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ORIGINAL ARTICLE

Endotoxins potentiate COX-2 and RANKL expression in compressed PDL cells

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Abstract
Objective This study aims to demonstrate in vitro the synergistic effect of orthodontic forces and periodontal pathogens on cyclooxygenase-2 regulation and the subsequent receptor activator of nuclear factor kappa-B ligand (RANKL) production from periodontal ligament (PDL) cells.
Materials and methods In comparison to a control group, three experimental groups were formed from human primary PDL cells stressed with compressive forces, bacterial endotoxins, or a combination of both. Gene expression of cyclooxygenase-2 and RANKL was analysed with RT real-time PCR. The prostaglandin E2 production was determined with ELISA. A co-culture of PDL cells and an osteoclast-progenitor cell line was used in order to demonstrate the osteoclast formation effect caused by the simultaneous combined stress.
Results The simultaneous combined stress resulted in a 56-fold up-regulation of cyclooxygenase-2 gene expression with a subsequent noticeable rise in the prostaglandin E2 in the culture medium. The RANKL/osteoprotegerin gene expression ratio was 50-fold up-regulated and the osteoclast formation assay revealed 153.5±15.7 tartrate-resistant acid phosphatase (TRAP)-positive cells per well compared with 42.3±3.8 TRAP-positive cells per well of the control group.
Conclusion The synergistic action of periodontal pathogens and orthodontic forces leads to an increased expression of cyclooxygenase-2 from PDL cells that intensify the RANKL production which in turn induces osteoclast differentiation and subsequent osteoclastogenesis.
Clinical relevance The present study puts an emphasis on the detrimental effect of orthodontic forces on patients with an active periodontal disease by underlining the significance of cyclooxygenase-2 activity and RANKL binding on the osteoclastogenesis process.

Keywords Periodontitis · PDL cells · Orthodontic tooth movement · RANKL · COX-2 · OPG

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Introduction

During orthodontic tooth movement, continuous force application results in bone resorption at the compression side whereas bone apposition takes place at the tension side [1]. Multiple signalling pathways, such as the canonical Wnt or FAK pathways are triggered when a mechanical force is applied to periodontal ligament (PDL) cells [2, 3]. Pro-inflammatory cytokines (interleukin (IL)-1β and IL-6), angiogenic factors (IL-8 and VEGF), prostaglandin E2 (PGE2), matrix metalloproteinases (MMPs), and receptor activator of nuclear factor kappa-B ligand (RANKL) are generated as a consequence of the former signalling cascade activation [1, 4].

The interaction between RANKL proteins located on PDL cell membranes and RANK receptors located on cell membranes of osteoclast precursors promote differentiation of the latter to mature osteoclasts. Osteoprotegerin (OPG) is an antagonist that competes with RANK for the RANKL binding which in turn inhibits osteoclast differentiation [5, 6]. Thus, an appropriate balanced expression of RANKL and OPG is vital for osteoclastogenesis regulation during orthodontic tooth movement [6].

In a relative manner, the presence of gram-negative periodontal-pathogen bacteria stimulates PDL cells to produce

pro-inflammatory cytokines and chemokines. This stimulation occurs because of lipopolysaccharides (LPS) binding to Toll-like receptors that induce transcription of cytokine and chemokine genes via different transcription factors and signalling cascades [7, 8]. Moreover, stimulation of PDL cells with LPS from periodontal-pathogen bacteria affected the balance of the RANKL/OPG system indicating an influence on the activity of bone-resorbing osteoclasts. In patients suffering from gingival inflammation or periodontitis, the gingival crevicular fluid exhibits a positive correlation between the RANKL/OPG ratio and the clinical parameters of periodontal pocket depth and attachment loss resulting from bone resorption [9].

In patients with active periodontal disease, a further loss of bone and tooth attachment may occur as a result of orthodontic tooth movement. In periodontal pockets, persisting periodontal-pathogens accentuate the expression of bone-resorbing factors from PDL cells. Such expression takes place anyway during orthodontic treatment at the compression side.

