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Effects of nicotine and lipopolysaccharide on the expression of matrix metalloproteinases, plasminogen activators, and their inhibitors in human osteoblasts

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ABSTRACT

Objective: Lipopolysaccharide (LPS) from periodontopathic bacteria can initiate alveolar bone loss through the induction of host-derived cytokines. Smoking increases the risk and severity of periodontitis. We examined the effects of nicotine and LPS on the expression of matrix metalloproteinases (MMPs), plasminogen activators (PAs), and their inhibitors, including tissue inhibitors of metalloproteinases (TIMPs) and PA inhibitor-1 (PAI-1), in osteoblasts.

Methods: The cells were cultured with or without 10^{-4} M nicotine and 100 ng/ml LPS for 12 days or with 100 μg/ml polymyxin B, 10^{-4} M D-tubocurarine, 10 μmol/ml NS398, or 10^{-6} M celecoxib in the presence of either nicotine or LPS for 12 days. The gene and protein expression levels for MMPs, PAs, TIMPs, and PAI-1 were examined using real-time PCR and ELISAs, respectively. PGE_2 production was determined using an ELISA.

Results: The addition of nicotine and/or LPS to the culture medium increased the expression of MMP-1, -2, and -3 and tissue-type PA (tPA); decreased the expression of TIMP-1, -3, and -4; and did not affect expression of TIMP-2 or PAI-1. In the presence of D-tubocurarine or polymyxin B, neither nicotine nor LPS stimulated the expression of MMP-1. In the presence of NS398 or celecoxib, the stimulatory effects of nicotine and LPS on MMP-1 expression were unchanged, but they were unable to stimulate PGE_2 production.

Conclusion: These results suggest that nicotine and LPS stimulate the resorption process that occurs during turnover of osteoid by increasing the production of MMPs and tPA and by decreasing the production of TIMPs. Furthermore, they suggest that the stimulatory effect of nicotine and LPS on PGE_2 production is independent of their stimulatory effect on MMP-1 expression.

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1. Introduction

Periodontal disease comprises a group of infections that lead to inflammation of gingival tissue, the destruction of periodontal tissue, and the accompanying loss of alveolar bone with eventual exfoliation of the teeth.[1] During the course of periodontal disease, the population of gram-negative bacteria increases by as much as 80%; these microorganisms, including *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans*, and *Bacteroides forsythus*, colonize the gingival groove, forming subgingival plaque.[2]

The cell walls of gram-negative bacteria are populated by lipopolysaccharide (LPS) macromolecules, complex glycolipids composed of a hydrophilic polysaccharide protein and a hydrophobic domain (lipid A), which is responsible for most of the biological effects of LPS. LPS stimulates the production of many local factors, including tumour necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and prostaglandin E_2 (PGE_2), in macrophages, fibroblasts, and osteoblasts in inflamed periodontal tissues.[3–5] These factors promote the resorption of bone and cartilage by increasing the production of proteases and the formation of osteoclasts.[6–9] However, despite many rigorous studies,[10–13] the molecular mechanism by which LPS induces bone matrix turnover remains unknown.

Tobacco smoking is the major risk factor associated with chronic destructive periodontal disease. Smoking-associated periodontal disease is typically characterized by the destruction of tooth-supporting tissue, with ensuing clinical symptoms of bone loss, attachment loss, and pocket formation.[14] Nicotine, a major component of the particulate phase of cigarette smoke, is generally thought to be addictive. It has a variety of detrimental effects on periodontal cells. *In vitro*, nicotine inhibits gingival fibroblast growth, inhibits the production of fibronectin and collagen, and promotes collagen breakdown.[15] It inhibits mineralized nodule formation by osteoblasts[16] but may stimulate osteoclast differentiation and resorption of calcium phosphate, the major component of bone.[17]

We previously examined the effect of nicotine on bone matrix turnover in osteoblasts.[18] By assessing nicotine-induced changes in the expression of matrix metalloproteinases (MMPs), plasminogen activators (PAs), and their inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) and PA inhibitor-1 (PAI-1), we found that nicotine stimulates the resorption process by increasing the production of MMP-1, -2, -3, and -13 and tissue-type PA (tPA).

