ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(12): 874–882 **CN** 31-1940/Q

Nicotine Treatment Induces Expression of Matrix Metalloproteinases in Human Osteoblastic Saos-2 Cells

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Abstract Tobacco smoking is an important risk factor for the development of severe periodontitis. Recently, we showed that nicotine affected mineralized nodule formation, and that nicotine and lipopolysaccharide stimulated the formation of osteoclast-like cells by increasing production of macrophage colony-stimulating factor (M-CSF) and prostaglandin E_2 (PGE_2) by human osteoblastic Saos-2 cells. In the present study, we examined the effects of nicotine on the expression of matrix metalloproteinases (*MMPs*), tissue inhibitors of matrix metalloproteinases (*TIMPs*), the plasminogen activation system including the component of tissue-type plasminogen activator (*tPA*), urokinase-type PA (*uPA*), and PA inhibitor type 1 (*PAI*-1), α7 nicotine receptor, and c-*fos*. We also examined the effect of the nicotine antagonist D-tubocurarine on nicotine-induced expression of *MMP*-1. Gene expression was examined using real-time polymerase chain reaction (PCR) to estimate mRNA levels. In addition, expression of the *MMP*, *TIMP*, *uPA*, *tPA*, and *PAI*-1 proteins was determined by Western blotting analysis. Nicotine treatment caused expression of *MMP*-1, 2, 3, and 13, but not *MMP*-14, to increase significantly after 5 or 10 d of culture; *MMP*-14 expression did not change through day 14. Enhancement of *MMP*-1 expression by nicotine treatment was eliminated by simultaneous treatment with D-tubocurarine. In the presence of nicotine, expression of *uPA*, *PAI*-1, or *TIMP*-1, 2, 3, or 4 did not change over 14 d of culture, whereas expression of *tPA* increased significantly by day 7. Nicotine also increased expression of the α7 nicotine receptor and c-*fos* genes. These results suggest that nicotine stimulates bone matrix turnover by increasing production of *tPA* and *MMP*-1, 2, 3, and 13, thereby tipping the balance between bone matrix formation and resorption toward the latter process.

Key words nicotine; matrix metalloproteinase; tissue inhibitor of matrix metalloproteinase; plasminogen activation system

Use of combustible tobacco may be the most important preventable risk factor for accelerated periodontal bone loss [1]. Direct heat from the cigarette, the vasoactive response to nicotine, or one of the many tobacco smoke by-products may be responsible for this fact, but the mechanism by which smoking induces periodontal attachment loss is presently unknown [2–4]. Nicotine has been shown to have detrimental effects on periodontal cells in a variety of ways. In an *in vitro* study, nicotine inhibited growth of gingival fibroblasts and production of fibronectin and collagen, and promoted collagen breakdown. Nicotine-exposed cells exhibited decreased growth and protein content, damaged cell membranes, atypical shapes, and vacuoles [5]. However, the pathogenesis of this process at the molecular level in osteoblasts remains unclear.

Bone resorption involves the simultaneous removal of

Received: July 12, 2006 Accepted: October 15, 2006
This study was supported by the grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology to promote multi-disciplinary research projects and the Sato Fund of the Nihon University School of Dentistry and by the Promotion and Mutual Aid Corporation for Private Schools of Japan
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DOI: 10.1111/j.1745-7270.2006.00240.x

both inorganic and organic constituents of bone. Dissolution of the mineral phase occurs under the ruffled border membrane of osteoclasts in an acid environment created by the vacuolar-type ATPase, which pumps protons [6]. The proteolytic mechanisms responsible for degrading the organic matrix of bone are less well understood, but may involve lysosomal cysteine proteinases, matrix metalloproteinases (MMPs), or serine proteinases.

The MMPs comprise a family of structurally and functionally related proteolytic enzymes that degrade extracellular matrix components such as collagens, elastin, glycoproteins, proteoglycans, and glucosaminoglycans [7]. At least 20 distinct members of the MMP family have been identified thus far, and they have been classified into four subgroups based on substrate specificity [8]. These subgroups are the collagenases (MMP-1, 8, and 13), gelatinases (MMP-2 and 9), membrane-type MMPs (MMP-14, 15, 16, and 17), stromelysins (MMP-3 and 10) and stromelysin-like MMPs (MMP-7, 11, and 12). Conversely, tissue inhibitors of matrix metalloproteinases (TIMPs) are secreted as endogenous inhibitors that down-regulate MMP activity. Four mammalian TIMPs (TIMP-1, 2, 3, and 4) have been cloned and analyzed for their primary structure and function [9,10]. In the plasminogen/plasmin pathway, generation of plasmin from the precursor zymogen, plasminogen, is induced by two plasminogen activators (PAs), tissue-type PA (tPA) and urokinase-type PA (uPA), and is inhibited by PA inhibitor type-1 (PAI-1) [11,12].

