J Periodont Res 2009; 44: 73–80

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01081.x

Tumor necrosis factor-α-stimulated membrane type 1-matrix metalloproteinase production is modulated by epidermal growth factor receptor signaling in human gingival fibroblasts

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Smith PC, Guerrero J, Tobar N, Cáceres M, González MJ, Martínez J. Tumor necrosis factor-α-stimulated membrane type 1-matrix metalloproteinase production is modulated by epidermal growth factor receptor signaling in human gingival fibroblasts. J Periodont Res 2009; 44: 73–80. © 2009 The Authors. Journal compilation © 2009 Blackwell Munksgaard

Background and Objectives: Membrane type 1-matrix metalloproteinase (MT1-MMP) is a collagenolytic enzyme involved in connective tissue remodeling. In periodontal tissues, either cytokines or growth factors regulate the production of proteolytic enzymes. Mice deficient in epidermal growth factor receptor (EGFR) show a reduced expression of MT1-MMP, suggesting that this receptor may play an important role in MT1-MMP production. The present study evaluated the role of the inflammatory cytokine tumor necrosis factor-α (TNF-α) and EGFR in the production of MT1-MMP in gingival fibroblasts.

Material and Methods: Primary cultures of human gingival fibroblasts were cultured over plastic or a type I collagen matrix and stimulated with TNF-α and EGF. A selective EGFR inhibitor (AG1478) was used to interfere with this signaling pathway. Production of MT1-MMP and activation of proMMP-2 were studied using Western blot and gelatin zymography, respectively. Activation of EGFR signaling was assessed through immunoprecipitation and Western blot. Expression of EGFR ligands was determined through reverse transcriptase-polymerase chain reaction.

Results: Treatment of gingival fibroblasts cultured over a collagen matrix with TNF-α stimulated proMMP-2 activation and MT1-MMP production. However, after using AG1478, both responses were inhibited. Tumor necrosis factor-α induced EGFR transactivation and stimulated the expression of the mRNA for the EGFR ligands heparin binding-epidermal growth factor (HB-EGF) and transforming growth factor-α (TGF-α).

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Key words: tumor necrosis factor-α; epidermal growth factor receptor; membrane type 1-matrix metalloproteinase; fibroblast

Accepted for publication December 11, 2007

Conclusions: The present study shows that TNF-α may stimulate MT1-MMP production through transactivation of EGFR. Tumor necrosis factor-α may also modulate the expression of the EGFR ligands TGF-α and HB-EGF. Production of MT1-MMP by TNF-α requires interaction with EGFR, suggesting that tissue remodeling is controlled by cross-communication between diverse signaling pathways in gingival fibroblasts.

Periodontitis is a chronic inflammatory disease that results in the selective breakdown of the collagenous matrix involved in periodontal attachment. Therefore, degradation of this molecular component is a key step in connective tissue destruction (1). Although our understanding of the metabolic pathways that mediate the remodeling of the extracellular matrix continues to grow, the molecular mechanisms of periodontal attachment loss still remain largely undefined. Fibroblasts are the predominant cell type in periodontal tissues and they synthesize and degrade collagen fibrils (1). Moreover, histopathological studies have demonstrated that these cells play a significant role in connective tissue breakdown during periodontal disease progression (2).

Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases that are capable of degrading most of the structural components of the extracellular matrix (3). The MMP gene family encodes a total of 24 homologous proteinases, classified into collagenases, gelatinases, stromelysins, membrane type-matrix metalloproteinases and other MMPs depending on their substrate specificity and molecular structure (4). Membrane type 1-MMP (MT1-MMP/MMP-14) was first described as the cellular receptor and activator of MMP-2. In various tumors its expression coincides with the presence of the activated form of MMP-2 (5). Mice deficient in MT1-MMP develop a severe impairment of growth, skeletal dimorphisms, scarring of joints and periskeletal tissues, reduced bone formation, enhanced bone resorptive activity and altered dental root formation (6,7). At the cellular level, MT1-MMP acts as a powerful collagen-degrading enzyme (6). The MMPs are synthesized as zymogens, which require extracellular activation for complete biological activity (3). Matrix metalloproteinase catalytic activity is also blocked by natural inhibitors such as α_2-macroglobulin and tissue inhibitors of MMPs (TIMPs; 4). Additionally, MMP production is a tightly regulated system that finely modulates connective tissue remodeling (8).

