

Cigarette smoke condensate inhibits collagen gel contraction and prostaglandin E₂ production in human gingival fibroblasts

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Background: Granulation tissue remodeling and myofibroblastic differentiation are critically important events during wound healing. Tobacco smoking has a detrimental effect in gingival tissue repair. However, studies evaluating the effects of cigarette smoke on these events are lacking.

Material and Methods: We used gingival fibroblasts cultured within free-floating and restrained collagen gels to simulate the initial and final steps of the granulation tissue phase during tissue repair. Collagen gel contraction was stimulated with serum or transforming growth factor- β 1. Cigarette smoke condensate (CSC) was used to evaluate the effects of tobacco smoke on gel contraction. Protein levels of alpha-smooth muscle actin, β 1 integrin, matrix metalloproteinase-3 and connective tissue growth factor were evaluated through Western blot. Prostaglandin E₂ (PGE₂) levels were determined through ELISA. Actin organization was evaluated through confocal microscopy.

Results: CSC reduced collagen gel contraction induced by serum and transforming growth factor- β 1 in restrained collagen gels. CSC also altered the development of actin stress fibers in fibroblasts cultured within restrained collagen gels. PGE₂ levels were strongly diminished by CSC in three-dimensional cell cultures. However, other proteins involved in granulation tissue remodeling and myofibroblastic differentiation such as alpha-smooth muscle actin, β 1 integrin, matrix metalloproteinase-3 and connective tissue growth factor, were unmodified by CSC.

Conclusions: CSC may alter the capacity of gingival fibroblasts to remodel and contract a collagen matrix. Inhibition of PGE₂ production and alterations of actin stress fibers in these cells may impair proper tissue maturation during wound healing in smokers.

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Gingival and periodontal wound healing are significantly affected by tobacco smoking. Therefore, the clinical

outcome of periodontal therapy is profoundly modified by tobacco smoking (1–3). Although several

advances have occurred in this field (4,5), the cellular and molecular mechanisms underlying the poor

healing responses observed in smokers remain poorly understood.

Granulation tissue development is a fundamental event necessary to achieve proper wound healing (6). During the initial steps of granulation tissue formation, fibroblasts migrate from the borders of the initial lesion to populate the wound (7). During tissue repair, fibroblasts differentiate into a specific phenotype known as myofibroblasts (8). These cells express alpha-smooth muscle actin (α -SMA), an actin isoform that enhances cell contraction facilitating tissue remodeling (9,10). Another important feature that characterizes myofibroblasts is the formation of enhanced focal adhesions enriched in β 1-integrins (11). Myofibroblastic differentiation is controlled by the mechanical tension exerted by the remodeling matrix and by cytokines and growth factors present in the wound environment (11). Transforming growth factor-beta1 (TGF- β 1) is a growth factor that stimulates myofibroblastic differentiation through the induction of the myofibroblast marker α -SMA (12) and facilitates the remodeling and contraction of the extracellular matrix (13). Myofibroblastic differentiation and granulation tissue contraction are events controlled by different molecules that may be present in the wound environment. These molecular agents may be released by fibroblasts that secrete growth factors and cytokines involved in the modulation of myofibroblastic differentiation and granulation tissue contraction. This group of molecules includes prostaglandin E₂ (PGE₂), connective tissue growth factor (CTGF) and matrix metalloproteinase-3 (MMP-3) (14–17).

It has been proposed that cigarette smoke may impair wound healing affecting cell migration and myofibroblastic differentiation (18–22). However, most of these studies have been performed using cells cultured over two-dimensional cell culture plates. Culture of cells within collagen gels allows a more physiologically relevant cell culture system, as cells are able to receive signals in a tissue-like environment (23). Therefore, it is tempting to

speculate whether cigarette smoke may alter cell responses in a three-dimensional cell culture environment. In the present study, we have assessed whether cigarette smoke condensate (CSC) may alter the remodeling of collagen gels populated with human gingival fibroblasts (GFs). We have also evaluated if CSC may alter the production of several mediators involved in granulation tissue maturation and contraction, including CTGF, MMP-3 and PGE₂.

