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Cigarette smoke condensate affects the collagen-degrading ability of human gingival fibroblasts

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Background and Objective: Cigarette smoke condensate, the particulate matter of cigarette smoke, is composed of thousands of chemicals, including nicotine. Cigarette smoking is a risk factor for periodontal disease. This study investigated the influence of cigarette smoke condensate on the collagen-degrading ability of human gingival fibroblasts and its mechanism.

Material and Methods: Human gingival fibroblasts were exposed for 72 h to various concentrations of total particulate matter cigarette smoke condensate. Cell proliferation and cytotoxicity were evaluated using water-soluble tetrazolium-1 and lactate dehydrogenase, respectively. The collagen-degrading ability of human gingival fibroblasts was evaluated in collagen-coated six-well plates. Conditioned media and membrane extracts were collected for zymography and western blot analyses of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs).

Results: Cell proliferation decreased and cytotoxicity increased in human gingival fibroblasts with increasing concentrations of cigarette smoke condensate. Cell proliferation decreased by more than 50% ($p < 0.05$) when the concentrations of total particulate matter cigarette smoke condensate were above 200 μg/mL, and cytotoxicity increased to more than 30% ($p < 0.05$) when the concentrations of total particulate matter cigarette smoke condensate were above 400 μg/mL. Cigarette smoke condensate increased the collagen-degrading ability of human gingival fibroblasts, especially at a concentration of 100 μg/mL (1.5-fold increase, $p < 0.05$) compared with the control. Cigarette smoke condensate increased the production of proMMP-1, proMMP-2, MMP-14 and TIMP-1, and decreased the production of TIMP-2, in conditioned media. Furthermore, compared with the control group, cigarette smoke condensate increased the production of MMP-2, MMP-14 and TIMP-2 in membrane extracts, especially at concentrations of 50–100 μg/mL.

Conclusion: Cigarette smoke condensate affects human gingival fibroblast proliferation and is toxic at total particulate matter cigarette smoke condensate concentrations of ≥ 400 μg/mL. Cigarette smoke condensate can increase the collagen-degrading ability of human gingival fibroblasts by altering the production and localization of MMPs and TIMPs.

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The negative heath consequences of smoking are widely recognized, but about 20% of people in the USA continue to use tobacco (1). Cigarette smoke condensate, the particulate matter of cigarette smoke, is composed of thousands of chemicals (e.g. nicotine, cadmium, phenol, anthracyclic hydrocarbons, nitrosamines, heavy metals and chemical carcinogens), which are powerful inducers of inflammatory responses and are toxic to multiple cell types (2).

Secondary to bacterial plaque, cigarette smoking is a major risk factor for periodontal disease (3,4) and even promotes its development (5–8). It is evident that patients who smoke have greater bone loss, greater attachment loss and deeper periodontal pockets than nonsmoking patients (9). Furthermore, the treatment outcome of periodontal disease is also less favorable in smokers (9).

The health of periodontal tissues depends on the normal functions of periodontal cells. The attachment, migration, growth and differentiation of periodontal cells are critical steps in the repair and regeneration of periodontal tissues. Human gingival fibroblasts are the main cellular component of periodontal connective tissues. Therefore, the normal functions of human gingival fibroblasts are very important to periodontal health.

During the development of periodontal disease, collagen degradation occurs. Collagen is the major extracellular matrix component of the gingiva. The major extracellular matrix-degrading enzymes produced by the human gingival fibroblasts are the matrix metalloproteinases (MMPs). These enzymes are a group of zinc-dependent endopeptidases that include the collagenases, gelatinases, stromelysins and membrane-associated MMPs. The MMPs play important roles in both physiological and pathological conditions. Several members of the MMP family have been shown to be involved in periodontal tissue destruction, and these include MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13 and MMP-14 (10,11). The MMPs are mainly modulated by the tissue inhibitors of metalloproteinases (TIMPs), which are secreted by various cell types and are found in most body fluids and tissues. The balance of MMPs/TIMPs is believed to be important to periodontal health, and tobacco may affect this balance (12).

Nicotine, one of the pharmacologically active agents in cigarette smoke condensate, has been used to study the effects of smoking on periodontal tissue. Zhou *et al.* (13) showed that nicotine (250 μg/mL) can increase human gingival fibroblast-mediated collagen degradation, in part, through the activation of membrane-associated MMPs. Furthermore, Almasri *et al.* (14) showed that nicotine (250 μg/mL), *Porphyromonas gingivalis* lipopolysaccharide, or a combination of both agents, can increase the production of multiple cytokines and growth factors in human gingival fibroblasts. Other studies have demonstrated that nicotine affects gingival blood flow (15,16), cytokine production (17) and certain functions of connective tissue cells (e.g. fibroblasts) such as cell morphology, attachment to substrates, and protein synthesis and secretion (18,19). However, nicotine is just one of the thousands of chemicals in cigarette smoke condensate. Currently, there are little data on the effects of cigarette smoke condensate on human gingival fibroblasts. Thus, the aim of this study was to examine the effects of cigarette smoke condensate on the collagen-degrading ability of human gingival fibroblasts, as well as on the production of multiple MMPs and TIMPs.

Material and methods

Cell culture

Human gingival fibroblasts were cultured from explants of clinically healthy gingival connective tissue removed from a patient undergoing crown-lengthening surgery, as described previously (13), at Indiana University School of Dentistry and with Institutional Review Board approval. Briefly, the tissue was transported from the clinic to the laboratory in 1% phosphate-buffered saline solution, washed with 70% ethanol and then rinsed in 1% phosphate-buffered saline to remove the ethanol. The washing and rinsing steps were repeated and then the tissue was minced into small fragments of approximately 1 mm^3. The tissue pieces were then placed in cell-culture dishes, air-dried and incubated for 5–7 d at 37°C and 5% CO_2 in low-glucose (1 g/L) Dulbecco's modified Eagle's minimal essential medium (Hyclone, Logan, UT, USA) supplemented with 15% fetal bovine serum (Hyclone), 4 mM L-glutamine (Hyclone), 100 U/mL of penicillin, 50 μg/mL of gentamicin and 2.5 μg/mL of fungizone (Invitrogen, Carlsbad, CA, USA). The cells that grew out of the explants were subcultured and maintained. Cells at passages 3–8 were utilized in these experiments.

