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Up-regulation of osteolytic mediators in human osteosarcoma cells stimulated with nicotine

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Background and Objective: Cigarette smoking is a major risk factor in the development and further progression of periodontal diseases. However, little is known about how nicotine influences the expression of osteolytic mediators in cigarette smoking-associated periodontal diseases. The aim of this study was to investigate the expression of interleukin-1, interleukin-8, receptor activator of nuclear factor-κB ligand (RANKL), gelatinases and tissue-type plasminogen activator in U2OS cells (from the human osteosarcoma cell line) stimulated with nicotine.

Material and Methods: Differences in the expression of interleukin-1, interleukin-8 and RANKL mRNAs, in response to exposure to various concentrations of nicotine (0, 0.125, 0.25, 0.5 and 1 mM) were evaluated in U2OS cells using the reverse transcripttion–polymerase chain reaction.In addition, the levels of interleukin-1, interleukin-8 and RANKL proteins were determined using enzyme-linked immunosorbent assays. The gelatinolytic and caseinolytic activities in nicotine treated-U2OS cells were demonstrated using gelatin and casein zymography, respectively.

Results: Nicotine was found to increase the expression of interleukin-1, interleukin-8 and RANKL mRNA and protein in U2OS cells ($p < 0.05$). The gelatin zymograms revealed that matrix metalloproteinase (MMP)-2 and MMP-9 were secreted by U2OS cells. The secretion of MMP-2 and MMP-9 occurred in a dose-dependent manner that was dependent on the concentration of nicotine ($p < 0.05$). Casein zymography exhibited a caseinolytic band with a molecular weight of 70 kDa, indicative of the presence of tissue-type plasminogen activator. Tissue-type plasminogen activator was also found to be up-regulated by nicotine in a dose-dependent manner ($p < 0.05$).

Conclusion: Taken together, the results of the present study indicated that smoking modulation of bone destruction in periodontal disease may involve various osteolytic mediators, such as interleukin-1, interleukin-8, RANKL, MMP-2, MMP-9, and tissue-type plasminogen activator.

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Cigarette smoking is associated with an increased incidence of destructive periodontal disease that leads to increased alveolar bone loss (1,2). However, little is known regarding the pathogenesis of smoking-associated periodontal diseases. It is likely that nicotine, a major component of cigarette smoke, influences the immunological surveillance or defense mechanisms. Indeed, nicotine has been reported to be present on the root surface (3) and has been detected in the saliva and gingival crevicular fluid of smokers (4). Our studies have shown

that nicotine is cytotoxic to human periodontal ligament fibroblasts through inhibiting cell viability and attachment via intracellular thiol depletion (5,6). Nicotine has also been found to induce c-*fos* in human periodontal ligament fibroblasts (7), to induce cyclooxygenase-2 (8,9), and heme oxygenase-1 (10) in human gingival fibroblasts, and to induce extracellular signal-regulated protein kinase in human osteosarcoma cells (11). However, little is known about how nicotine influences the expression of osteolytic mediators in cigarette smoking-associated periodontal diseases.

Pro-inflammatory cytokines are believed to contribute to the pathogenesis of periodontal disease. They are important mediators of cell function and make significant contributions to inflammatory responses. These cytokines alter the expression of proteolytic enzymes, such as matrix metalloproteinases (MMPs) and plasminogen activators, which are believed to be important in periodontal disease progression and tissue breakdown.

Interleukin-1 has a central role in the regulation of immunological and inflammatory reactions. The biological activity of interleukin-1 molecules seems to be directly relevant to periodontal destruction, such as periodontal attachment loss, destruction of collagen and bone resorption (12). Interleukin-8 is a chemokine produced by a variety of tissue and blood cells and is a potent inducer of neutrophil chemotaxis and activation (13). The amounts of interleukin-8 have been shown to be higher in gingival fibroblasts derived from periodontal disease sites than in cells from healthy sites (14).

A recently identified tumor necrosis factor family molecule, receptor activator of nuclear factor-κB ligand (RANKL), plays a critical role in the development of osteoclasts that result in bone resorption (15). Recently, Lu *et al.* (16) reported that the levels of RANKL were prominent in gingival crevicular fluid and gingival tissues from chronic periodontitis lesions.