Based on the reports that smoking influences the onset and progress of periodontitis [13,14], we hypothesized that alveolar bone resorption is greater in smokers than that in non-smokers under the same conditions. This hypothesis is consistent with our previous findings that nicotine inhibits mineralized nodule formation by osteoblasts [15] and that nicotine and lipopolysaccharide stimulate the formation of osteoclast-like cells by increasing production of macrophage colony-stimulating factor (M-CSF) and prostaglandin E_2 (PGE_2) by osteoblasts [16]. However, the effects of nicotine on the proteolytic mechanisms responsible for degrading the organic matrix of bone are unclear. Therefore, we investigated the mechanism by which nicotine promotes osteoblast-mediated degradation of the organic constituents of bone by determining the effect of nicotine on expression of *MMP*-1, 2, 3, 13, and 14; *TIMP*-1, 2, 3, and 4; *tPA*; *uPA*; *PAI*-1; α7 nicotine receptor; and c-*fos* in human osteoblastic Saos-2 cells *in vitro*. We also examined the effect of the nicotine antagonist D-tubocurarine on nicotine-induced expression of *MMP*-1.

Materials and Methods

Cell culture

Saos-2 human osteosarcoma cells [17,18] were obtained from the RIKEN Bioresource Center (Tsukuba, Japan) and used as osteoblasts. The cells were maintained in a growth medium consisting of Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Rockville, USA) containing 10% (*V*/*V*) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, USA) and 1% (*V*/*V*) penicillin-streptomycin solution (Sigma Chemical, St. Louis, USA) at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2.

For experimental treatment, the cells were seeded onto 100-mm tissue-culture plates at a density of 5×10^6 cells/cm^2. After overnight incubation, the cells were cultured for up to 14 d with DMEM containing 10% FBS and 0, 10^{-5}, 10^{-4}, or 10^{-3} M nicotine (Wako Fine Chemicals, Osaka, Japan). These nicotine concentrations were chosen based on a previous report by Tipton and Dabbous [19], who examined the effect of nicotine on the proliferation of human gingival fibroblasts *in vitro* and on extracellular matrix production by these cells, and on our previous studies that examined the effect of 10^{-3} and 10^{-4} M nicotine on mineralized nodule formation by Saos-2 cells *in vitro* [15], and that examined the effect of 10^{-3} M nicotine and 1 or 10 μg/ml lipopolysaccharide on the formation of osteoclast-like cells by increasing M-CSF and PGE_2 production [16].

The effect of the nicotine antagonist D-tubocurarine (Wako Fine Chemicals) on nicotine-induced changes in *MMP*-1 expression was examined by adding 10^{-3} M D-tubocurarine to the culture medium, as described in previous reports [20]. The cells were cultured with or without 10^{-3} M nicotine for up to 14 d.

Real-time PCR

Cells were plated on six-well microplates at a density of 6×10^3 cells/cm^2 and cultured with 0, 10^{-5}, 10^{-4}, or 10^{-3} M nicotine for up to 14 d. Total RNA was isolated from the cultured cells on days 3, 5, 7, 10, and 14 using an RNeasy mini kit (Qiagen, Valencia, USA). The mRNA was converted into complementary DNA (cDNA) using an RNA PCR kit (GeneAmp, Perkin-Elmer, Branchburg, USA), and the resulting cDNA mixture was diluted five-fold in sterile distilled water.

Two microliters of the diluted cDNA were subjected to real-time PCR using SYBR Green I dye. The reactions were performed in 25 μl of an SYBR® premixed *Ex Taq*™

solution (TaKaRa, Shiga, Japan) containing 20 μM sense and antisense primers (shown in **Table 1**). The primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, USA). The PCR assays were performed on a Smart Cycler (Cepheid, Sunnyvale, USA) and were analyzed using Smart Cycler software (version 1.2d). The PCR protocol for *MMP*-1, 2, and 3, *TIMP*-3, *tPA*, *PAI*-1, α7 nicotine receptor, and c-*fos* consisted of 40 cycles of two steps (95 ºC for 3 s and 60 ºC for 20 s). For *MMP*-13 and 14, *TIMP*-1, 2, and 4, and *uPA*, the protocol consisted of 40 cycles of three steps (95 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s). All real-time PCR reactions were carried out in triplicate, and the specificity of the PCR products was verified by melting curve analysis. Gene expression levels were normalized by dividing the resulting mRNA values by the value for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA isolated at the same time.