Measurement of human gingival fibroblast proliferation using the water-soluble tetrazolium-1 assay

Mitochondrial dehydrogenase activities were measured using the water-soluble tetrazolium-1 assay (Roche Applied Science, Penzberg, Germany), as described previously (20). Human gingival fibroblasts were detached by incubation for 5 min with 0.25% trypsin (Invitrogen), pelleted, resuspended in Dulbecco's modified Eagle's minimal essential medium containing 15% fetal bovine serum and then seeded (50,000 cells/well) in six-well plates containing 2 mL of Dulbecco's modified Eagle's minimal essential medium and 15% fetal bovine serum. The plates were incubated for 24 h to allow the cells to attach. The medium was then removed and the human gingival fibroblasts were exposed to 1 mL of various concentrations of particulate matter (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0 μg/mL) of cigarette smoke condensate (Murty Pharmaceuticals, Lexington, KY, USA) diluted in serum-free Dulbecco's modified Eagle's minimal essential medium. The positive control was serum-free Dulbecco's modified Eagle's minimal essential medium containing untreated human gingival fibroblasts and the negative control was serum-free Dulbecco's modified Eagle's minimal essential medium without cells. After 72 h, the media in the six-well

plates were removed and the cells were washed with 2 mL of serum-free Dulbecco's modified Eagle's minimal essential medium, three times for 15 min each wash. The cell proliferation reagent water-soluble tetrazolium-1 (100 μL of water-soluble tetrazolium-1 and 900 μL of serum-free Dulbecco's modified Eagle's minimal essential medium) was added and the plate was incubated for 2 h at 37°C and 5% CO_2. A 100-μL sample from each well of the six-well plates was placed in a 96-well plate and the absorbance of the samples against the negative control as the blank were measured using a microplate reader (Titertek, Multiskan MCC; Flow Laboratories, Mclean, VA, USA) at 450 nm. The experiments were repeated six times and the mean value was calculated. The absorbance values of each sample were compared with the untreated cell control, by percentage, in the following equation:

Cell proliferation (%) =
(absorbance value of cigarette smoke condensate treatment/
absorbance value of no cigarette smoke condensate treatment) × 100%

Measurement of cigarette smoke condensate cytotoxicity using the lactate dehydrogenase assay

Cell membrane integrity was monitored using the permeability assay based on the determination of the release of lactate dehydrogenase into the media. The Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Mannheim, Germany), which measures the conversion of tetrazolium salt into a red formazan product, was used as described previously (21). Cells were treated with the same range of concentrations of cigarette smoke condensate for 72 h as described above for the water-soluble tetrazolium-1 assay. The high control (total cell death) was generated by adding 1.9 mL of serum-free Dulbecco's modified Eagle's minimal essential medium and 100 μL of lysis solution to the control cells, as described by the manufacturer, after 72 h, which gave the maximum release of lactate dehydrogenase. The low control consisted of serum-free Dulbecco's modified Eagle's minimal essential medium from untreated control cells after 72 h and gave the minimal release of lactate dehydrogenase. Serum-free Dulbecco's modified Eagle's minimal essential medium without human gingival fibroblasts was utilized as the background level of the assay. After 72 h, media from each of the wells was transferred to a 96-well plate, and 100 μL of reconstituted mix, as per the manufacturer's instructions (Roche), was added to each well and the plates were incubated for 15 min at room temperature (23°C). The absorbance was recorded at 490 nm in a microplate reader (Titertek, Multiskan MCC; Flow Laboratories). The experiments were repeated seven times and the mean value was calculated. The percentage release of lactate dehydrogenase from the treated cells was calculated by comparing it to the maximum release of lactate dehydrogenase achieved by the lysis solution used in the control cells. To determine the cytotoxicity, the absorbance values of the background were subtracted from that of the experimental samples and the cytotoxicity was calculated using the following equation:

Cytotoxicity (%)
= (experiment value-low control)
/(high control − low control) × 100%

Collagen degradation

The collagen-degrading ability of human gingival fibroblasts was determined using a reconstituted collagen type I assay system, as previously described by Birkedal-Hansen *et al.* (22). Briefly, rat tail tendon type I collagen was dissolved in 13 mM HCl and then mixed rapidly on ice with one-fifth of the volume of a neutralizing phosphate buffer (40 mL of 0.2 M NaH_2PO_4 buffer, pH 7.4, 40 mL of 0.1 M NaOH, 8.3 mL of 5 M NaCl) to yield a final collagen concentration of 300 μg/mL. Aliquots of 1.5 mL per well (450 μg of collagen per well) were dispensed into six-well plates and incubated at 37°C for 2 h. The collagen gels were dehydrated overnight in a laminar flow hood, washed three times for 30 min with sterile water and then air-dried in the hood. Human gingival fibroblasts at 90% confluence were detached by incubation for 5 min with 0.25% trypsin (Invitrogen), pelleted, resuspended in Dulbecco's modified Eagle's minimal essential medium without serum and then seeded as a single colony (75,000 cells/well) in the center of a six-well plate coated with collagen. After the cells attached, 2 mL of serum-free Dulbecco's modified Eagle's minimal essential medium containing various concentrations of particulate matter (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0 μg/mL) of cigarette smoke condensate was added to each well. Serum-free medium without cigarette smoke condensate was utilized as the negative control. After 3 d, the cells were removed by incubation with 500 μL of 0.1% Triton X-100 and 200 μL of 0.25% trypsin (Invitrogen). The wells were then stained with Coomassie Blue to visualize the cleavage of collagen. The six-well plates were scanned and the collagen degradation was analyzed using NIH IMAGEJ software (version 1.37, Scion Corporation, Frederick, MD, USA). The experiments were repeated six times.