Cyclooxygenase-2 (COX-2) is an enzyme responsible for prostanoid formation and is, thus, primarily involved in inflammatory responses including periodontal inflammatory responses. COX-2 is an inducible enzyme that is expressed in various cell types of the periodontium, such as osteoblasts, osteoclasts, gingival fibroblasts, cementoblasts, and PDL cells [10–12]. COX-2 is the key enzyme for PGE2 production which is particularly involved in periodontal pathogenesis. LPS from gram-negative pathogens and IL-1β induce the up-regulation of COX-2. Indeed, in patients with gingivitis and periodontitis PGE2 levels were found to be elevated [13–15]. Furthermore, a large quantity of PGE2 in the crevicular gingival fluid is a predictor of periodontal attachment loss [16].

However, the synergistic effect of compression loads and periodontal-pathogens on COX-2 activity and RANKL production from PDL cells is not well investigated. Therefore, the objectives of this study are to investigate in vitro the expression of COX-2 and RANKL from PDL cells under the stress of compression loads, endotoxins of *Aggregatibacter actinomycetemcomitans*, or the simultaneous combination of them, and to demonstrate the synergistic role of the combined stress in overexpression of COX-2 and RANKL.

Materials and methods

Isolation and cultivation of PDL cells

Caries-free third molars from three donors (male, 18–25 years old) were extracted for orthodontic reasons after obtaining patient consent. Human PDL tissue was scraped from the middle third of the molar, sliced into small pieces, and cultured for 1 week in Dulbecco's minimal essential medium high glucose (Sigma-Aldrich, Munich, Germany) supplemented with 10 % FCS, 1 % antimycotics and antibiotics, 100 μM vitamin C, and 1 % L-glutamine at 37 °C in an atmosphere of 100 % relative humidity and 5 % CO_2. PDL cells were identified by confirmation of specific marker gene expression, such as fibromodulin, scleraxis, periostin, OPG, RANKL, Runx2, and alkaline phosphatase, which are expressed in PDL cells (Fig. 1a), [17–19]. Proliferating spindle-shaped PDL cells (Fig. 1b) from the extracts were sub-cultivated and stored in liquid nitrogen until use.

Bacterial strain and cell lysate preparation

A. actinomycetemcomitans bacterial strain (DSM 11123) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The strain was cultivated anaerobically (75 % N_2, 10 % CO_2, and 15 % H_2) at 37 °C on 5 % sheep blood agar plates enriched with hemin (5 mg/l) and menadione (1 mg/l) for 3 to 5 days. A single colony of *A. actinomycetemcomitans* was inoculated with brain-heart infusion broth and developed until the late logarithmic growth phase. At this phase, the bacteria were harvested by centrifugation, washed in phosphate-buffered saline and resuspended at a concentration with OD at 690 nm of 1, which is equivalent to approximately 1×10^8 colony-forming units (CFU)/ml [8]. The suspension was then heated at 60 °C for 10 min.

Experimental design

PDL cells at the fourth passage were seeded into five six-well plates with each well containing 70,000 cells and cultivated for 4 days. Four distinct groups were established. The control group received no intervention for 4 days. Experimental groups were as follows:

1. PDL cells were grown for three days until confluence. A compressive force (2 g/cm^2) was exerted applying a glass cylinder upon adherent growing PDL cells as described by Kanzaki et al. [17]. The compressive force was then applied for 24 h.
2. PDL cells were stressed with heat-inactivated *A. actinomycetemcomitans* for 4 days (1×10^7 cells/ml).
3. PDL cells were stressed with heat-inactivated *A. actinomycetemcomitans* for 3 days (1×10^7 cells/ml). The fourth day, they were stressed with heat-inactivated *A. actinomycetemcomitans* and a compression load (2 g/cm^2) simultaneously.

In order to evaluate dependence of RANKL expression from PGE2-synthesis, COX-2 activity was inhibited in an additional experimental group of PDL cells. PDL cells were grown for three days until confluence and in presence of