Tumor necrosis factor-α (TNF-α) is one of the key mediators of immune and inflammatory responses (9) and it has been found in significant quantities in inflamed periodontal tissues (10,11). Importantly, MT1-MMP production is stimulated by TNF-α in human dermal and rheumatoid synovial fibroblasts (12,13). Epidermal growth factor receptor (EGFR) signaling is a critical regulator of MT1-MMP production, as has been demonstrated in EGFR-deficient mice. In the absence of functional EGFR, mice develop a phenotype characterized by alterations in lung development and a reduced production of MT1-MMP (14). These data strongly suggest that EGFR signaling may play a significant role in MT1-MMP regulation.

In view of the fact that TNF-α is an inflammatory cytokine abundantly expressed in gingival tissues and that EGFR regulates the production of MT1-MMP in lung cells, we studied whether these factors may interact in gingival fibroblasts (GFs) and to what extent they may regulate MT1-MMP production in this cell type. Herein we show that in GFs, both MMP-2 activation and MT1-MMP production are strongly stimulated by TNF-α when cells are cultured over type I collagen. Interestingly, inhibition of EGFR signaling by AG1478 downregulates both proMMP-2 activation and MT1-MMP production in TNF-α-stimulated cells. In addition, exposure of GFs to TNF-α stimulates EGFR transactivation and the mRNA expression of the EGFR ligands heparin binding-epidermal growth factor (HB-EGF) and transforming growth factor-α (TGF-α). These results show that TNF-α may stimulate the EGFR signaling pathway to control MT1-MMP production in GFs.

Material and methods

Antibodies and reagents

Monoclonal antibody against MT1-MMP (MAB918) was purchased from R&D Systems (Minneapolis, MN, USA). Anti-β-actin antibody was supplied by Sigma (St. Louis, MO, USA). The selective EGFR inhibitor AG1478 was purchased from Calbiochem (San Diego, CA, USA). Recombinant TNF-α was purchased from R&D Systems. Recombinant EGF was obtained from Calbiochem. Polyclonal antibodies against a peptide of residues 1176–1186 of the carboxy-terminus of the epidermal growth factor receptor (anti-C EGFR), used to immunoprecipitate EGFR, and the AG10 monoclonal antibody, used to assess tyrosine phosphorylation, were kindly provided by Dr Alfonso González (Pontificia Universidad Católica de Chile) and have been previously characterized (15,16).

Cell culture

Primary cultures of human GFs were established by the explant method (17). Tissue explants were obtained from the retromolar tissue of one male and two female patients 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 used 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 months. Cells were cultured in α-modified Eagle's medium (α-MEM; Gibco BRL, Grand Island, NY, USA), containing 10% fetal bovine serum (FBS; Gibco BRL), 100 μg/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma) and 50 μg/mL gentamycin, at 37°C in a 5% CO_2 atmosphere. All experiments were performed using cells between the fourth and tenth passages.

Type I collagen matrix

A type I collagen matrix was prepared by mixing nine parts of a collagen solution (1.0 mg/mL) and one part of neutralizing buffer (2.2 g $NaHCO_3$, 4.77 g Hepes, in 100 mL NaOH 0.05 N). Collagen was obtained from rat tail tendon as previously described (18). After collagen polymerization, 3×10^5 cells were seeded over the collagen matrix.

Cell harvesting from collagen matrices

For harvesting cells from collagen-coated plates, each cell culture dish was rinsed with phosphate-buffered saline (PBS) and incubated for 10 min at 37°C with 0.3 mL of 0.05% trypsin–0.53 mM EDTA solution, followed by a 20–30 min incubation with 0.35 mL of collagenase (5 mg/mL). After cells were completely dispersed, enzymatic activity was blocked by the addition of 0.5 mL of serum. The dispersed cells were counted with a hemocytometer before collection by centrifugation at 19,000*g* for 10 min at 22°C and lysed in 0.05 mL of extraction buffer (2% Triton X-100, 160 mM KCl, 40 mM Tris-HCl, 20 mM EGTA, 10 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin and 1 mM benzamidine). Samples were boiled for 3 min at 95°C, and equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were transferred to appropriate membranes and processed for Western blotting. Membranes were exposed to primary antibodies against MT1-MMP (1:1000) and β-actin (1:3000) for 1h, followed by secondary antibodies coupled to horseradish peroxidase, and finally developed with an enhanced chemiluminescence kit.