MMP-2 and MMP-9, sometimes referred to as type IV collagenase and gelatinase, respectively, are members of the family of MMPs and are thought to play an important role in the degradation of extracellular components. They are of particular interest because they have been implicated in the pathogenesis of periodontitis (17,18). Plasminogen activator-generated plasmin is an activator of several MMPs (19) and might participate in the degradation of extracellular bone matrix (20). Previous studies have suggested that plasminogen activators could play an important role in periodontal inflammatory processes (21,22).

The exact mechanism and sequence of events associated with the activation of osteoclastic activity to produce bone destruction in cigarette smoking-associated periodontal diseases has not been fully elucidated. Osteoblasts are responsible for the synthesis and degradation of the extracellular matrix and thus play a major role in maintaining the health and integrity of the alveolar bone. The aim of this study was to investigate the expression of the osteolytic mediators interleukin-1, interleukin-8, RANKL, gelatinases and tissue-type plasminogen activator in cells of the human osteosarcoma cell line, U2OS, stimulated with nicotine.

Material and methods

Cell culture

U2OS cells (American Tissue Type Collection HTB 96; ATCC, Manassas, VA, USA), derived from human osteogenic sarcoma, were cultured in Dulbecco's modified Eagle's minimal essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), 100 μg/mL of streptomycin and 100 mg/mL of penicillin at 37°C in a humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every third day.

Treatment

Cells arrested in G0 by serum deprivation (0.5% fetal calf serum; 48 h) were used in the experiments. Prior to treatment, the cells were washed with serum-free Dulbecco's modified Eagle's minimal essential medium and exposed immediately to various concentrations (0, 0.125, 0.25, 0.5 and 1 mM) of nicotine (Sigma, St Louis, MO, USA) for the indicated incubation times. The viability of cells exposed to these elutes were, in general, cytoatatic to U2OS cells during a 24 h culture period, as assessed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (11). Total RNA was isolated at the end of 6 h. Samples of the conditioned medium were collected for zymography after 24 h of incubation.

Reverse transcription–polymerase chain reaction

Total RNA was prepared using TRIzol reagent (Gibco), according to the manufacturer's instructions. The concentration of RNA solution was quantified by spectrophotometry with an absorbance wavelength of 260 nm, and the purity was assessed by the optical density $(OD)_{260}/OD_{280}$ ratio. Complementary DNA was synthesized from RNA in a 15 μL reaction mixture containing 100 mg of random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco). The reaction mixture was diluted with 20 μL of water, and 3 μL of the diluted reaction mixture was used for polymerase chain reaction (PCR). The PCR mixture contained 10 pmol of forward and reverse primers and 2 units of Taq DNA polymerase. Amplification was performed for 25 cycles for glyceraldehyde-3-phosphate dehydrogenase and for 30 cycles for interleukin-1, interleukin-8, or RANKL, in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C and 1 min of extension at 72°C. The sequences of primers used were as follows. Glyceraldehyde-3-phosphate dehydrogenase: forward 5′-TCCTCTGACTTCAACAGCGACACC-3′, reverse 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′; interleukin-1: forward 5′-GAGAGCATGGTGGTAGTAGCAACC-3′, reverse: 5′-CCCTGCCAAGCACACCCAGTAGTC-3′; inter-

leukin-8: forward 5′-CGATGTCAGTGCATAAAGACA-3′, reverse 5′-TGAATTCTCAGCCCTCTTCAAAAA-3′; and RANKL: forward 5′-GCTTGAAGCTCAGCCTTTTGCTCAT-3′, reverse 5′-GGGGTTGGAGACCTCGATGCTGATT-3′.

When the cells were probed for interleukin-1, interleukin-8 and RANKL mRNA production by reverse transcription (RT)-PCR, a 564 bp band for interleukin-1, a 225 bp band for interleukin-8 and a 557 bp band for RANKL were noted. These bands were consistent with the size as designed by primers. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with glyceraldehyde-3-phosphate dehydrogenase mRNA was quantified by the photographed gels using a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± standard deviation.

