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Regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by basic fibroblast growth factor and dexamethasone in periodontal ligament cells

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Background and Objectives: In this study, we investigated the effect of basic fibroblast growth factor (bFGF) and dexamethasone (Dex) on mRNA expressions of collagen (COL) type I, III and X, matrix metalloproteinases (MMP)-1, -2, -3 and -9 and tissue inhibitors of metalloproteinases (TIMP)-1 and -2, and also on mineralization and morphology of periodontal ligament (PDL) cells.

Material and Methods: Periodontal ligament cells were obtained from premolar teeth extracted for orthodontic reasons. Periodontal ligament cells were cultured with Dulbecco's modified Eagle's medium containing: (1) 5% fetal bovine serum (FBS); (2) 5% FBS + ascorbic acid (AA, 50 μg/mL); (3) 5% FBS + Dex (10^{-7} M) + AA; (4) 5% FBS + bFGF (10 ng/mL) + AA; or (5) 5% FBS + Dex (10^{-7} M) + bFGF + AA. Cells within each group were evaluated for gene expression profile using semi-quantitative reverse transcriptase-polymerase chain reaction for COL I, III and X, MMP-1, -2, -3 and -9 and TIMP-1 and -2 on days 14 and 21 and for biomineralization by von Kossa stain *in vitro* on day 21. Images of PDL cells were examined using a phase contrast microscope.

Results: Basic fibroblast growth factor stimulated MMP-1, MMP-3 and MMP-9 mRNA expressions and inhibited TIMP-2 mRNA expression. Treatment of cells with Dex + bFGF led to downregulation of MMP-1, MMP-3 and MMP-9 transcripts. Whilst AA alone and Dex alone induced biomineralization of PDL cells, bFGF blocked the mineralization activity of the cells. In the Dex + bFGF group, more mineral nodules were noted when compared to AA alone and Dex alone groups.

Conclusion: The addition of Dex to culture reversed bFGF-mediated inhibition of mineralization. Use of combined bFGF and Dex to regulate PDL cell function may be a good therapeutic option to obtain periodontal regeneration.

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Significant progress has been made towards understanding the factors and cells involved in regeneration of periodontal tissues. However, cellular/molecular events, and the function and contribution of cells, including PDL cells, osteoblasts and cementoblasts, in the regeneration process are still not completely understood (1–4).

The periodontal ligament (PDL) is a non-mineralized connective tissue which is present between two mineralized tissues, alveolar bone and cementum. Several studies have demonstrated that PDL cells have the capacity to function as osteoblasts and cementoblasts under regenerative conditions (5–11). Characterization studies of PDL cells *in vitro* showed that these cells have osteoblast-like features, including synthesis and expression of alkaline phosphatase (ALP) and osteopontin (OPN), 1,25-dihydroxyvitamin D_3-induced bone-gla protein synthesis, responsiveness to parathyroid hormone (PTH) and dexamethasone (Dex)-induced PTH-mediated cAMP synthesis (12). Moreover, PDL cells have the capacity to produce mineralized nodules *in vitro* (13–15). In the periodontal regeneration process, progenitor PDL cells need to migrate and attach to the root surface, proliferate and differentiate and then synthesize extracellular matrix proteins to organize functional attachment tissues (16,17).

Numerous growth and differentiation factors have been investigated as potential therapeutic agents for periodontal tissue regeneration (18–20). Basic fibroblast growth factor (FGF2; bFGF) is involved in wound healing by facilitating the migration and proliferation of endothelial cells in angiogenesis, regulating fibroblast proliferation and collagen (COL) synthesis and collagenase production in remodeling. Basic fibroblast growth factor stimulates proliferation and differentiation of chondrocytes *in vitro* and promotes cartilage repair *in vivo*. Basic fibroblast growth factor is also a potent mitogen for bone cells derived from embryonic chick calvaria, newborn mouse calvaria, bovine bone and rat calvaria (21–25). In our previous study, we demonstrated that bFGF induces proliferation of cementoblasts and alters biomineralization behavior of these cells by regulating mineralized tissue-associated genes, including genes for bone sialoprotein (BSP), osteocalcin (OCN), OPN and matrix-gla protein (MGP) (26). Takayama *et al.* (27) reported that PDL cells express bFGF receptors (bFGFRs) and respond to bFGF sensitively in periodontal tissues. *In vitro* studies demonstrated that bFGF alters biological potentials of periodontal ligament cells (28), and *in vivo* studies revealed that topical application of bFGF stimulates periodontal tissue regeneration (29–32).