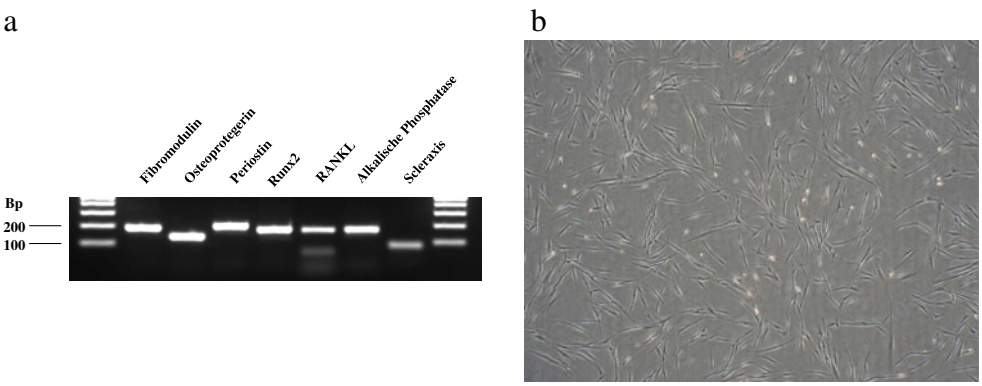

Fig. 1 Confirmation of PDL cells by **a** identification of gene marker expression and **b** spindle-shaped cell morphology

10 μM celecoxib and heat-inactivated *A. actinomycetemcomitans* (1×10^7 cells/ml). The cells were then additionally stimulated by compression load (2 g/cm^2) for 24 h simultaneously. We show in this manuscript results from one representative donor performed in triplicates. Similar results have been observed in the other donor cell lines.

Total RNA isolation and RT real-time PCR

On the fourth day, the cell monolayers of all groups were rinsed with 1 ml Tri-Reagent (Sigma-Aldrich, St. Louis, MO) and further processed according to manufacturer's recommendations in order to obtain the total RNA of each group. The cDNA synthesis (QuantiTect®Reverse Transcriptase, Qiagen, Germany) comprised 1 μg of total RNA of each group. Real-time PCR was performed by Jump Start SybrGreen I Kit (Sigma-Aldrich, St. Louis, MO) with triplets for each cDNA in Mastercycler® ep realplex (Eppendorf AG, Hamburg, Germany). The real-time amplifications included an initial step of 10 min at 95 °C (polymerase heat activation), followed by 45 cycles at 95 °C for 10 s (denaturation), 60 °C for 8 s (annealing), and 72 °C for 8 s (elongation and data collection). Gene expression was calculated according to the ΔΔCT-method of Livak and Schmittgen [20]. Appropriate intron spanning primers for PCR amplification of RNA-polymerase polypeptide A, COX-2, OPG, and RANKL (Table 1) were chosen using the online Universal ProbeLibrary Assay Design Center from Roche (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) in order to avoid co-amplification of genomic DNA.

Reverse transcription PCR

cDNA from isolated PDL cells were analysed for expression of specific expression markers, with reverse transcription PCR. The PCR reaction was prepared in a total volume of 20 μl with 50 ng cDNA, 2 μl 10×PCR buffer, 0.2 mM dNTP-Mix, 5 U of Taq-DNA polymerase (New England Biolabs, Ipswich, MA), and PCR oligonucleotides (MWG-Eurofins, Germany; Table 1) for specific amplification of fibromodulin, periostin, scleraxis, alkaline phosphatase, OPG, RANKL, and Runx2. The prepared PCR reaction was set in a thermocycler. The PCR program included an initial denaturation step of 10 min at 95 °C, followed by 35 cycles at 95 °C for 15 s (denaturation), and 60 °C (annealing and elongation). The amplicons were separated by 2 % agarose gel electrophoresis at 120 V for 40 min after amplification. The fluorescence dye (Gel Red) stained DNA bands were visualised with UV light at 256 nm and analysed for presence, specificity, and predicted length.

Quantification of PGE2 with ELISA

The collected deep-frozen cell culture mediums (−80 °C) of all groups were thawed on ice and used for quantification of PGE2. The production of PGE2 was measured using the Prostaglandin E2 EIA Monoclonal Kit (IMGENEX, San Diego CA) according to the manufacturer's protocol. The analysis of PGE2 content in the conditioned medium was correlated to the cell number in order to avoid misinterpretation of data due to altered cell proliferation.

Western Blot

PDL cells were lysed and solubilised with CelLytic™ M Cell Lysis-Reagent (Sigma-Aldrich, Munich, Germany) and western blotted. The primary antibody used in this study was rabbit polyclonal anti-RANKL antibody (www.antibodies-online.com, Aachen, Germany). The secondary antibody was anti-rabbit IgG-horseradish-peroxidase (www.antibodies-online.com, Aachen, Germany). After the membrane was washed thoroughly, the immuno-positive bands were detected by chemiluminescence using the Luminata™ Crescendo Western HRP Substrate (EMD Millipore Corporation, Billerica, MA).

