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Nicotine induces cell proliferation in association with cyclin D1 up-regulation and inhibits cell differentiation in association with p53 regulation in a murine pre-osteoblastic cell line

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ABSTRACT

Recent studies have suggested that nicotine critically affects bone metabolism. Many studies have examined the effects of nicotine on proliferation and differentiation, but the underlying molecular mechanisms remain unclear. We examined cell cycle regulators involved in the proliferation and differentiation of MC3T3-E1 cells. Nicotine induced cell proliferation in association with p53 down-regulation and cyclin D1 up-regulation. In differentiated cells, nicotine reduced alkaline phosphatase activity and mineralized nodule formation in dose-dependent manners. Furthermore, p53 expression was sustained in nicotine-treated cells during differentiation. These findings indicate that nicotine promotes the cell cycle and inhibits differentiation in association with p53 regulation in pre-osteoblastic cells.

Smoking has been implicated as a risk factor for many diseases, including osteoporosis and periodontal disease [1,2]. Nicotine is a component of cigarette smoke that causes addiction. Serum nicotine concentrations during smoking range from 90 to 1000 nM, and saliva nicotine concentrations in long-term snuff users can reach 9.6 mM [3–5]. Some studies suggest that nicotine may directly affect osteoblasts [6–11]. Although low concentrations of nicotine (0.1–1 μM) increase cell proliferation, high concentrations of nicotine (above 1 mM) inhibit cell proliferation [9,10]. Several researchers have reported the effects of nicotine on osteogenic differentiation. Alkaline phosphatase (ALP) activity decreased in mouse pre-osteoblastic cell lines and osteosarcoma cell lines after treatment with nicotine (60 μM–3.6 mM) [6,12,13]. Laroche et al. reported that serum osteocalcin levels are significantly lower in smokers than in nonsmokers [14].

The molecular mechanisms underlying the actions of nicotine on osteoblasts at the cellular level are poorly understood. In osteoblasts, Walker et al. found that nicotine induces c-*fos* expression during proliferation [9]. In epithelial cells, Dasgupta et al. have shown that nicotine can increase cyclin D1 expression [15]. Cyclin D1 is an important factor in the regulation of cellular proliferation [16]. The association of cyclin D1 with the cyclin-dependent kinases (cdk) 4 and cdk6 results in phosphorylation of retinoblastoma (Rb) protein, which releases the transcription factor E2F to activate S-phase-specific genes [17]. p53, a tumor suppressor, plays a vital role in promoting cell cycle arrest and has also been implicated in cell differentiation [18,19]. Recent studies have demonstrated that p53 is a novel regulator of osteogenic proliferation and differentiation [20,21].

It remains to be elucidated how nicotine evokes the intracellular signaling cascade in osteoblasts during proliferation or differentiation. This study examined the expression of cell cycle regulators during proliferation and differentiation of a well-characterized pre-osteoblastic cell line treated with nicotine.

Materials and methods

Reagents and antibodies. (−)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt (nicotine) and *N*,2,3,3-tetramethylbicyclo[2.2.1]-heptan-2-amine hydrochloride (mecamylamine) were obtained from Sigma–Aldrich (USA). Antibodies against cdk4 (C-22), cdk6 (DCS-83), cyclin D1 (HD11) and β-actin (C-4) were obtained from Santa Cruz Biotechnologies (USA). p53 antibody (MAB462) was obtained from R&D (USA).

Cell culture. The mouse pre-osteoblastic cell line MC3T3-E1 (a kind gift from Dr. T. Ogasawara) was maintained in growth medium, consisting of α-modified minimum essential medium

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(α-MEM) (WAKO, Japan) with 10% fetal bovine serum (FBS) (Bio-West, France), incubated at 37 °C in humidified air including 5% CO_2, and was passaged every 7 days. The growth medium was changed every 3 days. For the osteoblastic differentiation assay, the cells were cultured in growth medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) (conditioned medium). The conditioned medium was changed every 3 days.

