

Nicotine increases the collagen-degrading ability of human gingival fibroblasts

J. Zhou, B. L. Olson, L. J. Windsor

Department of Oral Biology, Indiana University
School of Dentistry, Indianapolis, IN, USA

Zhou J, Olson BL, Windsor LJ. Nicotine increases the collagen-degrading ability of human gingival fibroblasts. *J Periodont Res* 2007; 42: 228–235. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard

Background and Objective: The objective of this study was to determine the effects that nicotine and the combination of nicotine and *Porphyromonas gingivalis* supernatant have on human gingival fibroblast-mediated collagen degradation.

Material and Methods: Human gingival fibroblasts were cultured with 25–500 µg/ml of nicotine in collagen-coated six-well plates. On days 1–5, the conditioned media was collected for zymography and western blot analyses of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). The cells were then removed and the collagen cleavage visualized by Coomassie blue staining. To examine the combined effect, 250 µg/ml of nicotine and 10% v/v culture supernatant of *P. gingivalis* ATCC 33277 were added to the human gingival fibroblasts. The mRNA levels of multiple MMPs and TIMPs were monitored.

Results: Nicotine increased the human gingival fibroblast-mediated collagen cleavage. The MMP-14 and MMP-2 produced by the nicotine-treated human gingival fibroblasts more readily underwent zymogen activation. Nicotine treatment resulted in TIMP-2 redistribution to the cell surface. The mRNAs of multiple MMPs and TIMPs were unaltered by nicotine. An additive collagen cleavage effect was observed when the human gingival fibroblasts were treated with both nicotine and *P. gingivalis*.

Conclusion: Nicotine increased human gingival fibroblast-mediated collagen degradation, in part through the activation of membrane-associated MMPs. Nicotine and *P. gingivalis* had an additive effect on human gingival fibroblast-mediated collagen degradation.

Jing Zhou, 1121 West Michigan Street,
DS Room 271, Indianapolis, IN 46202, USA
Tel: +1 317 2742126
Fax: +1 317 2781411
e-mail: jzhou2@iupui.edu

Key words: collagen-degrading ability; gingival fibroblasts; matrix metalloproteinases; nicotine

Accepted for publication July 18, 2006

Tobacco use has been recognized to be a significant risk factor for the development and progression of periodontal disease. The relative risk for periodontal disease in tobacco users is considerably higher than in nontobacco users (1). Tobacco use is associated with increased probing depths, loss of periodontal attachment, loss of alveolar bone and a higher rate of tooth loss (1). There are over 4000 harmful chemicals and gases in cigarette smoke

(2). Nicotine is a major component and the most pharmacologically active agent in tobacco. It is likely to be a major contributing factor to the initiation and/or exacerbation of periodontal disease.

Previous studies have demonstrated that nicotine affects gingival blood flow (3–5), cytokine production (6), and the functions of neutrophils and other immune cells (7–9). Nicotine has also been reported to affect connective

tissue cells, such as fibroblasts, in regard to cell morphology, attachment to substrates, and protein synthesis and secretion (10–12). Yet, limited information is available about how nicotine influences the collagen-degrading ability of gingival fibroblasts. Gingival fibroblasts are the major connective tissue cells in the gingiva (13). These cells 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 gingiva. The major extracellular matrix-degrading enzymes produced by these fibroblasts are the matrix metalloproteinases (MMPs). These enzymes are a group of zinc-dependent endopeptidases that include the collagenases, gelatinases, stromelysins, membrane-associated MMPs and other MMPs (14). The MMPs are regulated at the level of expression and activation, as well as by the tissue inhibitors of metalloproteinases (TIMPs) (14). Several studies have shown that nicotine can modulate the mRNA expression and enzymatic activity of MMPs (15–20).

Clinically, smoking is consistently associated with poor oral hygiene. Smokers usually have an increased accumulation of plaque and calculus (21). Interestingly, smoking does not increase the presence of periodontal pathogens (22). However, whether smoking enhances the detrimental effects of the periodontal pathogens is presently unknown. One of the major pathogens implicated in adult periodontitis is *Porphyromonas gingivalis*, a gram-negative, anaerobic, nonmotile and nonsporulating coccobacillus (23). *P. gingivalis* culture supernatant, which contains numerous virulence factors, has been reported to stimulate epithelial and fibroblast cells to degrade an underlying collagen Type I fibril bed (24–26) and to increase the expression and/or activation of certain MMPs from the host cells (26–29).

Therefore, the objective of this study was to examine the effects that nicotine and the combination of nicotine and *P. gingivalis* supernatant have on the collagen-degrading ability of human gingival fibroblasts. The expression of multiple MMPs and TIMPs were also monitored.

Material and methods

Cell culture and bacteria supernatant

Human gingival fibroblast cells were cultured from healthy gingival tissue explants, as described previously (26). The cells were cultured in low-glucose (1 g/l) Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA)

supplemented with 15% fetal bovine serum (Hyclone), 4 mM L-glutamine (Hyclone), 100 U/ml penicillin, 50 µg/ml gentamicin and 2.5 µg/ml fungizone (Invitrogen, Carlsbad, CA, USA). Cells at passages 3–12 were utilized in these experiments. Cells at these passages displayed normal growth rates, and there were no passage-dependent variations in the results.

P. gingivalis ATCC 33277 supernatant was obtained as a generous gift from Dr J. Katz (University of Alabama at Birmingham School of Dentistry, Birmingham, AL, USA). *P. gingivalis* were cultured in supplemented brain heart infusion growth medium, as described previously (26). The collected supernatant was filtered twice through 0.2-µm membranes and then stored at –20°C until utilized. A previous study (26) has shown that this supernatant can enhance human gingival fibroblast-mediated collagen degradation. Although bacterial cultures might also enhance this degradation, they introduce more variability into the system. Therefore, supernatant was utilized in these studies.