Osteoclasts formation assay

As a model of osteoclast-like cells, the murine macrophage cell line RAW 264.7 (CLS Cell Lines Service, Eppelheim,

Table 1 Overview of intron-spanning PCR primers used in the PCR. All PCR primers were used at an annealing temperature of 60 °C

Genes	Accession number	5′-forward primer-3′	5′-reverse primer-3′
Fibromodulin	NM_002023.3	gggacgtggtcactctctg	ctgggagagggagaagagc
Periostin	NM_006475.2	gaaccaaaaattaaagtgattgaagg	tgacttttgttagtgtgggtcct
Scleraxis	BK000280.1	acacccagcccaaacagat	tctttctgtcgcggtcctt
Alkaline phoshatase	NM_000478.4	agaaccccaaaggattcttc	cttggcttttccttcatggt
RNA polymerase A	NM_000937.3	ttgtgcaggacacactcaca	caggaggttcatcacttcacc
OPG	AF134187.1	gaagggcgctaccttgagat	gcaaactgtatttcgctctgg
RANKL	AF019047	tgattcatgtaggagaattaaacagg	gatgtgctgtgatccaacga
COX-2	NM_000963	cttcacgcatcagttttt	tcaccgtaaatatgatttaagtccac

Germany) was used. This cell line is a well-established cell model for osteoclast formation assays [21]. After the intervention was applied to the experimental group, the cell medium was carefully removed and cells were washed with PBS. RAW264.7 cells (60,000 cells/well) were subsequently seeded in the PDL cells/cultures. The co-culture was grown for 3 days at 37 °C with 5 % CO_2 in a CO_2 incubator. Then, tartrate-resistant acid phosphatase (TRAP) staining was performed

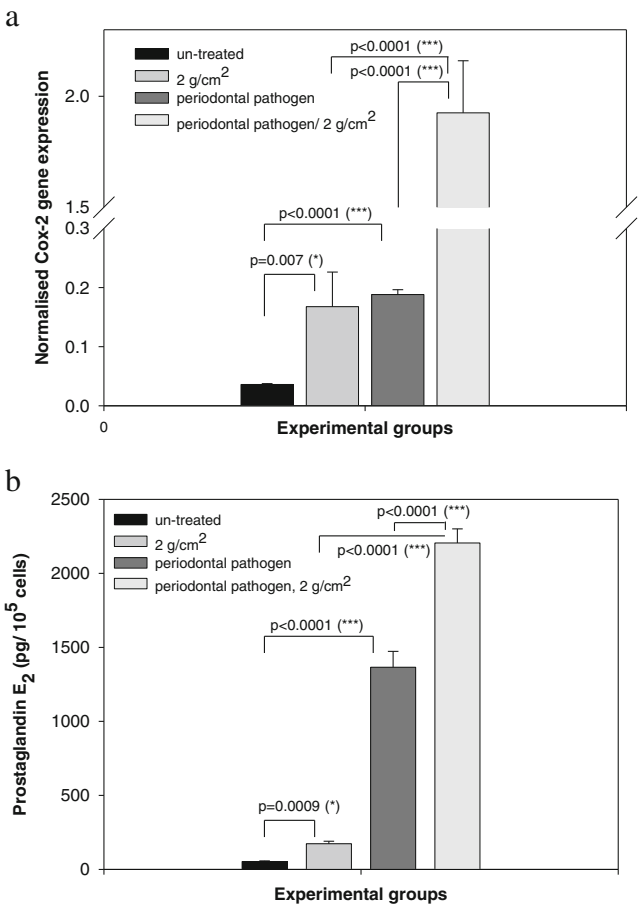

Fig. 2 Determination of COX-2 expression and PGE2 concentration after 24 h of treatment. **a** Gene expression analysis of COX-2 in control PDL cells, in compressed PDL cells, in bacteria-stressed PDL cells, and in both compressed and bacteria-stressed PDL cells (sample size was n=6 in each group). Statistical p values are indicated between groups. **b** The concentration of PGE2 in the cell culture was determined for each experimental group after treatment for 24 h (sample size, n=6). Statistical p values are indicated between groups