Material and methods

Cell culture

Primary cultures of human GFs were obtained by the explant method (24). Tissue explants were harvested from the retromolar tissue of four male adult individuals undergoing the extraction of third molars at the institutional clinical practice, School of Dentistry, Pontificia Universidad Católica de Chile. Tissue samples were obtained with the informed consent of the patients. The Ethical Committee, Faculty of Medicine, Pontificia Universidad Católica de Chile approved the protocol for obtaining tissue. No history of inflammation of the retromolar tissue was reported. Patients did not report relevant pre-existing medical or drug histories during the last 6 mo. Smokers were excluded from this study. Cells were cultured in α -minimal essential medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT, USA) and Normocin (Invivogen, San Diego, CA, USA) at 37°C in a 5% CO₂ atmosphere. All experiments were performed using cells expanded between passages 4 and 10.

Three-dimensional cell cultures

Type I collagen solution was obtained from Sprague–Dawley rat tail tendons as previously described (25). GF were embedded within a collagen solution (1 mg/mL) and neutralized with 4× Dulbecco's minimal essential medium. After this, the col-

lagen cell mixture was incubated at 37°C for 1 h to allow complete polymerization. Both restrained and floating collagen gels were generated according to Grinnell *et al.* (26). To generate restrained matrices, gels were maintained attached to the borders and bottom of the cell culture dish for 24 h in the presence of 10% FBS. After this, gels were released from the cell culture dish by gentle manipulation using a spatula. Floating gels were generated through gentle release of their attachment from the cell culture plate straight after polymerization. After this, stressed or floating gels were exposed to 1 or 10% FBS, 10 ng/mL TGF- β 1, CSC (50, 100 or 150 μ g/mL) (Murty Pharmaceuticals, Lexington, KY, USA) or cytochalasin D (Sigma, St. Louis, MO, USA). To determine the extent of collagen contraction, samples were photographed using a digital camera (Fujifilm finepix JZ500; Cypress, CA, USA). Collagen gel contraction was determined as the percentage of the area occupied by the gel at the end of the experiment compared to the total area of the cell culture dish. Collagen gel areas were calculated using the IMAGE J software provided by the National Institutes of Health (NIH, Bethesda, MD, USA).

Western blotting

To extract proteins from fibroblasts cultured within the collagen cultures, gels were washed with ice-cold phosphate buffered saline and lysed in a buffer containing 60 mM Tris HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 1 mM dithiothreitol and 0.01% bromophenol blue at 4°C. To facilitate protein extraction from the gels, samples were heated at 95°C for 5 min. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride transfer membrane (PerkinElmer Life Sciences, Boston, MA, USA). Membranes were exposed to primary antibodies against α -SMA clone 1A4, catalog number A2547 (Sigma), CTGF catalog number sc-14939 (Santa Cruz Biotechnology Inc., Santa

Cruz, CA, USA), MMP-3 catalog number MAB3369 (Millipore Corporation, Billerica, MA, USA), β 1 integrin catalog number sc-59827 (Santa Cruz Biotechnology) and secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA). These samples were then developed. As α -SMA and integrins are components of the cell cytoskeleton, their protein levels were analyzed in the proteins extracted from cell lysates. Given that MMP-3 and CTGF are secreted proteins, we determined their levels through Western blot of the conditioned media.

Lactate dehydrogenase release assessment

To estimate the amount of viable cells at the end of the experiments, cell culture medium was collected to assess lactate dehydrogenase (LDH) release due to cell death. Fifty μ L of medium was transferred to a 96-well plate and mixed with 50 μ L of the LDH substrate mix (Cytotox 96 non-radioactive cytotoxicity assay; Promega, Madison, WI, USA). The plate was then covered and incubated for 30 min at room temperature and protected from light. Fifty μ L of stop solution was then added and the absorbance at 490 nm was registered. These data were used to normalize the amount of cell lysate analyzed through Western blot in Fig. 3 and in ELISA assays in Fig. 4 (data not shown).

ELISA assays

Protein levels for inflammatory mediators were detected in the cell culture media using an ELISA assay for PGE₂ (Life Technologies, Carlsbad, CA, USA).

Confocal microscopy

Collagen gels were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton. F-actin was stained with Alexa Fluor 568 phalloidin (Invitrogen Molecular Probes, Carlsbad, CA, USA) and

nuclei detected using DAPI staining (Invitrogen Molecular Probes). Confocal microscopy was performed using a Nikon C1 Plus (Nikon, Japan) confocal microscope equipped with 409, 488 and 543 nm laser lines. Images were obtained using a 40 \times oil immersion objective (1.3 NA).

Statistical analysis

All experiments were carried out in triplicate and every experiment was repeated three or more times. Data points and error bars in the figures represent averages and standard errors. Where error bars cannot be seen, the data points overlapped. For statistical comparison between conditions under observation, the ANOVA test followed by the Bonferroni *post-hoc* test was used. The GRAPHPAD

PRISM (La Jolla, CA, USA) software was used. Statistical significance was set at $p < 0.05$.

