J Periodont Res 2009; 44: 386–394

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01114.x

Cigarette smoke condensate stimulates urokinase production through the generation of reactive oxygen species and activation of the mitogen activated protein kinase pathways in human gingival fibroblasts

R. Gonzalez[1], R. Arancibia[1], M. Cáceres[1], J. Martínez[1], P. C. Smith[2]

[1]Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile and [2]Faculty of Odontology, University of Chile, Santiago, Chile

Gonzalez R, Arancibia R, Cáceres M, Martínez J, Smith PC. Cigarette smoke condensate stimulates urokinase production through the generation of reactive oxygen species and activation of the mitogen activated protein kinase pathways in human gingival fibroblasts. J Periodont Res 2009; 44: 386–394. © 2009 The Authors. Journal compilation © 2009 Blackwell Munksgaard

Background and Objective: Tobacco smoking is a significant risk factor for periodontal disease. It has been suggested that smoking may alter connective tissue remodeling in the periodontium. In the present study, we investigated whether cigarette smoke condensate modulates the production of the serine protease urokinase in human gingival fibroblasts.

Material and Methods: Primary cultures of human gingival fibroblasts were stimulated with cigarette smoke condensate. Urokinase production was evaluated through casein zymography and western blotting. Plasmin activation was assessed by means of a radial diffusion assay. The roles of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and reactive oxygen species in cigarette smoke condensate-stimulated urokinase production were studied using distinct selective inhibitors (SP600125, PD98059, *N*-acetyl cysteine). Reactive oxygen species production was determined using a fluorometric assay. Activation of ERK and JNK pathways were evaluated using western blots.

Results: In gingival fibroblasts, cigarette smoke condensate potently stimulated urokinase production and plasmin activation. Cigarette smoke condensate-stimulated urokinase production was dependent on the activity of ERK/JNK pathways and was inhibited by the reactive oxygen species scavenger, *N*-acetyl cysteine. Cigarette smoke condensate strongly stimulated ERK and JNK phosphorylation and the generation of reactive oxygen species.

Patricio C. Smith, Faculty of Odontology, University of Chile, Olivos 943, Casilla 1903, Santiago, Chile
Tel: +56 2 978 1433
Fax: +56 2 201 4030
e-mail: patricio.smith@gmail.com

Key words: cigarette; mitogen-activated protein kinase; reactive oxygen species; urokinase; fibroblasts

Accepted for publication May 19, 2008

Conclusion: Cigarette smoke condensate stimulates urokinase production and plasmin activation in gingival fibroblasts. Moreover, cigarette smoke condensate-stimulated urokinase production depends on both the activation of ERK/JNK pathways and on the generation of intracellular reactive oxygen species. These results show that cigarette smoke may alter connective tissue remodeling by inducing production of the urokinase-type plasminogen activator through specific signaling pathways.

Tobacco smoking has been recognized as an important risk factor for the development and progression of periodontal disease (1,2). Smoking is associated with an increased risk for loss of periodontal attachment and bone destruction (1,2). Smoking also has negative effects on the response to surgical and nonsurgical periodontal therapy, regenerative procedures and dental implants (3,4).

Fibroblasts are the predominant cell type in gingival connective tissues and are actively involved in the formation and destruction of connective tissue in both health and disease (5). To achieve these functions, fibroblasts secrete a large repertoire of proteolytic enzymes and extracellular matrix glycoproteins and proteoglycans (5). Extracellular matrix is actively degraded by several groups of enzymes, which include the serine protease urokinase-type plasminogen activator. Urokinase-type plasminogen activator is able to convert plasminogen into plasmin, another serine protease with a wide spectrum of activity and responsible for the degradation of fibrin matrices and conversion of latent matrix metalloproteinases (MMPs) into their active forms (6,7). Urokinase-type plasminogen activator is expressed by gingival fibroblasts during wound healing and in periodontitis-affected gingival tissues (8–10). It has been observed that altered production of urokinase-type plasminogen activator may lead to aberrant wound repair or to the perpetuation of chronic inflammatory reactions in several diseases or conditions in which connective tissue remodeling plays a significant role (9,11,12). Therefore, factors regulating the production of urokinase-type plasminogen activator may critically modulate the evolution of tissue repair and inflammation.

The effects of smoking on periodontal tissues have been attributed to several mechanisms, including a decreased immune response (13), altered neutrophil function (14), decreased blood flow of gingival tissues (15) and an altered metabolism of molecular components of the extracellular matrix (16). These cellular effects also extend to other important functions of fibroblasts, such as cell adhesion, cell migration, cell proliferation and collagen synthesis (16–18). More recent studies have identified several effects of the components of nicotine and cigarette smoke on the activity and expression of extracellular matrix-degrading enzymes (19–21). Gingival fibroblasts exposed to nicotine show increased levels of collagenase activity (16). Neutrophil elastase activity is also increased in the gingival crevicular fluid of smokers when compared with that of nonsmokers (22). Moreover, nicotine may also stimulate the collagen-degrading activity of human gingival fibroblasts (21). Cigarette smoke contains more than 4000 different constituents, which could act individually or collectively as pathogenic agents for different diseases (23) The particulate phase, or cigarette smoke condensate, is composed of major toxicants such as nicotine, phenol, anthracyclic hydrocarbons, nitrosamines, heavy metals and chemical carcinogens, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Several previous studies have analysed the effect of isolated nicotine on gingival or periodontal ligament fibroblasts under cell culture conditions (16,17,24). However, the contribution of cigarette smoke to periodontal tissue damage has not been studied in detail.