Collagen-degradation assays

Human gingival fibroblast-mediated collagen degradation was assessed with a reconstituted collagen Type I assay system, as described previously (26). Briefly, a thin layer of rat tail tendon Type I collagen was coated on six-well plates (450 µg of collagen per well). Human gingival fibroblasts at 90% confluence were collected and seeded as single colonies (50,000 cells per well) in the center of the collagen-coated wells. After the cells attached, 2 ml of serum-free Dulbecco's modified Eagle's medium containing nicotine (Sigma Chemical Co., St Louis, MO, USA) at different concentrations (0, 25, 50, 100, 150, 250 and 500 µg/ml) was added. After 4 d, the conditioned media from the human gingival fibroblasts was collected for zymography before the cells were removed with 500 µl of 0.1% Triton X-100 (Sigma Chemical Co.) and 200 µl of 0.25% trypsin (Invitrogen). The plates were then stained with Coomassie blue to

visualize collagen cleavage. In certain wells, an MMP inhibitor (GM6001, 100 nM; Chemicon, Temecula, CA, USA) was also included in the cell-mediated collagen-degradation assays.

A previous study demonstrated that 10% (v/v) *P. gingivalis* culture supernatant increases the collagen-degrading ability of human gingival fibroblasts (26). Nicotine at 250 µg/ml was demonstrated to be able to increase the human gingival fibroblast-mediated collagen degradation without obvious cell damage. Therefore, 10% (v/v) *P. gingivalis* culture supernatant and 250 µg/ml of nicotine were utilized to analyze the combined effect of *P. gingivalis* and nicotine on human gingival fibroblast-mediated collagen degradation. Human gingival fibroblasts were cultured in serum-free Dulbecco's modified Eagle's medium (C), in serum-free medium containing 10% (v/v) *P. gingivalis* supernatant (P), in serum-free medium containing 250 µg/ml of nicotine (N), and in serum-free medium containing both 10% (v/v) *P. gingivalis* supernatant and 250 µg/ml of nicotine (N + P). The combination of 150 µg/ml of nicotine and 10% (v/v) *P. gingivalis* supernatant was also examined. The human gingival fibroblasts were removed and the collagen degradation visualized with Coomassie blue staining on day 1 through to day 5. The experiments were repeated at least three times.

Zymography

After a 4-d experimental period, the conditioned media from the human gingival fibroblasts were collected for zymography, as described previously (26). Briefly, the human gingival fibroblast-conditioned media were mixed with nonreducing loading buffer (without α-mercaptoethanol) and resolved at 200 V in 10% sodium dodecyl sulfate polyacrylamide gels copolymerized with 1 mg/ml of gelatin. The gels were then washed before incubation in 1× Tris buffer (50 mM Tris, pH 7.5, 3 mM NaN₃, 5 mM CaCl₂, 1 µM ZnCl₂) at 37°C overnight. These gels were then stained with Coomassie blue to visualize the proteolytic bands.

Western blot analyses

Western blots were performed to examine the levels of MMP-1, MMP-2, MMP-3, MMP-14, TIMP-1 and TIMP-2 in conditioned media, as well as to examine the levels of MMP-2, MMP-14 and TIMP-2 in cell membrane extracts. Human gingival fibroblasts were cultured for 2 d, with or without nicotine (250 µg/ml), before the conditioned media were collected and concentrated 16-fold with Centrprep centrifugal filters (Millipore, Bedford, MA, USA). Cell membrane extracts were prepared utilizing the Mem-PER Mammalian Membrane Protein Extraction Reagent Kit (Pierce, Rockford, IL, USA). The concentrations of the total protein in the samples were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples with equal amounts of total protein were mixed with reducing loading dye and then denatured by boiling before being resolved in 10% sodium dodecyl sulfate polyacrylamide gels at 200 V. The proteins on the gels were transferred to nitrocellulose membranes at 0.3 A for 1 h in blotting buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 10% methanol). The membranes were then blocked with 5% milk for 1 h in phosphate-buffered saline containing 0.1% Tween-20 (pH 7.4) and then incubated overnight at 4°C with the following primary antibodies: monoclonal Ab-5 (5 µg/ml, Clone III12b; NeoMarkers, Fremont, CA, USA) for MMP-1, monoclonal Ab-2 (5 µg/ml, Clone VB3; NeoMarkers) for MMP-2, monoclonal Ab-2 (5 µg/ml, Clone IID4; NeoMarkers) for MMP-3, monoclonal Ab-2 (5 µg/ml, Clone IC3; NeoMarkers) for TIMP-2, polyclonal Ab 815 (0.3 µg/ml; Chemicon) for MMP-14, and polyclonal Ab 2315 (5 µg/ml; a gift from Dr K. Bodden, Daphne, AL, USA) for TIMP-1. After washing three times with phosphate-buffered saline (pH 7.4) containing 0.1% Tween-20, the membranes were incubated with antimouse or antirabbit secondary antibodies (Amersham, Piscataway, NJ, USA) for 1 h at room temperature. An ECL™ kit (Amer-

sham) was utilized to develop the membranes according to the manufacturer's protocol. Western blot analyses were repeated three to five times for each protein.