Results

Cigarette smoke condensate inhibits collagen gel contraction

To evaluate the effects of CSC on collagen gel contraction, fibroblasts were cultured within restrained collagen gels and stimulated with 1% FBS for 24 h. FBS 10% was used as a positive control of gel contraction. Collagen gels were also stimulated with CSC (50, 100 and 150 μ g/mL) or 10 μ M cytochalasin D. These CSC concentrations were selected from a previous study in which we determined that GF cell viability is not affected by these range of concentra-

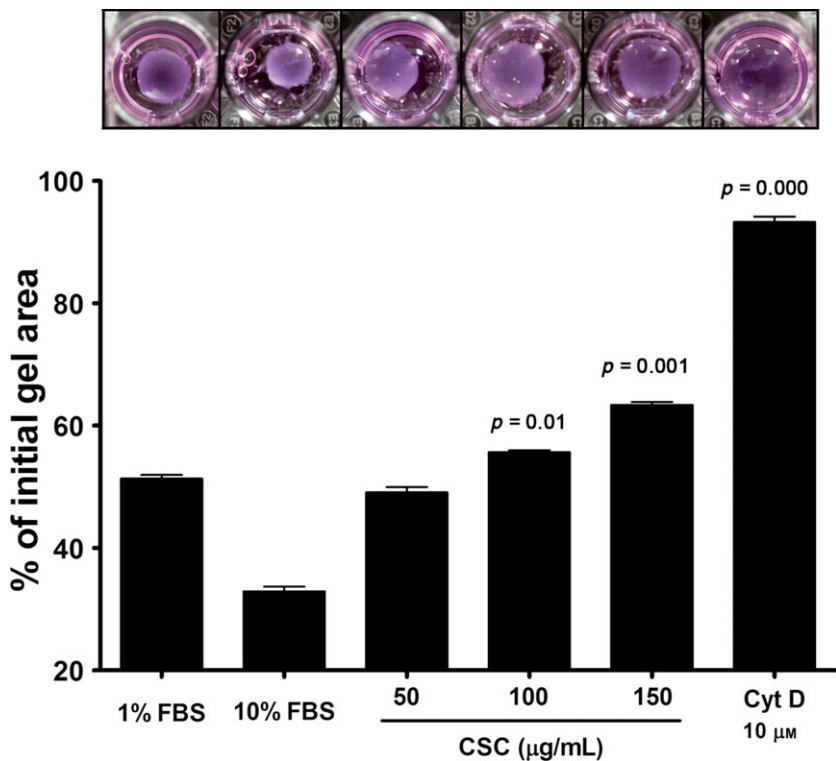


Fig. 1. Effects of CSC on FBS-stimulated collagen gel contraction. Human gingival fibroblasts were cultured within restrained collagen gels. After polymerization, gels were incubated in 1% FBS to stimulate contraction, and exposed to 50, 100 and 150 μ g/mL CSC or 10 μ M Cyt D. FBS 10% was used as a positive control of collagen gel contraction. Gel contraction was evaluated after 24 h. Graph represents the average and SEM of changes in collagen gel area for each experimental condition. Data were obtained from at least three independent experiments and analyzed using ANOVA test and Bonferroni *post-hoc* test. *p* values in the graph derive from comparisons between 1% FBS and each indicated condition. CSC, cigarette smoke condensate; Cyt D, cytochalasin D; FBS, fetal bovine serum.

tions (20). Cigarettes used to generate CSC have 6.4% nicotine. Nicotine has been detected in saliva of smokers at concentrations ranging between 0.9 and 4.6 $\mu\text{g}/\text{mL}$ (27). In chronic smokers, serum nicotine concentrations vary between 0.04 and 0.072 $\mu\text{g}/\text{mL}$, and in tissues it can reach levels ranging from 0.6 to 1 $\mu\text{g}/\text{mL}$ (28,29). Therefore, CSC dilutions selected to stimulate cells represented physiologically relevant concentrations of this agent. As shown in Fig. 1, a higher collagen gel area was detected in the case of gels incubated with 100 $\mu\text{g}/\text{mL}$ CSC (56.2%; standard error of the mean [SEM]: 0.05), 150 $\mu\text{g}/\text{mL}$ CSC (61.2%; SEM: 0.07) and cytochalasin D (92.3%; SEM 0.1) when compared to control (1% FBS) gels (52.1%; SEM 0.08). Cytochalasin D strongly inhibited collagen gel contraction confirming previous studies that have