An *in vivo* study has shown that platelet derived growth factor (PDGF) and Dex embedded collagen matrix when applied to periodontal defects, induced regeneration of the periodontium in monkey (33). Dexamethasone has been demonstrated to increase the PTH-mediated cAMP response, when compared with cells treated with PTH alone in periodontal ligament cells *in vitro* (12). Pitaru *et al.* (34) indicated that bFGF has the capacity to stimulate both the growth and the biochemical functions of rat stromal bone marrow cells, and the addition of bFGF resulted in a considerable increase in formation of mineralized tissue in the presence of Dex. It has been demonstrated that bFGF enhances growth and osteogenic phenotype expression of Dex-stimulated human bone marrow-derived bone-like cells, by inducing the proliferation of the osteogenic lineage. It has also been noted that bFGF with Dex stimulated mineral nodule formation in the same cell group (35). In addition, it has been shown that bFGF has a significant role in stimulating proliferation and osteogenic expression of stromal bone marrow osteoprogenitor cells and that bFGF increased the level of glucocorticoid receptors in Dex-treated cultures derived from young rats (36,37).

Basic fibroblast growth factor plays key role in extracellular matrix remodeling by controlling a number of proteolytic activities in various cell types and may affect orchestration of matrix–enzymes interactions. Palmon *et al.* (38) assessed the dose-dependent effect of bFGF administration on the levels of gene expression of *COL I* and *III* and *matrix metalloproteinase (MMP)-1* in PDL cells. Their results indicated that bFGF exhibits an inverse time- and dose-dependent effect on the gene expression of *COL I* and *MMP-1*, in that it simultaneously downregulates the gene expression of *COL I* and upregulates the gene expression of *MMP-1*. Moreover, bFGF had no dose-dependent effect on *COL III* gene expression. The effect of bFGF on the expressions of the three genes was modulated by the time of incubation with bFGF. Shimazu *et al.* (39) investigated the effect of bFGF on *MMP-3* gene expression in PDL cells and its mechanism. They observed that bFGF increased the release of the cell-associated proteoglycans into the medium and MMP-3 mRNA levels in a concentration-dependent manner as examined by reverse transcriptase-polymerase chain reaction (RT-PCR). Their findings suggested that bFGF induces MMP-3 expression in PDL cells through the activation of the MEK2 in mitogen-activated protein kinase pathway.

The role of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in developmental or regenerative processes of periodontium is still not entirely understood. To provide initial insights, the purpose of this study was to investigate the effects of bFGF alone or in combination with Dex on collagen type I, III and X, MMP-1, -2, -3 and -9 and TIMP-1 and -2 mRNA expressions and the morphology and mineralization of PDL cells *in vitro*.

Material and methods

Cell isolation and culture

Human PDL cells were isolated from healthy periodontal ligaments of premolars extracted for orthodontic reasons (12–14). All patients gave written informed consent before providing the samples. After extraction, the teeth were placed in biopsy medium [Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beit Haemek, Israel) with 10% fetal bovine serum (FBS), 250 μg/mL gentamicin sulfate, 5 μg/mL amphotericin B, 100 units/mL penicillin and 100 μg/mL streptomycin]. Only periodontal ligament attached to the middle third of the root was removed with a scaler to

avoid contamination with gingival and apical tissues. The PDL tissues were cut into small pieces, rinsed with biopsy medium and placed into tissue culture dishes. The PDL tissues were incubated in biopsy medium in a humidified atmosphere of 95% air and 5% CO_2 at 37°C overnight. The following day, biopsy medium was replaced with culture medium (DMEM with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin). After reaching confluency, cells were passaged with 0.25% trypsin and 0.1% EDTA. Periodontal ligament cells were used between the fourth and seventh passage for all experiments.

Assay of DNA synthesis

The effects of Dex alone, different concentrations of bFGF (Sigma-Aldrich Chemie GmbH, Munich, Germany) Dex + bFGF on DNA synthesis were assayed by [^{3}H]thymidine incorporation. The concentrations of bFGF used were 0.1, 1, 10 and 100 ng/mL. Doses selected were based on previous studies that examined the effect of bFGF on periodontal ligament cells (40). Assays of DNA synthesis were performed as previously described (5). Serum-free DMEM was used as a negative control. Cells were plated in 24-well plates (Corning, Amsterdam, the Netherlands) at a density of 5×10^4 cells/well in triplicate and incubated for 24 h in DMEM containing 10% FBS and antibiotics. Media were aspirated, cells were rinsed twice with phosphate-buffered saline (PBS), and serum-free DMEM was added with and without bFGF at selected doses. Cells were incubated for 20 h, and then 3 μCi/mL methyl-[^{3}H]thymidine (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA) was added to each well for the last 4 h of the 24 h experiment. Next, cells were rinsed with PBS and then incubated with 5% trichloroacetic acid (TCA) for 1 h at 4°C. The TCA was removed and 1% sodium dodecyl sulfate (SDS) added to the wells and incubated for 1 h at 55°C. The precipitate was transferred into scintillation liquid, and activity was counted in a scintillation counter (Wallac 1410; Pharmacia, Turku, Finland; 26).

