

Differential uPA Expression by TGF- β 1 in Gingival Fibroblasts

ABSTRACT

Transforming Growth Factor- β 1 (TGF- β 1) plays a key role in connective tissue remodeling and inflammation. Under pathological conditions, like periodontal disease, fibroblasts may display an altered response to this growth factor. To investigate this question, we have studied whether TGF- β 1 may differentially regulate the expression of urokinase at the protein level in primary cultures of fibroblasts derived from healthy gingiva, granulation tissue from gingival wounds, and chronic periodontal disease. We observed that TGF- β 1 may repress urokinase expression in healthy gingival fibroblasts and promote its production in granulation-tissue fibroblasts. A significant correlation was found between expression of the myofibroblast marker α -smooth-muscle actin and stimulation of urokinase production by TGF- β 1. Immunostaining of gingival wounds showed that myofibroblasts were involved in urokinase production. TGF- β 1-stimulated urokinase expression was blocked after inhibition of the c-jun-NH₂ terminal kinase signaling pathway. We propose that stimulation of urokinase production by TGF- β 1 is involved in the responses of activated fibroblasts to tissue injury.

KEY WORDS: gingival, myofibroblast, fibroblast, TGF- β 1, urokinase.

INTRODUCTION

Fibroblasts play a central role in the remodeling of extracellular matrix (ECM) (Bartold *et al.*, 2000). During chronic inflammation and wound healing, gingival fibroblasts become "activated" and acquire several properties giving rise to a heterogeneous cell population known as granulation-tissue fibroblasts (Häkkinen and Larjava, 1992). Granulation-tissue fibroblasts are characterized by the expression of α -smooth-muscle actin (α -SMA) and differ from normal fibroblasts in proliferation rate, morphology, response to cytokines, and synthesis of ECM proteins (Desmoulière *et al.*, 2003).

The urokinase-type plasminogen activator (uPA) is a serine-protease highly expressed in inflamed and normally healing gingival tissues (Kinby *et al.*, 1999; Xiao *et al.*, 2001). uPA converts plasminogen into plasmin, another serine-protease that degrades fibrin and activates matrix metalloproteinases (Andreasen *et al.*, 1997). Transforming Growth Factor β 1 (TGF- β 1) plays a prominent role in gingival wound-healing and inflammation (Steinsvoll *et al.*, 1999; Kuru *et al.*, 2004). Interestingly, many of the cell responses to this growth factor are modulated by the stage of differentiation of the cell (Akhurst and Derynck, 2001). In the case of human gingival granulation-tissue fibroblasts, TGF- β 1 stimulates the over-expression of proteoglycans (Häkkinen *et al.*, 1996). Although regulation of uPA production is a critical step in inflammation and wound-healing, the molecular clues controlling this event are poorly understood. In the present study, we investigated whether TGF- β 1 may regulate uPA production at the protein level in gingival fibroblasts derived from distinct physiological conditions.

MATERIALS & METHODS

Cell Cultures

Human gingival fibroblast primary cultures were established by the explant method (Häkkinen and Larjava, 1992). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco) supplemented with antibiotics. Cells were used between the fourth and tenth passages.

Gingival Tissue Samples and Patient Selection

All gingival samples were obtained, with informed consent, from 33 patients attending a dental practice in Santiago, Chile. The protocol was approved by the Ethical Committee of the Faculty of Medicine of the University of Chile. No relevant medical or drug histories were cited.

Granulation-tissue fibroblasts from periodontal disease (GT-PD) were obtained from marginal gingiva from patients affected by moderate to advanced periodontal disease during the extraction of hopeless teeth (n = 14) (nine women, five men; mean age, 40 \pm 8 yrs). Sites selected for biopsy showed 4 mm or more of probing depth, 3 mm or more of attachment loss, and bleeding upon

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probing. These cells were compared with gingival fibroblasts from healthy marginal gingiva (HMG) obtained during crown-lengthening surgery ($n = 6$) (three women, three men; mean age, 34 ± 13 yrs). Sites selected for biopsy demonstrated a probing depth of 3 mm or less, absence of attachment loss, and no bleeding upon probing. Granulation-tissue fibroblasts from wounds (GT-W) were obtained from wounds created in the attached gingiva of human volunteers as described previously ($n = 4$) (three men, one woman; mean age, 33 ± 5 yrs) (Larjava *et al.*, 1993). After 10 days of healing, granulation tissue was harvested and processed for cell culture. GT-W fibroblasts were compared with fibroblasts derived from healthy attached gingiva (HAG) obtained during the extraction of asymptomatic retained teeth ($n = 9$) (seven women, two men; mean age, 27 ± 12 yrs).