identified an important role for actin in the remodeling of collagen matrices (30). From this experiment, we selected 100 $\mu\text{g}/\text{mL}$ CSC as a physiologically relevant concentration of this compound for further experiments. As TGF- β 1 stimulates both collagen gel contraction and myofibroblastic differentiation (31), we used this cytokine as a stimulus to evaluate both responses. To this end, GF were cultured within floating and restrained collagen gels that were stimulated with 10 ng/mL TGF- β 1 and/or 100 $\mu\text{g}/\text{mL}$ CSC. As shown in Fig. 2A neither CSC nor TGF- β 1 were able to modify the area of floating collagen gels. However, CSC reduced collagen gel areas at 12 (89%; SEM: 3.5), 24 (75.2%; SEM: 5.2), 29 (73.2%; SEM: 5.3) and 48 h (71%; SEM: 5.6) compared to cells stimulated with TGF- β 1 at the same time points (Fig. 2B).

Cigarette smoke condensate does not alter α -smooth muscle actin, matrix metalloproteinase-3, connective tissue growth factor or tissue growth factor-beta1 integrin levels

To evaluate the effects of CSC on myofibroblastic differentiation and the production of molecules involved in granulation tissue remodeling, we analyzed changes in the protein levels of α -SMA, β 1 integrin, MMP-3 and CTGF. Floating and restrained collagen gels were incubated in the presence of 10 ng/mL TGF- β 1 and/or 100 $\mu\text{g}/\text{mL}$ CSC for 48 h. At this time point, α -SMA and β 1 integrin levels were analyzed in the cell lysate and MMP-3 and CTGF were detected in the cell culture media through Western blot. Figure 3A shows representative Western blot assays for α -SMA, β 1-integrin, MMP-3 and CTGF. Protein levels

