Periodontal Ligament Fibroblast Interleukin-6 Release after Exposure to Nicotine

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Purpose: To determine the influence of nicotine upon the release of interleukin-6 (IL-6) from human periodontal ligament fibroblasts (HPLF).

Methods and Materials: The HPLF were exposed to nicotine concentrations of 125 to 1500 μg/ml for 24 hours (h). IL-6 release and cell growth were determined after exposure to the different nicotine concentrations after 24 hours. IL-6 levels were measured by enzyme-linked immunosorbent assay (ELISA). Cell counts were determined with a Coulter counter.

Results: HPLF cell counts were significantly higher than untreated cells after nicotine exposure of 375 μg/ml and significantly lower than untreated cells for cells treated with 1500 μg/ml nicotine. After 24 h of cell exposure to nicotine concentrations of 250 and 375 μg/ml, HPLF release of IL-6 per cell was significantly higher than that of untreated HPLF. HPLF treated with 1500 μg/ml, nicotine released significantly less IL-6 per cell than did untreated cells.

Conclusions: These results indicate that nicotine can increase IL-6 release from HPLF.

Key words: nicotine, fibroblasts and Interleukin-6

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INTRODUCTION

Tobacco exposure is a recognized risk factor for a number of diseases (Newcomb and Carbone, 1992). In the oral cavity, cigarette smoking is a known risk factor for periodontal disease, alveolar bone loss and tooth loss while smokeless tobacco usage is a risk factor for oral cancer as well as gingival recession (Newcomb and Carbone, 1992; Zambon et al, 1996; Kianne and Chestnutt, 2000; Bergstrom, Eliasson et al, 1991; Osterberg and Mellstrom, 1986; Sundstrom et al, 1982; Offenbacher and Weathers, 1985). However, it is still unclear which agents and mechanism(s) impact on oral tissues in tobacco users. With a wide variety of substances present in tobacco and tobacco smoke, any one of them could potentially participate in these disease processes.

Nicotine is a major component and the most pharmacologically active agent in tobacco and is a potential suspect in the above disease processes. Several authors have reported growth inhibitory effects of nicotine at different concentrations on HPLF as determined by various growth or viability assays (Offenbacher and Weathers, 1985; Alpar et al, 1998; Chang et al, 2002; James et al, 1999; Giannoppoulou et al, 1999). However, there is still a need for further examination of the cytotoxic effect of nicotine on HPLF because of the variability of these previously reported results.

The cytokine, IL-6 is a mediator of the host response to tissue injury and infection. It is a pleiotrophic cytokine that induces antibody secretion in B cells and activation of T cells. In the oral cavity, it is produced in inflamed gingival tissues, periodontal disease and is involved in the acceleration of bone resorption (Dongari-Bagtzoglou et al, 1996; Mundy, 1991; Bartold and Haynes, 1991). Macrophages as well as epithelial cells and fibroblasts produce IL-6. However, information is currently not available concerning the influence of nico-

tine exposure upon the release of IL-6 from HPLF. It is speculated that nicotine may increase the release of IL-6 contributing to the inflammatory aspects of periodontal disease.

Therefore, the purpose of this study was to examine the effects of nicotine upon HPLF IL-6 release.

MATERIALS AND METHODS

Cell Cultures

A cell culture line of HPLF obtained originally from a periodontally and systemically healthy patient was used in this study. HPLF cells were grown in 25cm^2 culture flasks containing Dulbecco's modified Eagle's media (BioWhittaker, Walkerville, MD) containing 4.5g/L of glucose supplemented with 0.1mM non-essential amino acids, 2.0mM L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B (Gibco, Carlsbad, CA) and 10 % fetal bovine serum (Sigma Aldrich Co., St. Louis, MO.). The culture flasks were placed in an incubator at 37°C, in a humidified atmosphere of 5 % CO_2 and checked daily for morphology and growth. The media in the flasks were changed three times per week, removing old media, rinsing with Hank's balanced salt solution (HBSS, Gibco, Carlsbad, CA) and replacing with the media described above. Once the HPLF cell culture became confluent, the old media was removed from the flasks, the cells were washed with HBSS, 0.05 % trypsin- 0.53mM EDTA (Gibco) was added for one minute after which the trypsin was removed, the cells were dislodged with fresh media, and the cells utilized for the cell growth assays.

