ELSEVIER

European Journal of Pharmacology 383 (1999) 387–393

ejp

www.elsevier.nl/locate/ejphar

Effects of nicotine on cultured cells suggest that it can influence the formation and resorption of bone

Sachiko Yuhara [a], Satoshi Kasagi [a], Atsuto Inoue [a], Eri Otsuka [a], Shigehisa Hirose [b], Hiromi Hagiwara [a,*]

[a] *Research Center for Experimental Biology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan*
[b] *Department of Biological Sciences, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan*

Received 25 February 1999; received in revised form 19 July 1999; accepted 30 July 1999

Abstract

The acute effects of nicotine [1-methyl-2-(3-pyridyl)pyrrolidine] on the formation and resorption of bone were examined in cultures of clonal rat calvarial osteogenic cells (ROB-C26) and clonal mouse calvarial preosteoblastic cells (MC3T3-E1), as well as in osteoclast-like cells formed during coculture of mouse bone marrow cells and clonal stromal cells from mouse bone marrow, ST2 cells, at concentrations that occur in the saliva of smokeless tobacco users. Nicotine stimulated the rate of deposition of Ca^{2+} by ROB-C26 cells, as well as the alkaline phosphatase activity of these cells, in a dose-dependent manner. However, both activities decreased in MC3T3-E1 cells that had been exposed to nicotine. These results indicate that nicotine affected osteoblastic differentiation in osteoblast-like cells. By contrast, nicotine reduced, in a dose-dependent manner, the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) and the formation of pits on slices of dentine, both of which are typical characteristics of osteoclasts. Our results suggest that nicotine might have critical effects on bone metabolism.

Keywords: Nicotine; Osteoblast; Osteoclast-like cell; Mineralization; Pit formation

1. Introduction

The loss of bone mass in osteoporosis results from an imbalance between the formation and resorption of bone, which, in turn, depends on the interactions between osteoblasts and osteoclasts (Riggs, 1987). Tobacco smoking has been suggested to be a risk factor for osteoporosis (Seeman et al., 1983; Aloia et al., 1985; Stevenson et al., 1989; Broulik and Jarab, 1993; Looker et al., 1998) and has been associated with loss of alveolar bone (Christen et al., 1979; Daniell, 1983). Nicotine is a major component of tobacco. However, the mechanism of action of nicotine on the metabolism of osteoblasts and osteoclasts is still poorly understood.

Osteoblasts are bone-forming cells. The formation of bone involves a complex series of events that include the proliferation and differentiation of osteoprogenitor cells and result eventually in the formation of a mineralized extracellular matrix. The sequential expression of type I collagen, alkaline phosphatase and osteocalcin, and the deposition of Ca^{2+} are known as markers of osteoblastic differentiation. Nicotine has a direct regulatory effect on the growth and differentiation of cultured osteoblast-like cells, such as cells derived from embryonic chick calvariae (Ramp et al., 1991) and rat osteosarcoma cells (UMR 106-01) (Fang et al., 1991). However, the effects of nicotine on osteoblastic metabolism have been obscured by the absence of a suitable cellular model in vitro. In an attempt to establish an appropriate model in this study, we used clonal rat calvarial osteogenic cells (ROB-C26) (Yamaguchi and Kahn, 1991) and clonal mouse calvarial preosteoblastic cells (MC3T3-E1) (Sudo et al., 1983).

Osteoclasts are multinucleated cells (MNCs) that are responsible for resorption of bone. The osteoclastic resorption of bone consists of several processes (Suda et al., 1997): the development of osteoclasts from hematopoietic progenitor cells; the fusion of osteoclasts; the attachment of osteoclasts to the surface of the bone; and the secretion of acids and lysosomal enzymes into the space beneath the osteoclast. To our knowledge, there have been no reports

* Corresponding author. Tel.: +81-45-924-5830; fax: +81-45-924-5832.
E-mail address: hhagiwar@bio.titech.ac.jp (H. Hagiwara)

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00551-8

on the effects of nicotine on the formation of osteoclast-like cells and the bone resorption activity by such cells.

In the present study, we demonstrated that nicotine regulates the alkaline phosphatase activity of and the deposition of Ca^{2+} by ROB-C26 and MC3T3-E1 cells in two well-characterized model systems that have been used for studies of osteoblastic differentiation. Furthermore, we obtained evidence that nicotine might decrease the formation of tartrate-resistant acid phosphatase (TRAP)-positive MNCs and the bone resorption activity of osteoclast-like cells. The effects of nicotine on cultured osteoblasts and osteoclasts might provide clues to the mechanism of action of this drug in vivo.