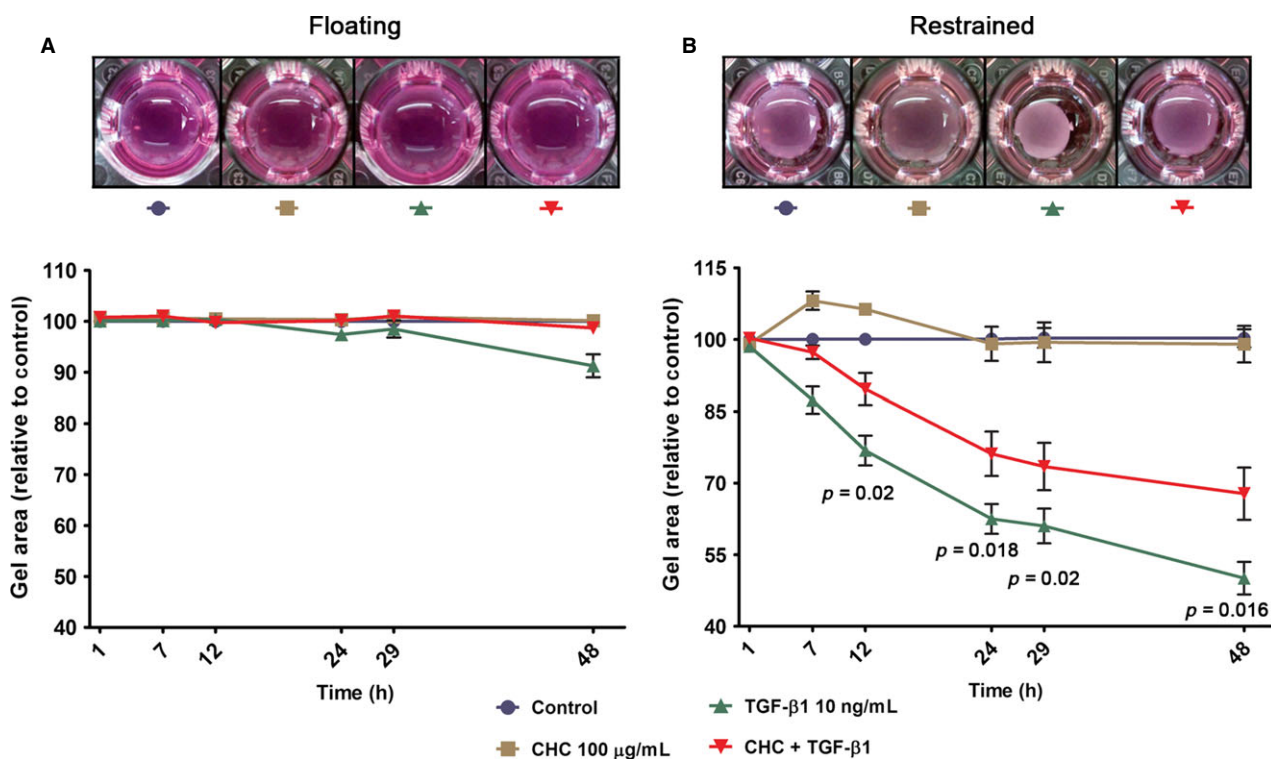


Fig. 2. Effects of CSC on TGF- β 1-stimulated collagen gel contraction. Human gingival fibroblasts were cultured within floating (A) or restrained (B) collagen gels and exposed to 100 $\mu\text{g}/\text{mL}$ CSC and/or 10 ng/mL TGF- β 1. Gel contraction was analyzed after 1, 7, 12, 24, 29 and 48 h. Graph represents the average and SEM of changes in collagen gels areas for each experimental condition. Data were obtained from three independent experiments and analyzed using ANOVA test and Bonferroni post-test. p values in the graph derive from comparisons between TGF- β 1 (control) and TGF- β 1 plus CSC at each indicated time point. CSC, cigarette smoke condensate; TGF, transforming growth factor.

were quantified by densitometry of each band and were further normalized against LDH levels. Graphs in Fig. 3B, 3C, 3D and 3E show average and SEM for each protein after normalization against LDH. It is interesting to men-

tion that CSC was not able to modify their protein levels neither in floating nor in restrained collagen gels. However, CTGF levels were increased in the cell culture media of restrained collagen gels.

Cigarette smoke condensate inhibits prostaglandin E₂ production

As PGE₂ may regulate the contraction of fibroblasts (32,33), we evaluated the effects of CSC on the protein levels of this mediator. Floating and restrained collagen gels were allowed to contract in the presence of 50, 100 and 150 µg/mL CSC. After 48 h, PGE₂ levels were measured in the cell culture media. In both floating and restrained collagen gels, CSC diminished PGE₂ levels. However, reductions in PGE₂ levels reached statistical significance only in the case of restrained collagen gels. Under these conditions, normalized PGE₂ levels were reduced from 1 to 0.18 (SEM: 0.019); 0.09 (SEM: 0.018) and 0.08 (SEM: 0.012) when exposed to 50, 100 or 150 µg/mL respectively. These data suggest that CSC may alter PGE₂ levels in restrained three-dimensional cell cultures (Fig. 4).

Cigarette smoke condensate alters actin cytoskeleton distribution

As the actin cytoskeleton is an important mediator of cell contraction in fibroblasts (26), we evaluated the effects of CSC on the distribution of F-actin in GF cultured within collagen gels. Floating and restrained collagen gels were generated and CSC (100 µg/mL) was used to alter collagen gel contraction. As shown in Fig. 5, in restrained collagen gels fibroblasts displayed a more elongated cell shape with the development of strong actin fibers when compared to floating collagen gels. Interestingly, CSC strongly altered the formation of actin stress fibers in restrained but not in floating collagen gels. This result was consistently observed in at least three independent experiments (Fig. 5).