Cell Growth Assays

The cells were adjusted to a cell density of 1 x 105/ml for seeding. The cells were then transferred to 24 well cell culture clusters (Costar, Cambridge, MA.), placing 1ml of the cell suspension per well in the 24 well plates and incubated at 37°C, in a humidified atmosphere of 5% CO_2. After incubating for 24 h, cells from three wells of the 24 well plates were counted using a Coulter counter (Coulter Electronics Ltd., Luton, England) to determine cell growth. Briefly, the media above these cells were removed, cells were washed with HBSS, and trypsin was added for one minute. The trypsin was removed and then a balanced electrolyte solution (Isoton, Coulter Diagnostics, Hialeah, FL) was added to the cells. This cell suspension was counted three times and an average cell density determined before the cells were treated with the test agents. After counting the cells, the media was removed from all the wells, the cells were washed with HBSS, the cells were exposed to nicotine (Sigma, St. Louis, MO) concentrations of 125, 250, 375, 500, 1000 or 1500μg/ml in cell culture media using three wells per nicotine concentration and incubated for 24 h. Three wells with untreated cells served as a negative control. After 24 h, the supernatants above the cells from the 24 well plates were removed, saved in small microtiter tubes and frozen at – 20°C for later analysis by ELISA. In order to assess the number of cells in each well, the cells in the wells were washed once with HBSS, trypsin was added for one minute, the trypsin was removed, Isoton was added to each well, and the cells counted using a Coulter counter as described above.

Cytokine Assay

IL-6 concentrations were determined in nicotine treated samples of 125, 250, 375, 500, 1000, or 1500μg/ml, as previously saved, after 24 h of incubation. Untreated cell samples served a negative control. Three samples per nicotine concentration and control were analyzed.

For the cytokine assay, IL-6 was measured using ELISA with matched antibody pairs (R & D Systems, Minneapolis, MN). Briefly, a 96 well plate was coated with 100μl/well of capture antibody (diluted to a working concentration of 5μg/ml in phosphate buffered saline, PBS) against IL-6 and incubated at 37°C overnight. After this, the wells were washed with wash buffer (0.05% Tween 20 in PBS, pH 7.4) three times, and the plates dried by inverting and blotting on clean paper towels. The plate was blocked with 300μl /well of block buffer (1% bovine serum albumin, BSA, 5% sucrose and 0.05% sodium azide in PBS), covered and incubated at room temperature for one hour. The block buffer was removed, and the wash steps were repeated to completely remove the block buffer. Frozen samples (supernatant saved from the cell growth studies) were thawed to room temperature and optimally diluted in 0.1% BSA, and 0.05% Tween 20 in Tris buffered saline pH 7.3 (20mM Trizma base and 150mM NaCl). Three wells (100μl/ well) were used per sample. In addition, IL-6 standards were serially diluted in the same solution as the samples. Three wells per standard were used (100ul/well). The plates were covered and incubated for 2 h at room temperature. After 2 h, the wash step was repeated. Biotinylated antibody to IL-6 (100μl; diluted to a working concentration of 25ng/ml in BSA, Tween 20 and Tris buffered saline) was added to each well and incubated for 2 h. The plate was again washed after 2 h, and then 100μl of steptavidin-HRP (Zymed, San Francisco, CA., 1/20,000 dilution of a 1:25mg/ml solution) was added to each well and incubated for 20 min at room temperature in the absence of light. The wash step was repeated again after 20 min; 100μl of freshly prepared substrate solution (0.5 M citrate buffer, pH 5 containing 10mg O-phenylenediamine, OPD; Sigma Aldrich Chemical Co.,St. Louis, Mo. and 14 μl of 30% H_2O_2 /20ml of citrate buffer) was added to each well, and the plates incubated for 15min in the dark. 100μl of 0.5 M H_2SO_4 (Sigma) was added to each well to stop

the color change. The optical density of each well was determined using a microplate reader (Molecular Devices Corp., Menlo Park, CA) set at 490nm. A standard curve was developed from the standards to determine the level of IL-6 in the unknown samples.

Statistical Analysis

The effect of nicotine concentration on cell growth and IL-6 per cell was assessed using analysis of variance (ANOVA). Pair-wise comparisons were adjusted for multiple testing using Tukey's method at a 95% overall confidence level.

RESULTS

Effect of nicotine on periodontal ligament fibroblast cell growth

With cells exposed to nicotine concentrations of 125, 250 and 375μg/ml after 24 h, HPLF growth was numerically higher than the untreated cells (Fig 1). Cell growth with exposure to 375μg/ml nicotine after 24 h increased significantly ($p < 0.05$) over that of untreated cells. After cell exposure to 500, 1000 and 1500μg/ml nicotine exposure, cell growth was numerically lower than untreated cells. Cell growth decreased significantly ($p < 0.05$) over that of untreated cells with exposure to 1500μg/ml of nicotine after 24 h.

