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Low magnitude high frequency vibration induces RANKL via cyclooxygenase pathway in human periodontal ligament cells *in vitro*

Sutiwa Benjakul[a], Chidchanok Leethanakul[b], Suwanna Jitpukdeebodintra[c,*]

[a] Orthodontic Section, Faculty of Dentistry, Thammasat University, Thailand
[b] Orthodontic Section, Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Thailand
[c] Department of Oral Biology, Faculty of Dentistry, Prince of Songkla University, Thailand

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ABSTRACT

Objective: This study aimed to examine the effects of PGE2 on RANKL expression in response to vibration and vibration in combination with compressive stress and characterise this transduction pathway in periodontal ligament (PDL) cells.

Methods: Cultured human PDL cells obtained from extracted premolar teeth (from six individuals) were subjected to three cycles of vibration (0.3 g, 30 Hz for 20 min every 24 h; V), compressive stress (1.5 g/cm^2, 48 h; C) or vibration in combination with compressive stress (VC). To investigate whether the expression of RANKL and PGE2 was COX-dependent, PDL cells were treated with indomethacin prior to the onset of mechanical stimulation. RANKL and OPG expressions were examined by quantitative real-time polymerase chain reaction (qPCR). Quantification of PGE2, soluble RANKL (sRANKL) and OPG productions were measured using enzyme-linked immunosorbent assay (ELISAs).

Results: All mechanical stresses (V, C and VC) significantly increased PGE2 and RANKL. OPG was not affected by vibration, but was downregulated in compressed cells (C and VC). Indomethacin abolished induction of RANKL and downregulated OPG in response to all mechanical stresses.

Conclusion: These results suggest that vibration, compressive stress and vibration in combination with compressive stress induce RANKL expression in human PDL cells by activating the cyclooxygenase pathway.

1. Introduction

Vibration with low magnitude high frequency (LMHF) can be used as an adjunctive method to accelerate orthodontic tooth movement.[1] Several studies have reported vibration effectively accelerates the rate of tooth movement,[1,2] though conflicting results have also been reported.[3,4] Moreover, the mechanisms that surrounding cells response to vibration were still unknown. Therefore, it is of interest to examine the mechanisms that the cells respond to vibration during the mimicking of orthodontic force application *in vitro*.

Mechanical stress stimulates several intracellular signalling molecules in the mechanotransduction pathways that alter gene expression and protein synthesis in periodontal ligament (PDL) cells.[5–7] Cyclooxygenase pathway; cyclooxygenase (COX)-2/ prostaglandin E2 (PGE2), is the major mechanotransduction pathway in PDL cells responded to mechanical stress.[5] PGE2 is one of an inflammatory cytokine produced by mechanically stimulated PDL cells. There were studies showed that PGE2 has an autocrine and paracrine functions that activates receptor activator of nuclear factor kappa-B ligand (RANKL) expression which promotes bone resorption.[5,8] The balance among RANKL and osteoprotegerin (OPG) in PDL cells regulates remodeling of alveolar bone during tooth movement.[9] A previous report showed upregulation of RANKL in PDL cells exposed to compressive stress was dependent on the cyclooxygenase pathway.[5,6] Our previous studies demonstrated that LMHF vibration upregulates COX-2, PGE2, interleukin (IL)-6, IL-8 and RANKL, and further increases the levels of these osteoclastogenic factors in compressed PDL cells.[10,11] However, the precise mechanisms that PDL cells respond to vibration on RANKL/OPG expression have not yet been determined. We proposed that the expression of RANKL in response to vibration may depend on the cyclooxygenase pathway.

This study aimed to examine the signalling pathway and involvement of PGE2 on RANKL expression in response to vibration and vibration in combination with compressive stress in human PDL cells. Indomethacin; a non-selective COX inhibitor was used to investigate the mechanism involved.[5]

* Corresponding author. Department of Oral Biology, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand.
E-mail address: suwanna.ji@psu.ac.th (S. Jitpukdeebodintra).