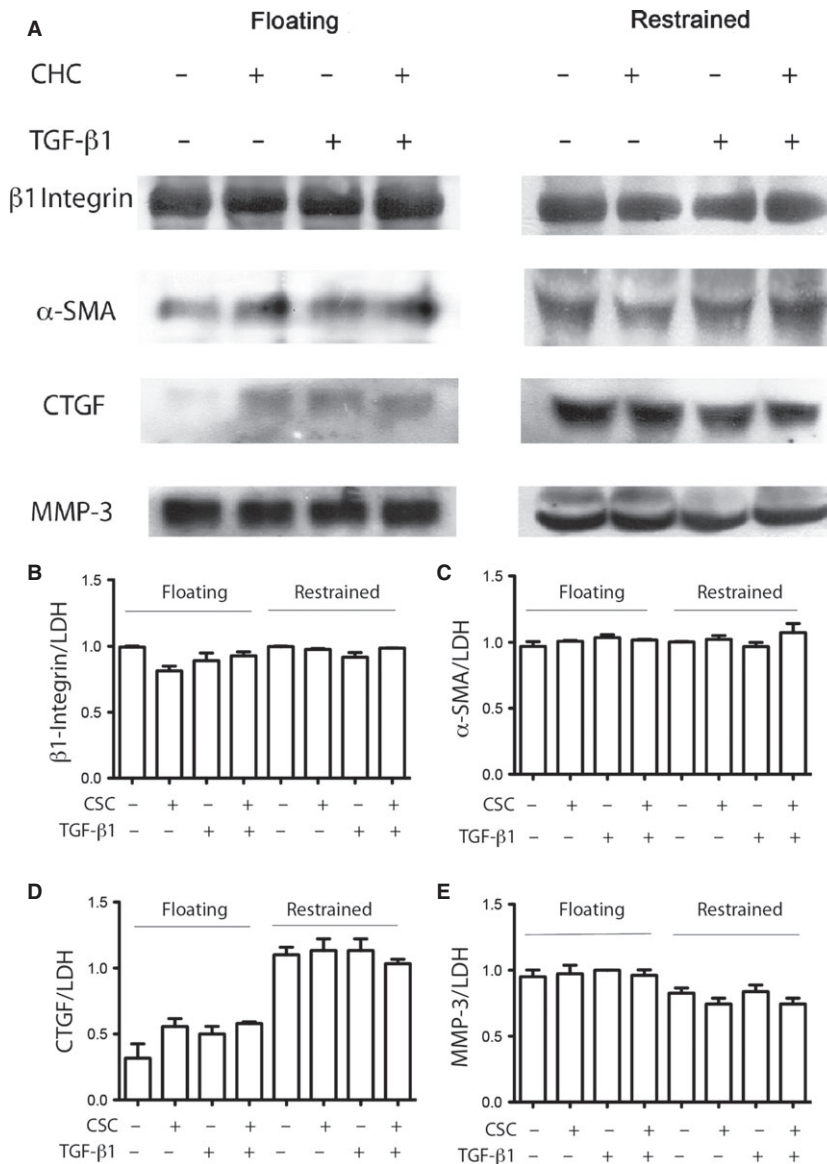


Fig. 3. Effects of CSC on $\beta 1$ integrin, α -SMA, CTGF and MMP-3 protein levels. (A) Human gingival fibroblasts were cultured within floating or restrained collagen gels. After collagen polymerization gels were incubated in the presence of 100 µg/mL CSC and/or 10 ng/mL TGF- $\beta 1$. After 48 h, $\beta 1$ integrin, α -SMA, CTGF or MMP-3 protein levels were analyzed through Western blot. Western blot images are representative of three independent experiments. Western blot bands were analyzed through densitometry and normalized against LDH levels detected in the cell culture media. Graphs show average and SEM for $\beta 1$ integrin (B), α -SMA (C), CTGF (D) and MMP-3 (E). CSC, cigarette smoke condensate; CTGF, connective tissue growth factor; LDH, lactic dehydrogenase; MMP, matrix metalloproteinase; α -SMA, alpha-smooth muscle actin; TGF, transforming growth factor.

Discussion

Our study has analyzed whether CSC may alter the remodeling of collagen gels populated by GF representing different stages of the wound healing process. We observed that CSC altered the contraction of restrained collagen gels, inhibited the release of PGE₂ and the

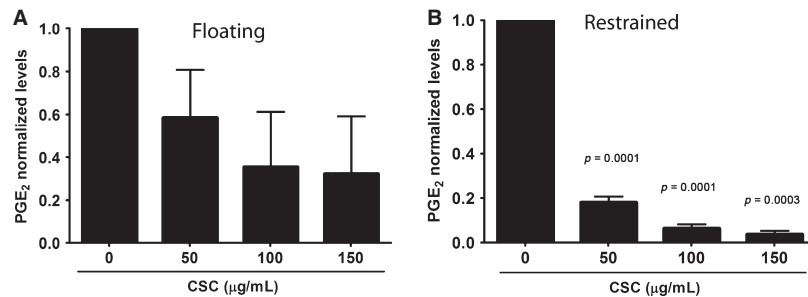


Fig. 4. Effects of CSC on PGE₂ levels. Human gingival fibroblasts were cultured within floating (A) or restrained (B) collagen gels and after polymerization were exposed to 50, 100 and 150 µg/mL CSC. After 48 h, PGE₂ protein levels were detected through an ELISA assay. Graph represents average and standard error of protein levels normalized to control. Data were obtained from three independent experiments and analyzed using ANOVA and Bonferroni post-test. *p* values in the graph derive from comparisons between control and CSC-stimulated groups. CSC, cigarette smoke condensate; PGE, prostaglandin E₂.

development of F-actin fibers. However, other molecular mediators presumably involved in granulation tissue development, including α -SMA, β 1 integrin, MMP-3 and CTGF, were not affected by CSC.