2. Materials and methods

2.1. Culture of osteoblastic cells

ROB-C26 cells were a generous gift from Dr. Akira Yamaguchi (Nagasaki University, Nagasaki, Japan). MC3T3-E1 cells and mouse clonal stromal cells from bone marrow (ST2 cells) were supplied by the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in 55-cm^2 dishes in α-modified minimum essential medium (α-MEM), supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 in air at 37°C. After reaching 70% confluence, cells were detached by treatment with 0.05% trypsin, replated in either 55-cm^2 dishes or 12-well (area of each well, 3.8 cm^2) plates at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and various concentrations of nicotine. Fresh medium was supplied to cells at 3-day intervals. ROB-C26 and MC3T3-E1 cells exhibited no changes in morphology during exposure to nicotine at 10 to 250 μg/ml.

2.2. Measurement of alkaline phosphatase activity

Osteoblastic cells were subcultured in 12-well plates (3.8 cm^2/well) in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and nicotine at various concentrations. The cells were washed with 10 mM Tris–HCl, pH 7.2, and sonicated in 1 ml of 50 mM Tris–HCl, pH 7.2, that contained 0.1% Triton X-100 and 2 mM $MgCl_2$ for 15 s with a sonicator (Ultrasonic Disruptor UD-201; Tomy, Tokyo, Japan). The alkaline phosphatase activity of the sonicate was determined by an established technique with *p*-nitrophenyl phosphate as the substrate (Hagiwara et al., 1996). Concentrations of protein were determined with BCA protein assay reagent (Pierce Chemical, Rockford, IL, USA), with bovine serum albumin as the standard.

2.3. Quantitation of Ca^{2+}

Osteoblastic cells were subcultured in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and nicotine at various concentrations. The amount of Ca^{2+}, in hydroxyapatite, in the cell layer was measured as follows. The layers of cells in 12-well plates (3.8 cm^2/well) were washed with phosphate-buffered saline (pH 7.4; PBS; 20 mM sodium phosphate and 130 mM NaCl) and incubated with 1 ml of 2 N HCl overnight with gentle shaking. The Ca^{2+} ions in the samples were quantitated by the *o*-cresolphthalein complexone method with a Calcium C kit (Wako, Osaka, Japan) (Hagiwara et al., 1996). This kit is specific for Ca^{2+} and has a limit of detection of 1 μg/ml. We used the Ca^{2+} solution (20 mg/dl) in the kit as the standard solution.

2.4. Formation of osteoclast-like cells in vitro

Bone marrow cells were collected from the femurs and tibias of 6-week old male ddY mice as described by Takahashi et al. (1988). ST2 cells were cocultured (2×10^4 cells/well) with bone marrow cells (5×10^4 cells/well) in α-MEM that contained 10% fetal bovine serum, 10^{-8} M 1α,25$(OH)_2$ vitamin D_3, and 10^{-7} M dexamethasone in 48-well plates (0.98 cm^2/well). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Fresh medium was supplied at 2-day intervals.

2.5. Localization of TRAP

After coculture for 6 days, adherent cells were fixed and stained for TRAP activity as described by Udagawa et al. (1990). TRAP-positive MNCs [TRAP(+)MNCs] with three or more nuclei were counted under a microscope (BH; Olympus, Tokyo, Japan).

2.6. Quantitation of bone resorption

The formation of pits on slices of dentine was monitored to determine the capacity for bone resorption of osteoclastic cells formed. Bone marrow cells and ST2 cells were cocultured for 6 days on collagen gel-coated dishes (55 cm^2). Cocultures were treated with 0.2% collagenase (Wako) at 37°C for 20 min to detach osteoclast-like cells from the dishes. The osteoclast-like cells were plated on