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2. Materials and methods

2.1. Isolation and culture of human PDL cells

The protocol for this experiment was accepted by the Institutional Ethics Committee Board of the Prince of Songkla University (EC5803-06-P-LR). Informed consent was obtained from all donors. Human PDL cells were taken from the middle third of the tooth root of healthy and non-carious premolar teeth of six individuals (3 females and 3 males; at age 18–20 yrs), extracted in the course of orthodontic treatments. PDL cells were cultured in normal culture medium (NCM, 10% foetal bovine serum supplemented Dulbecco's modified essential medium; Gibco BRL, Grand Island, NY, USA) as described in our previous study.[10] Cellular morphology, expression of the PDL cell-specific genes periostin, fibromodulin and scleraxis, and ability to initiate calcification after culture in osteogenic medium were assessed as previously described.[10] All experiments were done in triplicate using third to fifth passage PDL cells from six independent donors.

2.2. Application of mechanical stress and COX-2 inhibitor

PDL cells from each donor were transferred into 6-well plates (1×10^5 cells/well) and cultured in NCM. Cells were allowed to reach 70–80% confluence, then the cell cycle was synchronized as previously described.[10] Cells were randomly allocated to: control without mechanical stress (Con), vibration (V), compressive stress (C) and vibration in combination with compressive stress (VC).

The mechanical stimulation protocol was based on our previous study.[10] A GJX-5 vibration calibrator (Beijing Sending Technology, Beijing, China)—which generates mechanical vibration perpendicular to the bottom of the 6-well plate—was used to apply vibration at low-magnitude (0.3 g), 30 Hz for 20 min every 24 h for three cycles, with a total experimental time of 48 h. Compressive stress was applied as previously described,[10] with slight modifications. An acrylic mass contained in a glass cylinder that exerted 1.5 g/cm^2 of total force was placed over the cells in each well of the 6-well plate to generate compressive stress for 48 h. Cells in the VC group were treated by mounting the compressive device on the GJX-5 and vibrated using the same vibration protocol. Cells in the control group were placed on the platform for the same periods of time, but the machine was not switched on.

To study the involvement of PGE2 in vibration-induced RANKL expression, 10 μM of the non-specific COX inhibitor indomethacin (Sigma-Aldrich, St Louis, MO, USA) was added to the medium 30 min before the experiment to permit this compound to penetrate the cells and block it respective pathways. This concentration was previously reported as an effective dose.[6,11]

Samples were collected for analysis immediately after the experiments ended. The expression of RANKL and OPG were analysed using quantitative real-time polymerase chain reaction (qPCR). The levels of PGE2, soluble RANKL (sRANKL) and OPG in the culture media were quantified using ELISAs.

2.3. RNA extraction and quantitative real-time PCR

Total RNA was extracted from each cultured cells and was reverse transcribed into complementary DNA (cDNA), then quantitative real-time PCR was performed on a Rotor-Gene® Q (Qiagen Inc., Qiagen Str. 1, Hilden, Germany) using the SensiFAST™ SYBR No-ROX Kit (Bioline Inc., Taunton, MA, USA) as previously described (10). Primer sequences and PCR conditions are shown in Table 1. All primers were listed in our previous study.[10] PCR was generated at 95 °C for 2 min, followed by the cycles of denaturing (95 °C, 5s), annealing at the optimal temperature for each primer pair (see Table 1, 10 s), and extension (72 °C, 20 s). GAPDH was used as an internal control. Gene expression levels were analysed using the $2^{-\Delta\Delta Ct}$ method. Values were shown as fold changes in experimental groups relative to the control. Melting curves analysis were performed to confirm the purity of the amplification products, as previously described.[10]

2.4. PGE2, sRANKL, and OPG quantification

PGE2, sRANKL, and OPG secreted from the cells were quantified by the DuoSet® ELISA Development kit (R&D Systems Co., Minneapolis, MN, USA) on Multiskan GO microplate spectrophotometer. The levels of protein were analysed by comparing to the standard curve and were normalized to total protein content, as previously described.[10] Values were shown as fold changes in experimental groups relative to the control.