Morphology

Images of PDL cells treated with DMEM containing: (1) 5% FBS; (2) 5% FBS + ascorbic acid (AA, 50 μg/mL); (3) 5% FBS + Dex (10^{-7} M) + AA; (4) 5% FBS + bFGF (10 ng/mL) + AA; and (5) 5% FBS + Dex + bFGF + AA were examined visually for morphology, using a phase contrast microscope (Nikon TS100F, Tokyo, Japan).

Isolation of RNA

Periodontal ligament cells were plated in 60 mm cell culture dishes (Corning) at 3×10^4 cells/cm^2 and treated after 24 h as described above to determine gene expressions. Total RNAs from different treatment groups were isolated at passage four to seven, using a monophasic solution of phenol and guanidine isothiocyanate (Invitrogen, Life technology, Carlsbad, CA, USA; 26). Medium was aspirated from Petri dishes and cells were washed with PBS (Biological Industries). After addition of isolation solution, cells were scraped with a cell scraper (Corning) which had been cleaned with RNase Zap (Ambion, Austin, TX, USA) and rinsed with diethyl pyrocarbonate-treated distilled water (DEPC-dd H_2O). One-fifth volume of chloroform was added to the homogenate, and the samples were incubated at room temperature for 2–3 min. The suspension was spun at 10,400*g* at 4°C for 15 min. The RNA-containing aqueous phase was removed, and isopropanol was added (half of the volume of the aqueous phase). Samples were incubated at room temperature for 10 min and spun at 10,400*g* for 15 min. The supernatant was removed, and the RNA pellet was washed with 75% ethanol. The precipitated RNA was dissolved in DEPC-treated dd H_2O. Total RNA was quantified at 260 nm by spectrophotometer. The RNA samples were stored at −70°C.

Synthesis of cDNA for RT-PCR

Synthesis of cDNA was performed according to the RT enzyme (Promega, Madison, WI, USA) procedure. Reactions were carried out in 20 μL synthesis mixtures containing 1× reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTP, 1 μL (500 μg/mL) random hexamer, 1 unit/μL recombinant RNasin (Ribonuclease Inhibitor; Promega), 1 μL RT enzyme and 1 μg total RNA. The RNA samples and primers were first combined in a separate tube in a 5 μL volume, incubated at 70°C for 5 min, and held on ice until their addition to the reaction mixture, which contained the other reaction components. When combined, primers were annealed at 25°C for 5 min and then were incubated at 42°C for 60 min before heating at 70°C for 15 min. The cDNA samples were stored at −20°C until used for RT-PCR.

RT-PCR

Semi-quantiative RT-PCR was performed using primers for COL I, III and X, MMP-1, -2, -3 and -9 and TIMP-1 and -2, which may play important roles in periodontal tissue remodeling, and S15 rRNA primers (for normalization). The primers and RT-PCR protocols used are shown in Tables 1 and 2, respectively. Amplification reactions were performed in a final volume of 25 μL containing 0.5 μL cDNA, 25 mM of each dNTPs (Larova; Biochemie GmbH, Teltow, Germany), 50 pmol of each forward and reverse primer, 1.5 mM $MgCl_2$ (Bioron GmbH, Ludwingshafen, Germany), 1× reaction buffer [(160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl (pH 8.8) and 0.1% Tween-20)] (Bioron GmbH) and 1 U of Taq DNA polymerase (Bioron GmbH). The RT-PCR products (18 μL) resolved by electrophoresis in 1% agarose gels were stained with ethidium bromide. Gel photographs were taken using a gel documentation system (UVP Gel Documantation System-8000, UVP Ltd, Cambridge, UK). A representative experiment is shown (Fig. 3A), while results were reproduced in three separate experiments. Scion image analysis program (Bethesda, MD, USA) was used to quantify the data, and normalization calculations (gene/S15 rRNA) were performed.

Mineralization assay

Cells were plated at 3×10^4 cells/cm^2 in 24-well plates in DMEM containing 10% FBS. After 24 h, the medium was

Table 1. Synthetic oligonucleotide primers for RT-PCR

Primer	Sequence	Length (oligomer)	End-products (bp)
COL I	F 5′-GCAACATTGGATTCCCTGGACC-3′	22	510
	R 5′-GTTCACCCTTTTCTCCCTTGCC-3′	22	
COL III	F 5′-CCTCCAACTGCTCCTACTCG-3′	20	536
	R 5′-TCGAAGCCTCTGTGTCCTTT-3′	20	
COL X	F 5′-CAGGGATTCCAGGAACAAAA-3′	20	417
	R 5′-CTCCAGGATCACCTTTTGGA-3′	20	
MMP-1	F 5′-GATGGGAGGCAAGTTGAAAA-3′	20	424
	R 5′-CTGGTTGAAAAGCATGAGCA-3′	20	
MMP-2	F 5′-ATGACAGCTGCACCACTGAG-3′	20	425
	R 5′-CTCCTGAATGCCCTTGATGT-3′	20	
MMP-3	F 5′-TCATTTTGGCCATCTCTTCC-3′	20	476
	R 5′-AGTGCCCATATTGTGCCTTC-3′	20	
MMP-9	F 5′-TGCCAGTTTCCATTCATCTTCCAA-3′	24	518
	R 5′-CTGCGGTGTGGTGGTGGTT-3′	19	
TIMP-1	F 5′-GCTTCTGGCATCCTGTTGTT-3′	20	462
	R 5′-TTTGCAGGGGATGGATAAAC-3′	20	
TIMP-2	F 5′-GCAGCAAACACATCCGTAGA-3′	20	462
	R 5′-TCCTTCTCACTGACCGCTTT-3′	20	
S15 rRNA	F 5′-CTTCCGCAAGTTCACCTACC-3′	20	430
	R 5′-TGTGCGCCTTTATTAGCTGAG-3′	21	