Although urokinase-type plasminogen activator is a physiologically relevant source of extracellular proteolysis, studies evaluating the effect of cigarette smoke components on urokinase-type plasminogen activator expression or activity are limited. The present study was designed to evaluate the effects of cigarette smoke condensate on urokinase-type plasminogen activator production in primary cultures of human gingival fibroblasts. Because urokinase-type plasminogen activator production is tightly regulated by the mitogen-activated protein kinase pathways (9,25,26), we explored the role played by these signaling routes in cigarette smoke condensate-stimulated urokinase-type plasminogen activator production.

Material and methods

Cell culture

Primary cultures of human gingival fibroblasts were established using the explant method (27). Tissue explants were obtained from the retromolar tissue of two female and two male nonsmoking donors (average age 20 ± 3 years) undergoing extraction of third molars at a private dental practice in Santiago, Chile. Informed consent was obtained from all patients before biopsy was performed. The protocols utilized in this study were approved by the Ethics Committee of the Faculty of Dentistry of the University of Chile. No previous history of inflammation in the retromolar tissue was reported. No relevant pre-existing medical or drug histories were cited during the last 6 mo. Cells were cultured in alpha-minimal essential medium (Gibco Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL, Hyclone, Logan, UT, USA), 100 μg/mL of penicillin (Sigma, St Louis, MO, USA), 100 μg/

mL of streptomycin (Sigma) and 50 μg/mL of gentamycin (Sigma) at 37°C in a 5% CO_2 atmosphere. All experiments were performed using cells between the fourth and tenth passages.

Cigarette smoke condensate preparation

Cigarette smoke condensate (Murty Pharmaceutical, Lexington, KY, USA) was prepared using a Phipps-Bird 20-channel smoking machine. The particulate matter from a Kentucky standard cigarette 1R3F was collected on Cambridge glass fiber filters, and the amount obtained was determined by the weight increase of the filter. Cigarette smoke condensate thus prepared was dissolved in dimethylsulfoxide to give a final concentration of 4% (w/v). Aliquots of cigarette smoke condensate were kept at −80°C before experimental use.

Reactive oxygen species determination

Production of reactive oxygen species was detected using a fluorescence assay. Briefly, cells were loaded with 10 μM 2′,7′-dicholoro-dihydrofluorescein diacetate (DCDHF-DA; Gibco Invitrogen) in serum-free phenol-red-free medium (Gibco Invitrogen) for 30 min at 37°C. Then, cells were stimulated with cigarette smoke condensate and lysed with 0.1 N NaOH at different time points. Fluorescence was monitored using a microplate fluorometer (Spectra Max; Molecular Devices, Sunnyvale, CA, USA) using wavelengths of 480 and 530 nm for excitation and emission, respectively (28). DCDHF-DA is an uncharged cell-permeable molecule. Inside cells, this probe is cleaved by nonspecific esterases, forming carboxydichlorofluoroscein, which is oxidized in the presence of reactive oxygen species.

Casein zymography and radial diffusion assay

For these assays, cells were plated in 24-well plates (60,000 cells per each experimental condition). The day after plating, cells were left in serum-free medium and, after 18 h, were stimulated with the appropriate inhibitors and cigarette smoke condensate for 48 h. Control cells were stimulated with the vehicle (dimethylsulfoxide). Urokinase-type plasminogen activator-secreted activity of cell cultures was determined by caseinolytic zymography (29). Briefly, aliquots of culture medium, normalized for the protein content measured in the cell lysate, were subjected to electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel under nonreducing conditions. Sodium dodecyl sulfate was removed by extensive washing in 2.5% Triton X-100. To detect degradation of casein after plasminogen activation, gels were placed over a 1% agarose gel containing 0.5% casein and 1 μg/mL of plasminogen (Calbiochem, San Diego, CA, USA) and then incubated in a humidified chamber at 37ºC for 24 h. This two-step procedure allows the reduction, in a plasminogen-independent manner, of nonspecific bands that may degrade casein. Plasmin-dependent proteolysis was detected as a clear area in a white–blue field after staining with 2% $CuSO_4$. Quantification of these bands was performed by densitometric analysis. In selected experiments, cells were previously exposed to SP600125 (Biolmol, Plymouth Meeting, PA, USA) [a c-Jun N-terminal kinase (JNK) inhibitor, 1–5 μM for 30 min before stimulation with cigarette smoke condensate)], PD98059 (Sigma) (a mitogen-activated protein kinase kinase-1 inhibitor, 5–25 μM for 30 min before stimulation with cigarette smoke condensate) or *N*-acetyl cysteine (Sigma) (a reactive oxygen species quencher, 5 and 10 μM before stimulation with cigarette smoke condensate). A radial diffusion assay to study plasminogen activation was carried out in 1% agarose gels containing 0.5% casein and 2 μg/mL of plasminogen. In brief, aliquots of culture medium, normalized for the number of cells, were applied to holes previously punched in the gels and the gels were incubated at 37°C for 16 h. Gels lacking plasminogen were used as controls. The diameters of the radial zones of caseinolysis were measured using densitometric analysis (30).

