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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.

Keywords: Basic fibroblast growth factor; Heparan sulfate; Periodontal ligament cells; Shedding; Syndecan

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 (Bikfalvi et al., 1997; Nugent and Iozzo, 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 (Costor, Cambridge, MA, USA). The explants were cultured in α-MEM supplemented with 10% FCS, 50 U/ml penicillin G, and 50 μg/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% CO_2. 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 10^5 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/Biotecx 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 H_2O). 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 μl reverse transcription mixture containing 52.5 mM Tris–HCl; pH 8.3, 75.5 mM KCl, 3 mM $MgCl_2$, 0.5 mM each dNTPs, 1 mM dithiothreitol, 1.1 U/μl RNase inhibitor (Takara Biomedicals, Shiga, Japan), 55 ng/μl random hexamer, 5 U/μl 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 μl of DEPC-treated H_2O.

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′GCCTGACCAAGGAAAGCAAAGTC3′
GlcNAcT-1	546 bp	Sense	5′CTAAGCTGCAGGGAAATAAA3′
		Antisense	5′TTGCTGTCTGTTGTTTGAAG3′
HS2ST	443 bp	Sense	5′AGGATTTTATCATGGACACG3′
		Antisense	5′TCTTTCCTGTGCGATAGAGT3′
NDST-1	450 bp	Sense	5′ATCTTCTGCCTGTTCAGCGT3′
		Antisense	5′CTCATTGGCCTTGAAGAAGC3′
NDST-2	580 bp	Sense	5′CATGAAGGTGGCTGAGTTG3′
		Antisense	5′CGGATTAAGCAGCACTGTCA3′
Epimerase	624 bp	Sense	5′AGGTGGTTAGGTTGATTGCG3′
		Antisense	5′GCAGTTGATTGATGTGGGTG3′
Syndecan-1	472 bp	Sense	5′CTTTGAAACCTCGGGGAGAATAC3′
		Antisense	5′TCCAGGCAGAAGTCAGAGAAGCAG3′
Syndecan-2	395 bp	Sense	5′GGAGCTGATGAGGATGTAGA3′
		Antisense	5′CACTGGATGGTTTGCGTTCT3′
Syndecan-3	292 bp	Sense	5′GCTTCTTTTCCCTTTTACCCTCCGC3′
		Antisense	5′TGTTCCCCAACTTCTCTCTGCCAAG3′
Syndecan-4	245 bp	Sense	5′GGGCAGGAATCTGATGACTTTGAG3′
		Antisense	5′GCTGGACATTGACACCTTGTTGC3′

PCR was performed in a 50 μl mixture containing 5 μl of each sample of derived cDNA, 10 mM Tris–HCl; pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 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 predenaturation at 94 °C, PCR was performed in a DNA thermal cycler (MJ Research Inc., Watham, 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 μl 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 (Corning Laboratory Sciences Company, Corning, 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-αMEM 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′-tetramethylbenzidene 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, Hitachinaka, 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-1 (IGF-1), 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 5×10^3 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, Carlshad, CA, USA) or Alesa Fluor 568-labeled streptavidin (Molecular Probes). The samples were mounted on coverslipped, then viewed using an OLYMPUS 1×70 microscope (Tokyo, Japan) and photographed.

2.6. FACS analysis

HPDL cells were incubated in 10% FCS-αMEM 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),

