J Periodont Res 2004; 39; 388–397
Printed in the UK. All rights reserved

JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2004.00750.x

The involvement of platelet-derived growth factor receptors and insulin-like growth factor-I receptors signaling during mineralized nodule formation by human periodontal ligament cells

Eiji Nemoto[1], Mitsuru Shimonishi[2], Yasutaka Nitta[3], Hidetoshi Shimauchi[1]

[1]Division of Periodontology and Endodontology, [2]Division of Operative Dentistry, and [3]Division of Maxillofacial Surgery, Tohoku University School of Graduate Dentistry, Sendai, Japan

Nemoto E, Shimonishi M, Nitta Y, Shimauchi H. The involvement of platelet-derived growth factor receptors and insulin-like growth factor-I receptors signaling during mineralized nodule formation by human periodontal ligament cells. J Periodont Res 2004; 39; 388–397. © Blackwell Munksgaard 2004

Background and objective: Periodontal ligament cells are regarded to have the capacity to differentiate into cementoblasts or osteoblasts, and are capable of forming a mineralized nodule *in vitro*. However, the precise mechanisms are unclear. Here we evaluated the possible involvement of growth factor receptors, such as the platelet-derived growth factor receptor (PDGFR), insulin-like growth factor-I receptor (IGF-IR), and epidermal growth factor receptor (EGFR) on periodontal ligament cells and their ligands during periodontal ligament cells differentiation *in vitro*.

Methods: Human periodontal ligament cells were differentiated via culturing in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate for mineralized nodule formation, characterized by von Kossa staining. Expressions of receptors and their ligands were analyzed by flow cytometry/reverse transcription-polymerase chain reaction.

Results: During the differentiation, PDGFR-α was held at a lower level compared with the control. PDGFR-β, however, was maintained at a slightly higher level that was reversed to the control level when mineralized nodules formed. In contrast, IGF-IR and EGFR were not substantially different from the control. The mineralized nodule formation was strongly inhibited by a PDGFR kinase blocker (AG1295 and AG1296), partially inhibited by an IGF-IR kinase blocker (I-Ome-AG538 and AG1024), and not inhibited by an EGFR kinase blocker (AG99). PDGF-A, PDGF-C, PDGF-D, IGF-I, and IGF-II, but not PDGF-B, were expressed on the control as well as dexamethasone/ascorbic acid-treated periodontal ligament cells during mineralized nodule formation; however, the pattern of their expressions was quite different.

Eiji Nemoto, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
Tel: +81 22 717 8336
Fax: +81 22 717 8339
e-mail: e-nemoto@umin.ac.jp

Key words: insulin-like growth factor; mineralization; periodontal ligament cells; platelet-derived growth factor

Accepted for publication April 13, 2004

Conclusion: These findings suggest that a pathway of PDGFs/PDGFR and IGFs/IGF-IR on periodontal ligament cells are involved during mineralized nodule formation, and that PDGFs and IGFs expressed by periodontal ligament cells may contribute to the formation.

Periodontal diseases are infectious diseases and are characterized by the irreversible destruction of periodontal support tissues, i.e. cementum, bone, and periodontal ligament. The ultimate goal for periodontal therapy is the regeneration of these lost tissues. Though the exact cells responsible for regeneration of the periodontal tissue remain unknown, periodontal ligament cells are regarded to have the capacity to differentiate into cementoblasts or osteoblasts depending on need, and to form the cementum or alveolar bone (1). Periodontal ligament cells *in vitro* have been shown to possess osteoblast-like properties, including a high level of alkaline phosphatase expression, production of a cyclic AMP in response to parathyroid hormone (2), and synthesis of bone-associated proteins in response to 1,25-dihydroxyvitamin D_3 (2). Furthermore, when cultured with ascorbic acid, dexamethasone, and β-glycerophosphate, periodontal ligament cells are capable of producing cementum-like mineralized nodules that are morphologically different from bone-like mineralized nodules formed by osteoblastic cells (3, 4). It was reported that the alkaline phosphatase level increases and non-collagenous extracellular matrix proteins such as osteopontin and bone sialoprotein are synthesized during mineralized nodule formation *in vitro* (5–8). However, limited studies have demonstrated the possible involvement of growth factors and their receptors during mineralized nodule formation by periodontal ligament cells *in vitro* (8).

Among various growth factors, platelet-derived growth factors (PDGFs) regulate diverse cellular functions in connective tissue cells, and are important for normal embryonic development (9–12). The PDGF family consists of four members, PDGF-A (12), PDGF-B (12), and newly identified PDGF-C (11) and PDGF-D (9, 10), which form four functional homodimers, PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD, as well as the heterodimer PDGF-AB as endogenous cell products. PDGF-A and PDGF-B chains are expressed on most cell types (12) such as fibroblasts (12, 13), osteoblasts (12, 14), endothelial cells (12), and macrophages (12, 15), and PDGF-C and PDGF-D are expressed in various tissues (9–11). However, little is known regarding the expression of PDGF-A, PDGF-B, PDGF-C, and PDGF-D on periodontal ligament cells. The two PDGF receptors (PDGFR), which are protein tyrosine kinase receptors, have different ligand-binding capacities. The PDGF-A and PDGF-C chains selectively bind to PDGFR-α, whereas PDGF-D preferentially binds to PDGFR-β, and PDGF-B displays a similar affinity for both receptors (9–12). Recently, it has been shown that PDGF-CC and PDGF-DD bind to PDGFR-α/β heterodimers as well (9, 16). Most tissues express insulin-like growth factor-I (IGF-I) and IGF-II, and local production is thought to be important in the regulation of growth and differentiation (17, 18). Osteoblasts or connective tissue cells such as dermal fibroblasts and connective tissue stromal cells express IGF-I and IGF-II (18). Both IGF-I and IGF-II interact with IGF-I receptor (IGF-IR), a transmembrane heterotetramer with tyrosine kinase activity (17, 18). IGF-II can also bind to the IGF-IIR with high affinity, which exists as a single transmembrane chain with a small intracellular domain lacking tyrosine kinase activity and is thought to function as a clearance receptor for IGF-II (18). Therefore, most biological actions of IGF-I and IGF-II are mediated by IGF-IR. PDGF-AA, PDGF-BB, and IGF-I have been shown to have mitogenic and chemotactic effects on periodontal ligament cells (19–22), but little is known about IGF-II action toward periodontal ligament cells. It was also reported that the combination of PDGF-BB and IGF-I stimulates periodontal regeneration in various animal models (23–25), and in a human clinical trial (26) via an unidentified mechanism. Epidermal growth factor receptor (EGFR) (also termed Erb1/HER1) belongs to a family of four closely related receptor tyrosine kinases (ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4), which are expressed in a variety of tissues of epithelial, mesenchymal and neuronal origin. There they play fundamental roles in development, proliferation and differentiation (27). In periodontal ligament cells, epidermal growth factor (EGF) was reported to stimulate proliferation accompanied by autophosphorylation of EGFR and subsequent activation of extracellular-related kinase (ERK) 1/2 (28).

