Stimulation of Alkaline Phosphatase Activity by PGE₂ through Induction of IGF-1 in Human Dental Pulp Cells

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Abstract
Prostaglandin E₂ (PGE₂), which is considered to be an important local factor in bone metabolism, stimulates alkaline phosphatase (ALP) activity in dental pulp (HDP) cells at lower concentrations. Moreover, insulin-like growth factors (IGFs) are widely expressed abundant autocrine and paracrine factors that regulate the proliferation and differentiation of various cells. PGE₂ is also a potent stimulator of IGFs synthesis in bone formation. In this study, we examined the effect of PGE₂ on IGF induction in HDP cells. PGE₂ increased IGF-1 and IGFBP-5 mRNA level and production as compared to the control, and IGF-1 and IGFBP-5 significantly increased the ALP activities in a dose-dependent manner. On the other hand, the addition of the anti-IGF-1 antibody decreased ALP activity by PGE₂. These findings suggest that PGE₂ stimulates the formation of tertiary dentin through induction of IGF-1 and IGFBP-5 expressions in HDP cells.

Key word: PGE₂, IGF-1, ALP, dental pulp cells

Introduction
Dental pulp is a loose connective tissue characterized by complete enclosure within a mineralized tissue, dentin (1). Human dental pulp (HDP) cell cultures produce mineralized tissue, and morphological studies have demonstrated odontoblast-like cytodifferentiation of HDP cells in culture (2).

When inflammation occurs in dental pulp, prostaglandin E₂ (PGE₂) is produced by HDP cells (3). PGE₂ plays a significant role in the local control of bone metabolism, stimulating both the formation and resorption of bone. In vivo and in vitro studies have shown that PGE₂ is largely involved in the regulation of bone formation (4-7) although its effect varies depending on the experimental conditions (5). PGE₂ is known to induce bone resorption in organ culture, particularly at high concentrations, but PGE₂ induced bone formation in fetal rat calvariae at low concentrations (4, 5). However, the effect of PGE₂ on HDP cells has not been established. Our previous study investigated the effect of PGE₂ on alkaline phosphatase (ALP) activity, a marker enzyme for odontoblast differentiation in HDP cells (8). Low concentrations of PGE₂ (0.1 μM) increased ALP activity and mRNA levels, and calcified nodule formation was observed in HDP cells. However, high concentrations of PGE₂ (10 μM) decreased ALP activity (8). These findings suggest that low concentrations of PGE₂ promotes mineralization in HDP cells.

On the other hand, growth factors play an important role in the mineralization of dental pulp. McCarthy et ale reported that PGE₂ stimulated insulin-like growth factor (IGF)-I expression in rat calvaria cells (9, 10). IGF-1 is a ubiquitous growth factor that is involved in the regulation of metabolism, cell growth, differentiation (11), bone formation and stimulation of collagen production in vitro and in vivo (12-14). From these reports, we assume that IGFs participate in mineralization in HDP cells.

Thusfar six IGF-binding proteins (IGFBPs) have been characterized. IGFBPs are thought to modulate IGF action by prolonging IGF stability, possibly by sequestering the growth factor and protecting it from proteolytic degradation, and by influencing ligand-receptor interactions. Consequently, IGFBPs may delay, potentiate, or inhibit IGF activity (15). Although the exact function of IGFBPs in HDP cells is unknown, IGFBP-5 is known to increase bone cell growth and enhance the action of IGFs (16).

This study investigated the role of IGFs in the promotion of ALP activity by PGE₂, and attempted to clarify the mechanism(s) involved in the mineralization of HDP cells.
Materials and Methods

Cell culture

HDP cells were prepared by outgrowth from normal dental pulp obtained from the third molar extracted under aseptic conditions from a 20-year-old patient during orthodontic treatment. The patient gave informed consent before providing the sample. After the dental pulp was extracted, the tissue was minced, placed on a 35-mm tissue-culture dish and then covered with a sterile glass coverslip.