Assessment of cell proliferation. The cells were inoculated at 4×10^3 cells per well in 96-well plates. To assess the effects of nicotine on cell proliferation, the cells were incubated in growth medium or conditioned medium for the indicated times with nicotine at the indicated concentrations. To assess the effects of mecamylamine, the cells were incubated in growth medium with nicotine at 1 μM in the presence or absence of mecamylamine at 1 μM, which was added 1 h before nicotine treatment. The sample cells were quantified using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA), according to the manufacturer's instructions. Briefly, 20 μl of MTS solution reagent was added to 100 μl of culture medium in each well. After incubation for 2 h at 37 °C, the absorbance was measured at 490 nm using a Model 680 XR plate reader (BIO RAD, USA). The measurements are represented by the means of at least three independent experiments, with each data point based upon six replicates.

Western blot analysis. To detect cyclin D1, cdk4, and cdk6, the cells were inoculated at 50×10^4 cells in a 100-mm-diameter dish and allowed to proliferate for 24 h. Their growth was then arrested by incubation for 48 h in α-MEM containing 0.5% FBS [22]. Growth-arrested cells were treated with growth medium for the indicated times plus nicotine at 0 and 1 μM. To assess the effects of mecamylamine, growth-arrested cells were treated with growth medium for 6 days plus nicotine at 1 μM in the presence or absence of mecamylamine at 1 μM, which was added 1 h before nicotine treatment. To detect p53, 50×10^4 cells were inoculated in a 100-mm-diameter dish. For the proliferation assay, the cells were treated with growth medium plus nicotine at 1 μM for 0, 6, 12, 24, 36, and 48 h. For the differentiation assay, after reaching confluence, the cells were incubated in conditioned medium with nicotine at 0 and 1 μM for 2 and 8 days. Cells were sampled at the indicated times in each experiment. The analysis was performed as described previously [23]. Briefly, the cells were rinsed with ice-cold PBS and lysed with a lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 8.0], 10 μg/ml aprotinin, 0.1 M NaF, 2 mM Na_3VO_4, and 1 mM PMSF). The lysates were incubated on ice for 15 min and centrifuged at 15,000*g* for 10 min at 4 °C. The protein content was quantified by the Lowry method (BIO RAD). Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (BIO RAD). After the blocking of nonspecific binding by soaking the filters in 5% skim milk (Sigma–Aldrich), the desired proteins were immunodetected with their respective antibodies, followed by visualization using an ECL Plus Western blotting detection system (GE Healthcare, UK), according to the manufacturer's instructions. The bands were scanned by an LAS-3000mini luminescent image analyzer (Fuji Film, Japan). β-actin was used as a loading control.

Measurement of ALP activity. The cells were inoculated at 2×10^4 cells in 24-well plates. After reaching confluence, the cells were incubated in conditioned medium with nicotine at 0, 1, and 100 μM for 7 days. The ALP activity was assayed (Wako) as described previously [24] and normalized by the amount of protein as determined using the Lowry method (BIO RAD). The measurements are represented by the means of at least three independent experiments, with each data point based upon four replicates.