Effect of nicotine on the release of IL-6 from HPLF cells

With cells exposed to nicotine concentrations of 125, 250 and 375μg/ml after 24 h, HPLF IL-6 release per cell was numerically higher than untreated cells (Fig 2). Cell release of IL-6 per cell after exposure to 250 and 375μg/ml was significantly ($p < 0.05$) higher than untreated cells. After cell exposure to 500, 1000 and 1500μg/m of nicotine, IL-6 release per cell was numerically less than untreated cells. Cell release of IL-6 per cell was significantly ($p < 0.05$) less than untreated cells after 24 h exposure to 1500μg/ml nicotine.

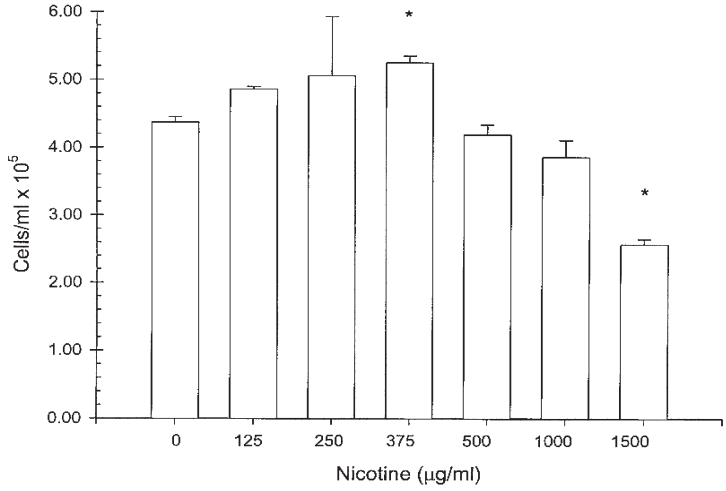

Fig. 1 Mean number of cells (1ml x 105) after nicotine exposure of 0, 125, 250, 375, 500, 1000 and 1500μg/ml for 24 hours. Error bars indicate 1 standard deviation (SD) of the mean (n=3).

*Asterisk indicates significantly different than untreated samples.

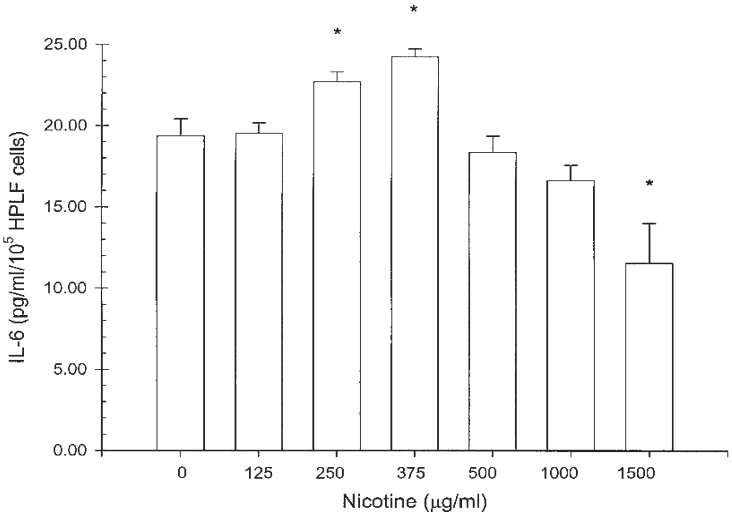

Fig. 2 Mean IL-6 (pg/ml/105 HPLF) after nicotine exposure of 0, 125, 250, 375, 500, 1000 and 1500μg/ml for 24 hours by ELISA. Error bars indicate 1 standard deviation (SD) of the mean (n=3).

*Asterisk indicates significantly different than untreated samples.

DISCUSSION

Nicotine is the principal, pharmacologically active agent in cigarette smoke and smokeless tobacco. The range of concentrations of nicotine encountered in cigarettes or smokeless tobacco can affect HPLF viability and metabolism. Cigarettes can deliver up to 1900μg nicotine per cigarette (Henningfield et al, 1993). If one smokes one pack of cigarettes per day, one can be exposed to 38,000μg of nicotine. Smokeless tobacco contains from 0.59 to 3.35% (w/w) nicotine (Tilashalski et al, 1994), and the saliva of a smokeless tobacco user can contain up to 1560μg/ml nicotine (Hoffman and Adam, 1981). Our study looked at the concentration range of nicotine of 125 to 1500μg/ml.

