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Short communication

Interleukin-1 and interleukin-8 in nicotine- and lipopolysaccharide-exposed gingival keratinocyte cultures

G. K. Johnson[1,2], J. M. Guthmiller[3], S. Joly[2], C. C. Organ[2], D. V. Dawson[2,4]

[1]Department of Periodontics, University of Iowa College of Dentistry, Iowa City, IA, USA, [2]Dows Institute for Dental Research, University of Iowa College of Dentistry, Iowa City, IA, USA, [3]Department of Periodontics, University of North Carolina at Chapel Hill, NC, USA and [4]Department of Preventive and Community Dentistry, University of Iowa College of Dentistry, Iowa City, IA, USA

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Background and Objective: Tobacco use is associated with increased periodontal destruction in both cigarette smokers and smokeless tobacco users. Gingival keratinocytes are the first cells in contact with microbial and tobacco components and play a key role in the innate immune response to these agents. The objective of this study was to evaluate the effect of nicotine and bacterial lipopolysaccharide (LPS) alone and in combination on gingival keratinocyte production of interleukin-1α (IL-1α) and interleukin-8 (IL-8).

Material and Methods: Gingival keratinocyte cultures were established from 10 healthy, non-tobacco-using subjects. The cells were stimulated for 24 h with 1 μM or 1 mM nicotine and/or 10 μg/mL *Escherichia coli* or *Porphyromonas gingivalis* LPS. Interleukin-1α and IL-8 proteins were quantified using ELISAs.

Results: Compared with untreated cultures, 1 mM nicotine stimulated production of IL-1α ($p < 0.001$); *E. coli* and *P. gingivalis* LPS increased IL-8 production ($p = 0.0014$ and $p = 0.0232$, respectively). A combination of nicotine and LPS produced the highest cytokine quantities. Amounts of IL-1α and IL-8 following 1 mM nicotine and LPS exposure were significantly greater than in untreated cultures ($p < 0.001$). Interleukin-8 was also responsive to 0.1 μM nicotine combined with *E. coli* or *P. gingivalis* LPS compared with control cultures ($p < 0.0001$ and $p = 0.0029$, respectively). Both cytokines tended to be elevated following the combined treatment relative to nicotine or LPS treatment alone.

Conclusion: These results demonstrate that nicotine and LPS differentially regulate IL-1 and IL-8 production by gingival keratinocytes. Combined treatment tended to elevate cytokine production further, which may have implications for the progression of periodontitis in tobacco users.

Georgia K. Johnson, DDS, MS, Dental Science Building S452, University of Iowa, Iowa City, IA 52242-1001, USA
Tel: +31 9 3357 238
Fax:+31 9 3357 239
e-mail:georgia-johnson@uiowa.edu

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Tobacco use is associated with pathological alterations in the periodontium. Cigarette smoking is a major risk factor in periodontitis and, based on the Third National Health and Nutrition Examination Survey (NHANES), current smokers are about four times as likely as subjects who have never smoked to have periodontitis (defined as ≥ 1 site with clinical attachment level ≥ 4 mm from the cemento-enamel junction and ≥ 4 mm probing depth) (1). The most common findings in smokeless tobacco users are localized gingival recession and white mucosal lesions at placement sites (2). In addition, findings from the NHANES data set indicate that smokeless tobacco users are twice as likely as non-tobacco users to have severe periodontal disease (defined as at least 1 tooth with 6 mm attachment loss at an interproximal surface; 3). The mechanisms of smoking-related periodontal destruction are twofold, including: (a) alterations in the periodontal microflora (4–8); and (b) effects on the immune response which influence vascular function, neutrophil/monocyte activities, antibody production and adhesion molecule expression, as well as release of cytokines and inflammatory mediators (9–12). Development of recession and mucosal lesions in smokeless tobacco users is related to mechanical and chemical effects and probably involves similar alterations of the immune response to those implicated in smoking-related periodontal destruction.