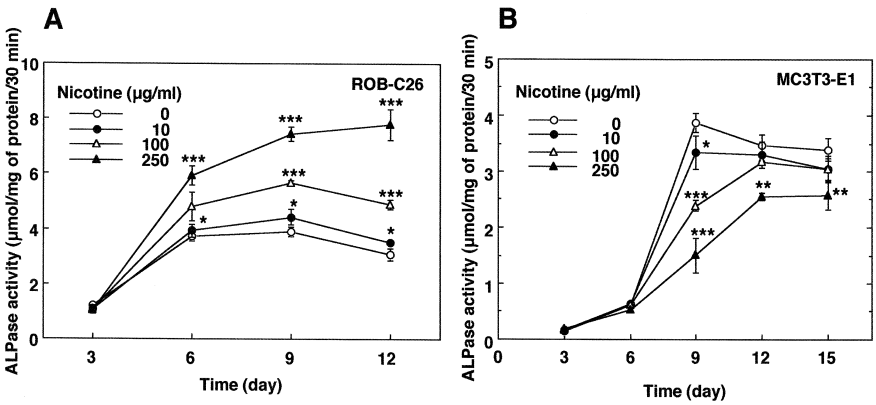

Fig. 1. Effects of nicotine on the alkaline phosphatase activity in ROB-C26 (A) and MC3T3-E1 (B) cells. ROB-C26 and MC3T3-E1 cells were cultured in 12-well plates with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and nicotine at various concentrations. Fresh medium with nicotine was supplied at 3-day intervals. At the times indicated, alkaline phosphatase activity was measured as described in Section 2. Values represent the means ± S.D. of results from three wells. Data are representative of results from three separate experiments that yielded similar results. $^{*}P < 0.05$ vs. controls; $^{**}P < 0.01$ vs. controls; $^{***}P < 0.001$ vs. controls.

dentine slices (4 mm in diameter), which had been placed in 96-well plates (0.48 cm^2/well) that contained 0.2 ml α-MEM supplemented with 10% fetal bovine serum. After incubation for 48 h, cells were removed from the dentine slices, and then resorption pits that had been formed were stained with Mayer's hematoxylin. The numbers and the total areas of pits were counted under a microscope (Olympus).

2.7. Drugs

(±)Nicotine [1-methyl-2-(3-pyridyl)pyrrolidine], 1α, 25$(OH)_2$ vitamin D_3, dexamethasone, pyruvic acid (sodium salt), and β-NADH were purchased from Sigma (St. Louis, MO, USA). Sodium tartrate was purchased from Wako; α-MEM, penicillin/streptomycin antibiotic mixture, and fetal bovine serum were obtained from Life Technologies.

3. Results

3.1. Effects of nicotine on the differentiation and mineralization of osteoblastic cells

Fig. 1 summarizes the time courses of dose-dependent changes in the alkaline phosphatase activity in ROB-C26 and MC3T3-E1 cells in response to nicotine. The alkaline phosphatase activity was increased in control cultures of both lines of cells. ROB-C26 cells that had been exposed to nicotine had higher alkaline phosphatase activity than

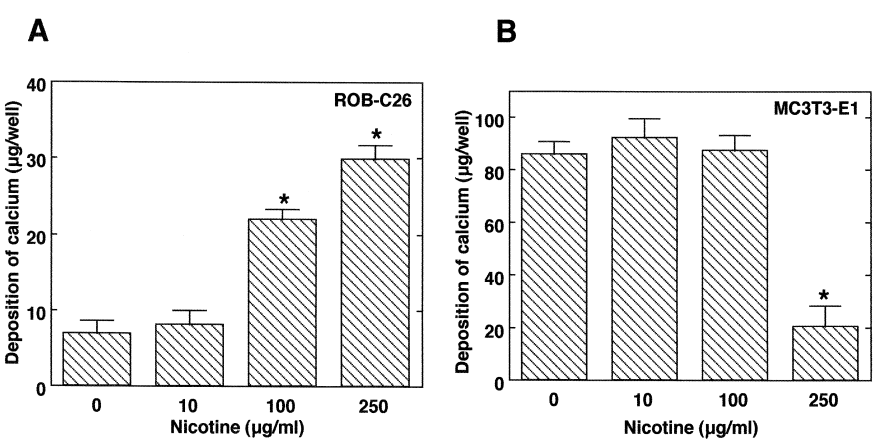

Fig. 2. Effects of nicotine on the mineralization by ROB-C26 (A) and MC3T3-E1 (B) cells. Cells in 12-well plates (3.8 cm^2/well) were cultured with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and nicotine at various concentrations. Fresh medium with nicotine was supplied at 3-day intervals. On day 14, the deposition of Ca^{2+} was measured. Quantitative analysis of Ca^{2+} ions derived from hydroxyapatite was performed as described in Section 2. Data are means ± S.D. of results from four wells and are typical of results of three separate experiments. $^{*}P < 0.01$ vs. 0 μg/ml nicotine.