Detection of mitogen-activated protein kinase activation through western blotting

Gingival fibroblasts were plated in six-well plates (400,000 cells per each experimental condition). The day after plating, cells were left in serum free-medium and then stimulated with cigarette smoke condensate at different time points. Then, cells were lysed in buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, in the presence of 2 mM phenylmethylsulfonyl fluoride (Sigma), 2 μg/mL of pepstatin (Sigma), 2 μg/mL of leupeptin (Sigma) and 1 mM sodium orthovanadate (Sigma), at 4ºC. The cell lysate was centrifuged (19,000 *g*, 10 min, 4ºC). Proteins were resolved by electrophoresis through a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to poly(vinylidene difluoride) transfer membranes (Perkin Elmer Life Sciences, Boston, MA, USA). The membranes were blocked by incubation in a Tris-buffered saline solution containing 5% nonfat dry milk and 0.1% Tween 20. Then, membranes were exposed to primary antibodies against extracellular signal-regulated kinase (ERK), phospho-ERK, phospho-JNK (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), JNK (Upstate Biotechnology, Lake Placid, NY, USA) and β-actin (Sigma). Membranes were washed for 5 min three times in Tris-buffered saline/0.1% Tween 20 before addition of the specific horseradish peroxidase-conjugated secondary antibody. The membranes were incubated for 1 h and washed three times, for 5 min each wash, in Tris-buffered saline/0.1% Tween 20. Binding was detected using an enhanced chemiluminescence kit (Perkin Elmer Life Sciences). The autoradiograms were scanned and analysed using molecular imaging software (Eastman Kodak Company,

Rochester, NY, USA) that can calculate relative quantity using the corresponding lane background signal for normalization.

Detection of urokinase-type plasminogen activator production through western blotting

To evaluate the production of urokinase-type plasminogen activator at the protein level, 1×10^6 cells were plated in 60-mm culture dishes and stimulated with cigarette smoke condensate or the appropriate inhibitors in the absence of serum. After 48 h the cell culture media (2 mL/treatment) were collected and concentrated up to 200 μL in an ultracentrifuge tube (Millipore, Bedford, MA, USA) at 1500 *g* for 20 min at 4ºC. Concentrated media were resolved by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel under reducing conditions and transferred to a poly(vinylidene difluoride) transfer membrane (Perkin Elmer Life Sciences). Membranes were exposed to primary antibodies against urokinase-type plasminogen activator (American Diagnostica, Temecula, CA, USA) and to secondary antibodies coupled to horseradish peroxidase, and then developed using an enhanced chemiluminescence kit (Perkin Elmer Life Sciences), as previously described.

Statistics

Statistical significance for each data set was tested using the Student's *t*-test, analysis of variance and the Kruskall–Wallis test, with the significance level set at $p < 0.05$.

Results

Cigarette smoke condensate stimulates urokinase-type plasminogen activator activity and plasmin generation in gingival fibroblasts

Human gingival fibroblasts were stimulated with a range of cigarette smoke condensate concentrations in serum-free alpha-minimal essential medium for 48 h, and cell culture media were analysed using casein zymography. As shown in Fig. 1A, cigarette smoke condensate stimulated a dose-dependent increase in urokinase-type plasminogen activator activity. This stimulus reached statistical significance after analysing this response in three independent experiments ($p < 0.05$; Fig. 1B). To identify whether this effect on the activity of urokinase-type plasminogen activator might induce plasmin activation, cell culture media derived from control and cigarette smoke condensate-stimulated cells were analysed using the radial diffusion assay. As shown in Fig. 1C,D, cigarette smoke condensate stimulated plasmin activation in the conditioned media of human gingival fibroblasts ($p < 0.05$).

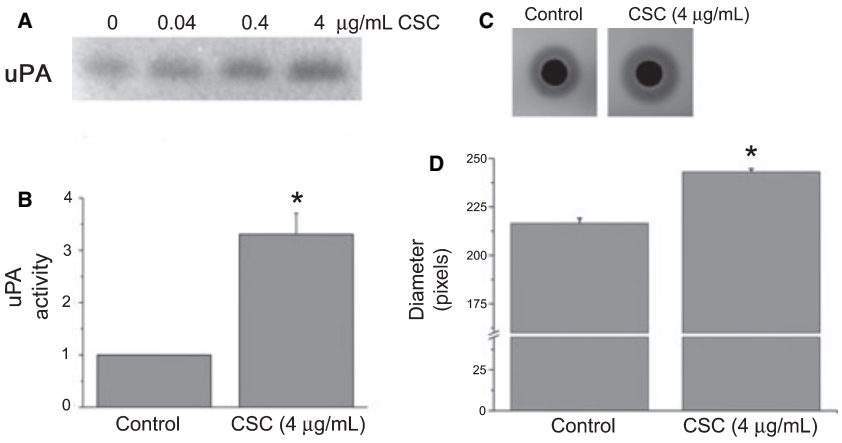

Fig. 1. Cigarette smoke condensate stimulates urokinase-type plasminogen activator production and plasmin activation in human gingival fibroblasts. (A) Serum-starved human gingival fibroblast cultures were stimulated with a range of cigarette smoke condensate concentrations (0.04–4 μg/mL). After 48 h, urokinase-type plasminogen activator activity present in the cell culture media was analysed using casein zymography, as described in the Material and methods. (B) A quantitative analysis of urokinase-type plasminogen activator bands was performed. Data obtained from three independent experiments were expressed as the average and standard error. Statistical analysis was performed using the Student's *t*-test. The asterisk indicates statistically significant differences. (C) Cell culture media derived from control and cigarette smoke condensate-stimulated cells (4 μg/mL), from three independent experiments, were analysed using the radial diffusion assay as described in the Material and methods. (D) A quantitative analysis of the plasmin-derived proteolytic activity was performed. Data were expressed as the average and standard error. The asterisk indicates statistically significant differences. CSC, cigarette smoke condensate; uPA, urokinase-type plasminogen activator.

Reactive oxygen species, stimulated by cigarette smoke condensate, regulate urokinase-type plasminogen activator production

To identify the generation of intracellular reactive oxygen species by cigarette smoke condensate, gingival fibroblasts were loaded with DCDHF-DA. Then, the cells were stimulated with 4 μg/mL of cigarette smoke condensate in serum-free medium and reactive oxygen species generation was detected in the cell lysate, as described in the Material and methods. After 30 min of stimulation with cigarette smoke condensate, we observed a statistically significant increase in the fluorescence activity detected in the cell lysate when compared with unstimulated cells ($p < 0.05$) (Fig. 2A). After this time point, a decrease in the generation of intracellular reactive oxygen species was detected up to 3 h of observation.

