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Cigarette smoke extract modulates human β-defensin-2 and interleukin-8 expression in human gingival epithelial cells

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Background and Objective: Human gingival epithelial cells (HGECs) are continually exposed to oral bacteria and to other harmful agents. Their responses to stimuli are critical in maintaining periodontal homeostasis. The aim of this study was to investigate the modulating effect of cigarette smoke extract (CSE) on the innate immune responses of HGECs.

Material and Methods: Toll-like receptor (TLR) expression of HGECs was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). The effect of CSE or nicotine on the expression of the antimicrobial peptide human β-defensin-2 (hBD-2) and the pro-inflammatory cytokine interleukin (IL)-8 in stimulated HGEC cultures was evaluated by RT-PCR and enzyme-linked immunosorbent assay.

Results: The HGECs expressed mRNA of TLRs 1, 2, 3, 5, 6, 9, 10, and minimally of TLR4, but not of TLRs 7 or 8. Stimulation of HGECs with highly purified TLR2, 3 or 5 ligands led to expression of hBD-2 and of IL-8. Enhancement of hBD-2 and IL-8 was observed in HGECs after combined stimulation with *Porphyromonas gingivalis* lipopolysaccharide (TLR2 ligand) and tumour necrosis factor-α, compared with stimulation using either agent alone. After CSE exposure, hBD-2 expression was markedly reduced in stimulated HGEC cultures, whereas IL-8 expression was markedly increased. These effects were also observed, but were markedly attenuated, upon nicotine treatment.

Conclusion: Human gingival epithelial cells play a critical role in orchestrating the innate immune responses of periodontal tissue via TLR signalling. Our results represent the first demonstration that CSE can modulate HGEC function by suppressing hBD-2 and enhancing IL-8 production, and this may be, in part, a possible mechanism which promotes periodontal disease.

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It is well recognized that cigarette smoke can alter cell function and promote disease development and severity (1–5). Epidemiological investigations indicate that cigarette smoking is a leading risk factor for periodontitis. Smokers exhibit a greater number of diseased sites than non-smokers, as well as greater loss of alveolar bone and increased tooth loss (6,7). Disease severity increases with the intensity and duration of smoking exposure (8–10).

It is thought that the immune-modulating effect of smoking on host response to bacterial plaque is associated with a more aggressive periodontal breakdown (11). Early studies demonstrated impaired phagocytosis by *in situ* smoke-exposed oral polymorphonuclear neutrophils (12). In a recent study of human experimental gingivitis, a significantly higher amount of interleukin (IL)-8 at baseline in smokers than in non-smokers was observed, while the anti-inflammatory cytokine IL-4 was depressed in smokers (13). On balance, smoking appears to inhibit host defense against microbial infection while promoting or amplifying inflammatory reactions. However, the specific mechanisms by which smoking promotes disease occurrence and severity are not yet well understood.

In the oral cavity, the epithelium is the first tissue that encounters cigarette smoke. Very little is known about the effect of smoking on gingival epithelial cells, although it is well recognized that these cells play an integral role in innate immune defense. They are strategically located between bacterial plaque biofilms and the underlying periodontal connective tissues and bone. To recognize invading pathogens, gingival epithelial cells express pattern recognition receptors, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domains (NODs; 14,15). These cells not only serve as a physical barrier, but also actively respond to microbes by synthesizing pro-inflammatory cytokines, such as IL-1, IL-8 and tumour necrosis factor-α (TNF-α), as well as important antimicrobial peptides. Human β–defensin-2 (hBD-2) is one such peptide. It is under intensive study because it is inducible when epithelial cells are exposed to bacteria and pro-inflammatory cytokines, thus suggesting that it has a specialized role in the innate epithelial defense mechanisms (16,17). It causes microbial death through disruption of bacterial membrane integrity. It is detectable in gingival crevicular fluid (18) and is significantly more abundant in the epithelial layer of periodontitis lesions than in healthy periodontal tissue (19).

In the present study, we aimed to investigate the immune-modulating effect of cigarette smoke extract (CSE) on primary human gingival epithelial cell (HGEC) responses, specifically IL-8 and hBD-2 expression.

