

- denaturing high-performance liquid chromatography. *Congenit Anom* 45:125–131
- Westling L, Mohlin B, Bresin A (1998) Craniofacial manifestations in the Marfan syndrome: palatal dimensions and a comparative cephalometric analysis. *J Craniofac Genet Dev Biol* 18:211–218
- Yamada S, Murakami S, Matoba R, Ozawa Y, Yokokoji T, Nakahira Y, Ikezawa K, Takayama S, Matsubara K, Okada H (2001) Expression profile of active genes in human periodontal ligament and isolation of PLAP-1, a novel SLRP family gene. *Gene* 275:279–286
- Yokoi T, Saito M, Kiyono T, Iseki S, Kosaka K, Nishida E, Tsubakimoto T, Harada H, Eto K, Noguchi T, Teranaka T (2007) Establishment of immortalized dental follicle cells for generating periodontal ligament in vivo. *Cell Tissue Res* 327:301–311
- Yuan X, Werner JM, Lack J, Knott V, Handford PA, Campbell ID, Downing AK (2002) Effects of the N2144S mutation on backbone dynamics of a TB-cbEGF domain pair from human fibrillin-1. *J Mol Biol* 316:113–125
- Zhang X, Soda Y, Takahashi K, Bai Y, Mitsuru A, Igura K, Satoh H, Yamaguchi S, Tani K, Tojo A, Takahashi TA (2006) Successful immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells. *Biochem Biophys Res Commun* 351:853–859

Basic fibroblast growth factor regulates expression of heparan sulfate in human periodontal ligament cells

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Abstract

Heparan sulfate (HS) proteoglycan is a widely distributed biological molecule that mediates a variety of physiological responses in development, cell growth, cell migration, and wound healing. We examined the effects of basic fibroblast growth factor-2 (FGF-2), which is known to modulate extracellular matrix (ECM) production of various cell types, on the production of HS proteoglycan by human periodontal ligament (HPDL) cells. We also examined the effects of FGF-2 on the expression of syndecans, a major family of membrane-bound HS proteoglycans. Treatment of HPDL cells with FGF-2 for 72 h resulted in a pronounced increase in the level of HS in the culture supernatant in a dose-dependent manner. However, reverse transcription-polymerase chain reaction data (RT-PCR) revealed that FGF-2 marginally reduced the gene expression of syndecan-1, -2, and -4, and did not alter the level of syndecan-3 mRNA. Furthermore, FGF-2 did not have an effect on the mRNA expression of enzymes associated with HS biosynthesis. Interestingly, FACS analysis revealed that the syndecan family displayed diverse alterations in response to FGF-2. FGF-2 barely altered the expression of syndecan-1, but decreased the expression of syndecan-2 and -4 on HPDL cells. Moreover, dot blot analysis showed that FGF-2 did not alter the level of syndecan-1 and -2, but enhanced the level of syndecan-4 in culture supernatants of FGF-2-stimulated HPDL cells. These results suggest that the FGF-2-activated increase in the level of HS in conditioned medium may be a result of shedding of syndecan-4 from the HPDL cell surface. Taken together, FGF-2 may differentially regulate the expression of HS proteoglycans in a HS-proteoglycan-subtype-dependent manner. The diversity of the expression patterns of HS proteoglycans may be associated with the FGF-2-induced biological functions of HPDL cells.

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1. Introduction

A wide range of extracellular matrix and growth factors participate during the wound-healing process. The basic fibroblast growth factor-2 (FGF-2) is one such growth factor detected in the early phase of wound healing. FGF-2 mediates various biological responses including cellular proliferation, angiogenesis, and tissue repair (Birkhøj et al., 1997; Nugent and Iozzoli, 2000). FGF-2 also modulates the expression of

glycosaminoglycans and proteoglycans, as well as collagen and non-collagenous protein, which are main components of connective tissue. However, the details of the regulatory effects of FGF-2 on the expression of glycosaminoglycans and proteoglycans in cells have not been fully defined.

The syndecans are a family of cell surface heparan sulfate (HS) proteoglycans which comprise of a core protein and glycosaminoglycan side chains. Four members of the syndecan family have been identified to date. Whereas syndecan-4 expression is ubiquitous, syndecan-1, -2, and -3 are mainly present in epithelial cells, fibroblasts, and neural cells, respectively. The HS chains of the syndecans are responsible for their biological

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functions, such as extracellular matrix assembly and growth factor binding. The HS chains of syndecans are also known to function as co-receptors of the FGF receptor. It has been reported that when tissue was injured, syndecan expression was increased in epithelial cells, endothelial cells, and fibroblasts (Elenius et al., 1997; Gallo et al., 1996), and shed syndecan ectodomain accumulated in wound fluid (Fitzgerald et al., 2000; Kainulainen et al., 1998; Park et al., 2000). Recent studies have also revealed impaired wound healing in syndecan-deficient mice (Echtermeyer et al., 2001; Ishiguro et al., 2000; Stepp et al., 2002), suggesting an essential role for the syndecans in wound repair.

Periodontal tissue is a tooth-supporting tissue and consists of periodontal ligament, gingiva, cementum, and alveolar bone. The periodontal ligament cells have the potential to secrete a variety of extracellular matrices and support teeth with these molecules. Furthermore, they play key roles in regeneration events following periodontal tissue breakdown caused by progression of periodontal diseases. Interestingly, we revealed that topical application of FGF-2 to periodontal tissue defects activates regeneration of tissue with new cementum and ligament tissue formation (Murakami et al., 1999), which may be a result of FGF-2 increasing cell growth and modulating extracellular matrices. In addition, we explained the stimulatory effects of FGF-2 on the expression of the high molecular type of hyaluronan, a non-sulfated glycosaminoglycan, by human periodontal ligament (HPDL) cells via the up-regulation of hyaluronan synthase (HAS)1 and HAS2 (Shimabukuro et al., 2005). Here we examined the production of HS and the expression pattern of the syndecan gene family by HPDL cells in response to FGF-2.

2. Experimental procedures

2.1. Reagent

Human recombinant FGF-2 was provided by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan).

2.2. HPDL cells

HPDL cells were isolated from healthy periodontal ligaments of first premolar teeth of individuals undergoing tooth extraction for orthodontic treatment in accordance with the method of Somerman et al. (1988), with minor modifications. All the patients gave informed consent before providing the samples. Healthy periodontal tissue was removed from the center of the root surface with a surgical scalpel. The tissue was minced then transferred to Leighton tubes (Costar, Cambridge, MA, USA). The explants were cultured in α -MEM supplemented with 10% FCS, 50 U/ml penicillin G, and 50 μ g/ml streptomycin (henceforth denoted standard medium), with medium changed every 2 or 3 days. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. When the cells growing out from the explants had reached confluence, they were separated by treatment with a solution containing 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid, collected by centrifugation, and cultured in standard

medium in culture dishes until confluency. The cells were then trypsinized and split at a 1:3 ratio. Experiments were carried out with cells from the fourth to fifth passages. In this study, we established 3 cell lines from different volunteers, and all cell lines were used in each experiment. All these cell lines provided similar results in each experiment of this study.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) for quantitation of mRNA levels

HPDL cells were seeded at a density of 10⁵ cells/dish in 600-mm dishes and grown to confluency in standard medium. Following 24 or 48 hours of activation in the absence or presence of FGF-2 (50 ng/ml), total RNA was isolated from HPDL cells by RNAzol™ (Cinna Biotech Laboratories Inc., Friendswood, TX, USA), according to the manufacturer's instructions. The precipitated RNA was resuspended in 0.1% diethylpyrocarbonate-treated distilled water (DEPC-treated H₂O). This RNA sample was heatdenatured at 65 °C for 10 min and chilled on ice. Complementary DNA (cDNA) synthesis and amplification via PCR were performed as described previously. cDNA synthesis was carried out in a 40 μ l reverse transcription mixture containing 52.5 mM Tris-HCl; pH 8.3, 75.5 mM KCl, 3 mM MgCl₂, 0.5 mM each dNTPs, 1 mM dithiothreitol, 1.1 U/ μ l RNase inhibitor (Takara Biomedicals, Shiga, Japan), 55 ng/ μ l random hexamer, 5 U/ μ l M-MLV reverse transcriptase (Gibco, Gaithersburg, MD, USA) and RNA. The mixture was incubated at 37 °C for 60 min. At the end of the reverse transcription, all samples were heated at 99 °C for 5 min to inactivate the enzyme and were diluted into 110 μ l of DEPC-treated H₂O.

Oligonucleotide PCR primers specific for HS biosynthesis-related enzymes, syndecan-1, -2, -3, and -4, and hypoxanthine phosphoribosyl transferase (HPRT) were synthesized at Takara Shuzo Co. Ltd. (Table 1).

Table 1
Primers utilized for reverse transcription-polymerase chain reaction

Primers	Size	Sequence
HPRT	303 bp	Sense: 5'CGAGATGTGATGAAGGAGATGGG3'
		Antisense: 5'GCCTGACCAAGGAAAGC AAAATGC3'
GleNAc1-1	546 bp	Sense: 5'CTAAGCTGCAGGGAAAATAAAA3'
		Antisense: 5'TTGCTGTCGTGTGTTTGAAG3'
HS2ST	443 bp	Sense: 5'AGGATTTTATCATGGACACG3'
		Antisense: 5'TCTTCTCTGTGCGATAGAGT3'
NDST-1	450 bp	Sense: 5'ATCTTCGCTGTTCCAGGT3'
		Antisense: 5'CTCATTGGCCCTGAAGAAAGC3'
NDST-2	580 bp	Sense: 5'CATGAAGGTGGCTGAGTTG3'
		Antisense: 5'CGGATTAAGCAGCAGCTGTCA3'
Epimerase	624 bp	Sense: 5'AGGTGGTTAGGTTGATTGCG3'
		Antisense: 5'GCAGTTGATTGATGTTGGGTG3'
Syndecan-1	472 bp	Sense: 5'CTTTGAAACCTCGGGGAGAATAC3'
		Antisense: 5'TCCAGGCAGAAGTCAGAGAAGCAG3'
Syndecan-2	395 bp	Sense: 5'GGAGCTGATGAGGATGTAGA3'
		Antisense: 5'CACTGGATGGTTTGCCTCT3'
Syndecan-3	292 bp	Sense: 5'GCTTCTTTCCCTTTTACCCTCCGC3'
		Antisense: 5'TGTTCCCCAACCTCTCTGCCC AAG3'
Syndecan-4	245 bp	Sense: 5'GGGCAGGAATCTGATGACTTTGAG3'
		Antisense: 5'GCTGGACATTGACACCTTGTTC3'

PCR was performed in a 50 μ l mixture containing 5 μ l of each sample of derived cDNA, 10 mM Tris-HCl; pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.15 mM of each dNTP, 1.25 mM Ampli Taq Gold™ (Perkin Elmer Cetus Co., Emeryville, CA, USA) and 0.2 mM of each primer. After 9 min of pre-denaturation at 94 °C, PCR was performed in a DNA thermal cycler (MJ Research Inc., Waltham, MA, USA) for 21, 24, 27, 30, 33 and 36 cycles to optimize the cycle number to maintain exponential conditions of amplification. Each cycle consisted of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, polymerization at 72 °C for 90 s. The 7 μ l samples were analyzed on 2% agarose ethidium bromide gels run at 100 V for 25–30 min.

2.4. Determination of the level of HS in culture supernatants

HPDL cells were seeded at a density of 5×10^5 cells in 100-mm culture dishes (Coming Laboratory Sciences Company, Coming, NY, USA) and grown in standard medium. When the monolayers were confluent, the cells were rinsed twice with Hanks' balanced salt solution and incubated in 10% FCS- α MEM in the absence or presence of FGF-2 (1–50 ng/ml). At the end of the incubation periods, the supernatants were collected and stored at -20 °C until determination of HS levels. The HS levels in culture supernatants were measured by an enzyme-linked immunosorbent assay (HS assay kit, Seikagaku Co., Tokyo, Japan), following the manufacturer's instructions. Samples or HS standards were added to the wells which were coated with an anti-HS antibody, and incubated at 4 °C for 24 h. After washing, bound HS was detected by incubating with a biotinylated-antibody specific for HS and horse radish peroxidase (HRP)-conjugated streptavidin for 1 h at room temperature. The plates were washed, loaded with 3,3',5,5'-tetramethylbenzidine substrate solution and incubated at room temperature before the reaction was stopped with stop solution. Absorbance was measured by a microplate reader (MTP-32; Corona Electric, Hinachinaka, Japan) set at 450 nm/630 nm. The HS levels in samples were calculated from the HS standard curve.

In order to determine the optimal dose of each cytokine for comparison in this study, we preliminarily examined the proliferative responses of HPDL cells induced by the cytokines. A non-saturating dose, which induced nearly equal responses among the cytokines, was selected as an optimal dose of each cytokine in this study. The doses for comparison of FGF-2, epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin like growth factor-I (IGF-I), platelet-derived growth factor-BB (PDGF-BB), and transforming growth factor- β (TGF- β) were 50, 100, 50, 50, 50, and 10 ng/ml, respectively.

2.5. Immunocytochemical analysis

HPDL cells were seeded at a density of 5×10^5 cells in 35-mm glass bottom dishes (Matsunami Glass Ind. Ltd., Kishiwada, Japan) and cultured until confluency. At the time of the assay, culture medium was replaced with fresh medium, supplemented with 10% FCS, in the absence or presence of FGF-2 (50 ng/ml). Cells were cultured at 37 °C for the indicated times.