To identify the role of reactive oxygen species generation in cigarette smoke condensate-stimulated urokinase-type plasminogen activator production, gingival fibroblasts were incubated for 1 h with the reactive oxygen species quencher, *N*-acetyl cysteine, and then stimulated with 4 μg/mL of cigarette smoke condensate. After 48 h, the cell culture

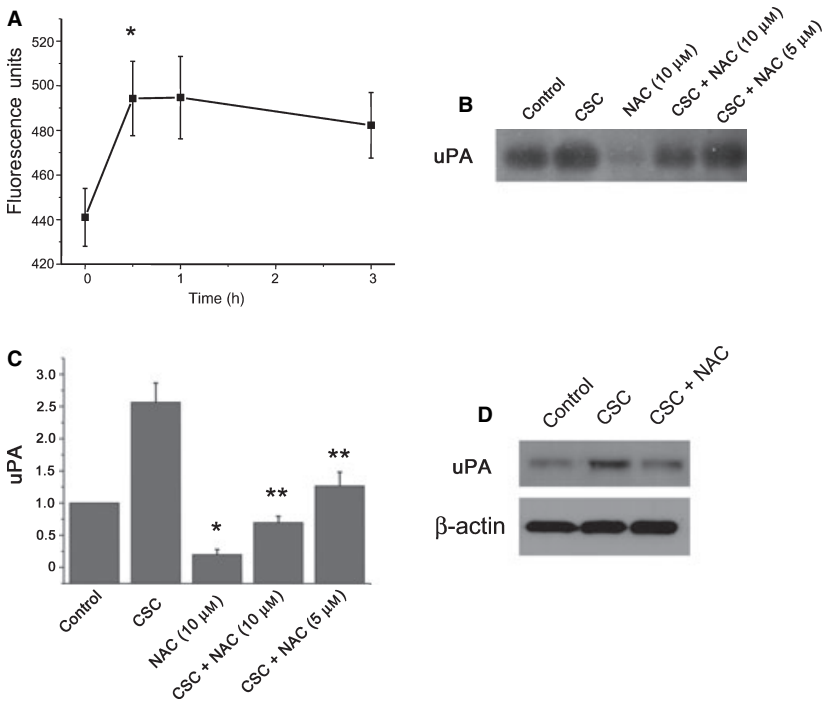

Fig. 2. Cigarette smoke condensate stimulates urokinase-type plasminogen activator production through the generation of reactive oxygen species. (A) Serum-starved gingival fibroblasts were stimulated with 4 μg/mL of cigarette smoke condensate and the production of reactive oxygen species was detected using a fluorescence assay, as described in the Material and methods. The asterisk indicates statistically significant differences between control and cigarette smoke condensate-stimulated cells. Data were derived from three independent experiments and are expressed as the average and standard error. (B) Serum-starved gingival fibroblasts were incubated in the presence of 5 and 10 μM *N*-acetyl cysteine and consequently stimulated with 4 μg/mL of cigarette smoke condensate. After 48 h, cell culture media were analysed using casein zymography. (C) A quantitative analysis of urokinase-type plasminogen activator bands, displayed in (B), was performed. Data from three independent experiments were expressed as the average and standard error. *, statistically significant differences between control and *N*-acetyl cysteine-treated cells ($p < 0.05$). **, statistically significant differences between *N*-acetyl cysteine + cigarette smoke condensate and cigarette smoke condensate-stimulated cells. (D) Serum-starved gingival fibroblasts were incubated in the presence of 10 μM *N*-acetyl cysteine and consequently stimulated with 4 μg/mL of cigarette smoke condensate. After 48 h, the presence of urokinase-type plasminogen activator in the cell culture media were determined by western blotting, as described in the Material and methods. β-actin, determined in the cell lysate by western blotting, was used as a loading control. CSC, cigarette smoke condensate; NAC, *N*-acetyl cysteine; uPA, urokinase-type plasminogen activator.

media were analysed using casein zymography. In accordance with the results observed in Fig. 1A, cigarette smoke condensate induced a significant increase in urokinase-type plasminogen activator activity (Fig. 2B,C). As shown in Fig. 2B,C, in the absence of cigarette smoke condensate stimulation, 10 μM *N*-acetyl cysteine induced a statistically significant decrease in urokinase-type plasminogen activator activity when compared with control cells ($p < 0.05$). When cells were stimulated with 4 μg/mL of cigarette smoke condensate, both 5 and 10 μM *N*-acetyl cysteine induced a statistically significant decrease in urokinase-type plasminogen activator activity when compared with cigarette smoke condensate-stimulated cells ($p < 0.05$). To confirm the role of reactive oxygen species in cigarette smoke condensate-stimulated urokinase-type plasminogen activator production, cells were exposed to 10 μM *N*-acetyl cysteine and the production of urokinase-type plasminogen activator was assessed in the cell culture media through western blotting. As demonstrated in Fig. 2D, cigarette smoke condensate induced an increase in urokinase-type plasminogen activator production that was effectively inhibited by *N*-acetyl cysteine.

