

Nicotine favors osteoclastogenesis in human periodontal ligament cells co-cultured with CD4⁺ T cells by upregulating IL-1 β

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Abstract. Periodontitis, which is the main cause of tooth loss, is one of the most common chronic oral diseases in adults. Tooth loss is mainly a result of alveolar bone resorption, which reflects an increased osteoclast formation and activation. Osteoclast formation in periodontal tissue is a multistep process driven by osteoclastogenesis supporting cells such as human periodontal ligament (PDL) cells and CD4⁺ T cells. Inflammatory cytokines, such as interleukin-1 β (IL-1 β), can induce osteoclastogenesis by affecting the expression of receptor activator of NF- κ B ligand (RANKL) and osteoprotegerin (OPG) in human PDL cells. Nicotine, the major component in tobacco smoking and a specific agonist of the α 7 nicotinic acetylcholine receptor (α 7 nAChR), has been proven to regulate the expression of inflammatory cytokines in smoking-associated periodontitis. In this study, we investigated the mechanism(s) through which nicotine affects osteoclastogenesis in human PDL cells co-cultured/non-co-cultured with CD4⁺ T cells. Human PDL cells were stimulated with nicotine (10⁻⁵ M) and/or α -bungarotoxin (α -BTX, specific antagonist of α 7 nAChR, 10⁻⁸ M) before being co-cultured with CD4⁺ T cells. Compared with mono-culture systems, stimulation with nicotine caused an increased secretion of IL-1 β in serum of human PDL cell-CD4⁺ T cell co-culture, and the expression of RANKL in human PDL cells was further upregulated co-cultured with CD4⁺ T cells, while no differences were observed in the expression of OPG between the co-culture and mono-culture systems. Our data suggested that nicotine upregulated IL-1 β secretion, further upregulated RANKL expression in smoking-associated periodontitis, which may aid in the better understanding of the relationship between nicotine and alveolar bone resorption.

Introduction

Periodontitis is one of the most common chronic infectious diseases in adults. It often coincides with increased alveolar bone resorption, causing tooth loss and eventually exfoliation. Smoking is recognized as the primary behavioral risk factor for periodontitis (1-4). However, the mechanisms underlying the effects of smoking on periodontal tissue destruction remain unclear (5). Compared with non-smoking periodontitis, the height and density of alveolar bone in the periodontal tissue of smoking-associated periodontitis is lower, and closely related to smoking dose and time (6). Nicotine, the main toxic component in tobacco, was confirmed as the main effect of smoking on periodontal tissue destruction. Previous studies have demonstrated that daily quantitative injection of nicotine causes significantly heavier alveolar bone resorption in rats than injection of saline (7,8). As the specific agonist of α 7 nicotinic acetylcholine receptor (α 7 nAChR), nicotine also upregulated the expression of α 7 nAChR in rat periodontal tissue and human periodontal ligament (PDL) cells, and these effects could be partially suppressed by pretreatment with α -BTX (8,9). A number of studies suggested that CD4⁺ T cell-mediated immune responses are critical to the process of periodontitis; large amounts of CD4⁺ T cells infiltrated periodontal tissue and the proportion of CD4⁺ T cells in peripheral blood was significantly changed in periodontitis (10-12). Other studies showed CD4⁺ T cell-mediated immune responses regulated alveolar bone resorption in periodontitis (13-16).

The receptor activator of NF- κ B ligand (RANKL)/RANK/osteoprotegerin (OPG) axis is the key factor for regulating differentiation of osteoclast and bone resorption (17,18). Nicotine affected the balance of the RANKL/RANK/OPG axis (19) and also upregulated the level of interleukin-1 β (IL-1 β) in human PDL cells (9) and gingival crevicular fluid (GCF) (20,21). Upregulated expression of IL-1 β can further affect the balance of the RANKL/RANK/OPG axis, causing alveolar bone resorption in periodontitis (22).

We hypothesized that nicotine could alter the secretion of IL-1 β in serum of human PDL cell-CD4⁺ T cell co-culture, further affecting the expression of RANKL in human PDL cells. Therefore, in this study, we investigated the stimulated human PDL cell-CD4⁺ T cell co-culture with nicotine for 72 h and analyzed the effects of this stimulation on the expressions of RANKL and OPG and the secretion of IL-1 β , examining

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whether the effects of nicotine were affected by the presence of CD4⁺ T cells.

Materials and methods

Cell culture. Cultures of human PDL cells from three healthy young patients undergoing first premolar tooth extractions at the Department of Maxillofacial Surgery, were explanted from the middle-third of the root surface and grown in RPMI-1640 medium supplemented with 15% fetal calf serum (FCS) and antibiotics (penicillin, 50 U/ml and streptomycin, 50 mg/ml). Experiments were performed in 6-well plates that were then placed in a 37°C humid atmosphere of 5% CO₂ and 95% O₂. Passage-5 cells were used for all the experiments. This study was independently reviewed and approved by the Ethics Committee of the School of Stomatology, The Fourth Military Medical University.