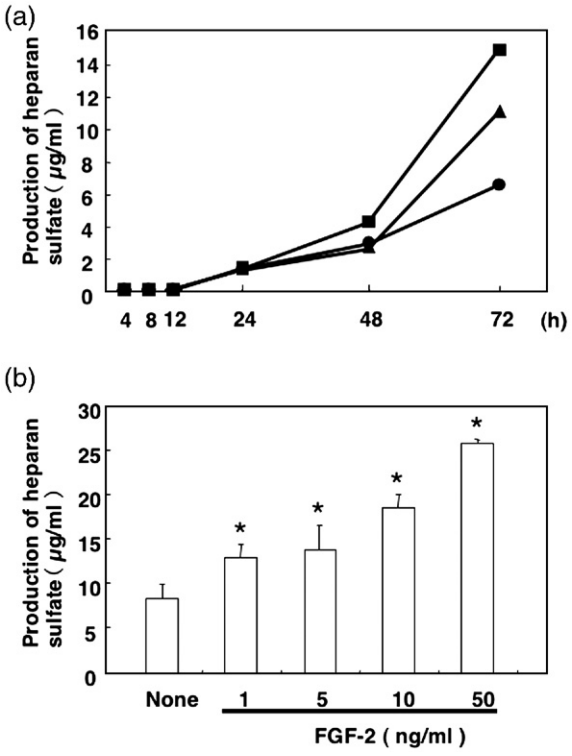


Fig. 1. Effect of FGF-2 on the production of HS by HPDL cells. HPDL cells were cultured in the absence (●) or presence of FGF-2 (5: ▲, 50: ■ ng/ml) and cultured for the indicated times (a.). HPDL cells were cultured in the absence or presence of FGF-2 (1, 5, 10, 50 ng/ml) for 72 h (b.). Conditioned medium 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).

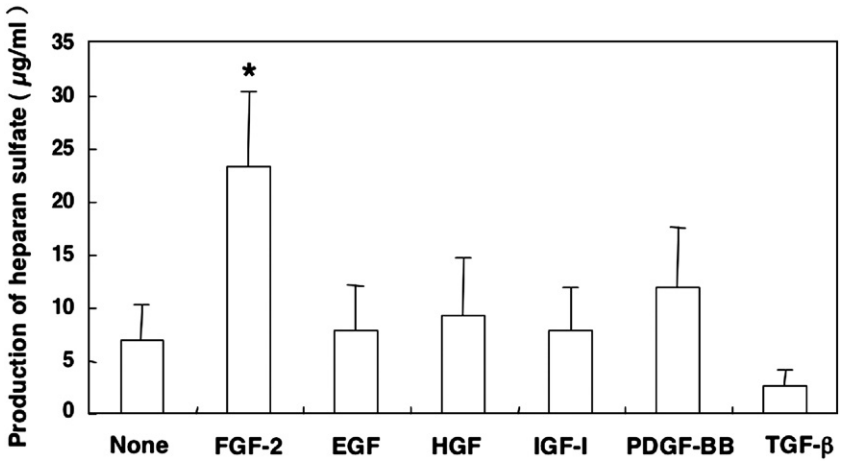


Fig. 2. Effect of various cytokines on HS production by HPDL cells. HPDL cells were cultured without any cytokine or with FGF-2 (50 ng/ml), EGF (100 ng/ml), HGF (50 ng/ml), IGF-1 (50 ng/ml), PDGF-BB (50 ng/ml), or TGF-β (10 ng/ml). Culture supernatants were removed after 72 h of exposure to the various 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-μl 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 ± 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 μg/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

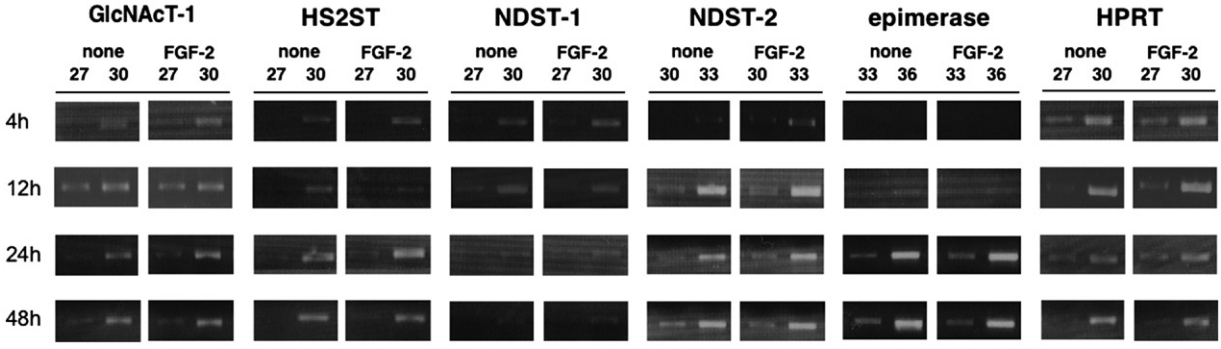


Fig. 3. mRNA expression of HS synthetic enzymes in HPDL cells. The expression of mRNA of HS-related enzymes in HPDL cells cultured in the absence or presence of FGF-2 (50 ng/ml) for 24 h was measured. Total RNA was recovered and RT-PCR performed to analyze the mRNA expression of GlcNAcT-1, HS2ST, NDST-1, 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.