In the present study, we hypothesized that the mineralized nodule formation by periodontal ligament cells induced by dexamethasone/ascorbic acid may be mediated through growth factor receptors such as PDGFR-α, PDGFR-β, IGF-IR, and EGFR. We investigated the possible involvement of these growth factor receptors and their ligands on periodontal ligament cells during differentiation *in vitro*.

Material and methods

Reagents

Phycoerythrin-conjugated monoclonal antibody (mAb) for human PDGFR-α [αR1, mouse immunoglobulin G2a (IgG2a)], PDGFR-β (28D4, mouse IgG2a), IGF-IR α subunit (1H7, mouse IgG1), EGFR (EGFR.1, mouse IgG2b) and isotype-matched control IgG conjugated with phycoerythrin were purchased from BD Biosciences PharMingen (San Diego, CA, USA).

Tyrphostin: AG1295, AG1296, AG1024, I-Ome-AG538, and A46 (AG99) were purchased from Calbiochem (San Diego, CA, USA). Ascorbic acid, β-glycerophosphate, dexamethasone, *p*-nitrophenyl phosphate and Cell Dissociation Solution® were purchased from Sigma (St. Louis, MO, USA). Multiple tissue cDNA (MTC™) panels were purchased from BD Biosciences Clontech (Palo Alto, CA, USA).

Cells

Human periodontal ligament cells were obtained by informed consent from the periodontal ligaments of fully erupted third molar teeth of 15 healthy individuals (age between 16 and 23) without clinical signs of inflammation in the periodontal tissues. Periodontal ligaments were dissected from the middle third of the root with a sharp blade, cut into small pieces, and cultured in tissue culture dishes containing a culture medium composed of α-MEM (minimal essential medium) with 10% heat-inactivated fetal bovine serum (Flow Laboratories, McLean, VA, USA), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B, with a medium change every 3 days until confluent cell monolayers formed. After confluency, the cells were passaged with 0.25% trypsin–0.1% EDTA. Periodontal ligament cells from at least three different donors were selected randomly and used for the fourth and seventh passage in all experiments.

Fluorescence-activated cell sorter (FACS)

Periodontal ligament cells in 24-well multiplates were collected using Cell Dissociation Solution® (no enzymatic), so as to avoid possible proteolysis destruction of cell surface proteins, processed by passing through a nylon mesh filter (94 μm of mesh size), washed with washing buffer (phosphate-buffered saline containing 1% bovine serum albumin) three times, and used for staining. A total of 10^5 periodontal ligament cells were stained with each phycoerythrin-conjugated mAb or isotype-matched control IgG at 4°C for 20 min followed by washing them with washing buffer three times. Staining was analyzed on a FACScan® (BD Biosciences, San Jose, CA, USA). Measurements were collected for 5000 events, which were stored in list mode and then analyzed with Lysis II software (Becton Dickinson, Franklin Lakes, NJ, USA). The arithmetic mean was used in the computation of the mean fluorescence intensity.

Mineralized nodule formation

Confluent periodontal ligament cells in 24-well multiplates were cultured in α-MEM with 10% fetal bovine serum supplemented with ascorbic acid (50 μg/ml), dexamethasone (1 μM), and β-glycerophosphate (10 mM) with a medium change every 3 days in all experiments except that of RNA extraction in which the medium was changed every 4 days, and cultured up to day 21. For the inhibition experiments with tyrphostin, each tyrosine kinase blocker dissolved in dimethyl sulfoxide was added to the well at the same time as the medium changes. Final percentage (v/v) of dimethyl sulfoxide was 0.1% in all samples.

Von Kossa staining

Periodontal ligament cells on 24-well multiplates were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline for 10 min, stained with 5% (w/v) silver nitrate in distilled water for 1 h, treated with 5% (w/v) sodium thiosulfate for 2 min, and then washed with distilled water. Periodontal ligament cells were counterstained with Mayer's hematoxylin solution (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). The stained periodontal ligament cells were digitally photographed with an OLYMPUS IX70 microscope (Olympus America, Inc., Melville, NY, USA) using the phase contrast mode equipped with digital imaging device, Penguin 600 CL (Pixera Corp., Los Gatos, CA, USA).

Alkaline phosphatase assay

Periodontal ligament cells cultured on 24-well multiplates were washed with phosphate-buffered saline. The activity was assayed by adding 1 mg/ml of *p*-nitrophenyl phosphate as a substrate in 0.1 M glycine buffer (pH 10.0) containing 1 mM $MgCl_2$ in a final volume of 1 ml for 10 min at 37°C. Supernatants were harvested, mixed with NaOH (final 0.25 N) to stop the reactions, and read spectrophotometrically at 405 nm.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay

When periodontal ligament cells cultured in six-well multiplates were confluent, the media were changed to α-MEM with 10% fetal bovine serum supplemented with ascorbic acid, dexamethasone, and β-glycerophosphate (referred to as day 0), and cultured with a medium change every 4 days up to 21 days. Total cellular RNA was extracted from periodontal ligament cells-culture by Isogen® (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions, at days 0, 5, 9, 13, 17, and 21, which correspond to 1 day after media changes. Reverse transcription of the RNA samples to cDNA was done using TaKaRa RNA PCR™ Kit (AMV) Ver.2.1 (Takara Bio, Shiga, Japan). To transcribe total RNA into cDNA, 1 μg of RNA, 250 U/ml reverse transcriptase XL isolated from avian myeloblastosis virus, 5 mM $MgCl_2$, 1 mM dNTP mixture, 1000 U/ml RNase inhibitor, and 2.5 μM Random 9 mer were mixed in a PCR buffer (total volume of 20 μl). The reaction mixture was incubated for 10 min at 30°C, 30 min at 42°C, followed by 5 min at 95°C. The primers used for PCR are given in Table 1. Amplification was performed in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) with the cycle program shown in Table 1. The number of cycles for PDGF-A, PDGF-B, PDGF-C, PDGF-D, IGF-I, IGF-II, and β-actin within the exponential range of PCR amplification were selected for analysis data which correspond to 20, 30, 20, 20, 25, 20, and 15 cycles, respectively. Amplified samples were visualized on 2% agarose gels stained with ethidium

