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ORIGINAL ARTICLE

Suppressive effects of nicotine on the cytodifferentiation of murine periodontal ligament cells

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OBJECTIVES: Tobacco smoking has been suggested to be one of the important risk factors of developing periodontal disease. Although epidemiological studies have shown the detrimental effects of smoking on periodontal disease, the effects of smoke compounds on gingival tissue are not well understood. The aim of this study was to evaluate the effects of nicotine, which is the major component of the thousands of chemicals that constitute cigarette smoke, for cytodifferentiation of murine periodontal ligament (MPDL) cell.

MATERIALS AND METHODS: Expression of nAChR subunits on MPDL cells was examined using RT-PCR. The effects of nicotine on gene expression of extracellular matrices and osteoblastic transcription factors were evaluated by quantitative RT-PCR. Mineralized nodule formation of nicotine-treated MPDL cells was characterized by alizarin red staining.

RESULTS: Murine periodontal ligament cells expressed several subunits of nAChR, which have functional calcium signals in response to nicotine. Gene expression of extracellular matrices and osteoblastic transcription factors were reduced in nicotine-treated MPDL cells. In addition, mineralized nodule formation was inhibited in MPDL cells in the presence of nicotine.

CONCLUSION: Our findings indicate that nicotine may negatively regulate the cytodifferentiation and mineralization of MPDL cells.

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Keywords: periodontal ligament cells; nicotine; cytodifferentiation; mineralization

Introduction

The periodontal ligament (PDL) is a connective tissue that surrounds the root of the tooth and attaches the root to the alveolar bone to provide mechanical support. The PDL also plays a nutritive and sensory role. In addition, PDL is regarded as a reservoir of multipotential mesenchymal stem cells that can differentiate into mineralized tissue-forming cells, such as osteoblasts and cementoblasts (Seo *et al*, 2004), and plays an important role in periodontal tissue remodeling and regeneration.

Cigarette smoking is known to be one of the most important risk factors in periodontal disease (Martinez-Canut *et al*, 1996; Ryder, 2007). Previous reports have shown that more clinical attachment loss and alveolar bone loss have been observed in smokers than in non-smokers (Grossi *et al*, 1994; Grossi *et al*, 1995). Tobacco smoke consists of thousands of chemicals (Lofroth, 1989) which individually, and collectively, can affect periodontal tissue. Nicotine is the main component of tobacco smoke and a selective agonist of the nicotinic acetylcholine receptor (nAChR). The main route of nicotine exposure is via inhalation of tobacco smoke. During inhalation, a high dose of nicotine (in excess of 10^{-3} M) would be exposed to the epithelial surface of the oral cavity, the bronchus and the lungs (Feyerabend *et al*, 1982; Seow *et al*, 1994). In particular, nicotine concentrations in the saliva of long-term snuff users can reach 9.6 mM (Hoffmann and Adams, 1981; Sato *et al*, 2008).

Therefore, we hypothesized that nicotine affects the characteristics of PDL directly and has detrimental effects on periodontal tissue, such as disease progression and the ineffectiveness for periodontal treatment. In this study, we investigated the gene expression of nAChR subunits and calcium influx via these receptors using a PDL clone obtained from murine PDL (MPDL) tissue (Yamada *et al*, 2007). We then examined the gene expression of alkaline phosphatase (ALP), collagen type I, bone sialoprotein (BSP), osterix, and runx2 in nicotine-treated MPDL. Further, the effects of nicotine on mineralization in MPDL were evaluated.

Materials and methods

Culture of MPDL

We have established a murine PDL clone cell, MPDL22, isolated from the PDL tissue of the molar teeth extracted from 2.5-week-old BALB/c mice (Yamada

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et al, 2007). MPDL22 cells were maintained in α-MEM (Nikken, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA) and 100 ng ml^{-1} fibroblast growth factor-2 (FGF-2; Kaken, Kyoto, Japan) at 37°C in a humidified atmosphere of 5% CO^2. After the cells reached confluence, we replaced the culture medium (α-MEM supplemented with 10% FCS and FGF-2) with the mineralization medium (α-MEM supplemented with 10% FCS, 10 mM β-glycerophosphate, and 50 μg ml^{-1} ascorbic acid). We replaced the mineralization medium every 2 days with or without nicotine (10^{-8} M to 10^{-3} M), which was prepared in PBS and neutralized to pH 7.2.

