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RUNNING TITLE: The Role of PGE$_2$ and EPs in Cementoblasts

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Abstract

Objective: PGE$_2$ is an important factor in pathogenesis of periodontal disease because of bone resorbing activity and association with attachment loss. PGE$_2$ and PGE receptor subtypes (EPs) play an important role in modulating bone metabolism via osteoblasts. However, little is known about the effects of PGE$_2$ on cementoblasts. The aims of this study were to determine the expression of EPs on matured cementoblasts and to examine the effect of PGE$_2$ and EPs on their cellular function.

Design: Expression of EPs in immortalized mouse cementoblasts (OCCM-30), which were characterized as matured cementoblasts, was determined using RT-PCR. Then effects of PGE$_2$ and EP agonists on mineralization were examined by studying nodule formation with alizarin red S (ALZ) staining. Alkaline phosphatase (ALP) activity with PGE$_2$, EP4 agonist was examined by Bessey-Lowry enzymologic method. Effects of PGE$_2$-EP4 pathway on expression levels of osteocalcin (OCN) and matrix metalloproteinase (MMP)-13 mRNA were examined by real-time RT-PCR.

Results: OCCM-30 expressed EP1, 2, 3 and 4 mRNA. PGE$_2$ and EP4 agonist caused downregulation of mineralized nodule formation and ALP activity in OCCM-30. OCN mRNA expression was suppressed and MMP-13 mRNA expression was stimulated via PGE$_2$-EP4 pathway in OCCM-30.

Conclusions: Cementoblasts may downregulate their mineralization ability and upregulate MMP-13.
production through PGE$_2$-EP4 pathway and may contribute to destruction of connective tissue attachment under inflammatory condition.
Introduction

Cementum, a thin mineralized tissue covering the tooth root surface, assists in anchoring teeth to surrounding alveolar bone. It also contributes to the maintenance of structural stability and physiological function of the dentition. Several studies have demonstrated that cementoblasts share many characteristics with osteoblasts, including similar molecular properties and the ability to promote mineralization. In the periodontal disease area, osteoblasts function as osteoclastogenesis controlling cell rather than as bone forming cell. It is considered that cementoblasts may be responsible for destruction of connective tissue attachment. However, little has known about the functions of cementoblasts under inflammatory condition. Therefore, it is important to study the mechanisms controlling function of cementoblasts in order to assist in enhancing of our understanding the pathogenesis of periodontal disease.

It is well known that a variety of products such as prostaglandins E₂ (PGE₂), cytokines and chemokines from inflammatory cells and periodontal tissue resident cells may contribute to this destructive process. Especially, PGE₂ is focused as an important factor involved in the pathogenesis of periodontal disease, because of powerful stimulator of bone resorption. In fact, high levels of PGE₂ within the gingival crevicular fluid have been associated with marked attachment loss in patients with periodontitis.

Various biological actions of PGE₂ are mediated by PGE specific G-protein-coupled receptors. PGE receptors (EPs) are divided into 4 subtypes, EP1, EP2, EP3 and EP4.
Recently we reported that cementoblasts at proliferative culture stage were stimulated alkaline phosphatase (ALP) activity with PGE2 treatment. However, there is no data about expression of EPs mRNA in cementoblasts and detail report about roles of PGE2 and EPs on cellular function of matured cementoblasts.

In this study, to determine the effect of PGE2 and EPs on function of cementoblast, we investigated the expression of EPs in cementoblasts and examined the role of PGE2 on function of matured cementoblasts such as mineralization and ALP activity, osteocalcin (OCN) and matrix metalloproteinase (MMP)-13 mRNA expressions.

