addition, 5-bromo-2′-deoxyuridine incorporation in periodontal ligament cells treated with fibroblast growth factor-1 showed a peak level of incorporation at 60 min in the time-course experiments.

### Expression of early growth response-1 mRNA and protein

Fibroblast growth factor-1 affected the expression of early growth response-1 at both mRNA and protein levels in periodontal ligament cells. The peak expression of early growth response-1 mRNA was observed after treating cells with 100 ng of fibroblast growth factor-1 (Fig. 2, left) and periodontal ligament cells showed this peak at 15 min (Fig. 2, right). Western blot analysis (Fig. 3) showed that exposure of periodontal ligament cells to Dulbecco's modified Eagle's medium

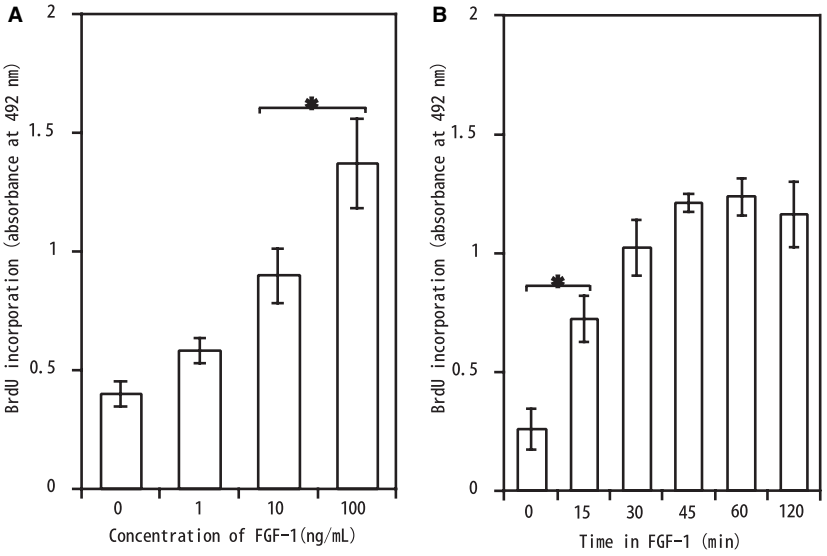


*Fig. 1.* 5-Bromo-2′-deoxyuridine incorporation of periodontal ligament cells (mean ± standard deviation, $n = 15$). 5-Bromo-2′-deoxyuridine incorporation was measured by indirect enzyme-linked immunosorbent assay. (A) 5-Bromo-2′-deoxyuridine incorporation was directly correlated with increasing doses of fibroblast growth factor-1 (*$p < 0.05$). (B) In time-course experiments, 5-bromo-2′-deoxyuridine incorporation of periodontal ligament cells cultured in Dulbecco's modified Eagle's medium supplemented with 10 ng of fibroblast growth factor-1 peaked at 60 min. BrdU, bromo-2′-deoxyuridine; FGF-1, fibroblast growth factor-1.

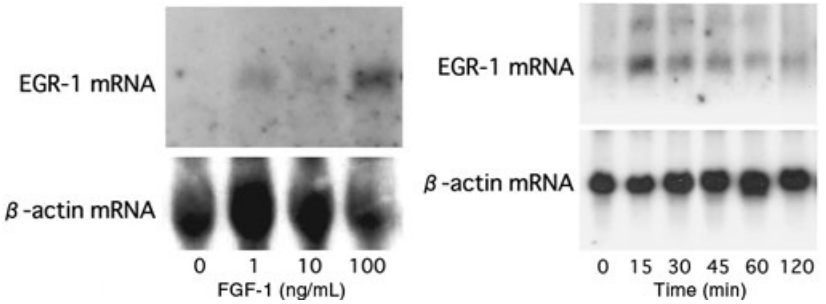


*Fig. 2.* Northern blot analyses of the dose–response effect of fibroblast growth factor-1 on early growth response-1 mRNA and of the effect over time of incubation of a fixed concentration of fibroblast growth factor-1 on early growth response-1 mRNA. Peak expression of early growth response-1 mRNA was observed in periodontal ligament cells treated with 100 ng of fibroblast growth factor-1 (left panel). Expression of early growth response-1 mRNA peaked 15 min after treatment with 10 ng of fibroblast growth factor-1 (right panel). EGR-1, early growth response-1; FGF-1, fibroblast growth factor-1.