Detection of nAChRs in MPDL by RT-PCR
MPDL22 cells were seeded at a density of 10^5 cells per dish in 60 mm dishes and grown to confluency. Following the culture of MPDL cells, total RNA was extracted using the RNAbee (TEL-TEST, Friendswood, TX, USA), according to the manufacturer's instructions. cDNA synthesis and amplification via PCR were performed, as described previously (Yamada *et al*, 2007). The primers used for PCR were prepared according to published results (Kageyama-Yahara *et al*, 2008). After denaturation at 94°C for 5 min, each PCR cycle consisted of 94°C for 30 s, 60°C for 45 s and 68°C for 45 s. Amplified products were analyzed by electrophoresis at 100 V for 30 min on 1.5% TAE agarose gels containing 0.5 μg ml^{-1} ethidium bromide. Murine brain samples were used as a positive control for nAChR subunits.

RT-PCR for ALP, collagen type I, BSP, osterix and runx2 mRNA
RNA samples were obtained from MPDL22 cells in α-MEM containing 1% FCS 2 days after nicotine treatment (10^{-3} M). Total RNA extract (0.4 mg) was reverse-transcribed using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) to generate the single-stranded cDNA. PCRs were carried out using the ABI 7300 Fast Real-Time PCR System (Applied Biosystems) with Power $SYBR^R$ Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. All reactions were run in triplicate. The primer sequences used for RT-PCR were as follows; ALP, (sense) 5′-ACA CCT GAC TGT GGT TAC TGC TGA-3′, (antisense) 5′-CCT TGT AGC CAG GCC CGT TA-3′; collagen type I, (sense) 5′-ATG CCG CGA CCT CAA GAT G-3′, (antisense) 5′-TGA GGC ACA GAC GGC TGA GTA-3′; BSP, (sense) 5′-TGG AGA CTG CGA TAG TTC CGA AG-3′, (antisense) 5′-CGT AGC TAG CTG TTA CAC CCG AGA G-3′; Osterix, (sense) 5′-CGC ATC TGA AAG CCC ACT TG-3′, (antisense) 5′-CAG CTC GTC AGA GCG AGT GAA-3′; runx2, (sense) 5′-CAC TGG CGG TGC AAC AAG A-3′, (antisense) 5′-TTT CAT AAC AGC GGA GGC ATT TC-3′.

Proliferation assay
MPDL22 (5×10^3 cells per well) were incubated in 96-well plates in α-MEM containing 1% FCS in the presence of nicotine or FGF-2 (100 ng ml^{-1}) for 48 h. Cell proliferation was measured using the non-radioactive colorimetric assay WST-1 system (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions and the OD450/650 measured after 2 h on a microplate reader (Bio-Rad, Hercules, CA, USA).

Intracellular calcium determination
The cells (2×10^4 cells per well) were incubated onto 96-well plates for 24 h and washed in PBS containing 1 mM Ca^{2+}, 1 mM $MgCl_2$ and 0.1% bovine serum albumin (BSA). Cells were incubated with 200 μl of 1 mg ml^{-1} Fluo3-AM (Dojindo, Kumamoto, Japan) for 30 min in the dark. Cells were then rinsed three times in PBS wash solution, as described above, to remove any extra Fluo3-AM. Cells were stimulated with nicotine and levels of calcium influx were measured by FluoroScan (Thermo Fisher Scientific Inc, Waltham, MA, USA). The excitation wavelength was 353 and 373 nm, and the emission wavelength was 510 nm. Non-selective nAChR antagonist, D-tubocurarine, was obtained from Sigma (St. Louis, MO, USA). In some experiments, MPDL22 cells were pretreated with 10^{-4} M D-tubocurarine, which was the most effective concentration for inhibiting nicotine-induced calcium influx in preliminary experiment, for 30 min before calcium measurement.