SDS-PAGE and Western blotting

After nicotine treatment, cells were cultured in the absence of nicotine and FBS for an additional 24 h. The culture medium was then collected, dialyzed using a PD-10 column (Amersham Biosciences, Piscataway, USA), and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gels (8.3 cm×6.5 cm×0.75 mm) with a discontinuous Tris-glycine buffer system [21]. Media samples containing 20 μg of extracellular protein were dissolved in 10 μl of sample buffer containing 1% SDS, 2 M urea, 15 mg/ml dithiothreitol, and bromophenol blue, and heated at 95 ºC for 5 min before loading onto the gel. Gels were run at 150 V for 60 min.

Gel-separated proteins were transferred to a membrane using a semi-dry electrotransfer unit with a continuous buffer system consisting of 39 mM glycine, 48 mM Tris, 0.0375% SDS, and 20% (*V*/*V*) methanol at a constant amperage of 0.8 mA/cm^2 for 60–90 min. On completion of the transfer, the transfer membrane was treated with 25% (*V*/*V*) blocking reagent in Tris-buffered saline (TBS) (10 mM Tris, 145 mM NaCl, pH 7.4) at 4 ºC for 18 h. The sheet was washed in TBS containing Tween-20 (TBS-Tween) and then incubated at room temperature for 90 min with rabbit polyclonal IgG antibodies (all from Santa Cruz Biotechnology, Santa Cruz, USA) against MMP-1, 2, 3 or 13, tPA (Santa Cruz), or anti-β-tubulin (internal standard) that were diluted 1:200 in distilled water containing 10% (*V*/*V*) blocking reagent. The membranes were washed in TBS-Tween and incubated at room temperature for 60 min with appropriate biotin-conjugated secondary antibodies that were diluted 1:10,000 in distilled water containing 10% blocking agent. The membranes were then washed in TBS-Tween and phosphate-buffered saline (PBS, Nissui Pharmaceutical, Tokyo, Japan) and

Table 1 Polymerase chain reaction (PCR) primers used in the experiments

Target	GenBank accession No.	Forward primer (5′→3′)	Reverse primer (5′→3′)
MMP-1	NM_002421	AGCTTGGCCACTCGCTCGGTCTG	GTCTCGGGATGCATGCTCGATTGC
MMP-2	NM_004530	GCCGCCTTTAACTGGAGCAA	TTCCAGGCATCTGCGATGAG
MMP-3	NM_002422	ATTCCATGGAGCCAGGCTTTC	CATTTGGGTCAAACTCCAACTGTG
MMP-13	NM_002427	TCCCAGGAATTGGTGATAAAGTAGA	CTGGCATGACGCGAACAATA
MMP-14	NM_004995	AGCCCACCCATTGAAGTCTCC	AATCTAGCCGAACTGCCAGCAC
TIMP-1	NM_003254	CAGCGTTCTGAGATCAAGATGACCA	AGTGATGTGCAAGAGTCCATCCTG
TIMP-2	NM_003255	GCGGTCAGTGAGAAGGAAGTGGA	GAGGAGGGGGCCGTGTAGATAAAC
TIMP-3	NM_000362	AGGCAAGCAGCTAGACTGGTGAA	AACTGGATGGGCAGCAGGAC
TIMP-4	NM_003256	AGGACCTGTCCTTGGTGCAGA	GCCGTCAACATGCTTCATACAGA
tPA	NM_000930	GTCAGATTCCAGTCAGTGTG	GTTGCTCGTGATGGTTTTG
uPA	NM_002658	GCTGCTGACCCACAGTGGAA	TCGTGTAGACGCCTGGCTTG
PAI-1	NM_000602	GACAATGGAAGAGCAACATG	ACCTCGATCTTGACCTTTTG
c-*fos*	NM_005252	CAGCGAGCAACTGAGAAGCC	CGCTGTGAAGCAGAGCTGG
α7-nicotine receptor	NM_000746	CTCTACTATGGCCTCAACCTGCTG	GATGTTGCGGGCATGATCTC
GAPDH	NM_002046	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *MMP*, matrix metalloproteinase; *PAI*-1, plasminogen activator type 1; *TIMP*, tissue inhibitors of matrix metalloproteinase; *tPA*, tissue-type plasminogen activator; *uPA*, urokinase-type plasminogen activator.