Gelatinolytic zymography

Conditioned media from cell cultures were analyzed for gelatin degradation activity by SDS-PAGE, under non-reducing conditions. Volumes of conditioned media loaded in each lane were normalised after quantifying the protein content from the cellular lysate. The gel contained 1 mg/mL gelatin and 10% acrylamide. Electrophoresis was carried out at 4°C. After a brief wash with water, the SDS in the acrylamide gel was extracted by incubation with 2.5% Triton X-100. Gelatinolytic activities were developed in a buffer containing 5 mM $CaCl_2$, 150 mM NaCl and 50 mM Tris pH 8.0 at 37°C for 16 h. The gelatinolytic activities were visualised by staining with Coomasie Blue R-250. The ratio of active 62 kDa MMP-2 in the total MMP-2 was determined using scanning densitometry with Kodak molecular imaging software (Rochester, NY, USA). Molecular weights of the gelatinolytic activities were confirmed using a prestained molecular weight standard (Bio-Rad, Hercules, CA, USA).

Immunoprecipitation of epidermal growth factor receptor

Before each treatment, cells were starved in serum-free α-MEM for 24 h. The EGFR was immunoprecipitated by the anti-C antibody as previously described (16) from cell extracts prepared in 50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 mg/mL pepstatin, 2 μg/mL leupeptin and 1 mM sodium orthovanadate (all from Sigma) at 4°C. Immunocomplexes were resolved by 10% SDS-PAGE and analyzed by immunoblot with antibody AG10 for EGFR tyrosine phosphorylation and antibody EGFR984 for EGFR total mass (15). Immunoreactive bands were detected using the enhanced chemiluminiscence detection kit (Amersham Corp., Arlington Heights, FL, USA).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated with TRIzol (Gibco BRL) from appropriately stimulated cells. Cloned DNA was synthesized for 1 h (42°C) with moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) using oligo-dT (Gibco BRL), and RT-PCR analyses were carried out as described previously (19). Primer sequences were as follows: *tgf*-α forward 5′ ACA GAC CTT CCT ACT TGG C 3′; *tgf*-α reverse 5′ CGT GGT TAG AGG ATA CAG C 3′ (amplified PCR product of 428 bp); *hb-egf* forward 5′ GGA CCC TCC CAC TGT ATC 3′; *hb-egf* reverse 5′ CCG TGC TCC TCC TTG TTT 3′ (amplified PCR product of 156 bp); *gapdh* forward 5′ ACC ACA GTC CAT GCC ATC AC 3′; and *gapdh* reverse 5′ TCC ACC ACC CTG TTG CTG TA 3′ (amplified PCR product of 452 bp).

In all cases the annealing temperature was 58°C. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, and DNA was visualised by ethidium bromide staining.

Statistics

Statistical significance for each data set was evaluated using paired Student's *t*-test, with the significance level set at $p < 0.05$.

Results

Tumor necrosis factor-α and type I collagen regulate proMMP-2 activation and MT1-MMP production in human GFs

It has been proposed that activation of proMMP-2 is controlled by MT-MMPs, including MT1-MMP (5). Therefore, to indirectly assess the

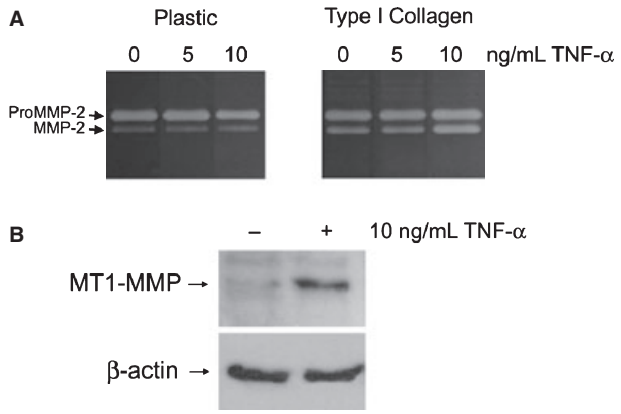

Fig. 1. Tumor necrosis factor-α stimulates proMMP-2 activation and MT1-MMP production in gingival fibroblasts. (A) Primary cultures of human GFs were seeded over plastic or a type I collagen matrix. Sixteen hours after cell attachment, cells were stimulated with TNF-α (5 or 10 ng/mL) in serum-free αMEM. After 48 h the conditioned medium was analyzed to identify the 72 and 62 kDa bands corresponding to pro- and active MMP-2 through gelatin zymography according to the Material and methods section. (B) Gingival fibroblasts cultured over a type I collagen matrix were stimulated with 10 ng/mL TNF-α in serum-free αMEM. After 48 h, cells were harvested from the collagen matrix after digestion with collagenase and tripsin–EDTA as described in the Material and methods section. The presence of MT1-MMP in the cell lysate was assessed through Western blotting. β-Actin was used as a loading control.