Nicotine, one of the most studied active ingredients in smoked and smokeless tobacco, can directly modify production of cytokines and inflammatory mediators by various cell types found in periodontal tissues, including fibroblasts, mononuclear cells, neutrophils, osteoblasts and keratinocytes (9–14). Previously, we demonstrated that nicotine, at concentrations in the range of those found in saliva of tobacco users (1 mM), increased interleukin-1α (IL-1α) and interleukin-1β (IL-1β) production by cultured gingival keratinocytes (15). Gingival keratinocytes are the first cells in contact with the myriad of chemicals found in tobacco products and are early producers of cytokines and chemokines, including IL-1 and interleukin-8 (IL-8), in response to bacterial plaque (16). As a proinflammatory cytokine, IL-1 activates macrophages and osteoclasts, increases release of proteolytic enzymes and triggers other aspects of the immune response (17). This includes stimulation of IL-8 secretion, which in turn plays a role in recruitment and activation of neutrophils (18). Cytokine production by gingival keratinocytes contributes to the development of the innate immune response to bacterial plaque and noxious agents.

Interactions between nicotine and bacteria, resulting in enhanced proinflammatory cytokine production, may contribute to increased periodontal destruction in tobacco users with poor oral hygiene (19,20). Hence, the goal of the present study was to evaluate IL-1α and IL-8 production by gingival keratinocytes in response to nicotine and lipopolysaccharide (LPS) alone and to nicotine in combination with LPS.

Material and methods

Gingival keratinocyte cultures

Gingival keratinocyte cultures were established from healthy gingival tissues discarded during clinical crown elongation surgeries using methodology previously described (15). Ten healthy, non-tobacco-using subjects under the age of 50 years contributed tissues for this study. These individuals had no history of systemic conditions requiring regular use of antibiotics or non-steroidal anti-inflammatory drugs within the past 3 mo. The protocol for this study was approved by the University of Iowa's Institutional Review Board for the Use of Human Subjects in Research.

Nicotine and bacterial LPS treatment

Keratinocytes (passage 2–3) were seeded (2.5×10^4 cells per well) into 48-well plates (Costar, Cambridge, MA, USA) for assessments of cytokine protein. The cultures were maintained in a serum-free keratinocyte growth medium (Clonetics Corporation, San Diego, CA, USA) containing 0.15 mM calcium, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 30 μg/mL bovine pituitary extract and 0.1 ng/mL epidermal growth factor.

When the cultures reached approximately 60% confluence, the wells were divided into nine groups and exposed to one of the following treatments: 0.1 μM nicotine; 1 mM nicotine; 10 μg/mL *Escherichia coli* LPS (Sigma Chemical, St Louis, MO, USA); 10 μg/mL *Porphyromonas gingivalis* LPS (kindly provided by Dr Jack Dyer, University of Nebraska Medical Center, College of Dentistry, Lincoln, NE, USA; 21); nicotine (0.1 μM or 1 mM) combined with LPS (*E. coli* or *P. gingivalis*) treatment; or medium alone (control). Nicotine dilutions were freshly prepared using nicotine free base (98–100% nicotine; Sigma Chemical, St Louis, MO, USA) and medium. These concentrations fall within the range of nicotine concentrations present in serum (0.1 μM; 22) and in saliva (1 mM) of smokers and smokeless tobacco users (23,24). The cells were exposed to the treatments for 24 h based on previous studies demonstrating that 24 h was the optimal time period for protein response (15). The keratinocyte nature of the cells was confirmed based on histological and ultrastructural features and immunostaining for high molecular weight cytokeratins.

Supernatants were collected from the cultures, and protease inhibitors (antipain, 1 μg/mL; aprotinin, 1 μg/mL; leupeptin, 1 μg/mL; and *N*-ethylmaleimide, 125 μg/mL; Sigma Chemical, St Louis, MO, USA) were added. The cells were detached from the wells by trypsin–EDTA exposure for 10–14 min at 37°C, and an aliquot of the cell suspension was counted in a Coulter counter (Coulter Electronics, Hialeah, FL, USA). The remaining cells were sonicated in a buffer containing 0.01% Triton® X-100 (Sigma Chemical, St Louis, MO, USA) detergent and protease inhibitors. The cell supernatants and lysates were stored at −80°C until the time of assay.

Interleukin-1α and IL-8 assays

In keratinocytes, the majority of IL-1α is cell associated (25); therefore, IL-1α was evaluated in the cell lysates using commercially available ELISAs

performed according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Interleukin-8 levels in the culture supernatants were also assessed by ELISA (Endogen, Woburn, MA, USA). These assays were performed in duplicate (two separate culture wells from each of the ten subjects). For each treatment condition, mean mediator levels were calculated and expressed as picograms per 10^4 cells.