Material and methods

Reagents

Medium for HGEC cultures was serum-free keratinocyte growth medium, which was purchased from Clonetics (Walkersville, MD, USA). Dispase was purchased from B-M Biochemicals (Indianapolis, IN, USA). Highly purified TLR ligands, including lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (TLR2 ligand), poly(I:C) (TLR3 ligand), LPS from *Escherichia coli* K12 strain (TLR4 ligand), flagellin from *Salmonella typhimurium* (TLR5 ligand), loxoribine (guanosine analogue, TLR7 ligand) and single-stranded poly(U) oligonucleotide complexed with LyoVec (TLR8 ligand), were obtained from InvivoGen (San Diego, CA, USA). Cytosine-phosphate-guanosine oligodeoxynucleotide (CpG ODN) (TLR9 ligand) was obtained from Coley Pharmaceutical Group (Wellesley, MA, USA). Tumour necrosis factor-α was purchased from R&D Systems (Minneapolis, MN, USA). (–)-Nicotine hydrogen tartrate salt was purchased form Sigma (St Louis, MO, USA).

Human gingival epithelial cells

Gingival tissue samples were collected from subjects who had clinically healthy periodontium and no history of periodontitis. All subjects were lifetime non-smokers. The gingival biopsies were obtained at the time of crown-lengthening procedure for prosthodontic reasons at the Periodontal Clinic, Faculty of Dentistry, Chulalongkorn University. Before operation, ethical approval was obtained from the ethics committee of the Faculty of Dentistry, and written informed consent was obtained from each subject. The HGECs from gingival tissues were obtained following an established procedure (20). In brief, the excised tissues were immediately washed with Dulbecco's phosphate buffered saline in 35 mm tissue culture dishes, and digested in 0.2% dispase for 24 h at 4°C. The separated epithelial layer was then washed, minced with scissors into fragments (1–3 mm^2) and cultured in growth medium. After a confluent monolayer of cells was reached, HGECs were trypsinized, washed twice and then transferred to new tissue culture flasks. The HGEC cultures at passage two to four were used throughout the study.

Messenger RNA expression of TLRs in HGECs

Total RNA from HGECs was isolated by using a RNeasy Mini kit (Qiagen, Hilden, Germany). One microgram of Dnase I-treated total RNA was reverse transcribed using ImProm-II Reverse Transcription System for reverse transcriptase-polymerase chain reaction (RT-PCR; Promega, Madison, WI, USA). Toll-like receptors 1–10 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were amplified using specific primers purchased from Proligo (Singapore). The PCR conditions have been described previously (21,22), and the primer sequences were as follows: TLR1, forward 5′-CGTAAAACTGG AAGCTTTGCAAGA-3′, reverse 5′-CCTTGGGCCATTCCAAATAAGT CC-3′; TLR2, forward 5′-GGCCAGC AAATTACCTGTGTG-3′, reverse 5′-CCAGGTAGGTCTTGGTGTTCA-3′; TLR3, forward 5′-ATTGGGTC-TGGGAACATTTCTCTTC-3′, reverse 5′-GTGAGATTTAAACATTCCT CTTCGC-3′; TLR4, forward 5′-C-TGCAATGGATCAAGGACCA-3′, reverse 5′-TCCCACTCCAGGTAAGT GTT-3′; TLR5, forward 5′-CCTCATG ACCATCCTCACAGTCAC-3′, reverse 5′-GGCTTCAAGGCACCAG-CCATCTC-3′; TLR6, forward 5′-ACTGACCTTCCTGGATGTGG-3′, reverse 5′-TGGCACACCATCCT GAGATA-3′; TLR7, forward 5′-ACA AGATGCCTTCCAGTTGC-3′, reverse 5′-ACATCTGTGGCCAGGTA AGG-3′; TLR8, forward 5′-CAGA ATAGCAGGCGTAACACATCA-3′, reverse 5′-AATGTCACAGGTG CATTCAAAGGG-3′; TLR9, forward

5′-GCGAGATGAGGATGCCCTGCC CTACG-3′, reverse 5′-TTCGGCCGT-GGGTCCCTGGCAGAAG-3′; TLR10, forward 5′-GGCCAGAAACTGTGG TCAAT-3′, reverse 5′-AACTTCCTGG CAGCTCTGAA-3′; and GAPDH, forward 5′-TCATCTCTGCCCCCTCT GCTG-3′, reverse 5′-GCCTGCTCAC CACCTTCTTG-3′).

