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ORIGINAL ARTICLE

Effects of nicotine on cell growth, migration, and production of inflammatory cytokines and reactive oxygen species by cementoblasts

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Abstract *Background/purpose:* Cigarette smoking is an important risk factor in the pathogenesis of periodontal disease. However, little is known about the effect of nicotine, the major component of cigarette smoke, on cementoblasts. The aim of this study was to investigate the pathological effects of nicotine on the murine immortalized cementoblast cell line (OCCM.30).
Materials and methods: Cell viability was judged by using the Alamar Blue reduction assay. Cell migration was evaluated by transwell and wound-healing assays. The protein concentrations of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were measured by using enzyme linked immunosorbent assay (ELISA). The semiquantitative 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) fluorescence technique was used to detect the intracellular level of reactive oxygen species (ROS).
Results: Concentrations of nicotine $>$ 1.5mM demonstrated cytotoxicity to cementoblasts ($P < 0.05$). Nicotine attenuated cell migration in a dose-dependent manner ($P < 0.05$). In addition, nicotine augmented the production of IL-6 and TNF-α in a dose-dependent manner ($P < 0.05$). The concentration of 1mM nicotine enhanced the generation of intracellular ROS in a time-dependent manner ($P < 0.05$).
Conclusion: Taken together, these results suggest that nicotine could inhibit the growth and migration of cementoblasts. In addition, nicotine could also induce the generation of inflammatory cytokines and ROS by cementoblasts.

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Introduction

Studies have demonstrated that the use of tobacco products is a risk factor in the incidence and severity of periodontal disease.[1,2] Cigarette smoke contains a complex mixture of substances including nicotine, various nitrosamines, trace-elements, and a variety of poorly characterized substances. Nicotine is one of the over 4000 chemical constituents in cigarette smoke.[3] Previous studies have reported that nicotine could be detected in blood, saliva, and gingival crevicular fluid from smokers.[4,5] *In vitro* study also has shown that nicotine can be detected on the root surface of periodontally involved teeth.[6]

Nicotine has been shown to alter some cellular functions. Previously, our studies have shown that nicotine is cytotoxic to human periodontal ligament fibroblasts through inhibiting cell viability and attachment via intracellular thiol depletion.[7,8] Nicotine has also been found to induce c-fos in human periodontal ligament fibroblasts,[9] to induce cyclooxygenase-2,[10,11] and heme oxygenase-1[12] in human gingival fibroblasts, and to induce extracellular signal-regulated protein kinase in human osteosarcoma cells.[13] In addition, nicotine could influence the expression of osteolytic mediators, such as interleukin-1 (IL-1), IL-8, receptor activator of nuclear factor-$\kappa\beta$ ligand (RANKL), matrix metalloproteinase (MMP)-2, MMP-9, and tissue-type plasminogen activator.[14] Despite the above evidence, the mechanisms of nicotine-induced inflammation still remain to be elucidated.

Cementum is a mineralized tissue, similar in composition and properties to bone, that is synthesized by cementoblasts during tooth root formation and plays an essential role in anchoring the tooth to the surrounding alveolar bone. Cementoblasts not only function as support cells for periodontium, but also control development, maintenance, and regeneration of periodontal tissues. Interestingly, little is known about the effect of nicotine on cementoblasts.

Oxidative stress is a signal for the activation of pathways which control cell death and survival as well as proliferation and differentiation through mitogen-activated protein kinases.[15] In addition, the excess generation of reactive oxygen species (ROS) can damage cells and tissues, resulting in the development of inflammation.[16] In the present study, the effects of nicotine on the murine immortalized cementoblast cell line (OCCM.30) were determined through measuring cytotoxicity, cell migration, the production of inflammatory mediators, and ROS formation.

Materials and methods

Cell culture

Immortalized murine cementoblasts (OCCM.30) were a generous gift provided by Professor Chi-Cheng Tsai from Dr. Somerman Laboratory (University of Washington, Seattle, WA, USA). Cementoblasts were obtained from the root surface of the first mandibular molar of osteocalcin (OC) large T-antigen transgenic mice.[17] Cultured cells were termed "OC-CM" to indicate cementoblasts derived from OC large T-antigen transgenic mice. OC-CM subclone 30 cells (OCCM.30), which express bone sialoprotein and osteopontin mRNAs, indicative of cementoblasts *in situ*, were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% fetal calf serum (FCS) and antibiotics (Gibco BRL). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cell layers were treated with 0.25% trypsin and 0.05% EDTA for 5 minutes.

Cytotoxicity assay

Alamar Blue is an oxidation-reduction indicator for eukaryotic cells. It was used for monitoring a cell proliferation assay based on the reduction of tetrazolium salts by the mitochondrial cytochrome oxidase system.[18] Briefly, cells were seeded 2×10^4 cells per well into 96-well culture plates for 24 hours. The culture medium was replaced with fresh DMEM with various concentrations (0mM, 0.25mM, 0.5mM, 1mM, 1.5mM, 2mM, 3mM, and 4mM) of nicotine (Sigma Chemical Co., St. Louis, MO, USA). Trypsinization after a 24 hour incubation period, 25 μL of Alamar Blue dye (BUF012A/B Alamar Blue, AbD serotec, Oxford, UK) was added to each well for 2 hours at 37°C. An amount of 100 μL of the solution in each well was transferred to a 96-well tissue culture plate. Plates were read in a plate reader (Metertech Co., Taipei, Taiwan) at 570 nm with a reference wavelength of 600 nm. Cell viability was represented in terms of optical density expressed as percentage of Alamar Blue absorbance.

Wound healing migration assay

Cells were seeded into a 6-well culture dish and grown in culture medium containing 10% FCS to a nearly confluent cell monolayer. Wounds were made to the confluent monolayer of cells with a sterile 200 μL plastic pipette tip to create a denuded area. Floating cells were removed by two washings with phosphate buffered saline (PBS) and replaced with culture medium containing 1% FCS, and then

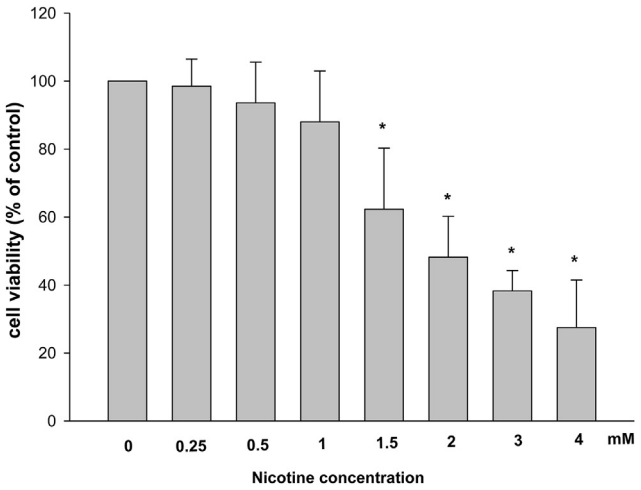

Figure 1 Cytotoxicity of various concentrations of nicotine on cementoblasts as measured by the Alamar Blue assay. Each point and bar represent a mean ± SD.* Denotes significant differences from control values with $P < 0.05$.