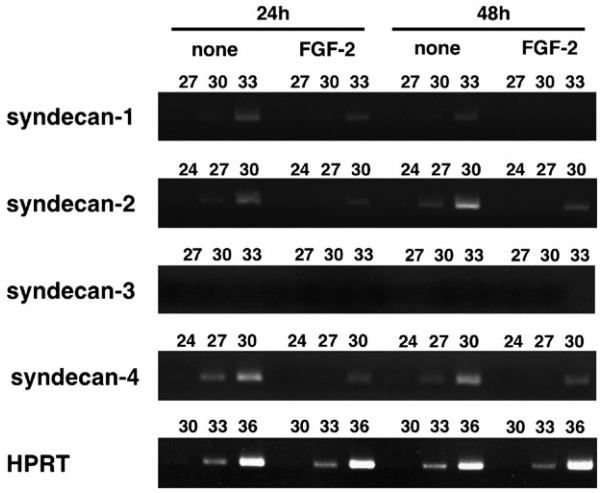


Fig. 4. mRNA expression of the syndecan gene family in HPDL cells. The expression of mRNA of the syndecan gene family in HPDL cells cultured in the absence or presence of FGF-2 (50 ng/ml) for 24 h and 48 h was measured. Total RNA was recovered and RT-PCR performed to analyze the mRNA expression of syndecan-1, -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 epimerazation, deacetylation/*N*-sulfation, and *O*-sulfation (Prydz and Dalen, 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,