Toll-like receptor ligation on HGECs after treatment with different TLR ligands and/or TNF-α

Human gingival epithelial cells (1.2×10^5 cells/mL) in 48-well plates (Corning Inc., Corning, NY, USA) were treated with the following alternatives: (1) various single TLR ligands: *P. gingivalis* LPS (50 μg/mL), poly (I:C) (100 μg/mL), *E. coli* LPS (10 μg/mL), *S. typhimurium* flagellin (5 μg/mL), loxoribine (100 μM), ssPolyU (5 μg/mL) and CpG ODN 2006 (10 μg/mL); (2) TNF-α (30 ng/mL); or (3) TLR ligand and cytokine combination: *P. gingivalis* LPS (50 μg/mL) plus TNF-α (30 ng/mL). After treatment with individual TLR ligand and/or TNF-α for 24 h, the cells and culture supernatants were collected for measurement of hBD-2 and IL-8.

Detection of hBD-2 expression by RT-PCR and real-time PCR

Total RNA of treated HGECs was isolated and reverse transcribed as described above. The hBD-2 was amplified using specific primer pair (forward 5′-CCAGCCATCAGCCAT-GAGGGT-3′, reverse 5′-GGAGCCCT-TTCTGAATCCGCA-3′) for RT-PCR, and the PCR conditions were as described previously (23). The specific hBD-2 primer pair for real-time PCR is (5′-TGATGCCTCTTCCAGGTG-TTT-3′ and 5′-GGATGACATA-TGGCTCCACTCTT-3′); the hBD-2 probe is (5′-6FAM-TGGTGGTATA-GGCGATCCTGTTACCTGC-TAM-RA-3′; 24). These were purchased from Operon (Cologne, Germany). Quantitative real-time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA, USA) by using QuantiTect Probe PCR Kit (Qiagen). The 25 μL reaction mixture contained 1 μL (50 ng total RNA) of cDNA template, 2× QuantiTect Probe PCR Master Mix, 0.4 μM (final concentration) primer pairs and 0.1 μM TaqMan Probe. In all reactions, HotStarTaq DNA polymerase was activated by an initial denaturation at 95°C for 15 min, followed by 45 cycles of two-step amplification procedure composed of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. For real-time PCR, hBD-2 mRNA expression was quantified using the comparative cycle-threshold (Ct) method. The Ct of the housekeeping gene GAPDH was subtracted from the Ct of hBD-2 to obtain ΔCt. The normalized fold changes of the hBD-2 mRNA expression were expressed as $2^{-\Delta\Delta Ct}$, where ΔΔCt equals (ΔCt sample – ΔCt control).

Preparation of CSE

Cigarette smoke extract was prepared as previously described (25), with modification. Briefly, the mainstream smoke from five cigarettes ('Krongtip 90,' Thailand Tobacco Monopoly, filtered type, Bangkok, Thailand) was bubbled through 30 mL of prewarmed (37°C) keratinocyte growth medium at a constant negative pressure of 290 mmHg. Medium containing CSE was filtered through 0.2 μm filters. Several aliquots were prepared and stored at −70°C until use. Each aliquot was used once.

Cigarette smoke extract/nicotine treatment of stimulated HGECs

Preliminary experiments were conducted to determine non-toxic concentrations of CSE and nicotine, in order to ensure that any of their observed effects on HGEC function did not result from cellular toxicity or cell death. Viability and appearance of treated and untreated cells were assessed after 24 h exposure to CSE or nicotine and compared by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay and Trypan blue exclusion. The cells showed 100% viability with CSE at 1:8, 1:4 and 1:2 dilution and nicotine at 0.1, 0.3 and 1 mM compared with control cells. These concentrations of CSE and nicotine were used throughout the study.

A non-toxic concentration of CSE or nicotine was added to HGEC cultures (1.2×10^5 cells/mL) in the presence of *P. gingivalis* LPS and TNF-α. After 24 h, the cells and culture supernatants were collected for measurement of hBD-2 and IL-8.