Mineralization assay
Histochemistry for staining calcified nodules was performed using the alizarin red staining method (Dahl, 1952). Cell layers were washed twice with PBS and then fixed in dehydrated ethanol. After fixation, the cell layers were stained with 1% alizarin red S in 0.1% NH_4OH (pH 6.3–6.5) for 5 min. The dishes were washed with H_2O and then observed microscopically, digitized and analyzed using the WinRoof software program (Mitani Corporation, Fukui, Japan).

Statistical analysis
Results were analyzed for statistical significance using the Student's *t*-test. Differences were considered significant at P values < 0.05.

Results

Expression of nAChRs mRNA and calcium signaling of nAChR in MPDL22 cells
To determine whether nAChRs were expressed in MPDL cells, mRNA was extracted from MPDL22 cells and analyzed by RT-PCR using mouse nAChR-specific primers. As shown in Figure 1a, MPDL22 cells were positive for α3, α4, α7 and β2 nAChR mRNA. To investigate whether the nAChRs expressed were functional, we analyzed cytozolic Ca^{2+} levels using Flou-3AM. When nicotine was used as the agonist for nAChR, it elicited cytozolic Ca^{2+}. Pretreatment with non-selective nAChR antagonist, D-tubocurarine, reduced nicotine-induced calcium mobilization (Figure 1b).

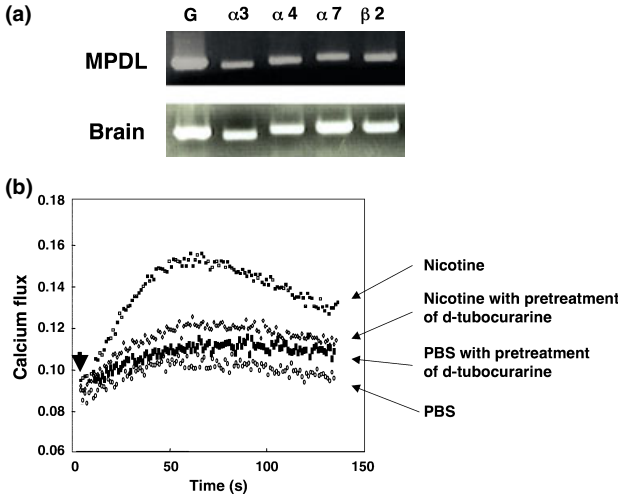

Figure 1 MPDL22 cells express functional nAChRs. **(a)** Expression of nAChR mRNA in MPDL22 cells was examined by RT-PCR. The number of PCR cycles was 33 and 24 for nAChRs and GAPDH, respectively. G: GAPDH. **(b)** Effects of nicotine on calcium mobilization in MPDL22 cells were monitored by measuring the fluorescence ratio (F340/380). Cells were pretreated for 30 min in the presence or absence of non-selective nAChR antagonist, D-tubocurarine (10^{-4} M), before addition of nicotine (10^{-3} M) or PBS. The arrowhead indicates the time of application of the stimulators. The results of one representative experiment from three identical experiments are shown

Effects of nicotine on MPDL22 proliferation

To investigate the effects of nicotine on the proliferation response of MPDL cells, cells were cultured for 48 h in a medium containing 1% FCS with or without nicotine. As previous studies revealed that FGF-2 induced the proliferation of PDL (Takayama *et al*, 1997), FGF-2 concentration of 100 ng ml^{-1}, which was the most optimal concentration for MPDL proliferation in preliminary experiment, was used as a positive control of this assay. The exposure of 10^{-8} M to 10^{-3} M nicotine did not induce significant proliferative responses in MPDL22 cells (Figure 2).