Detection of uPA and Plasminogen Activator Inhibitor (PAI-1)

Conditioned media (CM) derived from cell cultures stimulated with TGF- β 1 (US Biological, Swampscott, MA, USA) or Epidermal Growth Factor (EGF) (Calbiochem, La Jolla, CA, USA) was processed for uPA and PAI-1 detection through Western blot analysis as previously described (Smith *et al.*, 2004).

Signal Transduction Studies

Mitogen-activated protein kinase (MAPK) activation was assessed through Western blot analysis with antibodies against p-JNK (Upstate Biotechnology, Lake Placid, NY, USA) or JNK2 (Santa Cruz, CA, USA) as previously described (Smith *et al.*, 2004). Inhibition of MAPK pathways was assessed with the following reagents: SP600125 (Biomol, Plymouth, PA, USA), PD98059 and SB203580 (Calbiochem).

Casein Zymography and Radial Diffusion Assays

uPA-secreted activity and radial diffusion assay of cell cultures were determined as previously described (Santibáñez *et al.*, 1995). Quantification of caseinolytic bands was performed by densitometric analysis. Variations in uPA activity after TGF- β 1 or EGF treatment were calculated after normalization against the non-stimulated uPA activity.

Immunofluorescence of Cultured Cells

Cells were processed for immunofluorescence as described previously (Smith *et al.*, 2004) with antibodies against α -SMA (Sigma, St. Louis, MO, USA), vimentin (Sigma), and human cytokeratin (Dako, Carpinteria, CA, USA). Actin fibers were stained with Alexa Fluor-Phalloidin (Molecular Probes, Eugene, OR, USA). We quantified α -SMA-expressing cells by scoring α -SMA-positive/-negative cells within a total of 200 cells for each individual sample under study. For this purpose, we considered α -SMA cells as positive when immunostaining was clearly detected in association with the actin cytoskeleton.

Immunohistochemistry

Urokinase and α -SMA immunohistochemistry was performed on formalin-fixed, paraffin-embedded gingival tissue biopsies by the peroxidase-antiperoxidase technique (Dako), with diaminobenzidine as chromogenic substrate. Mouse monoclonal antibodies against human uPA (American Diagnostica, Stamford, CT, USA) and α -SMA (Sigma) were used. Harris hematoxylin was used as counterstain. We performed negative controls by replacing primary antibodies with non-immune serum. As positive controls, uPA and α -SMA expression were detected in migrating epithelial cells from gingival wounds and in blood vessels, respectively (data not shown).

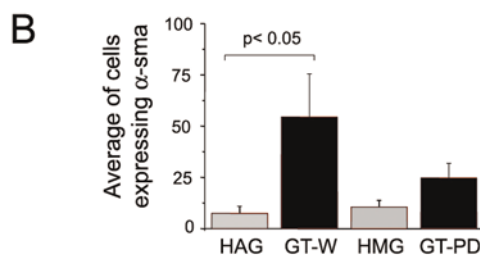
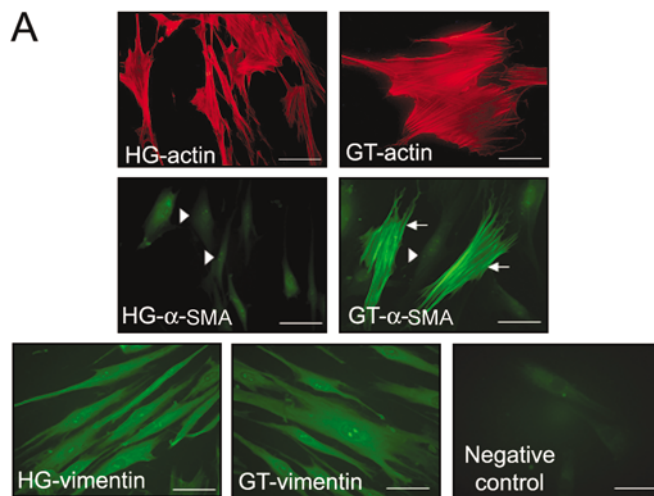