Table 2. Amplification conditions for RT-PCR

Gene	Protocol		Cycles
COL I, III and X	Denaturation	94°C 45 s	30
S15 rRNA	Annealing	56°C 50 s	
	Elongation	72°C 90 s	
MMP-1, -2 and -3	Denaturation	94°C 45 s	30
TIMP-1 and -2	Annealing	54.5°C 50 s	
S15 rRNA	Elongation	72°C 90 s	
MMP-9	Denaturation	94°C 45 s	36
S15 rRNA	Annealing	42°C 30 s	
	Elongation	72°C 90 s	

72°C 10 min to finalize extension.

changed to DMEM containing: (1) 5% FBS; (2) 5% FBS + AA (50 μg/mL); (3) 5% FBS + Dex (10^{-7} M) + AA; (4) 5% FBS + bFGF (10 ng/mL) + AA; or (5) 5% FBS + Dex + bFGF + AA supplemented with 10 mM β-glycerophosphate. Cell-induced mineralization was determined on day 21 with von Kossa staining (41,42). The mineralization patterns of PDL cells treated with different components were evaluated using a phase contrast microscope (Sigma-Aldrich Chemie GmbH) on day 21. The data shown here represent results obtained from three separate experiments.

Statistical analysis

For DNA synthesis assay, the statistical analysis used was one-way analysis of variance (ANOVA) and Tukey–Kramer multiple comparison tests.

Results

Assay of DNA synthesis (Fig. 1)

To determine the mitogenic response of PDL cells to bFGF at different concentrations (0.1, 1, 10 and 100 ng/mL), Dex alone and Dex + bFGF (10 ng/mL), a [^{3}H]thymidine incorporation assay was performed. Basic fibroblast growth factor stimulated DNA synthesis significantly vs. control cells (in serum-free DMEM). A significant increase in proliferation of PDL cells was noted at a concentration of 10 ng/mL bFGF. Based on these results, we selected 10 ng/mL bFGF for gene expression and mineralization experiments. While we observed no effect of Dex on DNA content of PDL cells, increased mitotic activity was noted when combined with bFGF (10 ng/mL). All treatments, except for Dex only, significantly increased the DNA content of PDL cells when compared with the control group.

Morphology of cells (Fig. 2; days 7, 14 and 21)

Differences were observed in the morphology of PDL cells treated with

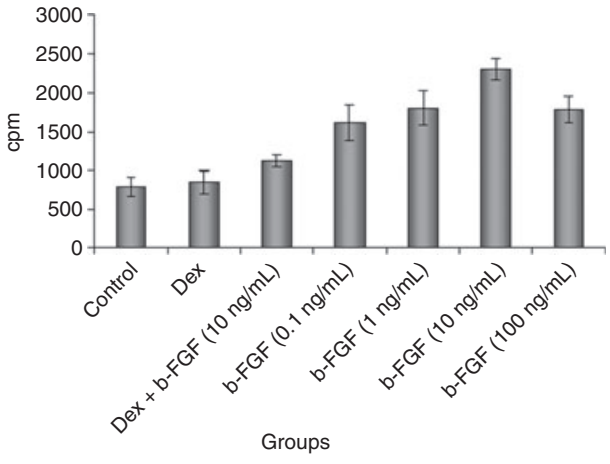

Fig. 1. Effect of different bFGF concentrations (0.1, 1, 10 or 100 ng/mL), Dex (10^{-7} M) and Dex (10^{-7} M) + bFGF (10 ng/mL) on DNA synthesis of PDL cells assessed using [^{3}H]thymidine incorporation. Each bar represents the mean ± SD (modified from Hakki *et al.* 40). *$p < 0.05$: 10 ng/mL bFGF > 0.1 ng/mL bFGF = 1 ng/mL bFGF = 100 ng/mL bFGF > Dex (10^{-7} M) + bFGF (10 ng/mL) > Dex (10^{-7} M) = control (0% FBS).