2.5. Statistical analysis

SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) was employed for statistical analyses. All data are mean ± standard deviation of the six independent biological replicates (six donor cell lines), each assessed in triplicate. To detect differences between groups, Kruskal-Wallis test and Mann-Whitney *U* test were used to assess differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of mechanical stress and indomethacin on PGE2 secretion

PGE2 secretion was significantly upregulated in PDL cells exposed to all mechanical stresses (V, C and VC; $P < 0.05$; Fig. 1A).

Pretreatment with indomethacin significantly inhibited the upregulation of PGE2 induced by mechanical stress in all experimental groups (V, C and VC; $P < 0.05$; Fig. 1A).

3.2. Effects of mechanical stress and indomethacin on RANKL and OPG mRNA and protein expression

RANKL expression significantly increased in all mechanical stress groups (V, C and VC) compared to control cells ($P < 0.05$; Fig. 1B). OPG expression had no significant change under vibration (Fig. 1D), while it significantly down-regulated in the cells under compressive stress (C and VC) compared to control cell ($P < 0.05$; Fig. 1D).

Soluble RANKL (sRANKL) was significantly upregulated in the cells exposed to all mechanical stresses (V, C and VC) compared to control cells ($P < 0.05$; Fig. 1C). The production of OPG was not significantly affected under vibration (Fig. 1E), while the cells in groups C and VC expressed significantly lower levels of OPG ($P < 0.05$; Fig. 1E).

Indomethacin significantly inhibited the upregulation of RANKL and sRANKL induced by mechanical stress in all experimental groups (V, C and VC; $P < 0.05$; Fig. 1B and C). The mRNA expression and protein production of OPG were significantly reduced by indomethacin in all groups ($P < 0.05$; Fig. 1D and E). Therefore, indomethacin-treated cells exposed to mechanical stimuli had significantly higher RANKL/OPG ratios than cells not treated with indomethacin and exposed to mechanical stimuli ($P < 0.05$; Fig. 1F).

4. Discussion

Our previous studies demonstrated LMHF vibration upregulated COX-2, PGE2, IL-6, IL-8 and RANKL, and further increased the levels of these osteoclastogenic factors in compressed PDL cells.[10,11] In addition, we found that the cyclooxygenase pathway is required to induce IL-6 and IL-8 in compressed PDL cells either compressive stress alone or in combination with vibration. On the other hand, the expression of IL-6 and IL-8 in response to vibration alone is the cyclooxygenase pathway independent.[11] Although, previous reports showed upregulation of RANKL in PDL cells exposed to compressive stress was dependent on the cyclooxygenase pathway,[5,6] the mechanisms by which the cellular

Table 1
Primers used for real-time PCR.

Gene (Accession number) & Sequences	Exon-exon junction spanning	Product (BP)	Annealing temperature (°c)	Annealing cycles
RANKL (NM_033012.3) F: 5′-TCCCATCTGGTTCCCATAAA-3′ R: 5′-GGTGCTTCCTCCTTTCATCA-3′	6, 7	260	60	40
OPG (NM_002546.3) F: 5′-GAAGGGCGCTACCTTGAGAT-3′ R: 5′-GCAAACTGTATTTCGCTCTGG-3′	2, 3	102	62	35
GAPDH (NM_002046.5) F: 5′-GCACCGTCAAGGCTGAGAAC-3′ R: 5′-ATGGTGGTGAAGACGCCAGT-3′	4, 5	142	62	35

F, Forward primer; R, Reverse primer.

responses to vibration and vibration in combination with compressive stress on the expression of RANKL are unclear. We proposed that the expression of RANKL in response to vibration may depend on the cyclooxygenase pathway. In order to determine whether the upregulation of RANKL observed in this model is related to the cyclooxygenase pathway, the non-selective COX inhibitor indomethacin was used to examine the effect of PGE2 on RANKL expression in response to vibration and vibration in combination with compressive stress in human PDL cells.