Figure 1. Characterization of healthy and granulation-tissue fibroblasts. **(A)** At passage 4, human gingival fibroblasts obtained from healthy and granulation-tissue gingiva were stained with Alexa Fluor-phalloidin or antibodies recognizing α -SMA or vimentin. Arrowheads indicate α -SMA-negative cells. Arrows indicate α -SMA-positive cells. Bar = 20 μ m. **(B)** The average of α -SMA expression was obtained after we counted positively stained cells in cell cultures derived from HAG, GT-W, HMG, and GT-PD patients, and analyzed them by Student's *t* test.

Statistical Analysis

Differences in the expression of α -SMA and TGF- β 1-stimulated uPA production were evaluated by the Student's *t* test. The relationship between TGF- β 1-stimulated uPA activity and α -SMA expression was analyzed by Pearson correlation. Significance was determined by Bonferroni corrections.

RESULTS

Characterization of Gingival Fibroblasts

Actin stress fibers were detected in both healthy and granulation-tissue fibroblasts (Fig. 1A). Granulation-tissue cells displayed a more extended cytoplasm when compared with healthy fibroblasts. α -SMA expression was low in HMG and HAG cultures, while it was more frequently detected in granulation-tissue cultures. Noteworthy, GT-W fibroblasts were frequently positive for this marker. The mesenchymal origin of the cells was confirmed after staining for vimentin. Quantification of myofibroblasts in each culture showed that α -SMA expression was significantly higher in GT-W fibroblasts as compared with HAG fibroblasts ($p < 0.05$) (Fig. 1B). A slightly higher, but non-significantly different, expression level of α -SMA was detected in GT-PD fibroblasts as compared with HMG fibroblasts.