Table 1. Primer pairs used for polymerase chain reaction amplifications

Gene (fragment)	Primer sequences	Denaturation/annealing/extension °C (s)	Cycle	Reference
PDGFR-α (891 bp)	5′-ATCAATCAGCCCAGATGGAC-3′ 5′-TTCACGGGCAGAAAGGTACT-3′	94 (60)/60 (60)/72 (60)	30	29
PDGFR-b (688 bp)	5′-AATGTCTCCAGCACCTTCGT-3′ 5′-AGCGGATGTGGAAAGGCATA-3′	94 (60)/58 (60)/72 (60)	30	29
IGF-1R (537 bp)	5′-CAACCACGAGGCTGAGAAGC-3′ 5′-CAGCATAATCACCAACCCTC-3′	94 (60)/55 (60)/72 (60)	30	30
EGFR (530 bp)	5′-AATGTGAGCAGAGGCAGGGA-3′ 5′-GGCTTGGTTTGGAGCTTCTC-3′	94 (30)/55 (60)/72 (105)	30	31
PDGF-A (237 bp)	5′-CCCCTGCCCATTCGGAGGAAGAG-3′ 5′-TTGGCCACCTTGACGCTGCGGTG-3′	94 (60)/62 (60)/72 (60)	15, 20, 25, 30	29
PDGF-B (435 bp)	5′-GATCCGCTCCTTTGATGATC-3′ 5′-GTCTCACACTTGCATGCCAG-3′	94 (60)/60 (60)/72 (60)	15, 20, 25, 30	29
PDGF-C (350 bp)	5′-TGGATACAACTTACGTTTGATGAA-3′ 5′-CGAATAAGGTCTTCCAAGGTACT-3′	94 (40)/58 (40)/72 (60)	15, 20, 25, 30	32
PDGF-D (918 bp)	5′-GTGCAGAGTCCTAGATTCCC-3′ 5′-GAGGTGGTCTTGAGCTGCAG-3′	94 (60)/59 (60)/72 (60)	15, 20, 25, 30	33
IGF-I (395 bp)	5′-AAATCAGCAGTCTTCCAACC-3′ 5′-CTTCTGGGTCTTGGGCATGT-3′	95 (35)/58 (40)/72 (40)	15, 20, 25, 30	34
IGF-II (196 bp)	5′-CTCTCCGTGCTGTTCTCTCC-3′ 5′-CGGGCCAGATGTTGTACTTT-3′	94 (45)/56 (45)/72 (60)	15, 20, 25, 30	35
β-actin (336 bp)	5′-ATTGGCAATGAGCGGTTCCGC-3′ 5′-CTCCTGCTTGCTGATCCACATC-3′	94 (60)/55 (60)/72 (60)	15, 20, 25	

EGFR, epidermal growth factor receptor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor.

bromide and photographed under UV light.

Cell proliferation assay

The number of periodontal ligament cells was determined using Cell counting Kit-8™ (Dojindo Laboratories, Kumamoto, Japan) composed of 5 mM WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt), 0.2 mM 1-methoxy methylphenazinium methylsulfate, and 0.15 M NaCl. Periodontal ligament cells cultured on 24-well multiplates were washed with phosphate-buffered saline followed by the addition of 1 ml of 10% of WST-8 solution. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 100 μl of 0.1 M HCl into the well, respectively. The supernatants were measured at 450 nm using a VERSAmax™ Tunable Microplate Reader (Molecular Devices Co., Sunnyvale, CA, USA). The proliferative response was determined as described below. Periodontal ligament cells on 24-well multiplates were cultured in α-MEM containing 10% fetal bovine serum. Eighteen hours before culture termination, 1 μCi [^{3}H]thymidine (Amersham Biosciences Corp, Piscataway, NJ, USA) was added to each well, and cells treated by trypsin were harvested with a cell harvester onto a glass fiber filter. The radioactivity was measured using a liquid scintillation β counter.

Statistical analysis

All experiments in this study were performed at least three times to test the reproducibility of the results, and representative findings are shown. In some experiments, experimental values are given as means ± standard error. The statistical significance of differences between two means was evaluated by one-way ANOVA. *p*-values less than 0.05 were considered significant.

Results

Expression of PDGFR-α, PDGFR-β, IGF-IE, and EGFR on periodontal ligament cells

Flowcytometric analysis revealed that confluent periodontal ligament cells expressed PDGFR-α, PDGFR-β, and IGF-IR; however, only weak expression of EGFR was observed (Fig. 1A). PDGFR-α, PDGFR-β, IGF-IR, and EGFR mRNA were also expressed in confluent periodontal ligament cells assessed by RT-PCR, although the EGFR signal was weak (Fig. 1B). We examined whether the expression of these receptors was modulated at different stages of culture. Figure 1(C) shows that both PDGFR-α and -β started decreasing upon being recultured with the initiation of DNA synthesis and cell proliferation, and then increased to a plateau level on days 4–6. This correlated with the point at which the cells became confluent and showed a low proliferative response. On the other hand, IGF-IR and EGFR expressions were relatively stable, and marked changes were not observed during the culture (Fig. 1C).

Expression of PDGFR-α, PDGFR-β, IGF-IR, and EGFR on periodontal ligament cells during mineralized nodule formation

It is well known that periodontal ligament cells produce mineralized nodules in the presence of dexamethasone and