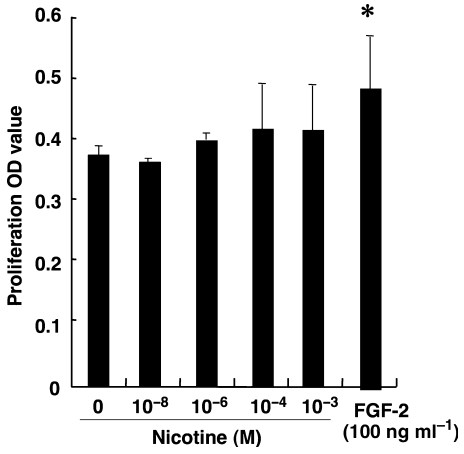

Figure 2 Effects of nicotine on proliferation of MPDL cells. MPDL cells were cultured with various doses of nicotine or FGF-2 (100 ng ml^{-1}) for 2 days. Values are means ± s.d. of four determinations. $^*P < 0.05$ compared with medium only

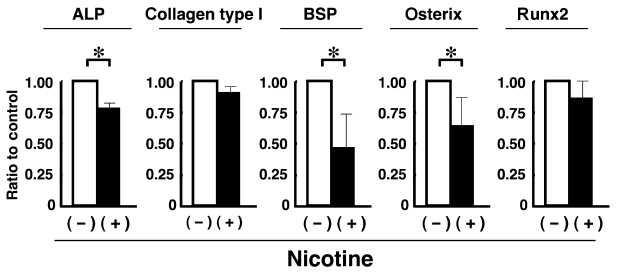

Figure 3 Analysis of mRNA expression of ECM in MPDL cells by RT-PCR. RNA samples were obtained from MPDL cells 2 days after nicotine treatment (10^{-3} M). The relative expression of each gene was standardized against the amount in HPRT as control and the expression of ECM in MPDL cells without nicotine was taken as 1.0. Values are means ± s.d. of three or four determinations. $^*P < 0.05$ compared with non-treated

Effects of nicotine on mRNA expression of ALP, BSP and osterix in MPDL22 cells

We then examined the effects of nicotine (10^{-3} M) on gene expression of the extracellular matrix (ECM) and osteoblastic transcription factors (Figure 3). MPDL22 cells, which were treated with nicotine, showed significantly decreased mRNA expression of ALP, BSP and osterix. In addition, the expression of collagen type I and *runx2* were also decreased compared with the control, but not significantly.

Effects of nicotine on mineralized nodule formation in MPDL22 cells

We cultured MPDL22 cells with or without nicotine in mineralization medium and then examined mineralized nodule formation on day 12. As shown in Figure 4a,b, nicotine reduced alizarin red staining intensity. Even a low concentration of nicotine (10^{-8} M) decreased mineralized nodule formation by the MPDL22 cells. Inhibitory effects of nicotine on the mineralization were partly abrogated by pretreatment with D-tubocurarine (Figure 4c). Next, we examined the time dependency of period of nicotine treatment on the inhibition of mineralization of MPDL cells (Figure 5). As shown in Figure 4, treatment with 10^{-3} M nicotine inhibited mineralization of MPDL22 cells. Interestingly, treatment with the same concentration of nicotine only for the first 8 days still considerably inhibited the mineralization. Similarly, nicotine treatment for both first 4 days (sample ID 4: day 0–4) and for the following 4 days (sample ID 5: day 4–8) still inhibited the mineralized nodule formation significantly.

Discussion

Little is known about the expression of nAChR subunits in hard tissue-forming cells. We previously reported that human dental pulp cells express nAChRs (Yanagita *et al*, 2008). In addition, α4 nAChR was expressed in human primary osteoblasts (Walker *et al*, 2001) and a recent study has reported human growth plate chondrocytes express α5, α7, β1 and ε nAChR subunits (Kawakita *et al*, 2008). In this study, we found that