The culture medium used was α-Minimal Essential Medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) and antibiotics comprising 100 mg/ml Penicillin G (Meiji Seika, Tokyo, Japan), 1 mg/ml Kanamicine (Meiji Seika, Tokyo, Japan) and 1 mg/ml Fungisone (Gibco, Grand Island, NY). Cultures were maintained in an atmosphere of 5% CO₂ in air at 37°C.

When cell growth from the explant had reached confluence, the cells were detached with 0.05% trypsin (Gibco, Grand Island, NY) in phosphate-buffered saline (PBS) and subcultured in culture flasks. For the experiment, HDP cells from 6 to 9 passages were plated at 2 × 10⁵ cells/ml medium.

Treatment of cells with PGE₂

HDP cells were cultured in 24-well plates and 10-cm cell culture dishes at a seeding density of 1 × 10⁵ cells/ml medium without FCS for 6 days; the medium was changed every other day. The medium was supplemented with 0.1 μM PGE₂ (Biosciences Inc., La Jolla, CA).

RNA preparation

HDP cells, treated with or without PGE₂, were homogenized using TRIzol reagent (Life Technologies, Gaithersburg, MD). The organic phase containing protein was extracted with chloroform, and 2-propanol was added to the remaining aqueous phase for RNA precipitation. The precipitate was collected by centrifugation (15 000 rpm), and the RNA pellet was dissolved in diethylpyrocarbonate-treated H₂O (DEPC H₂O), measured by its absorbance at 260 nm, and stored at −80°C.

Reverse transcription-polymerase chain reaction for IGF-
1, IGF-2 and IGFBP-5

cDNA synthesis and amplification by reverse transcription-polymerase chain reaction (RT-PCR) were carried out with a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT). The PCR mixture was subjected to amplification with a GeneAmp PCR system 9600 (Perkin-Elmer) set at 94°C for 1 min, 60°C for 1 min and 72°C for 30 cycles, respectively. The primers for IGF-1, IGF-2, IGFBP-5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized. The PCR primers for amplification were designed with reference to the reported IGF-1, IGF-2, IGFBP-5 (17) and GAPDH cDNA sequences (18). The primers were as follows: 5' - CTTTTATTTCAACAAGCCCCACAGG - 3' (forward primer for IGF-1), 5' - TCCTTAGATCACAGCTCCGGA - 3' (reverse primer for IGF-1), 5' - CGCGGCCCTTCTACTCCAGCA - 3' (forward primer for IGF-2), 5' - AGCACAGTACGTCTCCAGGAGG - 3' (reverse primer for IGF-2), 5' - TTCTCGGCTCTTCTTCTTCACTG - 3' (forward primer for IGFBP-5), 5' - GCCGAGCAAGTCAAGATCGAGA - 3' (reverse primer for IGFBP-5), 5' - GCCGAGAATGAGATGAGT - 3' (forward primer for GAPDH) and 5' - ATGGACTGTGGTCATGAG - 3' (reverse primer for GAPDH).

PCR fragments were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Slot-blot analysis

The cell lysates (100 μl) were placed on a nitrocellulose membrane using a slot-blot manifold system, and the membrane was blocked with 5% non-fat dry milk in TBS for 30 min at room temperature. The membrane was then incubated with monoclonal antibodies against IGF-1, IGF-2 (R&D Systems Inc., Minneapolis, MN) and IGFBP-5 (Austral Biologicals, San Ramon, CA) for 12 h at room temperature and then washed. The membrane was then incubated with goat anti-mouse IgG antibody (BioRad, Hercules, CA), washed again, and the reactive bands were observed with an ECL plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK).

Treatment of IGF-1 and IGFBP-5

HDP cells were cultured in 24-well plates at a seeding density of 1 × 10⁵ cells/ml medium without FCS for 6 days, and the medium was changed every other day. The
medium was supplemented with 0, 10 and 100 ng/ml concentrations of IGF-1 (Sigma Chemical Co., St. Louis, MO) and IGFBP-5 (Genzyme Techne, MPLS, MN).