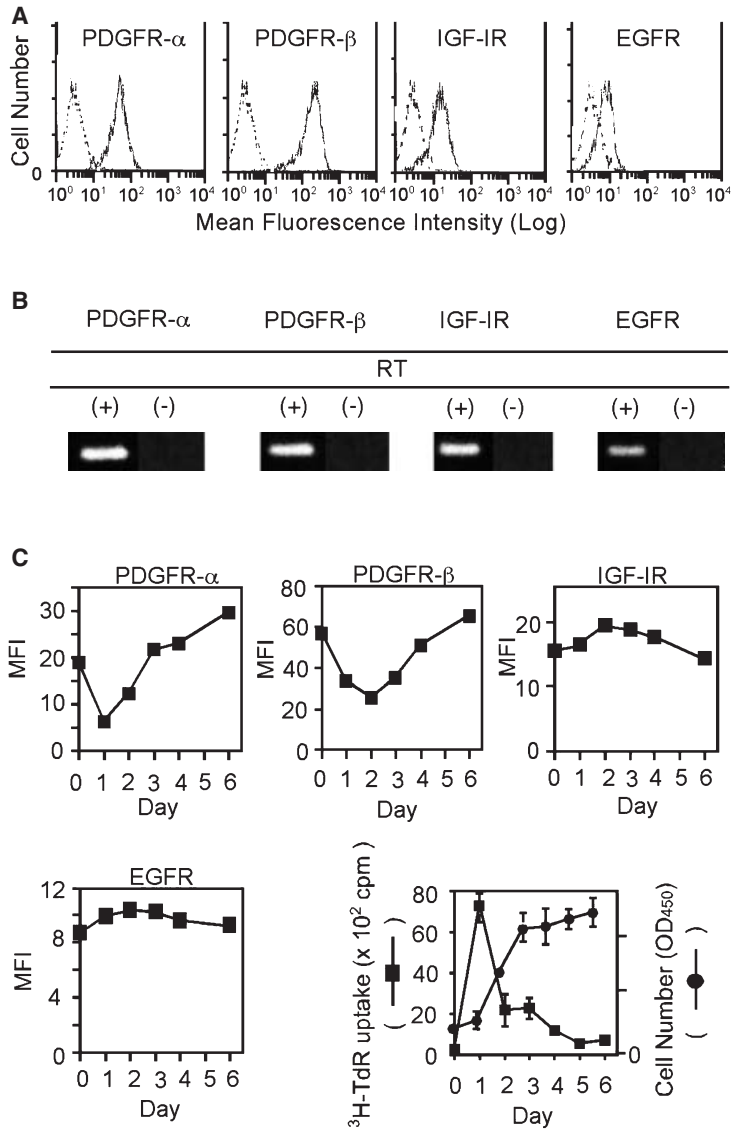

Fig. 1. Expression of platelet-derived growth factor receptor-α (PDGFR-α), PDGFR-β, insulin-like growth factor-I receptor (IGF-IR), and epidermal growth factor receptor (EGFR) on periodontal ligament cells. (A) Periodontal ligament cells were collected from confluent monolayers using Cell Dissociation Solution®. Expressions of PDGFR-α, PDGFR-β, IGF-IR, and EGFR on the cell surface were assessed by flow cytometry as described in Materials and Methods. Isotype-matched antibody was used as the negative control (broken line). (B) Total RNA was extracted from confluent monolayers. Expression of PDGFR-α (891 bp), PDGFR-β (688 bp), IGF-IR (537 bp), and EGFR (530 bp) mRNA were assessed by reverse transcription-polymerase chain reaction (RT-PCR) (+). For the negative control, RT was omitted from the RT-PCR reaction (–). (C) After periodontal ligament cells were harvested from confluent cultures (day 0), they were recultured in 24-well plates at 5×10^4 cells/well in 1 ml culture medium. At the times indicated, the cells were collected, stained with each monoclonal antibody, and analyzed by FACS, or [^{3}H]thymidine ([^{3}H]Tdr) incorporation into cells and cell number were counted as described in Materials and Methods and expressed as the mean ± SE of cpm or OD_{450} of triplicate assays. Findings are representative of three independent experiments with three different donors. MFI, mean fluorescence intensity.

ascorbic acid (3, 4). We investigated the possible change of growth factor receptor expression in periodontal ligament cells during mineralized nodule formation. Figure 2 shows that PDGFR-α was held at a lower level compared with the non-mineralizing (NON and GP groups) periodontal ligament cells. PDGFR-β decreased slightly in the early phase, and then remained at a slightly higher level. This increase correlated with the point at which alkaline phosphatase activity was markedly increased. The expression of PDGFR-β finally reversed to the non-mineralizing periodontal ligament cells level when the mineralized nodules were formed. In contrast to PDGFRs, the expression of IGF-IR and EGFR were substantially no different from that of the non-mineralizing periodontal ligament cells. IGF-IR expression was slight enhanced in the early phase and was kept at this level during the culture. EGFR expression was substantially unchanged during the culture. In this experiment, mineralized nodules were first detected at around day 15, the number and size gradually increased. Alkaline phosphatase activity increased gradually in a time-dependent manner on even non-mineralizing periodontal ligament cells. This response was markedly enhanced in the presence of dexamethasone and ascorbic acid (Fig. 2). This data suggested that these receptors could be involved in mineralized nodule formation.

Effect of blockers on PDGFR kinase, IGFR kinase, and EGFR kinase on mineralized nodule formation by periodontal ligament cells

Next, we examined whether selective tyrosine kinase blockers for each receptor could inhibit the mineralized nodule formation induced by dexamethasone/ascorbic acid. As shown in Fig. 3(A), AG1295 and AG1296, selective blockers for both PDGFR-α and -β kinase, markedly inhibited mineralized nodule formations in a concentration-dependent manner compared to the positive control (POS.). There were no mineralized nodules observed in the high concentration (100 μM) of both blockers, and there was still considerable inhibition in the low concentration (1 μM) of blockers. AG1024 and I-Ome-AG538, selective blockers for the IGF-IR kinase, partially inhibited mineralized

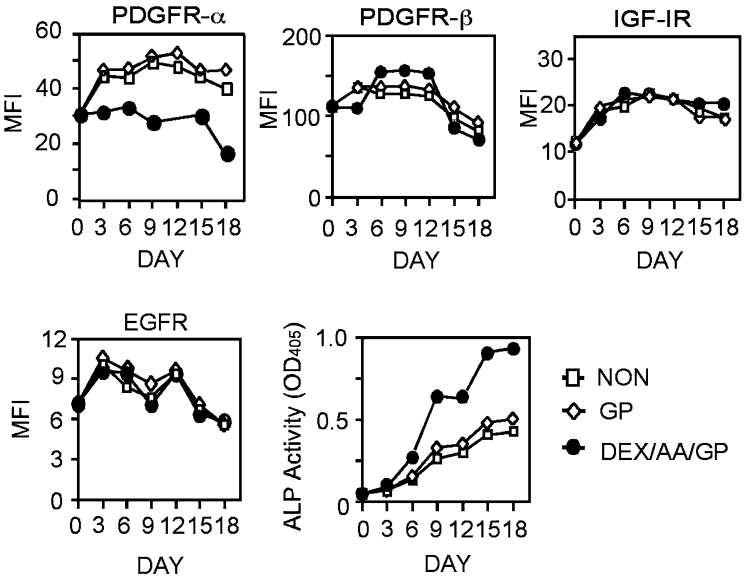

Fig. 2. Expressions of platelet-derived growth factor receptor-α (PDGFR-α), PDGFR-β, insulin-like growth factor-I receptor (IGF-IR), and epidermal growth factor receptor (EGFR) on periodontal ligament cells during mineralized nodule formation. Confluent periodontal ligament cells were cultured in α-MEM with 10% fetal bovine serum supplemented with nothing (NON), β-glycerophosphate (GP), or dexamethasone, ascorbic acid, and β-glycerophosphate (DEX/AA/GP) for 18 days. At the times indicated, the cells were collected, stained with anti PDGFR-α, -β, IGF-IR, and EGFR monoclonal antibody, and analyzed using FACS, or alkaline phosphatase (ALP) activity on periodontal ligament cells was measured as described in Materials and Methods. Findings are representative of three independent experiments with three different donors. MFI, mean fluorescence intensity.

nodule formations in a concentration-dependent manner, and AG1024 exhibited the formation more effectively than I-Ome-AG538 (Fig. 3). On the other hand, no inhibition of the formation was observed in any concentration of AG99, a selective blocker for the EGFR kinase. These data suggest that PDGFR-α, PDGFR-β, and IGF-IR, but not EGFR on periodontal ligament cells are involved in mineralized nodule formation by periodontal ligament cells.