Gelatin zymography

Human gingival fibroblasts were cultured for 3 d in serum-free Dulbecco's modified Eagle's minimal essential medium containing various concentrations of cigarette smoke condensate particulate matter (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0 μg/mL). The conditioned media was collected. The cell-membrane extracts were then prepared utilizing the Mem-PER Eukaryotic Membrane Protein Extraction Regent Kit and PAGEprepTM Protein Clean-Up and Enrichment Kit (Pierce, Rockford, IL, USA). The concentrations of total protein in the concentrated media and membrane extracts were determined according to the Bio-Rad protein assay protocol (Bio-Bad Laboratories, Hercules, CA, USA). The same protein concentrations from the conditioned human gingival fibroblast media and cell-membrane extracts were mixed with nonreducing loading buffer

(without beta-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 with solution 1 (50 mM Tris, pH 7.5, 3 mM NaN_3, 2.5% Triton X-100), solution 2 (50 mM Tris, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100) and solution 3 (50 mM Tris, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$), for 20 min each. The gels were then incubated in fresh solution 3 at 37°C overnight and stained with Coomassie Blue to visualize the proteolytic bands. The gels were scanned and the proteolytic bands analyzed using NIH IMAGEJ software (version 1.37). The experiments were repeated three times.

Western blot analyses

Western blots were performed to examine the levels of production of MMP-1, MMP-3, MMP-14, TIMP-1 and TIMP-2 in the conditioned media, as well as to examine the levels of MMP-14 and TIMP-2 in the cell-membrane extracts. The same concentrations of total protein from untreated media and from cigarette smoke condensate-treated media and membrane extracts were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels at 200 V. The proteins on the gels were transferred to polyvinylidene difluoride membranes at 0.08 amps overnight using blotting buffer (2 mM Tris–HCl, pH 8.3, 192 mM glycine and 10% methanol). The membranes were then incubated in 5% dried skimmed milk prepared in phosphate-buffered saline containing 0.1% Tween-20 (pH 7.4) for 1 h to block nonspecific binding. The membranes were then incubated with primary antibodies at 4°C overnight. Antibodies used in this study were monoclonal Ab-5 (5 μg/mL, Clone III12b; Neomarkers, Fremont, CA, USA) for MMP-1, monoclonal Ab-2 (5 μg/mL, Clone IID4; Neomarkers) for MMP-3, monoclonal Ab-1 (5 μg/mL, Clone 102B1; Neomarkers) for TIMP-1, monoclonal Ab-1 (5 μg/mL, Clone T2-101; Neomarkers) for TIMP-2 and polyclonal Ab-2 (0.3 μg/mL; Chemicon, Temecula, CA, USA) for MMP-14. The membranes were then washed three times with phosphate-buffered saline containing 0.1% Tween-20 (pH 7.4) and incubated with anti-mouse or anti-rabbit secondary immunoglobulins (Amersham, Piscastaway, NJ, USA) for 1 h at room temperature (23°C). The membranes were then developed using the ECL™ kit (Amersham) according to the manufacturer's protocol. The western blot analyses for the MMPs/TIMPs were repeated three times.

Statistical analyses

The data were presented as mean and standard deviation. Statistical analyses were performed using one-way analysis of variance and Tukey's test in the STATISTICAL PACKAGE FOR SOCIAL SCIENCE SPSS 11.5 (SPSS Inc., Chicago, IL, USA) (13). The level of significant was set at a p-value of < 0.05.

Results

Morphology of human gingival fibroblasts

The human gingival fibroblasts attached and spread rapidly to take on a typical long spindle-shaped parallel-alignment appearance in the six-well plate without cigarette smoke condensate (Fig. 1A). At low concentrations of cigarette smoke condensate, the human gingival fibroblasts remained normal in appearance (Fig. 1B). By contrast, cigarette smoke condensate-treated human gingival fibroblasts showed a rounded appearance with a haphazard arrangement and detached from the six-well plate at higher concentrations (above 200 μg/mL; Fig. 1C). When the concentrations of cigarette smoke condensate reached 800 μg/mL, almost no human gingival fibroblasts were still attached (data not shown).

Measurement of cell proliferation using the water-soluble tetrazolium-1 assay

After exposure to cigarette smoke condensate at 800, 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL, the amount of cell proliferation was

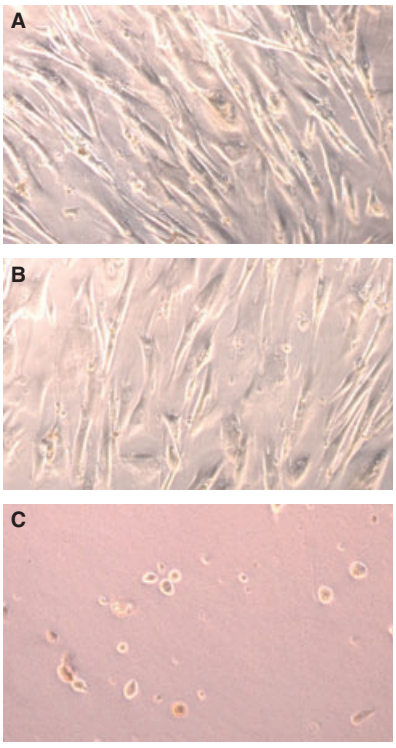

Fig. 1. (A) Cigarette smoke condensate-free human gingival fibroblasts (negative control). Human gingival fibroblasts attached to the plate typically displayed a long spindle-shaped parallel-alignment appearance (magnification ×100). (B) Human gingival fibroblasts treated with 3.125 μg/mL of cigarette smoke condensate displayed a normally typical long spindle-shaped parallel-alignment appearance (magnification ×100). (C) Human gingival fibroblasts treated with 200 μg/mL of cigarette smoke condensate displayed a round appearance with a haphazard arrangement and most of the human gingival fibroblasts had become detached from the plate (magnification ×100).

