ORIGINAL ARTICLE

Effects of nicotine on differentiation, prostaglandin E2, and nitric oxide production in cementoblasts

Yi-Juai Chen a,b, Shiuan-Shinn Lee c, Fu-Mei Huang a, Yu-Chao Chang a,d*

a School of Dentistry, Chung Shan Medical University, Taichung, Taiwan
b Department of Dentistry, Da Chien Health Medical System, Miaoli County, Taiwan
c School of Public Health, Chung Shan Medical University, Taichung, Taiwan
d Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan

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Abstract  Background/purpose: Cigarette smoking is an important risk factor in the pathogenesis of periodontal disease. Little is known about the effects of nicotine, the major component of cigarette smoke, on cementoblasts. The aim of this study was to investigate the cytopathologic effects of nicotine on murine immortalized cementoblast cell line (OCCM.30).

Materials and methods: Cell viability was judged by using the tetrazolium bromide reduction assay. Cell differentiation was examined by alkaline phosphatase assay. The production of prostaglandin E2 (PGE2) and nitric oxide (NO) were evaluated using an enzyme-linked immunosorbent assay and Griess reaction, respectively. Inducible nitric oxide synthase (iNOS) was evaluated by western blot.

Results: Nicotine demonstrated cytotoxicity to cementoblasts in a dose-dependent manner (P < 0.05). Nicotine was found to inhibit alkaline phosphatase activity in a time-dependent manner (P < 0.05). In addition, nicotine increased the secretion of PGE2 in a dose-dependent manner (P < 0.05). Nicotine was found to induce NO generation and iNOS expression in a dose-dependent manner (P < 0.05).

Conclusion: Our results suggest that nicotine could inhibit cementoblast growth and differentiation. In addition, nicotine could also induce the inflammatory effects by the augmentation of PGE2 secretion and iNOS/NO expression.

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* Corresponding author. School of Dentistry, Chung Shan Medical University, 110, Section 1, Chien-Kuo N. Road, Taichung, Taiwan.
E-mail address: cyc@csmu.edu.tw (Y.-C. Chang).

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Introduction

Studies have been reported that the role of cigarette smoking is a major modifiable environmental risk factor for periodontal disease. In addition, cigarette smokers are found to increase three to six times more likely to have periodontitis than nonsmokers. Xenobiotics derived from cigarette smoking are the important negative effect on the response to periodontal therapy.

Tobacco smoke contains more than 4000 different chemical compounds, the most studied of which is nicotine. Nicotine, a major component of cigarette smoke/cigarette smoke extract, has been detected on the root surface of periodontally involved teeth and may alter some cellular functions (e.g., inhibiting cell viability and attachment). Periodontitis is a chronic inflammatory disease. Previous studies have reported that nicotine could induce inflammatory mediators, such as cyclooxygenase-2, interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α). In addition, reactive oxygen species levels are related to bony destruction in periodontitis. Recently, we found that nicotine can upregulate the generation of reactive oxygen species in cementoblasts. Despite the lines of evidence cited above, the cytopathologic effects of nicotine remain to be elucidated.

Cementum is a mineralized tissue lining the surface of tooth root, and shares numerous similarities with bone in terms of composition, mechanical properties, and disease responsiveness. Cementoblasts are highly differentiated with a capacity to form reparative cementum. The stimulation of cementoblast proliferation and differentiation is important to regenerate damaged periodontal tissues. However, little is known about the cytopathologic effects of nicotine on cementoblasts. In the present study, the effects of nicotine on murine immortalized cementoblast cell line (OCCM.30) were determined by measuring cytotoxicity, alkaline phosphatase (ALP) assay, prostaglandin E2 (PGE2) production, nitric oxide (NO) generation, and inducible nitric oxide synthase (iNOS) expression.

Materials and methods

Cell culture

Immortalized murine cementoblasts (OCCM.30) were a generous gift from Dr Somerman’s laboratory (University of Washington, Seattle, WA, USA). Cells were grown in Dulbecco’s modified Eagle’s medium (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% fetal calf serum and antibiotics (Gibco BRL). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Confluent cell layers were treated with 0.25% trypsin and 0.05% EDTA for 5 minutes.

Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) solution was prepared in 5 mg/mL phosphate-buffered saline (PBS) prior to use and filtered through a 0.22-μm filter. Nicotine in medium was prepared from a 1 mg/mL stock solution of pure nicotine (Sigma Chemical Co.) in distilled water, which was prepared fresh prior to each experiment. The cells were incubated with or without different concentrations of nicotine for 24 hours. Briefly, 2 × 104 cells were seeded to a 96-well plate in 100 μL medium and left overnight to attach. Serial dilutions of nicotine in 100-μL volumes were added, and cells were treated for 20 hours. After treatment, 50 μL of the MTT solution (2 mg/mL in PBS) was added to each well and incubated for another 4 hours at 37°C. To each well, 50 μL of dimethyl sulfoxide was added. Plates were then shaken until the crystals were dissolved. Reduced MTT was then measured spectrophotometrically in a dual beam microtiter plate reader at 570 nm with a 650-nm reference.

Measurement of ALP activity

ALP activity was evaluated using a substrate assay as described previously. The cells were also incubated with or without 0.5 mM nicotine for a 3-day culture period. Briefly, cell lysates were sonicated on ice bath; this was followed by centrifugation at 1500 g for 5 minutes, and the ALP activity in supernatant was measured using ALP assay mixtures containing 0.1 M 2-amino-2-methyl-1-propanol, 1 mM MgCl2, and 8 mM p-nitrophenyl phosphate disodium. After incubation at 37°C for 30 minutes, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. The OD of control cells was considered to be 100%. The relative ALP activity was calculated using the following formula: [optical density (OD) of experimental sample/OD of control cells] × 100%. Each value was expressed as the mean ± standard deviation.

Measurement of PGE2

The protein concentrations of PGE2 were measured with enzyme-linked immunosorbent assay (ELISA). Briefly, 5 × 104 cells were incubated with or without nicotine for the indicated periods. The cells were also incubated with or without different concentrations of nicotine for 24 hours. The cytokine levels in culture medium were measured using the ELISA kit (eBiosciences, San Diego, CA, USA) according to the manufacturer’s instructions.

Nitrite assay

The nitrite concentration in the supernatant was measured as an indicator of NO production detected by the Griess reaction. The cells were incubated with or without different concentrations of nicotine for 8 hours. Briefly, 5 × 104 cells were seeded in 24-well plates for 24 hours, treated with different concentrations of nicotine. Fifty microliters of the culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl-ethylenediamine dihydrochloride in distilled water) and then shaken for 10 minutes at room temperature. The absorbance at 540 nm was determined with a microplate reader. The nitrite concentration was compared with a standard curve generated with known concentrations of sodium nitrite.
Western blot for iNOS

For western blot analysis, cell lysates were collected as described previously. The cells were incubated with or without different concentrations of nicotine for 8 hours. Briefly, cells were solubilized with sodium dodecyl sulfate-solubilization buffer (5mM EDTA, 1mM MgCl2, 50mM Tris-HCl, pH 7.5 and 0.5% Triton X-100, 2mM phenylmethylsulfonyl fluoride, and 1mM N-ethylmaleimide) for 30 minutes on ice. Then, cell lysates were centrifuged at 12,000 g at 4°C, and the protein concentrations were determined with Bradford reagent using bovine serum albumin as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3% bovine serum albumin for 2 hours, rinsed, and then incubated with primary antibodies anti-iNOS (sc-650; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000) in PBS containing 0.05% Tween 20 for 2 hours. After three washes with Tween 20 for 10 minutes, the membranes were incubated for 1 hour with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above, and then treated with 1:1000 streptavidin–peroxidase solution for 30 minutes. After a series of washing steps, protein expression was detected by chemiluminescence using an enhanced chemiluminescence detection kit (Amersham Biosciences UK Limited, Buckinghamshire, UK), and relative photographic density was quantitated using scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000; Alpha Innotech Corp., San Leandro, CA). Each densitometric value was expressed as the mean ± standard deviation.

Statistical analysis

Three replicates of each concentration were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was carried out with the Student t test, and a value of P < 0.05 was considered statistically significant.

Results

Nicotine was cytotoxic to OCCM.30 cells from concentrations higher than 0.5mM over a 24-hour culture period in a dose-dependent manner (P < 0.05). Fig. 1 shows the percentage of cell survival rate compared with controls following treatment with various concentrations of nicotine. The 50% inhibition concentration of nicotine was about 1mM.