Cell monolayers were fixed with acetone or 2% paraformaldehyde for 10 min, then blocked with 1% bovine serum albumin

(BSA) and 10% goat serum in PBS at room temperature for 30 min. The primary antibodies used were: mouse anti-human syndecan-1 antibody (Serotec Ltd., Kidlington, Oxford, UK); goat anti-human syndecan-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA); goat anti-syndecan-4 antibody (Santa Cruz Biotechnology Inc.); mouse monoclonal biotinylated anti-hyaluronan antibody (10E4; Seikagaku Co.). Monolayers were washed three times with PBS to remove unbound antibody, then incubated for 30 min at 37 °C with Alexa Fluor 488-labeled polyclonal anti-mouse or goat IgG antibodies (Molecular Probes, Carlsbad, CA, USA) or Alexa Fluor 568-labeled streptavidin (Molecular Probes). The samples were mounted on coverslipped, then viewed using an OLYMPUS 1 \times 70 microscope (Tokyo, Japan) and photographed.

2.6. FACS analysis

HPDL cells were incubated in 10% FCS- α MEM in the absence or presence of FGF-2 (50 ng/ml). At the time of the assay, cells were incubated with mouse anti-human syndecan-1 antibody (Serotec Ltd.), goat anti-human syndecan-2 antibody (Santa Cruz Biotechnology Inc.), and biotin-conjugated mouse anti-syndecan-4 antibody (Santa Cruz Biotechnology Inc.) as primary antibodies. The cells were washed three times with PBS and incubated for 30 min at 37 °C with or without FITC-labeled polyclonal anti-mouse antibody (Caltag Laboratories, Burlingame, CA, USA).

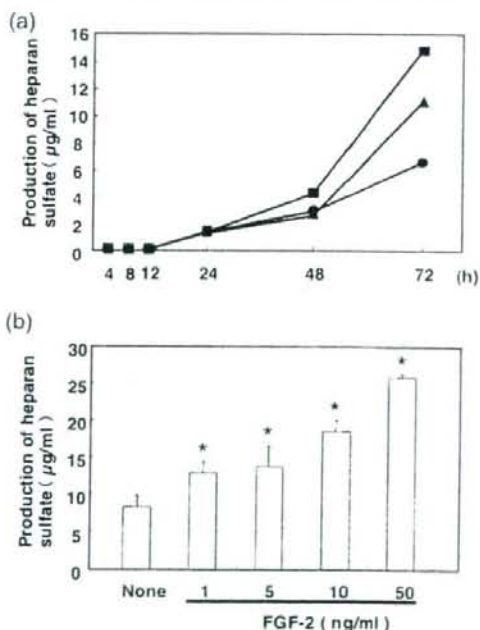


Fig. 1. Effect of FGF-2 on the production of HS by HPDL cells. HPDL cells were cultured in the absence (●) or presence of FGF-2 (1 (▲), 50 (■) ng/ml) and cultured for the indicated times (a). HPDL cells were cultured in the absence or presence of FGF-2 (1, 5, 10, 50 ng/ml) for 72 h (b). Conditioned medium was removed and the level of HS analyzed using an enzyme-linked immunosorbent assay. Results of one representative experiment from three separate experiments are shown. (* $p < 0.05$ compared to unstimulated control).

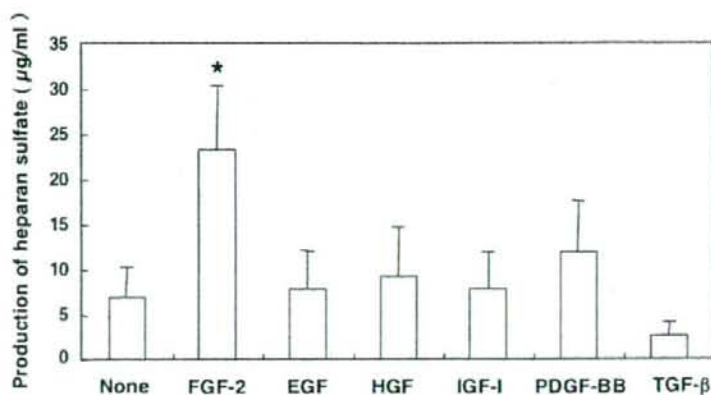


Fig. 2. Effect of various cytokines on HS production by HPDL cells. HPDL cells were cultured without any cytokine or with FGF-2 (50 ng/ml), EGF (100 ng/ml), HGF (50 ng/ml), IGF-1 (50 ng/ml), PDGF-BB (50 ng/ml), or TGF- β (10 ng/ml). Culture supernatants were removed after 72 h of exposure to the various cytokines, and the HS concentration measured by enzyme-linked immunosorbent assay. Results of one representative experiment from three separate experiments are shown. (* $p < 0.05$ compared to unstimulated control).

FITC-labeled polyclonal anti-goat antibody (Santa Cruz Biotechnology Inc.), or FITC-labeled streptavidin (BD Biosciences, San Diego, CA).

2.7. Dot blot analysis

Medium from FGF-2-stimulated or unstimulated HPDL cells was collected, and a 400- μ l sample spotted onto a nitrocellulose membrane. The membrane was blocked for 1 h with PBS containing 10% BSA, incubated overnight at 4 °C with a mouse monoclonal antibody to syndecan-1, -2, or -4, or a goat polyclonal antibody to glypican-1 (Santa Cruz), -2 (R&D Systems, Inc., Minneapolis, MN, USA), -3 (Santa Cruz), -6 (R&D Systems, Inc.), or perlecan (Santa Cruz), washed with PBS containing 1% BSA, and then incubated with HRP-conjugated rabbit anti-mouse serum or anti-goat serum. Immunoreactive bands were identified using a chemiluminescent kit (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA), and were scanned densitometrically and analyzed using NIH image 1.63.

2.8. Statistical analysis

Data are expressed as mean \pm standard deviation, and were compared by ANOVA and *post-hoc* Scheffé's comparisons.

3. Results

3.1. FGF-2 induced production of HS by HPDL cells, but did not affect the production of HS biosynthesis-associated enzymes

Although FGF-2 has been reported to modulate extracellular matrix production, the effects of FGF-2 on glycosaminoglycan production by HPDL cells have not been fully elucidated (Shimabukuro et al., 2005). Focusing on HS production by HPDL cells, we first analyzed the concentration of HS in conditioned medium of FGF-2-stimulated HPDL cells. HS (1.29 μ g/ml) was detected in the culture supernatants after 24 h of stimulation. The concentration of HS was found to gradually increase during 72 h of stimulation, with the increase

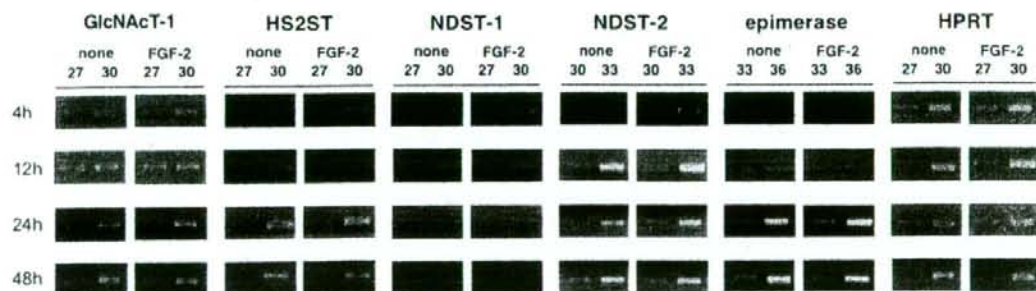


Fig. 3. mRNA expression of HS synthetic enzymes in HPDL cells. The expression of mRNA of HS-related enzymes in HPDL cells cultured in the absence or presence of FGF-2 (50 ng/ml) for 24 h was measured. Total RNA was recovered and RT-PCR performed to analyze the mRNA expression of GlcNAcT-1, HS2ST, NDST-1, NDST-2, epimerase, and HPRT in HPDL cells. Results of one representative experiment from three separate experiments are shown. The number of PCR amplification cycles is shown above each lane.

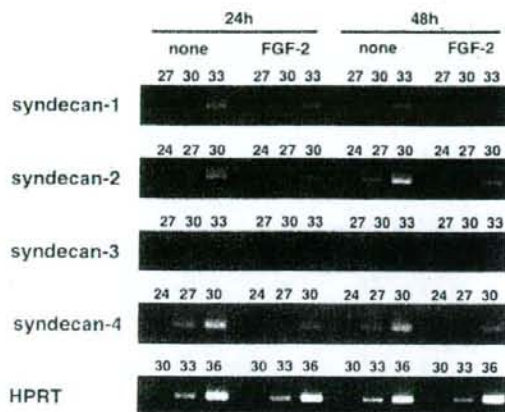


Fig. 4. mRNA expression of the syndecan gene family in HPDL cells. The expression of mRNA of the syndecan gene family in HPDL cells cultured in the absence or presence of FGF-2 (50 ng/ml) for 24 h and 48 h was measured. Total RNA was recovered and RT-PCR performed to analyze the mRNA expression of syndecan-1, -2, -3, and -4, and HPRT in HPDL cells. Results of one representative experiment from three separate experiments are shown. The number of PCR amplification cycles is shown above each lane.

between 48 and 72 h being particularly pronounced. In addition, the amount of HS produced was dependent on the dose of FGF-2 (Fig. 1).

The effects of other cytokines on the production of HS were then investigated, as production of HS can be modulated by various factors. In contrast to the marked effect of FGF-2, PDGF-BB slightly stimulated and TGF- β weakly suppressed the production of HS by HPDL cells, and the other cytokines examined (EGF, HGF, and IGF-1) did not alter HS production (Fig. 2). However, minor effects of PDGF-BB and TGF- β were not statistically significant.

HS synthesis is extended with *N*-acetyl-glucosamine (GlcNAc) from the four initial monosaccharides, and the growing glycosaminoglycan chains are modified by epimerization, deacetylation/*N*-sulfation, and *O*-sulfation (Prydz and Dahlen, 2000). Thus, we used semi-quantitative RT-PCR to examine the gene expressions of enzymes involved in the biosynthesis of HS 4, 12, 24 and 48 h after FGF-2 stimulation. However, we found that gene expressions of *N*-acetylglucosaminyltransferase (GlcNAcT)-1, heparan sulfate 2-*O*-sulfotransferase (HS2ST), *N*-deacetylase/*N*-sulfotransferase (NDST)-1, NDST-2, epimerase,

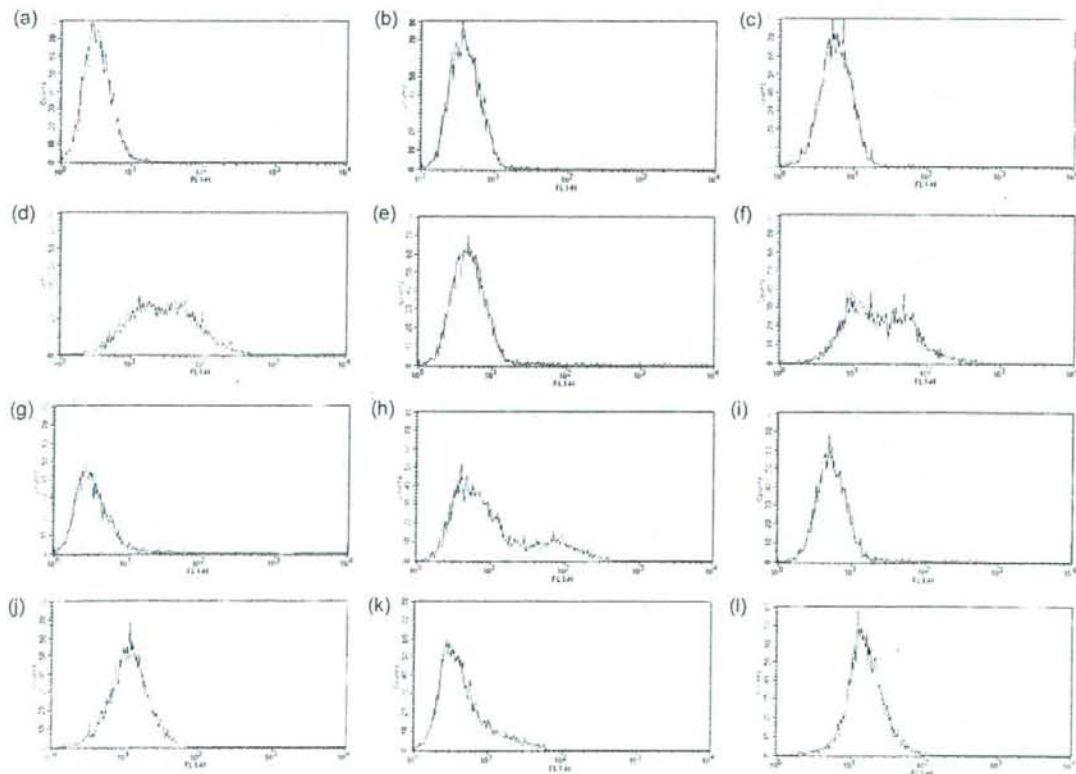


Fig. 5. FACS analysis of syndecan expression on HPDL cells. HPDL cells were incubated in 10% FCS-rMEM in the absence (gray line) or presence of FGF-2 (50 ng/ml) (black line) for 24 h (a, d, g, j), 48 h (b, e, h, k) or 72 h (c, f, i, l). At the time of the assay, cells were incubated without (a, b, c) or with the following primary antibodies: mouse anti-human syndecan-1 antibody (d, e, f); goat anti-human syndecan-2 antibody (g, h, i); mouse anti-syndecan-4 antibody (j, k, l). The cells were washed three times with PBS and incubated for 30 min at 37 °C with or without a biotinylated polyclonal anti-mouse or anti-goat antibody. After washing with PBS, staining was achieved with streptavidin-FITC.

and HPRT were not altered in FGF-2-stimulated HPDL cells at any time points examined (Fig. 3).