Several studies have examined the effect of nicotine on HPLF growth. Alpar, Leyhausen, et al. (1998) measured HPLF growth by DNA content at nicotine concentrations of 0.48 to 62 mM (7.8 to 1 x 10^4μg/ml) after 24h of incubation and found that concentrations greater than 7.8 mM (1265μg/ml) inhibited cell growth. James et al (1999) measured HPLF cell growth with a Coulter counter after nicotine exposure over 14 days upon the growth of HPLF at concentrations of $5x10^{-3}$ to $1x10^4$μg/ml nicotine. They found that nicotine at concentrations greater than 1000μg/ml inhibited cell growth. In addition, Giannopoulou et al (1999) found that after 24 and 48 h, HPLF growth measured by DNA synthesis was inhibited by nicotine concentrations above 0.1μg/ml. Finally, Chang et al (2002) determined that nicotine at a concentration of 25uM (4.1μg/ml) and above inhibited cell proliferation of HPLF as measured by DNA synthesis, while a dose of 5mM (810μg/ml) and above indicated cell toxicity as measured by the MTT assay. Thus a number of studies have documented the inhibition of HPLF growth by nicotine at various concentrations by cell growth assays.

In this study at the highest level of nicotine tested, cell growth was clearly reduced compared to untreated cells. In comparing this study with others, one finds that results vary from study to study which may depend on the heterogeneity of fibroblasts and their subpopulation (Mariotti and Cochran, 1990) as well as the concentrations of nicotine tested and the method used to measure cell growth or viability. Our study compares with the HPLF growth studies with nicotine of James et al (1999) and Alpar et al (1998).

In this study, we also found that the lower concentrations tested, 125, 250 and 375μg/ml of nicotine and particularly 375μg/ml, stimulated cell growth. We did not note any increases in growth in prior published HPLF studies with nicotine, but Checchi et al (1999) noted an increase after 24h in the viability of human gingival fibroblasts (HGF) from older smokers and non-smokers exposed to 60μg/ml of nicotine as measured by an MTT assay. In addition, Peacock et al (1993) found that exposure of HGF to a low level of nicotine, 0.025μM (4.1μg/ml), caused a significant increase in cell numbers after 48h of incubation as measured by the MTT assay. The increase in cell growth that we noted compares more closely with the results obtained by Checchi et al (1999). This increase in cell numbers could be caused by the up-regulation of the production of growth factors such as basic fibroblast growth factor (Carty et al, 1996) and platelet-derived growth factor (Cucina et al, 2000) or enhanced expression of growth receptors such as epidermal growth factor receptor (Mathur et al, 2000).

In our study, we noted a significant increase in IL-6 release over untreated cells after 24 h exposure to nicotine concentrations of 250 and 375μg/ml. The only study that we can compare with this is that of Wendell and Stein (2001) who examined the effects of nicotine and lipopolysaccharides (LPS) on HGF. HGF obtained from a patient with periodontal disease were exposed to nicotine concentrations of 1nM (1.6 x 10^{-10}μg/ml), 1uM (0.16μg/ml) and 1mM (162μg/ml). They detected a significant increase in IL-6 release after 24h with 1nM nicotine and 48h exposure to all three nicotine concentrations. The 1mM nicotine concentration is close to the lowest concentration of nicotine we tested, 125 μg/ml. In this study, we did not find an increase in IL-6 after 24h at this lowest concentration. The differences we noted could be due to the fact that we were utilizing HPLF and not HGF and that our HPLF were obtained originally from a periodontally healthy patient, while Wendell and Stein (2001) utilized HGF obtained originally from a subject with periodontal disease. Dongari-Bagtzoglou and Ebersole (1998) found that HGFs from periodontal lesions produced more IL-6 than healthy controls. In addition, Kent et al (1998) detected higher quantities of IL-6 from diseased HGF before and after stimulation with IL-1 a than by healthy HGF. This increase in IL-6 could be due to signal transduction through a surface receptor. This receptor binding could affect the activation of transcription nuclear factor-kB (NK-kB). NF-kB activation has been linked to the release of IL-6 in cultured lung synovial fibroblasts and alveolar macrophages (Carter et al, 1998; Yoshida et al, 1999).

After 24 h of exposure to the highest concentration of nicotine tested, 1500μg/ml, there was a significantly lower level of IL-6 secretion compared to untreated cells. Since the number of cells decreased with increasing nicotine concentration, this was probably the result of the cytotoxic effects of nicotine on the synthesis and/or release of IL-6.

In summary, this study demonstrates that nicotine can increase the release of IL-6 from HPLF.

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