Determination of IL-8 and hBD-2 by enzyme-linked immunosorbent assay (ELISA)

The supernatants of stimulated HGECs were harvested and assessed for IL-8 and hBD-2 production by ELISA (R&D Systems, Minneapolis, MN, USA and Phoenix Pharmaceuticals Inc., Belmont, CA, USA, respectively).

Statistical analysis

Statistical comparisons among treatment conditions, with respect to production of hBD-2 and IL-8, were conducted using SPSS version 11.5 software (SPSS, Chicago, IL, USA). Student's paired *t*-test was used for normally distributed data, and the non-parametric Wilcoxson signed rank test was used for non-normally distributed data. A value of $p < 0.05$ was considered statistically significant.

Results

Toll-like receptor activation of HGECs

Previous studies showed conflicting data of TLR expression in HGECs(14,15,26). We therefore systematically investigated TLR expression and their responses to specific ligands. In HGECs derived from healthy periodontal tissues, we observed mRNA expression of TLRs 1, 2, 3, 4, 5, 6, 9 and 10, but not of TLRs 7 and 8 (Fig. 1A). However, TLR4 mRNA was minimally expressed. The results were reproducible in all four HGEC lines (four donors). In order to characterize the functional relevance of TLRs in HGECs, expression of the

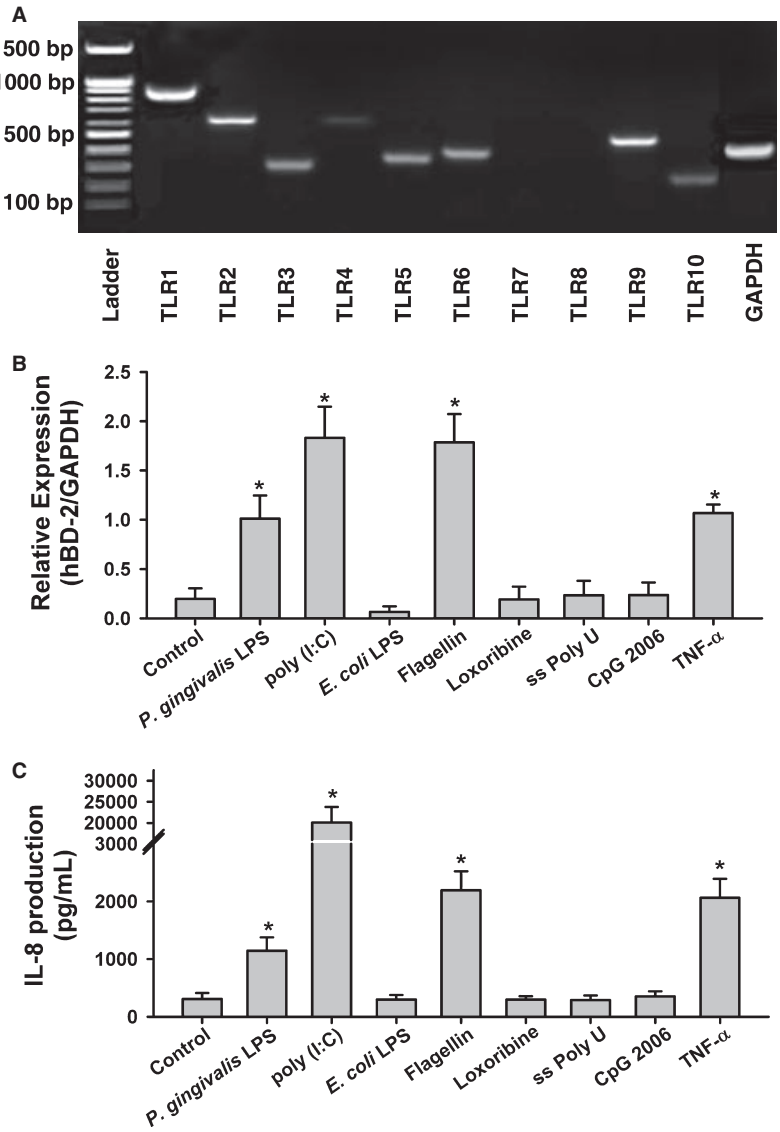

Fig. 1. Toll-like receptor expression and activation in HGECs. (A) Expression of mRNA for TLRs 1–10 was measured in cultured HGECs by RT-PCR. As an internal control, GAPDH mRNA was used. (B) Human gingival epithelial cells were stimulated with the following ligands: *P. gingivalis* LPS (TLR2 ligand), poly(I:C) (TLR3 ligand); *E. coli* LPS (TLR4 ligand); *S. typhimurium* flagellin (TLR5 ligand); loxoribine (TLR7 ligand); poly(U) (TLR8 ligand); and CpG ODN 2006 (TLR9 ligand). Control and stimulated HGECs were harvested after 24 h, and mRNA expression of hBD-2 was analysed by RT-PCR. Tumour necrosis factor-α-stimulated HGECs were used as a positive control. As an internal control, GAPDH mRNA was used. Data represent the mean ratios of hBD-2:GAPDH ± SEM ($n = 4$, $^*p < 0.05$, compared with unstimulated control). (C) For assessment of IL-8 production, culture supernatants of control and stimulated HGECs were harvested after 24 h and then assayed by ELISA. Data shown are means ± SEM of four separate experiments ($^*p < 0.05$, compared with unstimulated control).