then incubated for 30 min at room temperature with horseradish peroxidase-conjugated streptavidin diluted with PBS. Immunoreactive proteins were visualized using a commercial chemiluminescence kit (Amersham Life Science, Buckinghamshire, UK) and autoradiography with X-ray film (Eastman Kodak, New Haven, USA). As a control, membranes were exposed to diluted normal rabbit serum; the dilution factor was the same as that used for the primary antibodies. Pre-stained molecular weight standards that were run on the same gel and transferred were used to estimate protein size.

Statistical analysis

All experiments were performed in triplicate. Each value represents the mean±SD. The significance of differences was determined using Bonferroni's modification of Student's *t*-test. Differences with $P<0.05$ were considered significant.

Results

Effect of nicotine on expression of *MMP* genes

We used real-time PCR to measure expression of the genes encoding *MMP*-1, 2, 3, 13 and 14 in Saos-2 cells cultured for up to 14 days with 0, 10^{-5}, 10^{-4} or 10^{-3} M nicotine (**Fig. 1**). In the absence of nicotine, levels of mRNAs encoding *MMP*-1, 3 and 13 increased gradually through 14 days of culture. The amount of *MMP*-2 mRNA increased gradually through day 10, but then decreased on day 14, and the amount of *MMP*-14 mRNA did not change through day 10 of culture and then increased slightly on day 14.

When Saos-2 cells were cultured with 10^{-5} to 10^{-3} M nicotine, the amount of *MMP*-1 mRNA was significantly higher than that in control cells on day 5, 7, 10 and 14. At 10^{-4} or 10^{-3} M nicotine, mRNA levels for *MMP*-2 and 3 were also significantly higher than in the control cells. This difference was observed on day 5, 7 and 14 for both *MMP*-2 and 3, and also on day 10 for *MMP*-2. For *MMP*-1, 2 and 3, the increased expression was most evident at 10^{-3} M nicotine on day 14. At 10^{-3} M nicotine, the level of *MMP*-13 mRNA expression was significantly higher than that in control cells on day 10 and 14, and this difference was most pronounced on day 14. Nicotine did not appear to affect expression of *MMP*-14 mRNA.