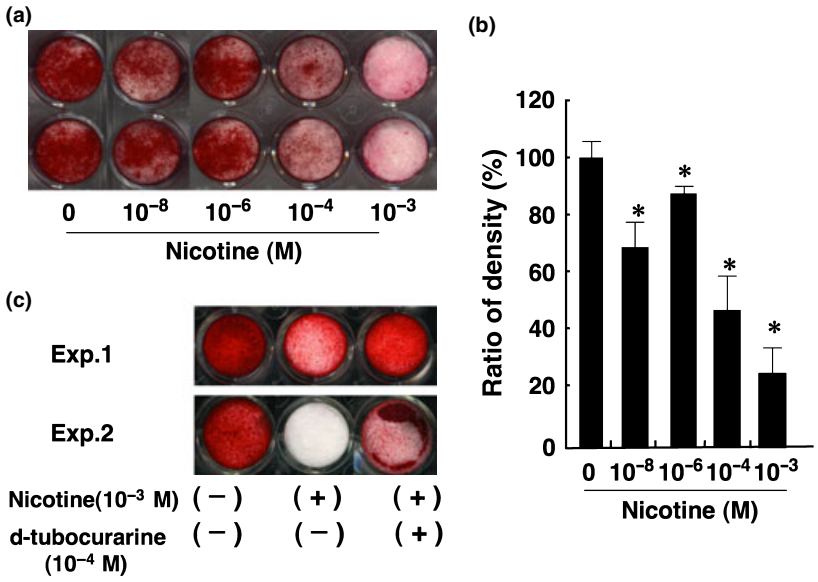

Figure 4 Effects of nicotine on mineralization of MPDL cells. **(a)** Effects of nicotine (10^{-8} M to 10^{-3} M) on mineralization in MPDL cells were examined by alizarin red staining. Alizarin red staining was performed after 12 days of culture in mineralization medium. Results of one representative experiment out of three identical experiments are shown. **(b)** The relative expression value of alizarin staining is shown in **(a)** and was quantitated and normalized to alizarin staining without nicotine. **(c)** Effects of D-tubocurarine on nicotine-dependent inhibition of the mineralization was examined. Alizarin red staining was performed after 12 days of culture in mineralization medium with nicotine (10^{-3} M) in the presence or absence of D-tubocurarine (10^{-4} M). Results of one representative experiment out of three identical experiments are shown. $^*P < 0.05$ compared with medium only

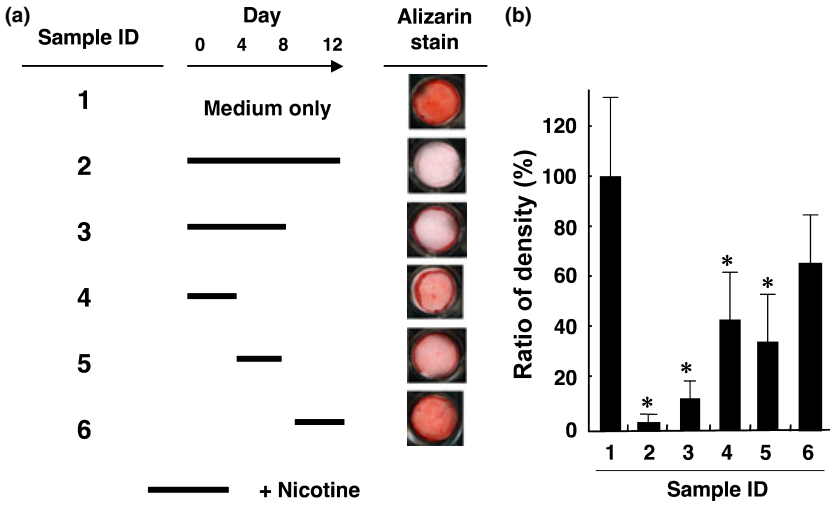

Figure 5 Effect of nicotine at differential stages of mineralization of MPDL cells. **(a)** Nicotine (10^{-3} M) was added to mineralization medium for the indicated periods and alizarin red staining was performed after 12 days of culture. **(b)** The relative expression value of the alizarin staining is shown in **(a)** and was quantitated and normalized to alizarin staining without nicotine. Results of one representative experiment out of two identical experiments are shown. $^*P < 0.05$ compared with medium only

expression of α3 α4, α7 and β2 nAChR subunits at mRNA level, which are reportedly the most abundantly expressed subtypes in murine brain and ganglion cells (Gahring and Rogers, 2005; Gotti *et al*, 2006) are detected in MPDL cells by RT-PCR. Unfortunately, we did not reveal the nAChR subunit expression at protein level in this study, and the detection of nAChR subunit proteins by immunoblot or immunohistochemistry remained to be the future work. Furthermore, nicotine elicited a transient calcium influx and D-tubocurarine, a non-selective nAChR antagonist, inhibited the calcium signals. This suggests that the effects of smoking on MPDL cells can be mediated via nAChR.