3.2. FGF-2 modulated syndecan expression by HPDL cells

Syndecan proteoglycan is the principal source of cell surface HS. To study the effects of FGF-2 on mRNA expression of the syndecan family in HPDL cells, RT-PCR was performed. In unstimulated HPDL cells, the mRNAs of syndecan-1 and -2 were weakly expressed and that of syndecan-4 was moderately expressed; syndecan-3 mRNA was not detected. When the HPDL cells were stimulated with FGF-2 for 24 and 48 h, the gene expression of syndecan-1, -2, and -4 was suppressed (Fig. 4).

HS proteoglycans on the cell surface are known to be shed from the cell surface into culture medium by proteolytic enzymes (Bemfield et al., 1999; Hooper et al., 1997). Next, we used FACS and immunocytochemical analyses to examine whether surface expression of HS proteoglycans on HPDL cells could be altered by FGF-2 treatment. Since syndecan-3 mRNA was not detected in either unstimulated or FGF-2-stimulated HPDL cells, we examined the expression of syndecan-1, -2, and -4 on HPDL cells. As shown in Fig. 5 FACS analysis revealed that FGF-2 lowered the expressions of syndecan-2 and -4, after 48 h and 72 h stimulation with FGF-2 and did not alter the expression of syndecan-1 on the surface of HPDL cells (Fig. 5) at any time point examined.

All three HPDL cell lines showed the decreased syndecan-2 and -4 expression in response to FGF-2 although the extent of these syndecan expression was slightly different among the cell lines. In order to confirm these alterations in HS expression on FGF-2-stimulated HPDL cells, immunocytochemical experiments were also performed. Consistent with the results of FACS

experiments, expression of syndecan-2 and -4 on HPDL decreased after FGF-2 stimulation (data not shown).

Using dot blot analysis, we further examined the role of shedded syndecan in the enhanced secretion of HS in the culture supernatant of FGF-2-stimulated HPDL cells. The amounts of syndecan-1 and -2 detected in the culture supernatants of unstimulated HPDL cells were changed during the culture period. Medium from HPDL cells stimulated with FGF-2 showed no change in the amounts of syndecan-1 and -2. However, a significant increase in the amount of syndecan-4 was observed (Fig. 6). Although glypican-1, -2, -3, -6 and perlecan were also investigated, these HS proteoglycans were not detectable and no differences were observed between FGF-2-stimulated and unstimulated HPDL (data not shown).

4. Discussion

HS proteoglycan, which is composed of a core protein and HS chains, is prevalent on the cell surface and basement membrane, and has been shown to regulate various cell behaviors. It mediates signaling via interaction with matrix molecules, growth factors, or matrix metalloproteinases (MMPs). The syndecan family of four transmembrane proteoglycans is a main source of HS at cell surfaces. In this study, FGF-2 enhanced the concentration of HS in the culture medium of HPDL cells, and differentially regulated the expression of syndecan family members on the cell surface (Fig. 5). Of particular note is the fact that the level of HS in conditioned medium of FGF-2-stimulated HPDL cells was elevated in the presence of specific regulation of syndecan family members (Fig. 6). These observations suggest that individual syndecan family members may play distinct roles in response to FGF-2 (Kim et al., 1994; Lories et al., 1997).

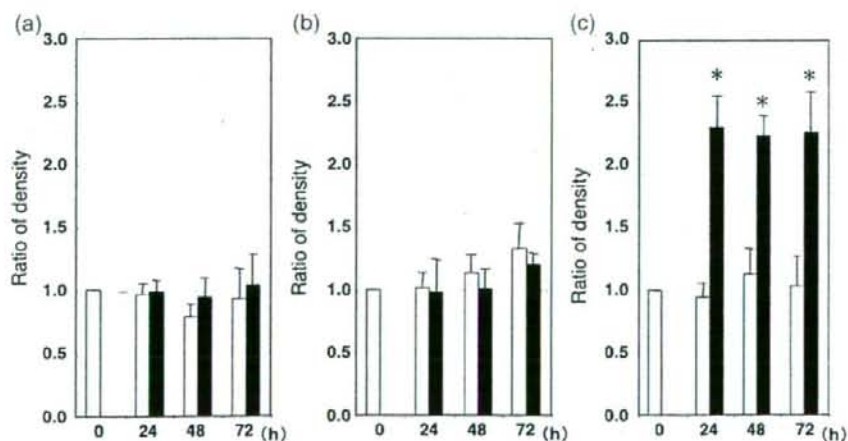


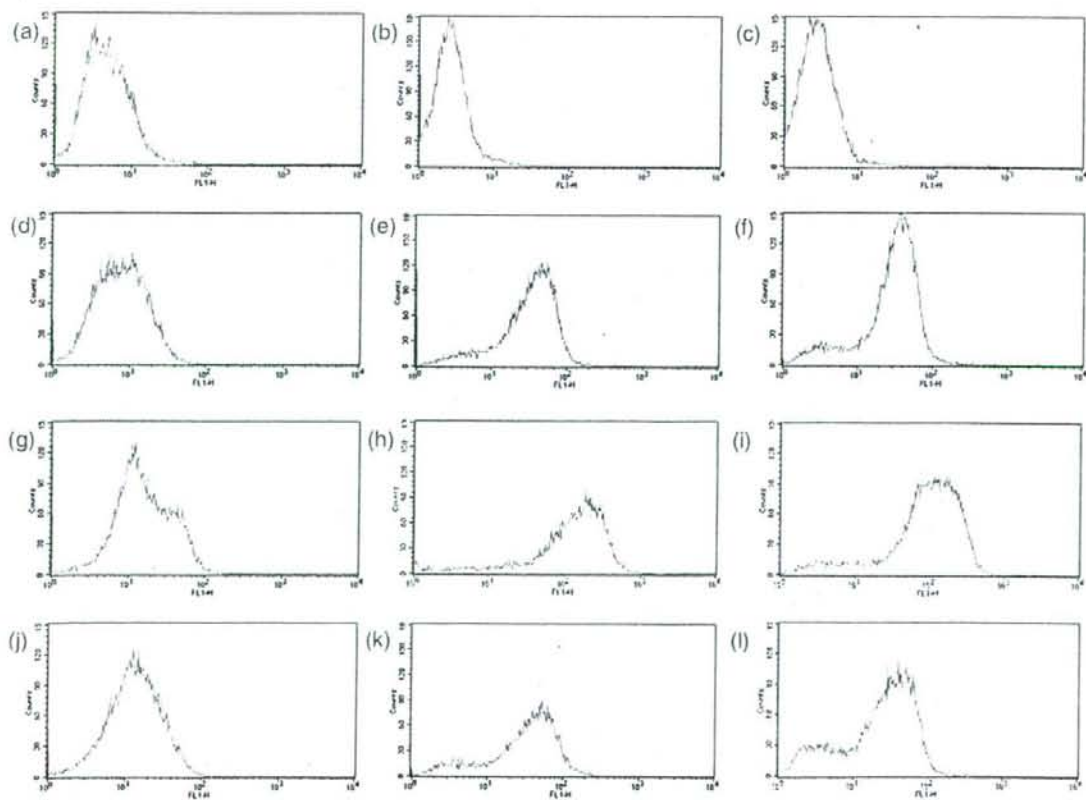
Fig. 6. Dot blot analysis of shedded syndecan in the culture supernatant of HPDL cells. Medium from FGF-2 (50 ng/ml)-stimulated (closed column) or unstimulated (open column) HPDL cells was collected after 24, 48, or 72 h. Nitrocellulose membrane was spotted with each sample, and blocked with 10% BSA to minimize non-specific binding. The membrane was incubated with HRP-conjugated rabbit anti-mouse serum for 1 h following overnight treatment with a mouse monoclonal antibody to syndecan-1 (a), -2 (b), or -4 (c). Immunoreactive bands were visualized by a chemiluminescent reaction. Data were expressed as a ratio of densitometric units relative to the value at hour 0. The experiments were performed by using these different HPDL cell lines. Mean ratios and standard deviations of the data obtained from all these cell lines are expressed in this figure ($p < 0.05$ compared to unstimulated control).

HS, a highly-sulfated glycosaminoglycan, is synthesized via multiple processes during which a series of enzymes is involved in the polymerization and modification of the HS chain. The expression of these enzymes has been reported to be regulated by several factors (Clasper et al., 1999; Moreira et al., 2003). However, FGF-2 did not activate the gene expression of the HS biosynthetic enzymes examined in this study (Fig. 3). In addition, the gene expressions of syndecan (Fig. 4) and glypican (data not shown), both of which are major HS proteoglycans on the HPDL cell surface, were not increased. These results suggest that up-regulation of the biosynthesis of HS proteoglycan does not play a major role in the elevation of the level of HS in FGF-2-stimulated HPDL cell culture medium. However, the fact that no changes in the above-mentioned genes were observed may be a timing issue. Thus, the timing may be one aspects that needs further studies.

Membrane-bound proteins such as CD43, CD44, tumor necrosis factor- α receptor, IL-6 receptor, and the syndecans are cleaved by proteolytic enzymes, sheddase or secretase, and subsequently function as soluble factors (Hooper et al., 1997). The syndecans and glypican are major cell surface proteoglycans and can be shed by proteolytic cleavage of their core proteins (David et al., 1990; Kato et al., 1998). In this study, FACS analysis revealed FGF-2-induced reduction of syndecan-2 and -4 expression on the HPDL cell surface (Fig. 5). Furthermore, dot blot

analysis demonstrated an increased level of syndecan-4 in conditioned medium of FGF-2-activated HPDL cells (Fig. 6). These results suggest that FGF-2 treatment results in the loss of cell surface syndecan-4, with a concomitant increase in the level of syndecan-4 in the conditioned medium. Therefore, the current findings support the hypothesis that syndecan-4 is shed from FGF-2-activated HPDL cell surfaces. However, the mechanism of the release of each syndecan from the cell surface seems to differ with the individual syndecan family member, as the expression of each syndecan was altered differently.

Shedding of syndecan is accelerated by various factors via several intracellular signaling molecules, including extracellular signal-regulated kinase and protein kinase C, and involves the proteolytic activity responsible for cleavage of syndecan ectodomain which is regulated by MMPs and tissue inhibitors of metalloproteinases (TIMPs) (Endo et al., 2003; Fitzgerald et al., 2000; Subramanian et al., 1997). In fact, it has been reported that FGF-2 modulates the activities of some MMPs and TIMPs (Liu et al., 2002; Pintucci et al., 2003; Yasui et al., 2004). Interestingly, TIMP-3 has been reported to inhibit shedding of syndecan-1 and -4 ectodomain (Fitzgerald et al., 2000), and MMP-7 has been reported to be associated with syndecan shedding (Li et al., 2002). However, exogenous addition of these molecules to the culture medium did not affect the release of HS



Appendix data 1

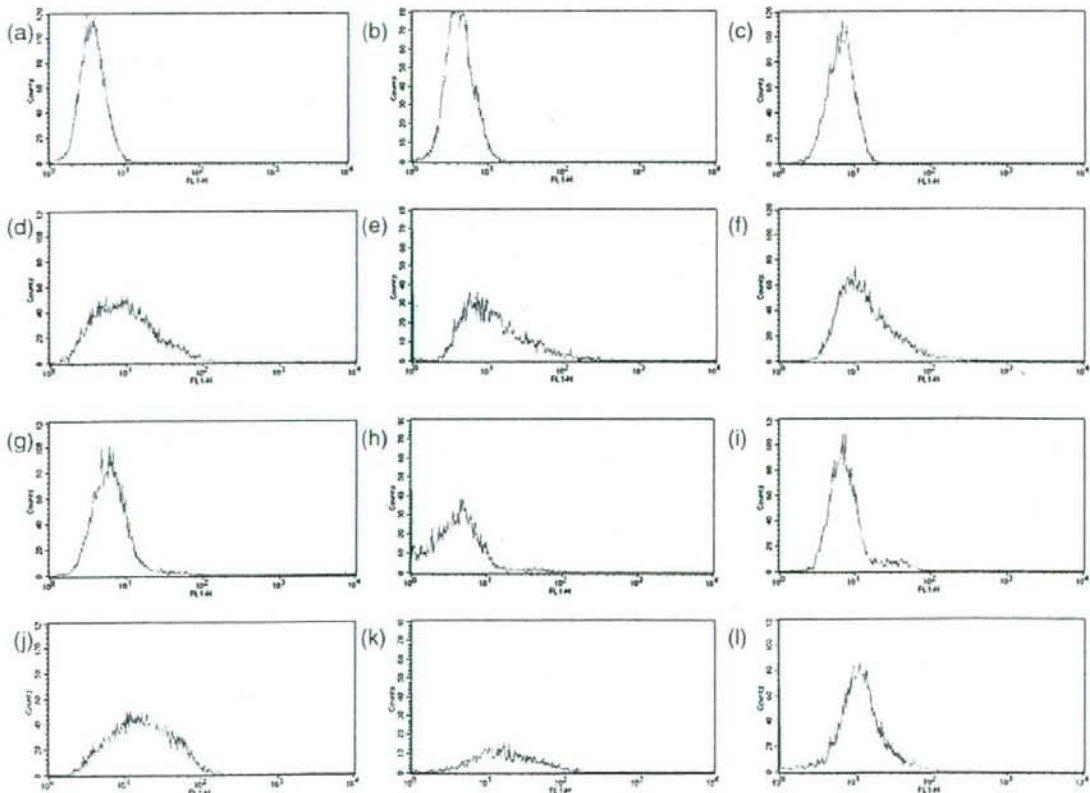
from HPDL cells (data not shown). Further investigation is needed to clarify the mechanism by which cleavage of syndecan family members, particularly syndecan-4, occurs.

Whereas an increased level of syndecan-4 in conditioned medium of FGF-2-activated HPDL cells and a suppression of syndecan-4 expression on FGF-2-stimulated HPDL cells were observed, FGF-2 did not change the level of syndecan-2 in conditioned medium, but decreased its surface expression. HS proteoglycans on the cell surface are constitutively internalized (Yanagishita and Hascall, 1984), and FGF-2 is also internalized by both HS proteoglycan and FGF receptor (Reiland and Rapraeger, 1993; Roghani and Moscatelli, 1992). Thus, surface syndecan-2 on HPDL cells may be internalized from the cell surface following treatment of HPDL cells with FGF-2.

