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Effects of nicotine on proliferation and osteoblast differentiation in human alveolar bone marrow-derived mesenchymal stem cells

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ABSTRACT

Aims: Nicotine is a risk factor for various diseases, including osteoporosis, oral cancer, and periodontal disease. Numerous studies have elucidated the effects of nicotine on cell proliferation and differentiation. The purpose of this study was to determine the effects of nicotine on the proliferation and osteoblast differentiation of human alveolar bone marrow-derived mesenchymal stem cells (hABMMSCs).
Main methods: In this study, we treated hABMMSCs with different doses (1 μM to 5 mM) of nicotine. The survival and proliferation of hABMMSCs were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay and crystal violet assay. TUNEL and propidium iodide (PI) double staining assay were also performed. The effect of nicotine on osteoblast differentiation of hABMMSCs was determined by measuring calcium accumulation using alizarin red-sulfate (AR-S) staining, measurement of alkaline phosphatase (ALP) activity, and semi-quantitative PCR of osteoblast markers.
Key findings: The survival and proliferation of hABMMSCs did not differ when they were exposed to nicotine at concentrations ranging from 1 μM to 100 μM; however, cell proliferation increased when the cells were exposed to nicotine at concentrations of 1–2 mM and decreased significantly when exposed to 5 mM of nicotine. A number of cells were stained by PI but not by TUNEL, and membrane vacuolization was observed in hABMMSCs treated with 5 mM nicotine. Calcium accumulation; ALP activity; and mRNA levels of ALP, bone sialoprotein (BSP), collagen type I α 1 (Col1αI), and runt-related transcription factor 2 (Runx2) were significantly decreased by treatment with 2 mM of nicotine, while osteocalcin transcripts decreased by treatment with 1 to 2 mM of nicotine.
Significance: These results suggest that nicotine has a bimodal effect on the proliferation and osteoblast differentiation in hABMMSCs.

Introduction

Smoking has been implicated as a risk factor for various diseases such as osteoporosis, oral cancer, and periodontal disease (Greer and Poulson, 1983; Qandil et al., 1997). Cigarettes contain a large number of toxic compounds, including nicotine, which causes addiction. Many of the undesirable health effects of smoking have been attributed to nicotine. Several studies have examined the in vitro effects of nicotine on cell proliferation, attachment, and osteoblast differentiation in various cell types. For example, the production of collagen and non-collagenous proteins in fibroblasts is reported to decrease after nicotine treatment (Giannopoulou et al., 2001). In addition, nicotine inhibits cellular growth and stimulates alkaline phosphatase (ALP) activity in rat osteoblast-like cells (Fang et al., 1991). Nicotine can penetrate the soft tissue of the oral cavity, adhere to the tooth surface, penetrate the lung and oral mucosal membranes, and enter the blood stream. Smoking has been associated with the incidence of alveolar bone loss (Bolin et al., 1986) and implant failure caused by alveolar bone loss (Hinode et al., 2006).

Bone tissue is continuously broken down, lost, and replaced by the formation of new bone. New bone formation requires the recruitment of mesenchymal stem cells (MSCs), which are capable of proliferation and differentiation (Fang and Hall, 1997). MSCs are multipotent stem

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cells that can differentiate into a variety of cell types, including osteoblasts. In order to understand the mechanism whereby nicotine influences bone loss, several studies have used osteoblast-related cells to examine the effect of nicotine on cell differentiation (Sato et al., 2008). Furthermore, MSCs have been isolated from human alveolar bone marrow, cultured successfully, and found to have a capacity to differentiate and resemble osteoblasts. However, these alveolar bone marrow derived mesenchymal stem cells (ABMMSCs) have shown poor chondrogenic or adipogenic potential, unlike MSCs isolated from iliac (Matsubara et al., 2005). Therefore, these studies suggest the possibility that ABMMSCs can be used as models of nicotine-induced alveolar bone loss. However, the effect of nicotine on the proliferation and osteoblast differentiation of human ABMMSCs (hABMMSCs) has not yet been reported. Therefore, we sought to verify the hypothesis that alveolar bone loss and implant failure caused by cigarette smoking may be related to the suppression of cell proliferation or osteogenesis. In this study, we used hABMMSCs to evaluate the effect of nicotine on cell proliferation and osteogenesis.

Materials and methods

hABMMSCs culture

In our previous study, we had isolated and characterized MSCs from the human alveolar bone marrow (Kim et al., 2011); we used these hABMMSCs for the current set of experiments. Cells were cultured in α-MEM containing 20% FBS (Gibco, Invitrogen Corporation, Carlsbad, CA) and 1% antibiotics (penicillin G: 10,000 units/mL and amphotericin B: 25 μg/mL; Gibco), in a humidified CO_2 incubator at 37 °C. The cells of passages 3–6 were used in this study. To induce osteoblast differentiation, the cells had to be nearly 90% confluent, and osteoblast differentiation was induced in each experiment by treating the cells with osteoblast-induction stimulants (OS) containing 50 μg/mL ascorbic acid (Sigma-Aldrich Co., St. Louis, MO), 10 mM β-glycerophosphate (Sigma-Aldrich Co.), and 100 nM dexamethasone (Sigma-Aldrich Co.).