Cell viability assay

For each subject, the effect of nicotine and LPS on keratinocyte viability was evaluated using a commercial assay for mitochondrial dehydrogenase (MTS; CellTiter Aqueous non-radioactive cell proliferation assay, Madison, WI, USA). This assay is based upon the conversion of a tetrazolium compound by mitochondrial dehydrogenase enzymes present in viable cells to a colored formazan product. Keratinocytes were seeded (7.5×10^3 cells per well) into 96-well tissue culture plates (Corning Life Sciences, Lowell, MA, USA) and exposed to the experimental treatments previously described, and the assay was performed according to the manufacturer's instructions.

Statistical analysis

A linear mixed modeling approach was employed to take account of the repeated measures nature of the design, specifying treatment as a fixed effect and cell line as a random effect. Analyses were carried out using the average value of duplicate measurements of the 10 subjects. Linear contrasts were used to test comparisons of interest. Adjustment for multiple comparisons was made using the Bonferroni method, based upon an overall type I error level of 0.05 and 28 planned comparisons. Therefore, a p-value < 0.0018 was accepted as statistically significant. Standard model assumptions were assessed for validity.

Results

Nicotine or LPS treatment

The high dose of nicotine (1 mM) strongly upregulated the production of IL-1α ($p < 0.0001$) compared with medium alone (Fig. 1A). Interleukin-1α concentrations in these nicotine-exposed cultures were approximately 2.5 times greater than in control cultures. Neither the low concentration of nicotine (0.1 μM) nor LPS treatment had a significant impact on IL-1α protein amounts (Fig. 1A). Interleukin-8 secretion following high- or low-dose nicotine treatment was not significantly different from that seen with the control cells (medium alone). In contrast, both *E. coli* and *P. gingivalis* LPS increased IL-8 production relative to the control cultures (joint p-value = 0.0017). Results were significant when the control was compared with *E. coli* LPS ($p = 0.0014$) and suggestive when *P. gingivalis* was compared with the control cultures ($p = 0.0232$), although not significant after correction for multiple comparisons (Fig. 1B).

Nicotine and LPS combined treatment

A combination of nicotine and LPS treatment resulted in the greatest production of both cytokines. When *E. coli* LPS or *P. gingivalis* LPS and the high nicotine dose (1 mM) were combined, IL-1α and IL-8 production were significantly increased compared with control conditions ($p < 0.0001$,

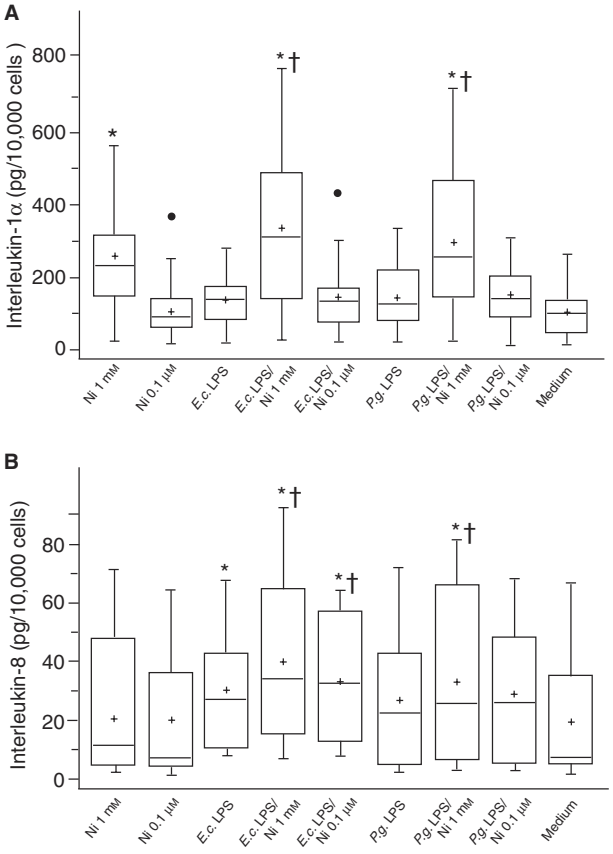

Fig. 1. Box plot diagrams showing the differences in IL-1α (A) and IL-8 (B) protein production by gingival keratinocytes exposed to nicotine (Ni) at 1 mM or 0.1 μM, *E. coli* (*E.c.*) or *P. gingivalis* (*P.g.*) LPS (10 μg/mL), alone or in combination. Vertical boxes represent 25th–75th percentiles; error bars represent 10th and 90th percentiles; the line within the box plot represents the median value; and + represents the mean value. Values represent the mean of duplicates of 10 subjects. Outliers are represented by black dots. For IL-1α, in each case the associated standard error is 45.36 because the design is balanced and the variance is a pooled estimate. For IL-8, the associated standard error is 7.67. *Significantly different from medium alone ($p < 0.0018$). †Significantly different from respective LPS only ($p < 0.0018$; A). † Significantly different from dose-matched nicotine only ($p < 0.0018$; B).