ERK and JNK activities regulate cigarette smoke condensate-stimulated urokinase-type plasminogen activator production in gingival fibroblasts

To identify the role of the ERK and JNK pathways in cigarette smoke condensate-stimulated urokinase-type plasminogen activator production, serum-starved gingival fibroblasts were exposed to a range of concentrations of the selective inhibitors for mitogen-activated protein kinase kinase-1 (PD98059; 10–30 μM) and JNK (SP600125; 1–5 μM). After 30 min of incubation with the above-mentioned inhibitors, gingival fibroblasts were stimulated with 4 μg/mL of cigarette smoke condensate for 48 h and the cell culture was analysed using casein zymography. In the absence of cigarette smoke condensate stimulation, treatment with 5 μM SP600125 was able to inhibit urokinase-type plasminogen activator production at statistically significant levels ($p < 0.05$). However, 30 μM PD98059 did not affect the basal urokinase-type plasminogen activator level at significant levels (Fig. 3A–C). Cigarette smoke condensate-stimulated urokinase-type plasminogen activator production was inhibited by 30 μM PD98059 and by 5 and 2.5 μM SP600125 ($p < 0.05$) (Fig. 3B,C).

To confirm the role of the ERK and JNK pathways in cigarette smoke condensate-stimulated urokinase-type plasminogen activator production, cells were exposed to 5 μM SP600125 and 30 μM PD98059 and urokinase-type plasminogen activator production was assessed in the conditioned media by western blotting. As demonstrated in Fig. 3D, cigarette smoke condensate induced an increase in urokinase-type plasminogen activator production that was down-regulated by both inhibitors.

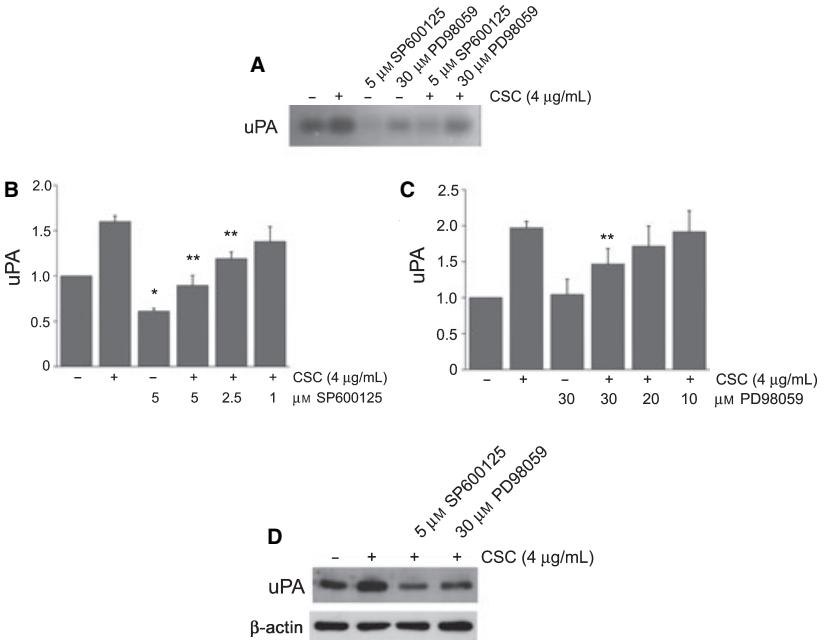

Fig. 3. Mitogen-activated protein kinase kinase-1 and c-Jun N-terminal kinase inhibitors interfere with the production of cigarette smoke condensate-stimulated urokinase-type plasminogen activator. (A) Serum-starved gingival fibroblasts were incubated in the presence of SP600125 or PD98059 and subsequently stimulated with 4 μg/mL of cigarette smoke condensate. Conditioned media derived from each experimental condition were analysed using casein zymography, as described in the Material and methods. Serum-starved gingival fibroblasts were incubated in the presence of a range of concentrations of SP600125 (1–5 μM) (B) or PD98059 (10–30 μM) (C) and subsequently stimulated with 4 μg/mL of cigarette smoke condensate. Conditioned media derived from each experimental condition were analysed using casein zymography. Caseinolytic bands were digitalized and quantified, and are expressed in the graphs as the average and standard error. Data were obtained from three independent experiments. *, statistically significant differences between control cells and SP600125-treated cells ($p < 0.05$). **, statistically significant differences between cigarette smoke condensate and SP600125-treated or PD98059-treated cells ($p < 0.05$). (D) Serum-starved gingival fibroblasts were incubated in the presence of 5 μM SP600125 or 30 μM PD98059 and consequently stimulated with 4 μg/mL of cigarette smoke condensate. After 48 h, the presence of urokinase-type plasminogen activator in the conditioned media was determined using western blotting, as described in the Material and methods. β-actin, determined in the cell lysate using western blot, was used as a loading control. CSC, cigarette smoke condensate; uPA, urokinase-type plasminogen activator.

Cigarette smoke condensate stimulates ERK and JNK activation in gingival fibroblasts

To identify whether cigarette smoke condensate may stimulate the activation of the ERK and JNK pathways, serum-starved gingival fibroblasts were treated with 4 μg/mL of cigarette smoke condensate and phosphorylation of the above-mentioned kinases was evaluated at several time points by western blotting. As shown in Fig. 4A, cigarette smoke condensate induced a rapid increase in the phosphorylation of ERK, which lasted between 15 and 60 min of stimulation. In the case of JNK, cigarette smoke condensate induced the phosphorylation of JNK after 5 min of stimulation and this response was observed for up to 180 min of exposure to this compound.

Discussion

Regulation of urokinase-type plasminogen activator production is a critical step in the turnover of extracellular matrix proteins (31). Tobacco smoking is considered to be a significant risk factor for periodontal disease progression and also adversely affects the host response to periodontal therapy. Considering this evidence, we analysed whether cigarette smoke condensate may modulate urokinase-type plasminogen activator production in gingival fibroblasts. To our knowledge, the present study is the first to show that urokinase-type plasminogen activator production and plasmin activation are significantly stimulated by cigarette smoke condensate in primary cultures of human gingival fibroblasts. Our results also show that this effect is mediated by variations in the intracellular redox state of gingival fibroblasts and through the activation of the ERK1/2 and JNK signaling pathways.