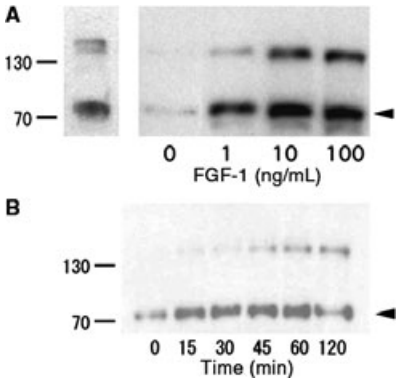


*Fig. 3.* Western blot analyses of the dose–response effect of fibroblast growth factor-1 on early growth response-1 protein and of the effect over time of incubation of a fixed concentration of fibroblast growth factor-1 on early growth response-1 protein. The numbers to the left refer to molecular weight (kDa), and the left lane in (A) is the positive control for early growth response-1, which was expressed in periodontal ligament cells treated with Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Western blot analyses confirmed that treatment of periodontal ligament cells with Dulbecco's modified Eagle's medium supplemented with fibroblast growth factor-1 induced expression of early growth response-1 (arrowhead). (A) Early growth response-1 protein expression was detected even when stimulated with low concentrations of fibroblast growth factor-1 (arrowhead). (B) Expression of early growth response-1 protein peaked at 60 min when periodontal ligament cells were incubated with 10 ng of fibroblast growth factor-1 (arrowhead).

supplemented with fibroblast growth factor-1 induced expression of early growth response-1 protein with an apparent molecular weight of 82 kDa (Fig. 3, arrowhead). In Dulbecco's modified Eagle's medium containing fibroblast growth factor-1, the expression of early growth response-1 protein was observed upon stimulation with an amount of fibroblast growth factor-1 as low as 1 ng (Fig. 3A). After treatment with 10 ng of fibroblast growth factor-1, the peak expression of early growth response-1 protein was seen at 60 min (Fig. 3B).

Fibroblast growth factor-1 increased the expression of both high-molecular-weight and low-molecular-weight early growth response-1 protein in the

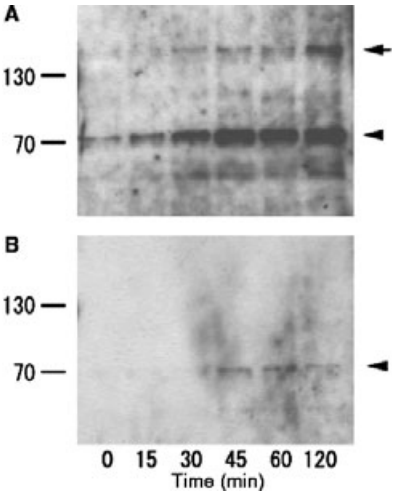


*Fig. 4.* Western blot analyses for the localization of early growth response-1 protein in cytoplasmic and nuclear fractions. (A) Expression of both high-molecular-weight (arrow) and low-molecular-weight (arrowhead) early growth response-1 protein gradually increased in cytoplasmic extracts after stimulation with fibroblast growth factor-1. (B) In nuclear extracts, only low-molecular-weight early growth response-1 protein was detected, initially detectable at 30 min and peaking at 60 min.

cytoplasmic fraction (Fig. 4A). However, in the nuclear fraction, expression only of low molecular-weight early growth response-1 protein was seen, from 45 min onwards (Fig. 4B).

### Immunocytochemical observation of early growth response-1

Early growth response-1 protein was localized, by immunocytochemical methods, in and around the nuclear region after stimulation with as little as 10 ng of fibroblast growth factor-1 (Fig. 5, bottom set of panels, left). We also observed strong reactions in the nuclear region, starting 30 min after culture with fibroblast growth factor-1, in the time-course experiments (Fig. 5, bottom set of panels, right).

## Discussion

Fibroblast growth factor-1 is the prototypic fibroblast growth factor involved in the proliferation, migration and differentiation of various cell types (27,28). Expression of early growth response-1 is induced by fibroblast growth factor-1, and is thus very important for cell growth during development, wound healing and regeneration in the oral tissues. To characterize the proliferative capacity of periodontal ligament cells and to determine whether fibroblast growth factor-1 influences the transcription and expression of early growth response-1, we investigated the effects of exogenous fibroblast growth factor-1 on the expression of early growth response-1 in human periodontal ligament cells.