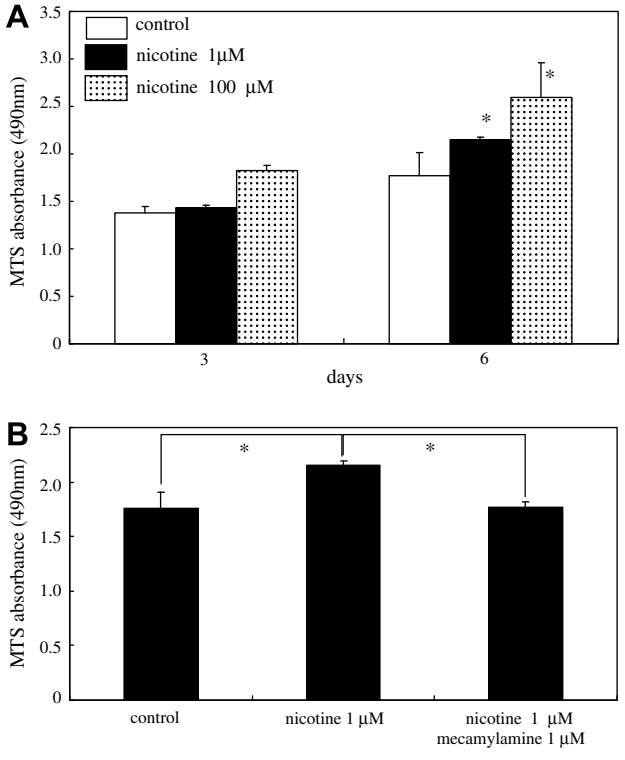

Fig. 1. Effect of nicotine on proliferation. (A) The cells were cultured with nicotine (0, 1, and 100 μM) for 3 and 6 days. Open bars, control (unstimulated cells); filled bars, 1 μM (nicotine-stimulated cells); dot bars, 100 μM (nicotine-stimulated cells). (B) The cells were cultured with nicotine for 6 days in the presence or absence of mecamylamine (1 μM), a nicotinic acetylcholine receptor subunit inhibitor. Error bars represent ± SD. $^*P < 0.05$ compared with control.

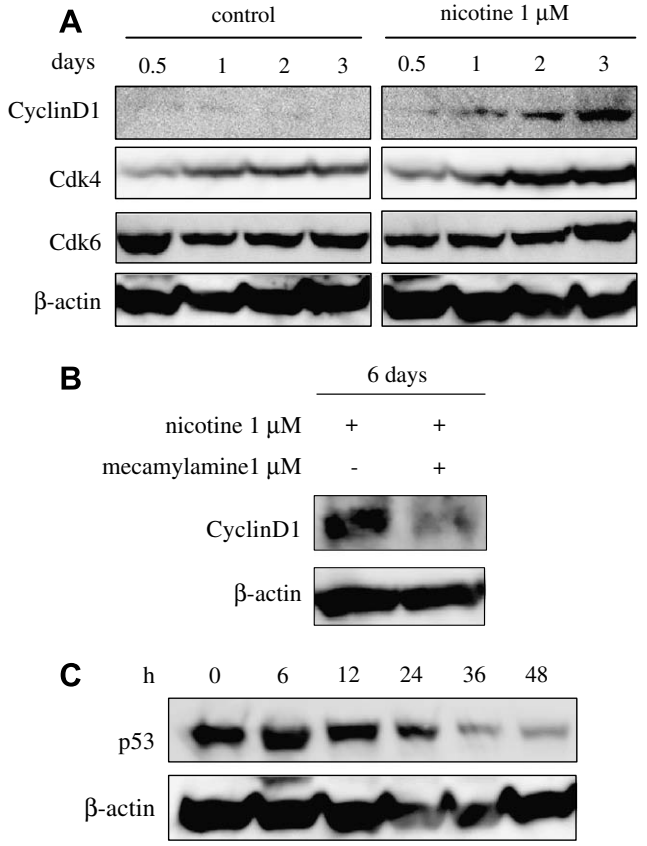

Fig. 2. Time-dependent expression of cell cycle regulators. (A) Time-dependent expression of cyclin D1, cdk4, and cdk6. Growth-arrested cells were cultured with nicotine (0 and 1 μM). (B) Growth-arrested cells were cultured with nicotine (1 μM) in the presence or absence of mecamylamine (1 μM). (C) Time-dependent expression of p53. The cells were cultured with nicotine (1 μM).

Mineralization assay. The cells were inoculated at 10×10^4 cells in 6-well plates. After reaching confluence, the cells were incubated in conditioned medium with nicotine at 0, 1, and 100 μM for 21 days. Mineralization of the cells was determined by von Kossa staining and alizarin red staining. The cells were fixed with 95% ethanol and stained with $AgNO_3$ by the von Kossa method to detect phosphate deposits in bone nodules [25]. At the same time, the order plates were fixed with ice-cold 70% ethanol and stained with alizarin red to detect calcification.

Statistical analysis. The data are presented as means ± SD. Statistical significance was assessed by Student's *t*-test using SPSS 14.0J. Differences were considered to be significant at $P < 0.05$.

Results and discussion

Nicotine induces cell proliferation via nicotinic acetylcholine receptors in a time- and dose-dependent manner

Since the addition of nicotine has been shown to induce cell proliferation in primary osteoblasts [9], we examined the proliferative effects of nicotine at the indicated doses on days 3 and 6 in MC3T3-E1. Nicotine significantly increased the osteoblast proliferation rate (Fig. 1A). The proliferative effects of nicotine at 1 μM were abrogated by the nicotinic acetylcholine receptor antagonist mecamylamine at 1 μM on day 6 (Fig. 1B).