Glypicans are HS proteoglycans which are anchored to cell membrane via a glycosyl phosphatidyl inositol linkage. They comprise a family of six genes in mammals. Perlecan is also one of HS proteoglycan, known to be an important component of basement membrane like collagen type IV and laminin and takes part in wound healing and angiogenesis. As both HS proteoglycan are known to be cleaved from cell membrane and secreted (Bernfield et al., 1999; Haeker et al., 2005), we examined

their expression in the culture supernatants by dot blot analysis. However, increased expressions of glypican-1, -2, -3, -6 and perlecan were not detected in the culture supernatants of FGF-2-stimulated HPDL. Thus, it is unlikely that increase of HS in the culture supernatants of FGF-2 stimulated HPDL can be explained by shedding of those glypicans and perlecan.

It has been reported that syndecan expression was elevated at the wound edge and in lesions surrounding injured tissue, and was transiently decreased in cells migrating into the wound area (Elenius et al., 1991; Gallo et al., 1996; Iseki et al., 2002). Impaired wound healing and angiogenesis in mice lacking syndecan-4 (Fechter et al., 2001), and delayed migration of corneal epithelial cells in mice lacking syndecan-1 (Stepp et al., 2002) have been observed. Over-expression of syndecan-1 and prolonged shedding is associated with delayed wound healing (Elenius et al., 2004), and reduced proliferation rate at the wound edge was also noted. Moreover, a recent study showed that cleavage of syndecan-1 was involved in cell migration (Endo et al., 2003). Also, it has been demonstrated that soluble syndecan ectodomain promotes cell growth (Yang et al., 2002), and that syndecan ectodomains accumulate in wound fluid (Subramanian et al., 1997). These findings suggest that,



Appendix data 2.

not only the cell surface, but also the shed syndecans are closely associated with wound healing. Therefore, it is possible that FGF-2-induced shedding and accumulation of HS proteoglycan are associated with the effects of FGF-2 on acceleration of wound healing and subsequent tissue regeneration.

A close relationship between the actions of HS and FGF has also been reported (Rapraeger et al., 1991; Yayon et al., 1991). Shed proteoglycan can bind HS-binding proteins such as FGF-2 and modulate the functional effects via regulation of binding activity to receptors (Kato et al., 1998). Whether exogenous HS proteoglycans prevent or activate FGF-2 binding to FGF receptors (Bernfield et al., 1999; Kato et al., 1998; Mali et al., 1993; Modrowski et al., 2000) is dependent on the source and composition of the HS proteoglycan. Although the detailed mechanism by which shed HS or HS proteoglycan regulate FGF-2 activity remains elusive, released HS appears to modulate the interaction between FGF-2 and its receptor.

FGF-2 has been recognized to play a critical role in tissue regeneration. Indeed, it is detected at the wound (Crowley et al., 1995; Flaumenhaft et al., 1992; Murakami et al., 1999). It is postulated that in injured tissue, release of FGF-2, which is trapped to HS at the cell surface, is enhanced through proteolytic enzymes (Flaumenhaft et al., 1989). In addition, HS itself is a major constituent of tissue matrices and appears to play modulatory roles in tissue remodeling. Furthermore, we have previously reported that FGF-2 prompted regeneration of periodontal tissue that had been destroyed by the progression of periodontal diseases (Murakami et al., 1999, 2003; Takayama et al., 2001). The present observation that FGF-2 increases the level of HS in the HPDL cell culture supernatant suggests positive or negative feedback regulation during wound healing and regeneration processes in damaged periodontal tissues.

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Appendix A

Appendix data 1 and 2 FACS analysis of syndecan expression on HPDL cells. HPDL cells (the other cells than that in Fig. 5) were incubated in 10% FCS- α MEM in the absence (gray line) or presence of FGF-2 (50 ng/ml) (black line) for 24 h (a, d, g, j), 48 h (b, e, h, k) or 72 h (c, f, i, l). At the time of the assay, cells were incubated without (a, b, e) or with the following primary antibodies: mouse anti-human syndecan-1 antibody (d, e, f); goat anti-human syndecan-2 antibody (g, h, i); mouse anti-syndecan-4 antibody (j, k, l). The cells were washed three times with PBS and incubated for 30 min at 37 °C with or without a

biotinylated polyclonal anti-mouse or anti-goat antibody. After washing with PBS, staining was achieved with streptavidin-FITC.

References

- Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., Zako, M., 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem. Allied Res. India* 68, 729–777.
- Bikfalvi, A., Klein, S., Pintucci, G., Rifkin, D.B., 1997. Biological roles of fibroblast growth factor-2. *Endocr. Rev.* 18, 26–45.
- Clasper, S., Vekkenis, S., Fiore, M., Plevanski, M., Wordsworth, P., David, G., Jackson, D.G., 1999. Inducible expression of the cell surface heparan sulfate proteoglycan syndecan-2 (fibrolycan) on human activated macrophages can regulate fibroblast growth factor action. *J. Biol. Chem.* 274, 24113–24123.
- Crowley, S.T., Ray, C.J., Nawaz, D., Mirajack, R.A., Horwitz, L.D., 1995. Multiple growth factors are released from mechanically injured vascular smooth muscle cells. *Am. J. Physiol.* 269, H1641–H1647.
- David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J.J., Van den Berghe, H., 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.* 111, 3165–3176.
- Echterneyer, T., Streit, M., Wilcox-Adelman, S., Samcella, S., Denhez, F., Detmar, M., Gottrick, P., 2001. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J. Clin. Invest.* 107, R9–R14.
- Elenius, K., Vainio, S., Lantto, M., Salmivirta, M., Thesleff, I., Jalkanen, M., 1991. Induced expression of syndecan in healing wounds. *J. Cell Biol.* 114, 585–595.
- Elenius, K., Paul, S., Allison, G., Sun, J., Klagesbrun, M., 1997. Activation of HLR4 by heparin-binding FGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J.* 16, 1268–1278.
- Elenius, V., Gotte, M., Reizes, O., Elenius, K., Bernfield, M., 2004. Inhibition by the soluble syndecan-1 ectodomains delays wound repair in mice over-expressing syndecan-1. *J. Biol. Chem.* 279, 41928–41935.
- Endo, K., Iikino, T., Miyamoto, H., Kinsen, H., Yoshizaki, T., Furukawa, M., Sato, H., 2003. Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration. *J. Biol. Chem.* 278, 40764–40770.
- Fitzgerald, M.L., Wang, Z., Park, P.W., Murphy, G., Bernfield, M., 2000. Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase. *J. Cell Biol.* 148, 811–824.
- Flaumenhaft, R., Moscatelli, D., Salsela, O., Rifkin, D.B., 1989. Role of extracellular matrix in the action of basic fibroblast growth factor: matrix is a source of growth factor for long-term stimulation of plasminogen activator production and DNA synthesis. *J. Cell. Physiol.* 140, 75–81.
- Flaumenhaft, R., Abe, M., Mignatti, P., Rifkin, D.B., 1992. Basic fibroblast growth factor-induced activation of latent transforming growth factor beta in endothelial cells: regulation of plasminogen activator activity. *J. Cell Biol.* 118, 901–909.
- Gallo, P., Kim, C., Kokenyesi, R., Adzick, N.S., Bernfield, M., 1996. Syndecans-1 and -4 are induced during wound repair of neonatal but not fetal skin. *J. Invest. Dermatol.* 107, 676–683.
- Hacker, U., Nybakken, K., Perrimon, N., 2005. Heparan sulphate proteoglycans: the sweet side of development. *Nat. Rev. Mol. Cell Biol.* 6, 530–541.
- Hooper, N.M., Kauran, F.H., Turner, A.J., 1997. Membrane protein secretases. *Biochem. J.* 321, 265–279.
- Iseki, K., Hagino, S., Mori, T., Zhang, Y., Yokoyama, S., Takaki, H., Tase, C., Mizukawa, M., Watanabe, A., 2002. Increased syndecan expression by pleiotrophin and FGF receptor-expressing astrocytes in injured brain tissue. *Glia* 39, 1–9.
- Ishiguro, K., Tadomatsu, K., Kojima, T., Muramatsu, H., Nakamura, I., Ito, M., Nagasawa, T., Kobayashi, H., Kusumoto, K., Saito, H., Muramatsu, T., 2000. Syndecan-4 deficiency impairs the fetal vessels in the placental labyrinth. *Dev. Dyn.* 219, 539–544.
- Kaimäläinen, V., Wang, H., Schick, C., Bernfield, M., 1998. Syndecans, heparan sulfate proteoglycans, maintain the proteolytic balance of acute wound fluids. *J. Biol. Chem.* 273, 11563–11569.
- Kao, X.L., Wang, H., Kaimäläinen, V., Fitzgerald, M.J., Ledbetter, S., Omori, D.M., Bernfield, M., 1998. Physiological degradation converts the soluble syndecan-

- 1 ectodomain from an inhibitor to a potent activator of FGF-2. *Nat. Med.* 4, 691–697.
- Kim, C.W., Goldberger, O.A., Gallo, R.L., Bernfield, M., 1994. Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns. *Mol. Biol. Cell* 5, 797–805.
- Li, Q., Park, P.W., Wilson, C.I., Parks, W.C., 2002. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transendothelial efflux of neutrophils in acute lung injury. *Cell* 111, 635–646.
- Liu, J.F., Crepin, M., Liu, J.M., Barrault, D., Ledoux, D., 2002. FGF-2 and TPA induce matrix metalloproteinase-9 secretion in MCF-7 cells through PKC activation of the Ras-ERK pathway. *Biochem. Biophys. Res. Commun.* 293, 1174–1182.
- Lories, V., Cassiman, J.J., Van den Berghe, H., David, G., 1992. Differential expression of cell surface heparan sulfate proteoglycans in human mammary epithelial cells and lung fibroblasts. *J. Biol. Chem.* 267, 1116–1122.
- Mali, M., Fleming, K., Miettinen, H.M., Jalkanen, M., 1993. Inhibition of basic fibroblast growth factor-induced growth promotion by overexpression of syndecan-1. *J. Biol. Chem.* 268, 24215–24222.
- Modrowski, D., Basle, M., Lomri, A., Marie, P.J., 2000. Syndecan-2 is involved in the mitogenic activity and signaling of granulocyte-macrophage colony-stimulating factor in osteoblasts. *J. Biol. Chem.* 275, 9178–9185.
- Morcia, C.R., Porcianatto, M.A., Dietrich, C.P., Nader, H.B., 2003. Effect of bradykinin and PMA on the synthesis of proteoglycans during the cell cycle of endothelial cells in culture. *Int. J. Immunopharmacol.* 3, 293–298.
- Murakami, S., Takayama, S., Ikezawa, K., Shimabukuro, Y., Kitamura, M., Nozaki, T., Terashima, A., Asano, T., Okada, H., 1999. Regeneration of periodontal tissues by basic fibroblast growth factor. *J. Periodontol.* 34, 425–430.
- Murakami, S., Takayama, S., Kitamura, M., Shimabukuro, Y., Yarugi, K., Ikezawa, K., Sato, T., Nozaki, T., Okada, H., 2003. Recombinant human basic fibroblast growth factor (bFGF) stimulates periodontal regeneration in class II furcation defects created in beagle dogs. *J. Periodontol.* 38, 97–103.
- Nugent, M.A., Jozzo, R.V., 2000. Fibroblast growth factor-2. *Int. J. Biochem. Cell Biol.* 32, 115–120.
- Park, P.W., Pier, G.B., Preston, M.J., Goldberger, O., Fitzgerald, M.L., Bernfield, M., 2000. Syndecan-1 shedding is enhanced by LISA, a secreted virulence factor of *Pseudomonas aeruginosa*. *J. Biol. Chem.* 275, 3057–3064.
- Pinnac, G., Yu, P.J., Sharony, R., Baumann, F.G., Saponara, F., Frasca, A., Galloway, A.C., Moscatelli, D., Mignatti, P., 2003. Induction of stromelysin-1 (MMP-3) by fibroblast growth factor-2 (FGF-2) in FGF-2 microvascular endothelial cells requires prolonged activation of extracellular signal-regulated kinases-1 and -2 (ERK-1/2). *J. Cell. Biochem.* 90, 1015–1025.
- Prydz, K., Dalen, K.T., 2000. Synthesis and sorting of proteoglycans. *J. Cell Sci.* 113, 193–205.
- Ruppraeger, A.C., Krifka, A., Olwin, B.B., 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* 252, 1705–1708.
- Reiland, J., Ruppraeger, A.C., 1993. Heparan sulfate proteoglycan and FGF receptor target basic FGF to different intracellular destinations. *J. Cell Sci.* 105, 1085–1093.
- Roghani, M., Moscatelli, D., 1992. Basic fibroblast growth factor is internalized through both receptor-mediated and heparan sulfate-mediated mechanisms. *J. Biol. Chem.* 267, 22156–22162.
- Shimabukuro, Y., Ichikawa, T., Takayama, S., Yamada, S., Takedachi, M., Terakura, M., Hashikawa, T., Murakami, S., 2005. Fibroblast growth factor-2 regulates the synthesis of hyaluronin by human periodontal ligament cells. *J. Cell. Physiol.* 203, 557–563.
- Somerman, M.J., Archer, S.Y., Imb, G.R., Foster, R.A., 1988. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J. Dent. Res.* 67, 66–70.
- Stepp, M.A., Gibson, I.F., Gada, P.H., Iglesia, D.D., Pajooesh-Ganji, A., Pal-Ghosh, S., Brown, M., Aquino, C., Schwartz, A.M., Goldberger, O., Hinkes, M.T., Bernfield, M., 2002. Defects in keratinocyte activation during wound healing in the syndecan-1-deficient mouse. *J. Cell Sci.* 115, 4517–4531.
- Subramanian, S.V., Fitzgerald, M.L., Bernfield, M., 1997. Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. *J. Biol. Chem.* 272, 14713–14720.
- Takayama, S., Murakami, S., Shimabukuro, Y., Kitamura, M., Okada, H., 2001. Periodontal regeneration by FGF-2 (bFGF) in primate models. *J. Dent. Res.* 80, 2075–2079.
- Yanagishita, M., Hascall, V.C., 1984. Metabolism of proteoglycans in rat ovarian granulosa cell culture. Multiple intracellular degradative pathways and the effect of chloroquine. *J. Biol. Chem.* 259, 10270–10283.
- Yang, Y., Yacoby, S., Liu, W., Langford, J.K., Pumphrey, C.Y., Thetis, A., Epstein, J., Sanderson, R.D., 2002. Soluble syndecan-1 promotes growth of myeloma tumors in vivo. *Blood* 100, 610–617.
- Yasai, H., Andoh, A., Bamba, S., Inatomi, O., Ishida, H., Fujiyama, Y., 2004. Role of fibroblast growth factor-2 in the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human intestinal myofibroblasts. *Digestion* 69, 34–44.
- Yayon, Y., Klagsbrun, M., Esko, J.D., Leder, P., Ornitz, D.M., 1991. Cell surface heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 64, 841–848.