Expression of PDGFs and IGFs on periodontal ligament cells during mineralized nodule formation

Since PDGFR-α, PDGFR-β, and IGF-IR on periodontal ligament cells were involved in mineralized nodule formation (Fig. 3), the ligands for these receptors should be co-expressed on periodontal ligament cells. To examine whether periodontal ligament cells can express PDGF-A, PDGF-B, PDGF-C, PDGF-D, IGF-I, and IGF-II mRNA during mineralized nodule formation, transcript levels were determined using semi-quantitative RT-PCR by normalizing the amount of PCR product for PDGFs and IGFs against that for β-actin (Figs 4B and C). Furthermore, the relative values of mRNA vs. β-actin from three independent experiments with three different donors were statistically analyzed by a paired *t*-test ($p < 0.05$) (data not shown). Figure 4(A) shows that PDGF-A, -B, -C, -D, IGF-I, and -II nucleotide were detected in the human multi-tissues cDNA library as template cDNA with each specific primer used in this study. As shown in Figs 4(B) and (C), PDGF-A and -C mRNA were expressed on both non-mineralizing (N) and mineralizing (M) periodontal ligament cells during the culture. Mineralizing periodontal ligament cells exhibited a slight increase or decrease expression of PDGF-A or PDGF-C, respectively, compared with non-mineralizing periodontal ligament cells, although a significant difference of PDGF-A or PDGF-C was observed only on days 5 and 17, or day 9, respectively. PDGF-B mRNA was not detected at all during the culture on both periodontal ligament cells. PDGF-D mRNA gradually increased and reached a plateau level on around day 13 in non-mineralizing periodontal ligament cells. Mineralizing stimulation appeared to accelerate this response and declined just after reaching a plateau with a significant difference on days 1, 17, and 21. IGF-I mRNA, which was expressed as faint band on day 1, was markedly up-regulated during the culture in non-mineralizing periodontal ligament cells. On the other hand, the mineralizing stimulation strongly suppressed this response during the culture ($p < 0.05$). IGF-II mRNA, which was expressed as a faint band on day 1, was significantly ($p < 0.05$) (Fig. 4C) up-regulated upon mineralizing stimulation of periodontal ligament cells compared with non-mineralizing periodontal ligament cells during the culture, with a peak response around day 13 (Fig. 4C). These findings suggested that the autocrine pathways of PDGFs and IGFs might exist for the formation and that coordinated expression of PDGFs and IGFs also might be involved.

Discussion

We demonstrated that PDGFR-α, -β, and IGF-IR were expressed on periodontal ligament cells at protein and mRNA levels. These findings confirmed previous reports that PDGFR-α and -β are present on cultured human periodontal ligament cells at the protein level (36) and that PDGF-AA, -AB, -BB, and IGF-I have mitogenic activity in human periodontal ligament cells *in vitro* (22, 36). In contrast to these results, it was reported that cultured periodontal ligament cells express PDGFR-β but not PDGFR-α (37). Since the expression of PDGFR-α and -β are higher in dense than in sparse cultures in human fibroblasts (38), and a similar response was observed in this study (Fig. 1C), the discrepancy may be explained by the fact that the expression of PDGFR-α and -β could be modulated at different

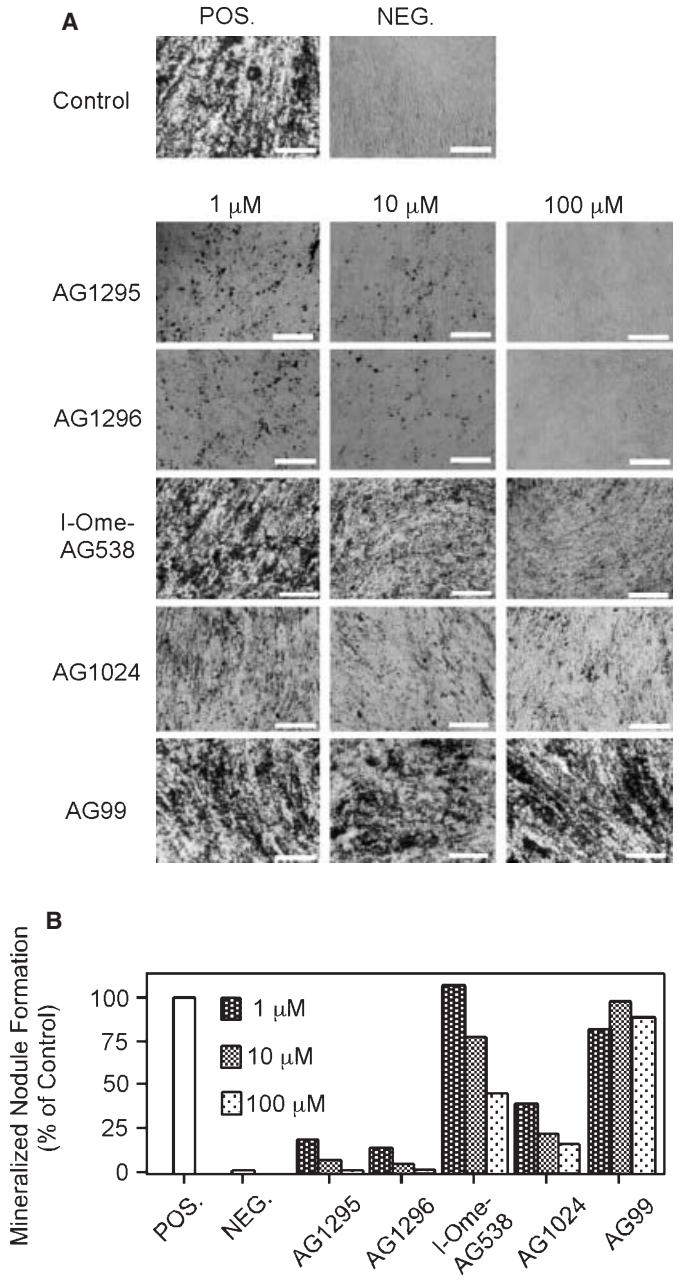

Fig. 3. Effect of tyrphostins on mineralized nodule formation by periodontal ligament cells. (A) Confluent periodontal ligament cells were cultured in medium containing dexamethasone, ascorbic acid, and β-glycerophosphate for 21 days in the absence (POS.) or presence of the indicated concentration of tyrphostins, AG1295, AG1296, AG538, AG1024, and AG99, and subjected to von Kossa staining. For the negative control (NEG.), periodontal ligament cells were cultured in medium containing β-glycerophosphate only. Findings are representative of three independent experiments with three different donors. Scale bars represent 500 μm. (B) The images of (A) were converted to binary using NIH Image software and the nodule areas stained with silver nitrate were quantified.

stages or by the degree of confluency of culture. EGFR is suggested to be expressed on undifferentiated periodontal ligament cells, to act as a negative regulator of osteoblastic differentiation in periodontal ligament cells (39), and to be down-regulated on differentiation *in vitro* (39). This possibly explains our finding that only part of the population of periodontal ligament cells expressed only low amounts of EGFR (Fig. 1).