antimicrobial peptides hBD-2 and IL-8 was determined after stimulation with a variety of highly purified TLR ligands. Tumour necrosis factor-α-treated HGECs were used as a positive control. In line with the observed expression of their respective TLRs 2, 3, and 5, *P. gingivalis* LPS, poly(I:C) and *S. typhimurium* flagellin induced hBD-2 and IL-8 in the epithelial cells (Fig. 1B,C). *Escherichia coli* LPS, a TLR4 ligand, did not induce either hBD-2 or IL-8, consistent with our observation of only minimal expression of TLR4. On the contrary, CpG ODN 2006, a potent TLR9 activator, had no effect, even though HGECs clearly expressed TLR9 mRNA (Fig. 1B,C).

Cigarette smoke extract suppresses epithelial hBD-2 but stimulates IL-8 expression

The immune-modulating effect of CSE was evaluated in HGECs that had been stimulated with the combination of *P. gingivalis* LPS and TNF-α. As depicted in Fig. 2A,B, the combination of the TLR2 ligand *P. gingivalis* LPS and TNF-α exerted an enhancing effect on both hBD-2 and IL-8 expression compared with those of the substances applied individually ($p < 0.05$). Figure 2C shows that treatment of stimulated HGECs with a non-toxic concentration of CSE (1:2 dilution) led to a large (approximately 80%) and statistically significant reduction in hBD-2 mRNA expression, compared with the response in stimulated cells with no CSE treatment ($p < 0.05$). The suppressed hBD-2 production was also confirmed at protein level (Fig. 2D). In contrast, CSE at the same concentration significantly enhanced IL-8 production (approximately 53% enhancement; $p < 0.05$; Fig. 2E). Cigarette smoke extract alone had no effect on either hBD-2 or IL-8 expression in unstimulated HGEC culture. Other concentrations of CSE (1:4 and 1:8 dilution) were also tested on HGEC production of hBD-2 and IL-8 (data not shown) but the optimal effect was found at the 1:2 dilution.

Nicotine suppresses epithelial hBD-2 but stimulates IL-8 expression

We next investigated whether the effect of CSE could be due to nicotine, the major component of CSE. Similar to the CSE results, treatment of stimulated HGECs with nicotine at non-toxic concentrations (0.1, 0.3 and 1 mM) led to a decrease in hBD-2 expression (Fig. 3A). Nicotine at higher concentrations (0.3 and 1 mM) significantly reduced hBD-2 mRNA expression (approximately 31% reduc-