Effect of nicotine on expression of *TIMP* genes

We also assessed the effect of nicotine treatment on the

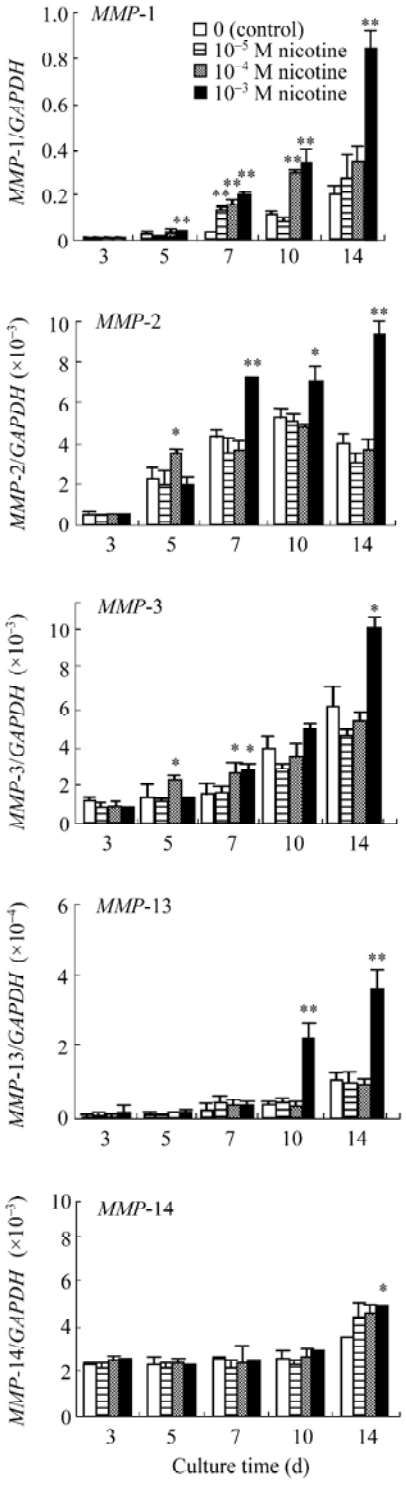

Fig. 1 Effect of nicotine on matrix metalloproteinases (*MMPs*) gene expression

Saos-2 cells were cultured with 0 (control), 10^{-5}, 10^{-4} or 10^{-3} M nicotine for up to 14 d. Expression of the genes encoding *MMP*-1, 2, 3, 13 and 14 was determined by real-time polymerase chain reaction (PCR) on day 3, 5, 7, 10 and 14. Each bar indicates the mean±SD of three separate experiments; * $P<0.05$, ** $P<0.01$, nicotine treatment versus control. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

expression of the genes encoding *TIMP*-1, 2, 3 and 4 in Saos-2 cells. The cells were cultured for up to 14 d with 0, 10^{-5}, 10^{-4} or 10^{-3} M nicotine, and levels of *TIMP* mRNA were analyzed by real-time PCR (**Fig. 2**).

In the absence of nicotine, the amount of *TIMP*-1 mRNA increased gradually through day 10 of culture and decreased slightly on day 14, and the amount of *TIMP*-2 mRNA increased gradually through day 7 of culture and decreased slightly on day 10. The amount of *TIMP*-3 mRNA increased gradually through day 7 of culture and decreased on day 10. In contrast, the amount of *TIMP*-4 mRNA decreased gradually through day 10 of culture and increased slightly on day 14.

Nicotine treatment did not appear to affect expression of *TIMP*-1, 2, 3 or 4 compared to that in control cells through day 14 of culture.

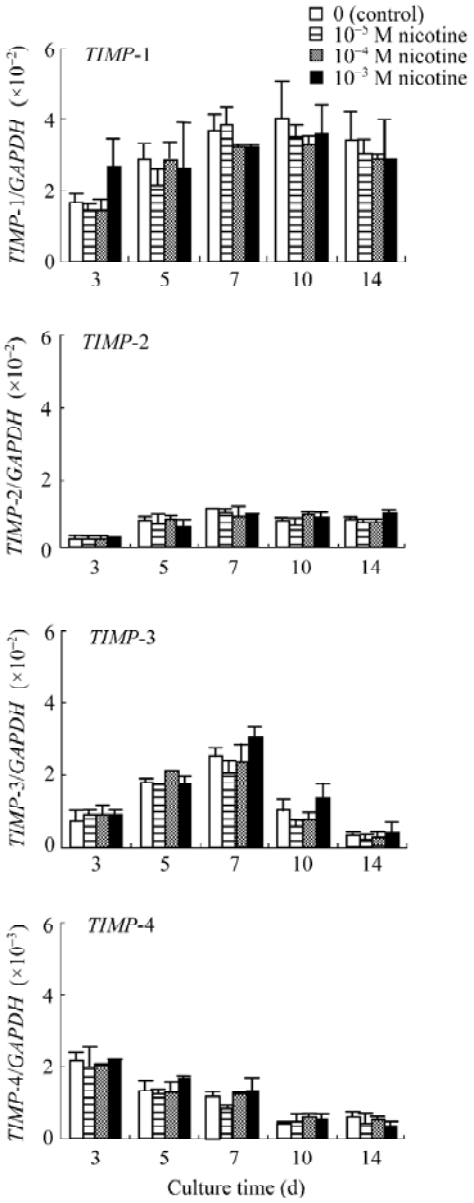

Fig. 2 Effect of nicotine on tissue inhibitors of matrix metalloproteinases (*TIMPs*) gene expression

Saos-2 cells were cultured with 0 (control), 10^{-5}, 10^{-4}, or 10^{-3} M nicotine for up to 14 d. Expression of the genes encoding *TIMP*-1, 2, 3, and 4 was determined by real-time polymerase chain reaction (PCR) on day 3, 5, 7, 10, and 14. Each bar indicates the mean±SD of three separate experiments. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Effect of nicotine on expression of the *tPA*, *uPA*, and *PAI*-1 genes

Expression of the *tPA*, *uPA*, and *PAI*-1 genes was assessed similarly, using real-time PCR (**Fig. 3**). Levels of *tPA* mRNA increased gradually through 5 days of culture