Materials and methods

Cell line and cell culture

OCCM-30 cells used for these studies were established by isolating tooth root surface cells from transgenic mice containing a SV40 large T-antigen under control of an OCN promoter and were characterized as highly differentiated cementoblasts. OCCM-30 cells were maintained in DMEM (NISSUI PHARMACEUTICAL CO., LTD., Tokyo, Japan) supplemented with 10 mM HEPES (pH 7.2) (Sigma-Aldrich, Tokyo, Japan), 10% fetal bovine serum (FBS) (Invitrogen Corporation, N.Y. U.S.A.) and 100 U/mL Penicillin-Streptomycin (Invitrogen Corporation, N.Y., U.S.A) at 37 °C in a humidified atmosphere of 5% CO2.
Reagents

PGE\textsubscript{2} was purchased from Advanced Magnetics Inc. (Massachusetts, USA). ONO-DI-004 (EP1 agonist), ONO-AE1-259-01 (EP2 agonist), ONO-AE-248 (EP3 agonist), ONO-AE1-329 (EP4 agonist) and ONO-AE3-208 (selective EP4 antagonist) were kindly provided from ONO Pharmaceuticals Co. Ltd. (Tokyo, Japan).

I. Gene Expression Experiments

i. Extraction of total mRNA

Expression of EPs: OCCM-30 cells were plated into 60 mm culture dishes (4×10\textsuperscript{5} cells/dish) and cultured in \(\alpha\)-MEM containing 10% FBS and ascorbic acid (AA) (50 \(\mu\)g/ml). At confluence, total RNA was extracted with TRIzol\textsuperscript{\textregistered} Reagent (Invitrogen).

Expression of OCN and MMP-13: OCCM-30 cells were plated in 6 well plates (4×10\textsuperscript{5} cells/well) and maintained in \(\alpha\)-MEM containing 10% FBS and AA (50 \(\mu\)g/ml). Upon reaching confluence, cells were switched to \(\alpha\)-MEM containing 2% FBS and AA (50 \(\mu\)g/ml) with PGE\textsubscript{2} (300 nM) or each EP agonist (1 \(\mu\)M). To determine the effects of PGE\textsubscript{2}-EP4 pathway, the cells were pretreated with EP4 antagonist (1 \(\mu\)M) for 2 hours prior to the addition of PGE\textsubscript{2} (300 nM). After treatment for 7 days, total RNA was extracted with TRIzol\textsuperscript{\textregistered} Reagent. After digesting contaminating genomic DNA with DEOXYRIBONUCLEASE I (SIGMA-Aldrich), total RNA was purified with Quiaquick\textsuperscript{\textregistered} PCR Purification Kit (QIAGEN KK, Tokyo, Japan).
ii. RT-PCR

cDNAs were synthesized from 1 μg of total RNA using Rever Tra Dash (TOYOBO CO. LTD., Osaka, Japan). Aliquots of total cDNA were amplified with KOD-Plus-DNA Polymerase (TOYOBO CO. LTD.). The amplification was performed using a MyCycler™ thermal cycler (BIO-RAD, Tokyo, Japan). PCR followed using primer pairs, annealing temperatures and reaction cycles listed in Table 1. PCR products were reduced on 1.5% agarose gels, electrophoresed at 100 mV and visualized by ethidium bromide.

iii. Quantitative real-time RT-PCR

cDNA was synthesized from 1 μg of total RNA as described above. Real-time RT-PCR was performed in the Light Cycler System (light cycler quick system 350S, Roche Diagnostics GmbH) using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) and specific primers for OCN and MMP-13 gene. The primer pairs and annealing temperatures used here listed in Table 1.

Reaction product was quantified (the LightCycler software version 3.5, Roche Diagnostics GmbH) with GAPDH as the reference gene. Each experiment was repeated four times with comparable results.

II. Mineralization

Mineral nodule formation was detected by alizarin red S (ALZ), which stains for calcium.
OCCM-30 cells were plated in 24 well plates (5×10^4 cells/well) and cultured in α-MEM supplemented with 10% FBS and AA (50 μg/ml) for 24 hours. Then medium was changed to α-MEM containing 2% FBS, AA (50 μg/ml), and sodium β−glycerophosphate (10 mM) with PGE_2 (300 nM) or each EP agonist (1 μM). After 3 weeks, the cells were fixed in a 3.5% formaldehyde neutral buffer solution, and then stained with ALZ.