activity of MT1-MMP in GF cultures, MMP-2 activation was evaluated through gelatin zymography. The GFs were seeded over plastic or type I collagen and stimulated with 5.0 or 10 ng/mL TNF-α. After 48 h, the gelatinolytic activity present in the conditioned media was analyzed using gelatin zymography. As shown in Fig. 1A, when cells were seeded over plastic, TNF-α was unable to induce proMMP-2 activation measured as the appearance of a 62 kDa band; however, when cells were plated over a type I collagen matrix, TNF-α stimulated proMMP-2 activation. Given these results, we evaluated whether TNF-α was able to specifically stimulate MT1-MMP production. The GFs were seeded over type I collagen and then treated with 10 ng/mL TNF-α for 48 h. As demonstrated in Fig. 1B, Western-blotting of the cell lysate revealed that TNF-α strongly stimulated MT1-MMP production. The effect of TNF-α on MT1-MMP expression was evaluated through semi-quantitative RT-PCR. This assay showed that TNF-α was able to stimulate *mt1-mmp* gene expression (data not shown).

A selective inhibitor of the EGFR kinase activity (AG1478) inhibits TNF-α-stimulated MT1-MMP production and proMMP-2 activation

To analyze whether EGFR signaling is involved in the TNF-α-dependant MT1-MMP production, we took advantage of the capacity of the selective inhibitor Tyrphostin (AG1478) to block the intrinsic kinase activity of this receptor. To this end, GFs were seeded over a type I collagen matrix and after 12 h, 1 μM AG1478 was added to αMEM in the absence of serum. After 45 min, cells were stimulated with 10 ng/mL TNF-α or 10 ng/mL EGF for 48 h. The MT1-MMP production was evaluated through Western blotting of the cell lysate. As shown in Fig. 2A, MT1-MMP production was stimulated by both TNF-α and EGF. Treatment of cells with AG1478 strongly inhibited MT1-MMP production in TNF-α-stimulated cells. Treatment of cells with AG1478 alone did not induce any change in MT1-MMP production (data not shown). Figure 2B shows a quantitative analysis of four independent experiments, in which we observed that MT1-MMP production, stimulated by TNF-α, was inhibited at statistically significant levels when cells were exposed to AG1478 ($p < 0.05$). Analysis through gelatin zymography of the conditioned media derived from these experiments demonstrated that MMP-2 activation was also inhibited by addition of AG1478 (Fig. 2C). Figure 2D shows a quantitative analysis of four independent experiments, in which we observed that AG1478 inhibited TNF-α-stimulated proMMP-2 activation at statistically significant levels ($p < 0.05$).

Tumor necrosis factor-α stimulates EGFR phosphorylation in human GFs

Several studies have identified EGFR as a common signal transducer of diverging extracellular stimulants such as growth factors, integrins, cytokines and G-protein-coupled receptors (20). Therefore, it was tempting to speculate whether TNF-α would stimulate EGFR activation in GFs. To this end, GFs were seeded over a type I collagen matrix and treated with 10 ng/mL TNF-α or 10 ng/mL EGF for 10 min. To interfere with the EGFR signaling activity, cells were exposed for 45 min to 1 μM AG1478 in serum-free αMEM before the TNF-α stimulation. After TNF-α or EGF stimulation, EGFR was immunoprecipitated, and EGFR tyrosine phosphorylation was detected by Western blotting. As shown in Fig. 3A, TNF-α and EGF stimulated EGFR phosphorylation, while AG1478, which is a well-known inhibitor of the tyrosine kinase activity of this receptor, completely blocked its activation. Quantitative analysis of this experiment, performed in triplicate, demonstrated that activation of EGFR by TNF-α and inhibition of this response by AG1478 were statistically significant ($p < 0.05$; Fig. 3B).