Fibroblast Growth Factor-2 Regulates Expression of Osteopontin in Periodontal Ligament Cells

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Osteopontin is a protein found in the bone-related matrix and plays multiple regulatory roles in mineralizing and non-mineralizing tissue. In osteogenic cell-lines, the expression of osteopontin increases with the progression of differentiation, but both the expression and function of osteopontin vary with the cell type and its activation state. In this study, we examined the expression of osteopontin by clones established from mouse periodontal ligament, in response to inorganic phosphate and fibroblast growth factor (FGF)-2, which can induce periodontal tissue regeneration. The involvement of inorganic phosphate in the expression of osteopontin during the course of cell differentiation of a clone MPDL22 was confirmed by addition of fosfarnet, an inorganic phosphate transport inhibitor. Although FGF-2 decreased the mRNA expression of almost every bone-related protein in MPDL22, FGF-2 upregulated the expression of osteopontin in MPDL22 at both mRNA and protein levels. Interestingly, FGF-2 enhanced the concentration of osteopontin in the culture supernatant of MPDL22, whereas inorganic phosphate did not. The FGF-2-induced osteopontin in the culture supernatant seems to be involved in cell survival activity. An immunohistochemical study showed that the FGF-2-induced osteopontin was mainly present in perinuclear matrices while the inorganic phosphate-induced osteopontin was associated with extracellular matrices in addition to perinuclear matrices. The present results indicated that FGF-2 induces unique expression of osteopontin, which may play a role different from the other bone-related proteins during the process of periodontal tissue regeneration by FGF-2.

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Osteopontin is a non-collagenous phosphoprotein found in bone extracellular matrix and is known as an osteogenesis marker. However, several lines of reports have revealed that its roles for the regulation of matrix mineralization and bone cell function is controversial although the detailed mechanism regulating the bioactivity of osteopontin in calcified tissues has not been fully elucidated. In addition to those functions as a calcified tissue-related molecule, broad biological functions of osteopontin have also been demonstrated in association with tumor biology (Senger et al., 1983), tissue injury and repair (Giachelli et al., 1994; Liaw et al., 1998), dystrophic calcification (Srivatsa et al., 1997), and inflammation (Patarca et al., 1989; Nau et al., 1997). In fact, osteopontin is synthesized by a variety of cell types, such as epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, immune cells like T cells, NK cells, macrophages in addition to osteoblasts and osteoclasts (O'Regan and Berman, 2000; Denhardt et al., 2001b).

Periodontal tissue, which is a tooth-supporting apparatus, consists of gingiva, alveolar bone, cementum and periodontal ligament (PDL). PDL plays an important role as a reservoir of mesenchymal stem cells (Seo et al., 2004). Thus, the cells in PDL tissue are critical participants during the tissue remodeling and regeneration process when breakdown of periodontal tissue caused by bacterial biofilm or mechanical stress is healed. We found that topical application of fibroblast growth factor (FGF)-2 to experimentally created alveolar bone defects prompted significant periodontal tissue regeneration accompanying the formation of new bone and cementum (Murakami et al., 1999, 2003; Takayama et al., 2001). *In vitro* studies revealed that FGF-2 can promote strong growth of PDL cells, and regulate the production of extracellular matrix, which play important roles for desirable periodontal tissue regeneration (Takayama et al., 1997; Shimabukuro et al., 2005). Interestingly, we recently found that FGF-2 suppressed the

expression of bone-related proteins, such as type I collagen, osteocalcin and bone sialoprotein, but prompted the expression of osteopontin by PDL cells.

In this study, utilizing PDL clones obtained from mouse PDL (MPDL) tissues (Yamada et al., 2007), we investigated the expression of osteopontin by PDL cells in detail. In particular, we focused on FGF-2-dependent expression of osteopontin in comparison with inorganic phosphate, which facilitates osteogenic differentiation, and examined its possible biological roles.

Materials and Methods

Materials

Tissue culture supplies and plastic Leighton tubes were obtained from Corning Coster (New York, NY). Alpha Modification of

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Eagles Medium (α -MEM) was a product of ICN Biomedicals, Inc. (Costa Mesa, CA). Bovine calf serum (FCS) was purchased from JRH Biosciences (Lenexa, KS). Bovine serum albumin and β -glycerophosphate (BGP) and bisbenzimidazole (Hoechst 33258) were products of Sigma (St. Louis, MO). Human recombinant FGF-2 was obtained from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Goat anti-mouse osteopontin antibody and goat anti- β -actin antibody was obtained from R & D Systems, Inc. (Minneapolis, MN).

Isolation of mouse periodontal cell clones

MPDL cell clones were isolated as previously described (Yamada et al., 2007). Cell cloning was done twice using the limiting dilution method in α -MEM supplemented with 10% FCS and 100 ng/ml FGF-2. Twenty-nine MPDL clonal cell lines were obtained and classified by alkaline phosphatase (ALPase) activity. We selected two of these clones: one clone, MPDL22 was characterized by a high level of alkaline phosphatase (ALPase) and the other clone, MPDL6 exhibit low level in ALPase even in the presence of BGP and ascorbic acid. MPDL22 cells were positive for collagen type I, Runx2, Mx2, Dix5, and Osterix mRNA (Yamada et al., 2007).

Assay of ALPase activity

ALPase activity was investigated according to the procedure of Bessey and Lowry (1946). The PDL clones were seeded in 24-well culture dishes (1×10^5 cells/well) with standard medium containing 50 μ g/ml ascorbic acid and 10 mM BGP. They were cultured for indicated days in this medium, which was changed every 2 days. After washed twice with saline, the cells were homogenized in a glass homogenizer in 1 ml of 0.9% NaCl, 0.2% Triton X-100 at 0–4°C and centrifuged for 15 min at 12,000g. ALPase activity in the supernatant was determined using *p*-nitrophenyl phosphate (pNP) as substrate. The supernatant was assayed in a 0.5 M Tris/HCl buffer (pH 9.0) containing 0.5 mM pNP and 0.5 mM MgCl₂. The reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by addition of 0.25 volume of 1 N NaOH. Hydrolysis of pNP was monitored as change in A₄₁₀ with a spectrometer (Hitachi Instruments Service Co., Ltd., Tokyo, Japan). *p*-Nitrophenol was used as a standard. One unit of activity was defined as the amount hydrolyzing 1 nmol of *p*-NP in 30 min.

Cellular DNA content

DNA content was measured by a modification of the method of Labarca and Paigen (1980). The PDL clones were seeded in 24-well culture dishes (1×10^5 cells/well) with standard medium containing 50 μ g/ml ascorbic acid and 10 mM BGP. They were cultured for 20 days in this medium, which was changed every 2 day. Alteration

of DNA content of the cells was determined as follows. The PDL clones were washed with PBS and then homogenized at 0–4°C in 1 ml of 2 M NaCl/25 mM Tris-HCl (pH 7.4). After centrifugation at 12,000g for 10 min, 25 μ l of 5 μ g/ml bisbenzimidazole was added to 100 μ l of the supernatant. The fluorescent spectra at emission 458 nm after excitation at 356 nm was monitored by a spectrophotometer. The concentration of DNA in the samples was determined by a standard curve made at various concentrations of calf thymus DNA.

Alizarin red staining

Histochemical staining of calcium was performed by modification of the alizarin red staining method (Dahl, 1952). The cell layers were washed twice with saline and then fixed with dehydrated ethanol. After fixation, the cell layers were stained with 1% alizarin red S in 0.1% NH₄OH (pH 6.5) for 5 min, then washed with H₂O.

Detection of osteopontin mRNA by RT-PCR

MPDL clones were seeded at a density of 1×10^6 cells/dish in a 60 mm dish and grown to confluence in standard medium. Following 24 h activation of MPDL with or without FGF-2 (100 ng/ml), total RNA was isolated from each cell by RNAzol™ (Cinna/Biotech Laboratories, Inc., Friendswood, TX) according to the manufacturer's instructions. The precipitated RNA was resolved in 0.1% diethylpyrocarbonate-treated distilled water (DEPC-treated H₂O). cDNA synthesis and amplification via PCR were performed according to the methods described by Takayama et al. (1997). For PCR analysis, we incubated a 40 ml cDNA synthesis reaction mixture per RNA sample at 37°C for 60 min. Each 40 μ l cDNA synthesis reaction mixture contained: 5.2 μ l of DEPC-treated H₂O; 4 μ l 10 \times PCR buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl; Perkin Elmer Cetus, Norwalk, CT); 6 μ l 25 mM MgCl₂; 4 μ l of each 10 mM deoxynucleotide-triphosphate (Takara Shuzo Co. Ltd., Kyoto, Japan); 0.4 μ l 20 U/ml RNase inhibitor (Perkin Elmer Cetus); 1 ml 50 U/ml M-MLV reverse transcriptase; 4 μ l 0.25 μ g/ml RNA sample. After incubation, all samples were heated to 94°C for 5 min to inactivate the reverse transcriptase.

Oligonucleotide PCR primers specific for type I collagen, *Pebp2 α A/Cbfa1*, *Osf-2/Til-1/Cbfa1*, bone sialo protein, osteonectin, osteopontin, osteocalcin, and β -actin mRNA were synthesized by Clontech Laboratories, Inc. (Palo Alto, CA) (Table 1). The cDNA samples prepared above were amplified by adding to a PCR reaction mixture which included 10 mM Tris-HCl buffer (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.15 mM dNTP mixture, 1.25 U AmpliTaq Gold™ (Perkin Elmer), and 0.2 μ M sense and antisense oligonucleotide primers. The PCR reaction mixture was overlaid with mineral oil (Aldrich Chemical Company,

TABLE 1. Primers utilized for reverse transcription-polymerase chain reaction

Primers	Size (bp)	Sequence
Type I collagen	268	Sense 5'-TCTCCACTCTTCTAGTTCCT-3' Antisense 5'-TTGGGTCATTTCCACATGC-3'
<i>Pebp2αA/Cbfa1</i>	303	Sense 5'-AGGATTTTATCATGGACACG-3' Antisense 5'-TCTTCTCTGTGCGATAGAGT-3'
<i>Osf-2/Til-1/Cbfa1</i>	386	Sense 5'-GAGGGCACAAGTTCTATCTGGA-3' Antisense 5'-GGTGGTCCCGGATGATCTC-3'
Bone sialo protein	430	Sense 5'-ACCGGCCACGCTACTTTCITTT-3' Antisense 5'-GACCCCGAGCTCGTTTTCA-3'
Osteonectin	453	Sense 5'-CGCCCCCTGCTGGATTCCG-3' Antisense 5'-GATCACACAGATCCCTTGTGATGTCCTGC-3'
Osteopontin	485	Sense 5'-CTGCTAGTACACAAGCAGACA-3' Antisense 5'-CATGAGAAATTCGGAATTCAG-3'
Osteocalcin	264	Sense 5'-CTGGCCCTGGCTGCGCTGTG-3' Antisense 5'-GGTCTCTAAATAGTGATACCGTAGATGCG-3'
β -actin	388	Sense 5'-AGCAAGAGAGGTATCCT-3' Antisense 5'-ATGAGGTAGTCTGTCCAGGT-3'

Inc., Milwaukee, WI) and subjected to amplification for two or three different number of cycles using a DNA Thermal cycler 480 (Perkin Elmer). After initial denaturation at 94°C for 4 min, each cycle consisted of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min. PCR products were analyzed by electrophoresis at 100 V for 30 min on 1.5% TAE agarose gel (NIPPON GENE Co., Ltd., Toyama, Japan) containing 0.5 µg/ml ethidium bromide.

Protein extraction from plasma membrane

Cells (1×10^8) were collected in PBS by scraping cells and washed with ice cold PBS. Resuspended cells were homogenized on ice for 30–50 times and centrifuged at 700g for 10 min at 4°C, and then the supernatant was collected and centrifuged at 10,000g for 30 min at 4°C. The supernatant was used as the cytosol fraction. The pellet was resuspended, centrifuged at 1,000g for 5 min and used as plasma membrane protein.