CD4⁺ T cell sort and culture. Peripheral venous blood was derived from healthy non-smoking volunteers. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation (Ficoll-Hypaque solution; Sigma, USA), stained with CD4 fluorescein isothiocyanate antibody (BD Biosciences, USA) and incubated at 4°C for 30 min. CD4⁺ T cell sorting was performed by flow cytometry. Only samples with >90% CD4⁺ T cells were grown in RPMI-1640 medium supplemented with 15% FCS and antibiotics (penicillin, 50 U/ml and streptomycin, 50 mg/ml) for further experiments.

To determine the effects of nicotine and/or α -BTX, human PDL cells and CD4⁺ T cells were divided into two systems: the mono-culture and the co-culture system (ratio 1:4 human PDL cells:CD4⁺ T cells), then incubated for 72 h. We used a semi-permeable membrane (0.4 μ m; Millipore, Germany) to establish the human PDL cell-CD4⁺ T cell co-culture system. Each system was divided into four groups, and received one of the following treatments randomly: i) no treatment; ii) nicotine (10⁻⁵ M); iii) α -BTX (10⁻⁸ M); iv) α -BTX (10⁻⁸ M) followed by nicotine (10⁻⁵ M) after 30 min.

ELISA analysis. Supernatants of mono-culture human PDL cells, mono-culture CD4⁺ T cells and human PDL cell-CD4⁺ T cell co-culture challenged as described above were collected and centrifuged. Concentrations of IL-1 β were quantified using highly sensitive enzyme-linked immunoassay from R&D Systems (Abingdon, UK) according to the manufacturer's instructions, and normalized to the number of cells. Culture supernatants were thawed only once and assayed in the same run.

RNA analysis and real-time quantitative PCR. We then determined the constancy of the transcriptional alterations following stimulation with nicotine for 72 h. Total mRNA of human PDL cells was isolated with RNeasy kit (Omega, USA). mRNA concentration was measured by using NanoDrop Spectrophotometer (Thermo-Fischer Scientific, USA), and reverse-transcribed to cDNA by using iScript Select cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed with iQ SYBR-Green Supermix (Bio-Rad) and primers for RANKL and OPG (Takara, Ohtsu, Japan), and were then analyzed with the 2^{- $\Delta\Delta$ Ct} method. β -actin served

Table I. Primers used for real-time quantitative PCR.

β -actin	F: 5'-TGGCACAGCACAATGAA-3' R: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'
RANKL	F: 5'-TGATGTGCTGTGATCCAACGA-3' R: 5'-AAGATGGCACTCACTGCATTTATAG-5'
OPG	F: 5'-GAAGGTGAGGTTAGCATGTCC-3' R: 5'-CAAAGTAAACGCAGAGAGTGTAGA-3'

F, forward; R, reverse; OPG, osteoprotegerin; RANKL, receptor activator of NF- κ B ligand.

as the housekeeping gene. Real-time PCR was performed on an ABI PRISM 7500 (Applied Biosystems, USA). Primer sequences are listed in Table I, all PCR efficiencies were comparable.

Western blot analysis. Whole-cell protein lysates of mono-culture human PDL cells and human PDL cell-CD4⁺ T cell co-culture as described above were collected on ice and resuspended in SDS-sample buffer. Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples containing equal amounts of protein were separated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a minigel apparatus. The proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% fat-free dry milk in TBST (TBS containing 0.1% Tween-20), washed with TBST (3x5 min), and incubated with rabbit anti-human RANKL and OPG (Santa Cruz Biotechnology, Inc., USA), dilution 1:500, and with mouse anti-human β -actin (Abcam, Cambridge, MA, USA), dilution 1:500, respectively. Then, the membranes were incubated overnight with primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, dilution 1:10,000. Immunoreactive bands were visualized with the Western-Light chemiluminescent detection system (Peiqing, Shanghai, China).

Statistical analysis. All data are from experiments performed in triplicate and recorded as the means \pm standard error (SE). The significant differences in mRNA and protein expressions of differences between groups were analyzed through one way-ANOVA and Tukey's test using SPSS 18.0 software. A P-value <0.05 was considered to indicate statistically significant differences.