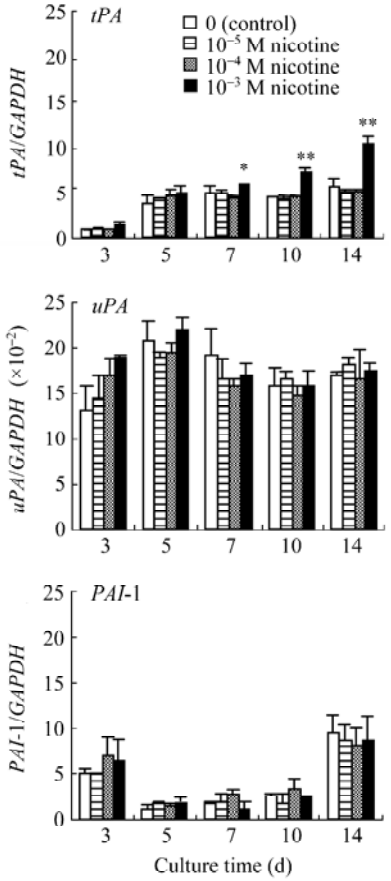

Fig. 3 Effect of nicotine on expression of plasminogen activator (*PA*) and PA inhibitor type 1 (*PAI*-1) genes

Saos-2 cells were cultured with 0 (control), 10^{-5}, 10^{-4}, or 10^{-3} M nicotine for up to 14 d. Expression of the genes encoding *tPA*, *uPA*, and *PAI*-1 was determined by real-time polymerase chain reaction (PCR) on day 3, 5, 7, 10, and 14. Each bar indicates the mean±SD of three separate experiments; * $P<0.05$, ** $P<0.01$, nicotine treatment versus control. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

in the absence of nicotine and then remained constant from day 7 to 14. In cells treated with 10^{-3} M nicotine, expression of *tPA* mRNA occurred at a significantly higher level than that in control cells on day 7, 10, and 14, and this difference was most pronounced on day 14.

In the absence of nicotine, the amount of *uPA* mRNA increased gradually through day 5 of culture and decreased slightly on day 7, and the amount of *PAI*-1 mRNA increased gradually from day 5 to 14. Nicotine treatment did not affect the amount of *uPA* or *PAI*-1 mRNA compared with that in control cells through day 14 of culture.

Effect of nicotine on the expression of MMP and tPA proteins

We also examined the effect of nicotine on the expression of tPA and MMP proteins in Saos-2 cells. The cells were cultured for up to 14 days in the presence or absence of 10^{-3} M nicotine, and protein levels of tPA and MMP-1, 2, 3, and 13 in the culture media were determined by Western blotting. On day 14 of culture, levels of all five proteins were markedly higher in cells cultured in the presence of nicotine than in the control cells, which is consistent with our results demonstrating that nicotine increases mRNA levels for these proteins (**Fig. 4**).

Effect of nicotine on expression of the α7 nicotine receptor and c-*fos* genes

Expression of the α7 nicotine receptor and c-*fos* genes in Saos-2 cells cultured for up to 3 d in the presence or absence of 10^{-3} M nicotine was determined by real-time PCR. On day 3 of culture, levels of the α7 nicotine receptor and c-*fos* mRNAs were significantly higher in nicotine-treated cells than in the control cells (**Fig. 5**).

Effect of D-tubocurarine on nicotine-induced expression of the *MMP*-1 gene

The effect of D-tubocurarine on nicotine-induced expression of the *MMP*-1 gene in Saos-2 cells was examined by real-time PCR. Cells were cultured in the presence of 10^{-3} M nicotine, 10^{-3} M D-tubocurarine, or both, for up to 14 days. In the absence of D-tubocurarine, nicotine treatment caused the amount of *MMP*-1 mRNA to increase on day 14 of culture, but simultaneous treatment with D-tubocurarine eliminated this effect on day 14 (**Fig. 6**).