At this point, it is unclear how the coordinated expression of the low level of PDGFR-α and the slightly high level of PDGFR-β shown in Fig. 2 could be associated with dexamethasone/ascorbic acid-induced periodontal ligament cells differentiation. Previous reports, which are consistent with this finding, have shown that a regenerating periodontal tissue exhibits an absent expression of PDGFR-α and a stronger positive expression of PDGFR-β compared to normal periodontal ligament tissue in humans (38), and that expression of PDGFR-α on rat calvarial osteoblastic cells is lower during mineralized nodule formation compared to when the formation is suppressed by the continuous treatment of PDGF-BB (40). Therefore, the coordinated expression of PDGFR-α and -β might have an important role in dexamethasone/ascorbic acid-induced periodontal ligament cells differentiation.

Tyrphostins are a family of synthetic protein tyrosine kinase blockers. AG1295 and AG1296 selectively inhibit both PDGFR-α and -β kinase and PDGF-dependent DNA synthesis with IC_{50} values below 5 and 1 μM in reversible mode (41), respectively. AG1296 shows complete insensitivity to the receptors that are structurally related to PDGFR such as EGFR, insulin receptors (InsR), and vascular endothelial growth factor receptors (41). AG1024 (42) and I-Ome AG538 (43) selectively inhibit IGF-IR autophosphorylation in intact cells with IC_{50} values of 7 μM and 3.4 μM, respectively. Since many of the structural features of IGF-IR are similar to those of the InsR (17), I-OMe AG538 inhibits the InsR with IC_{50} values similar to those for IGF-IR kinase inhibition (43), and AG1024 shows significantly lower IC_{50} for IGF-IR than for InsR (42). AG99 (A46) selectively inhibits EGFR kinase, ERK1 and ERK2 activity, and EGF-dependent DNA synthesis with an IC_{50} value of 10 μM (44). In the present study we demonstrated that mineralized nodule formation was strongly inhibited by AG1295 and AG1296, partially inhibited by I-Ome-AG538 and AG1024, and not inhibited by AG99, suggesting that a signaling pathway through PDGFR and IGF-IR, but not EGFR, could be

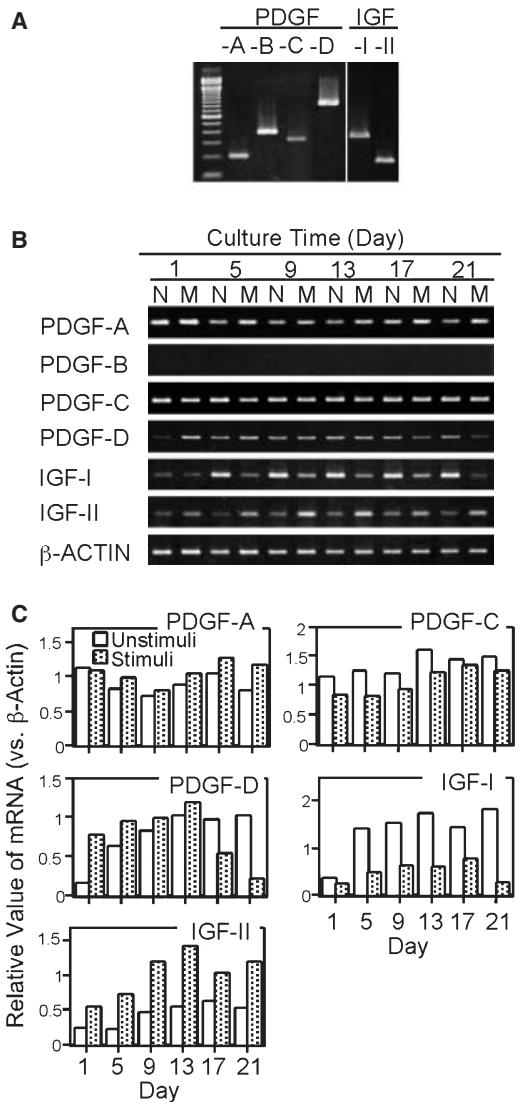

Fig. 4. Expression of platelet-derived growth factor-A (PDGF-A), PDGF-B, PDGF-C, PDGF-D, insulin-like growth factor-I (IGF-I), and IGF-II mRNA on periodontal ligament cells during mineralized nodule formation. (A) Polymerase chain reaction (PCR) showing PDGF-A, PDGF-B, PDGF-C, PDGF-D, IGF-I, and IGF-II using the human multi-tissue cDNA library as template cDNA. PCR product sizes are 237 bp, 435 bp, 350 bp, 918 bp, 395 bp, and 196 bp, respectively. (B) Confluent periodontal ligament cells were cultured in mineralizing (M) or non-mineralizing (N) condition for 21 days. Total cellular RNA was extracted at the indicated time. Expression of PDGF-A, PDGF-B, PDGF-C, PDGF-D, IGF-I, IGF-II, and β-actin mRNA were assessed by semiquantitative RT-PCR. Findings are representative of three independent experiments with three different donors. (C) Quantitation of mRNA levels in (B) via densitometry scanning using NIH Image software. Relative mRNA expression levels were normalized to β-actin expression. Findings are representative of three independent experiments with three different donors.

involved in dexamethasone/ascorbic acid-induced periodontal ligament cells differentiation. Since the IGF-IR kinase blocker used in this study can act on InsR kinase (42, 43), and IGF-II which can act through either IGF-IR or InsR (45) was induced in periodontal ligament cell differentiation (Fig. 4), the possible involvement of signaling via InsR for periodontal ligament cells differentiation cannot be excluded.

In the present study, IGF-I decreased and IGF-II increased compared to the non-mineralizing periodontal ligament cells during mineralized nodule formation. This differential expression of IGF-I and IGF-II has been observed during the dexamethasone-induced differentiation of osteoblasts from human bone marrow stromal cells (46), and IGF-II gene expression is up-regulated in the dexamethasone-induced myogenic differentiation of C2C12 myoblasts (47). Although IGF-I shows mitogenic activity in periodontal ligament cells (20), IGF-I was reported to have only a limited effect on periodontal ligament cells *in vitro* (19, 21) and *in vivo* (23). These reports and our findings suggest that IGF-II may play more important roles rather than IGF-I in mineralized nodule formation by periodontal ligament cells.