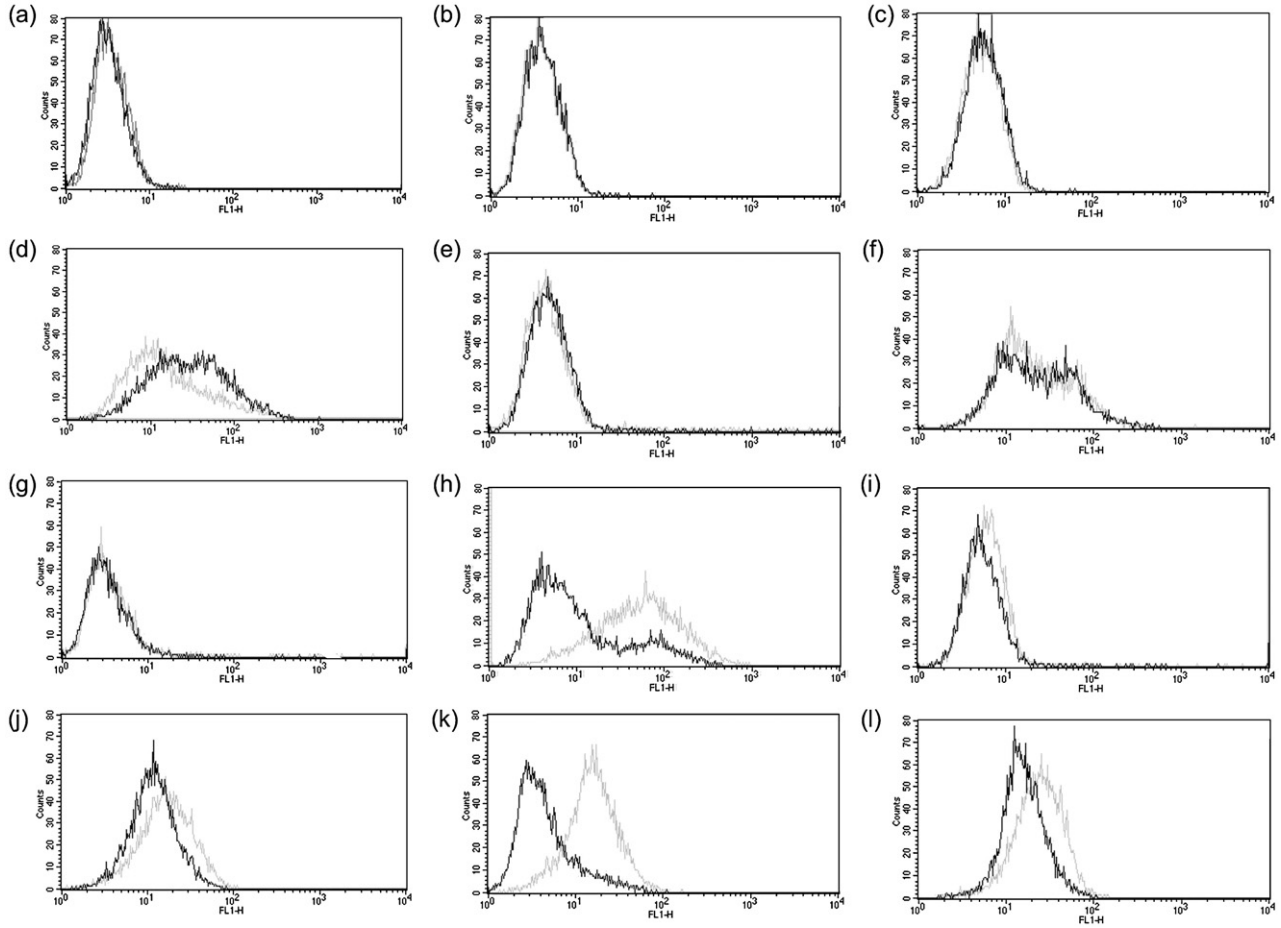


Fig. 5. FACS analysis of syndecan expression on HPDL cells. HPDL cells were incubated in 10% FCS-αMEM in the absence (gray line) or presence of FGF-2 (50 ng/ml) (black line) for 24 h (a, d, g, j), 48 h (b, e, h, k) or 72 h (c, f, i, l). At the time of the assay, cells were incubated without (a, b, c) 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 (Bernfield 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., 1992).