6.86 ± 5.36%, 7.32 ± 5.49%, 46.79 ± 26.39%, 90.29 ± 42.43%, 81.56 ± 24.71%, 80.43 ± 29.36%, 64.85 ± 30.10%, 60.09 ± 12.92% and 81.85 ± 38.24%, respectively when compared with the untreated control human gingival fibroblasts (100%; Fig. 2). Overall, the cigarette smoke condensate had a negative effect on the growth of the human gingival fibroblasts compared with the control. However, different concentrations of the cigarette smoke condensate had different effects on the growth of the human gingival fibroblasts. There

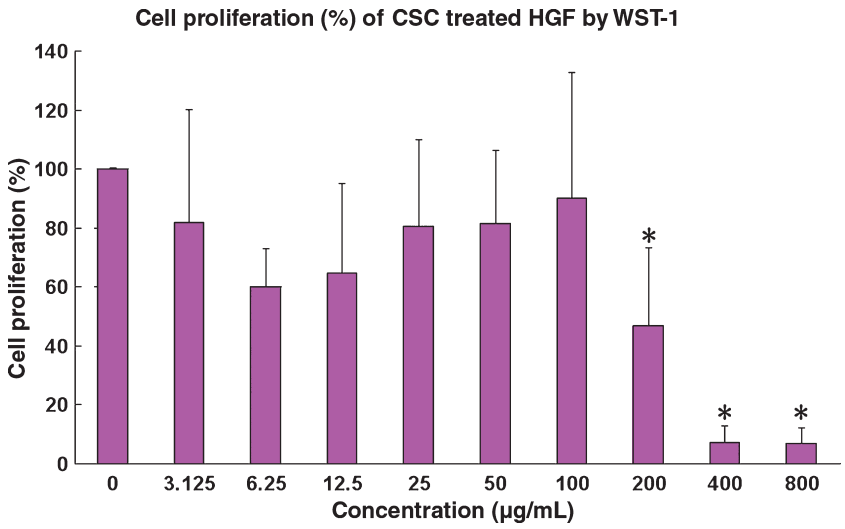

Fig. 2. Proliferation (%) of cigarette smoke condensate-treated human gingival fibroblasts, evaluated using water-soluble tetrazolium-1. The proliferation of cigarette smoke condensate-treated human gingival fibroblasts was lower than that of the untreated human gingival fibroblasts. Data were analyzed using one-way analysis of variance and Tukey's multiple comparison tests. *Significant differences from the control value at a p-value of < 0.05.

were no concentration-dependent relationships between the cigarette smoke condensate concentrations, and there was a negative effect on human gingival fibroblasts at cigarette smoke condensate concentrations of < 200 μg/mL. When the concentration of cigarette smoke condensate reached 200 μg/mL, cell proliferation was reduced to 46.79% and decreased significantly when compared with the untreated human gingival fibroblasts ($p = 0.023$).

Measurement of cytotoxicity of human gingival fibroblasts by lactate dehydrogenase

Generally, the cigarette smoke condensate had a toxic effect on the growth of human gingival fibroblasts, despite some variations (Fig. 3). After exposure to cigarette smoke condensate at 800, 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL, the amount of cytotoxicity was 70.43 ± 11.13%, 32.42 ± 24.62%, 10.59 ± 16.44%, 10.93 ± 19.66%, 8.97 ± 17.22%, −0.80 ± 4.52%, 4.43 ± 10.356%, 1.23 ± 6.49% and 4.22 ± 4.25%, respectively (Fig. 3). The cigarette smoke condensate at concentrations of 400 and 800 μg/mL demonstrated significant toxic effects on the human gingival fibroblasts ($p = 0.001$ and $p = 0.000$, respectively).

Collagen degradation

Untreated human gingival fibroblasts (control) cleaved the collagen underneath the cell colonies and formed a small transparent area (Fig. 4A. At low concentrations ($\leq$ 25 μg/mL) of cigarette smoke condensate, the area of collagen degradation increased a little but was not statistically significant (Fig. 4B) ($p > 0.05$). This increase in collagen degradation was particularly evident when the concentration of cigarette smoke condensate reached 100 μg/mL ($p = 0.002$). When the concentration of cigarette smoke condensate was $\geq$ 200 μg/mL, the collagen-degrading ability decreased, probably because of the effects of cigarette smoke condensate on the cells in regard to proliferation and toxicity.

Gelatin zymography

In the human gingival fibroblast-conditioned media, with or without cigarette smoke condensate, gelatinase-A (MMP-2, 72/62 kDa) bands were detected (Fig. 5A). The production of proMMP-2 (72 kDa) at low concentrations ($\leq$ 100 μg/mL) of cigarette smoke condensate showed a little variation among the different concentrations of cigarette smoke condensate (Table 1). At high concentrations ($\geq$ 200 μg/mL) of cigarette smoke condensate, the level of both the pro