The results of this study show that nicotine did not change the proliferative responses of MPDL cells. In contrast, it was reported that nicotine has cytotoxicity and inhibits proliferation of human gingival fibroblasts and periodontal ligament cells (Giannopoulou *et al*, 2001; Chang *et al*, 2002). On the contrary, a recent study showed that nicotine increased the proliferation rate of a murine preosteoblastic cell line, MC3T3-E1 (Sato *et al*, 2008). In addition, our previous study revealed that nicotine increased the DNA amounts in human dental pulp cells (Yanagita *et al*, 2008). Thus, the effect of nicotine on cellular proliferation is still controversial and may be cell-type dependent.

We examined the expression of some osteogenic markers, and revealed by RT-PCR that nicotine reduced the mRNA expression of ALP, BSP, and osterix (Figure 3). ALP is an enzyme marker of osteoblasts and participates in bone mineralization (Majeska and Wuthier, 1975). BSP is a small integrin-binding ligand N-linked glycoprotein, involved in the regulation of mineralization (Moses *et al*, 2006). Osterix, the osteoblast-specific transcription factor, has been identified as a modulator of bone formation and osteoblast differentiation (Nakashima and de Crombrugghe, 2003). Thus,

the inhibitory effects of nicotine on the above-mentioned mRNA expression are correlate with that on the mineralization of MPDL22 cells. On the contrary, nicotine-induced BSP and collagen type I mRNA expression but down-regulated osteopontin mRNA in a human osteosarcoma cell line (Tanaka *et al*, 2005). Another study documented that 24-h treatment of nicotine up-regulated collagen type I, ALP and osteocalcin in osteosarcoma cells, but after 48 or 72-h incubation mRNA expression of these three genes was downregulated by nicotine in a dose-dependent manner (Rothem *et al*, 2009). In the case of chondrocytes, nicotine decreased the expression of ALP (Kawakita *et al*, 2008). Thus, the effects of nicotine on the mRNA expression of bone-related ECM also appears to be cell-type dependent.

Alizarin red staining showed that mineralization of MPDL22 cells was inhibited by nicotine (Figure 4a,b), and the inhibition was partly abrogated by D-tubocurarine, a non-selective nAChRs antagonist (Figure 4c). Furthermore, nicotine treatment for the first and second 4 days (sample ID 4, 5: day 0–4, 4–8) inhibited the mineralization. This result suggests that the suppressive effect of nicotine on the cytodifferentiation and mineralization of MPDL22 cells is culture-phase dependent. The fact that nicotine treatment for the last 4 days (sample ID 6: day 8–12) did not reduce the mineralization may suggest that MPDL22 should be committed to terminally differentiate at day 8. As shown in Figure 1b, D-tubocurarine almost completely inhibited nicotine-induced calcium influx, although suppression of mineralization was not completely recovered by the reagent (Figure 4c). In the case of calcium influx experiment, the incubation time was quite short, whereas cells were cultured for 12 days in mineralization assay. It is difficult at this point to establish a correlation between the initial, transient calcium signals and the mineralization results done on day 12. Measurement of calcium levels in the cells at day 12 may need to be done to accurately determine if early, transient calcium signals are linked to mineralization via an unknown signaling cascade.

Recently nicotine has been reported to induce p53 in osteoblasts (Sato *et al*, 2008). p53 is originally identified as a tumor suppressor, and promotes cell cycle arrest (Vogelstein *et al*, 2000). Interestingly it has been demonstrated that p53 regulates osteogenic differentiation (Wang *et al*, 2006). This suggests that p53 may be involved in the nicotine-induced inhibition of the differentiation of MPDL cells. Further studies are required to clarify the nicotine-induced signal cascade through nAChRs that influences the mineralization.