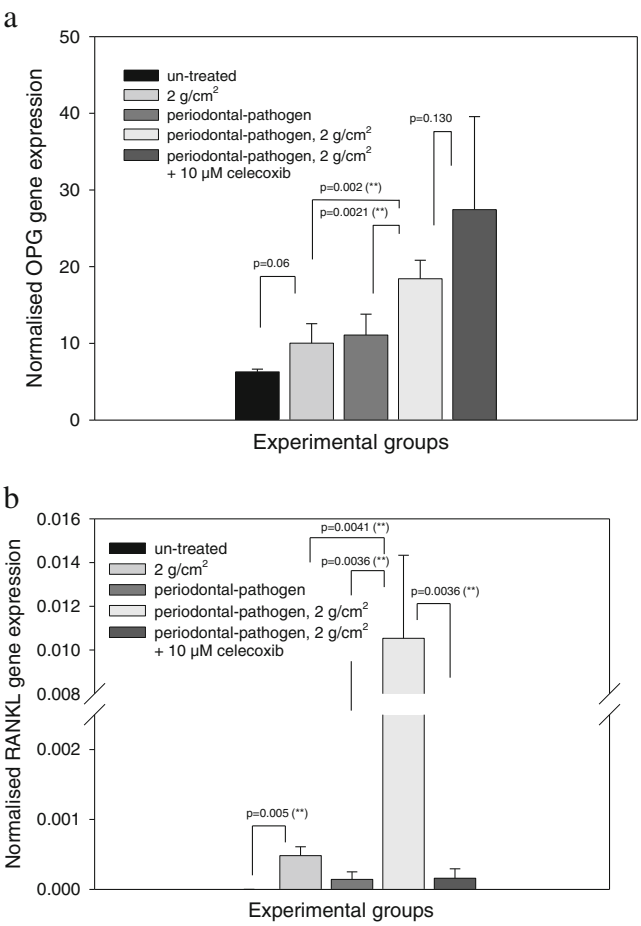

Fig. 3 **a** Gene expression analysis of OPG in control PDL cells, in compressed PDL cells, in bacteria-stressed PDL cells, in both compressed and bacteria-stressed PDL cells, and in celecoxib-treated and both compressed and bacteria-stressed PDL cells (sample size was n=6 in each group). Statistical p values are indicated between groups. **b** Gene expression analysis of RANKL in control PDL cells, in compressed PDL cells, in bacteria-stressed PDL cells, in both compressed and bacteria-stressed PDL cells, and in celecoxib-treated and both compressed and bacteria-stressed PDL cells (sample size was n=6 in each group). Statistical p values are indicated between groups

according to the protocol of Barka and Anderson [22]. The total number of TRAP-positive cells was determined with a light microscope (Olympus, IX50 Inverted System Microscope) at ×200 magnification.

Statistical analysis

Statistical analysis of the RT real-time PCR and ELISA data was performed using the un-paired Student's *t* test. Significance was established at $p<0.05$.

Results

Quantification of COX-2 gene expression and PGE2

In comparison with the control group, the application of a continuous compressive force (2 g/cm^2) for 24 h resulted in a 5-fold up-regulation of the COX-2 mRNA (Fig. 2a). This up-regulation was similar to that of the group stressed with heat-inactivated *A. actinomycetemcomitans*. However, the combination of compressive force and heat-inactivated bacteria resulted in a 56-fold up-regulation of COX-2 gene expression.

The conditioned media samples were analysed for PGE2 content (Fig. 2b). The culture medium of the control group demonstrated a 53.6±17.5 pg PGE2/1×10^5 cells. The group that was stressed with the compression load for 24 h exhibited an increase in PGE2 concentration of 172.9±17.5 pg PGE2/1×10^5 cells, whereas the group stressed with heat-inactivated *A. actinomycetemcomitans* exhibited a 1,365±183 pg PGE2/1×10^5 cells. The combination of the compressive load and heat-inactivated *A. actinomycetemcomitans* stress resulted in 2,205±132 pg PGE2/1×10^5 cells.