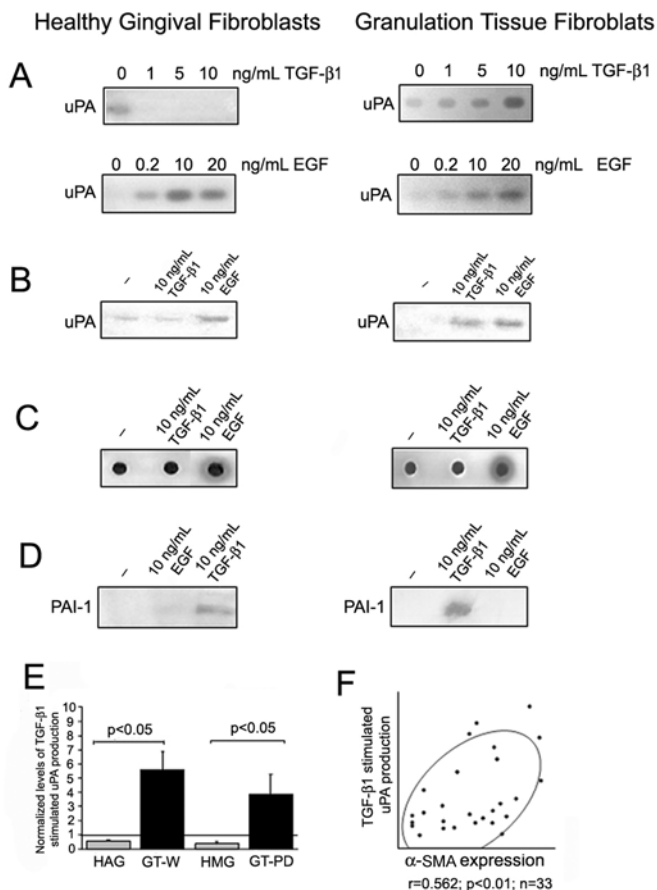


Figure 2. Regulation of uPA production by TGF- β 1 and EGF in healthy and granulation-tissue fibroblasts. **(A)** Serum-free cultures of gingival fibroblasts from healthy and granulation-tissue (80,000 cells) were stimulated with increasing concentrations of TGF- β 1 or EGF for 48 hrs in DMEM without serum. Conditioned medium, normalized after the quantification of total protein amounts in the cell lysate at the end of the experiment, was analyzed through casein zymography. **(B)** uPA protein levels were determined through Western blot analysis of the concentrated conditioned medium of 500,000 cells stimulated with 10 ng/mL TGF- β 1 or 10 ng/mL EGF. **(C)** uPA-derived proteolytic activity was determined in conditioned medium of cells stimulated with 10 ng/mL TGF- β 1 or 10 ng/mL EGF through a radial diffusion assay. **(D)** PAI-1 production was identified through Western blotting of concentrated conditioned medium of cells stimulated with 10 ng/mL TGF- β 1 or 10 ng/mL EGF. **(E)** TGF- β 1-stimulated uPA production, determined through casein zymography, in each individual was normalized against non-stimulated uPA production (indicated as 1 in the graph) and compared among the 4 groups under study. Bars indicate averages \pm standard deviations. Statistical analysis was performed with Student's *t* test ($n=33$). **(F)** We performed Pearson correlation analysis, comparing TGF- β 1-stimulated uPA production through casein zymography and α -SMA expression.

Regulation of uPA Expression by TGF- β 1 in Healthy and Granulation-tissue Fibroblasts

Sub-confluent cultures from healthy and granulation tissues were stimulated with increasing concentrations of TGF- β 1 for 48 hrs. Casein zymography of conditioned medium showed that TGF- β 1 inhibited the secreted uPA activity in healthy gingival fibroblasts (Fig. 2A). In contrast, granulation-tissue fibroblasts responded with a dose-dependent increase in secreted uPA activity after TGF- β 1 treatment (Fig. 2A). Analysis of the cell-

associated uPA activity in cell lysates did not show any significant changes after TGF- β 1 stimulation (data not shown). Interestingly, treatment of cells with a distinct growth factor such as EGF consistently stimulated uPA expression in both healthy and granulation-tissue fibroblasts. Regulation of uPA expression after TGF- β 1 or EGF stimulation was confirmed through Western blotting of the concentrated conditioned medium of healthy and granulation-tissue fibroblasts (Fig. 2B). We further evaluated, by means of a radial diffusion assay, the overall proteolytic activity derived from plasminogen activation in the conditioned medium of TGF- β 1-stimulated cells. Surprisingly, TGF- β 1 did not stimulate uPA-derived proteolytic activity in the conditioned medium from granulation-tissue cells (Fig. 2C). This result was explained by the observation that TGF- β 1, but not EGF, was able to stimulate the production of the uPA inhibitor PAI-1 in granulation-tissue cells (Fig. 2D).

After examining several cultures from healthy and granulation tissues, we observed that GT-W ($n = 4$) and GT-PD ($n = 14$) fibroblasts responded with a significant increase in uPA production, as compared with their respective controls ($p < 0.05$) (Fig. 2E). All of the cultures derived from HAG and HMG showed an inhibition or a non-responsive behavior after TGF- β 1 stimulation ($n = 15$). Although cells derived from both GT-W and GT-PD cultures responded to TGF- β 1 with an increase in uPA production, a higher response was observed in wound-derived cells. We performed a correlation analysis comparing TGF- β 1-stimulated uPA production and α -SMA expression in all cell cultures, regardless of their clinical origin. Pearson correlation analysis showed a measurable relationship between α -SMA expression and TGF- β 1-stimulated uPA production ($r = 0.562$; $p < 0.01$; $n = 33$) (Fig. 2F).

Signaling Pathways Involved in uPA Production

In healthy gingival fibroblasts, TGF- β 1 inhibited uPA production, and the MAPK inhibitors tested did not modify this response (Fig. 3A). In granulation-tissue fibroblasts, the JNK inhibitor SP600125 completely abrogated the stimulus of TGF- β 1 on uPA production, while blockade of the ERK (PD98059) and p38 (SB203580) pathways did not interfere in this response. These results were replicated in 3 independent experiments with different cell cultures. Inhibition of uPA production by SP600125 displayed dose-dependent behavior (Fig. 3B). In addition, we observed that JNK was activated by TGF- β 1 in granulation-tissue fibroblasts (Fig. 3C).