the control cells at all times examined. On day 12, nicotine at 10, 100, and 250 μg/ml had increased the alkaline phosphatase activity to 110%, 160%, and 250%, respectively, of the control activity (3.08 ± 0.11 μmol/mg of protein/30 min; $n = 4$) (Fig. 1A). By contrast, the alkaline phosphatase activity in MC3T3-E1 cells was reduced both time- and dose-dependently by nicotine. On day 9, the exposure to nicotine at 10, 100, and 250 μg/ml reduced alkaline phosphatase activity to 86%, 62%, and 39%, respectively, of the control activity (3.87 ± 0.17 μmol/mg of protein/30 min; $n = 4$) (Fig. 1B). Fig. 2 shows the dose-dependent changes in the deposition of Ca^{2+} by both lines of cells in response to nicotine. On day 15, exposure of ROB-C26 cells to nicotine at 10, 100, and 250 μg/ml increased the accumulation of Ca^{2+} to 120%, 320%, and 430% of levels (6.9 ± 1.6 μg/3.8-cm^2 well) in control cultures, respectively (Fig. 2A). By contrast, MC3T3-E1 cells that had been exposed to nicotine at 250 μg/ml accumulated very low levels (Fig. 2B).

3.2. Effects of nicotine on the formation and activity of osteoclast-like cells

Fig. 3 shows the effects of nicotine on the formation of osteoclast-like cells. TRAP is known as a marker enzyme of osteoclasts. In control cultures, TRAP-positive MNCs appeared after a 6-day coculture and there were 524 ± 72 ($n = 4$) cells of this type per 0.98-cm^2 well. By contrast, exposure to nicotine at 10 to 150 μg/ml decreased the formation of TRAP-positive MNCs in a dose-dependent manner. Few TRAP-positive MNCs were formed when the medium contained 150 μg/ml nicotine, but TRAP-positive mononucleated cells were detected in the culture. The supporting ST2 cells exhibited no changes in morphology or in growth rate in the presence of nicotine at 150 μg/ml. To examine the effects of nicotine on the bone-resorbing ability of osteoclast-like cells, we recovered osteoclast-like cells that had formed on the collagen gel by digestion with collagenase and then cultured them on dentine slices for 48 h. Fig. 4 shows that osteoclast-like cells in control culture formed resorption pits (140 ± 20/well; $n = 6$) on dentine slices within 48 h. The exposure of osteoclast-like cells to nicotine at 10, 100, and 250 μg/ml nicotine reduced the formation of pits on dentine slices to 60%, 40%, and 5% of levels in control cultures, respectively.

4. Discussion

Nicotine had significant effects on the metabolism of cultured osteoblasts and osteoclasts. The concentrations (0.06 to 1.5 mM) of nicotine that we used in this study were in the range of those (1 μM to 10 mM) in previously reported studies (Fang et al., 1991; Ramp et al., 1991). Blood concentrations of nicotine obtained from habitual cigarette smokers were in the range of 0.06–0.3 μM. However, the concentrations of nicotine in the saliva of clonic snuff users reached from 0.6 to 9.6 mM (Hoffmann and Adams, 1981). Therefore, our study showed the effects of high but physiological concentrations of this drug on cultured osteoblasts and osteoclasts.