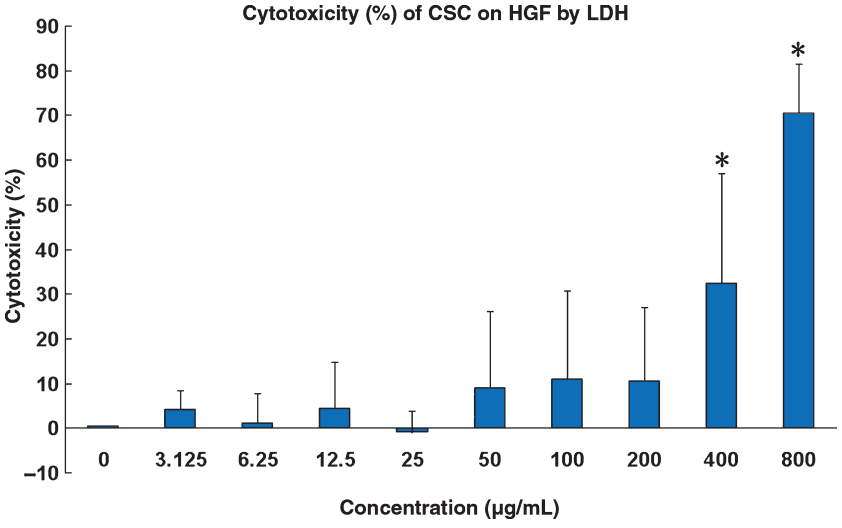

Fig. 3. Cytotoxicity (%) of cigarette smoke condensate-treated human gingival fibroblasts, evaluated using lactate dehydrogenase. The cytotoxicity of cigarette smoke condensate-treated human gingival fibroblasts was higher than that of cigarette smoke condensate-free human gingival fibroblasts. Data were analyzed using one-way analysis of variance and Tukey's multiple comparison tests. *Significant differences from the control value at a p-value of < 0.05.

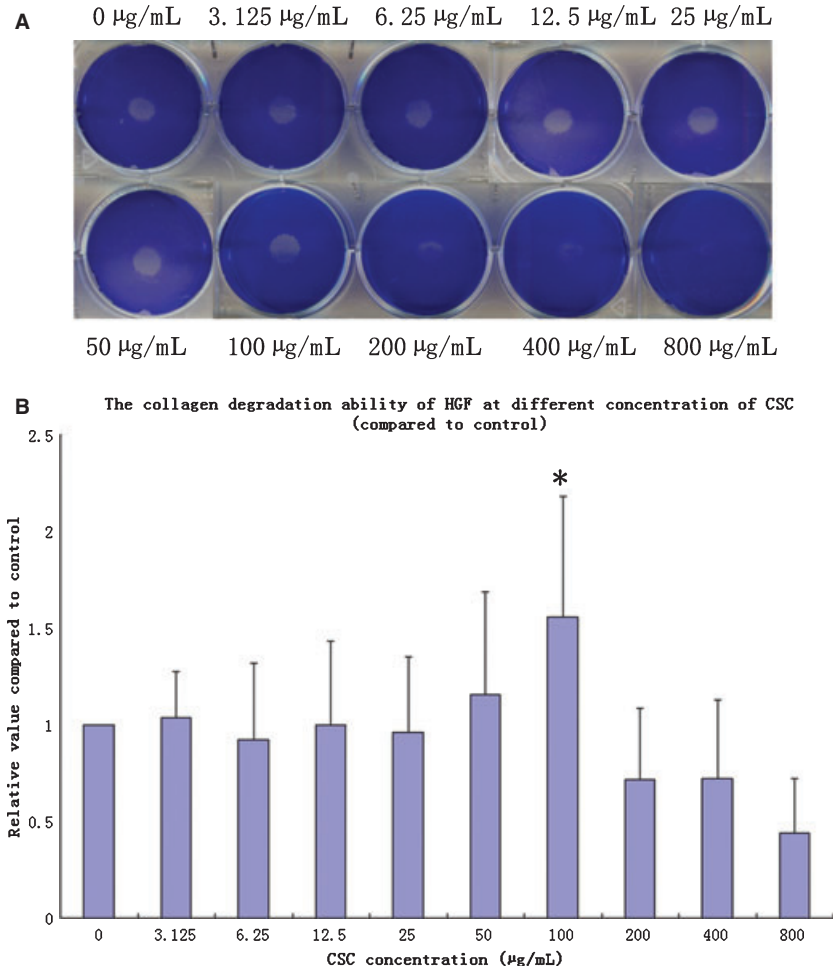

Fig. 4. (A) Effects of different concentrations of cigarette smoke condensate (0–800 μg/mL) on the collagen-degrading ability of human gingival fibroblasts. (B) Statistical analyses of human gingival fibroblasts at different concentrations of cigarette smoke condensate (0–800 μg/mL) compared with the control group (without cigarette smoke condensate). Data were analyzed using one-way analysis of variance and Tukey's multiple comparison tests. *Significant differences from the control at a p-value of < 0.05. CSC, cigarette smoke condensate.

and active forms of MMP-2 decreased compared with the untreated human gingival fibroblasts. However, at low concentrations (≤ 100 μg/mL) of cigarette smoke condensate, the active form of MMP-2 (62 kDa) increased with increasing concentrations of cigarette smoke condensate. This increase was especially evident when the concentration of cigarette smoke condensate reached 25 μg/mL. Furthermore, the production of MMP-9 (gelatinase B) could not be detected by zymography (data not shown).

Gelatinase-A (MMP-2, 72/62 kDa) bands were detected in the human gingival fibroblast membrane extracts with or without cigarette smoke condensate (Fig. 5B). The production of proMMP-2 (72 kDa) was not significantly different among the various concentrations of cigarette smoke condensate studied (Table 2). However, the active form of MMP-2 (62 kDa) increased more than 16-fold compared with the control at concentrations of cigarette smoke condensate above 50 μg/mL. Furthermore, the production of MMP-9 (gelatinase B) could not be detected by zymography of the membrane extracts (data not shown).