This *in vitro* study showed that indomethacin significantly inhibited mechanically-induced PGE2 production in human PDL cells which in turn abolished the induction of RANKL expression in all mechanical stress groups: vibration, compressive stress and vibration in combination with compressive stress. The effects of compressive stress on PDL cells in this study are similar to previous reports.[5,6,11] This study suggests that both vibration and compressive stress are likely to induce RANKL expression in PDL cells via the cyclooxygenase (COX/PGE2) signalling pathway. However, an agonist experiment using exogenous PGE2 at the same concentration as measured under mechanical stimuli in this study is required to further investigate whether the RANKL and OPG signals will be changed as the cells were mechanically changed.

PGE2 is an inflammatory mediator that responds to several stimuli.[12,13] Leethanakul et al.[2] showed that vibration increased IL-1β in orthodontic patients, as well as increasing the rate of tooth movement. IL-1β is an inflammatory cytokine that can induce RANKL expression and osteoclastic activity.[14] Based on this work and our previous studies,[2,10,11] we fulfill the current knowledge of the effects of vibration on PDL cells (Fig. 2), vibration may increase the production of

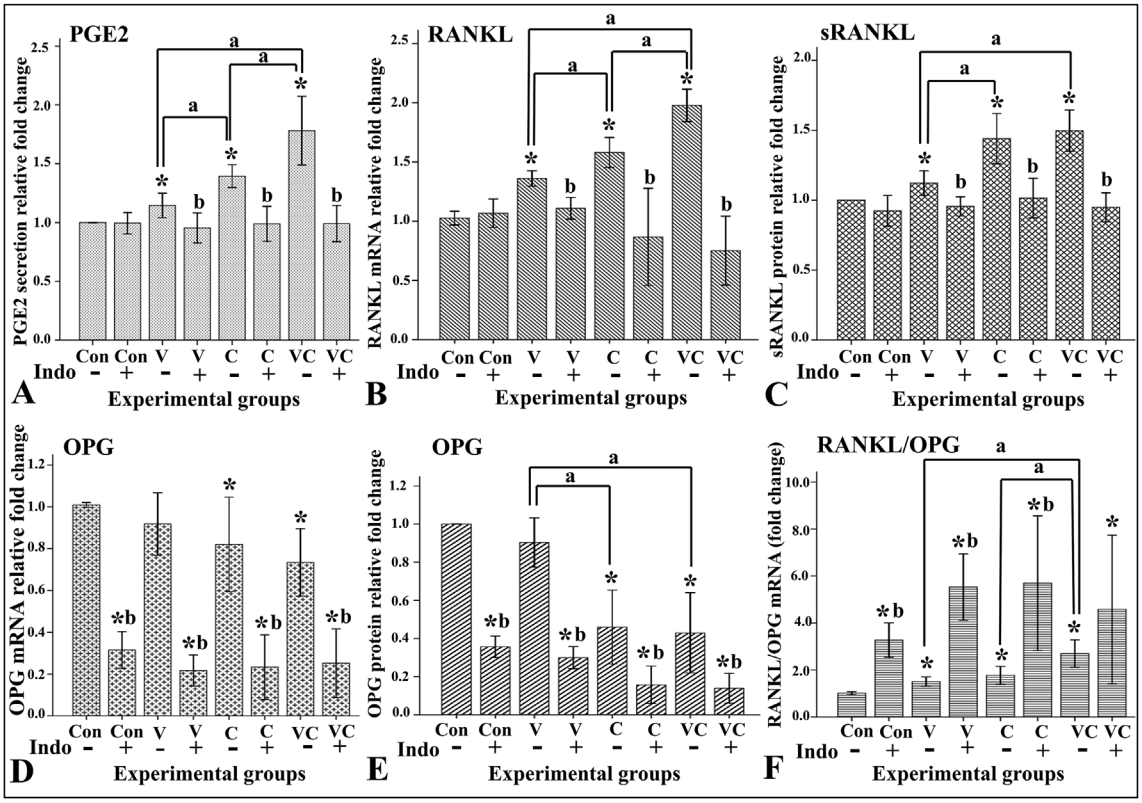