Results

IL-1 β secretion in serum of co-culture and mono-culture systems. The secretion of IL-1 β was subsequently quantified in co-culture and mono-culture systems by ELISA. The secretion of IL-1 β was significantly upregulated following stimulation with nicotine (P<0.01) in mono-culture human PDL cells, and the secretion of IL-1 β in mono-culture CD4⁺ T cells showed a similar tendency to mono-culture human PDL cells (P<0.01). Furthermore, the secretion of IL-1 β in human PDL cell-CD4⁺

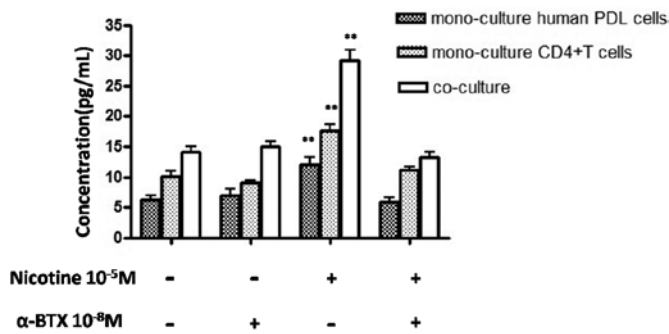


Figure 1. Effects of nicotine and/or α -BTX on IL-1 β secretion in mono-culture human PDL cells, mono-culture CD4⁺ T cells and human PDL cell-CD4⁺ T cell co-culture. **P<0.01, compared to the control.

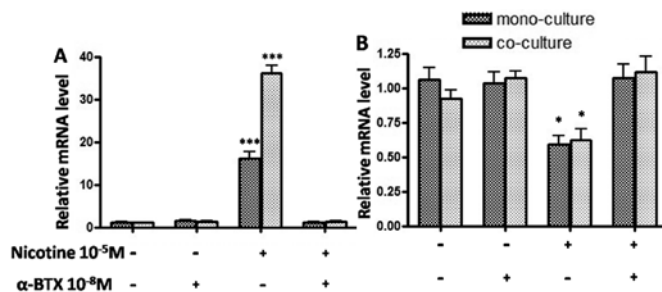


Figure 2. Effects of nicotine and/or α -BTX on (A) RANKL and (B) OPG gene expression in human PDL cells co-cultured/non-co-cultured with CD4⁺ T cells. *P<0.05, ***P<0.001, compared to the control.

T cell co-culture was significantly higher than both mono-culture systems (P<0.01) (Fig. 1).

Gene expressions of RANKL and OPG in human PDL cells. Subsequently, we examined whether nicotine regulated expressions of RANKL and OPG, thus potentially modulating alveolar bone metabolism. We used real-time PCR to amplify the mRNAs of RANKL and OPG in mono-culture human PDL cells and human PDL cell-CD4⁺ T cell co-culture. In the mono-culture system, gene expression of RANKL was significantly upregulated after stimulation with nicotine (P<0.001) (Fig. 2A), while OPG was significantly downregulated (P<0.05) (Fig. 2B). In the co-culture system, gene expression of RANKL was significantly higher than the mono-culture system after stimulation with nicotine (P<0.01) (Fig. 2A), while no differences were observed in OPG mRNA expression between the co-culture and mono-culture systems (P>0.05) (Fig. 2B). β -actin used as the housekeeping gene was unaffected by the different stimulations.

RANKL and OPG protein expression in human PDL cells. Since human PDL cells constitutively expressed RANKL and OPG on mRNA levels, protein levels of RANKL and OPG were assessed by western blot analysis. Protein levels showed a similar tendency to mRNA levels (Fig. 3). Consequently, the observed minor changes at the protein and mRNA levels do not evidently affect OPG, identifying this cytokine as a stable protein synthesized by human PDL cells.

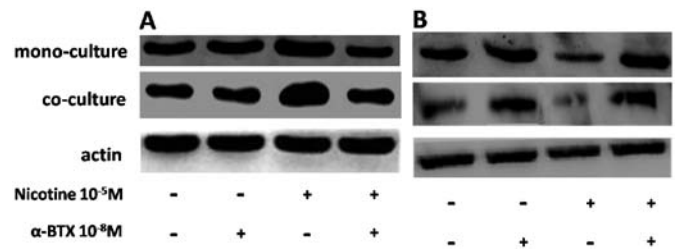


Figure 3. Effects of nicotine and/or α -BTX on (A) RANKL and (B) OPG expression at the protein level in human PDL cells co-cultured/non-co-cultured with CD4⁺ T cells.

Discussion

In the present study, we found that nicotine significantly upregulated RANKL expression and downregulated OPG expression in mono-culture human PDL cells; it also upregulated the secretion of IL-1 β in serum of mono-culture human PDL cells and mono-culture CD4⁺ T cells. The secretion of IL-1 β in human PDL cell-CD4⁺ T cell co-culture was significantly higher than in mono-culture systems, and the expression of RANKL was further upregulated in human PDL cells co-cultured with CD4⁺ T cells.