Western blotting analysis

MPDL clones which had been grown to subconfluence in standard medium, were cultured without FCS for 48 h, and then incubated in the presence or absence of FGF-2 or inorganic phosphate. At the end of the culture, cells were harvested in lysis buffer (150 mM NaCl, 1% Triton-X-100, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM sodium pyrophosphate, 10 mg of aprotinin/ml, and 10 mg of leupeptin/ml) and centrifuged at 10,000g. Protein was resolved on SDS-PAGE and transferred onto nitrocellulose membrane. Filters were incubated with 10% bovine serum albumin for 1 h and subsequently with goat anti-osteopontin antibody (R & D Systems, Inc.) or mouse monoclonal anti-β-actin antibody (mouse IgG 1 isotype) (Sigma) for 3 h at room temperature. Immune complex were detected using an enhanced chemiluminescence kit (ECL, Amersham, Little Chalfont, England).

Histochemical analysis

MPDL clones were cultured onto a coverglass (Matsunami Co., Osaka, Japan) mounted on a 35-mm plate. Cells were fixed for 10 min in 2% paraformaldehyde and either treated with PBS including with 0.1% Triton X to permeabilize cell membrane or not. After fixation, the cell monolayer was washed in PBS, incubated with goat anti-osteopontin antibody, rabbit anti-type I collagen antibody (R & D Systems, Inc.), rabbit anti-fibronectin antibody (Chemicon International, Temecula, CA) at 50 µg/ml in PBS for 5 h or overnight at room temperature, and then washed in PBS three times, 30 min each time. Then, cells were incubated with Alexa Fluor 488 conjugated donkey anti-goat antibody (Molecular Probes, Engle, OK) or Alexa Fluor 546 goat anti-rabbit IgG. Immunofluorescence was measured by confocal laser scanning microscopy (Olympus Co., Tokyo, Japan). Reagent controls consisted of cell monolayer processed as above but incubated with PBS.

Determination of osteopontin in culture supernatants

MPDL clones were placed in a 24 well plate (Corning Coster) at 1×10^4 cells in complete media. Following serum starvation for 48 h, MPDL clones were stimulated with or without FGF-2 (100 ng/ml) or inorganic phosphate (10 mM). At the end of the incubation period, the supernatants were collected and stored at -20°C until determination of osteopontin level. The osteopontin levels in culture supernatants were measured by using an enzyme-linked immunosorbent assay (ELISA) kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) according to the manufacturer's instructions.

Cell survival assay

Apoptosis was detected using a cell death detection ELISA^{plus} kit following the instruction manual. MPDL22 was treated with 2 ng/ml of camptothecin for 6 h at 37°C to induce apoptosis and centrifuged (200g). The cells were lysed with lysis buffer and the

lysate was centrifuged (200g) for 10 min. A 20 µl aliquot from the supernatant was transferred into the streptavidin-coated 96-well plate. Anti-histone-biotin and anti-DNA-peroxidase were added to each well, incubated for 2 h at room temperature, and then rinsed with incubation buffer. Color was developed with ABTS solution and absorbance was measured at 405 nm using a microplate reader (Model 550, Bio-Rad Laboratory, Hercules, CA). Quantitative determination of the amount of nucleosomes was calculated from the standard.

Results

MPDL22 exhibited expression of osteopontin

We established 29 clones from MPDL tissues. They were composed of heterogeneous phenotypes in terms of ALPase activity. One clone, MPDL22, showed cell differentiation in the presence of ascorbic acid and BGP and formed mineralized nodules in the presence of a high level of ALPase *in vitro* (Fig. 1). Another clone, MPDL6, showed impaired differentiation ability in the presence of a low level of alkaline phosphatase. Using these clones, we investigated the expression of osteopontin, an early differentiation marker of osteoblasts. Western blot analysis indicated that expression of osteopontin was elevated with a peak expression on day 14 and decreased afterward when MPDL22 was cultured in the presence of ascorbic acid and BGP (Fig. 2a). Consistent with the results of western blot analysis, immunohistochemical analysis revealed the increased expression of osteopontin by MPDL22 at day 12 (Fig. 2b). Compared with MPDL22, MPDL6 exhibited only a marginal increase of expression of osteopontin in both western blot and immunohistochemical analysis (Fig. 2a,b). β-actin expression which was used as an internal control did not altered between MPDL22 and MPDL6.

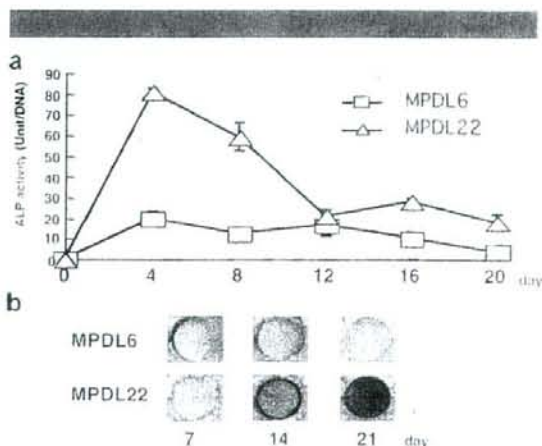


Fig. 1. Kinetics of ALPase activity and mineral deposition by MPDL6 and MPDL22 during cell differentiation. MPDL6 or MPDL22 was cultured in the presence of ascorbic acid (50 µg/ml) and BGP (10 mM) for indicated days. **a:** Cells were homogenized and centrifuged. ALPase activity in the supernatant was measured using pNP. Hydrolysis of pNP was monitored as a change in A₄₁₀ with a spectrometer. **b:** Cell monolayers were fixed with dehydrated ethanol and stained with 1% alizarin red S in 0.1% NH₄OH for 5 min. Results of one representative experiment out of three separate experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

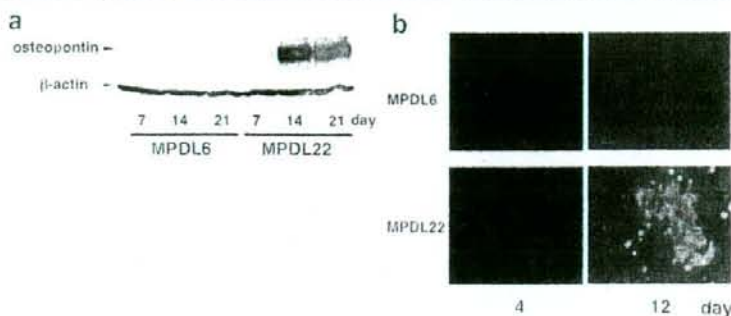


Fig. 2. Expression of osteopontin by MPDL6 and MPDL22 during cell differentiation. **a:** MPDL6 and MPDL22 were cultured in the presence of 50 $\mu\text{g/ml}$ ascorbic acid and 10 mM BGP for 7, 14, or 21 days. Samples were electrophoresed on 8% mini-gels, transblotted onto nitrocellulose membrane. Immunoreactive osteopontin was detected using a primary goat anti-osteopontin polyclonal antibody and chemiluminescence kit. Immunoreactive β -actin was also detected as an internal control. **b:** MPDL6 and MPDL22 were cultured in the presence of 50 $\mu\text{g/ml}$ ascorbic acid and 10 mM BGP for 4 or 12 days. Cell monolayers were fixed with 2% paraformaldehyde and incubated with or without goat anti-osteopontin polyclonal antibody after incubation with Alexa Fluor 488 conjugated anti-goat IgG antibody. Results of one representative experiment out of three separate experiments are shown.

Inorganic phosphate contributes to mineralization of MPDL22 and prompts its expression of osteopontin

During *in vitro* differentiation of osteoblasts, levels of ALPase activity and extracellular inorganic phosphate are increased (Bellows et al., 1992; Chung et al., 1992). Recently, inorganic phosphate has been found to regulate the *in vitro* differentiation of osteoblasts with elevation of bone-related gene and

mineralization through a inorganic phosphate transport system as a signaling molecule (Beck et al., 2003). The action of inorganic phosphate during the differentiation of PDL cells was confirmed by directly examining the effects of inorganic phosphate on mineralization by MPDL22. Inorganic phosphate elicited mineralized nodule formation by MPDL22 at day 7 as in the long-term culture with ascorbic acid and BGP (Fig. 3a). Inorganic phosphate was confirmed to be involved in the inorganic phosphate transport system by treatment with foscarnet, an inhibitor of inorganic phosphate transport. As shown in Figure 3b, the mineralized nodule formation of MPDL22 was blocked by foscarnet, suggesting the necessity of transportation of inorganic phosphate for biomineralization by MPDL22.

Then, we investigated whether inorganic phosphate regulates the expression of osteopontin in MPDL22 and MPDL6. Western blot analysis revealed that treatment of MPDL22 with inorganic phosphate resulted in the enhancement of expression of osteopontin in a dose-dependent manner (Fig. 4a) and that the inorganic phosphate-induced expression of osteopontin was also inhibited with foscarnet (Fig. 4b). In contrast to MPDL22, inorganic phosphate-treated MPDL6 exhibited low expression of osteopontin. A histochemical study also demonstrated abrogation of the elevation of inorganic phosphate-dependent osteopontin in MPDL6 and MPDL22 by foscarnet (Fig. 4c). Thus, inorganic phosphate upregulated the expression of osteopontin in association with the mineralization process by MPDL22 *in vitro*.

FGF-2 promotes osteopontin and its gene expression in MPDL22

We then examined the effect of FGF-2 on mRNA expression of bone-related molecules in MPDL22. While FGF-2 suppressed mRNA expression of bone-related molecules such as type I collagen, *Pebp2 α /Cbfa1*, *Osf-2/Ti1-Cbfa-1*, bone sialoprotein, osteonectin, and osteocalcin, expression of osteopontin was notably elevated in FGF-2-stimulated MPDL22 at both mRNA and protein levels in a dose-dependent manner (Figs. 5 and 6a,c). In contrast, MPDL6 exhibited relatively weak or little expression of osteopontin (Fig. 6b,d). Interestingly, western blot analysis revealed two osteopontin proteins showing slightly different molecular sizes (Fig. 6c,d), which probably suggests the stimulation-dependent posttranslational modification of osteopontin (Denhardt et al., 2001a) since

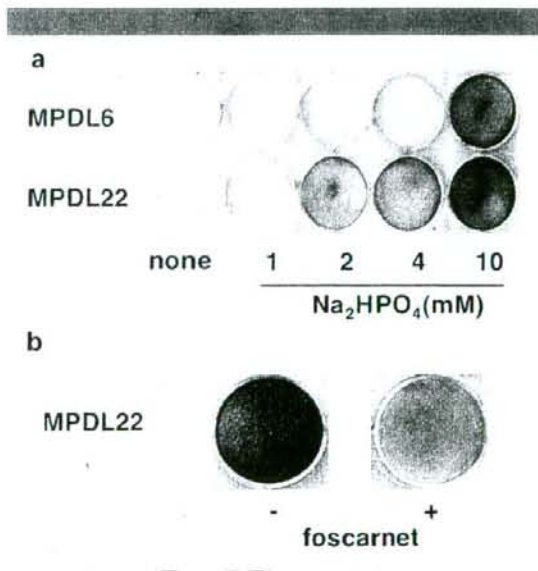


Fig. 3. Involvement of inorganic phosphate on mineralization by MPDL6 and MPDL22. MPDL6 and MPDL22 were cultured for 7 days in the presence or absence of Na_2HPO_4 (1–10 mM) (a) or with or without foscarnet (600 μM) in the presence of Na_2HPO_4 (10 mM) (b). MPDL22 were cultured for 21 days with or without foscarnet (600 μM) in the presence of Na_2HPO_4 (10 mM). Mineralized nodules of the cell monolayers were detected with Arizarin red staining as described in "Materials and Methods Section." Results of one representative experiment out of three separate experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

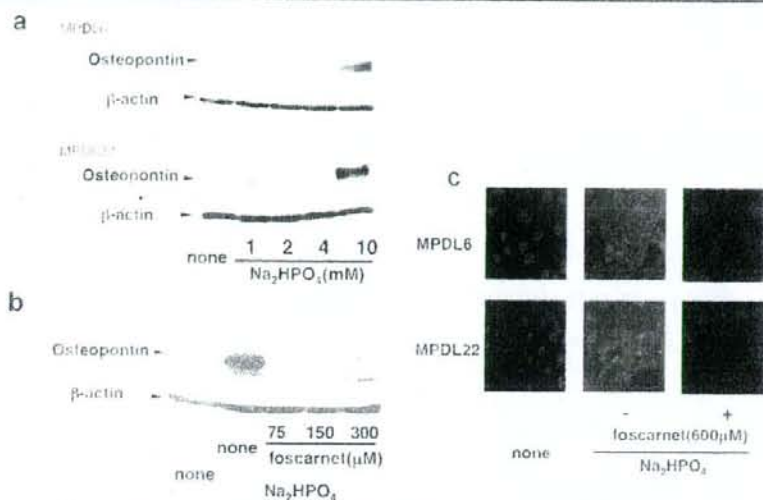


Fig. 4. Involvement of inorganic phosphate in expression of osteopontin by MPDL6 and MPDL22. MPDL6 or MPDL22 was cultured for 48 h in the presence or absence of Na_2HPO_4 (1–10 mM) (a) or with or without foscarnet at indicated concentration in the presence of Na_2HPO_4 (10 mM) (b,c). Samples were electrophoresed on 8% mini-gels, transblotted onto nitrocellulose membrane and immunoreactive osteopontin was detected using a primary goat anti-osteopontin antibody and chemiluminescence kit. β -actin was used as an internal control (a,b). Cell monolayers were fixed with 2% paraformaldehyde and incubated with or without anti-osteopontin polyclonal antibody following Alexa Fluor 488 conjugated anti-goat IgG antibody (c). Results of one representative experiment out of three separate experiments are shown.

alternative splicing of osteopontin in mice has not been reported.