Treatment of antibodies against IGF-1

HDP cells were cultured in 24-well plates at a seeding density of $1 \times 10^5$ cells/ml medium without FCS for 6 days, and the medium was changed every other day. The medium was supplemented with 0.1 mM PGE$_2$ and 250 ng/ml of polyclonal antibodies against IGF-1 (Sigma Chemical Co., St. Louis, MO).

Assay of ALP activity

HDP cells cultured in 24-well plates were rinsed twice with PBS, and glycine-NaOH buffer (0.1 M, pH 10.5) was added. p-Nitrophenylphosphate (8 mM) containing metal ions ($Mg^{2+}, Zn^{2+}$) was then added, and the mixture was incubated for 15 min at 37°C as described by Ohshima et al. (19). The enzyme reaction was terminated by the addition of NaOH (1 M), and the absorbance of released p-nitrophenol was determined at 405 nm in a microplate reader (MTP-32; Corona, Ibaraki, Japan).

Statistical analysis

All values were calculated as mean values ±SD. Statistical significance was determined by the Student’s t test.

Results

Effect of PGE2 on IGF-1, IGF-2 and IGFBP-5 gene expression

In our previous study, 0.1 µM PGE$_2$ increased ALP activity and mRNA levels, and calcified nodule formation was observed in HDP cells. Therefore, to examine the role of PGE$_2$ as a regulator of IGF-1, IGF-2 and IGFBP-5 gene expression, the effect of 0.1 µM PGE$_2$ on IGF-1, IGF-2 and IGFBP-5 mRNA levels was examined in HDP cells. Figure 1 shows that the PCR products corresponding to GAPDH were similar in all RNA preparations, with the amount of PCR product reflecting the level of mRNA expression. After 48 h incubation of HDP cells, bands representing PCR products from PGE$_2$-stimulated RNA preparations were more intense than those from non-stimulated preparations.

Slot-blot analysis

Since expression of IGF-1 and IGF-2 mRNA was increased by PGE$_2$, the protein derived from HDP cells was analyzed by slot-blot to determine whether it was IGF-1, IGF-2 or IGFBP-5. Figure 2 shows that PGE$_2$ increased the production of IGF-1 and IGFBP-5 compared with the control, but there was no such increase in IGF-2. Therefore, PGE$_2$ increased the levels of IGF-1 and IGFBP-5 protein in HDP cells compared with untreated cells.

Effect of IGF-1 and IGFBP-5 on ALP activity

The dose-dependent effect of IGF-1 and IGFBP-5 on ALP...
Dose-dependent effect of IGF-I and IGFBP-5 on ALP activity in HDP cells. HDP cells were incubated for 6 days, and fresh medium containing each IGF-I or IGFBP-5 was added every other day. Values are the means ±SD (n = 6). *p<0.01, **p<0.05: significant difference from the control (without IGF-I or IGFBP-5).

Effect of anti-IGF-1 antibodies on ALP activity
Since IGF-1 plus IGFBP-5 and a low concentration (0.1 μM) of PGE_2 significantly increased ALP activity, the effect of neutralizing anti-IGF-1 antibodies on PGE_2 stimulation of ALP activity in HDP cells was investigated to clarify whether the effect of PGE_2 is mediated through the action of IGF-1. As shown in Fig. 5, the addition of anti-IGF-1 antibodies blocked the effect of PGE_2 on mineralization of HDP cells.

Discussion
PGE_2 is a multifunctional modulator of bone metabolism, with both stimulatory and inhibitory effects on bone formation and resorption. The ability of low concentrations of PGE_2 (0.1 μM) to stimulate bone formation has been demonstrated in vitro (5). The effect of PGE_2 on ALP activity has previously been examined in HDP cells. PGE_2 at low concentrations (0.1 μM) increased ALP activity and mRNA levels compared with PGE_2 at high concentrations (10 μM). Furthermore, calcified nodules were observed with low concentrations of PGE_2 (8). The present study was based on previous observations that the effects of PGE_2 on bone formation might be due to a mechanism involving increased production of IGFs (9, 10). Therefore, in the present study, the induction of IGFs by low concentrations of PGE_2 was examined in HDP cells.