Expression of Urokinase in Gingival Wounds

Urokinase and α -SMA expression was assessed, through immunohistochemistry, in 3 normally healing gingival wounds. Expression of α -SMA was prominent in granulation-tissue fibroblasts (Figs. 4A, 4C). uPA expression was observed in keratinocytes, endothelial cells, and fibroblasts (Fig. 4B). After staining serial sections, we observed a spatial correlation between the distribution of α -SMA-positive fibroblasts and the expression of uPA in similar cell types (Figs. 4C, 4D). In healthy gingival tissues, α -SMA expression was occasionally detected in blood vessels and fibroblasts, while uPA expression was observed at low levels in endothelial cells or fibroblasts (Figs. 4E, 4F). Negative controls did not show any specific immunoreactivity (not shown).

DISCUSSION

Regulation of uPA production is a critical event that may affect

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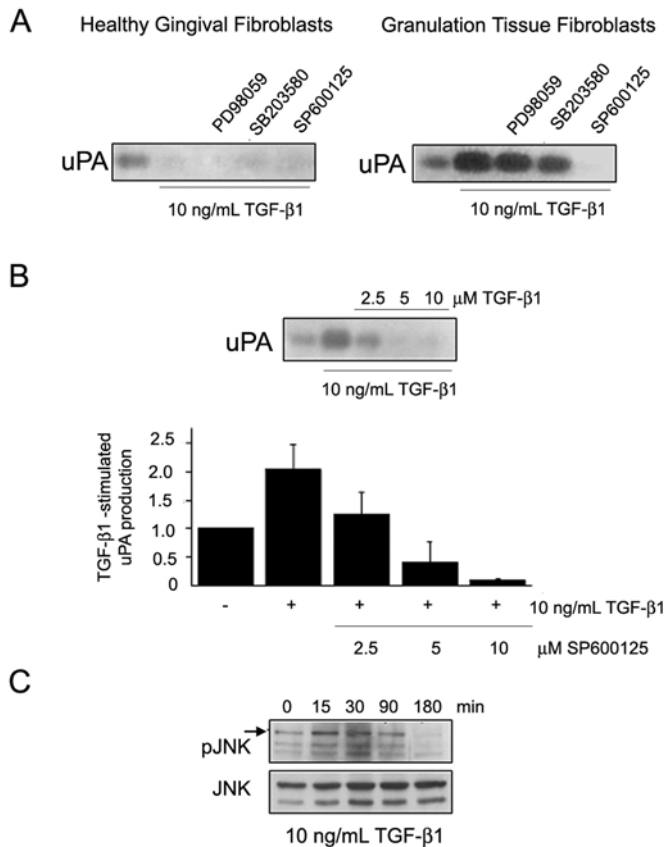


Figure 3. Signal transduction pathways involved in TGF- β 1-stimulated uPA production. **(A)** Healthy and granulation-tissue gingival fibroblasts (80,000 cells) were cultured in the presence of specific MAPK inhibitors: 40 μ M PD98059, 10 μ M SB203580, or 10 μ M SP600125 in serum-free DMEM. After 15 min, cell cultures were treated with 10 ng/mL TGF- β 1 for 48 hrs. Secreted uPA activity was evaluated by casein zymography. **(B)** Dose-response analysis of the effects of the JNK inhibitor SP600125 on TGF- β 1-stimulated uPA production determined through casein zymography in 3 independent experiments. **(C)** Gingival granulation-tissue fibroblasts (300,000 cells) were incubated with TGF- β 1 (10 ng/mL) in DMEM without serum for different periods of time, as indicated. The level of activated JNK (pJNK) was determined by Western blotting with specific antibodies.