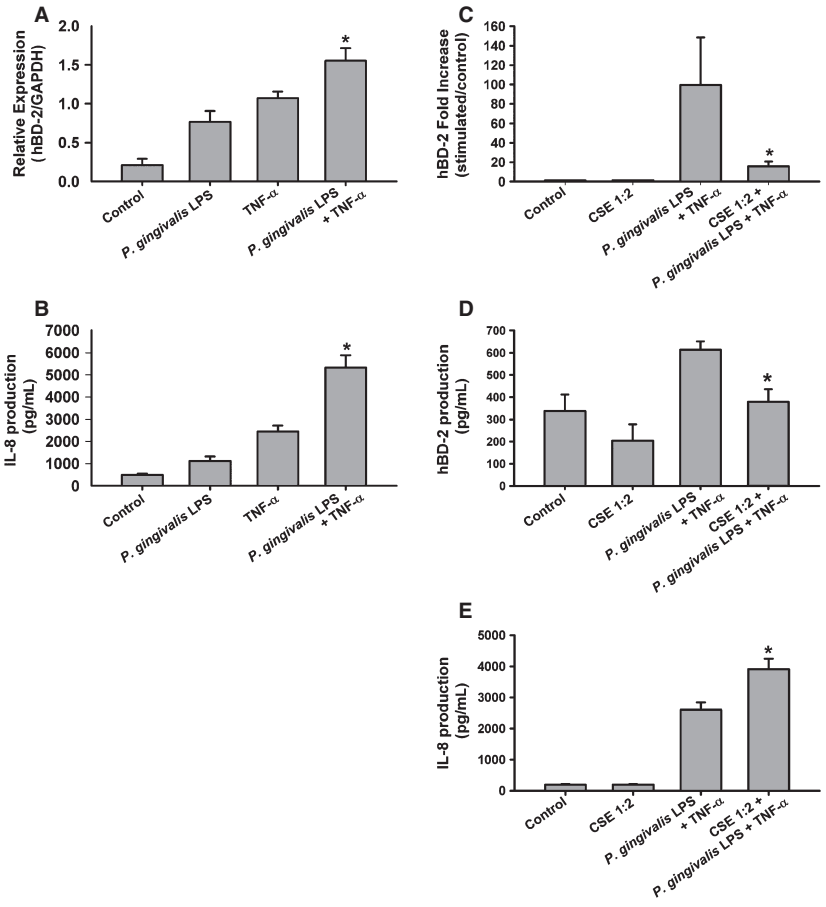

Fig. 2. The effect of CSE on expression of hBD-2 and IL-8 in HGECs upon stimulation with *P. gingivalis* LPS plus TNF-α. The combination of TLR2 ligand *P. gingivalis* LPS and TNF-α enhanced hBD-2 (A) and IL-8 expression (B). Data shown are means ± SEM of four separate experiments (*$p < 0.05$, compared with single stimulator). (C) Human gingival epithelial cells were stimulated with *P. gingivalis* LPS plus TNF-α in the presence or absence of a non-toxic concentration of CSE (1:2 dilution). For quantitative analysis of hBD-2 expression, stimulated HGECs with or without CSE treatment were harvested after 24 h and mRNA expression of hBD-2 was analysed by real-time PCR. As an internal control, GAPDH mRNA was used. Data are the mean ratios of hBD-2:GAPDH ± SEM from five separate experiments (*$p < 0.05$, compared with HGECs stimulated with the combination of *P. gingivalis* LPS plus TNF-α in the absence of CSE). For assessment of hBD-2 (D) and IL-8 production at a protein level (E), culture supernatants were harvested after 24 h and then assayed by ELISA. Data shown are means ± SEM of five separate experiments (*$p < 0.05$, compared with HGECs stimulated with the combination of *P. gingivalis* LPS plus TNF-α in the absence of CSE).

tion) compared with cultures with no nicotine treatment ($p < 0.05$; Fig. 3A). The suppressed hBD-2 production was also confirmed at protein level (Fig. 3B). In contrast, nicotine at 0.3 and 1 mM induced a slightly but statistically significant increase in IL-8 production (approximately 13% enhancement) compared with cultures with no nicotine treatment ($p < 0.05$; Fig. 3C). Thus, the directions of effect after nicotine treatment were the same as those after CSE treatment. However, the magnitudes of the nicotine effects were considerably smaller than those of the CSE effects.

Discussion

It is known that host immune responses employ pattern recognition receptors to recognize invasive microbial organisms. This recognition leads to release of mediators which limit and contain microbial invasion. Recent evidence suggests an emerging role of TLRs in peridontitis (27). In this study, HGECs from healthy gingival tissue clearly expressed mRNA of TLRs 1, 2, 3, 5, 6, 9 and 10, and minimally expressed TLR4, but did not express TLRs 7 and 8. This result was reproducible in all samples examined ($n = 4$). These findings agree with previous observations of Kusumoto *et al.*(14). In contrast, some investigators have reported clear expression of TLR4 and TLR7 in primary HGECs (15,28). This discrepancy requires further investigation.