Western blotting analyses

Both proMMP-1 (52 kDa) and active MMP-1 (42 kDa) were detected in the conditioned media (Fig. 6A). Compared to the control group, high levels of both pro and active MMP-1 were

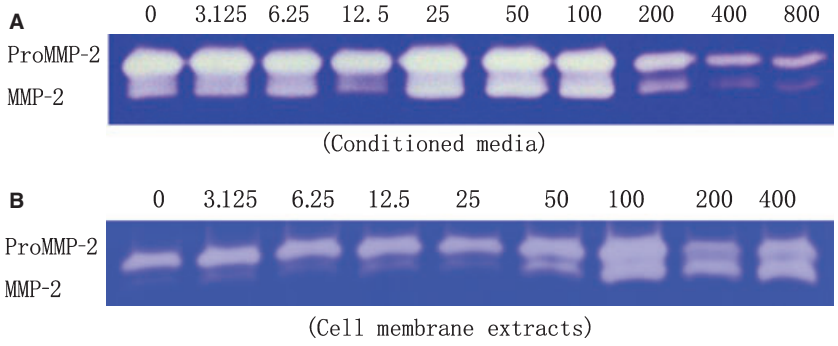

Fig. 5. (A) Gelatin zymography of the human gingival fibroblast-conditioned media treated with different concentrations of cigarette smoke condensate (0–800 μg/mL) for 72 h. (B) Gelatin zymography of membrane extracts from the human gingival fibroblasts treated with different concentrations of cigarette smoke condensate (0–400 μg/mL) for 72 h. MMP-2, matrix metalloproteinase-2.

Table 1. Expression of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in human gingival fibroblast-conditioned media at different cigarette smoke condensate concentrations compared with the control

	3.125 μg/mL vs. control	6.25 μg/mL vs. control	12.5 μg/mL vs. control	25 μg/mL vs. control	50 μg/mL vs. control	100 μg/mL vs. control	200 μg/mL vs. control	400 μg/mL vs. control	800 μg/mL vs. control
ProMMP-1	0.80 ± 0.04	0.98 ± 0.03	1.22 ± 0.02	0.86 ± 0.06	0.72 ± 0.04	1.43 ± 0.07	1.04 ± 0.07	1.56 ± 0.06	0.93 ± 0.01
MMP-1	0.77 ± 0.03	1.28 ± 0.05	1.17 ± 0.03	0.95 ± 0.03	0.77 ± 0.06	1.35 ± 0.07	0.65 ± 0.02	0.94 ± 0.02	0.89 ± 0.05
ProMMP-2	1.30 ± 0.34	1.12 ± 0.20	0.93 ± 0.22	1.77 ± 1.13	1.76 ± 1.21	1.45 ± 0.81	0.64 ± 0.50	0.44 ± 0.25	0.26 ± 0.15
MMP-2	1.09 ± 0.08	1.23 ± 0.09	0.80 ± 0.22	1.62 ± 0.53	1.44 ± 0.10	1.43 ± 0.28	0.83 ± 0.40	0.43 ± 0.30	0.30 ± 0.10
ProMMP-3	1.05 ± 0.02	1.04 ± 0.02	1.14 ± 0.02	1.14 ± 0.05	0.93 ± 0.04	1.21 ± 0.06	1.17 ± 0.08	0.96 ± 0.03	0.89 ± 0.06
MMP-3	0.96 ± 0.01	0.80 ± 0.02	0.90 ± 0.02	0.94 ± 0.02	0.80 ± 0.03	0.90 ± 0.11	0.22 ± 0.02	0.11 ± 0.01	0.15 ± 0.01
MMP-14	1.09 ± 0.01	1.08 ± 0.06	1.97 ± 0.09	1.17 ± 0.06	1.20 ± 0.02	1.57 ± 0.04	1.29 ± 0.02	1.18 ± 0.04	1.19 ± 0.11
TIMP-1	1.34 ± 0.01	3.49 ± 0.04	4.56 ± 0.06	4.35 ± 0.05	5.91 ± 0.05	6.45 ± 0.05	6.20 ± 0.16	3.83 ± 0.09	5.59 ± 0.09
TIMP-2	1.28 ± 0.01	1.02 ± 0.02	0.97 ± 0.06	0.92 ± 0.04	0.41 ± 0.02	0.31 ± 0.04	0.40 ± 0.02	0.44 ± 0.02	0.34 ± 0.01

ProMMP-2 and MMP-2 results are from zymography and the other results are from western blotting.

Table 2. Expression of pro-matrix metalloproteinase-2 (pro MMP-2), MMP-2, MMP-14 and tissue inhibitor of metalloproteinase-2 (TIMP-2)

	3.125 μg/mL vs. control	6.25 μg/mL vs. control	12.5 μg/mL vs. control	25 μg/mL vs. control	50 μg/mL vs. control	100 μg/mL vs. control	200 μg/mL vs. control	400 μg/mL vs. control
ProMMP-2	1.25 ± 0.02	1.26 ± 0.01	1.27 ± 0.03	1.07 ± 0.02	1.40 ± 0.02	1.68 ± 0.05	0.93 ± 0.03	1.25 ± 0.05
MMP-2	5.68 ± 0.29	5.36 ± 0.59	8.87 ± 0.88	5.19 ± 1.52	16.64 ± 2.20	31.88 ± 2.62	23.73 ± 2.32	27.16 ± 2.86
MMP-14	0.97 ± 0.02	1.04 ± 0.03	0.79 ± 0.06	1.13 ± 0.06	2.13 ± 0.14	1.23 ± 0.03	1.67 ± 0.06	0.78 ± 0.05
TIMP-2	1.46 ± 0.05	1.50 ± 0.01	1.74 ± 0.12	1.02 ± 0.03	2.98 ± 0.13	2.47 ± 0.11	0.88 ± 0.04	0.09 ± 0.01

ProMMP-2 and MMP-2 results are from zymography and the other results are from western blotting.

produced when the concentration of cigarette smoke condensate was at 100 μg/mL (Table 1).

ProMMP-3 (59/54 kDa) was detected in the conditioned media at all concentrations of cigarette smoke condensate, and the active forms of MMP-3 (49/44 kDa) were also detected (Fig. 6A; Table 1). However, the production of the active form of MMP-3 decreased sharply compared with the control at cigarette smoke condensate concentrations of > 100 μg/mL, possibly because of cell death.