PGE_2 was found to stimulate IGF-1 and IGFBP-5 mRNA levels in HDP cells in a time-dependent manner. Levels of IGF-1 mRNA increased after 48 h in HDP cells treated with PGE_2 compared with non-treated cells, while IGF-2 mRNA
levels did not change. Furthermore, IGF-1 and IGFBP-5 protein levels increased with PGE₂ after 96 h. These results are consistent with previous studies in osteoblasts (9, 20), and it is suggested that PGE₂ affects the synthesis and distribution of IGF-1 and IGFBP-5. PGE₂ increased IGF-1 production in primary cultures of cells from fetal rat calvariae enriched in osteoblasts (9). In the present study, similar effects of PGE₂ on IGF-1 levels were observed in HDP cells. This suggests that endogenous as well as exogenous PGE₂ may be important in maintaining IGF-1 production in HDP cells.

The differential effect of PGE₂ at low concentrations is mediated predominantly by an elevation in intracellular cAMP as a consequence of adenylate cyclase activation (21). Moreover, increased cAMP levels by PGE₁ also increased IGF-1 production in osteoblasts. It can be speculated that cAMP intervenes to elevate IGF-1 production by PGE₁ in HDP cells.

Pulp tissue has a high level of dentinogenesis, and ALP as a marker of mineralization could be important in repair mechanisms and healing after pulpal injury (22). In HDP cells, ALP activity increased in a dose-related fashion up to IGF-1 and IGFBP-5 concentrations of 100 ng/ml. Addition of IGFBP-5 to IGF-1 significantly increased ALP activity in HDP cells compared to the control. The exact function of IGFBP-5 in bone is not known, but it has been reported to stimulate bone cell growth and promote IGF-1 activity in bone cells (20). It has been demonstrated that IGF-1 induces the synthesis of IGFBP-5 in rat osteoblastic cells (23,24). Although there is a species difference between humans and rats, and a difference between osteoblastic cells and HDP cells, it is suggested that IGF-1 is associated with IGFBP-5 in HDP cells.

It has been reported that IGF-2 production is unaffected by PGE₂, whereas IGF-1 is induced by PGE₂ in osteoblasts (9). In this study, the potency of the stimulatory effect of PGE₂ on IGF-2 production was less than that of IGF-1. Therefore, it is suggested that IGF-1 participates in the increase in ALP activity with PGE₂ in HDP cells. It is also suggested that the high level of ALP activity induced by PGE₂ is mediated through the action of IGF-1. To clarify whether the effect of PGE₂ is mediated through the action of IGF-1, the effect of a neutralizing anti-IGF-1 antibody on PGE₂-stimulation of ALP activity was investigated in HDP cells. Anti-IGF-1 antibodies blocked IGF-1, which were synthesized by PGE₂, inhibiting ALP activity. Therefore, it is suggested that IGF-1 participate in the process in which ALP activity is increased by PGE₂.

HDP cells secrete autocrine and/or paracrine growth factors constitutively. The current findings clarify the role that IGF-1 plays in the regulation of HDP cell proliferation and differentiation.

When injuries such as attrition and abrasion to dentin and bacterial infection of pulp occur, a new mineralized scar tissue, tertiary dentin, is formed (1). However, the mechanism for this has not yet been fully described. HDP cells are capable of mineralization and are known to differentiate into odontoblasts (2). Because of the current findings, we speculate that IGF-1, induced by low concentrations of PGE₂ in HDP cells, promotes odontoblast differentiation and may be associated with the mechanism of tertiary dentin formation. Further studies are clearly required to prove the existence of IGF-1 in the neighborhood of odontoblasts and where tertiary dentin is being formed in vivo.

In conclusion, the present study demonstrates that PGE₂ stimulates the synthesis and secretion of IGF-1 and IGFBP-5 through a pathway that involves a transcriptional mechanism. Furthermore, IGFs participate in the increase in ALP activity by PGE₂. PGE₂ appears to stimulate mineralization and induce IGF-1 and IGFBP-5 formation in HDP cells.

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References