We demonstrated that PDGF-B was not expressed on periodontal ligament cells during the culture. It was reported that continuous treatment with PDGF-BB has been shown to decrease in biomineralization induced by fetal rat calvarial osteoblasts (40) and mouse cementoblasts (48). These reports and our finding suggested that PDGF-B has potential negative effect on periodontal ligament cells differentiation, although PDGF-BB shows mitogenic and chemotactic activities for periodontal ligament cells (19–22). Although PDGF-C and PDGF-D have been shown to induce proliferation of fibroblasts (9, 11), neither their actions toward periodontal ligament cells nor expressions on periodontal ligament cells were known. Previous studies show that the pattern of PDGF-C expression is distinct from those of PDGF-A and PDGF-B (11), and also PDGF-D has a localization that is distinct from that of PDGF-B (9, 10), indicating that they may provide distinct signaling to PDGFR-α-expressing and PDGFR-β-expressing cells, respectively. Here we first demonstrated that PDGF-C and PDGF-D as well as PDGF-A were expressed on cultured periodontal ligament cells, suggesting that an autocrine pathway of PDGFs might be involved in periodontal ligament cells differentiation. Furthermore, we found that PDGF-D expression on mineralizing periodontal ligament cells appeared to be enhanced at an early phase of mineralized nodule formation compared to normal

periodontal ligament cells, suggesting that PDGF-D may have an important role at the early phase of periodontal ligament cells differentiation. However, considering that non-mineralizing periodontal ligament cells substantially expressed PDGFs although the pattern of expressions was different from the dexamethasone/ascorbic acid-stimulated periodontal ligament cells, dexamethasone/ascorbic acid-dependent additional unknown factor(s) would be required in order to acquire mineralizing-tissue forming activity for periodontal ligament cells. It has been reported that the formation of, and linkage between, type I collagen and non-collagen matrix protein is crucial for mineral nucleation in extracellular matrix (49–52). Dexamethasone and ascorbic acid have been reported to induce expression of several proteins and to regulate the formation of extracellular matrix, which could affect mineralization *in vitro* (6–8). Cell adhesion to the extracellular Matrix is mediated by integrins, a family of heterodimeric transmembrane proteins comprising at least 16 α and eight β subunits in mammals (53), and integrins are required for growth factor-induced biological processes (54), suggesting that expressions of appropriate extracellular matrix and integrins on periodontal ligament cells might be involved in mineralized nodule formation as essential co-operating factor(s).

In conclusion, the present findings may provide a viewpoint to help clarify the mechanism of differentiation of periodontal ligament cells into acquiring a mineral forming activity, and also may lead to a prediction of optimal conditions, such as types of essential growth factor(s) and appropriate time of administration, for periodontal tissue regeneration.

Acknowledgements

We thank Dr S. Sugawara for the helpful discussions and Dr R. Kunii for preparing the digital images. This work was supported in part by Grants-in-Aid for Scientific Research (13557187), Encouragement of Young Scientists (14771215) from the Japan Society for the Promotion of Science, and TAKEDA science foundation. We also thank D. Mrozek (Medical English Service, Kyoto, Japan) for reviewing the article.

References

1. MacNeil RL, Somerman MJ. Development and regeneration of the periodontium: parallels and contrasts. *Periodontol 2000* 1999;**19:**8–20.
2. Nojima N, Kobayashi M, Shionome M, Takahashi N, Suda T, Hasegawa K. Fibroblastic cells derived from bovine periodontal ligaments have the phenotypes of osteoblasts. *J Periodont Res* 1990;**25:**179–185.
3. Cho MI, Matsuda N, Lin WL, Moshier A, Ramakrishnan PR. *In vitro* formation of mineralized nodules by periodontal ligament cells from the rat. *Calcif Tissue Int* 1992;**50:**459–467.
4. Arceo N, Sauk JJ, Moehring J, Foster RA, Somerman MJ. Human periodontal cells initiate mineral-like nodules in vitro. *J Periodontol* 1991;**62:**499–503.
5. Chien HH, Lin WL, Cho MI. Interleukin-1β-induced release of matrix proteins into culture media causes inhibition of mineralization of nodules formed by periodontal ligament cells *in vitro*. *Calcif Tissue Int* 1999;**64:**402–413.
6. Ramakrishnan PR, Lin WL, Sodek J, Cho MI. Synthesis of noncollagenous extracellular matrix proteins during development of mineralized nodules by rat periodontal ligament cells *in vitro*. *Calcif Tissue Int* 1995;**57:**52–59.
7. Lin WL, Chien HH, Cho MI. *N*-Cadherin expression during periodontal ligament cell differentiation in vitro. *J Periodontol* 1999;**70:**1039–1045.
8. Chien HH, Lin WL, Cho MI. Expression of TGF-β isoforms and their receptors during mineralized nodule formation by rat periodontal ligament cells *in vitro*. *J Periodont Res* 1999;**34:**301–309.
9. LaRochelle WJ, Jeffers M, McDonald WF *et al*. PDGF-D, a new protease-activated growth factor. *Nat Cell Biol* 2001;**3:**517–521.
10. Bergsten E, Uutela M, Li X, Pietras K *et al*. PDGF-D is a specific, protease-activated ligand for the PDGF β-receptor. *Nat Cell Biol* 2001;**3:**512–516.
11. Li X, Ponten A, Aase K *et al*. PDGF-C is a new protease-activated ligand for the PDGF α-receptor. *Nat Cell Biol* 2000;**2:**302–309.
12. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 1999;**79:**1283–1316.
13. Lasky JA, Coin PG, Lindroos PM, Ostrowski LE, Brody AR, Bonner JC. Chrysotile asbestos stimulates platelet-derived growth factor-AA production by rat lung fibroblasts in vitro: evidence for an autocrine loop. *Am J Respir Cell Mol Biol* 1995;**12:**162–170.
14. Rydziel S, Canalis E. Expression and growth factor regulation of platelet-derived growth factor B transcripts in primary osteoblast cell cultures. *Endocrinology* 1996;**137:**4115–4119.
15. Nagaoka I, Honma S, Someya A, Iwabuchi K, Yamashita T. Differential expression of the platelet-derived growth factor-A and -B genes during maturation of monocytes to macrophages. *Comp Biochem Physiol B* 1992;**103:**349–356.
16. Gilbertson DG, Duff ME, West JW *et al*. Platelet-derived growth factor C (PDGF-C), a novel growth factor that binds to PDGF α and β receptor. *J Biol Chem* 2001;**276:**27406–27414.
17. LeRoith D, Werner H, Beitner-Johnson D, Roberts CT Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev* 1995;**16:**143–163.
18. Cohick WS, Clemmons DR. The insulin-like growth factors. *Annu Rev Physiol* 1993;**55:**131–153.
19. Rutherford RB, TrailSmith MD, Ryan ME, Charette MF. Synergistic effects of dexamethasone on platelet-derived growth factor mitogenesis *in vitro*. *Arch Oral Biol* 1992;**37:**139–145.
20. Ivanovski S, Haase HR, Bartold PM. Isolation and characterization of fibroblasts derived from regenerating human periodontal defects. *Arch Oral Biol* 2001;**46:**679–688.
21. Matsuda N, Lin WL, Kumar NM, Cho MI, Genco RJ. Mitogenic, chemotactic, and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors in vitro. *J Periodontol* 1992;**63:**515–525.
22. Oates TW, Rouse CA, Cochran DL. Mitogenic effects of growth factors on human periodontal ligament cells in vitro. *J Periodontol* 1993;**64:**142–148.
23. Giannobile WV, Hernandez RA, Finkelman RD *et al*. Comparative effects of platelet-derived growth factor-BB and insulin-like growth factor-I, individually and in combination, on periodontal regeneration in Macaca fascicularis. *J Periodont Res* 1996;**31:**301–312.
24. Lynch SE, Williams RC, Polson AM *et al*. A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration. *J Clin Periodontol* 1989;**16:**545–548.
25. Cochran DL, Wozney JM. Biological mediators for periodontal regeneration. *Periodontol 2000* 1999;**19:**40–58.
26. Howell TH, Fiorellini JP, Paquette DW, Offenbacher S, Giannobile WV, Lynch SE. A phase I/II clinical trial to evaluate a combination of recombinant human