Enzyme-linked immunosorbent assay

It is known that mRNA expression does not always lead to protein production. The levels of interleukin-1, interleukin-8 and RANKL protein were determined using enzyme-linked immunosorbent assays (ELISAs). Briefly, 20 μL of conditioned medium was directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed according to the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer, and interleukin-1, interleukin-8 and RANKL levels were determined by extrapolation from a calibration curve using human interleukin-1 (BD Biosciences, San Diego, CA, USA), interleukin-8 (BD Biosciences) and RANKL (R&D Systems, Minneapolis, MN, USA) as standards, respectively. The amount of RANKL was expressed as pg/mL of protein. Each value was expressed as the mean ± standard deviation.

Zymography

The activity of MMP-2 and of MMP-9 in conditioned medium was measured using gelatin-zymogram protease assays, as described previously (23). Conditioned media were prepared with standard sodium dodecyl sulfate gel-loading buffer, which contained 0.01% sodium dodecyl sulfate but no beta-mercaptoethanol and was not boiled before loading. Then, prepared samples were subjected to electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gels containing 0.1% gelatin. Electrophoresis was performed at 150 V for 3 h in an OWL P-1 apparatus (Thermo Fisher Scientific, Inc., Portsmouth, NH, USA). After electrophoresis, gels were washed twice with 100 mL of distilled water, containing 2% Triton X-100, on a gyratory shaker for 30 min at room temperature to remove the sodium dodecyl sulfate. The gel was then incubated in 100 mL of reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$, 0.02% NaN_3) for 12 h at 37°C, stained with Coomassie brilliant blue R-250 and then destained with methanol–acetic acid water. The gelatin cleavage rate was analyzed from the photographed gels using a densitometer (AlphaImager 2000; Alpha Innotech). Each densitometric value was expressed as the mean ± standard deviation.

Visualization of tissue-type plasminogen activator activity was performed as described by Chang *et al.* (24). Briefly, 2% (w/v) casein and 20 mg/mL of plasminogen were added to 8% sodium dodecyl sulfate–polyacrylamide gels. Samples with a total protein content of about 20 mg were then loaded onto the gels. The tissue-type plasminogen activator activity of cells treated or untreated with nicotine was measured as described for gelatin zymography. The intensities of the bands obtained were determined using a densitometer (AlphaImager 2000; Alpha Innotech). Each densitometric value was expressed as the mean ± standard deviation.

Statistical analysis

Experiments were performed in triplicate throughout this study. All assays were repeated three times to ensure reproducibility. The results obtained from control and treated groups were statistically analyzed for significance using the paired Student's *t*-test.

Results

To examine the effect of nicotine on the expression of pro-inflammatory cytokines, U2OS cells were treated with various concentrations of nicotine. As shown in Fig. 1A, nicotine was found to increase the level of interleukin-1 in a dose-dependent manner ($p < 0.05$). From the AlphaImager 2000 analyses, the levels of the interleukin-1 mRNAs were increased by about 1.3-, 1.4-,

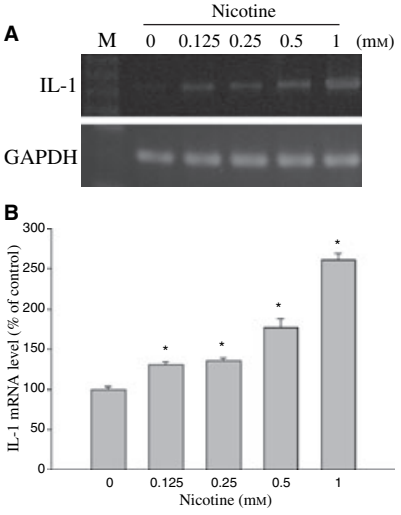

Fig. 1. (A) Analysis, by reverse transcription–polymerase chain reaction, of expression of the interleukin-1 mRNA gene in U2OS cells upon stimulation with various concentrations of nicotine. A DNA ladder (M) of known base pairs was used for identification of the PCR products. To monitor equal RNA loading, the glyceraldehyde-3-phosphate dehydrogenase gene was included as a control. (B) Following treatment with different concentrations of nicotine, interleukin-1 mRNA gene levels were measured using the AlphaImager 2000. The relative level of interleukin-1 mRNA gene expression was normalized against the glyceraldehyde-3-phosphate dehydrogenase mRNA signal and the control was set as 1.0. Optical density values represent the mean ± standard deviation. The asterisk (*) represents a significant difference from the control (p-value < 0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1, interleukin-1.