The effects of nicotine on ALP activity were determined using the substrate assay. As shown in Fig. 2, ALP activity was found to be significantly decreased after 0.5mM nicotine over the 3-day culture period (P < 0.05). The levels of ALP were 1065, 1128, 1272, 1503, 1671, and 1478 pg/mL at the concentrations of 0, 0.125, 0.25, 0.5, 1, and 1.5mM nicotine, respectively.

To evaluate the effects of nicotine on NO production in OCCM.30 cells, nitrite accumulation was examined using the Griess assay. As shown in Fig. 4, nicotine was found to induce nitrite generation in a dose-dependent manner (P < 0.05). The levels of NO were 2.9-, 3.4-, 4.7-, 6.5-, and 4.9-fold at the concentrations of 0.125, 0.25, 0.5, 1, and 1.5mM nicotine, respectively, as compared with the controls.

To investigate whether nicotine may induce NO production via the induction of the corresponding gene...
expression, iNOS expression was determined using western blots (Fig. 5A). Nicotine was found to induce significant iNOS protein expression in a dose-dependent manner \( (P < 0.05) \). As shown in Fig. 5B, the levels of iNOS was significantly increased about 2.2-, 2.8-, 3.7-, and 2.5-fold at the concentrations of 0.125, 0.25, 0.5, and 1mM nicotine as compared with the controls, respectively.

**Discussion**

Previous studies have shown that nicotine is a cytotoxic agent to periodontal ligament fibroblasts, \(^7\) human gingival fibroblasts, \(^8\) human buccal mucosal fibroblasts, \(^21\) osteoblasts, \(^22\) and cementoblasts. \(^23\) In this study, nicotine exhibited cytotoxicity to OCCM.30 cells. These have clearly shown the cytotoxic nature of nicotine. In addition, the cytotoxicity of nicotine is not cell type-specific.

ALP is a membrane-bound glycoprotein, which is one of the osteogenic differentiation markers considered to indicate the presence of osteoblast and the formation of new bone. Cementum is a mineralized tissue, similar in composition and properties to bone. To the best of our knowledge, this is the first report that nicotine could inhibit ALP activity in OCCM.30 cells. This drop in ALP is consistent with the reports of decreased ALP expression in the human osteosarcoma cell line Saos-2\(^24\) and MG63 cells. \(^25\) Taken together, cigarette smoking may impair the mineralization of collagenous bone matrix by cementoblasts.

PGE2 is one of the proinflammatory mediators has been implicated as a key mediator in the pathogenesis of periodontal disease. \(^26\) Previous studies have shown the upregulation of PGE2 by nicotine on human neutrophils\(^27\) and monocytes. \(^28\) Our results also demonstrated that nicotine could increase PGE2 secretion in OCCM.30 cells. Thus, the pathogenesis of periodontal diseases may be affected by alterations in the inflammatory response by cigarette smoking.

NO is a gaseous, colorless, highly reactive, short-lived free radical that plays the role of an intracellular...
Effects of nicotine on cementoblasts

messenger molecule with important immune functions. However, overexpression of NO could result in acute or chronic inflammatory diseases. NO is generated by iNOS conversion of l-arginine to l-citrulline. In addition, iNOS is involved in the regulation of inflammatory reactions. In the present study, we first found that nicotine could stimulate NO generation and iNOS expression in OCMC.30 Similar results were found that nicotine could induce iNOS expression and NO production in human gingival fibrobasts and HPV16-immortalized human periodontal ligament cells. Consistently, Wadhwa et al. reported that cigarette smokers with chronic periodontitis exhibited an increase in serum and saliva NO levels compared with nonsmokers with chronic periodontitis. Taken together, nicotine may lead to NO generation and result in inflammatory reaction during cigarette smoking.

These results indicate that nicotine is a cytotoxic agent to cementoblasts by inhibiting cell viability and differentiation. In addition, nicotine could upregulate the secretion of PGE2 and the generation of NO. However, the other signaling pathways responsible for the induction of other inflammatory mediators and NO generation by nicotine must be evaluated in further studies.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

References