The association between smoking and progression of periodontal diseases, including alveolar bone loss, has been well-investigated (Grossi *et al*, 1994; Grossi *et al*, 1995; Ryder, 2007). Nicotine alters the cellular functions of PDL cells and gingival fibroblasts *in vitro* (Chang *et al*, 2002). Smokers demonstrate impaired periodontal tissue regeneration compared with non-smokers (Stavropoulos *et al*, 2004). The inhibitory effect of nicotine on the mineralization of PDL cells partly accounts for the reason. Furthermore, smokers have been reported to suffer decrease of bone mass, increased risk of fracture and prolonged fracture repair (Krall and Dawson-Hughes, 1991, 1999). The important information is that the nicotine concentration in smokers' sera was 25–444 nM (Russell *et al*, 1980) and that serum nicotine levels of non-smokers exposed to secondhand smoke was around nM (5.9 ng ml^{-1}) (Pacifici *et al*, 1995). Figure 4a showed that a low concentration of nicotine circulating in the blood stream ($\sim$10 nM) can negatively influence the mineralization of PDL cells. These results suggest the possibility that secondhand smoke could be detrimental to periodontal health. In addition, inflammatory cytokines are detected in inflamed periodontal lesions, and some of those cytokines such as IL-1β affect ECM expression (Chien *et al*, 1999). Therefore, to investigate the effect of nicotine on cytokine-induce ECM expression should be carried out in the future.

In conclusion, we have shown that nicotine is a potent negative regulator of cytodifferentiation and mineralization in PDL cells. Our studies suggest that nicotine causes progressing periodontal diseases and unfavorable prognoses after periodontal treatments.

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Competing interests

None declared.

Ethical approval

Not required.

References

Chang YC, Huang FM, Tai KW, Yang LC, Chou MY (2002). Mechanisms of cytotoxicity of nicotine in human periodontal ligament fibroblast cultures in vitro. *J Periodontal Res* **37:** 279–285.

Chien HH, Lin WL, Cho MI (1999). Interleukin-1β-induced release of matrix proteins into culture media causes inhibition of mineralization of nodules formed by periodontal ligament cells in vitro. *Calcif Tissue Int* **64:** 402–413.

Dahl LK (1952). A simple and sensitive histochemical method for calcium. *Proc Soc Exp Biol Med* **80:** 474–479.

Feyerabend C, Higenbottam T, Russell MA (1982). Nicotine concentrations in urine and saliva of smokers and non-smokers. *Br Med J (Clin Res Ed)* **284:** 1002–1004.

Gahring LC, Rogers SW (2005). Neuronal nicotinic acetylcholine receptor expression and function on nonneuronal cells. *AAPS J* **7:** E885–E894.

Giannopoulou C, Roehrich N, Mombelli A (2001). Effect of nicotine-treated epithelial cells on the proliferation and

collagen production of gingival fibroblasts. *J Clin Periodontol* **28:** 769–775.

Gotti C, Zoli M, Clementi F (2006). Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol Sci* **27:** 482–491.

Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, Norderyd OM, Genco RJ (1994). Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* **65:** 260–267.

Grossi SG, Genco RJ, Machtei EE, *et al*. (1995). Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. *J Periodontol* **66:** 23–29.

Hoffmann D, Adams JD (1981). Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Res* **41:** 4305–4308.

Kageyama-Yahara N, Suehiro Y, Yamamoto T, Kadowaki M (2008). IgE-induced degranulation of mucosal mast cells is negatively regulated via nicotinic acetylcholine receptors. *Biochem Biophys Res Commun* **377:** 321–325.

Kawakita A, Sato K, Makino H, *et al*. (2008). Nicotine acts on growth plate chondrocytes to delay skeletal growth through the alpha7 neuronal nicotinic acetylcholine receptor. *PLoS One* **3:** e3945.