Quantification of RANKL, OPG, and RANKL/OPG gene expression ratio

The application of compressive force did not change the OPG expression significantly. However, the administration of heat-inactivated periodontal-pathogens in the cell culture of PDL cells enhanced the expression of OPG (Fig. 3a). In comparison to OPG, the gene expression of RANKL was significantly increased after application of compressive force for 24 h (Fig. 3b). The effect was further enhanced by a combination of heat-inactivated *A. actinomycetemcomitans* and application of compressive force to PDL cells. Moreover, we calculated the RANKL/OPG ratio which is criterion for determination of osteoclastogenesis: In the control group, the RANKL/OPG expression ratio was very slight with $8.1\times10^{-13}\pm9.8\times10^{-13}$ (Fig. 4a). After applying the compressive force for 24 h, a significant increase in RANKL/OPG gene expression ratio of $5.16\times10^{-5}\pm2.01\times10^{-5}$ was detected. The presence of heat-inactivated *A. actinomycetemcomitans* led to a rise in the RANKL/OPG gene expression ratio of $1.15\times10^{-5}\pm8.72\times10^{-6}$. The stress including both compressive force and heat-inactivated *A.*

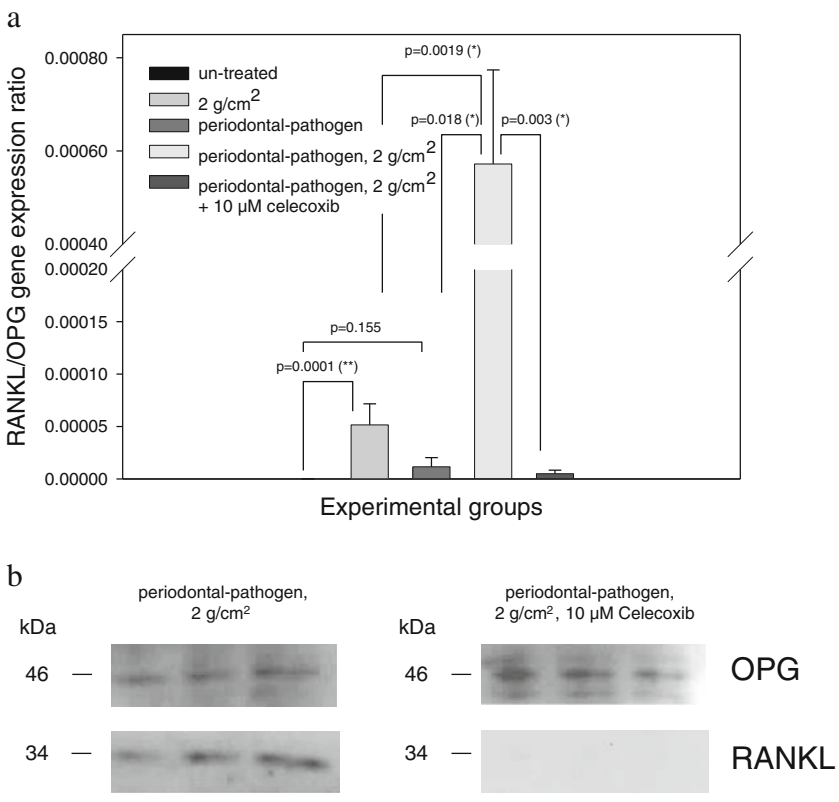

Fig. 4 **a** Gene expression analysis of RANKL/OPG ratio in control PDL cells, in compressed PDL cells, in bacteria-stressed PDL cells, in both compressed and bacteria-stressed PDL cells, and in celecoxib-treated and both compressed and bacteria-stressed PDL cells (sample size was $n=6$ in each group). Statistical *p* values are indicated between groups. **b** Western blot detection of OPG and RANKL. Treatment with 10 µM celecoxib demonstrated that RANKL expression in both bacteria- and compressive force-stressed PDL cells is mainly dependent on COX-2 activity

actinomycetemcomitans resulted in an approximately 50-fold increase in the RANKL/OPG gene expression ratio of $5.72\times10^{-4}\pm2.02\times10^{-4}$.