MMP-14 (64 kDa) was also detected in the conditioned media. Compared with the control group, cigarette smoke condensate concentrations of 12.5 and 200 μg/mL increased the production of MMP-14(Fig. 6A; Table 1). However, the production of MMP-14 increased by more than two-fold in the membrane extracts from human gingival fibroblasts at cigarette smoke condensate concentrations of 50 μg/mL (Fig. 6B; Table 2).

TIMP-1 (28.5 kDa) levels in the conditioned media increased at cigarette smoke condensate concentrations of ≥ 6.25 μg/mL. Generally, an increasing concentration of cigarette smoke condensate led to increased production of TIMP-1 compared with the control until cytotoxicity occurred (Fig. 6A; Table 1).

Generally, the production of TIMP-2 (21 kDa) in conditioned media decreased with increasing concentrations of cigarette smoke condensate (Fig. 6A; Table 1). However, the production of TIMP-2 in human gingival fibroblast membrane extracts increased at cigarette smoke condensate concentrations of 50 and 100 μg/mL (Fig. 6B; Table 2). The levels of TIMP-2 in the membrane extracts decreased at cigarette smoke condensate concentrations of ≥ 200 μg/mL.

Discussion

Cigarette smoke is a complex mixture that contains more than 6000 chemicals (2). Many of these chemicals are cytotoxic. Cytotoxicity is an important factor in understanding the mechanism of action of different chemicals on cells and tissues. Cytotoxicity is thought to play an important role in a number of pathological processes including inflammation and carcinogenesis. Cytotoxicity may also modulate the activity of other agents, including free radicals, cytokines and genotoxins (23).

In the present study carried out to determine the effects of smoke condensate on human gingival fibroblast proliferation, the doses of cigarette smoke condensate particulate matter ranged from 0–800 μg/mL. These concentrations were similar to human exposure, according to the following calculation. As previously reported (24,25), each cigarette yields 26.1 mg of cigarette smoke condensate. At a half pack to a full pack (10–20 cigarettes) smoked per day per person, the exposure of cigarette smoke condensate amounts to 261–522 mg. As the volume of saliva that one person secretes in 1 d is approximately 1–1.5 L, the cigarette smoke condensate exposure dose is 174–522 μg/mL. When the human gingival fibroblasts were exposed to cigarette smoke condensate doses above 800 μg/mL (1000, 2000 and 4000 μg/mL) in our preliminary

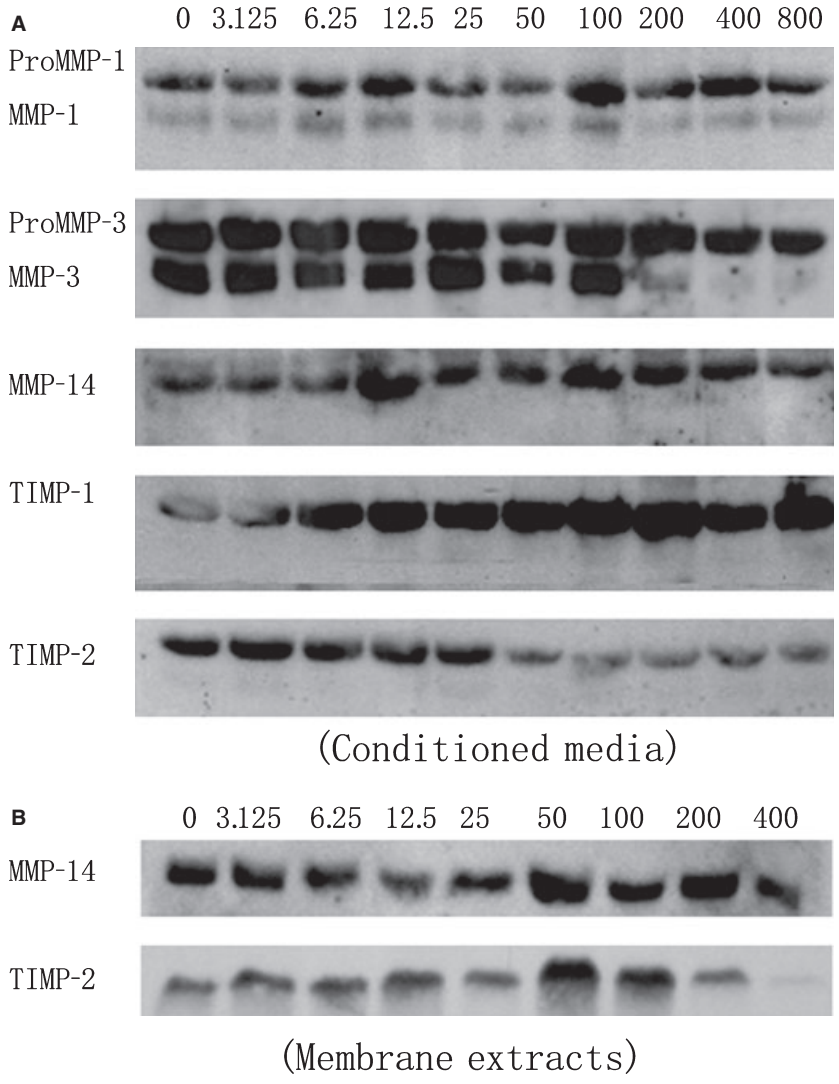

Fig. 6. (A) Western blot analyses for selected matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in human gingival fibroblast-conditioned media treated with different concentrations of cigarette smoke condensate (0–800 μg/mL) for 72 h. (B) Western blot analyses, for MMP-14 and TIMP-2, of the membrane extracts from human gingival fibroblasts treated with different concentrations of cigarette smoke condensate (0–400 μg/mL) for 72 h.

experiments, almost no viable human gingival fibroblasts were observed in the tissue culture plates (data not shown). This was the rationale for using the range of 0–800 μg/mL of cigarette smoke condensate in this study. Furthermore, in this study the treatment time of cigarette smoke condensate exposure to the human gingival fibroblasts was 72 h. In the preliminary experiments, 24 or 48 h was too short for the cigarette smoke condensate to affect the human gingival fibroblasts fully (data not shown). In addition, culture media should be regularly changed (at least every 3–4 d) to provide the necessary nutritients for the human gingival fibroblasts. Therefore, the treatment time could not be too long. This was the rationale for using 72 h as the treatment time. In this study, MMP-2 and MMP-9 were not examined by western blot analyses because gelatin zymography is a more sensitive measurement of their production. Furthermore, as almost no human gingival fibroblasts were still alive when the concentration of cigarette smoke condensate reached 800 μg/mL, there were no data for membrane extracts at total particulate matter cigarette smoke condensate concentrations of 800 μg/mL.