the evolution of cancer, wound healing, and inflammation (Carmeliet *et al.*, 1994; Harbeck *et al.*, 2004). TGF- β 1 stimulates uPA production during epithelial-mesenchymal transformation (Santibáñez *et al.*, 2000). However, in mesenchymal cells, contradictory studies have reported that TGF- β 1 may have a stimulatory, inhibitory, or null effect on uPA production in fibroblasts derived from lung, synovium, breast, or skin (Laiho *et al.*, 1986a,b; Hamilton *et al.*, 1991; Sieuwerts *et al.*, 1999). In the present study, we observed that healthy gingival fibroblasts either responded to TGF- β 1 with an inhibition of uPA production or, eventually, did not respond. In granulation-tissue fibroblasts, TGF- β 1 stimulated uPA and PAI-1 production, adding a crucial element to the cellular proteolytic balance. In spite of the fact that the radial diffusion assay showed that TGF- β 1 seems to be unable to stimulate plasminogen activation due to PAI-1 synthesis, we cannot rule out that temporal or spatial variations in the synthesis or distribution of uPA and PAI-1 by TGF- β 1-stimulated cells might result in plasminogen processing *in vivo*.

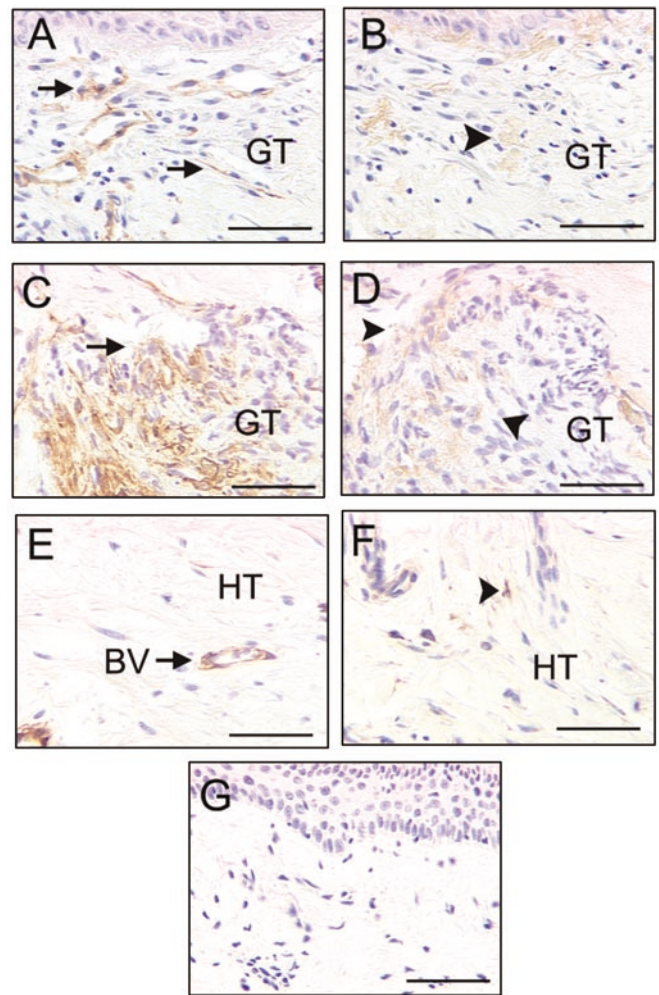


Figure 4. Immunolocalization of α -SMA expression and uPA production in sections of gingival wounds and healthy gingiva. **(A,C,E)** α -SMA expression. **(B,D,F)** uPA production. **(A-D)** Acute gingival wounds. **(E,F)** Healthy gingiva. GT = granulation tissue. HT = healthy tissue. BV = blood vessel. **(G)** Negative control section. Arrows indicate α -SMA staining; arrowheads indicate uPA staining. Bar = 50 μ m.

uPA is secreted as an inactive molecule and is converted to an active form that generates plasminogen activation. Plasmin is the main factor involved in uPA activation, thus leading to a reciprocal pro-enzyme activation phenomenon (Behrendt, 2004). From our data, we can suggest that EGF, which generates strong plasminogen activation in both cell types, may stimulate uPA synthesis and activation. It remains to be determined whether TGF- β 1 is able to induce uPA activation in gingival fibroblasts.

Plasminogen activation may also be achieved by tissue plasminogen activator (tPA) (Irigoyen *et al.*, 1999). Although previous studies have demonstrated that gingival fibroblasts may express tPA, our casein-zymography assays were not able to detect a 64-kDa band corresponding to this molecular species.