### III. Activity of ALPase

OCCM-30 cells were plated into 24 well plates (3×10^3 cells/well) and cultured in α−MEM containing 10% FBS and AA (50 μg/ml). After 4 days, medium was changed to α−MEM containing 2% FBS and AA (50 μg/ml) and cells were treated with PGE_2 (30 nM, 300 nM) or each EP4 agonist (0.1 μM, 1 μM). At 7 days after treatment with regents, the quantitative analysis of ALP activity was performed by Bessey-Lowry enzymologic method (Alkaline-phospha B test; Wako, Osaka, Japan). Each experiment was repeated four times with comparable results.

### Statistical analysis

Each experiment was repeated four times with comparable results. Data are expressed as means ± SD for each group. Statistical differences between groups were evaluated by the multivariate
analysis of variance (ANOVA) at 0.05 levels. The fisher’s test was used as the post hoc test at the 5% level of significance.

Results

Expression of EPs

EP1, EP2, EP3 (α, β, γ) and EP4 mRNAs were expressed in OCCM-30 cells (Fig. 1A).

Mineralization

PGE2 (300 nM) and EP4 agonist (1 μM) markedly suppressed mineral nodule formation by OCCM-30 cells (Fig. 1B). Other EP agonist did not affect mineral nodule formation in OCCM-30 cells.

Effects of PGE2 and EP agonists on ALP activity

ALP activity was significantly suppressed with 30 nM and 300 nM of PGE2-stimulation at 7 days in OCCM-30 cells. EP4 agonist (1 μM) also significantly suppressed ALP activity at 7 days (Fig. 2).

Effects of PGE2 and EP4 agonists on expression of OCN and MMP-13 mRNA

To further clarify the cell phenotype, as a cementum forming cell or a tissue destruction regulating cell, after long term incubation with PGE2, and EP4 agonist, transcripts for OCN (mineralization
related genes) and MMP-13 (an important inducible MMP involved in degradation of the collagenous matrix of bone and cartilage) were analyzed. PGE\textsubscript{2} (300 nM) and EP4 agonist (1 μM) significantly suppressed OCN mRNA expression and significantly increased mRNA levels of MMP-13 compared to that of control (Fig. 3A). The effects of PGE\textsubscript{2} on the expression levels of OCN mRNA and MMP-13 mRNA were eliminated completely by treatment with EP4 antagonist (Fig. 3B).

Discussion

In the present study we report that EP1, EP2, EP3 (α, β, γ), and EP4 are expressed in the mouse cementoblast cell line, OCCM-30 cells. To our knowledge, this is the first report showing the expression of EPs in cementoblasts at mRNA level. The finding indicates that all PGE\textsubscript{2} –EP pathways exist in cementoblasts.

ALZ staining showed suppressive effects of PGE\textsubscript{2} and EP 4 agonist on mineralization in OCCM-30 cells. Under the same condition, other EP agonists did not show suppressive effect of the mineral nodule formation in OCCM-30 cells. So in further examination, we focused on the effects of EP4 on the function of cementoblasts.

Numerous reports have highlighted the effects of PGE\textsubscript{2} on ALP activity and mineralization in osteoblasts.\textsuperscript{12,13} It is well known that the effects of PGE\textsubscript{2} on ALP activity were mediated by EP2 and EP4 pathway.\textsuperscript{14} Stimulation of both EP2 and EP4 causes an upregulation of adenylate
cyclase/cAMP system. In the present study, PGE\textsubscript{2} and EP4 agonist significantly downregulated ALP activity in OCCM-30 cells at 7 days after treatment. There are at least two explanations for this downregulation of ALP activity by PGE\textsubscript{2} and EP4 agonist. One possibility is that these changes are related to the stage of cementoblast differentiation. It is well known that ALP expression and activity are increase with osteoblast maturation and then decreased with osteoid mineralization. Another possibility is that incubation with PGE\textsubscript{2} may alter the cellular function of OCCM-30 from cementum forming cell into tissue destruction modulating cells.