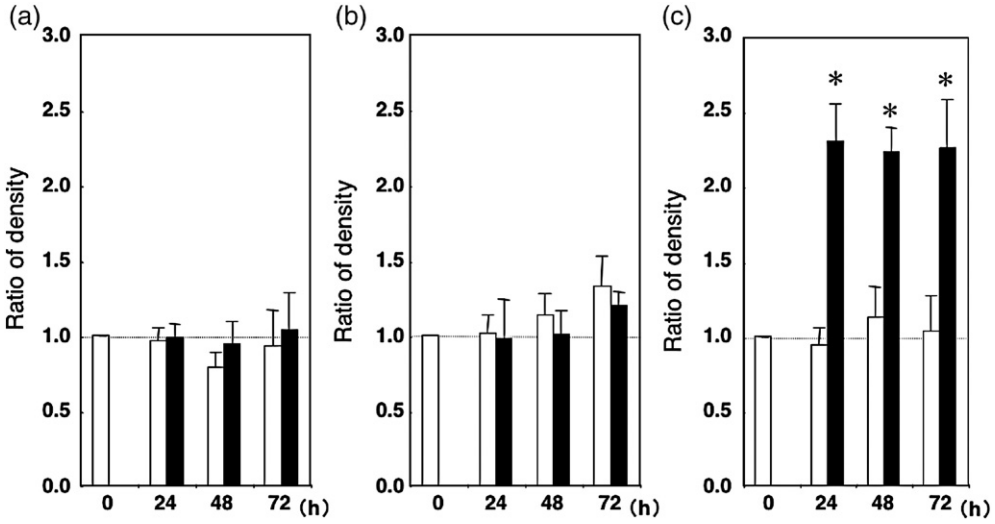
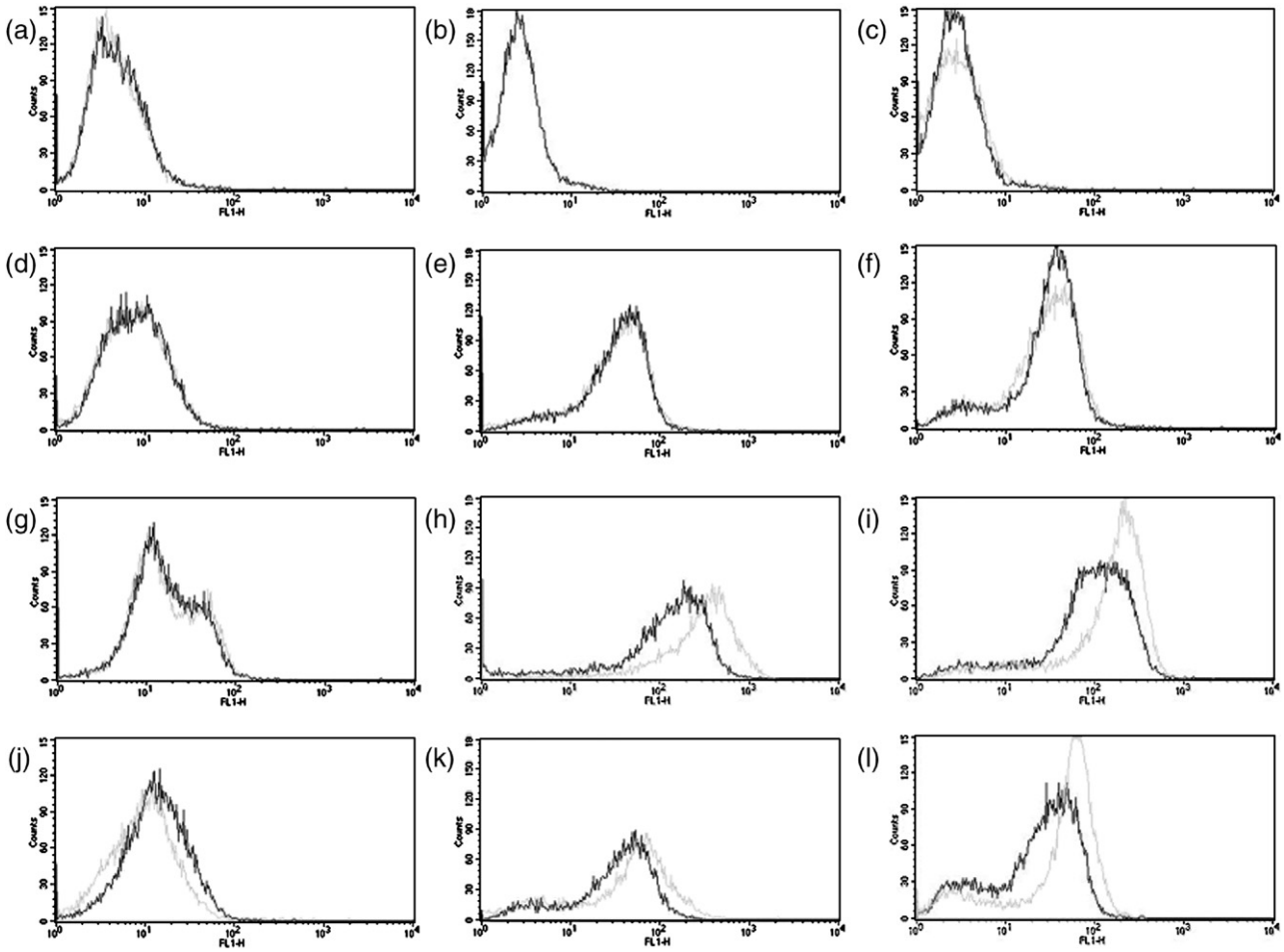


Fig. 6. Dot blot analysis of shedded syndecan in the culture supernatant of HPDL cells. Medium from FGF-2 (50 ng/ml)-stimulated (closed column) or unstimulated (open column) HPDL cells was collected after 24, 48, or 72 h. Nitrocellulose membrane was spotted with each sample, and blocked with 10% BSA to minimise 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