Reverse transcription-polymerase chain reaction

Total RNA was extracted according to the protocol of the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA) after culture of the human gingival fibroblasts, with or without nicotine (250 µg/ml), for 24 h. The mRNA and primers, as described previously (30), were mixed with the Qiagen OneStep RT-PCR mix (Qiagen), according to the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a Bio-Rad iCycler (Bio-Rad Laboratories). The RT was accomplished at 50°C for 30 min and terminated at 95°C for 15 min. PCR was then performed for 30 cycles with denaturation at 95°C, annealing at 50°C and extension at 72°C, for 1 min each. The PCR products were then resolved in 1% (w/v) agarose gels at 120 V and stained with ethidium bromide. The gels were photographed under ultraviolet light and then analyzed with NIH SCION IMAGE software (Version beta 4.03). The cyclophilin bands were utilized for standardization of the RT-PCR results. The relative expression levels between the nicotine-treated and untreated cells were then determined.

Cell viability assays

Cell viability assays were performed based on the cleavage of water-soluble tetrazolium by mitochondrial dehydrogenases in viable cells. After culturing the human gingival fibroblasts on collagen-coated plates with nicotine and/or *P. gingivalis* supernatant for 3 d, 10% water-soluble tetrazolium-1 (Roche Diagnostics, Mannheim, Germany) was added and incubated at 37°C for another 4 h. The medium was then collected and triplets of 100 µl/well of each sample were distributed in 96-well plates. The absorbance in the 96-well plates was then detected with an enzyme-linked immunosorbent

assay (ELISA) reader (Molecular Devices Co., Sunnyvale, CA, USA) at a wavelength of 450 nm. Absorbance values of each sample were compared with the untreated cell control by percentage and then the standard deviation was calculated. Means of the treatment groups ($n = 9$) were compared by one-way analysis of variance and Tukey's tests, at a significance level of 0.05. Statistical analyses of the data were performed utilizing SIGMASTAT software (version 3.0).

Results

Collagen degradation and zymography

Nicotine increased the collagen-degrading ability of the human gingival fibroblasts in a dose-dependent manner (Fig. 1A). The increase in collagen degradation was especially evident when the human gingival fibroblasts were treated with 150 and 250 µg/ml of nicotine (Fig. 1A). The collagen degradation decreased, as a result of cell death, when the human gingival fibroblasts were treated with 500 µg/ml of nicotine (data not shown). The MMP inhibitor, GM6001, completely blocked the collagen cleavage mediated by the human gingival fibroblasts treated with or without 150 µg/ml of nicotine (Fig. 1B).

Zymography of the conditioned media revealed that proMMP-2 (72 kDa) underwent activation more readily when the human gingival fibroblasts were treated with nicotine at 150 µg/ml or higher (Fig. 1C), as evident by the appearance of the activated form of MMP-2 (62 kDa).

Western blot

Western blot analyses were performed to monitor certain MMPs and TIMPs that were secreted into the media by human gingival fibroblasts which were either untreated or treated with 250 µg/ml of nicotine for 48 h. ProMMP-1 and proMMP-3 were detected in the conditioned media from the untreated control cells and were basically unaltered in the conditioned media from the nicotine-treated human

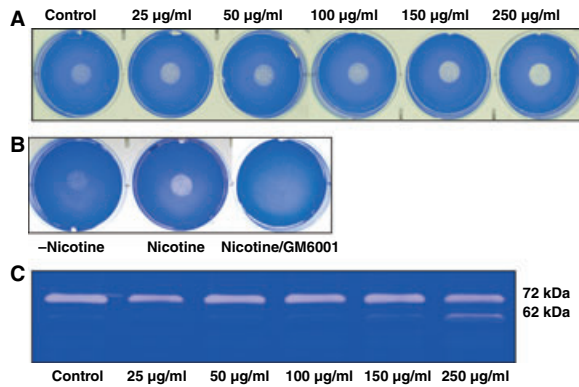


Fig. 1. Effect of nicotine on the collagen-degrading ability of human gingival fibroblasts. Human gingival fibroblasts were seeded as single colonies (50,000 cells/well) in six-well plates coated with Type I collagen. (A) Nicotine was added at different concentrations (25–250 µg/ml). On day 4, the human gingival fibroblasts were removed and the collagen cleavage visualized by Coomassie blue staining. (B) GM6001 inhibition of nicotine-stimulated collagen degradation by human gingival fibroblasts. –Nicotine, human gingival fibroblasts cultured in serum free media without nicotine. Nicotine, human gingival fibroblasts were cultured in media containing 150 µg/ml of nicotine. Nicotine + GM6001, human gingival fibroblasts were cultured in media containing nicotine (150 µg/ml) and GM6001 (100 nM). (C) Gelatin zymography of the human gingival fibroblast-conditioned media treated with/without nicotine (0–250 µg/ml) for 4 d.

gingival fibroblasts (Fig. 2). The TIMP-1 level was slightly decreased in the presence of nicotine (Fig. 2).

MMP-14, MMP-2 and TIMP-2 were detected in both the conditioned media and membrane extracts from the treated and the untreated human gingival fibroblasts (Fig. 3). Both the pro-enzymes and activated forms of MMP-14 and MMP-2 were detected in the conditioned media from the human gingival fibroblasts in the absence or

presence of nicotine. However, a lower-molecular-weight fragment of MMP-14 (43 kDa) was detected only in the membrane extracts of the nicotine-treated human gingival fibroblasts (Fig. 3). Also, a partially activated form of MMP-2 (68 kDa) was more prevalent in the membrane extracts of the nicotine-treated human gingival fibroblasts than in the untreated control (Fig. 3). TIMP-2, which plays a role in the MMP-2 activation mediated

by MMP-14, was increased in the membrane extracts and decreased in the conditioned media from the human gingival fibroblasts treated with 250 µg/ml of nicotine (Fig. 3).