Correlation between COX-2 activity and RANKL expression

In order to determine the correlation between COX-2 activity and RANKL expression, a highly specific COX-2 inhibitor was applied to PDL cells previously stressed with both a compressive load and heat inactivated *A. actinomycetemcomitans*. The complete absence of PGE2 in the culture medium confirmed the total inhibition of COX-2 activity (data not shown). Both the RT real-time PCR analysis of RANKL gene expression (Fig. 4a) as well as the protein analysis by Western Blot detection (Fig. 4b) demonstrated a significant reduction of RANKL expression.

Osteoclast formation assay

Co-culture of RAW264.7 cells with mechanically compressed PDL cells for 3 days resulted in 52.7±3.1 TRAP-positive osteoclast-like cells per well as compared with the control co-culture with 42.3±3.8 TRAP-positive cells per well (Fig. 5). The co-culture of RAW264.7 cells with PDL cells previously stressed with heat-inactivated *A. actinomycetemcomitans* led to a pronounced formation of 66.7±7.6 TRAP-positive stained osteoclast-like cells per well. Finally, an exceptional increase of 153.5±15.7 TRAP-positive cells per well was observed in the co-culture of RAW264.7 cells and PDL cells previously stressed with compression load and heat-inactivated *A. Actinomycetemcomitans* concurrently. The additional administration of 10 μM celecoxib to both compressed and bacterial stimulated PDL cells led to a significantly decreased formation with 66.8±4.2 TRAP-positive osteoclast-like cells in the co-culture.

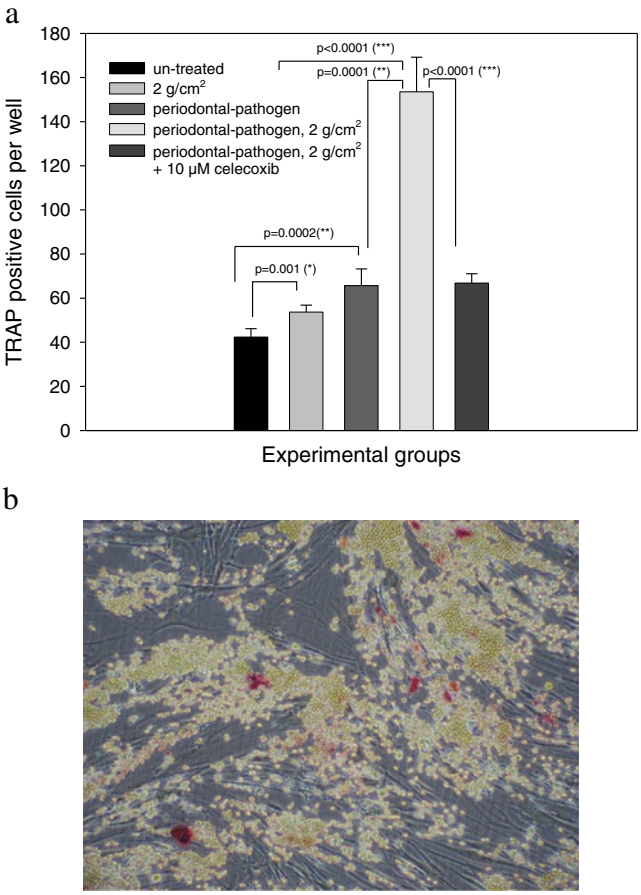

Fig. 5 Determination of osteoclastic differentiation of RAW264.7 in a 3-day co-culture with pre-treated PDL cells. **a** Determination of TRAP-positive stained RAW264.7 cells in co-culture with PDL cells (sample size was $n=6$). Statistical p values are indicated between groups. **b** Co-culturing of PDL cells (*spindle shaped*) and RAW264.7 cells (*round shaped*) for 3 days. Multinucleic RAW264.7 cells with osteoclast-like properties were red-stained by tartrate-resistant acid phosphatase staining (magnification, ×200)

Discussion

Nowadays, an increasing number of adult patients seek orthodontic treatment in order to improve occlusal function, periodontal health, and facial aesthetics. It is a common phenomenon that these patients present with clinical symptoms of active periodontal disease. Although orthodontic treatment can improve periodontal health by establishing favourable occlusal relationships and hygiene conditions, it may also lead to severe damage of the periodontium in the presence of periodontal pathogenic bacteria [23, 24].