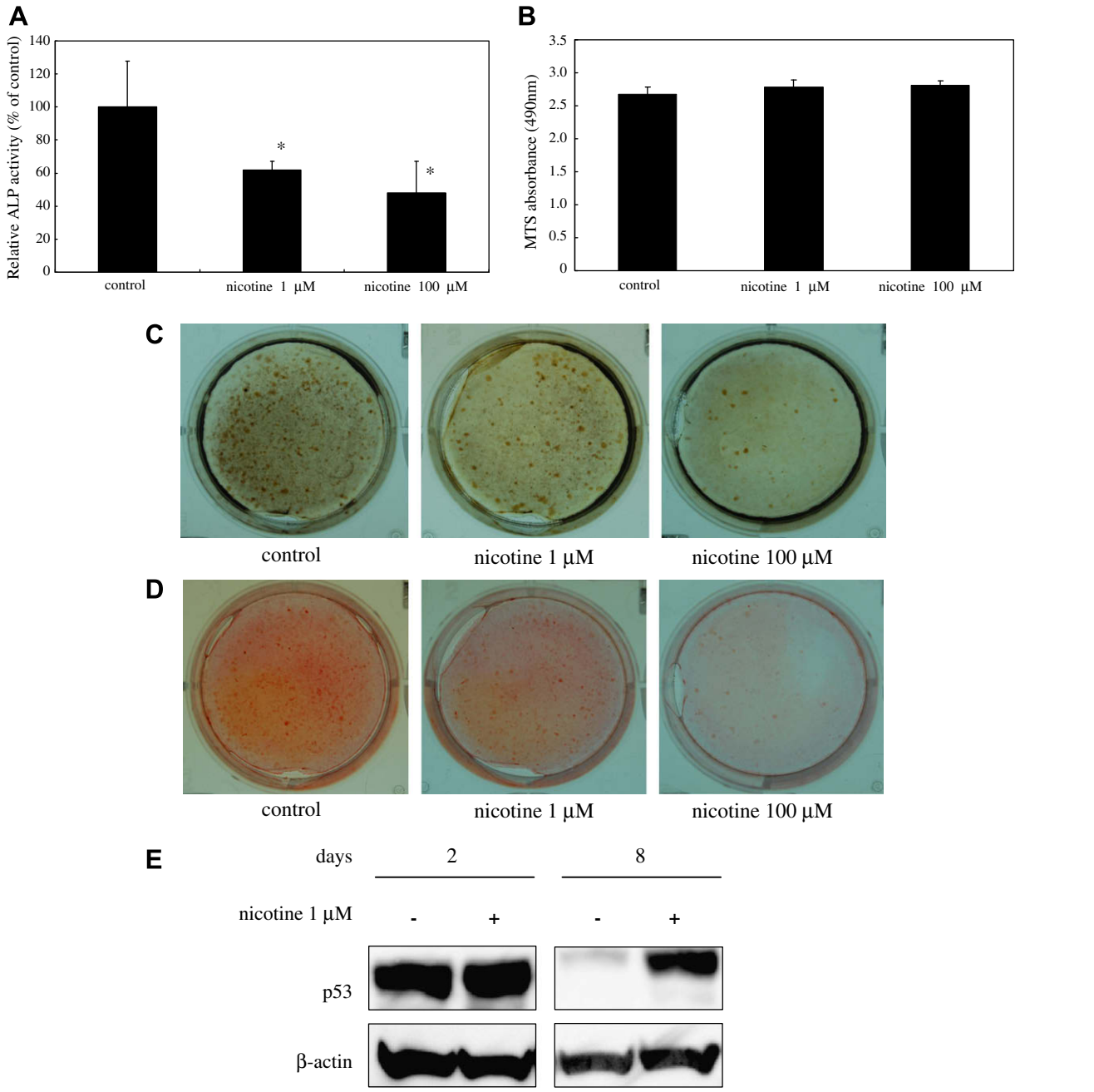

Fig. 3. Effect of nicotine on differentiation. (A) Effect of nicotine on ALP activity. After nicotine (1 and 100 μM) stimulation for 7 days, relative ALP activity was compared with controls. (B) Effect of nicotine on proliferation during differentiation. The cells were cultured with nicotine (0, 1, and 100 μM) for 21 days. (C, D) After nicotine (0, 1, and 100 μM) stimulation for 21 days, mineralization was assessed by von Kossa staining and alizarin red staining. (E) Time-dependent expression of p53. The cells were cultured with nicotine (0 and 1 μM). Error bars represent ± SD. $^*P < 0.05$ compared with control.