RT-PCR

RT-PCR was performed on the RNA extracted from the human gingival fibroblasts cultured for 24 h in the absence and presence of 250 µg/ml of nicotine. The mRNAs for MMP-1, MMP-2, MMP-3, MMP-10, MMP-11, MMP-7, MMP-12, MMP-14, MMP-15, MMP-16, TIMP-1 and TIMP-2 were detected in both the untreated and treated human gingival fibroblast cells (Fig. 4). The mRNAs of the MMPs and TIMPs from the treated human gingival fibroblasts were basically unaltered when compared with those from the untreated human gingival fibroblasts (Table 1).

Combined effect of nicotine and *P. gingivalis* on cell-mediated collagen degradation

Compared with the untreated control, *P. gingivalis* supernatant increased the human gingival fibroblast cell-mediated collagen degradation, as evident by the fact that all the collagen in the wells was dissolved on day 5 (Fig. 5, panel P). The increase in collagen cleavage induced by nicotine was limited to the area of collagen underneath the human gingival fibroblast cell colonies (Fig. 5, panel N). The combined treatment of 10% *P. gingivalis* supernatant and 250 µg/ml of nicotine resulted in a collagen cleavage pattern that was additive (Fig. 5, panel N + P). The combined treatment of 10% *P. gingivalis* supernatant and 150 µg/ml of nicotine also resulted in an additive effect in regard to collagen cleavage (data not shown).

Cell viability tests

Compared with the untreated control, 91.68% and 70.36% of human gingival fibroblast cells were viable after 3 d of treatment with 10% *P. gingivalis* supernatant and 250 µg/ml of nicotine, respectively (Fig. 6). After treatment

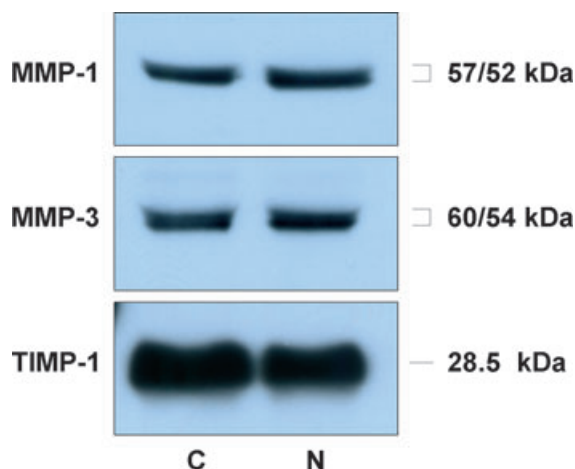


Fig. 2. Western blot analyses for matrix metalloproteinase (MMP)-1, MMP-3 and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the conditioned media from human gingival fibroblasts treated without (C) and with 250 µg/ml of nicotine (N) for 48 h.

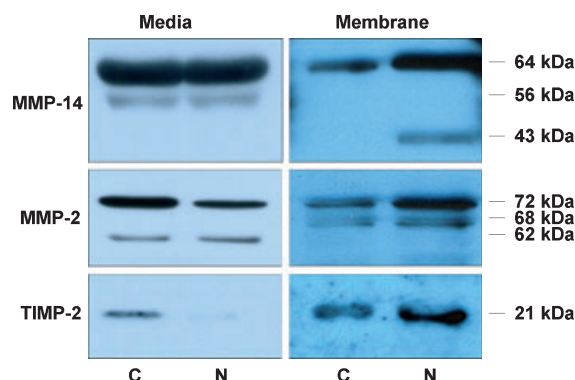


Fig. 3. Western blot analyses for matrix metalloproteinase (MMP)-14, MMP-2 and tissue inhibitor of metalloproteinase-2 (TIMP-2) in the conditioned media and membrane extracts from human gingival fibroblasts treated without (C) and with 250 µg/ml of nicotine (N) for 48 h.

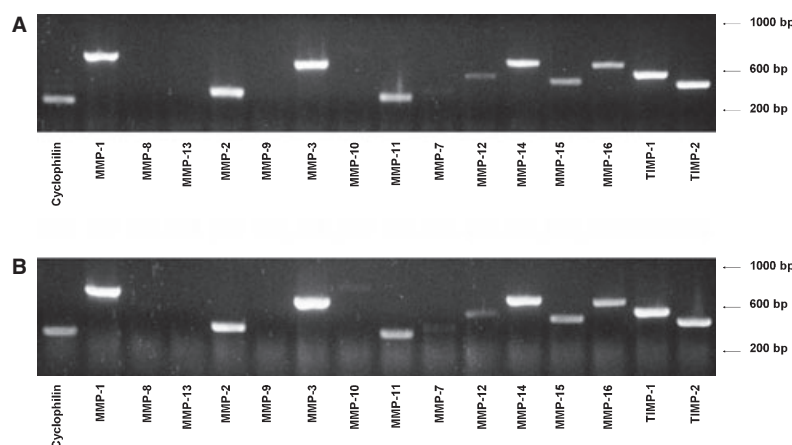


Fig. 4. Reverse transcription-polymerase chain reaction results of the RNA extracted from human gingival fibroblasts treated without (A) and with 250 µg/ml of nicotine (B) for 24 h.