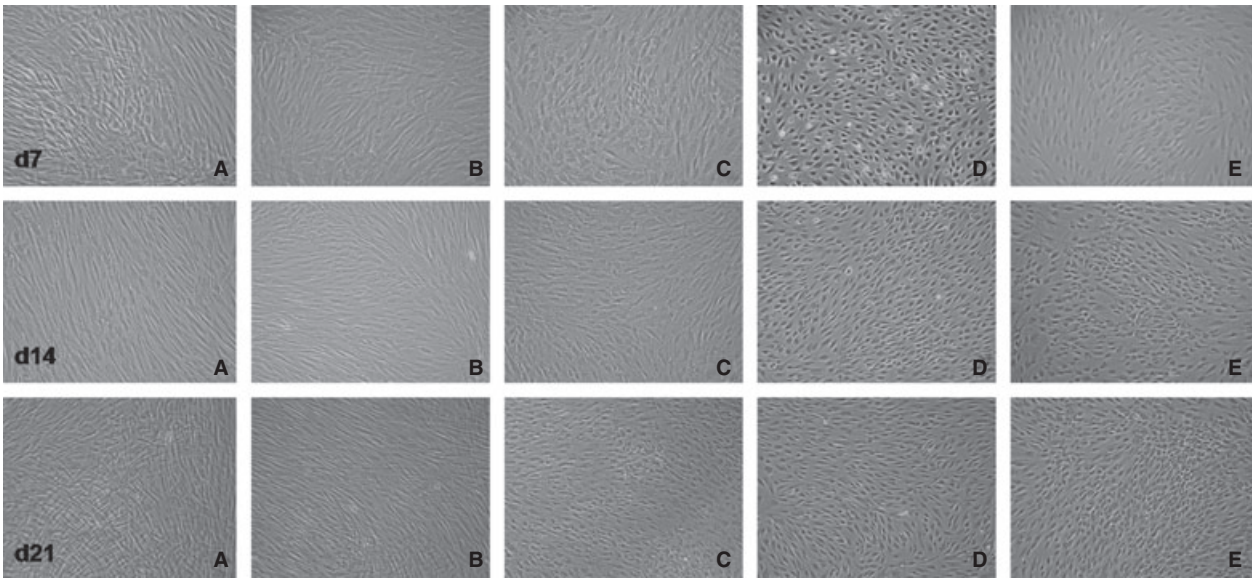

Fig. 2. Phase contrast micrographs of PDL cell cultures treated as follows: 5% FBS (A); 5% FBS + AA (B); 5% FBS + Dex + AA (C); 5% FBS + bFGF + AA (D); and 5% FBS + AA + Dex + bFGF (E) on days 7, 14 and 21 (top, middle and bottom panels, respectively) at ×10 magnification. Note cuboidal appearence of cells in C, D and E. Addition of Dex and/or bFGF resulted in alteration of the morphology of PDL cells to a more rounded appearence with a different pattern of extracellular matrix.

Dex, bFGF and Dex + bFGF. While cells treated with 5% FBS and 5% FBS + AA were spindle shaped, others looked cuboidal. Moreover, images suggested that Dex treatment results in more extracellular matrix. In the bFGF-treated groups, membranes of cells were more apparent and attachment types of cells looked different when compared with other groups.

Semi-quantitative RT-PCR experiments (Fig. 3; days 14 and 21)

Expression of COL I, III and X mRNA — While there was no apparent difference between the groups for COL I mRNAexpression, Dex increased COL III mRNA expression, and cells exposed to bFGF demonstrated increased mRNA expression for COL X. Treatment of cells with Dex + bFGF caused downregulation of the bFGF-mediated upregulation of COL X transcript.

Expression of MMP-1, -2, -3 and -9 mRNA — Basic fibroblast growth factor significantly stimulated MMP-1 and MMP-3 mRNA expression of PDL cells. Downregulation was noted for MMP-3 transcritps when Dex was combined with bFGF. Although consistent stimulation was observed at both time points, the induction of MMP-1 and MMP-3 mRNA expressions via bFGF was more marked at day 21 compared with day 14. No significant difference was observed for MMP-2 mRNA expression of PDL cells for each treatment. While induced MMP-9 mRNA expression was noted in the cells treated with Dex and bFGF vs. control cells, addition of Dex and bFGF decreased MMP-9 mRNA expression of PDL cells when compared with either treatment alone.

Expression of TIMP-1 and -2 mRNA — Whilst similar mRNA expressions for TIMP-1 were noted, decreased TIMP-2 transcripts were observed in the cells treated with bFGF. At day 24, the addition of Dex to bFGF prevented downregulation of TIMP-2 transcript.

Microscopic features of mineralization of PDL cells (Fig. 4; day 21)

Treatment with AA and Dex + AA promoted biomineralization of PDL cells. Basic fibroblast growth factor blocked the mineralization activity of PDL cells and, interestingly, addition of Dex was able to reverse this effect. Apparently, there were more mineralized nodules in the combined Dex + AA + bFGF group compared with the other treatments in which we observed mineralized nodule formation.