Increased expression of urokinase-type plasminogen activator has been associated with aberrant or altered tissue repair (32,33). Periodontal tissue destruction has also been attributed to increased levels of tissue-degrading enzymes, such as serine proteases and MMPs (34,35). The present study shows that cigarette smoke condensate may stimulate urokinase-type plasminogen activator production in gingival fibroblasts. We also observed that urokinase-type plasminogen activator production was associated with the generation of active plasmin. Several *in vitro* studies have evaluated whether nicotine or cigarette smoke condensate may stimulate the production of proteolytic enzymes in different cell types. Nicotine stimulates the expression of MMPs in arterial smooth muscle cells (20) and cigarette smoke condensate increases MMP levels in vascular cells (19,36). In lung epithelial cells and fibroblasts, cigarette smoke condensate stimulates the production of several MMP members, such as MMP-1 and MMP-14 (37,38). Stimulation of gingival fibroblasts with nicotine increases collagenase secretion (16) and the ability of these cells to degrade collagen matrices (21), suggesting that cigarette components may stimulate extracellular matrix remodelling. Plasmin has been identified as a potent source of MMP activation (7). Therefore, it could be proposed that cigarette smoke condensate-stimulated urokinase-type plasminogen activator production may constitute the first step of an amplifying signal that, by means of plasmin generation, might activate

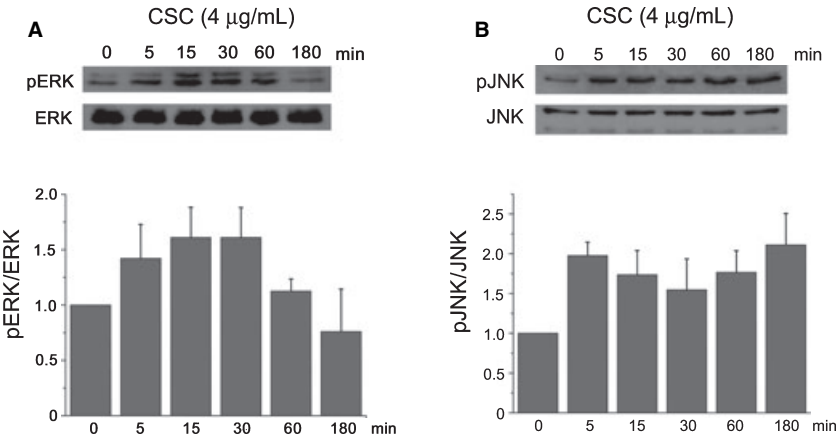

Fig. 4. Cigarette smoke condensate stimulates extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activation in gingival fibroblasts. (A) Serum-starved gingival fibroblasts were stimulated with 4 μg/mL of cigarette smoke condensate, and phosphorylated forms of both ERK and JNK were estimated in the cell lysate using western blotting, as described in the Material and methods. As a loading control, the presence of total ERK and JNK was determined in the cell lysate. (B) After image analysis, phosphorylated ERK and phosphorylated JNK bands were normalized against total ERK or JNK bands and expressed as the average and standard error. These data were derived from three independent experiments (B). CSC, cigarette smoke condensate; pERK, phosphorylated ERK; pJNK, phosphorylated JNK; uPA, urokinase-type plasminogen activator.

other proteolytic events in smoke-affected gingival tissues.

A significant finding of the present study is that cigarette smoke condensate stimulates the generation of reactive oxygen species at the intracellular level. Reactive oxygen species act as cellular messengers in diverse processes such as signal transduction, cell adhesion, gene expression, senescence and apoptosis (39). Both protein tyrosine kinases and protein tyrosine phosphatases are regulated by reactive oxygen species. Transient oxidation of thiols in protein tyrosine phosphatases leads to inhibition of these enzymes. Conversely, oxidation of protein tyrosine kinases leads to their activation by direct thiol group (SH) modification (40). Therefore, an increase in reactive oxygen species production after stimulation with cigarette smoke condensate might alter the balance between protein tyrosine kinases and protein tyrosine phosphatases, leading to activation of diverse signaling pathways. Our observations provide evidence that upon exposure to cigarette smoke condensate, both ERK and JNK pathways are activated. Previous studies have identified that cigarette smoke condensate may stimulate diverse signaling pathways, including ERK, nuclear factor-κB and early growth response factor-1, in epithelial cells and fibroblasts derived from the lung (37,41,42) Our results show that cigarette smoke condensate may stimulate the generation of reactive oxygen species and the activation of specific members of the mitogen-activated protein kinases to regulate urokinase-type plasminogen activator production in gingival fibroblasts.

Intracellular reactive oxygen species have been implicated in urokinase-type plasminogen activator production in carcinoma cells and human fetal membranes (43,44). In gingival fibroblasts and gastric cancer cells, exposure to H_2O_2 stimulates urokinase-type plasminogen activator expression, demonstrating that reactive oxygen species are involved in urokinase-type plasminogen activator production in these cells (45,46). It is well known that urokinase-type plasminogen activator production is tightly regulated at the transcriptional level. The urokinase-type plasminogen activator promoter contains an activator protein-1 (AP-1) enhancer element that is known to respond to diverse extracellular stimuli (47). AP-1 molecular components correspond to murine osteosarcoma viral oncogene (c-Fos) and c-Jun, and both of these transcription factors may associate to regulate urokinase-type plasminogen activator production (25). Previous studies have identified that both ERK and JNK pathways may also regulate urokinase-type plasminogen activator production in different cell lines and primary cultures (26,48,49). In the case of human gingival fibroblasts, we identified that urokinase-type plasminogen activator production, stimulated by epidermal growth factor and transforming growth factor-β, is dependent on the ERK and JNK pathways (9,50). The results of the present study, obtained under the stimulus of cigarette smoke condensate, confirm the role of ERK and JNK activity in urokinase-type plasminogen activator production. In the case of ERK activity, our results suggest that this pathway is mostly involved in the cigarette smoke condensate-stimulated response, in view of the fact that the higher concentration of PD98059 utilized did not affect the basal urokinase-type plasminogen activator production. By contrast, using SP600125, both basal and cigarette smoke condensate-stimulated urokinase-type plasminogen activator production were inhibited. Further studies should be carried out to establish the specific role of these signaling pathways in the control of urokinase-type plasminogen activator production in gingival fibroblasts.