in all instances; Fig. 1A and B). Interleukin-8 was also responsive to the low-dose nicotine combined with *E. coli* or *P. gingivalis* LPS when compared with medium alone ($p < 0.0001$ and $p = 0.0029$, respectively), although the latter was not significant after correction for multiple comparisons (Fig. 1B).

Comparisons were made between nicotine treatment alone and the combined nicotine and LPS treatments. For IL-1α, the comparison of high-dose nicotine alone vs. high-dose nicotine plus *E. coli* LPS was suggestive ($p = 0.0363$) of an enhanced effect. Findings for IL-8 were consistent with the strong effect of LPS treatment and the lack of effect of nicotine on this chemokine. Both LPS treatments combined with high-dose nicotine resulted in significantly higher IL-8 amounts compared with high-dose nicotine alone ($p < 0.0001$ for *E. coli* and $p = 0.0006$ for *P. gingivalis*. The combination low-dose nicotine and *P. gingivalis* LPS was elevated, albeit not significantly after adjustment ($p = 0.0043$), compared with low-dose nicotine alone.

The final comparison was made between LPS alone and LPS plus nicotine treatment. Given the highly significant effect of the high-dose nicotine treatment alone on IL-1α, it was not surprising that high-dose nicotine combined with either LPS treatment resulted in a higher mean IL-1α response than treatment with the respective LPS alone ($p < 0.0001$) in both instances (Fig. 1A). When the combination treatment was compared with the LPS alone treatment, there was a trend for higher IL-8 amounts for the high-dose nicotine plus LPS combination treatment compared with LPS alone; this was more pronounced for *E. coli* LPS ($p = 0.0026$) than for *P. gingivalis* LPS ($p = 0.0187$) and not significant after multiple comparisons adjustment (Fig. 1B).

Cell viability

Cell counts, as determined by a Coulter counter, were not significantly different among treatments at 24 h, nor was cell viability altered, as assessed by mitochondrial dehydrogenase assays (data not shown).

Discussion

Altered cytokine production is one of the proposed mechanisms to explain the increased periodontal destruction observed in tobacco users (11). In previous studies, nicotine (1 and 100 μM) increased IL-1α, IL-1β (26) and IL-8 secretion in cultured human oral epidermoid carcinoma cell lines (27). Mahanonda *et al.* (14) recently reported that high nicotine doses (0.3 and 1 mM) significantly enhanced human gingival epithelial cell production of IL-8 (pg/mL) by 13% compared with control cultures. Although IL-8 production in response to 1 mM nicotine was increased by 19% in the present study, this increase of 3.74 pg per 10,000 cells was not statistically significant ($p = 0.47$). A lack of impact of nicotine on IL-8 production was also reported in neonatal foreskin epidermal keratinocyte cultures exposed to relatively lower levels of nicotine (6 μM; 28). Differences among the studies may be related to varied nicotine doses as well as subject and cell variability.

Increased IL-8 production following gingival keratinocyte treatment with LPS has been previously reported (29). Like our study, Mahanonda *et al.* (14) reported relatively low baseline levels of IL-8 in unstimulated cultures and that LPS was a stronger stimulant of IL-8 than nicotine. Interestingly, the cytokine responses to *P. gingivalis*, a major periodontal pathogen, were not as robust as to the *E. coli* LPS, which is the converse of that observed by Mahanonda *et al.* (14). These investigators reported a lack of effect of *E. coli* LPS on IL-8 production. Other investigators have shown that gingival epithelial cells infected with *E. coli* or *P. gingivalis* produce IL-8 and that different strains of *P. gingivalis* have differential effects (30). Therefore, differences in the bacterial strains as well as the method of LPS preparation could explain discrepancies among studies.