In line with TLR mRNA expression, HGECs expressed hBD-2 and IL-8 in response to *P. gingivalis* LPS, poly(I:C), *S. typhymurium* flagellin, which are respective ligands for TLRs 2, 3 and 5. The pro-inflammatory cytokine TNF-α significantly enhanced *P. gingivalis* LPS-induced hBD-2 and IL-8 expression. These enhanced responses were consistent with the observed TNF-α-induced upregulation of TLR2 expression (data not shown). Other investigators have reported upregulation of hBD-2, but no effect on IL-8, in oral epithelial cell lines (HSC-2 and HO-1-u-1) treated with a variety of specific TLR ligands (TLRs 2, 3 and 4; 15,26). This difference in findings may be due to use of different cell types. We used primary epithelial cells derived from healthy gingival tissue, whereas the other research groups used oral cancer cells.

In the present study, treatment with CpG ODN 2006, a potent ligand for TLR9, did not induce hBD-2 or IL-8 despite detectable TLR9 expression in HGECs. This is consistent with previous observations (14). We recently reported that human gingival fibroblasts expressed TLR9, but showed no response to CpG ODN (29). The reason for this remains unclear and requires further investigation. The observed inability of the TLR4 ligand *E. coli* LPS to induce hBD-2 and IL-8 expression in HGECs could be attributable to the minimal expression of TLR4, the culture conditions, or both. In the HGEC medium, there was no added fetal bovine serum, a source of soluble cluster