Therefore to clarify this point, we examined the effects of application of PGE\textsubscript{2} and EP4 agonist on the expression of OCN (mineralization related genes) and MMP-13 (an important inducible MMP involved in degradation of the collagenous matrix of bone and cartilage) mRNA. PGE\textsubscript{2} and EP4 agonist decreased expression of OCN mRNA, whereas upregulated mRNA level for MMP-13. And EP4 antagonist eliminated the effects of PGE\textsubscript{2} on OCN and MMP-13 mRNA expressions.

Parathyroid hormone (PTH) and PTH-related protein downregulated BSP expression through increasing cAMP in cementoblasts and periodontal ligament cells. As described above, PGE\textsubscript{2} stimulates cAMP via EP2 and EP4 pathway. Thinking together, it is suggested that PGE\textsubscript{2} downregulate OCN expression mainly via EP4-cAMP pathway in OCCM-30 cells. MMP-13 is expressed by differentiated phenotypes of the osteoblastic lineage. Inflammatory cytokines such as TNF-\(\alpha\) and IL-1\(\beta\) induced MMP-13 expression in chondrocytes and osteoblasts. PGE\textsubscript{2} also stimulated MMP-13 production by osteoblasts via EP4 pathway and contribute to
bone resorption. Osteoblasts obtained from patients with rheumatoid arthritis are known to produce proinflammatory cytokines and PGE\textsubscript{2} and considered to be involved in tissue destruction.

Previously, we demonstrated that topical application of LPS to rat periodontal tissues in vivo enhanced the immuno-expression of cyclooxygenase-2 (COX-2; a synthetic enzyme of inducible PGE\textsubscript{2}) and proinflammatory cytokines including TNF-\(\alpha\), IL-1\(\beta\) and IL-1\(\alpha\) in cementoblasts and osteoblasts in vivo periodontal tissue. Moreover, we demonstrated that IL-1\(\alpha\)-stimulated IL-6 production from OCCM-30 was upregulated by COX-2 dependent PGE\textsubscript{2}. And it was reported that PGE\textsubscript{2} induced osteoclasts formation mainly via EP4 pathway in osteoblasts. Therefore we consider that exposure to PGE\textsubscript{2} changes cellular function of OCCM-30 from a cementum forming cell type to destruction modulating cell type like osteoblasts.

Recently Camargo et al. showed that PGE\textsubscript{2}, EP1 and EP3 activators increased mineralization of OCCM-30. The reason for the discrepancy from our data may be differentiation stage of cementblast with PGE\textsubscript{2} stimulation. In this study, we stimulated cementoblasts at high-cellular condition after confluency, which seemed to be more matured cells than their report. Using MC3T3-E1 cells, Suda et al. reported that the effect of PGE\textsubscript{2} is quite different between cells at confluency and those 5 days after confluency. It was also indicated that responsiveness of primary osteoblasts to PGE\textsubscript{2} may change during the culture period. According to their results, PGE\textsubscript{2}-addition 3 days before confluency stimulated mineralization but addition of PGE\textsubscript{2} 2 days after confluency markedly suppressed it. Although PGE\textsubscript{2} may stimulate cell differentiation of
immature cementoblasts via EP1 and EP3, it may inhibit mineralization of highly differentiated
cementoblasts via EP4. There is a possibility that this changing in balance of EP expression levels
with cellular maturation caused these differences in responsiveness to PGE2. Further studies are
needed to clarify this point.

In conclusion, in mature cementoblasts, OCCM-30, PGE2-EP4 pathway downregulated
mineralization ability and upregulated MMP-13 production. The present study suggests that
mature cementoblasts located along tooth root surface may downregulate their mineralization
ability and positively contribute to loss of connective tissue attachment through destruction of
collagen via MMP production under inflammatory condition.