The intriguing observation that the combination of nicotine and LPS produced the highest amounts of IL-1α and IL-8 supports clinical evidence that tobacco users with poor oral hygiene have more severe periodontal destruction than tobacco users with good oral hygiene (19,20). Both IL-1 and IL-8 are important in controlling infections; however, excessive production may favor tissue destruction. Although not significant after correction for multiple comparisons, it is interesting that IL-1α was higher in the high-dose combination treatment than with nicotine alone and that IL-8 was higher in the high-dose combination treatment than LPS alone. Collectively, these findings support an interactive effect of smoking and bacterial factors on the host response in periodontal disease. It is not clear whether this is an additive or synergistic phenomenon; the fact that we identified these trends despite the high variability in expression of mediators warrants further investigation.

In this study, nicotine was the main stimulant of IL-1α, and LPS was the primary stimulant for IL-8. In addition to the differential impact of nicotine and LPS on IL-1 and IL-8, cellular responses to nicotine and LPS treatments vary according to cell type. This contributes to the contradictory findings concerning the impact of nicotine on cytokines; some studies report that nicotine either suppresses or has no effect on proinflammatory cytokines. For example, nicotine (3 μM to 0.617 mM) had no effect on IL-1β secretion by peripheral blood mononuclear cells (31,32), and nicotine (0.617–6.17 mM) treatment of LPS-primed monocyte and gingival mononuclear cells inhibited IL-1 secretion (31–34). In human neutrophils, nicotine at concentrations comparable to those used in the present study did not affect IL-1β secretion, but IL-8 was strongly induced in a dose- and time-dependent manner (35). In contrast to this study, where nicotine did not significantly alter IL-8 production from keratinocytes, gingival fibroblast cultures treated with low nicotine concentrations (1 nM) released elevated amounts of both IL-6 and IL-8 proteins; the combination of high-dose nicotine (1 mM) and LPS synergistically upregulated IL-6 and IL-8

production (36). These diverse effects of nicotine and LPS on various cell types may reflect differences in receptors and regulatory pathways inherent to the cell type.

The presence of the LPS receptors, including Toll-like receptors 2 and 4, has been identified on gingival keratinocytes (14,37). These surface receptors regulate pathogen recognition, activate various transcriptional responses and regulate inflammatory responses (38). Arredondo *et al*. (39) have shown that nicotine binds to nicotinic acetylcholine receptors on human gingival keratinocytes, including nicotinic receptor α7 (nAChRα7), and that activation of these receptors upregulates gene expression of nuclear factor κB (NF-κB), which stimulates a proinflammatory response. In contrast, Osborne-Hereford *et al*. (40) did not identify any evidence of NFκB pathway activation in skin following nicotine exposure. In fact, these investigators demonstrated that nAChRα7 activation by nicotine led to downregulation of proinflammatory cytokine production in skin and proposed that alternative signaling mechanisms were affected. The dichotomous effects of nicotine on the immune system reflect, in part, differences between *in vitro* and *in vivo* models.

In contrast to this study showing increased IL-1α secretion and a lack of IL-8 production following nicotine exposure *in vitro*, studies using gingival crevicular fluid have shown similar or decreased amounts of IL-1α (41,42) and IL-1β in smokers (43–45). In an experimental gingivitis model, quantities of IL-8 in gingival crevicular fluid were elevated in smokers compared with non-smokers (46). Differences between cell culture and gingival crevicular fluid studies may be attributed to the fact that the keratinocyte represents only one of the cell types producing cytokines. In addition, nicotine is among the 2000–3000 noxious chemicals in tobacco products, which also include benzenes, nitrosamines, carbon dioxide and many others (11). In the context of virulence factors, bacterial components in addition to LPS interact with host cells and trigger cytokine expression and secretion. Finally, it is important to consider the responses of other cytokines, because ultimately it is the balance between proinflammatory and other regulatory cytokines that characterizes the host response.

In summary, we observed a significant stimulatory effect of nicotine on IL-1α production and of LPS on IL-8 secretion by human gingival keratinocytes. The trend of enhanced mediator secretion in response to the combination of LPS and nicotine exposure calls for future studies specifically designed to examine potential synergism and the underlying signaling mechanisms involved. Furthermore, additional *in vivo* studies to examine gingival crevicular fluid profiles of proinflammatory, T- and B-cell regulatory cytokines in smokers and non-smokers would provide insight into the impact of tobacco use on the host response to bacterial plaque.

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