Tumor necrosis factor-α stimulates TGF-α and HB-EGF mRNA expression in human gingival fibroblasts

To analyze whether TNF-α may affect the expression of EGFR ligands, we assessed the mRNA expression of HB-

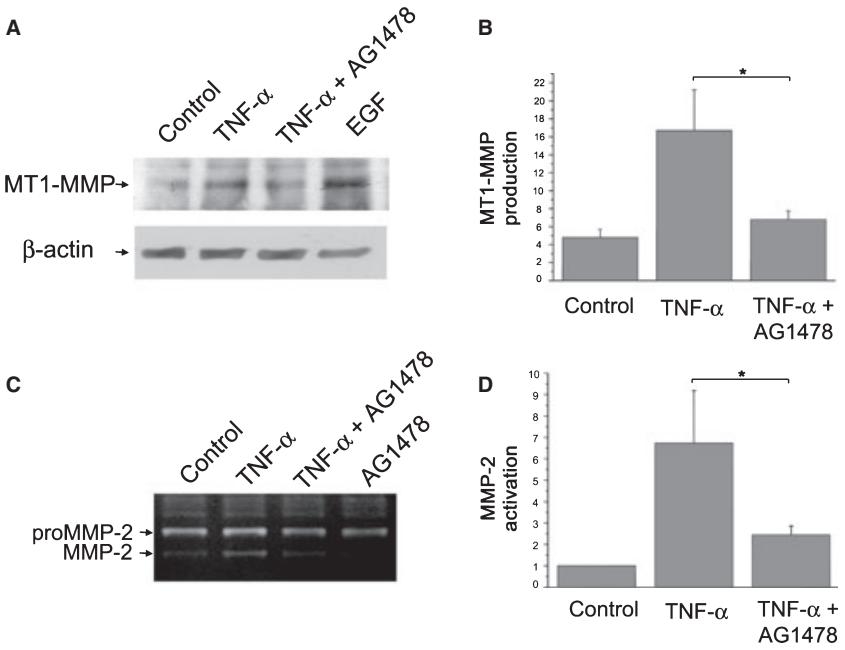

Fig. 2. An inhibitor of EGFR activity downregulates TNF-α-stimulated MT1-MMP production and proMMP-2 activation. (A) Human GFs seeded over a type I collagen matrix were exposed to a selective EGFR inhibitor (AG1478; 1 μM). Cells were then treated with 10 ng/mL TNF-α. As a positive control, cells were treated with 10 ng/mL EGF in serum-free αMEM. After 48 h, cells were harvested from the collagen matrix as described in the Material and methods section, and MT1-MMP production was evaluated through Western blotting of the cell lysate using β-actin as a loading control. (B) A quantitative analysis of MT1-MMP bands, normalised against β-actin, was performed. These data are expressed as the mean and standard error and were obtained from four paired independent experiments. Statistical analysis was performed using Student's *t*-test; $*p < 0.05$. (C) Conditioned media derived from cells cultured in the presence of TNF-α, AG1478 or a combination of both were analyzed through gelatin zymography as described in the Material and methods section. (D) Active MMP-2 bands (62 kDa), obtained from zymography, were digitalised and normalised against proMMP-2 bands (72 kDa). These data are expressed as the mean and standard error and were obtained from three paired independent experiments. Statistical analysis was performed using Student's *t*-test; $*p < 0.05$.

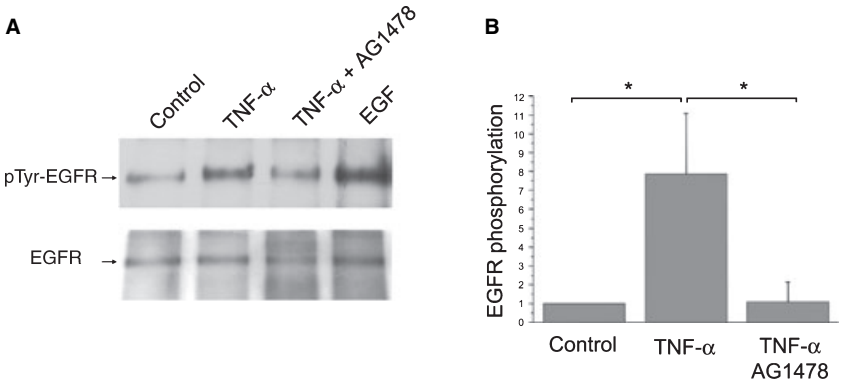

Fig. 3. Tumor necrosis factor-α transactivates EGFR in gingival fibroblasts. (A) Gingival fibroblasts seeded over a type I collagen matrix were maintained in serum-free medium for 12 h and exposed to 1 μM AG1478 for 30 min. Cells were stimulated with 10 ng/mL TNF-α or 10 ng/mL EGF and after 10 min were lysed, and EGFR was immunoprecipitated as described in the Material and methods section. The immune complexes were analyzed by immunoblotting with an antibody against phosphorylated tyrosines (pTyr), and an anti-EGFR antibody was used to determine total mass of the receptor. (B) The pTyr bands derived from Western blots were digitalised and normalised against total EGFR levels. Bars indicate normalised pTyr levels expressed as the mean and standard error. These data were obtained from three independent experiments. Statistical analysis was performed using paired Student's *t*-test; $*p < 0.05$.