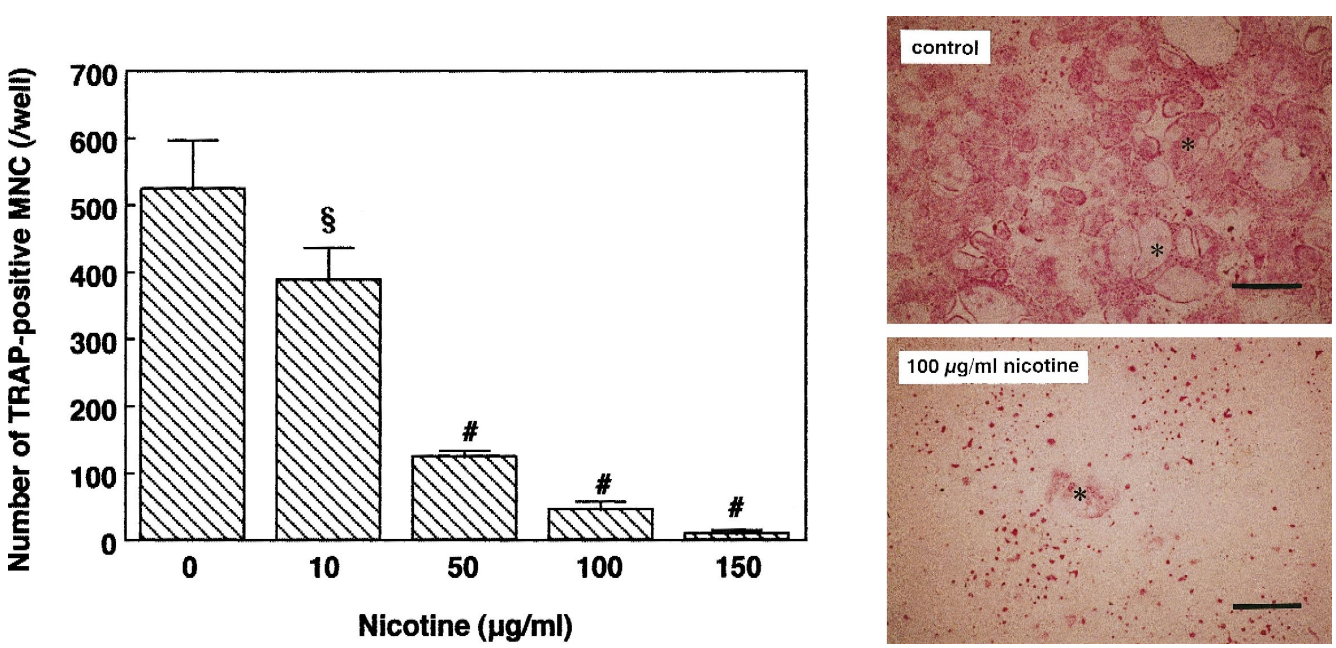

Fig. 3. Reduction in the formation of osteoclast-like cells by nicotine. Mouse bone marrow cells and ST2 cells were cocultured in 48-well plates (0.98 cm^2/well) for 6 days in α-MEM that contained 10% fetal bovine serum, 10^{-8} M vitamin D_3, 10^{-7} M dexamethasone, and nicotine at various concentrations. Fresh medium with nicotine was supplied at 2-day intervals. Asterisks indicate TRAP-positive MNCs. Bar, 500 μm. Numbers of TRAP-positive MNCs (more than three nuclei) were counted under a microscope. Data are means ± S.D. of results from six wells. $^{\S}P < 0.05$ vs. 0 μg/ml nicotine; $^{\#}P < 0.001$ vs. 0 μg/ml nicotine.