Krall EA, Dawson-Hughes B (1991). Smoking and bone loss among postmenopausal women. *J Bone Miner Res* **6:** 331–338.

Krall EA, Dawson-Hughes B (1999). Smoking increases bone loss and decreases intestinal calcium absorption. *J Bone Miner Res* **14:** 215–120.

Lofroth G (1989). Environmental tobacco smoke: overview of chemical composition and genotoxic components. *Mutat Res* **222:** 73–80.

Majeska RJ, Wuthier RE (1975). Studies on matrix vesicles isolated from chick epiphyseal cartilage. Association of pyrophosphatase and ATPase activities with alkaline phosphatase. *Biochim Biophys Acta* **391:** 51–60.

Martinez-Canut P, Guarinos J, Bagan JV (1996). Periodontal disease in HIV seropositive patients and its relation to lymphocyte subsets. *J Periodontol* **67:** 33–36.

Moses KD, Butler WT, Qin C (2006). Immunohistochemical study of small integrin-binding ligand, N-linked glycoproteins in reactionary dentin of rat molars at different ages. *Eur J Oral Sci* **114:** 216–222.

Nakashima K, de Crombrugghe B (2003). Transcriptional mechanisms in osteoblast differentiation and bone formation. *Trends Genet* **19:** 458–466.

Pacifici R, Altieri I, Gandini L, *et al*. (1995). Environmental tobacco smoke: nicotine and cotinine concentration in semen. *Environ Res* **68:** 69–72.

Rothem DE, Rothem L, Soudry M, Dahan A, Eliakim R (2009). Nicotine modulates bone metabolism-associated gene expression in osteoblast cells. *J Bone Miner Metab* **27:** 555–561.

Russell MA, Jarvis M, Iyer R, Feyerabend C (1980). Relation of nicotine yield of cigarettes to blood nicotine concentrations in smokers. *Br Med J* **280:** 972–976.

Ryder MI (2007). The influence of smoking on host responses in periodontal infections. *Periodontol* **43:** 267–277.

Sato T, Abe T, Nakamoto N, *et al*. (2008). 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. *Biochem Biophys Res Commun* **377:** 126–130.

Seo BM, Miura M, Gronthos S, *et al*. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364:** 149–155.

Seow WK, Thong YH, Nelson RD, MacFarlane GD, Herzberg MC (1994). Nicotine-induced release of elastase and eicosanoids by human neutrophils. *Inflammation* **18:** 119–127.

Stavropoulos A, Mardas N, Herrero F, Karring T (2004). Smoking affects the outcome of guided tissue regeneration with bioresorbable membranes: a retrospective analysis of intrabony defects. *J Clin Periodontol* **31:** 945–950.

Takayama S, Murakami S, Miki Y, *et al*. (1997). Effects of basic fibroblast growth factor on human periodontal ligament cells. *J Periodontal Res* **32:** 667–675.

Tanaka H, Tanabe N, Suzuki N, *et al*. (2005). Nicotine affects mineralized nodule formation by the human osteosarcoma cell line Saos-2. *Life Sci* **77:** 2273–2284.

Vogelstein B, Lane D, Levine AJ (2000). Surfing the p53 network. *Nature* **408:** 307–310.

Walker LM, Preston MR, Magnay JL, Thomas PB, El Haj AJ (2001). Nicotinic regulation of c-fos and osteopontin expression in human-derived osteoblast-like cells and human trabecular bone organ culture. *Bone* **28:** 603–608.

Wang X, Kua HY, Hu Y, *et al*. (2006). p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. *J Cell Biol* **172:** 115–125.

Yamada S, Tomoeda M, Ozawa Y, *et al*. (2007). PLAP-1/asporin: a novel negative regulator of periodontal ligament mineralization. *J Biol Chem* **282:** 23070–23080.

Yanagita M, Kashiwagi Y, Kobayashi R, Tomoeda M, Shimabukuro Y, Murakami S (2008). Nicotine inhibits mineralization of human dental pulp cells. *J Endod* **34:** 1061–1065.