Normally, orthodontic tooth movement induces a sterile inflammation process of the PDL in response to the orthodontic forces applied. The sterile inflammation is mediated by up-regulation of pro-inflammatory cytokines and prostanoids, which define the remodelling processes of the PDL and alveolar bone [1]. The application of appropriate orthodontic forces induces bone remodelling with resorption of the alveolar bone at the pressure side and appropriate bone apposition at the tension side. Osteoclastogenesis at the pressure side is induced by up-regulation of RANKL/OPG ratio, which favours the formation of bone resorbing osteoclasts [5].

Similar to the orthodontic forces applied during tooth movement, cell wall components of gram-negative periodontal-pathogen bacteria activate toll-like receptors of PDL cells [8], which in turn leads to an increased production of pro-inflammatory cytokines and COX-2 [15, 25–27]. Our data confirmed that heat-inactivated *A. actinomycetemcomitans* induced COX-2 production from PDL cells and, consequently, the concentration of PGE2 increased in the cell culture environment.

COX-2 is not only induced by bacterial stimulation but also by mechanical stress in osteoblasts and PDL cells. Static compressive force application to PDL cells was found to up-regulate COX-2 gene expression and enhance PGE2

production [17]. Orthodontic patients exhibit an increased quantity of PGE2 in the gingival crevicular fluid in the initial phase of orthodontic tooth movement. As expected, we found a significant up-regulation of COX-2 gene expression and PGE2 synthesis in mechanically stressed PDL cells in vitro. An even greater up-regulation of COX-2 was observed when compressive force application and heat-inactivated gram-negative bacteria were present concurrently.

Kanzaki et al. [17] first reported that under compressive forces RANKL expression is mediated by enhanced PGE2 production. Nukaga et al. [28] observed that PGE2 binds to constitutively expressed prostanoid receptors EP2 and EP4 in PDL cells. The stimulation of EP2 and EP4 by PGE2 activate the cAMP-dependent protein kinase A and protein kinase C which are involved in the activation of RANKL gene expression. Our data suggest that the up-regulated COX-2 gene expression and the subsequent PGE2 production are strongly associated with overexpression of RANKL in PDL cells stressed with compression load and *A. actinomycetemcomitans* endotoxins at the same time. This effect was also reflected by enhanced osteoclastogenesis in case of PDL cell and RAW264.7 co-culture. In order to demonstrate that the overexpression of RANKL is mainly dependent on de novo produced COX-2, we inhibited the COX-2 activity by administrating a specific inhibitor. We could demonstrate that RANKL expression in mechanically co-stimulated PDL cells is mainly dependent on PGE2 production by COX-2. This observation is in accordance with the findings of Sanuki et al. [29]. They reported about the promoting effect of compressive force in COX-2 expression and subsequently enhanced PGE2 synthesis in osteoblasts. The administration of a specific COX-2 inhibitor to an osteoblast/RAW264.7 co-culture system diminished the differentiation of RAW264.7 in osteoclast-like cells [29]. Thus, the findings of our experiment suggest that the enhanced RANKL production induced by compressive force and bacterial stimulation is mainly mediated by production of PGE2.

The results of the present cell study from one representative donor indicate the paramount effect of COX-2 and subsequent PGE2 production on the mediation of RANKL expression and subsequent osteoclastogenesis. These observations were found to be similar in PDL cell lines from other donors. In addition, our findings indicate that different kinds of stress, such as mechanical stress and the presence of periodontal pathogens do stimulate COX-2 and RANKL genes synergistically. Yamamoto et al. [30] arrived at similar observations on PDL cells, which were stimulated with *Porphyromonas gingivalis* and hydrostatic pressure. They reported that IL-8 production was induced by mechanical stress, and the simultaneous presence of compressive forces and *P. gingivalis* led to an overproduction of IL-8 in PDL cells.

In conclusion, the present investigation suggests that some mechanically regulated genes from PDL cells are additionally responsive to periodontal pathogenic gram-negative bacterial stimulation. The synergistic activation of COX-2 and RANKL genes and IL-8 [30] leads to an overexpression of RANKL, thus deteriorating osteoclastogenesis and bone resorption. Our findings may help to explain the clinical effect of periodontal attachment loss and enhanced alveolar bone resorption in orthodontically treated teeth within an active periodontal pocket.

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Conflict of Interest The authors declare that they have no conflict of interest.

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