Discussion

Growth factors are potent bioactive molecules responsible for the co-ordination of many cell functions and interactions during development, wound healing and regeneration of the tissues. In periodontium, bFGF is present in the extracellular matrix, as well as in the cementum, and can function as a local factor. Basic fibroblast growth factor is produced primarily by PDL fibroblasts and endothelial cells in the PDL, and bFGF levels may be decreased in tissues associated with chronic periodontal lesions (43). Basic fibroblast growth factor is involved in wound healing by facilitating the migration and proliferation of endothelial cells in angiogenesis, regulating fibroblast

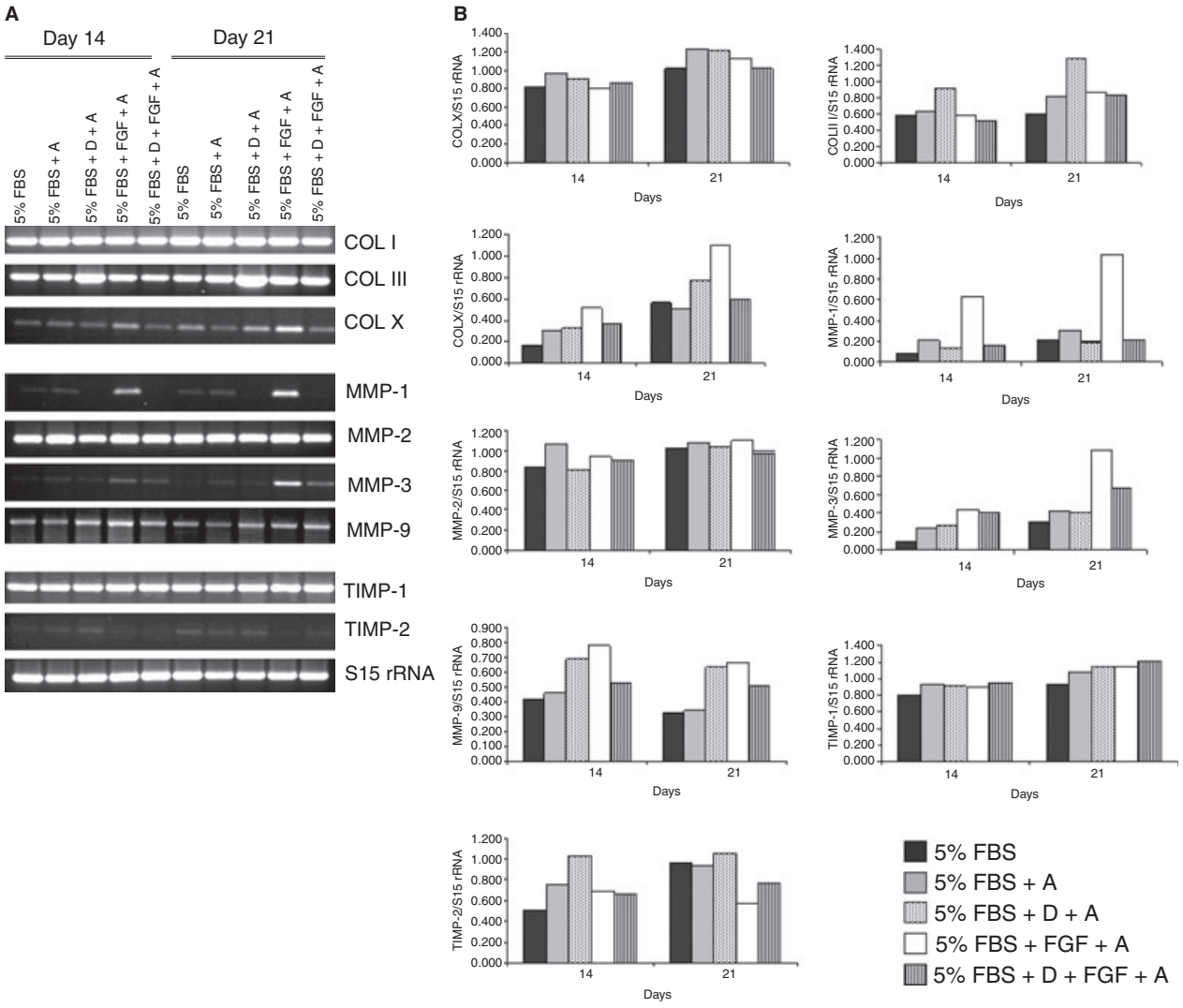

Fig. 3. (A) Effects of Dex (abbreviated to D) and/or bFGF on gene expression by PDL cells on days 14 and 21. Periodontal ligament cells were incubated in media containing 5% FBS, 5% FBS + AA, 5% FBS + Dex + AA, 5% FBS + bFGF + AA or 5% FBS + Dex + bFGF + AA, and RNA was isolated on days 14 and 21. Gene expression of *COL I, III and X, MMP-1, -2, -3* and *-9* and *TIMP-1* and *-2* was measured by semi-quantitative RT-PCR. Note the dramatically increased expression of MMP-1, MMP-3 and MMP-9 and decreased expression of TIMP-2 in cells exposed to bFGF on days 14 and 21. (B) Normalization expressed as gene/S15 rRNA.

proliferation and collagen synthesis and collagenase production in remodeling and repair of periodontal tissues. Basic fibroblast growth factor has a potent mitogenic effect on PDL cells, especially on undifferentiated cells (28).