Table 1. Reverse transcription-polymerase chain reaction results of the RNA extracted from the human gingival fibroblasts cultured in the absence and presence of nicotine (250 µg/ml)

	Treated/untreated ^a
Cyclophilin	1.00
MMP-1 (collagenase-1)	1.03 ± 0.055
MMP-8 (collagenase-2)	ND
MMP-13 (collagenase-3)	ND
MMP-2 (gelatinase-A)	0.95 ± 0.093
MMP-9 (gelatinase-B)	ND
MMP-3 (stromelysin-1)	1.06 ± 0.110
MMP-10 (stromelysin-2)	1.14 ± 0.298
MMP-11 (stromelysin-3)	0.96 ± 0.081
MMP-7 (matrilysin)	0.67 ± 0.635
MMP-12 (macrophage MMP)	1.01 ± 0.078
MMP-14 (MT MMP-1)	1.09 ± 0.150
MMP-15 (MT MMP-2)	1.02 ± 0.038
MMP-16 (MT MMP-3)	1.04 ± 0.073
TIMP-1	1.02 ± 0.043
TIMP-2	0.95 ± 0.089

MMP, matrix metalloproteinase; MT, membrane type; ND, not detected; TIMP, tissue inhibitor of metalloproteinase.

^aAverage ± standard deviation ($n = 6$).

with both nicotine and *P. gingivalis* supernatant, 58.11% of cells were viable. The combination of both treatments significantly decreased cell viability when compared with either treatment alone ($p = 0.038$ to nicotine and $p < 0.001$ to *P. gingivalis* supernatant).

Discussion

Nicotine enhances the collagen-degrading ability of human gingival fibroblasts. This was evident by the fact that nicotine increased the cleavage of Type I collagen coated on the six-well plates by the human gingival fibroblasts in a dose-dependent manner. The collagen degradation was limited to the area underneath the cell colonies, which suggests that the increased collagen cleavage was cell membrane associated. Before this study, Tipton & Dabbous (31) used [³H]-gly and [¹⁴C]-pro-labeled Type I collagen gels as substrates and demonstrated a significant increase in the collagenase activity of nicotine-treated gingival fibroblasts. However, the treatment of cardiac fibroblasts with nicotine led to a decrease in collagenase activity (19). These conflicting results may be caused by differences in the fibroblast type, the nicotine concentration, the treatment period and/or the detection method utilized.

The nicotine concentrations utilized in this study ranged from 25 to 500 µg/ml. This concentration range was chosen based on a previous study on cell tolerance (32) and the range of concentrations of nicotine detected in saliva from tobacco users. Although plasma nicotine levels measured after the use of tobacco products are only in the range of 15 to 73 ng/ml (33–38), saliva samples from smokeless tobacco users have been found to contain as much as 1560 µg/ml of nicotine (39). Smoking one cigarette can expose the oral cavity to 1000 µg/ml of nicotine (40). Therefore, the concentrations used in this study were closer to the saliva concentrations and more relevant to the local effect on the oral tissues.

The human gingival fibroblast-mediated collagen degradation was

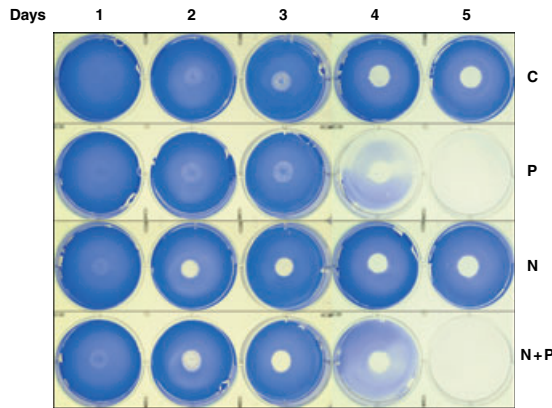


Fig. 5. Effect of nicotine and *Porphyromonas gingivalis* supernatant on the collagen-degrading ability of the human gingival fibroblasts. Human gingival fibroblasts were cultured in control serum-free Dulbecco's modified Eagle's medium (C), in medium containing 10% *P. gingivalis* supernatant (P), in medium containing 250 µg/ml of nicotine (N) or in medium containing 10% *P. gingivalis* supernatant plus 250 µg/ml of nicotine (N + P) for 5 d on six-well plates coated with Type I collagen. The human gingival fibroblasts were removed at days 1 through day 5, and the collagen cleavage visualized by Coomassie blue staining.

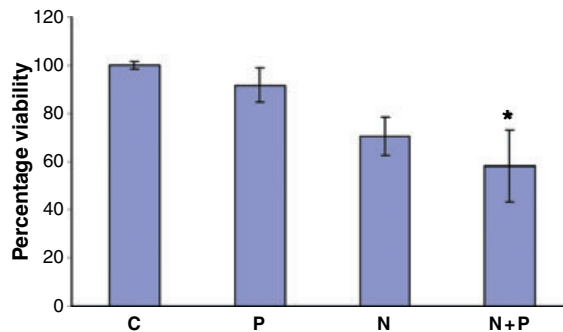


Fig. 6. Viability test for nicotine and/or *Porphyromonas gingivalis* supernatant-treated human gingival fibroblasts. Human gingival fibroblasts were cultured in control serum-free Dulbecco's modified Eagle's medium (C), in medium containing 10% *P. gingivalis* supernatant (P), in medium containing 250 µg/ml of nicotine (N) or in medium containing 10% *P. gingivalis* supernatant plus 250 µg/ml of nicotine (N + P) for 3 d. Water-soluble tetrazolium (10%) was added to the cultures which were incubated for 4 h before the light absorbance of the culture media was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 450 nm. Values for the absorbance from the samples were compared with the untreated cell control to determine percentage viability ($n = 9$). *The combination of both treatments significantly decreased cell viability when compared with nicotine ($p = 0.038$) and *P. gingivalis* supernatant ($p < 0.001$).