Cell proliferation assay

For the cell survival and proliferation assay, cells were seeded at 4×10^3 cells/well in a 96-well plate. In order to assess the effects of nicotine on cell survival and proliferation, the cells were incubated in growth medium for the indicated durations at the indicated concentrations of nicotine. The effect of nicotine on cell survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay and that on proliferation was determined by crystal violet assay. At the indicated time points, 100 μL of MTT solution (5 mg/mL in PBS) was added to the cultures. The absorbance of the solution was measured by using a microplate reader at 540 nm (Spectra MAX 250; Molecular Devices, Inc., Sunnyvale, CA). For the crystal violet assay, the cells were cultured for 3 days with 5 mM nicotine, after which the medium was removed and cells were washed twice with PBS buffer, fixed with 100 μL of acetone:methanol (1:1) at 4 °C, and stained with 0.5% crystal violet in 20% methanol for 10 min. After washing with water, crystal violet was solubilized with 200 μL of 1% sodium dodecyl sulfate solution, and the absorbance was measured using a microplate reader at 595 nm.

TUNEL assay

Apoptotic cells were detected in situ by using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (TUNEL POD kit, Roche Molec Biochemic, Mannheim, Germany). Briefly, hABMMSCs were seeded at 5×10^4 cells on circular coverglass slips (Fisher Scientific, Houston, TX), with diameter 12 mm, and treated with 5 mM of nicotine. The cells were fixed in 4% paraformaldehyde for 1 h and then incubated with 20 μg/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) for 30 min at room temperature. Next, the cells were washed in PBS and incubated with labeling solution for 1 h at 37 °C. The nuclei were then counterstained with propidium iodide (PI) (0.5 g/mL, Molecular Probes, Carlsbad, CA, USA) for 10 min at room temperature. After washing with PBS, the specimens were examined under a fluorescence microscope (DM IL LED Fluo, Leica, Germany).

Alkaline phosphatase (ALP) activity

For the ALP activity assay, cells were seeded at 5×10^4 cells/well in a 24-well plate and treated with nicotine and osteoblast differentiation reagents. After 5 days, the medium was removed, and the cells were detached by treatment with trypsin/EDTA and centrifuged for 10 min at 1500 rpm. Each 100 μL of the suspension was mixed with 200 μL of 0.1 M glycine NaOH buffer (pH 10.4), 100 μL of 15 mM p-nitrophenyl phosphate (p-NPP) (Sigma-Aldrich Co.), 100 μL of 0.1% (v/v) Triton X-100 in PBS, and 100 μL of distilled water and incubated for 30 min at 37 °C. The reaction was quenched by adding 600 μL of 0.1 N NaOH. The degree of p-NPP hydrolysis was determined using an ELISA reader (Spectra MAX 250; Molecular Devices, Inc.) at 410 nm, and p-nitrophenol (Sigma-Aldrich Co.) was used as the standard. Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL) according to the manufacturer's instructions; then, the quantity of p-NPP was normalized to that of the total protein. The ALP activity was recorded in terms of nM/30 min/mg of protein. The experiment was repeated 3 times.

Calcium accumulation assay

Calcium accumulation was evaluated by alizarin red-sulfate (AR-S; Sigma-Aldrich Co.) staining. Cells were seeded at a density of 5×10^4 cells/well in 24-well plates and cultured with continuous exposure to OS medium and nicotine for 21 days. In order to induce extracellular mineralization, 4 mM $NaHPO_4$ was added to the culture media, and the cells were then fixed with 70% (v/v) ice-cold ethanol for 1 h at 4 °C. After fixation, ethanol was removed, and the amount of calcium accumulated was measured by staining with 40 mM AR-S solution for 10 min at room temperature. The stained portions were photographed using a digital camera (Nikon D80; Nikon Corporation, Tokyo, Japan) and then solubilized using 10% (w/v) cetylpyridinium chloride (CPC) in 10 mM sodium phosphate (pH 7.0); the absorbance was then measured at 562 nm.

Observation of cell morphology

In order to assess the effects of nicotine on cell morphology, cells were seeded at 1×10^5 cells/well and incubated in growth medium in 6-well plates for the indicated durations at the indicated nicotine concentrations. After 5 days, hABMMSCs were stained with Wright staining solution (Sigma-Aldrich Co.) for 5 min and observed under a microscope (Nikon Eclipse MS100; Nikon Corporation, Tokyo, Japan).

Semi-quantitative PCR

RNA was isolated using a previously described protocol (Abdelmagid et al., 2007). Briefly, nicotine- and OS-treated hABMMSCs were harvested, homogenized in Trizol (Invitrogen Corporation, Carlsbad, CA), and separated into organic and aqueous layers by chloroform; the RNA was then recovered by isopropyl alcohol precipitation. Pellets were washed with ice-cold 70% ethanol, and the concentration of RNA was calculated using a spectrophotometer. One microgram of the extracted total RNA was reverse transcribed to produce cDNA. One microliter of the generated cDNA was amplified in 20 μL of PCR master mix. For each gene, a set of primers was designed, and the annealing temperature