FGF-2 but not inorganic phosphate upregulated osteopontin secretion in culture supernatants

Osteopontin functions differently at different sites of localization. Extracellular osteopontin in soluble form plays an

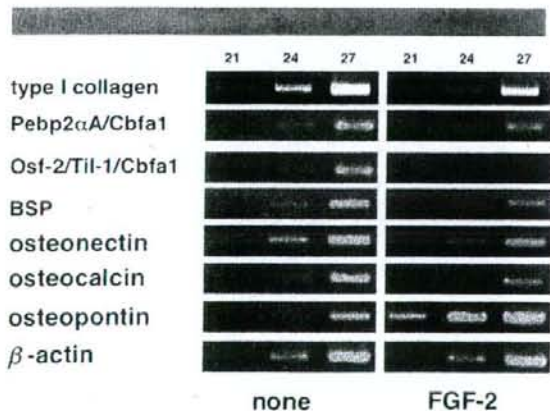


Fig. 5. Effects of FGF-2 on bone-related proteins by MPDL22. MPDL22 was cultured without or with FGF-2 (100 ng/ml) for 24 h. Total mRNA was isolated and RT-PCR was performed to examine the mRNA expression of type I collagen, *Pebp2 α A/Cbfa1*, *Osf-2/Til-1/Cbfa1*, bone sialoprotein (BSP), osteonectin, osteocalcin, osteopontin, and β -actin in MPDL22. Results of one representative experiment out of three separate experiments are shown. The numbers of PCR cycles used to amplify each mRNA in MPDL22 are shown above each lane.

important role in cell survival (Rogers et al., 1997; Scatena et al., 1998; Ophascharoensuk et al., 1999; Denhardt et al., 2001a; Lin and Yang-Yen, 2001; Rittling et al., 2002), chemotaxis and migration (Senger et al., 1996; Li et al., 2000) and cell proliferation and invasion (Castellone et al., 2004) via cell surface receptors. Thus, we quantitated the production of osteopontin in culture supernatants of FGF-2- and inorganic phosphate-activated MPDL22. Treatment of MPDL22 with FGF-2 for 48 h markedly enhanced osteopontin secretion in the culture supernatant (Fig. 7). Consistent with the data of MPDL6 shown previously, exposure of MPDL6 to FGF-2 exhibited a slight elevation of osteopontin in the culture supernatant. Interestingly, however, inorganic phosphate rather inhibited the osteopontin production in culture supernatant of either MPDL6 or MPDL22 (Fig. 7). Taken together, these results suggest that osteopontin induced by FGF-2 was localized in a manner different from that induced by inorganic phosphate.

FGF-2 evoked expression of osteopontin in the cytosol, but not on the cell surface of MPDL

FGF-2, which reversibly suppressed cell differentiation into hard tissue, stimulated MPDL22 to secrete osteopontin. The localization of FGF-2-induced osteopontin in MPDL was determined by histochemical analysis. Unexpectedly, no staining was detectable on the cell surface of both MPDL6 and MPDL22 stimulated with FGF-2, while inorganic phosphate vigorously activated expression of osteopontin on the cell surface (Fig. 8). Recently, osteopontin has been reported to be present not only in the extracellular matrix but also in perinuclear cytosol (Zohar et al., 1997). Thus, in order to detect the intracellular localization of osteopontin, permeabilized cells were stained with anti-osteopontin antibody. Forty-eight hours after FGF-2 stimulation, weak punctuate immunostaining pattern was detected in perinuclear cytoplasm of the MPDL22 (Fig. 9). In the permeabilized-cell experiment, MPDL6 expressed osteopontin at a lower level

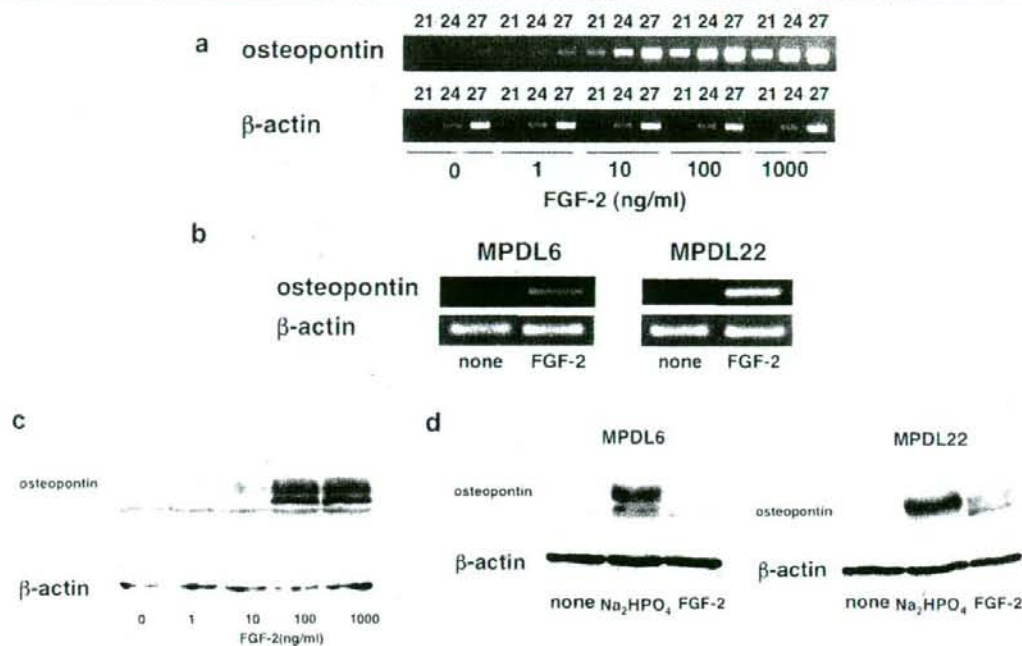


Fig. 6. Effects of FGF-2 on expression of osteopontin by MPDL6 and MPDL22. MPDL22 was cultured without or with FGF-2 (1–1,000 ng/ml) (a,c). In another experiment, MPDL6 and MPDL22 were cultured without or with FGF-2 (100 ng/ml) (b,d) or with Na_2HPO_4 (10 mM) (d). After 24 h of incubation, total mRNA was isolated from each cell and RT-PCR was performed to examine the mRNA expression of osteopontin, β -actin in MPDL6 (b) and MPDL22 (a,b). The numbers of PCR cycles used to amplify each mRNA in the MPDL cells are shown above each lane. Results of one representative experiment out of three separate experiments are shown. After 48 h of culture, protein was extracted as described in "Materials and Methods Section." Samples were electrophoresed on 8% mini-gels, transblotted onto nitrocellulose membrane and immunoreactive osteopontin was detected using a primary goat anti-osteopontin antibody and chemiluminescence kit (c,d). β -actin was used as an internal control (c,d). Results of one representative experiment out of three separate experiments are shown.

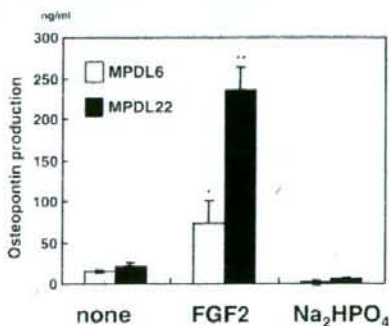


Fig. 7. Osteopontin production in culture supernatant of MPDL6 and MPDL22 in response to FGF-2 or inorganic phosphate stimulation. MPDL6 and MPDL22 were cultured as described in "Materials and Methods Section." Culture supernatants were removed after 48 h of culture and the concentration of osteopontin in the conditioned medium was determined by an ELISA assay as described in "Materials and Methods Section" ($^*P < 0.05$ and $^{**}P < 0.01$ compared to unstimulated control). Results are expressed as the mean \pm SD of three separate experiments.

than that of MPDL22. As in the case of MPDL22, without permeabilization, no staining was observed on FGF-2-stimulated MPDL6. In contrast, inorganic phosphate induced strong expression of osteopontin on non-permeabilized and permeabilized MPDL6 and MPDL22.

The localization of osteopontin in cytosol- or membrane-associated fraction of MPDL22 in response to FGF-2 or inorganic phosphate was further examined by western blot analysis. The biochemical analysis revealed that FGF-2 increased the expression of osteopontin in the cytosol but not in the membrane-associated fraction whereas inorganic phosphate enhanced the expression of osteopontin in both fractions (particularly in the cytosol fraction) (Fig. 10).

Osteopontin interacts with other extracellular matrices such as collagen and fibronectin on the cell surface (Aeschlimann et al., 1993, 1995; Heath et al., 2001). Thus, membrane-associated osteopontin which was observed in inorganic phosphate-induced MPDL22 may interact with such extracellular matrices on the cell surface. To test this possibility, double stainings of osteopontin/collagen and osteopontin/fibronectin in inorganic phosphate-stimulated MPDL22 were conducted. Unstimulated MPDL22 showed weak expression of osteopontin, collagen type I and fibronectin (data not shown). Activation of MPDL22 with inorganic phosphate elucidated the increased level of these matrices (Fig. 11a,b,e,f). Co-localization of osteopontin with collagen type I and, notably, with fibronectin was observed (Fig. 11d,h). In addition, treatment of

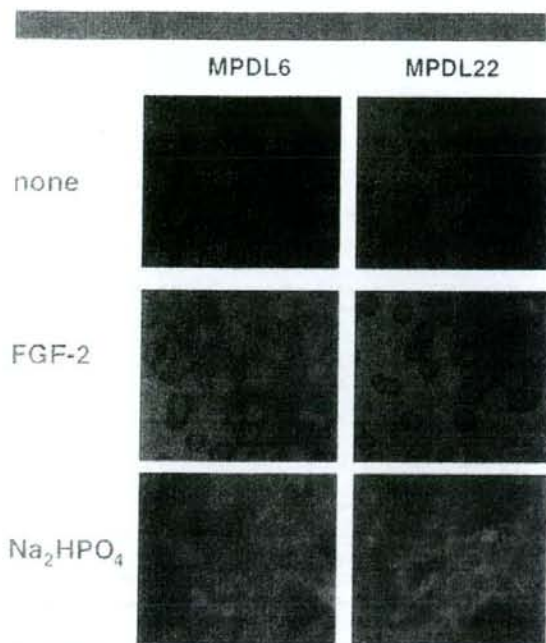


Fig. 8. Expression of osteopontin on MPDL6 or MPDL22 in response to FGF-2 or inorganic phosphate stimulation. MPDL6 and MPDL22 were cultured for 48 h in the presence or absence of FGF-2 (100 ng/ml) or Na_2HPO_4 (10 mM). Each cell monolayer was fixed with 2% paraformaldehyde and incubated with or without anti-osteopontin polyclonal antibody after incubation with Alexa Fluor 488 conjugated anti-goat IgG antibody. Results of one representative experiment out of three separate experiments are shown.

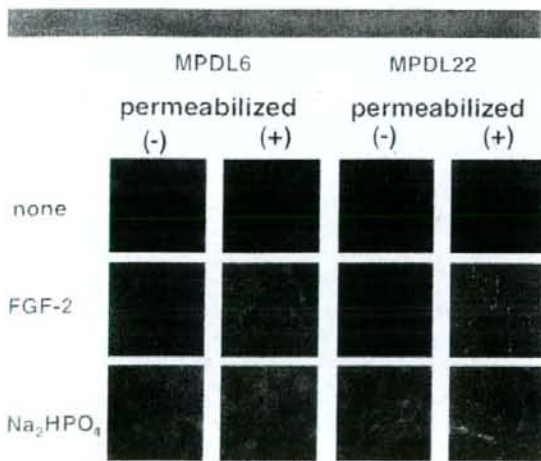


Fig. 9. Expression of osteopontin of permeabilized MPDL6 or MPDL22 in response to FGF-2 or inorganic phosphate stimulation. MPDL6 and MPDL22 were cultured for 48 h in the presence or absence of FGF-2 (100 ng/ml) or Na_2HPO_4 (10 mM). Each cell monolayer was fixed with 2% paraformaldehyde or 2% paraformaldehyde combined with 0.1% Triton X to permeabilize the monolayers and incubated with or without anti-osteopontin polyclonal antibody after incubation with Alexa Fluor 488 conjugated anti-goat IgG antibody. Results of one representative experiment out of three separate experiments are shown.

inorganic phosphate-stimulated MPDL22 with collagenase resulted in the prominent reduction of not only collagen but also osteopontin expressions of MPDL22 (data not shown). As a control, hyaluronidase treatment abrogated the expression of hyaluronan, but not that of osteopontin. These results suggest that osteopontin was anchored on the cell surface probably via collagen and fibronectin matrices.

Soluble osteopontin promoted cell survival of MPDL22

Soluble osteopontin interacts with several cell-surface receptors and modulates various cell functions. Osteopontin has been reported to promote the survival of endothelial cells (Scatena et al., 1998; Khan et al., 2002), epithelial cells (Rogers et al., 1997; Ophascharoensuk et al., 1999), and vascular smooth muscle cells (Weintraub et al., 2000). As shown in Figure 12, the conditioned medium of FGF-2-stimulated MPDL22 suppressed the apoptosis. The suppressive effect was partially but significantly inhibited by neutralizing anti-osteopontin antibody (Fig. 12). Although FGF-2 also has an anti-apoptotic effect (Debiais et al., 2004), the anti-FGF-2 neutralizing antibody did not alter the survival effect of the culture supernatant of FGF-2-stimulated MPDL22 (data not shown).

Discussion

In this study, we demonstrated that the localization and expression pattern of the osteopontin induced by inorganic phosphate and that induced by FGF-2 were different. Of interest, inorganic phosphate enhanced the formation of mineralized nodules via its influx into cells (Fig. 3), while FGF-2 suppressed it (Takayama et al., 1997). Osteopontin is an abundant acidic glycoprotein present in extracellular matrix of mineralized tissue albeit negatively related to mineralization (Ishijima et al., 2007). This suggests that the inorganic phosphate-induced osteopontin is probably relevant to regulation of mineralization. In contrast, osteopontin was not expressed on the cell surface but osteopontin secretion was elevated in the culture supernatant by MPDL22 in response to FGF-2 (Fig. 7). This implies that FGF-2-induced osteopontin may be associated with function(s) distinct from biomineralization.