1.8- and 2.6-fold after exposure to 0.125, 0.25, 0.5 and 1 mM nicotine, respectively (Fig. 1B). Similar results were also found for the nicotine-induced expression of interleukin-8 ($p < 0.05$). The AlphaImager 2000 results showed that the levels of the interleukin-8 mRNAs were increased by about 1.5-, 1.3-, 1.6- and 2.1-fold after exposure to 0.125, 0.25, 0.5 and 1 mM nicotine, respectively (Fig. 2B).

As shown in Fig. 3A, nicotine stimulated an increase in expression of RANKL mRNA in a dose-dependent manner ($p < 0.05$). The AlphaImager 2000 results showed that the levels of RANKL mRNAs increased by about 2.0-, 2.6-, 2.9- and 3.4-fold after exposure to 0.125, 0.25, 0.5 and 1 mM nicotine, respectively (Fig. 3B).

In addition, the RT-PCR results were confirmed by ELISA. Similar patterns were seen by ELISA. As shown in Table 1, nicotine was found to stimulate the production of inteleukin-1, interleukin-8 and RANKL proteins ($p < 0.05$).

Specific characterization of MMPs in the conditioned medium using gelatin zymography demonstrated that the 72 kDa MMP-2 and the 92 kDa MMP-9 were released by U2OS cells (Fig. 4A). Secretion of MMP-2 and MMP-9 was time-dependent during a 24 h culture period upon treatment with various concentrations of nicotine ($p < 0.05$). The results obtained from the AlphaImager 2000 (Fig. 4B) showed that the levels of MMP-2 increased by about 1.3-, 1.5-, 1.6- and 1.5-fold after exposure to 0.125, 0.25, 0.5 and 1 mM nicotine, respectively. The levels of MMP-9 increased by about 1.5-, 2.0-, 2.2- and 2.1-fold after exposure to 0.125, 0.25, 0.5 and 1 mM nicotine, respectively.

Casein zymography exhibited a caseinolytic band with a molecular weight of approximately 70 kDa, indicative of the presence of tissue-type plasminogen activator (Fig. 5A). Nicotine was found to up-regulate the production of tissue-type plasminogen activator in U2OS cells ($p < 0.05$). Data obtained from the AlphaImager 2000 showed that the levels of MMP-2 increased by about 1.2-, 2.1-, 5.3- and 8.4-fold after exposure to 0.125, 0.25, 0.5 and 1 mM nicotine, respectively (Fig. 5B).

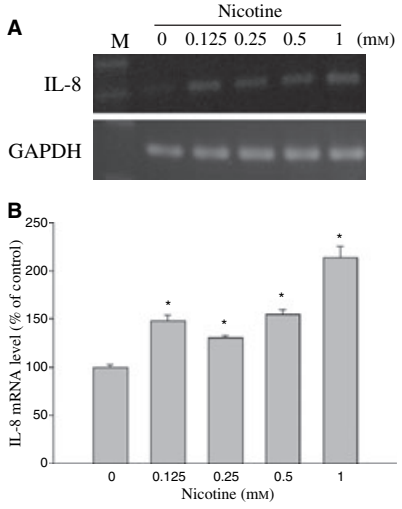

Fig. 2. (A) Analysis, by reverse transcription–polymerase chain reaction, of expression of the interleukin-8 mRNA gene in U2OS cells upon stimulation with various concentrations of nicotine. A DNA ladder (M) of known base pairs was used for identification of PCR products. To monitor equal RNA loading, the glyceraldehyde-3-phosphate dehydrogenase gene was included as a control. (B) Following treatment with different concentrations of nicotine, interleukin-8 mRNA gene levels were measured using the AlphaImager 2000. The relative level of interleukin-8 mRNA gene expression was normalized against the glyceraldehyde-3-phosphate dehydrogenase mRNA signal and the control was set as 1.0. Optical density values represent the mean ± standard deviation. The asterisk (*) represents a significant difference from the control (p-value < 0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-8, interleukin-8.