In conclusion, the present study is the first to report that cigarette smoke components may stimulate urokinase-type plasminogen activator production in a reactive oxygen species and mitogen-activated protein kinase-dependent manner *in vitro*. The above-described pathways should be considered as potential targets to control the effects of cigarette smoke in gingival cells or tissues.

Acknowledgements

This work was totally financed by a grant from the National Fund for Science and Technology (FONDECYT, Chile) to P. S. (# 1061065).

References

1. Tomar SL, Asma S. Smoking-attributible periodontitis in the United States. Findings from NHANES III. National Health and Nutritional Examination Survey. *J Periodontol* 2000;**71:**743–751.
2. Bergstrom J. Tobacco smoking and risk for periodontal disease. *J Clin Periodontol* 2003;**30:**107–113.
3. Johnson GK, Guthmiller JM. The impact of cigarette smoking on periodontal disease and treatment. *Periodontol 2000* 2007;**44:**178–194.
4. Strietzel FP, Reichart PA, Kale A, Kulkarni M, Wegner B, Küchler I. Smoking interferes with the prognosis of dental implant treatment: a systematic review and meta-analysis. *J Clin Periodontol* 2007;**34:**523–544.
5. Bartold PM, Walsh LJ, Narayanan AS. Molecular and cell biology of the gingiva. *Periodontol 2000* 2000;**24:**28–55.
6. Dano K, Andreasen PA, Grondhal-Hansen K, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation and cancer. *Adv Cancer Res* 1985;**44:**139–266.
7. Birkedal-Hansen H, Moore H, Bodden MK *et al*. Matrix metalloproteinases. A review. *Crit Rev Oral Biol Med* 1993;**4:**197–250.
8. Xiao Y, Bunn CL, Bartold PM. Immunohistochemical demonstration of the plasminogen activator system in human gingival tissues and gingival fibroblasts. *J Periodontal Res* 1998;**33:**17–26.
9. Smith PC, Martínez J. Differential uPA expression by TGF-β1 in gingival fibroblasts. *J Dent Res* 2006;**85:**150–155.
10. Kinnby B, Lindberg P, Lecander I, Matsson L. Localization of plasminogen activators and plasminogen inhibitors in human gingival tissues by immunohistochemistry and in situ hybridization. *Arch Oral Biol* 1999;**44:**1027–1034.
11. Schäfer BM, Maier K, Eickhoff U, Todd RF, Kramer MD. Plasminogen activation in healing human wounds. *Am J Pathol* 1994;**144:**1269–1280.
12. Weckroth M, Vaheri A, Myöhänen H, Tukiainen E, Sirén V. Differential effects of acute and chronic wound fluids on urokinase-type plasminogen activator, urokinase-type plasminogen activator receptor, and tissue-type plasminogen activator in cultured human keratinocytes and fibroblasts. *Wound Repair Regen* 2001;**9:**314–322.
13. Graswinckel JE, van der Velden U, van Winkelhoff AJ, Hoek FJ, Loos BG. Plasma antibody levels in periodontitis patients and controls. *J Clin Periodontol* 2004;**31:**562–568.
14. Guntsch A, Erler M, Preshaw PM, Sigusch BW, Klinger G, Glockmann E. Effect of smoking on crevicular polymorphonuclear neutrophil function in periodontally healthy subjects. *J Periodontal Res* 2006;**41:**184–188.
15. 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.
16. 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.
17. Gamal AY, Bayomy MM. Effect of cigarette smoking on human PDL fibroblasts attachment to periodontally involved root surfaces *in vitro*. *J Clin Periodontol* 2002;**29:**763–770.
18. Tanur E, McQuade MJ, McPherson JC, AlHashimi IH, Rivera-Hidalgo F. Effects of nicotine on the strength of attachment of gingival fibroblasts to glass and non-diseased human root surfaces. *J Periodontol* 2000;**71:**717–722.
19. Nordskog BK, Blixt AD, Morgan WT, Fields WR, Hellman GM. Matrix degrading and pro-inflammatory changes in human vascular endothelial cells exposed to cigarette smoke condensate. *Cardiovasc Toxicol* 2003;**3:**101–117.
20. 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.
21. Zhou JB, Olson L, Windsor J. Nicotine increases the collagen-degrading ability of human gingival fibroblasts. *J Periodontal Res* 2007;**42:**228–235.
22. Soder B. Neutrophil elastase activity, levels of prostaglandin E2 and matrix metalloproteinase-8 in refractory periodontitis sites in smokers and non-smokers. *Acta Odontol Scand* 1999;**57:**77–82.
23. Hoffmann D, Wynder EL. Active and passive smoking. In: Marquardt H, Schaefer SG, McClellan R, Welsch F, eds. *Toxicology*. New York: Academic Press, 1999: 879–898.
24. Giannopoulou C, Geinoz A, Cimasoni G. Effects of nicotine on periodontal ligament fibroblasts *in vitro*. *J Clin Periodontol* 1999;**26:**49–55.
25. Aguirre-Guiso JA, Alonso DF, Farias EF, Gomez DE, de Kier Joffè EB. Deregulation of signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. *Eur J Biochem* 1999;**263:**295–304.
26. Parra M, Lluis F, Miralles F, Caelles C, Muñoz-Cánoves P. The cJun N-terminal kinase (JNK) signaling pathway mediates induction of urokinase-type plasminogen activator (uPA) by the alkylating agent MNNG. *Blood* 2000;**96:**1415–1424.
27. Larjava H, Heino J, Kähäri V-M, Vuorio E. Characterization of one phenotype of human periodontal granulation-tissue fibroblasts. *J Dent Res* 1989;**68:**20–25.
28. Benhar M, Dalyot I, Engelberg D, Levitzki A. Enhanced ROS production in oncogenically transformed cells potentiates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation and sensitization to genotoxic stress. *Mol Cell Biol* 2001;**20:**6913–6926.
29. Martinez J, Fuentes M, Cambiazo V, Santibañez JF. Bone extracellular matrix stimulates invasiveness of estrogen-responsive human mammary MCF-7 cells. *Int J Cancer* 1999;**83:**278–282.
30. Santibañez JF, Maccioni RB, Martínez J. The secretion of urokinase-like plasminogen activator is inhibited by microtubule-interacting drugs. *Cell Biochem Funct* 1995;**13:**217–225.
31. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. *J Clin Invest* 1991;**88:**381–389.
32. Wysocki AB, Kusakabe AO, Chang S, Tuan TL. Temporal expression of urokinase plasminogen activator, plasminogen activator inhibitor and gelatinase-B in chronic wound fluid switches from a chronic to acute wound profile with progression to healing. *Wound Repair Regen* 1999;**7:**154–165.
33. Rogers AA, Burnett S, Lindholm C *et al*. Expression of tissue-type and urokinase-type plasminogen activator activities in chronic venous leg ulcers. *Vasa* 1999;**28:**101–105.
34. Olofsson A, Lindberg P, Lanke J, Matsson L, Kinnby B. Relationship between fibrinolytic activity and gingival inflammatory reaction in young individuals. *J Periodontal Res* 2003;**38:**104–108.
35. Beklen A, Tüter G, Sorsa T *et al*. Gingival tissue and crevicular fluid co-operation in adult periodontitis. *J Dent Res* 2006;**85:**59–63.
36. Wright JL, Tai R, Wang R, Wang X, Chung A. Cigarette smoke upregulates pulmonary vascular matrix metalloproteinases via TNF-α signaling. *Am J Physiol Lung Cell Mol Physiol* 2007;**292:**L125–L133.
37. Kim H, Liu X, Kohyama T *et al*. Cigarette smoke stimulates MMP-1 production by human lung fibroblasts through the ERK1/2 pathway. *COPD* 2004;**1:**13–23.
38. Ning W, Dong Y, Sun J *et al*. Cigarette smoke stimulates matrix metalloproteinase-2 activity via EGR-1 in human lung fibroblasts. *Am J Respir Cell Mol Biol* 2007;**36:**480–490.
39. D'Autreaux B, Toledano MB. ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 2007;**8:**813–824.