Fig. 1. Relative mRNA expression and protein levels for control (Con) PDL cells and cells stimulated with vibration (V), compressive stress (C) or vibration in combination with compressive stress (VC). Each experimental group was treated with (+) or without (−) 10 μM of the COX inhibitor indomethacin (Indo) 30 min before the experiment. (A) PGE2 production (values are relative fold changes compared to control cells; absolute values for control cells ranged from 114.62 ± 19.17 to 804.43 ± 15.35 pg/mg), (B) RANKL mRNA expression, (C) sRANKL (absolute values for control cells ranged from 2.02 ± 0.11 to 5.27 ± 0.01 pg/mg), (D) OPG mRNA expression, (E) OPG secretion (absolute values for control cells ranged from 3156.99 ± 164.22 to 28082.96 ± 1060.61 pg/mg) and (F) RANKL/OPG mRNA ratio. Values are mean ± SD of the six biological replicates (six donor cell lines), each assessed in triplicate ($n = 6$); * significant difference between experimental and control groups, [a] significant difference between mechanically stimulated groups, [b] significant difference between indomethacin treated- and non-treated groups in each mechanical stimulation; $P < 0.05$, Mann-Whitney U test.

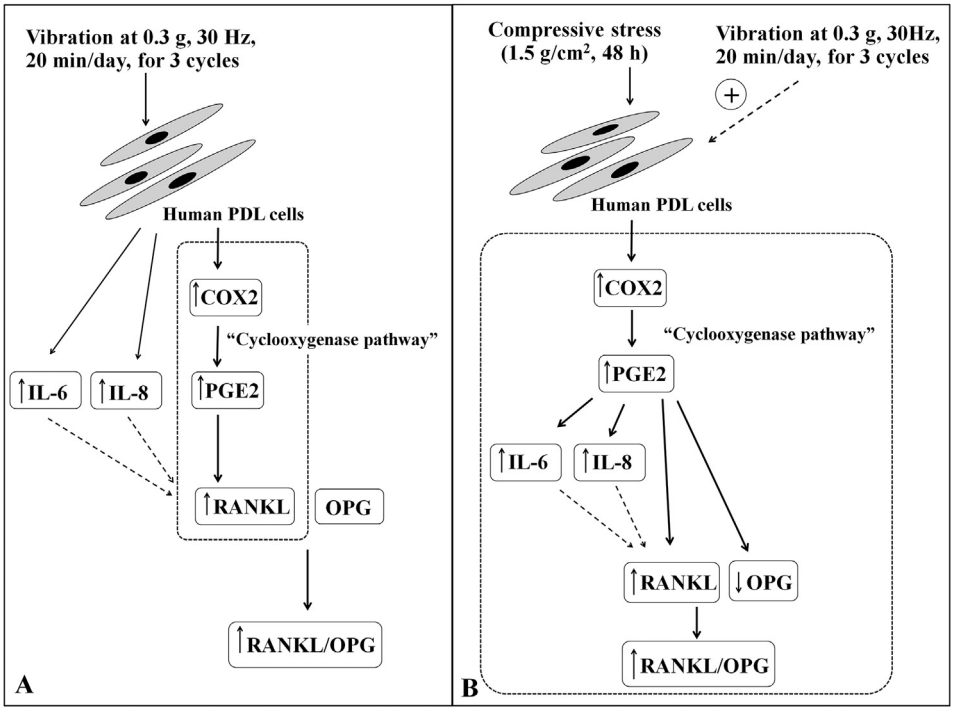

Fig. 2. Diagram shows effects of (A) vibration, (B) compressive stress, and vibration in combination with compressive stress on human PDL cells.