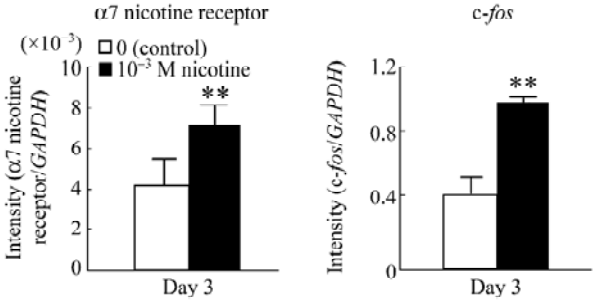

Fig. 5 Effect of nicotine on the expression of the α7 nicotine receptor and c-*fos* genes

Saos-2 cells were cultured in the presence or absence of 10^{-3} M nicotine, and levels of the α7 nicotine receptor and c-*fos* mRNAs were determined by real-time polymerase chain reaction (PCR) on day 3. Each bar indicates the mean±SD of three separate experiments; $^{**}P<0.01$, nicotine treatment versus control. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

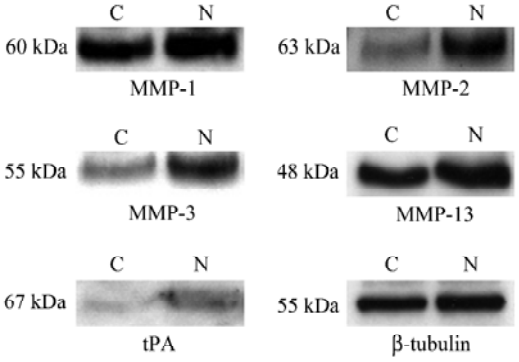

Fig. 4 Effect of nicotine on levels of matrix metalloproteinase (MMP) and tissue-type plasminogen activator (tPA) proteins

Saos-2 cells were cultured in the presence or absence of 10^{-3} M nicotine. Levels of MMP-1, 2, 3, and 14, tPA, and anti-β-tubulin proteins was determined by Western blotting analysis on day 14 of culture. C, control; N, nicotine-treated.

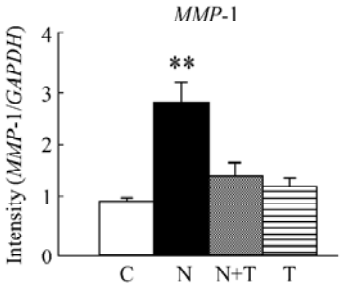

Fig. 6 Effect of D-tubocurarine on nicotine-induced expression of the matrix metalloproteinase (*MMP*)-1 gene

Saos-2 cells were cultured with 10^{-3} M nicotine (N), 10^{-3} M nicotine and 10^{-3} M D-tubocurarine (N+T), or 10^{-3} M D-tubocurarine (T). Expression of the *MMP*-1 gene was determined by real-time polymerase chain reaction (PCR) on day 14. Each bar indicates the mean±SD of three separate experiments; $^{**}P<0.01$, nicotine treatment versus control (C). *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Discussion

Several studies *in vitro* and *in vivo* have demonstrated that tobacco smoking is an important risk factor for the development of severe inflammatory periodontal disease. Furthermore, the degree of alveolar bone loss in smokers is reported to be greater than that in nonsmokers [22]. We previously showed that nicotine affects mineralized nodule formation in a simulated mouth environment [15], and that nicotine and lipopolysaccharide stimulate the formation of osteoclast-like cells by increasing production of M-CSF and PGE_2 by human osteoblastic Saos-2 cells [16].

Because the effect of nicotine on the proteolytic mechanisms responsible for the degradation of the organic matrix of bone is not well understood, we examined the effect of nicotine on the expression of *MMP*-1, 2, 3, 13 and 14 in Saos-2 cells, which we had used previously [15,16,23–26]. All five of these MMPs are abundant in osteoblasts [27,28]. We also examined expression of *TIMP*-1, 2, 3 and 4, *uPA*, *tPA* and *PAI*-1. We chose a treatment period of 14 d, based on our previous research [15,16], which examined the effects of nicotine on bone formation and the formation of osteoclast-like cells by Saos-2 cells. In determining the experimental concentration of nicotine, we assumed that nicotine directly stimulates the gingival epithelium; thus, we chose to use concentrations from 10^{-5} to 10^{-3} M. Our previous studies had already shown that 10^{-3} M nicotine does not kill Saos-2 cells [15,16].

Type I collagen is a major organic constituent of the extracellular matrix of bone. Of the non-collagenous bone matrix proteins, the major proteins include the chondroitin sulfate-type small proteoglycans, bone sialoprotein, osteopontin, osteocalcin, and SPARC/osteonectin [29]. MMP-1 and 13 cleave type I collagen, the most abundant protein present in the osteoid layer, into characteristic 3:1 length fragments, which subsequently denature into random-coil polypeptide chains at physiological temperature [30]. Digestion of type I collagen by MMP-1 and 13 thus appears to be the initial step in the bone resorption process. Subsequently, denatured collagen fragments can be degraded further by MMP-2. MMP-13 has broad substrate specificity [31] and may cleave other bone matrix proteins such as proteoglycans, glycoproteins, and native type IV and IX collagens in the osteoid layer.