Nicotine up-regulates cyclin D1 expression via nicotinic acetylcholine receptors and down-regulates p53 expression during proliferation

Next, signal transduction of the proliferative effects of nicotine was studied. Since nicotine resembles a mitogen [15], the contributions of cyclin D1, an important cell cycle regulator, and of cdk4 and cdk6, specific partners of D type cyclin, were examined by Western blot analysis. The expression of cdk4 increased during serum stimulation in the presence or absence of nicotine. The amount of cyclin D1 showed little change until day 3 in the absence of nicotine, while cyclin D1 dramatically increased until day 3 in the presence of nicotine (Fig. 2A). Since mecamylamine inhibits cell proliferation, the level of cyclin D1 in nicotine-treated osteoblasts in the presence or absence of mecamylamine (1 μM) was examined. The level of cyclin D1 in nicotine-treated osteoblasts was reduced in the presence of mecamylamine (Fig. 2B). These results suggest that nicotine binds nicotinic acetylcholine receptors and subsequently activates components of the cell cycle machinery.

Next, we examined p53 expression during proliferation in the presence of nicotine because osteoblasts derived from p53-deficient mice show an increased proliferation rate [20]. A previous study reported that p53 is strongly expressed in MC3T3-E1 [26]. We detected p53 protein in this cell line and also found that p53 was down-regulated during proliferation (Fig. 2C). Chen et al. have reported that the up-regulation of cyclin D1 and the down-regulation of p53 were associated with cell growth promotion [27]. Taken together, available evidence suggests that nicotine down-regulates p53 expression and up-regulates cyclin D1, thereby promoting cell cycle progression during proliferation.

Nicotine inhibits ALP activity and mineralization in association with sustained p53 expression during differentiation

Since nicotine directly affects differentiation in osteoblasts, we examined early osteogenic markers [6,12,13]. The level of ALP activity in bone tissues reflects osteoblastic differentiation [28]. Ramp et al. reported that nicotine at 3.6 mM inhibits collagen synthesis and ALP activity in primary cells from embryonic chick calvariae. Yuhara et al. reported that nicotine at 1.5 mM decreases ALP activity and Ca^{2+} accumulation in MC3T3-E1 [6,12]. In contrast, Gullihorn et al. reported that nicotine stimulates ALP activity on day 3 in MC3T3-E1 [29]. Thus, we investigated whether nicotine at the indicated doses directly affects ALP activity in MC3T3-E1 cultured in conditioned medium. As shown in Fig. 3A, ALP activity in MC3T3-E1 was significantly reduced by nicotine on day 7, after a transient increase in ALP activity on day 3 (data not shown).

Next, we tested whether nicotine affects mineralization. To examine the effect of nicotine on proliferation during differentiation, we investigated whether nicotine at the indicated doses affects proliferation in conditioned medium on day 21. Nicotine did not inhibit proliferation during differentiation (Fig. 3B). To evaluate the mineralization of osteoblasts, we used von Kossa staining and alizarin red staining. After 21 days, staining with both techniques showed that the mineralization of MC3T3-E1 cells was inhibited by nicotine in a dose-dependent manner (Fig. 3C and D). We also demonstrated that nicotine inhibited mineralization without decreasing proliferation. Our data indicate that nicotine inhibits matrix maturation and mineralization.

We then examined p53 expression during differentiation in the presence of nicotine because p53 is a negative regulator of differentiation in osteoblasts [20,21]. After 8 days, p53 expression was down-regulated in control, while p53 expression was sustained in nicotine-treated cells during differentiation (Fig. 3E). These results suggest that p53 may play a role in inhibiting differentiation of osteoblasts treated with nicotine.

In conclusion, our study showed that nicotine induced proliferation in association with cyclin D1 up-regulation and inhibited differentiation in association with p53 regulation in pre-osteoblastic cells.

Acknowledgments

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