Several *in vivo* studies have indicated that the local application of bFGF enhances periodontal regeneration, with new bone, cementum and PDL formation in comparison with control lesions, and neither epithelial downgrowth nor ankylosis and root resorption were observed in the bFGF-treated sites (30–32,44,45). These studies demonstrated that bFGF could be applied as one of the therapeutic modalities to actively induce periodontal tissue regeneration by increasing the number of undifferentiated mesenchymal cells which differentiate into osteoblasts and/or cementoblasts. They demonstrated that bFGF plays an important role in the physiology of connective tissue, participating in the healing and regeneration process and that bFGF has potential therapeutic applications owing to angiogenic and positive growth-promoting effects in the periodontium. Pitaru *et al.* indicated that aging results in a decrease in the pool of the mineralized tissue progenitors in the periodontium. Basic fibroblast growth factor may be a useful tool to increase the progenitor pool of the periodontium (46).

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) possess important roles in the metabolism of the major extracellular matrix proteins and different collagen types. The MMPs and TIMPs are important participants in various physiological processes involved in tissue remodeling and regeneration. The

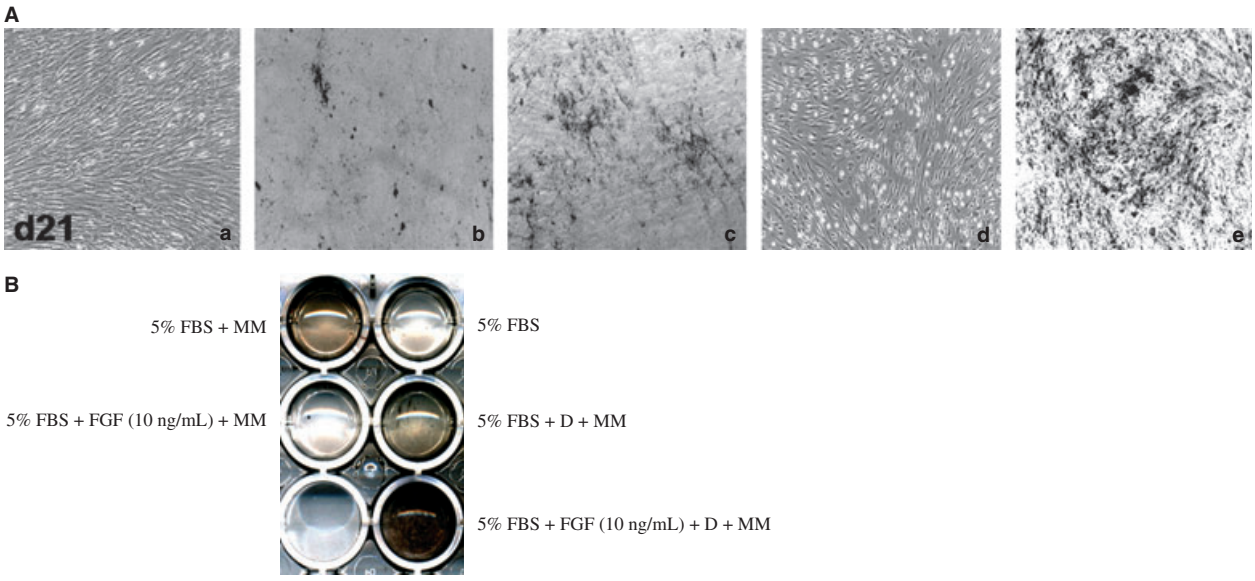

Fig. 4. (A) Microscopic features of PDL cell mineralization. (B) Macroscopic images of the plate. von Kossa staining was carried out to determine mineralized nodule formation on day 21. For these studies, cells were cultured in media containing 5% FBS, AA (50 μg/mL), 10 mM β-glycerophoshate and Dex (10^{-7} M), and bFGF (10 ng/mL) and Dex (10^{-7} M) + bFGF (10 ng/mL). Treatment with bFGF alone prevented nodule formation. While bFGF blocked mineralization of PDL cells, Dex + bFGF together induced mineralization of PDL cells. These results were noted in three separate experiments.Treatments shown are as follows: 5% FBS + β-glycerophosphate (Aa); 5% FBS + MM (ascorbic acid, β-glycerophosphate; Ab); 5% FBS + Dex + MM (Ac); 5% FBS + bFGF + MM (Ad); and 5% FBS + Dex + bFGF + MM (Ae).