mediated by the MMPs, as evident by the fact that it could be completely inhibited by the MMP inhibitor, GM6001. Western blot analyses revealed that the levels of the membrane-associated MMP-14, MMP-2 and TIMP-2 changed upon nicotine treatment. A low-molecular-weight fragment of MMP-14 (43 kDa), and a partially activated form of MMP-2

(68 kDa), were detected in the membrane extracts of the nicotine-treated human gingival fibroblasts. In addition, the fact that TIMP-2 was detected at a higher level in the membrane extracts suggests that a redistribution of TIMP-2 to the cell membrane had occurred. These findings can be interpreted utilizing the MMP-2 activation model proposed by Strongin *et al.* (41).

According to this model, the TIMP-2 molecule serves as a bridging molecule for the binding of latent proMMP-2 to active MMP-14 on the cell membrane. Subsequently, another active MMP-14 molecule on the cell membrane approaches this trimolecular complex and activates the proMMP-2 by cleaving its propeptide domain. Therefore, more TIMP-2 would be recruited to the cell membrane in order to enhance MMP-2 activation.

The mechanisms by which nicotine regulates the activation of the membrane-associated MMPs are presently unknown. One hypothesis is that nicotine affects the cytoskeleton and/or cell attachment receptors that may subsequently influence the distribution of the membrane-associated MMPs. It should be noted that the human gingival fibroblasts exposed to nicotine had more rounded edges (data not shown), which supports this hypothesis. Previous data suggest that nicotine modulates the expression and distribution of gingival fibroblast cell surface proteins, such as $\alpha 1$ integrin (42,43) and $\alpha 2$ integrin (44). It has also been shown that the activation of latent MMP-2 by MMP-14 is regulated by the organization of the actin cytoskeleton (45) and surface proteins, including the $\alpha 5\alpha 1$ integrin receptors (46). Therefore, nicotine might cause the re-organization of the cytoskeleton and/or the redistribution of the cell surface proteins, such as the integrins and membrane type-MMPs, which could subsequently affect the activation of latent MMP-2.

Although MMP-1 is believed to be the major collagenase produced by fibroblasts, and MMP-3 is involved in the superactivation of MMP-1, neither the amount nor the level of activation of MMP-1 or MMP-3 changed upon nicotine treatment. These findings help to explain why the coated collagen around the cell colonies was intact and only the collagen underneath the cell colonies was degraded.

The mechanisms of how nicotine affected the TIMP-1 level are unknown. The decreased TIMP-1 level in the conditioned media could be an indirect effect of nicotine that may be mediated through some TIMP-1-

degrading enzyme(s) that are induced by the nicotine treatment. The mechanism for the recruitment of TIMP-2 on the cell membrane is also unclear. It could simply be a response to the clustering of the MMP-14 to certain areas of the human gingival fibroblast cell surface, which could provide a higher MMP-14 concentration locally and increase the number of TIMP-2-binding sites. Alternatively, the increase of membrane-associated TIMP-2 could be attributed to integrin binding of TIMP-2 (47). It should be noted that a higher MMP-14 concentration could also result in enhanced collagen degradation, because MMP-14 has collagenolytic activity.

The mRNA profiles of multiple MMPs and TIMPs expressed by the human gingival fibroblasts were examined in the absence and presence of nicotine. Nicotine did not drastically affect the mRNA expression of the examined MMPs or TIMPs after 24 h, which suggests that the regulation of MMPs by nicotine may mainly be limited to the activation of the MMPs. Previous data vary about how nicotine affects MMP mRNA expression in other cell types. Nicotine has been reported to up-regulate MMP-1, MMP-2 and MMP-3 gene expression in arterial smooth muscle cells (16). Cigarette smoke condensate has been reported to up-regulate MMP-1, MMP-8 and MMP-9 gene expression in vascular endothelial cells (15). However, nicotine had no effect on the MMP-2 gene expression in artery endothelial cells (20). The regulation of MMPs by nicotine in different types of cells may vary according to the functions of these cells. As enzyme activity can also be regulated by processing and inhibitor levels and location, lack of change in expression does not necessarily equate with enzyme activity level.

The combined effect of nicotine and *P. gingivalis* supernatant on human gingival fibroblast-mediated collagen degradation was examined. An additive effect of this combination on the cell-mediated collagen degradation was observed. This can be explained by the fact that nicotine + *P. gingivalis* supernatant increase collagen degra-

dation through different MMPs. As demonstrated previously (26), and in this study, the treatment of human gingival fibroblasts with *P. gingivalis* supernatant resulted in the degradation of all the collagen in the well. This is believed to be accomplished mainly through the activation of secreted MMP-1, MMP-2, MMP-3 and MMP-14, as well as by the degradation of TIMP-1 (26). Although there are multiple virulence factors in *P. gingivalis* supernatant, the major virulence factors that are responsible for the regulation of the MMPs and TIMP-1 are believed to be the cysteine proteinases (gingipains) (24,26,48). Nicotine appeared mainly to regulate the activation of membrane-associated MMP-2 and MMP-14, as well as the redistribution of TIMP-2. A combined effect of nicotine + *P. gingivalis* supernatant on reducing the cell viability was also observed. This agrees with a previous study that reported a lethal enhancement of toxins from putative periodontal pathogens by nicotine (49). It should be noted that this increase in collagen degradation, coupled with a decrease in cell viability, indicates an even greater increase in collagen-degrading capacity per cell.