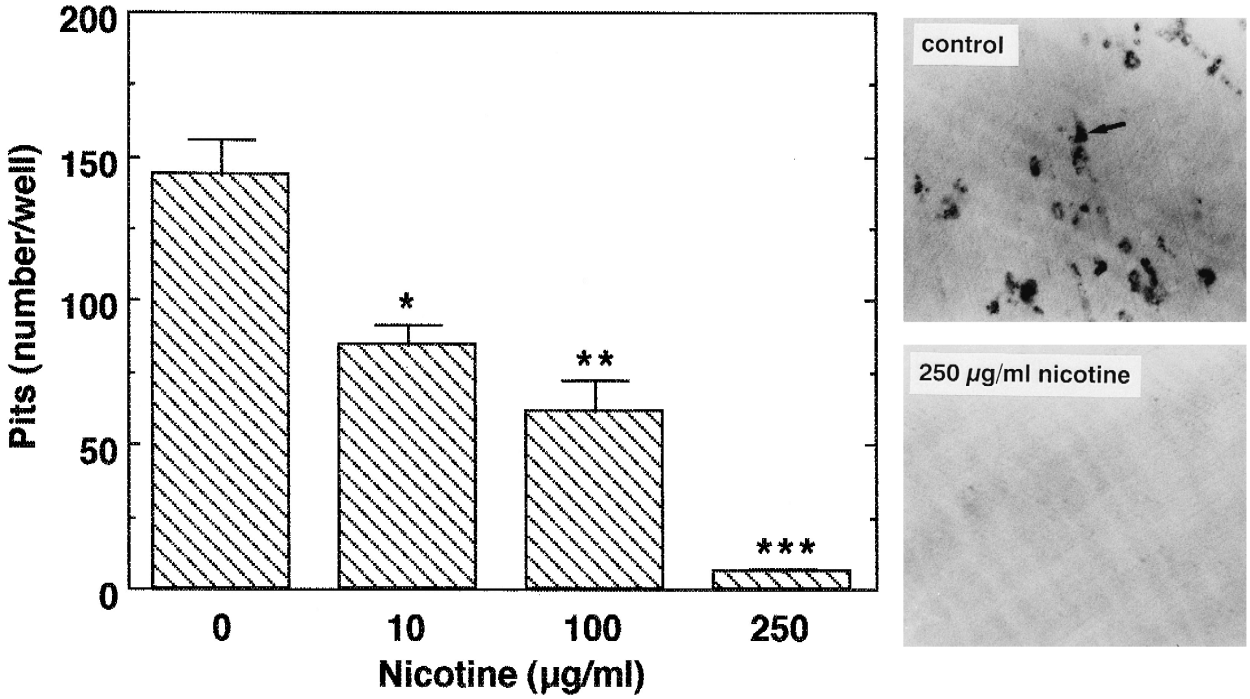

Fig. 4. Effects of nicotine on formation of pits in slices of dentine by mouse osteoclast-like cells. Mouse osteoclast-like cells were cultured in 96-well plates (0.48 cm^2/well) in the presence of ST2 cells (8×10^3 cells/well), and a slice of dentine was placed in each well. Nicotine was supplied to the osteoclast-like cells on each dentine slice at the concentrations indicated. After culture for 48 h, adherent cells were removed from the slices of dentine, and resorption pits formed on the surface of the dentine were stained with Mayer's hematoxylin. Arrow shows the pits on dentine slices. Bar, 330 μm. Values represent the means ± S.D. of results from six wells. Data are representative of results from three separate experiments that yielded similar results. $^{*}P < 0.05$ vs. 0 μg/ml nicotine; $^{**}P < 0.01$ vs. 0 μg/ml nicotine; $^{***}P < 0.001$ vs. 0 μg/ml nicotine.

In ROB-C26 cells, nicotine increased alkaline phosphatase activity in a dose-dependent manner (Fig. 1A) and increased the deposition of Ca^{2+} similarly (Fig. 2A). By contrast, nicotine decreased both activities in MC3T3-E1 cells (Figs. 1B and 2B). Thus, nicotine had different effects on the differentiation and mineralization of ROB-C26 and MC3T3-E1 cells. Ramp et al. (1991) reported that nicotine inhibits the synthesis of collagen and the alkaline phosphatase activity in osteoblast-like cells derived from embryonic chick calvariae . Fang et al. (1991) reported that nicotine stimulates alkaline phosphatase activity in rat osteoblastic osteosarcoma cells, UMR 106-01. The differences among the effects of nicotine on osteoblast metabolism in vitro might reflect differences in conditions for cell culture, differences among species, and the type of osteoblast model used including the stages of differentiation. A nicotinic receptor has been reported to mediate the expression of mRNA for neuronal nitric oxide synthase via the activation of a pathway that involves Ca^{2+} ions and protein kinase C (Nakamura et al., 1998). We (Otsuka et al., 1998) and other investigators (Hikiji et al., 1997; Kanematsu et al., 1997) have shown that nitric oxide influences the differentiation of osteoblast-like cells: nitric oxide increases the rate of differentiation of ROB cells (Otsuka et al., 1998) and decreases that of MC3T3-E1 cells (Kanematsu et al., 1997). The differences in the effects of nicotine in osteoblastic cells might in some way be associated with differences in nitric oxide-related pathways. Although the mechanism of action of nicotine in osteoblasts remains unclear, we have detected nicotine-binding sites on ROB-C26 cells (data not shown). Nicotine is thought to cause depolarization of the cell membrane after binding to its receptor and then to activate voltage-dependent Ca^{2+} channels that allow Ca^{2+} ions to enter the cell (Afar et al., 1994; Benowitz, 1996). Recently, voltage-dependent Ca^{2+} channel antagonist was reported to increase the alkaline phosphatase activity of osteoblastic cells (Kosaka and Uchii, 1998). Moreover, a Ca^{2+} signal has been implicated in the induction of osteoblastic differentiation (Takuwa et al., 1989; Eklou-Kalonji et al., 1998; Hiruma et al., 1998). These observations suggest that the effects of nicotine on osteoblastic metabolism might be mediated, at least in part, by changes in levels of Ca^{2+} ions in cells.