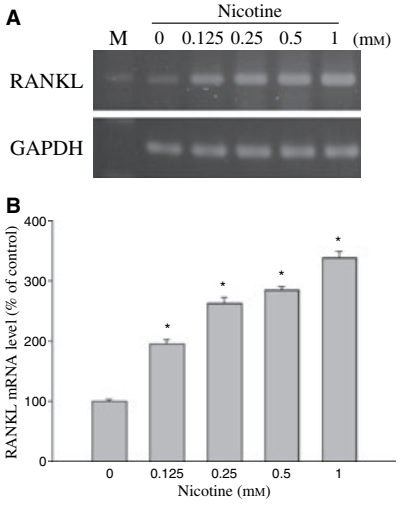

Fig. 3. (A) Analysis, by reverse transcription–polymerase chain reaction, of expression of the RANKL mRNA gene in U2OS cells upon stimulation with various concentrations of nicotine. A DNA ladder (M) of known base pairs was used for identification of PCR products. The glyceraldehyde-3-phosphate dehydrogenase gene was included as a control to monitor equal RNA loading. (B) Following treatment with different concentrations of nicotine, RANKL mRNA gene levels were measured using the AlphaImager 2000. The relative level of receptor activator of nuclear factor-κB ligand (RANKL) mRNA gene expression was normalized against the glyceraldehyde-3-phosphate dehydrogenase mRNA signal and the control was set as 1.0. Optical density values represent the mean ± standard deviation. The asterisk (*) represents a significant difference from the control (p-value < 0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

Periodontal disease is an oral disease characterized by the destruction of the tissues supporting the teeth, in which the bone surrounding the teeth (namely alveolar bone) is frequently involved. The detrimental effect of smoking on alveolar bone is reflected by results showing that the risk of more severe alveolar bone loss is greater in smokers than in nonsmokers, ranging from 3.25 to 7.28 for light and heavy smokers, respectively (25). Within 20 years, the marginal bone loss in smokers is 50% more than in nonsmokers (26).

Osteoblasts are the principal cells responsible for alveolar bone. The human osteosarcoma cell line, U2OS, used in this study, has been used as a model for osteoblasts because these cells express the osteoblast phenotype and have been widely used in dental research (11,23,24,27). Recently, the U2OS cell line was found to play an important role in the recruitment of immune cells and to contribute to the breakdown of alveolar bone (27). Osteoblasts not only provide structural support but may also function as

Table 1. Summary of interleukin-1, interleukin-8 and receptor activator of nuclear factor-κB ligand (RANKL) protein levels in U2OS cells following stimulation with different concentrations of nicotine

Nicotine (mM)	0	0.125	0.25	0.5	1
Interleukin-1 (pg/mL)	1.29 ± 0.07	1.50 ± 0.11*	1.63 ± 0.06*	1.63 ± 0.14*	1.88 ± 0.42*
Interleukin-8 (pg/mL)	74.22 ± 9.34	87.65 ± 13.53	97.61 ± 5.66*	101.11 ± 3.31*	127.08 ± 9.68*
RANKL (pg/mL)	117.48 ± 7.53	118.66 ± 11.50	145.53 ± 5.27*	177.48 ± 15.34*	202.42 ± 22.12*

The results were obtained using enzyme-linked immunosorbent assays.
*Significant differences from control values; $p < 0.05$.
Note: according to the manufacturer's instruction manuals, the minimum detectable concentrations of interleukin-1, interleukin-8 and RANKL are 0.8, 0.8, and 1.5 pg/mL, respectively.

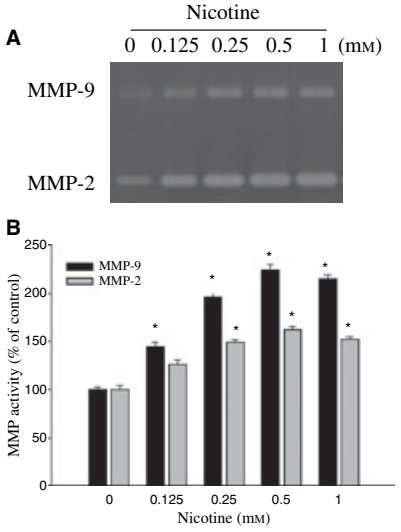

Fig. 4. (A) Gelatin zymogram of conditioned medium from U2OS cells following treatment with various concentrations of nicotine for 24 h of culture. (B) The levels of matrix metalloproteinase (MMP)-2 and MMP-9 from conditioned medium treated with nicotine were calculated from their gelatinolytic activity, as measured using the AlphaImager 2000. The asterisk (*) denotes a significant difference from the control (p-value < 0.05).