In summary, nicotine appears to increase the collagen-degrading ability of the human gingival fibroblasts, in part through the activation of membrane-associated MMP-2 and MMP-14, as well as by the redistribution of TIMP-2 to the cell surface. Nicotine and *P. gingivalis* supernatant appear to affect host-mediated collagen degradation through different MMPs, and an additive effect was observed. These findings add to the body of knowledge about the roles that nicotine or tobacco use plays in the development of periodontal disease. In addition to interfering with host inflammatory processes and immune responses, nicotine promotes the degradation of collagen. This effect of nicotine would alter the connective tissue turnover rate and disrupt the normal wound healing process. Nicotine and *P. gingivalis* might also add to each other's detrimental effects on the extracellular matrix and enhance their destructive effects on the gingiva. These findings

help to explain why tobacco use is a major contributing factor to the initiation and/or exacerbation of periodontal disease.

Acknowledgements

The authors would like to thank Dr Jannet Katz for kindly providing the *P. gingivalis* ATCC 33277 culture supernatant and Dr Kirby Bodden for the polyclonal Ab 2315 against TIMP-1.

References

1. Burgan SW. The role of tobacco use in periodontal diseases: a literature review. *Gen Dent* 1997;**45**:469–470.
2. Harris NO. Smoking (tobacco) cessation. In: Hams NO, Christen AG, eds. *Primary Preventive Dentistry*. Columbus, OH: McGraw-Hill/Appleton & Lange 1995: 597–600.
3. Morozumi T, Kubota T, Sato T, Okuda K, Yoshie H. Smoking cessation increases gingival blood flow and gingival crevicular fluid. *J Clin Periodontol* 2004;**31**:267–272.
4. Meekin TN, Wilson RF, Scott DA, Ide M, Palmer RM. Laser Doppler flowmeter measurement of relative gingival and forehead skin blood flow in light and heavy smokers during and after smoking. *J Clin Periodontol* 2000;**27**:236–242.
5. Johnson GK, Todd GL, Johnson WT, Fung YK, Dubois LM. Effects of topical and systemic nicotine on gingival blood flow in dogs. *J Dent Res* 1991;**70**:906–909.
6. Wendell KJ, Stein SH. Regulation of cytokine production in human gingival fibroblasts following treatment with nicotine and lipopolysaccharide. *J Periodontol* 2001;**72**:1038–1044.
7. Obeid P, Bercy P. Effects of smoking on periodontal health: a review. *Adv Ther* 2000;**17**:230–237.
8. Kinane DF, Chestnutt IG. Smoking and periodontal disease. *Crit Rev Oral Biol Med* 2000;**11**:356–365.
9. Palmer RM, Scott DA, Meekin TN, Poston RN, Odell EW, Wilson RF. Potential mechanisms of susceptibility to periodontitis in tobacco smokers. *J Periodont Res* 1999;**34**:363–369.
10. Peacock ME, Sutherland DE, Schuster GS et al. The effect of nicotine on reproduction and attachment of human gingival fibroblasts *in vitro*. *J Periodontol* 1993;**64**: 658–665.
11. Raulin LA, McPherson JC 3rd, McQuade MJ, Hanson BS. The effect of nicotine on the attachment of human fibroblasts to