platelet-derived growth factor-BB and recombinant human insulin-like growth factor-I in patients with periodontal disease. *J Periodontol* 1997;**68:**1186–1193.
27. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network. receptor heterodimerization in development and cancer. *EMBO J* 2000;**19:**3159–3167.
28. Matsuda N, Morita N, Matsuda K, Watanabe M. Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP kinases in response to epidermal growth factor, hypoxia, and mechanical stress in vitro. *Biochem Biophys Res Commun* 1998;**249:**350–354.
29. Basciani S, Mariani S, Arizzi M *et al*. Expression of platelet-derived growth factor-A (PDGF-A), PDGF-B, and PDGF receptor-α and -β during human testicular development and disease. *J Clin Endocrinol Metab* 2002;**87:**2310–2319.
30. Yang Y, Niu J, Guo L. The effects of antisense insulin-like growth factor-I receptor oligonucleotide on human cord blood lymphocytes. *J Mol Endocrinol* 2002;**28:**207–212.
31. Naruse I, Ohmori T, Ao Y, *et al*. Antitumor activity of the selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) Iressa (ZD1839) in an EGFR-expressing multidrug-resistant cell line *in vitro* and *in vivo*. *Int J Cancer* 2002;**98:**310–315.
32. Zwerner JP, May WA. PDGF-C is an EWS/FLI induced transforming growth factor in Ewing family tumors. *Oncogene* 2001;**20:**626–633.
33. Changsirikulchai S, Hudkins KL, Goodpaster TA *et al*. Platelet-derived growth factor-D expression in developing and mature human kidneys. *Kidney Int* 2002;**62:**2043–2054.
34. Chatzistamou I, Schally AV, Varga JL *et al*. Antagonists of growth hormone-releasing hormone and somatostatin analog RC-160 inhibit the growth of the OV-1063 human epithelial ovarian cancer cell line xenografted into nude mice. *J Clin Endocrinol Metab* 2001;**86:**2144–2152.
35. Shi S, Robey PG, Gronthos S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. *Bone* 2001;**29:**532–539.
36. Oates TW, Kose KN, Xie JF, Graves DT, Collins JM, Cochran DL. Receptor binding of PDGF-AA and PDGF-BB, and the modulation of PDGF receptors by TGF-β, in human periodontal ligament cells. *J Cell Physiol* 1995;**162:**359–366.
37. Parkar MH, Kuru L, Giouzeli M, Olsen I. Expression of growth-factor receptors in normal and regenerating human periodontal cells. *Arch Oral Biol* 2001;**46:**275–284.
38. Paulsson Y, Karlsson C, Heldin CH, Westermark B. Density-dependent inhibitory effect of transforming growth factor-β 1 on human fibroblasts involves the down-regulation of platelet-derived growth factor α-receptors. *J Cell Physiol* 1993;**157:**97–103.
39. Matsuda N, Kumar NM, Ramakrishnan PR, Lin WL, Genco RJ, Cho MI. Evidence for up-regulation of epidermal growth-factor receptors on rat periodontal ligament fibroblastic cells associated with stabilization of phenotype in vitro. *Arch Oral Biol* 1993;**38:**559–569.
40. Yu X, Hsieh SC, Bao W, Graves DT. Temporal expression of PDGF receptors and PDGF regulatory effects on osteoblastic cells in mineralizing cultures. *Am J Physiol* 1997;**272:**C1709–C1716.
41. Kovalenko M, Gazit A, Bohmer A *et al*. Selective platelet-derived growth factor receptor kinase blockers reverse *sis*-transformation. *Cancer Res* 1994;**54:**6106–6114.
42. Parrizas M, Gazit A, Levitzki A, Wertheimer E, LeRoith D. Specific inhibition of insulin-like growth factor-1 and insulin receptor tyrosine kinase activity and biological function by tyrphostins. *Endocrinology* 1997;**138:**1427–1433.
43. Blum G, Gazit A, Levitzki A. Substrate competitive inhibitors of IGF-1 receptor kinase. *Biochemistry* 2000;**39:**15705–15712.
44. Pai R, Ohta M, Itani RM, Sarfeh IJ, Tarnawski AS. Induction of mitogen-activated protein kinase signal transduction pathway during gastric ulcer healing in rats. *Gastroenterology* 1998;**114:**706–713.
45. Frasca F, Pandini G, Scalia P *et al*. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol Cell Biol* 1999;**19:**3278–3288.
46. Cheng SL, Zhang SF, Mohan S *et al*. Regulation of insulin-like growth factors I and II and their binding proteins in human bone marrow stromal cells by dexamethasone. *J Cell Biochem* 1998;**71:**449–458.
47. Yoshiko Y, Hirao K, Maeda N. Dexamethasone regulates the actions of endogenous insulin-like growth factor-II during myogenic differentiation. *Life Sci* 1998;**63:**77–85.
48. Saygin NE, Tokiyasu Y, Giannobile WV, Somerman MJ. Growth factors regulate expression of mineral associated genes in cementoblasts. *J Periodontol* 2000;**71:**1591–1600.
49. Christoffersen J, Landis WJ. A contribution with review to the description of mineralization of bone and other calcified tissues in vivo. *Anat Rec* 1991;**230:**435–450.
50. Desbois C, Karsenty G. Osteocalcin cluster: implications for functional studies. *J Cell Biochem* 1995;**57:**379–383.
51. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. *Proc Natl Acad Sci USA* 1993;**90:**8562–8565.
52. Veis A. Mineral–matrix interactions in bone and dentin. *J Bone Miner Res* 1993;**8**(Suppl 2)**:**S493–S497.
53. Giancotti FG, Ruoslahti E. Integrin signaling. *Science* 1999;**285:**1028–1032.
54. Eliceiri BP. Integrin and growth factor receptor crosstalk. *Circ Res* 2001;**89:**1104–1110.