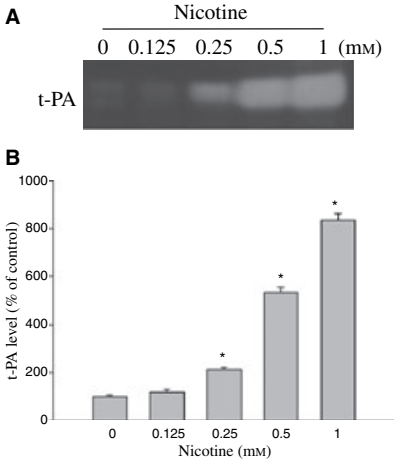

Fig. 5. (A) Casein zymogram of conditioned medium from U2OS cells following treatment with various concentrations of nicotine for 24 h of culture. (B) Levels of tissue-type plasminogen activator from conditioned medium treated with nicotine were calculated from their tissue-type plasminogen activator activity, as measured using the AlphaImager 2000. The asterisk (*) denotes a significant difference from the control (p-value < 0.05). t-PA, tissue-type plasminogen activator.

accessory immune cells and play an important role in the initial inflammatory reaction as well as in the amplification of the immune response.

In this study, nicotine was found to induce the release of interleukin-1 and interleukin-8 mRNA and protein from U2OS cells. Our results were in agreement with previous studies that nicotine can up-regulate interleukin-1 in human macrophages (28) and interleukin-8 in human gingival fibroblasts (29,30). Consistently, the concentration of interleukin-1 was higher in smokers than in nonsmokers (31). These results demonstrated that nicotine itself can stimulate the production of interleukin-1 and interleukin-8 in osteoblast cultures. Taken together, interleukin-1 and interleukin-8 release may play an important role in the pathogenesis of cigarette smoking-associated alveolar bone loss.

In the present study, nicotine was first found to increase the concentrations of RANKL mRNA and protein in U2OS cells. Recently, the RANKL gene was reported to be expressed more highly in smokers with periodontitis than in healthy controls (32). Thus, the increase of the RANKL mRNA and protein, favoring bone resorption, was promoted by smoking. Taken together, these results show a possible modulation, by smoking, of the mRNA levels of RANKL in alveolar bone destruction.

Moreover, nicotine was found to elevate the secretion of MMP-2 and of MMP-9 in U2OS cells. Our results were in agreement with previous studies that nicotine can increase MMPs in endothelial cells (33,34) and in human gingival fibroblasts (32,35). These results indicate that the up-regulation of MMP expression by nicotine is not cell type-specific. Nicotine may increase cell-mediated collagen degradation, in part through the activation of MMPs. At sites of inflammation, the plasmin-dependent pathway for activation of MMP is considered to be a significant mechanism for the induction of matrix degradation (36). To the best of our knowledge, nicotine was first found to stimulate tissue-type plasminogen activator secretion in U2OS cells. Taken together, the activation of proteolytic enzymes might play an important role in the pathogenesis of nicotine-induced bone destruction.

Interleukin-1 is known to modulate RANKL expression in periodontal ligament cells (37). Our previous studies also reported that MMP-9 and tissue-type plasminogen activator were up-regulated by interleukin-1 in U2OS cells (23,24). The interaction among pro-inflammatory cytokines, MMP-9, and tissue-type plasminogen activator is worthy of further investigation.

Cigarette smoking is recognized as the most important environmental risk factor in periodontitis and as a major risk factor that contributes to the pathogenesis of periodontal disease. It

would seem logical to expect that factors which are associated with tissue destruction should be higher in smokers than in nonsmokers. The biological basis for the effects of smoking on bone is not completely understood. Inhibition of oxidative metabolism and collagen synthesis have been reported as mechanisms for cigarette smoking associated-bone destruction (38,39). Our studies showed that another mechanism by which smoking may affect bone loss is through increasing the secretion of osteolytic mediators, such as interleukin-1, interleukin-8, RANKL, MMP-2, MMP-9 and tissue-type plasminogen activator.

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