Acknowledgments

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TABLE LEGEND

Table 1. Oligonucleotide primer sequences utilized in the RT-PCR.
FIGURE LEGENDS

Fig. 1. Expression of PGE receptor subtypes (EPs) mRNAs in OCCM-30 cells (A). Effects of PGE\textsubscript{2} and EP4 agonist on mineralization in OCCM-30 cells (B). Cells were plated into 60 mm culture dishes (4×10\textsuperscript{5} cells/dish) and cultured in α−MEM (10% FBS and 50 μg/ml AA). Total RNA was extracted from confluent cells and expression of mRNA for EPs was examined by RT-PCR analysis. OCCM-30 cells expressed EP1, EP2, EP3\textalpha, EP3\textbeta, EP3\textgamma, and EP4 mRNAs (A). Mineral nodule formation was detected by alizarin red S (ALZ), which stains for calcium. Cells were placed in a 24 well plate at a density of 5 × 10\textsuperscript{4} cells per well and cultured in α-MEM (10% FBS and 50 μg/ml AA, 10mM sodium β−glycerophosphate) with PGE\textsubscript{2} (300 nM) or each EP agonist (1 μM). After 3 weeks, cells were fixed in a 3.5% formaldehyde neutral buffer solution, and then stained with ALZ.

Fig. 2. Effects of PGE\textsubscript{2} and EP4 agonist on ALP activity in OCCM-30 cells. OCCM-30 cells were plated into 24 well plates (3×10\textsuperscript{3} cells/well) and cultured in α−MEM (10% FBS and 50 μg/ml AA). After 4 days, medium was changed to α-MEM (2% FBS, 50 μg/ml AA, 10mM sodium β−glycerophosphate) with PGE\textsubscript{2} (30, 300 nM) or each EP agonist (0.1, 1 μM). At 7 days after treatment with reagents, the quantitative analysis of ALP activity was performed. PGE\textsubscript{2} and EP4 agonist significantly suppressed ALP activity in OCCM-30 cells. Data are expressed as the mean ± SD of four independent experiments. *; Significantly different from the culture with control (p<0.05),
**; (p<0.01).

**Fig. 3.** Effects of EP4 on OCN and MMP-13 mRNA expression in OCCM-30 cells. OCCM-30 cells were plated in 6 well plates (4×10^5 cells/well) and maintained in α–MEM (10% FBS and 50 μg/ml AA). Upon reaching confluence, cells were switched to α–MEM (2% FBS and 50 μg/ml AA) with PGE2 (300 nM) and EP4 agonist (1 μM). To determine the effects of EP4 antagonists, the cells were pretreated with EP4 antagonist (1 μM) for 2 hours prior to the addition of PGE2 (300 nM). After treatment for 7 days, total RNA was extracted and used to synthesize cDNA, and quantitative real-time RT-PCR was used to check expression of OCN (mineralization related genes) and MMP-13 (an important inducible MMP involved in degradation of the collagenous matrix of bone and cartilage) mRNA. Results were normalized to GAPDH as a reference gene. Data are expressed as the mean ± SD of four independent experiments. *; Significantly different from the culture with control (p<0.05), **; (p<0.01).
Fig. 1

(A)

OCCM-30

|-----|-----|------|------|------|-----|-------|

(B)

Fig. 2

Left graph:

- **X10^-5** bar graph
- **Y-axis:** unit/cell/min
- **X-axis:** PGE\_2 (nM)
- Data points: 0, 30, 300
- **ALP**
- **Bars:** **,**
- Error bars shown

Right graph:

- **X10^-5** bar graph
- **Y-axis:** unit/cell/min
- **X-axis:** EP4 agonist (μM)
- Data points: 0, 0.1, 1
- **ALP**
- **Bars:** **,*
- Error bars shown
Fig. 3

(A) OCN -EP4 agonist- and MMP-13
rel. to control (mRNA OCN/GAPDH) and rel. to control (mRNA MMP-13/GAPDH)

(B) OCN -EP4 antagonist- and MMP-13
rel. to control (mRNA OCN/GAPDH) and rel. to control (mRNA MMP-13/GAPDH)

- Control
- PGE₂
- EP4 agonist
- EP4 antagonist

* Indicates significance levels.
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Ta: annealing temperature