The growth of periodontal ligament cells is maximal after stimulation with 10 ng/mL of fibroblast growth factor-1 (29). On the other hand, $^{3}$H-thymidine incorporation by periodontal ligament cells was greatest after treatment with 10 ng/mL of fibroblast growth factor-2 for 22 h (20). The increased 5-bromo-2′-deoxyuridine incorporation in this study confirmed that high concentrations of fibroblast growth factor-1 enhance the proliferative capacity on short-term culture.

Periodontal ligament cells treated with fibroblast growth factor-1 for 15 min began to express early growth response-1 mRNA and protein, thus supporting the hypothesis that expression of early growth response-1 is induced during the G0 and G1 phases of the cell cycle. As 5-bromo-2′-deoxyuridine incorporation in periodontal ligament cells treated with 10 ng/mL of fibroblast growth factor-1 showed a tendency to increase up to 45 min in this study, we investigated for 45 min in a dose–response experiment. As expression of early growth response-1 was directly correlated with fibroblast growth factor-1 dose, transcription of growth-related genes appear to be controlled by such growth factors. In addition, it has been shown that early growth response-1 protein specifically binds to the fibroblast growth factor-2 promoter (13,14). These data suggest that the growth of periodontal ligament cells is subject to autocrine/paracrine control under certain conditions. As high molecular-weight early growth response-1 protein was not detected in the nuclear fraction on western blotting, this suggests that

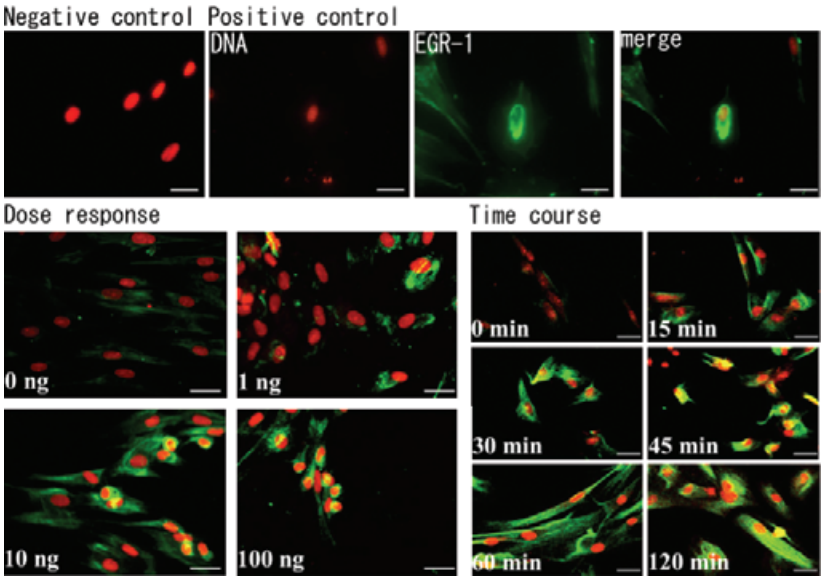


*Fig. 5*. Immunostaining for early growth response-1 protein in fibroblast growth factor-1-stimulated periodontal ligament cells (counterstaining with propidium iodide; magnification ×100; bar, 5 μm). The numbers in the bottom left corner indicate the concentrations of fibroblast growth factor-1 (ng) or treatment time (min). Counterstaining was observed in negative controls, and early growth response-1 protein expressed in periodontal ligament cells treated with 10% fetal calf serum for 30 min was used as a positive control (DNA, early growth response-1 and merge). Immunocytochemically, expression of early growth response-1 protein in periodontal ligament cells increased as a function of fibroblast growth factor-1 dose, and early growth response-1 protein was observed in the nuclear region after stimulation with as little as 10 ng of fibroblast growth factor-1. In the time-course experiment, anti-early growth response-1 reacted in and around the nucleus at 30 min after treatment with 10 ng of fibroblast growth factor-1.

early growth response-1 protein is modified in the plasma and that these post-translational modifications inhibit some further transcription of early growth response-1 protein in human periodontal ligament cells.

Our data show that early growth response-1 protein is expressed in periodontal ligament cells and that it is initially found in the cytoplasm before being translocated to the nucleus, following stimulation with fibroblast growth factor-1. Thus, early growth response-1 may play an important role in the growth of periodontal ligament cells, as it can be induced by very low concentrations of fibroblast growth factor-1. These data establish the inducibility of the the early growth response-1 gene in fibroblast growth factor-1-stimulated human periodontal ligament cells and suggest an important role for early growth response-1 in the growth of periodontal ligament cells during tissue regeneration and healing *in vivo*.

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