An interesting experimental model to study the development of granulation tissue is the culture of fibroblasts immersed within collagen gels (34). Two important approaches have been used to mimic the different stages that characterize granulation tissue maturation (35). Fibroblasts may be cultured within a floating collagen matrix simulating the early events observed during wound healing. Under these conditions, cells do not receive mechanical stimuli (35). Alternatively, fibroblasts may be cultured within restrained collagen gels and under these conditions, cells develop isometric tension. This experimental situation simulates the latest phase of granulation tissue (35). Therefore, our experimental setup allowed us to identify whether CSC was able to alter the remodeling behavior, cell morphology and biochemical mediators released during these two phases of tissue repair. It has been described that cigarette smoke extract may inhibit the remodeling capacity of pulmonary fibroblasts cultured within collagen gels (36). Carnevali *et al.* (36) used cigarette smoke extract that has both the particulate and volatile components present in cigarette smoke. Our observations were per-

formed using CSC that only has the particulate phase of cigarette smoke. It is interesting to note that volatile components such as acrolein and acetaldehyde may affect the actin cytoskeleton in GFs (19) and may inhibit the contraction of collagen gels (36). Therefore, the present study adds new data that proposes that particulate components present in CSC may also alter the remodeling capacity of fibroblasts. This may be important as volatile agents probably affect cell function for a brief period. On the other hand, particulate components may exert a long-lasting effect in periodontal tissues that probably comprises their biological and clinical response. These observations are in agreement with our experiments in which F-actin stress fibers were altered in restrained collagen gels exposed to CSC. In addition, collagen gel contraction was potently inhibited by cytochalasin D, which is able to sequester actin monomers and, thereby, inhibits F-actin polymerization (37). It is interesting to note that CSC did not affect cells cultured within floating collagen gels, neither in their contraction behavior, nor in the development and maturation of their actin cytoskeleton. This observation suggests that CSC may preferentially affect cells under mechanical stimulation. Owing to the complex regulation of actin polymerization during collagen remodeling (23), several signaling pathways, including

Rho and Rac might be targets of CSC. Accordingly, nicotine may alter the activation of Rac in migrating GF (38) and tobacco smoke has been found to modify F-actin kinetics in human neutrophils (39). Future studies should identify the molecular mechanism involved in the modulation of the actin cytoskeleton by cigarette smoke.

One important observation of our study was that CSC inhibits the production of PGE₂ in fibroblasts cultured within restrained collagen gels. On the other hand, when cells were cultured within floating collagen gels, PGE₂ levels were reduced. However, this response did not reach statistical significance. Again, it is important to highlight the role of mechanical stimulation on the generation of signals involved in PGE₂ production. PGE₂ corresponds to a lipid-derived mediator involved in wound healing and inflammation (40). PGE₂ inhibits myofibroblastic differentiation (16) and the contraction of pulmonary fibroblasts cultured within collagen gels (17). However, contradictory studies have identified conflicting data concerning the regulation of PGE₂ production by cigarette smoke. Cigarette smoke extract stimulated PGE₂ production in pulmonary fibroblasts cultured on rigid substrates (41). On the other hand, Carnevali *et al.* observed that cigarette smoke extract was not able to alter PGE₂ levels in pulmonary fibroblasts cultured within collagen gels (36). These differences may arise from the culture conditions of the cells under study as mechanical stimulation has been implicated in the regulation of PGE₂ levels (42,43). Otherwise, phenotypic differences due to the diversity of fibroblasts found in different tissues might explain these discrepancies in the modulation of PGE₂ (44,45).

CTGF is a well-recognized downstream protein induced after TGF- β 1 stimulation in several conditions and diseases, including wound healing and fibrosis (46). CTGF is involved in myofibroblastic differentiation and plays a role in granulation tissue remodeling and collagen contraction under the stimulus of TGF- β 1 (15).