Smoking influences the onset and progression of periodontitis[19,20] and nicotine and LPS stimulate the formation of osteoclast-like cells by increasing the production of macrophage colony-stimulating factor and PGE_2 by osteoblasts.[21] In osteoblasts, LPS also enhances the induction of nicotine-induced PGE_2 by increasing cyclooxygenase (COX)-2 expression.[22] Based on these results, we hypothesized that the resorption of alveolar bone during matrix turnover would be more pronounced in smokers who neglected oral hygiene than in non-smokers with poor oral hygiene or in smokers with appropriate oral hygiene. To test this hypothesis, we examined the effects of nicotine and LPS on the expression of MMP-1, -2, and -3; TIMP-1, -2, -3, and -4; tPA; urokinase-type PA (uPA); and PA inhibitor-1 (PAI-1) in human osteoblasts. We also examined the effects of nicotine and LPS on cell proliferation, alkaline phosphatise (ALPase) activity, PGE_2 production, and the expression of COX-1 and COX-2 in the cells.

2. Materials and methods

2.1. Cell culture

Normal human osteoblasts were purchased from Cambrex (Walkersville, MD, USA) and cultured in osteoblastic growth medium (OGM; Cambrex) supplemented with 10% foetal bovine serum (FBS), ascorbic acid, gentamicin sulphate, and amphotericin B at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

For experimental treatments, the cells were seeded onto 100-mm tissue culture plates at a density of 1.0×10^4 cells/cm^2. After overnight incubation, the cells were cultured in OGM with or without 10^{-4} M nicotine (Wako Fine Chemicals, Osaka, Japan) and/or 100 ng/ml LPS from *E. coli* 026-B6 (L 2654; Sigma–Aldrich, St. Louis, MO, USA) for 12 days. In some cases, cells treated with nicotine or LPS were simultaneously treated with 100 μg/ml polymyxin B (Sigma–Aldrich), 10^{-4} M D-tubocurarine (Wako Fine Chemicals), 10 μmol/ml NS398 (Wako Fine Chemicals), or 10^{-6} M celecoxib (Stellas Pharma Inc., Tokyo, Japan) for 12 days.

The experimental nicotine concentration was selected on the basis of research showing that nicotine may augment the destruction of the gingival extracellular matrix during periodontal inflammation,[15] that suppress osteogenesis through a decrease in alkaline phosphatase and type I collagen produced by osteoblasts,[16] and that stimulate resorption during bone matrix turnover.[18] The concentration was also selected on the basis of previous studies showing levels of nicotine in the crevicular fluid.[23,24] The experimental LPS concentration was selected based on research showing that LPS and IL-1 may play multiple roles in stimulating osteoclastic bone resorption,[25] that nicotine and LPS may stimulate the formation of osteoclast-like cells by increasing macrophage colony-stimulating factor and PGE_2,[21] and that LPS may stimulate the production of PGE_2 and Ep4 receptor in osteoblasts.[13] The concentrations of polymyxin B,[26,27] which inhibits the biological activity of LPS and which is an antagonist of protein kinase C, D-tubocurarine,[28] which is a nicotine antagonist, and NS398 and celecoxib,[25,29] which specifically inhibit COX-2, were chosen based on results described by Iwagaki et al.,[26] Walker et al.,[28] Suda et al.[25], and Chang et al.,[29] respectively.

2.2. Determination of cell proliferation

Cells were plated in 96-well microplates in OGM at a density of 1.0×10^4 cells/cm^2 and cultured for 12 days under the conditions described in Section 2.1. At the indicated time-points, the culture medium was changed to fresh medium that contained 10% (v/v) cell-counting reagent (Wako Fine Chemicals), and the plates were incubated for an additional 1 h. The absorbance at 450 nm of each well was measured using a microtiter plate reader (Titertek Multiskan Plus; Flow Laboratories, McLean, VA, USA). The relative cell numbers were calculated from the relative absorbance values using a standard curve.