40. Chiarugi P. PTPs versus PTKs: The redox side of the coin. *Free Radic Res* 2005; **39:**353–364.
41. Hellermann GR, Nagy SB, Kong X, Lockey RF, Mohapatra SS. Mechanism of cigarette smoke condensate-induced inflammatory response in human bronchial epithelial cells. *Respir Res* 2002;**3:** 22–29.
42. Mercer BA, Kolesnikova N, Soneto J, D'Armiento J. Extracellular regulated kinase/mitogen activated protein kinase is up-regulated in pulmonary emphysema and mediates matrix metalloproteinase-1 induction by cigarette smoke. *J Biol Chem* 2004;**279:** 17690–17296.
43. Kiguchi T, Niiya K, Shibakura M *et al*. Induction of urokinase-type plasminogen activator by the anthracycline antibiotic in human RC-K8 lymphoma and H69 lung-carcinoma cells. *Int J Cancer* 2001;**93:**792–797.
44. Lappas M, Permezel M, Rice GE. *N*-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-kappaB deoxyribonucleic acid-binding activity in human fetal membranes *in vitro*. *J Clin Endocrinol Metab* 2003;**88:**1723–1729.
45. Kim MH, Cho HS, Jung M *et al*. Extracellular signal-regulated kinase and AP-1 pathways are involved in reactive oxygen species-induced urokinase plasminogen activator receptor expression in human gastric cancer cells. *Int J Oncol* 2005;**26:**1669–1674.
46. Tanaka F, Ogura N, Abiko Y. Stimulation of plasminogen activator/plasmin system in gingival fibroblast cells by oxygen radicals. *Arch Oral Biol* 1997;**42:**263–270.
47. Besser D, Presta M, Nagamine Y. Elucidation of a signaling pathway induced by FGF-2 leading to uPA gene expression in NIH 3T3 fibroblasts. *Cell Growth Differ* 1995;**6:**1009–1017.
48. Witowsky J, Abell A, Johnson NL, Johnson GL, Cuevas BD. MEKK1 is required for inducible urokinase-type plasminogen activator expression. *J Biol Chem* 2003;**278:**5941–5946.
49. Benasciutti E, Pagès G, Kenzior O, Folk W, Blasi F, Crippa MP. MAPK and JNK transduction pathways can phosphorylate Sp1 to activate the uPA minimal promoter element and endogenous gene transcription. *Blood* 2004;**104:**256–262.
50. Smith PC, Santibañez JF, Morales JP, Martinez J. Epidermal growth factor stimulates urokinase-type plasminogen activator expression in human gingival fibroblasts. Possible modulation by genistein and curcumin. *J Periodontal Res* 2004;**39:**380–387.