Since osteopontin is unable to form organized multimeric structures, its interaction with other extracellular molecules is essential for its association with the insoluble extracellular matrix. Osteopontin binds extracellular matrices such as collagen, fibronectin, and osteocalcin and therefore interacts with the hydroxyapatite crystal with high affinity (Singh et al., 1990; Boskey et al., 1993; Kaartinen et al., 1999; Weintraub et al., 2000). Cell surface-associated fibronectin is capable of bind to osteopontin as heat-dissociable cross-linked products likely by effects of transglutaminase (Mukherjee et al., 1995). Therefore, an appropriate tight association of osteopontin with other extracellular matrices may be critical for its localization on cell surface. As shown in Figure 11, osteopontin is co-localized predominantly with fibronectin and partially with collagen. And collagenase treatment, which decreased the expression of collagen and fibronectin in inorganic phosphate-stimulated MPDL22, resulted in partial reduction of expression of osteopontin, while hyaluronidase treatment did not (data not shown). These results suggest that fibronectin and/or collagen, at least partially, mediates the osteopontin adhesion on cell surface and that expression of osteopontin on the cell surface is attributed to collagen-fibronectin complex. On the other hand, FGF-2 suppressed the expression of type I collagen by human PDL cells and MPDL 22 (Takayama et al., 1997). These results suggest the impaired adherence of FGF-2-induced osteopontin to the extracellular matrix on the cell surface. Taken together, the difference of extracellular



Fig. 10. Expression of osteopontin in cytosol or plasma membrane fraction of MPDL22 in response to FGF-2 or inorganic phosphate stimulation. MPDL22 (1×10^6), which had been cultured for 48 h in the presence or absence of FGF-2 (100 ng/ml) or inorganic phosphate (10 mM), were collected, homogenized on ice and centrifuged at 700g for 10 min at 4°C. The supernatant and pellet were used as the cytosol fraction and plasma membrane protein, respectively. Each fraction was subjected to western blot analysis as described in "Materials and Methods Section." Results of one representative experiment out of three separate experiments are shown.

matrix expression of MPDL22 in response to the stimulation with inorganic phosphate and FGF-2 may, in part, account for the distinct localization of osteopontin.

Osteopontin bound to the cell surface is phosphorylated and forms a heat-stable complex with fibronectin, while dephosphorylated osteopontin forms a heat-dissociable complex with fibronectin in culture supernatant (Singh et al., 1990). Furthermore, the inhibitory effect of osteopontin on mineralization was found to depend on the phosphorylation of osteopontin (Jono et al., 2000; Pampena et al., 2004). In this study, western blot analysis revealed a slight difference in molecular size between cell-surface-associated osteopontin and that detected in the culture supernatant (Fig. 10). This difference may be explained by posttranslational modification. Post-translational modification of osteopontin, such as phosphorylation, *N*- and *O*-glycosylation, and transglutaminase catalyzed contributes to its interaction with other extracellular matrices. Such interactions are thought to be necessary for bone mineralization (Glimcher, 1989; Saavedra, 1994; Sorensen and Petersen, 1995). Unfortunately, however,

western blotting analysis revealed similar immunoreactive staining with anti-phosphotyrosine excluding anti-phosphoserine and anti-phosphothreonine antibodies in both FGF-2- and inorganic phosphate-induced osteopontin (data not shown). In addition, *N*- and *O*-glycosidase treatment did not cause a prominent effect on molecular weight and exposure of tissue transglutaminase to FGF-2-activated MPDL22 unchanged expression of osteopontin on the cell surface (data not shown). Further investigation regarding other modifications affecting the property of osteopontin is needed.

Osteopontin is detected within cementum surface and PDL cells (McKee and Nanci, 1996; Lao et al., 2006) and implicated in cementogenesis and homeostasis of periodontal tissues (MacNeil et al., 1995a,b). Interestingly, increased gene expression of osteopontin in cementoblast was observed following stimulation with inorganic phosphate while collagen type I mRNA was decreased (Foster et al., 2006) and the effects were inhibited by the treatment of cementoblast with foscarnet. FGF-2 increased DNA synthesis and osteopontin expression but decreased gene expression of osteocalcin and

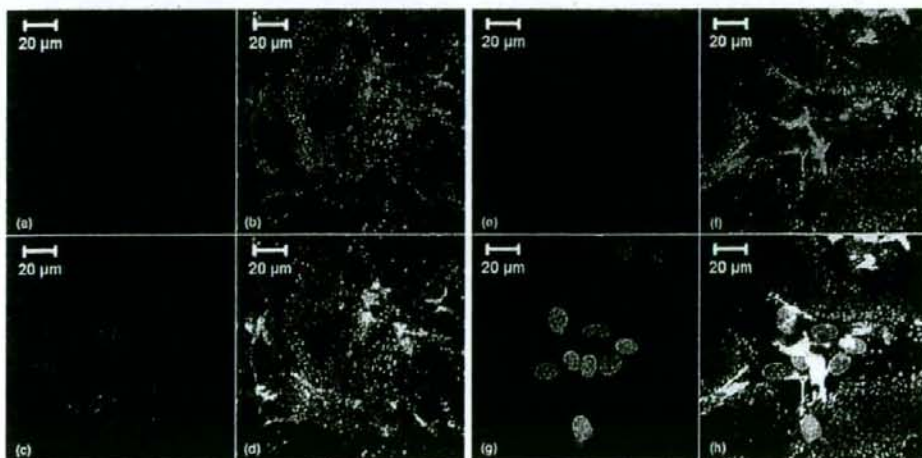


Fig. 11. Extracellular matrix expression of MPDL22 in response to inorganic phosphate stimulation. MPDL22 was cultured for 48 h in the presence of Na_2HPO_4 (10 mM). Cell monolayers were fixed with 2% paraformaldehyde and incubated with or without goat anti-osteopontin antibody (b,f), rabbit anti-Type I collagen (a) or anti-fibronectin (e) polyclonal antibody after incubation with Alexa Fluor 488 conjugated anti-goat IgG antibody (b,f) or Alexa Fluor 546 goat anti-rabbit IgG (a,e). DAPI staining was performed to stain nuclear (c,g). Parts (a-c) and (e-g) were merged into parts (d) and (h), respectively. Incubation of inorganic phosphate-stimulated MPDL22 with Alexa Fluor 488 conjugated anti-goat IgG antibody or Alexa Fluor 546 goat anti-rabbit IgG did not result in any staining. Results of one representative experiment out of three separate experiments are shown.

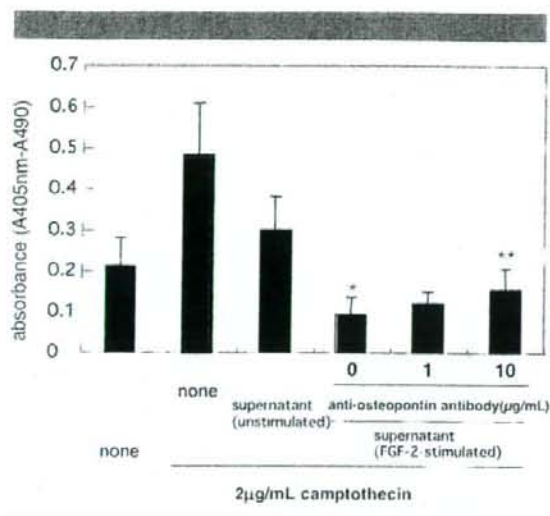


Fig. 12. Suppressive effect of FGF-2-induced osteopontin on apoptosis. Apoptosis was detected using a cell death detection ELISA^{Plus} kit following the instruction manual. MPDL22 was treated with 2 µg/ml of camptothecin for 4 h at 37°C to induce apoptosis. The cells were lysed with lysis buffer and the lysate was centrifuged. Twenty microliter from the supernatant was transferred into the streptavidin-coated 96-well plate and anti-histone-biotin and anti DNA-peroxidase were added to each well, incubated for 2 h. Color was developed with ABTS solution and absorbance was measured using a microplate reader (* $P < 0.05$ compared to unstimulated control, ** $P < 0.05$ compared to culture without anti-osteopontin antibody). Results are expressed as the mean \pm SD of three separate experiments.

bone sialoprotein with suppression of mineral nodule formation by cementoblast (Hakki et al., 2005). These results including our data suggest that mouse cementoblasts as well as MPDL22 are influenced by inorganic phosphate and FGF-2 to regulate expression of bone-related extracellular matrices. In contrast, FGF-2 stimulated osteocalcin mRNA and promoter activity with increased Runx2 phosphorylation in MC3T3-E1 preosteoblastic cells (Xiao et al., 2002). This indicates that effects of FGF-2 on hard tissue formation and mineralization may differ between each cell type. Similarly, functions of osteopontin appear to vary depending on the cell types despite mineralizing and non-mineralizing tissue.

Osteopontin is produced by injured epithelium and inflammatory infiltrates (Murry et al., 1994; O'Brien et al., 1994; Nau et al., 1997; Liaw et al., 1998; O'Regan et al., 1999). In addition, osteopontin promotes the survival of epithelial cells exposed to injury (Noiri et al., 1999; Denhardt et al., 2001b). A recent study using osteopontin knock-out mice has revealed abnormal matrix organization during skin incisional wound healing (Liaw et al., 1998). In this study, we demonstrated that FGF-2-induced osteopontin played roles in cell survival (Fig. 12) and migration of MPDL22 (data not shown). FGF-2 also activated cranial suture closure of cultured mouse calvaria with increased expression of osteopontin (Kim et al., 2003). Furthermore, the FGF-2-induced osteopontin amplified neovascularization via osteopontin-mediated recruitment of proangiogenic monocytes (Leali et al., 2003) and endothelial cell survival (Scatena et al., 1998; Khan et al., 2002). Interestingly, topical application of FGF-2 to experimentally induced periodontitis sites with alveolar bone defects prompted periodontal tissue regeneration. FGF-2 has been suggested to induce proliferation of PDL cells, modulation of extracellular

matrix production (Takayama et al., 1997) and angiogenesis, and thereby accelerates periodontal regeneration (Murakami et al., 1999). Taken together, these findings suggest that FGF-2-induced osteopontin is associated with the early phase of wound healing and that it shows beneficial and cross-talk effects with FGF-2 during the course of periodontal tissue regeneration.

Increased osteopontin levels have been demonstrated in inflammatory disease (Denhardt and Guo, 1993; Nau et al., 2000; Hashimoto et al., 2003; Leali et al., 2003; Agnholt et al., 2007), such as rheumatic heart valves (Rajamannan et al., 2005), pulmonary inflammation (Takahashi et al., 2001) and Crohn's disease (Agnholt et al., 2007). Numerous factors including phorbol esters, concanavalin A, and various growth factors and cytokines, such as IL-1, IL-2, IL-3, LPS, TNF- α , BMP, EGF, FGF-2, TGF- β , and PDGF, up-regulated osteopontin production (Denhardt and Guo, 1993; Denhardt and Noda, 1998; O'Regan and Berman, 2000). Osteopontin inhibits NO production and iNOS expression (Rollo et al., 1996; Guo et al., 2001) and regulates production of a variety of cytokines by T cells, monocytes and macrophages (Ashkar et al., 2000; Naldini et al., 2006; Agnholt et al., 2007). Moreover, osteopontin augmented the mitogenic action of PDGF (Takahashi et al., 2001). These results suggest that osteopontin contributes to regulate inflammatory response and subsequent tissue remodeling through cell adhesion, chemotaxis, cytokine regulation, and prevention of apoptosis (Hijiya et al., 1994; O'Regan and Berman, 2000; Denhardt et al., 2001b).

Osteopontin-deficient mice exhibit less accumulation of macrophage in inflammation in ischemic kidneys (Persy et al., 2003) and the abnormal wound repair (Liaw et al., 1998). Osteopontin expression was associated with infiltration and migration of T cell and monocytes (Patarca et al., 1989; Giachelli et al., 1994; O'Regan et al., 1999). It has been reported that chemotactic factor for monocyte infiltration and proinflammatory cytokines expression contribute proangiogenic activity of osteopontin (Leali et al., 2003; Naldini et al., 2006). Furthermore, osteopontin function as mediator of cell migration, chemotactic factor for macrophage and muscle cells (Liaw et al., 1994; Bendeck et al., 2000). Perimembranous osteopontin co-localization with CD44 was observed at leading edge in migrating fibroblasts (Zohar et al., 2000). Similarly, perinuclear osteopontin was present in migratory MPDL22 and its expression and CD44 expression was enhanced by stimulation with FGF-2 (data not shown). Identification of other functions of osteopontin in FGF-2-stimulated MPDL22 is thus of paramount interest in future studies.

Osteopontin can bind to several receptors including integrin $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_4\beta_5$, $\alpha_8\beta_1$, $\alpha_9\beta_1$, and certain variant forms of CD44 (Denhardt et al., 2001b). CD44 and $\alpha_v\beta_3$ has been reported to mediate cell motility (Chellaiah and Hruska, 2003). In particular, the interaction of osteopontin with the cell surface receptor CD44 has been reported to elicit a cell survival response and phosphatidylinositol 3-kinase/AKT signaling pathway accounted for its function (Lin et al., 2000; Lin and Yang-Yen, 2001). Interestingly, treatment of MPDL22 with FGF-2 resulted in an increase in the level of CD44 expression. This suggests that CD44 on MPDL22 is related to the osteopontin-dependent motility. Further investigation on this matter is now in progress.

The present study revealed that inorganic phosphate and FGF-2 differentially regulate the expression of osteopontin by PDL cells. These observations provide important insight into the roles of osteopontin in not only the late phase (mineralization) but also the early phase (cell proliferation, survival, and migration) of the periodontal regeneration process caused by PDL cells, although additional studies are necessary to delineate the detail functions of osteopontin released in response to FGF-2.