Posttranslational regulation of MMP activity depends on the interactions between MMPs and inhibitory TIMPs [30]. MMPs are secreted as latent, inactive proenzymes that are activated by the sequential cleavage of their N-terminal propeptide by other proteinases. Pro-MMP-13 is reportedly activated by MMP-2 and 14 [32], as well as by MMP-3 and plasmin [30]. In a reaction dependent on TIMP-2 binding, the membrane gelatinase MMP-14 activates pro-MMP-2 and pro-MMP-13 to produce MMP-2 and MMP-13 derivatives of decreasing molecular size, increasing molar enzymatic activity, and reduced the efficiency of binding to tissue inhibitors [33]. TIMP-3, unlike TIMP-1 and 2, binds to the extracellular matrix [34], and TIMP-4, the most recently discovered member of the TIMP family, blocks the activities of several MMPs implicated in arthritic cartilage erosion [35].

In light of these findings, we examined the effect of nicotine on the expression of *MMPs* and *TIMPs* in Saos-2 cells. Expression of *MMP*-1, 2, 3 and 13 increased significantly after day 5 or 10 of culture in the presence of nicotine, whereas nicotine did not affect *MMP*-14 expression over 14 days in culture. Nicotine induction of *MMP*-1 expression was blocked by simultaneous treatment with D-tubocurarine, showing that the effect was nicotine-specific. In contrast to the generally enhancing effect of nicotine on *MMP* expression, nicotine had no effect on the expression of *TIMPs* over 14 days in culture. Similar results were obtained using another osteoblastic cell line, the rat clonal line ROS 17/2.8 [36] (data not shown). These results indicate that nicotine may tip the balance between the actions of MMPs and TIMPs toward the breakdown of bone matrix proteins.

In the plasminogen/plasmin system, the inactive proenzyme plasminogen is converted to the proteolytic enzyme plasmin by two physiological PAs, tPA and uPA [11]. Plasmin is a broad-spectrum serine protease that can degrade several noncollagenous components of the extracellular matrix, including fibronectin, laminin, and the core protein of proteoglycans [37]. In addition, plasmin has been reported to cleave osteocalcin and release it from hydroxyapatite [38]. Plasmin may also participate indirectly in collagen breakdown by activating latent MMPs [39, 40].

Fibrinolysis may be inhibited at the plasmin level (primarily by α2-antiplasmin) or at the PA level. Inhibition of PAs is mediated by specific PAIs, principally PAI-1 [41]. Removal of fibrin from the vascular tree is believed to be carried out primarily by tPA, owing to the high specific affinity of tPA for fibrin [11]. On the other hand, uPA binds to a cellular receptor and may participate in pericellular proteolysis by degrading matrix components or by activating latent proteinases or growth factors [12]. Taken together, these data suggest that plasmin modulates degradation of the osteoid layer covering the bone surface; since it exposes resorption-stimulating minerals to the osteoclasts, this degradation may initiate the bone

resorption process [37,42].

Given these findings, we also examined the effect of nicotine on *PA* and *PAI*-1 expression. In nicotine-treated Saos-2 cells, expression of *tPA* increased significantly after 7 days of culture, whereas expression of *uPA* and *PAI*-1 did not change through 14 days of culture, suggesting that nicotine stimulates tPA production in osteoblasts. Furthermore, nicotine-induced *tPA* expression may activate pro-MMPs by increasing production of plasmin, which degrades noncollagenous matrix proteins in the bone extracellular matrix.

Confirmation that the observed changes in mRNA and protein expression were specific to nicotine stimulation is provided by two sets of results. First, we found that nicotine treatment caused a significant increase in expression of the genes encoding the $\alpha 2$ nicotine receptor [43] and the c-*fos* transcription factor [20]. Second, the nicotine antagonist D-tubocurarine [20] blocked the enhancement of *MMP*-1 gene expression by nicotine. Taken together, the results of this study suggest that nicotine stimulates turnover of the bone matrix by increasing production of *MMP*-1, 2, 3 and 13 and *tPA* in osteoblasts, which tips the balance between bone production and resorption toward the latter process. Degradation of bone matrix proteins by these enzymes in osteoblasts may promote the resorption of alveolar bone in the mouth of smokers.

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