MMPs are a family of proteolytic enzymes that mediate the degredation of extracellular matrix macromolecules. The TIMPs form bimolecular complexes with the active forms of MMPs and regulate matrix degradation by blockage of autolytic MMP activation. Their activities are strictly controlled at the level of gene expression, including transcription and translation, at the level of activation or at the step of inhibition by TIMPs (47–51).

Research regarding the effects of bFGF on extracellular matrix protein demonstrated that bFGF downregulated transcription levels of type I collagen and elastin while upregulating hyaluronan and heparin sulfate transcripts of PDL cells (38,52–54). Moreover, bFGF orchestrated degrading enzymes of connective tissues, i.e. MMPs. Basic fibroblast growth factor increased MMP-1 (38) and MMP-3 (39) mRNA expressions in PDL cells *in vitro*. In our study, bFGF upregulated MMP-1, MMP-3 and MMP-9 mRNA expressions and downregulated TIMP-2 mRNA expression, while there was no change in TIMP-1 mRNA expression with the treatments. Our results are consistent with other studies (38,39) for MMP-1 and MMP-3 transcripts.

In culture conditions, stromal bone marrow cells (SBMCs) and PDL cells may express the osteogenic phenotype when treated with differentiation factors. Dexamethasone, a synthetic glucocorticoid, is one of the differentiation factors. In the presence of vitamin C (ascorbic acid, AA), β-glycerophoshate and Dex, SBMCs (55), cementoblasts (26) and PDL cells (14) differentiate into mature osteoblasts and/or cementoblasts, which produce mineralized bone and cementum-like tissues. In our study, increased biomineralization of PDL cells was noted when the cells were treated with AA and Dex, as seen for SBMCs.

Martin *et al.* (56) reported that bFGF-pretreated human SBMCs respond to Dex induction by expressing the osteogenic phenotype both *in vitro* and *in vivo*, suggesting that bFGF may maintain the Dex-inducible osteoprogenitor pool in short-term cultures. Kotev-Emeth *et al.* (55) demonstrated that in a rat cultured SBMC population, bFGF acts mainly by maintaining the osteoprogenitor pool and by stimulating the growth and differentiation of the osteoprogenitor pool. These previous results demonstrated that bFGF and Dex had a synergistic effect in maintaining the proliferation and the osteogenic phenotype. Based on these findings, in the periodontal regeneration process, these two agents may be an ideal combination to maintain the proliferation and differentiation of PDL cells to the osteoblast/cementoblast phenotype.

Researchers have demonstrated that bFGF added to Dex-treated SBMC cultures enhanced proliferation of these cells and also increased the production of ALP, osteocalcin and mineralized bone-like tissue (34,35). In contrast to these findings, bFGF alone totally blocked mineralization of cementoblasts, and combination with Dex did not alter this response. Basic fibroblast growth factor alone and in combination with Dex downregulated BSP and osteocalcin mRNA expression and upregulated OPN and MGP mRNA transcripts of cementoblats (26). In this study, we observed that while Dex is capable of inducing PDL

cell biomineralization, bFGF alone inhibited this mineralization activity. Interestingly, the bFGF + Dex-treated group had more mineralized nodules when compared with the Dex-treated group. While PDL cells demonstrated a similar response to SBMCs, PDL cells behaved differently from the cementoblasts regarding mineralization as a response to these factors.

Correlating these mineralization results with the gene expression profile, increased MMP-1 and MMP-3 and decreased TIMP-2 may be involved in the degradation of the extracellular matrix which is required to initiate mineralization. The addition of Dex to bFGF downregulated the bFGF-induced MMP-1 and MMP-3 mRNA transcripts of PDL cells. In contrast to the effects on MMP-1 and MMP-3, both bFGF and Dex induced MMP-9 mRNA transcripts. Interestingly, the combination of bFGF and Dex treatment downregulated MMP-9 mRNA expression, an effect that was more apparent on day 14.

Basic fibroblast growth factor has pronounced regenerative potential in *in vivo* studies owing to the positive effects on proliferation, angiogenesis and extracellular matrix regulation, which are important steps for regeneration. *In vitro* studies, such as the present study, are useful to identify possible mechanisms by which agents may affect cell activity *in vivo* and thus provide guidence for clinical trials. However, *in vitro* studies have some limitations, especially regarding the effects of agents *in vitro* vs. *in vivo*. Our results indicated that the combination of bFGF with Dex significantly induced biomineralization of cells when compared with Dex alone. It must be noted that before considering the use of these two agents together clinically to improve regeneration of periodontal tissues, several points need to be investigated in order to clarify the role of bFGF and/or Dex on the signal transduction pathways that are involved in the differentiation and mineralization of PDL cells. Additional investigations into the mechanisms of action of these factors will help to clarify their potential as therapeutic agents and will provide information that will aid in designing predictable treatments for periodontal disease.

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