Besides its role as a protease, uPA may also stimulate proliferation of fibroblasts and chemotaxis of neutrophils, macrophages, and fibroblasts (Anichini *et al.*, 1994; Resnati *et al.*, 1996). Therefore, TGF- β 1-stimulated uPA production by granulation-tissue fibroblasts might promote the recruitment of

different cells to the site of tissue injury.

Resident tissue fibroblasts may become activated after tissue injury, and acquisition of this phenotype may further modulate the responses of these cells to growth factors (Desmoulière *et al.*, 2003). In this context, gingival granulation-tissue fibroblasts may develop an altered expression of several proteoglycans and collagens after TGF- β 1 stimulation (Häkkinen *et al.*, 1996). Our results, showing that granulation-tissue fibroblasts up-regulate uPA expression after TGF- β 1 treatment, are in accordance with the proposed distinct cell behavior among fibroblasts derived from different physiological conditions.

Gingival myofibroblasts play a significant role in the remodeling of the ECM (Arora and McCulloch, 1994). We observed that expression of the myofibroblast marker α -SMA varied greatly in the cell cultures under study, with lower levels in resting healthy tissues, intermediate levels in GT-PD, and higher levels in GT-W fibroblasts. Although both wound- and periodontitis-derived cells responded to TGF- β 1 with an increase in uPA production, a more expressive response was observed in wound-derived cells that displayed the higher levels of α -SMA. These findings suggest that chronic periodontal lesions represent a mixture of cell phenotypes in which myofibroblast differentiation is not totally accomplished.

Urokinase expression is tightly regulated at the transcriptional level, and several MAPK pathways have been involved in uPA expression (Miralles *et al.*, 1998; Parra *et al.*, 2000; Santibáñez *et al.*, 2000). Fibroblasts deficient in MEKK1, an upstream stimulator of ERK and JNK, are unable to up-regulate uPA expression after stimulation with growth factors (Witowsky *et al.*, 2003). We have recently reported that, in healthy gingival fibroblasts, EGF-stimulated uPA expression depends on the combined activity of ERK and JNK pathways (Smith *et al.*, 2004). Here, we observed that, in granulation-tissue cells, TGF- β 1-stimulated uPA expression was abolished after inhibition of the JNK pathway, suggesting that this route may be involved in the expression of uPA.

Immunostaining of normally healing gingival wounds showed that gingival granulation-tissue fibroblasts were involved in uPA expression. These results reinforce *in vitro* results showing that, in cell cultures enriched in myofibroblasts, TGF- β 1 stimulated uPA expression. Granulation-tissue fibroblasts have been identified as a prominent source of uPA during wound healing (Schäfer *et al.*, 1994). Interestingly, in response to cancer invasion, peritumoral stromal cells develop a tissular reaction known as 'cancer desmoplasia', and these cells are also involved in uPA production (Nielsen *et al.*, 1996).

Periodontal disease is characterized by alternating periods of tissue degradation and fibrosis. During wound healing, tissue remodeling allows for the replacement of the injured tissue. In both processes, it has been hypothesized that myofibroblasts play an active role. In the present study, we were able to determine that granulation-tissue fibroblasts respond to TGF- β 1 by increasing uPA production, a process which is possibly regulated by the JNK signaling pathway. We propose that these events are involved in the responses of activated gingival fibroblasts to tissue injury.

ACKNOWLEDGMENTS

This work received financial support from the Departamento de Investigación y Desarrollo, Universidad de Chile, grant number

02/10-2 (to JM), FONDECYT, grant number (1040734) (to JM), and from Colgate Palmolive Chile S.A. We thank Prof. Julieta González and Prof. Cecilia Allende (ICBM, Faculty of Medicine, University of Chile), Prof. Alejandro Oyarzún (Faculty of Odontology, University of Chile), and Dr. Juan F. Santibáñez (INTA, University of Chile) for helping us with immunohistochemistry, microphotography, and cell culture assays, respectively. We also acknowledge Dr. Marco Méndez (INTA, University of Chile), who performed the statistical analysis, and Dr. Mariana Cifuentes (INTA, University of Chile), for critical reading of this manuscript.

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