- glass and human root surfaces *in vitro*. *J Periodontol* 1988;**59**:318–325.
12. Chamson A, Frey J, Hivert M. Effects of tobacco smoke extracts on collagen biosynthesis by fibroblast cell cultures. *J Toxicol Environ Health* 1982;**9**:921–932.
 13. Giannopoulou C, Cimasani G. Functional characteristics of gingival and periodontal ligament fibroblasts. *J Dent Res* 1996;**75**:895–902.
 14. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;**92**:827–839.
 15. Nordskog BK, Blixt AD, Morgan WT, Fields WR, Hellmann GM. Matrix-degrading and pro-inflammatory changes in human vascular endothelial cells exposed to cigarette smoke condensate. *Cardiovasc Toxicol* 2003;**3**:101–117.
 16. Carty CS, Soloway PD, Kayastha S *et al*. Nicotine and cotinine stimulate secretion of basic fibroblast growth factor and affect expression of matrix metalloproteinases in cultured human smooth muscle cells. *J Vasc Surg* 1996;**24**:927–934.
 17. Arredondo J, Hall LL, Ndoye A *et al*. Central role of fibroblast alpha3 nicotinic acetylcholine receptor in mediating cutaneous effects of nicotine. *Lab Invest* 2003;**83**:207–225.
 18. Seroby N, Schraufstatter IU, Strongin A, Khaldoyanidi SK. Nicotinic acetylcholine receptor-mediated stimulation of endothelial cells results in the arrest of haematopoietic progenitor cells on endothelium. *Br J Haematol* 2005;**129**:257–265.
 19. Tomek RJ, Rimar S, Eghbali-Webb M. Nicotine regulates collagen gene expression, collagenase activity, and DNA synthesis in cultured cardiac fibroblasts. *Mol Cell Biochem* 1994;**136**:97–103.
 20. Zhang S, Day I, Ye S. Nicotine induced changes in gene expression by human coronary artery endothelial cells. *Atherosclerosis* 2001;**154**:277–283.
 21. Turnbull B. Smoking and periodontal disease. A review. *J N Z Soc Periodontol* 1995;**79**:10–15.
 22. Bouclin R, Landry RG, Noreau G. The effects of smoking on periodontal structures: a literature review. *J Can Dent Assoc* 1997;**63**:360–353.
 23. Travis J, Pike R, Imamura T, Potempa J. *Porphyromonas gingivalis* proteinases as virulence factors in the development of periodontitis. *J Periodont Res* 1997;**32**:120–125.
 24. DeCarlo AA Jr, Windsor LJ, Bodden MK, Harber GJ, Birkedal-Hansen B, Birkedal-Hansen H. Activation and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe *Porphyromonas gingivalis*. *J Dent Res* 1997;**76**:1260–1270.
 25. Birkedal-Hansen H, Wells BR, Lin HY, Caufield PW, Taylor RE. Activation of keratinocyte-mediated collagen (type I) breakdown by suspected human periodontopathogen. Evidence of a novel mechanism of connective tissue breakdown. *J Periodont Res* 1984;**19**:645–650.
 26. Zhou J, Windsor LJ. *Porphyromonas gingivalis* affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases. *J Periodont Res* 2006;**41**:47–54.
 27. Pattamapun K, Tiranathanagul S, Yongchaitrakul T, Kuwatanasuchat J, Pava-sant P. Activation of MMP-2 by *Porphyromonas gingivalis* in human periodontal ligament cells. *J Periodont Res* 2003;**38**:115–121.
 28. Chang YC, Lai CC, Yang SF, Chan Y, Hsieh YS. Stimulation of matrix metalloproteinases by black-pigmented Bacteroides in human pulp and periodontal ligament cell cultures. *J Endod* 2002;**28**:90–93.
 29. DeCarlo AA, Grenett HE, Harber GJ *et al*. Induction of matrix metalloproteinases and a collagen-degrading phenotype in fibroblasts and epithelial cells by secreted *Porphyromonas gingivalis* proteinase. *J Periodont Res* 1998;**33**:408–420.
 30. Song F, Windsor LJ. Novel nonmatrix-metalloproteinase-mediated collagen degradation. *Biochim Biophys Acta* 2005;**1721**:65–72.
 31. Tipton DA, Dabbous MK. Effects of nicotine on proliferation and extracellular matrix production of human gingival fibroblasts *in vitro*. *J Periodontol* 1995;**66**:1056–1064.
 32. Olson BL, Warner NA, Gregory RL, McDonald JL Jr, Zapata CA. Periodontal ligament fibroblast interleukin-6 release after exposure to nicotine. *Oral Biosci Med* 2005;**2**:15–19.
 33. Benowitz NL, Jacob P 3rd, Yu L. Daily use of smokeless tobacco: systemic effects. *Ann Intern Med* 1989;**111**:112–116.
 34. Ebert RV, McNabb ME, Snow SL. Effect of nicotine chewing gum on plasma nicotine levels of cigarette smokers. *Clin Pharmacol Ther* 1984;**35**:495–498.
 35. Hill P, Haley NJ, Wynder EL. Cigarette smoking: carboxyhemoglobin, plasma nicotine, cotinine and thiocyanate vs self-reported smoking data and cardiovascular disease. *J Chronic Dis* 1983;**36**:439–449.
 36. Herning RI, Jones RT, Benowitz NL, Mines AH. How a cigarette is smoked determines blood nicotine levels. *Clin Pharmacol Ther* 1983;**33**:84–90.
 37. Kozlowski LT, Frecker RC, Lei H. Nicotine yields of cigarettes, plasma nicotine in smokers, and public health. *Prev Med* 1982;**11**:240–244.
 38. Russell MA, Jarvis MJ, Devitt G, Fey-erabend C. Nicotine intake by snuff users. *Br Med J* 1981;**283**:814–817.
 39. Hoffmann D, Adams JD. Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Res* 1981;**41**:4305–4308.
 40. Benowitz NL, Jacob P 3rd. Daily intake of nicotine during cigarette smoking. *Clin Pharmacol Ther* 1984;**35**:499–504.
 41. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. *J Biol Chem* 1995;**270**:5331–5338.
 42. Snyder HB, Caughman G, Lewis J, Billman MA, Schuster G. Nicotine modulation of *in vitro* human gingival fibroblast beta1 integrin expression. *J Periodontol* 2002;**73**:505–510.
 43. Austin GW, Cuenin MF, Hokett SD *et al*. Effect of nicotine on fibroblast beta 1 integrin expression and distribution *in vitro*. *J Periodontol* 2001;**72**:438–444.
 44. Leonardi R, Lanteri E, Stivala F, Caltabiano M, Fenga C, Travali S. Alteration in alpha 2 integrin immunocytochemical expression on cultured human gingival fibroblasts following nicotine exposure. *Minerva Stomatol* 1999;**48**:495–499.
 45. Tomasek JJ, Halliday NL, Updike DL *et al*. Gelatinase A activation is regulated by the organization of the polymerized actin cytoskeleton. *J Biol Chem* 1997;**272**:7482–7487.
 46. Stanton H, Gavrilovic J, Atkinson SJ *et al*. The activation of ProMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form. *J Cell Sci* 1998;**111**:2789–2798.
 47. Seo DW, Li H, Guedez L *et al*. TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. *Cell* 2003;**114**:171–180.
 48. Grenier D, Mayrand D. Inactivation of tissue inhibitor of metalloproteinases-1 (TIMP-1) by *Porphyromonas gingivalis*. *FEMS Microbiol Lett* 2001;**203**:161–164.
 49. Sayers NM, Gomes BP, Drucker DB, Blinkhorn AS. Possible lethal enhancement of toxins from putative periodontopathogens by nicotine: implications for periodontal disease. *J Clin Pathol* 1997;**50**:245–249.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.