inflammatory cytokines such as PGE2, IL-6 and IL-8 directly, but the expression of RANKL in response to vibration in PDL cells either vibration alone or in combination with compressive stress is depended on the cyclooxygenase pathway. In addition, PDL cells respond to vibration and compressive stress on the expression of IL-6 and IL-8 in the difference pathway. Inflammatory cytokines are potent inducers of the alveolar bone remodeling.[15] These results may explain the effects of vibration on the acceleration of tooth movement. In addition, vibration may affect the expression of the other inflammatory cytokines that initiate the alveolar bone remodeling process, such as tumor necrosis factor alpha (TNFα).[15] Further *in vitro* and *in vivo* studies exploring the effects of vibration on the expression of other inflammatory mediators are required.

We found OPG mRNA and protein expression did not change in response to vibration, in agreement with our previous reports.[10,11] However, the effect of compressive stress and vibration in combination with compressive stress on OPG was different. In this study, OPG was significantly downregulated by compressive stress and vibration in combination with compressive stress, but OPG was not affected by these stimuli in our previous studies.[10,11] The larger sample size of this study may explain this variation. Indeed, the effect of compressive stress on OPG expression was still controversy; unchanged,[5] decreased[9] or increased.[16] Based on the available evidence, we suggest that vibration has no significant effect on OPG expression in PDL cells. Expression of OPG in response to compressive stress in PDL cells may be mediated by a complex mechanism involving several pathways and factors. The precise effects and mechanisms that regulate OPG expression in compressed PDL cells need to be further investigated.

Our study showed that blocking the COX/PGE2 pathway using indomethacin reduced OPG mRNA and protein expression in all groups. The effect of PGE2 on OPG expression is controversial. Some studies reported that inhibition of PGE2 synthesis upregulates OPG in PDL cells[17] and osteoblasts.[18] However, another report showed that neither PGE2 nor inhibition of PGE2 synthesis had significant effects on OPG expression in PDL cells.[5] Moreover, Tsuji et al.[19] reported that indomethacin suppressed intermittent tensile stress-induced upregulation of OPG in human PDL cells, suggesting a COX-dependent mechanism. These discrepancies may be due to cell types, culture conditions and concentrations of PGE2 and inhibitors used. The culture conditions and concentration of indomethacin used in this study may inhibit OPG expression directly, and/or it is possible that there are several signalling systems related to OPG expression and indomethacin may affect some pathways that have the inhibitory effect on the expression of OPG. Further studies are needed to determine the effect of various mechanical stimuli and indomethacin concentrations on OPG expression in PDL cells.

This study provides evidence that both vibration and compressive stress induce RANKL—at least in part—via activation of the COX/PGE2 pathway in PDL cells *in vitro*. However, several signalling molecules and proteins related to this signalling cascade may cross-talk with other signalling systems.[20] In addition, cellular responses depend on several factors. Further studies employing different mechanical stimulation protocols to define the involvement of other signalling molecules in the COX/PGE2 pathway and/or other signalling pathways are necessary to elucidate the mechanisms underlying the cellular responses to vibration. In addition, cellular responses to mechanical stimulation *in vivo* are likely to be more complex than the *in vitro*, further *in vivo* studies are required to determine whether vibration affects the orthodontic tooth movement via the COX/PGE2 pathway.

5. Conclusion

In this study, blocking COX/PGE2 abolish induction of RANKL in PDL cells under mechanical stresses. Therefore, the cyclooxygenase pathway is involved vibration- and compressive stress-induced RANKL expression in PDL cells.

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Conflicts of interest

The authors declare no conflict of interest.

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