Nicotine inhibited the formation of MNCs (Fig. 3) and the resorption of bone by osteoclast-like cells (Fig. 4). There are recent reports that osteoclast differentiation factor and macrophage colony-stimulating factor, synthesized by mesenchymal cells (e.g. ST2 cells), are important factors in the induction of the formation of and bone resorption by osteoclast-like cells (Lacey et al., 1998; Yasuda et al., 1998). Our preliminary examination indicated that nicotine did not affect the levels of expression of mRNAs for osteoclast differentiation factor and macrophage

colony-stimulating factor in ST2 cells. These suggest the direct effects of nicotine on osteoclasts. The mechanism for effects of nicotine on osteoclasts is unclear, but there are some reports that nitric oxide inhibits the formation of TRAP-positive multinucleated osteoclasts (MacIntyre et al., 1991; Chae et al., 1997; Holliday et al., 1997) and the osteoclastic resorption of bone (MacIntyre et al., 1991; Holliday et al., 1997). Therefore, the association of the nicotinic receptor with expression of nitric oxide synthase (Nakamura et al., 1998), nitric oxide-related pathways, might be involved in this mechanism. Tobacco smoking is known as a risk factor for osteoporosis and this connection suggests that osteoclastogenesis might be stimulated by tobacco smoking. By contrast, our results showed that nicotine by itself inhibited osteoclastogenesis in osteoclast-like cells in culture. In tobacco smokers, other components of tobacco, such as tar, nitric oxide, nitrosoamines, and quinolines, might act with nicotine on the metabolism of osteoclast to yield results different from those obtained in vitro. Experiments using smoking rats or mice or the direct injection of nicotine into bone might yield useful results in future studies.

5. Conclusion

It appears that nicotine regulates the differentiation and mineralization of osteoblastic cells and inhibits the differentiation and activation of osteoclast-like cells. These observations suggest a role for nicotine in modulating bone metabolism. Further studies are needed to elucidate the contribution of nicotine to bone metabolism in vivo.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, and by grants from the Smoking Research Foundation.

References

Afar, R., Trifaro, J., Quik, M., 1994. Nicotine-induced intracellular calcium changes are not antagonized by α-bungarotoxin in adrenal medullary cells. Brain Res. 641, 127–131.

Aloia, J.F., Chon, S.H., Vaswani, A., Yeh, J.K., Yuen, K., Ellis, K., 1985. Risk factors for postmenopausal osteoporosis. Am. J. Med. 78, 95–100.

Benowitz, N.L., 1996. Pharmacology of nicotine: addiction and therapeutics. Annu. Rev. Pharmacol. Toxicol. 36, 597–613.

Broulik, P.D., Jarab, J., 1993. The effect of chronic nicotine administration on bone mineral content in mice. Horm. Metab. Res. 25, 219–221.

Chae, H.-J., Park, R.-K., Chung, H.-T., Kang, J.-S., Kim, M.-S., Choi, D.-Y., Bang, B.-G., Kim, H.-R., 1997. Nitric oxide is a regulator of bone remodelling. J. Pharm. Pharmacol. 49, 897–902.

Christen, A.G., Armstrong, W.R., McDaniel, R.K., 1979. Intraoral leukoplakias, abrasion, periodontal breakdown, and tooth loss in a snuff dipper. J. Am. Dent. Assoc. 98, 584–586.

Daniell, H.W., 1983. Postmenopausal tooth loss. Contributions to edentulism by osteoporosis and cigarette smoking. Arch. Intern. Med. 143, 1678–1682.

Eklou-Kalonji, E., Denis, I., Lieberherr, M., Pointillart, A., 1998. Effects of extracellular calcium on the proliferation and differentiation of porcine osteoblasts in vitro. Cell Tissue Res. 292, 163–171.

Fang, M.A., Frost, P.J., Iida-klein, A., Hahn, T.J., 1991. Effects of nicotine on cellular function in UMR 106-01 osteoblast-like cells. Bone 12, 283–286.

Hagiwara, H., Inoue, A., Yamaguchi, A., Yokose, S., Furuya, M., Tanaka, S., Hirose, S., 1996. cGMP produced in response to ANP and CNP regulates proliferation and differentiation of osteoblastic cells. Am. J. Physiol. 270, C1311–C1318.