In this study, cigarette smoke condensate affected the proliferation of human gingival fibroblasts. This effect of the cigarette smoke condensate showed a gradual increase with increasing concentrations of cigarette smoke condensate, although some variations were observed. The negative effects of the cigarette smoke condensate varied at low concentrations. This may be a result of the fact that there are many different components in the cigarette smoke condensate and that each component has individual effects. Some of the components may promote the proliferation of cells, whereas other components may inhibit the proliferation of cells. Therefore, the net result is a balance of all the components. The results of this study showed the total effects of these components on the proliferation of human gingival fibroblasts. At high concentrations (more than 200 μg/mL), cell proliferation decreased with cigarette smoke condensate treatment. Luppi *et al.* (26) reported that low concentrations of cigarette smoke condensate increased proliferation of a bronchial epithelial cell line, whereas high concentrations were inhibitory as a result of cytotoxicity. In the current study, similar results were also observed. With the increasing of concentrations of cigarette smoke condensate, the cytotoxicity, as measured by lactate dehydrogenase, increased, although at low concentrations, the cytotoxicity varied greatly. When the concentrations of cigarette smoke condensate were more than 200 μg/mL, the toxic effect increase was dependent on the concentrations of the cigarette smoke condensate. Alonso *et al.* (27) reported that the lactate dehydrogenase in whole saliva could be useful as a biochemical marker of periodontal status. In that study, the values of salivary lactate dehydrogenase activity from individuals with periodontal disease were significantly higher than those obtained in people with a healthy periodontium.

In periodontal disease development, human gingival fibroblast migration plays an important role in cell attachment to the extracellular matrix (28). The control human gingival fibroblasts displayed a normal long spindle-shape with a parallel arrangement. However, the human gingival fibroblasts treated with cigarette smoke condensate produced a disordered alignment and some of the human gingival fibroblasts detached.

At low concentrations ($\leq$ 100 μg/mL) of cigarette smoke condensate, the collagen-degrading ability of the human gingival fibroblasts gradually increased with increases in the cigarette smoke condensate. The collagen degradation was limited to the area underneath the cell colonies, which suggests that the increased collagen cleavage was cell-membrane associated. In this study, the increase in the active form of MMP-2 and MMP-14 in the membrane extracts supports this point. Overall, an increase in MMP activity was observed. Although the family members involved differed, Nordskog *et al*. (29) showed that exposure of endothelial cells to cigarette smoke condensate resulted in the up-regulation of MMP-1, MMP-8 and MMP-9. Furthermore, Zhou *et al*. (13) reported that nicotine, an active agent in cigarette smoke condensate, could increase collagen degradation. Other studies have shown that nicotine could bind to the root surface in smokers and alter gingival and periodontal ligament fibroblast attachment and proliferation *in vivo* (30–33). Tipon & Dabbous (34) also suggested that nicotine itself may augment the destruction of the gingival extracellular matrix occurring during periodontal inflammation associated with smokeless tobacco use. However, the treatment of cardiac fibroblasts with nicotine leads to a decrease in collagenase activity (35). These contrasting results may be a result of the difference in the fibroblast type, the cigarette smoke condensate concentration and the treatment period, as well as the level of MMP production. In this study, there was a considerable increase in TIMP-1 in the conditioned media. This may be a result of the attempt to balance the increasing production of MMPs in the conditioned media.

In our study, TIMP-2 increased in the membrane extracts at higher concentrations of cigarette smoke condensate. This implies that a redistribution of TIMP-2 to the cell membrane may have occurred. These findings can be interpreted by the MMP-2 activation model proposed by Strongin *et al*. (36). In 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. This trimolecular complex activates proMMP-2 by the cleavage of its propeptide domain by MMP-14. Therefore, more TIMP-2 could be recruited to the cell membrane to enhance MMP-2 activation. In our studies, the active form of MMP-2 increased in the membrane extracts treated with cigarette smoke condensate, which suggests that this may have occurred.

The mechanism or mechanisms by which cigarette smoke condensate regulates the MMPs are currently unclear. Previous research has focused mainly on nicotine. Some studies have shown that nicotine can affect the production and distribution of gingival fibroblast cell-surface proteins (37) and the activation of latent MMP-2 by MMP-14 at higher concentrations (38). In our study, the human gingival fibroblasts exposed to cigarette smoke condensate had more rounded edges. This may imply that cigarette smoke condensate can also affect the cytoskeleton and/or cell-attachment receptors (38,39). Therefore, cigarette smoke condensate might cause the redistribution of cell-surface proteins, such as the integrins and the membrane type-MMPs, which could subsequently affect the activation of latent MMP-2 (13). As the cigarette smoke condensate is a complex mixture, it is difficult to say which components are involved. Future studies should be undertaken to investigate how the active components in cigarette smoke condensate influence the development of periodontal disease and the interaction of cigarette smoke condensate with bacteria, such as *P. gingivalis*.

In conclusion, cigarette smoke condensate is cytotoxic to human gingival fibroblasts at concentrations of $>$ 400 μg/mL and, at concentrations of $>$ 200 μg/mL, it affects the proliferation of human gingival fibroblasts. Cigarette smoke condensate may also destroy the balance and alter localization of the MMPs and TIMPs to promote the degradation of extracellular matrix, as seen in periodontal disease.

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