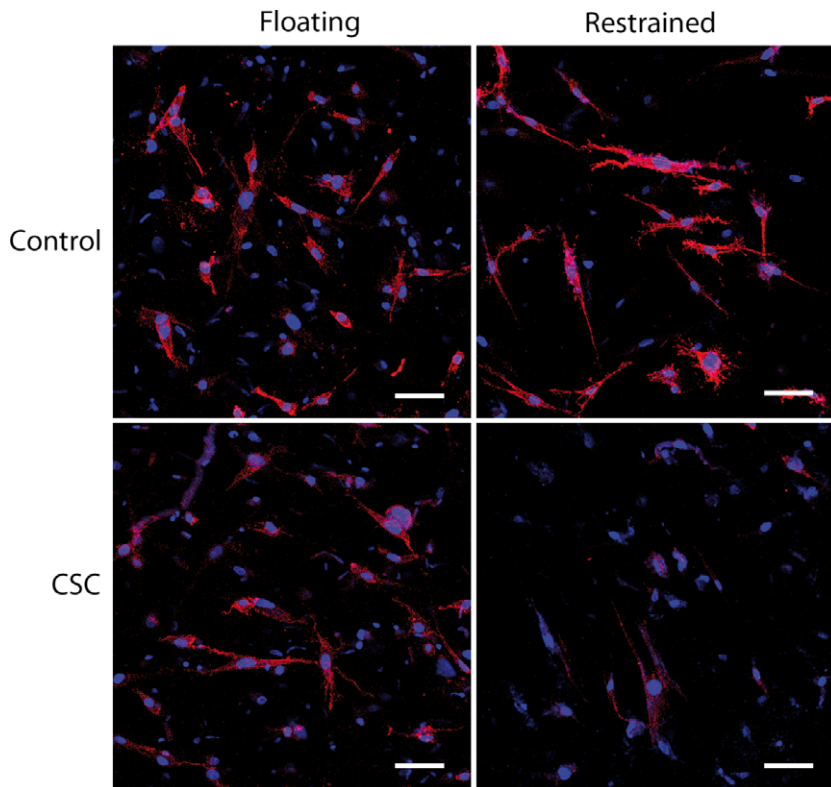


Fig. 5. Effects of CSC on actin cytoskeleton organization. Human gingival fibroblasts were cultured within floating or restrained collagen gels and collagen contraction was stimulated with 1% fetal bovine serum. Collagen gels were exposed to 100 $\mu\text{g}/\text{mL}$ CSC for 48 h. Actin cytoskeletal was stained using phalloidin, nuclei detected with DAPI and visualized through confocal microscopy. CSC, cigarette smoke condensate.

Tracheal explants exposed to cigarette smoke show enhanced levels of CTGF mRNA expression (47) and nicotine stimulates the production of CTGF in GF cultured in two-dimensional dishes (21). In our study, stimulation of GF with CSC did not modify CTGF levels, neither when cells were cultured within floating nor within restrained collagen lattices. It is important to consider that CTGF production is regulated by mechanical stimulation (48). Therefore, it is not clear why we could not detect changes in CTGF levels when cells were cultured within collagen gels in which different levels of strain were generated. A similar result was observed when α -SMA levels were evaluated in the presence of CSC. Previous studies have reported that CSC may reduce α -SMA levels in GF cultured over two-dimensional cell culture plates (20). Two-dimensional cell culture represents a condi-

tion in which cells are exposed to maximal tension (35). Arora *et al.* (31) identified that myofibroblastic differentiation is modulated by the tension that cells receive in different cell culture settings. Accordingly, α -SMA levels are prominently induced when cells are cultured in two dimensions, moderately expressed in restrained collagen gels and poorly regulated in floating collagen lattices (31). Consequently, we believe that the differences found between the study from Silva *et al.* (20) and the present report probably rely on the different mechanical conditions and, therefore in the strains generated in two- and three-dimensional cell culture systems.

Mice deficient in MMP-3 show deficiencies in wound contraction (14) and fibroblasts derived from these mice have defects in collagen gel remodeling (49). These studies strongly suggest that MMP-3 plays an

important role in wound healing. However, the precise target of MMP-3 remains elusive. Although we could not find changes in MMP-3 levels after CSC exposure, we observed that MMP-3 protein levels were stimulated when cells were cultured within restrained collagen gels. This observation is in accordance with studies showing that MMP-3 is modulated by mechanical stimuli in human synovial cells (50,51) and fibroblast-like synoviocytes (52). Further studies are needed to identify the precise role of MMP-3 during granulation tissue maturation and wound healing.

The present study has identified that CSC alters important responses during wound healing, including the remodeling of collagen matrices, development of actin stress fibers and production of PGE₂ levels. These observations may be important for the alterations observed during wound healing in smokers.

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