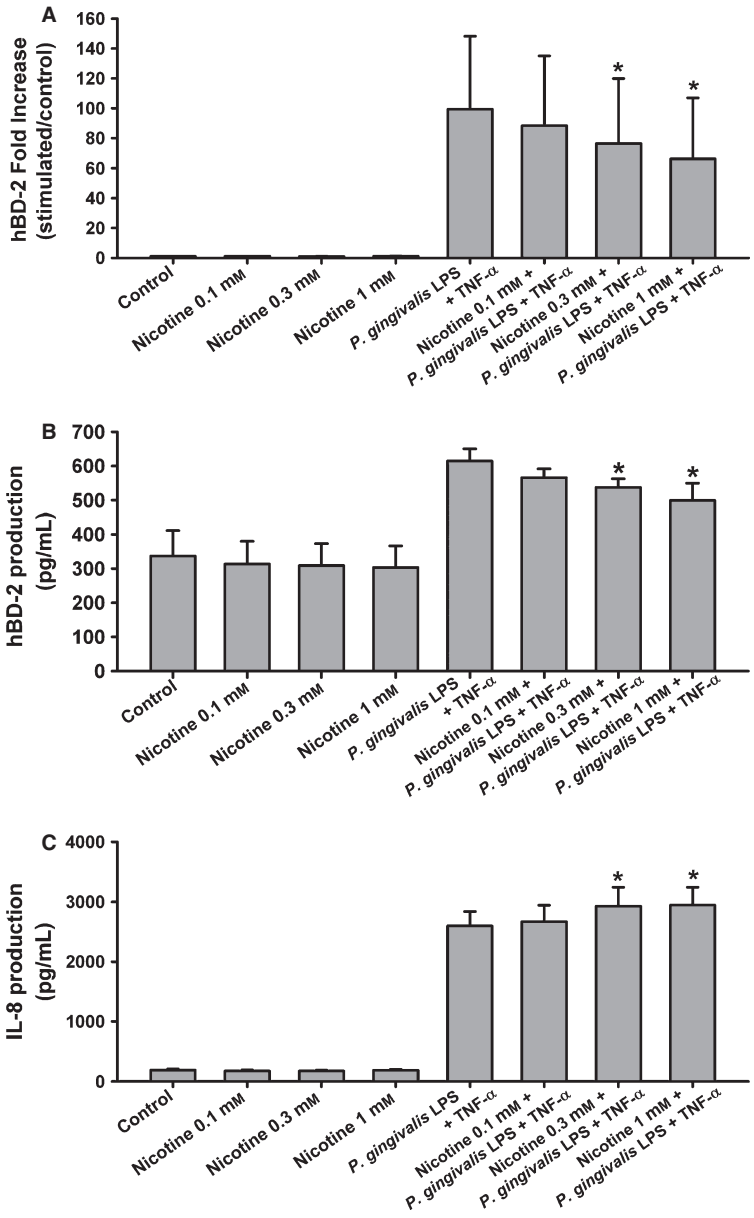

Fig. 3. The effect of nicotine on expression of hBD-2 and IL-8 in stimulated HGECs. Human gingival epithelial cells were stimulated with *P. gingivalis* LPS plus TNF-α in the presence or absence of nicotine (0.1, 0.3 and 1 mM). (A) For quantitative analysis of hBD-2 expression, stimulated HGECs with or without nicotine treatment were harvested after 24 h and mRNA expression of hBD-2 was analysed by real-time PCR. As an internal control, GAPDH mRNA was used. Data are means ± SEM of five separate experiments (*$p < 0.05$, compared with HGECs stimulated with the combination of *P. gingivalis* LPS plus TNF-α in the absence of nicotine). For assessment of hBD-2 (B) and IL-8 production at a protein level (C), culture supernatants of HGECs were harvested after 24 h and then assayed by ELISA. Data shown are means ± SEM of five separate experiments (*$p < 0.05$, compared with HGECs stimulated with the combination of *P. gingivalis* LPS plus TNF-α in the absence of nicotine).

of differentiation 14 and LPS-binding protein, which is required for TLR4 signalling (30). Our findings agree with previous studies showing that human intestinal epithelial cells express only low levels of TLR4 and respond only weakly to bacterial LPS (31,32).

Cigarette smoking is strongly associated with multiple diseases, including lung cancer, chronic obstructive pulmonary disease, heart disease, respiratory infection and periodontitis (6,7,33–35). Smoking induces and aggravates at least some of these diseases by initiating and promoting inflammatory host responses (35). To the best of our knowledge, no previous studies have assessed the immune-modulating effect of CSE on HGEC response. In the present study, we demonstrated that CSE inhibited antimicrobial hBD-2 expression but enhanced IL-8 expression in stimulated HGECs. The data suggest that the expression of these two mediators may be controlled by different pathways. A simultaneous enhancing and suppressing effect of CSE on mediator production has also be observed in another cell type. Treatment of human dendritic cells with CSE inhibited IL-12 but enhanced IL-10 production, thus promoting T-helper 2-mediated immunity (4).

Nicotine showed similar effects but to a much lesser extent, implying that non-nicotine components in CSE are primarily responsible for these immune-modulating effects. The observed effects of CSE and nicotine in this study were not due to cellular toxicity, since the concentrations used were in the non-toxic range. In addition, nicotine levels were similar to those in the saliva of smokers (36). Our results strongly suggest that smoking reduces the ability to kill bacteria, and may thereby allow overgrowth and invasion of periodontal pathogens. *Porphyromonas gingivalis* and *Tannerella forsythia* have been shown to colonize a larger proportion of tooth sites in smokers than in non-smokers (37). Also, our results suggest that smoking enhances the IL-8 response, supporting the *in vivo* observation of elevated IL-8 in the gingival crevicular fluid of smokers (13). Our findings are also consistent with previous observations of heavier cellular infiltration in the epithelial cell layer in the periodontitis lesions of smokers compared with non-smokers (38).

In conclusion, our study demonstrates the dynamic role of HGECs in the innate immune response of periodontal tissue. Human gingival epithelial cells express an array of TLRs which can respond to specific microbial components, leading to the production

of antimicrobial hBD-2 and recruitment of inflammatory cells via the production of IL-8. Continuous TLR triggering in HGECs by commensal organisms of dental plaque is likely to occur in clinically healthy periodontal tissue in order to protect against infection with periodontopathic bacteria. Disruption of oral homeostasis by cigarette smoke, via suppressing the hBD-2 and promoting the IL-8 response, is likely to promote the invasion of pathogenic bacteria and enhance chronic inflammation, the hallmark of periodontitis. Therefore, the present study provides mechanistic support for the previous observation that cigarette smoking is an important risk factor in periodontitis. However, much remains to be explored regarding the mechanisms of cigarette smoking and further research is required.

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