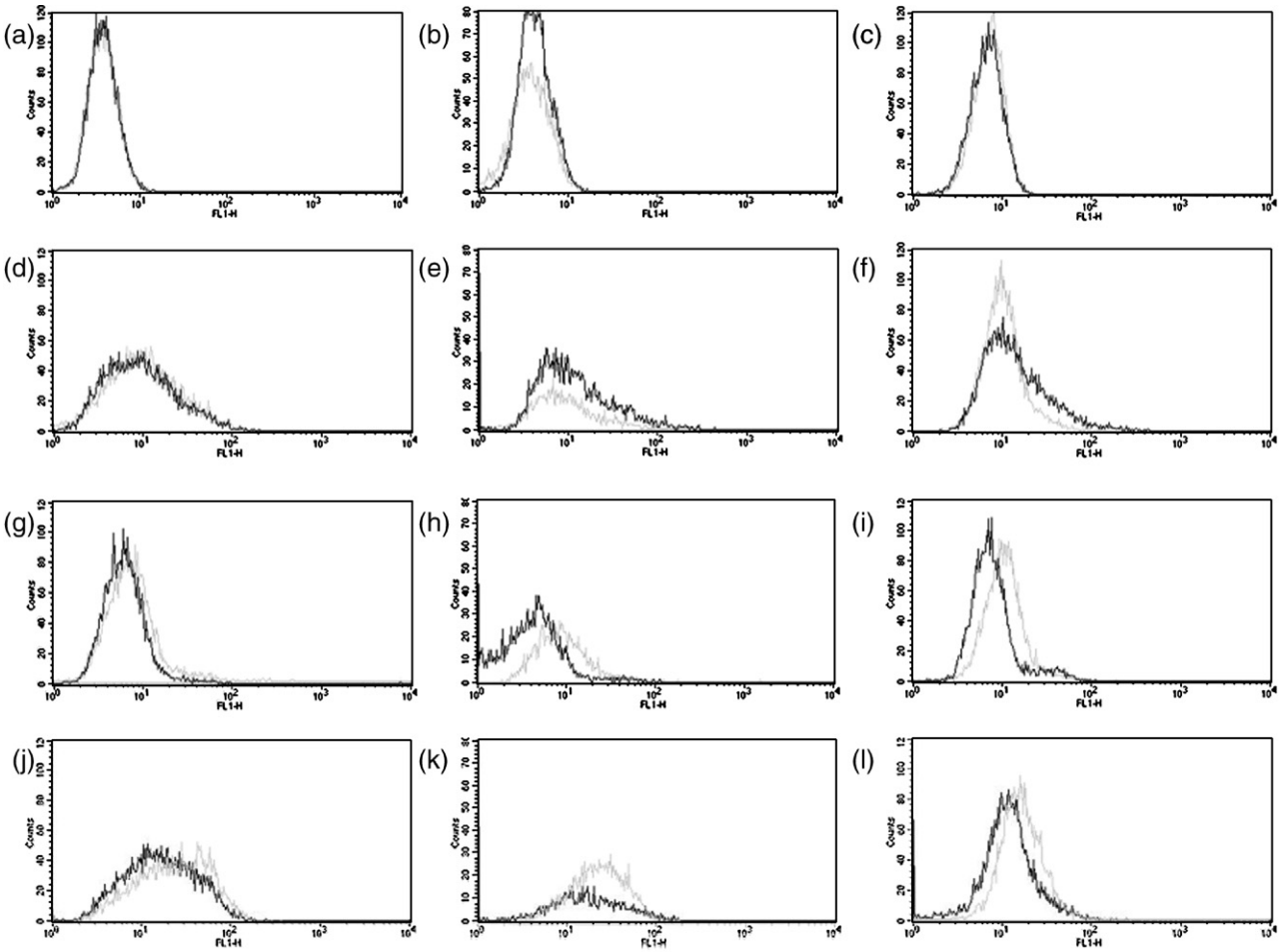
Appendix data 1.

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 cleaved from cell membrane and secreted (Bernfield et al., 1999; Hacker 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 (Echtermeyer 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-αMEM 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.

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