Hikiji, H., Shin, W.S., Oida, S., Takato, T., Koizumi, T., Toyo-oka, T., 1997. Direct action of nitric oxide on osteoblastic differentiation. FEBS Lett. 410, 238–242.

Hiruma, Y., Inoue, A., Shiohama, A., Otsuka, E., Hirose, S., Yamaguchi, A., Hagiwara, H., 1998. Endothelins inhibit the mineralization of osteoblastic MC3T3-E1 cells through the A-type endothelin receptor. Am. J. Physiol. 275, R1099–R1105.

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

Holliday, L.S., Dean, A.D., Lin, R.H., Greenwald, J.E., Gluck, S.L., 1997. Low NO concentrations inhibit osteoclast formation in mouse marrow cultures by cGMP-dependent mechanism. Am. J. Physiol. 272, F283–F291.

Kanematsu, M., Ikeda, K., Yamada, Y., 1997. Interaction between nitric oxide synthase and cyclooxygenase pathways in osteoblastic MC3T3-E1 cells. J. Bone Miner. Res. 12, 1789–1796.

Kosaka, N., Uchii, M., 1998. Effect of benidipine hydrochloride, a dihydropyridine-type calcium antagonist, on the function of mouse osteoblastic cells. Calcif. Tissue Int. 62, 554–556.

Lacey, D.L., Timms, E., Tan, H.-L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y.-X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., Boyle, W.J., 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93, 165–176.

Looker, A.C., Flegal, K.M., Mussolino, M.E., 1998. Cigarette smoking reduces femoral bone density in both men and women. Bone 23, S291.

MacIntyre, I., Zaidi, M., Alam, A.S.M.T., Datta, H.K., Moonga, B.S., Lidbury, P.S., Hecker, M., Vane, J.R., 1991. Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. Proc. Natl. Acad. Sci. U.S.A. 88, 2936–2940.

Nakamura, K., Takahashi, T., Taniuchi, M., Hsu, C.X., Owyang, C., 1998. Nicotinic receptor mediates nitric oxide synthase expression in the rat gastric myenteric plexus. J. Clin. Invest. 101, 1479–1489.

Otsuka, E., Hirano, K., Matsushita, S., Inoue, A., Hirose, S., Yamaguchi, A., Hagiwara, H., 1998. Effects of nitric oxide from exogenous nitric oxide donors on osteoblastic metabolism. Eur. J. Pharmacol. 349, 345–350.

Ramp, W.K., Lenz, L.G., Galvin, R.J.S., 1991. Nicotine inhibits collagen synthesis and alkaline phosphatase activity, but stimulates DNA synthesis in osteoblast-like cells. Proc. Soc. Exp. Biol. Med. 197, 36–43.

Riggs, B.L., 1987. Pathogenesis of osteoporosis. Am. J. Obstet. Gynecol. 156, 1342–1346.

Seeman, E., Melton, L.J., O'Fallon, Giggs, B.L., 1983. Risk factors for spinal osteoporosis in men. Am. J. Med. 75, 977–983.

Stevenson, J.C., Lees, B., Devenport, M., Cust, M.P., Ganger, K.F., 1989. Determinants of bone density in normal women: risk factors for future osteoporosis? Br. Med. J. 298, 924–928.

Suda, T., Nakamura, I., Jimi, E., Takahashi, N., 1997. Regulation of osteoclast function. J. Bone Miner. Res. 12, 869–879.

Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., Kasai, S., 1983. In

vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J. Cell Biol. 96, 191–198.

Takahashi, N., Yamana, H., Yoshiki, S., Roodman, G.D., Mundy, G.R., Jones, S.J., Boyde, A., Suda, T., 1988. Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology 122, 1373–1382.

Takuwa, Y., Ohue, Y., Takuwa, N., Yamashita, K., 1989. Endothelin-1 activates phospholipase C and mobilizes Ca^{2+} from extra- and intracellular pools in osteoblastic cells. Am. J. Physiol. 257, E797–E803.

Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T.J., Suda, T., 1990. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proc. Natl. Acad. Sci. U.S.A. 87, 7260–7264.

Yamaguchi, A., Kahn, A.J., 1991. Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif. Tissue Int. 49, 221–225.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N., Suda, T., 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and identical to TRANCE/RANKL. Proc. Natl. Acad. Sci. U.S.A. 95, 3597–3602.