Tobacco smoking aggravated periodontal tissue destruction, pocket formation, alveolar bone resorption in periodontitis (2-4,23,24). It also reduced alveolar bone height and bone mineral density and led to alveolar bone resorption in rats (25). Meanwhile, its main component, nicotine, also aggravated the alveolar bone resorption in rat experimental periodontitis, and it was positively related to the expression of $\alpha 7$ nAChR (8,9). In addition, local accumulation of excessive CD4⁺ T cells may be associated with periodontal tissue destruction in periodontitis (26). CD4⁺ T cells in peripheral blood of smoking-associated periodontitis was significantly higher than that of non-smokers (27). These findings suggested that CD4⁺ T cell-mediated immune responses play a critical role in the process of periodontitis.

The RANKL/RANK/OPG axis is significantly involved in osteoclast differentiation and bone remodeling (17,18). Any deregulation of this axis can alter bone metabolism, resulting in loss or gain of bone mass. Compared with periodontal health, RANKL is upregulated, whereas OPG is downregulated, in periodontitis, resulting in an enhanced RANKL/OPG ratio (28-30). Compared with health or gingivitis, GCF RANKL level is also significantly upregulated and OPG level is significantly downregulated in chronic and generalized aggressive periodontitis, and positively related to probing pocket depth and clinical attachment level (31). In smoking-associated periodontitis, the GCF levels of RANKL and OPG showed a similar tendency to chronic and generalized aggressive periodontitis (32). The RANKL level was higher in gingival biopsies of smokers with periodontitis than in controls, whereas the OPG level was lower (33). Smoking also affected the status of periodontal treatment; the untreated as well as the treated smokers exhibited higher RANKL and lower OPG concentrations than non-smokers in saliva (34). Nicotine downregulated the expression of OPG and upregulated the expression of RANKL in human PDL cells (19). High RANKL/OPG ratio could promote differentiation of

osteoclasts. These results suggested that nicotine may affect the RANKL/RANK/OPG axis involved in alveolar bone resorption of smoking-associated periodontitis.

An animal study showed that CD4⁺ T cells, but not CD8⁺ T cells or B cells, were identified as essential mediators of alveolar bone destruction (35). Extract of smokeless tobacco at low concentrations enhanced the production of IL-1 β in lymphocytes and also induced lymphocyte proliferation (36). IL-1 β could be part of host responses involved in the process of local periodontitis and promote differentiation of osteoclasts, causing periodontal tissue destruction and alveolar bone absorption (22). Gursoy *et al* (37) suggested that salivary IL-1 β could be referred to as the marker of periodontitis. Clinical studies showed that the GCF IL-1 β level is significantly associated with pocket depth and bleeding on probing in periodontitis (38). Smoking status affected the GCF IL-1 β level; GCF IL-1 β from deep bleeding sites of heavy smokers was significantly higher than that of non-smokers (20). Moreover, smoking could also affect the GCF IL-1 β level following periodontal therapy. IL-1 β concentration was significantly greater in smokers following therapy than in non-smokers (39). Any factors affecting the balance of the RANKL/RANK/OPG axis could lead to the imbalance of bone metabolism, both in physiological and pathological conditions. Nakao *et al* (40) suggested IL-1 β as an autocrine factor regulating compressive force-induced RANKL expression in human PDL cells. IL-1 β induced RANKL expression at the mRNA and protein levels, as well as RANKL activity, through partial suppression of prostaglandin E2 synthesis in human PDL cells directly or indirectly (41). Cell-cell interactions between PBMCs and periodontal ligament fibroblasts significantly favor the expression of osteoclastogenesis-related genes (RANKL, RANK, TNF- α and IL-1 β) and the ultimate formation of osteoclasts (42). CD4⁺ T cells also derive from PBMCs, indicating human PDL cell-CD4⁺ T cell co-culture could show similar effects as human PDL cell-PBMC co-culture. Stimulated human PDL cell-PBMC co-culture with IL-1 β has a long-lasting effect, leading to a significantly increased osteoclastogenesis, upregulating mRNA expression of intercellular adhesion molecule-1 (ICAM-1), macrophage colony stimulating factor (M-CSF) and IL-1 β , augmenting formation of TRACP⁺ multinucleated cells (43). These findings indicate that IL-1 β could affect the balance of the RANKL/RANK/OPG axis, particularly RANKL, causing bone resorption and inflammation in periodontitis.

In conclusion, our data indicate that nicotine could favor osteoclastogenesis in human PDL cells co-cultured with CD4⁺ T cells by upregulating IL-1 β . This may have implications for the better understanding of affected bone remodeling in smoking-associated periodontitis. Although we gained significant insights, these results are based on the responses of cell lines; further investigations are required to ascertain whether or not these results reflect processes in the intact body.

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