EGF and TGF-α, the more prevalent EGFR ligands identified in mesenchymal cells (21). To this end, GFs were cultured in serum-free medium for 12 h and subsequently, cells were stimulated with 10 ng/mL TNF-α. After 12 and 24 h, HB-EGF and TGF-α mRNA levels were analyzed by RT-PCR. As shown in Fig. 4A and B, both HB-EGF and TGF-α were expressed at low levels in unstimulated cells. After 12 h of TNF-α stimulation, the expression of both genes was induced at statistically significant levels (Fig. 4A and B). No further increases in the mRNA expression levels for HB-EGF and TGF-α were observed after 24 h of TNF-α stimulation (Fig. 4).

Discussion

Regulation of collagen-degrading enzymes is a critical step in the turnover of gingival connective tissues. The

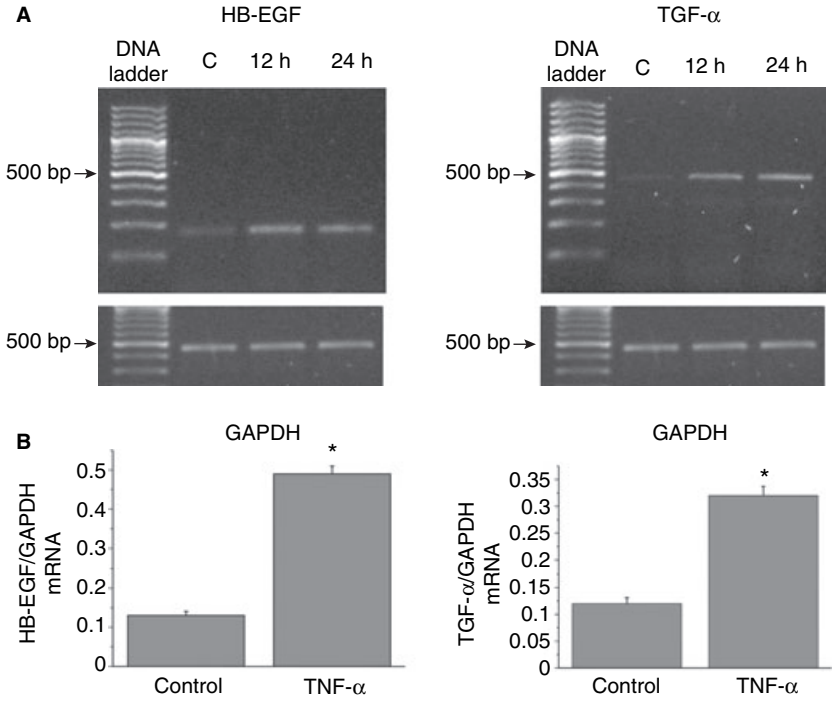

Fig. 4. Tumor necrosis factor-α stimulates HB-EGF and TGF-α mRNA expression in gingival fibroblasts. (A) Gingival fibroblasts seeded over a type I collagen matrix were maintained in serum-free medium for 12 h and stimulated with 10 ng/mL TNF-α. After this, cells were lysed and RNA was extracted as described in the Material and methods section. Expression levels of HB-EGF, TGF-α and GAPDH mRNA were analyzed through semi-quantitative RT-PCR. The PCR products were visualised after agarose electrophoresis. (B) The HB-EGF and TGF-α PCR product bands were digitalised and normalised against GADPH levels. Bars represent normalised HB-EGF and TGF-α mRNA expression levels expressed as the mean and standard error. These data were obtained from three independent experiments. Statistical analysis was performed using paired Student's *t*-test; $*p < 0.05$.

present study was designed to identify molecular mechanisms that regulate MT1-MMP production. Our results are the first to show that EGFR and TNF-α may regulate MT1-MMP production in GFs. This statement is supported by the observation that TNF-α-stimulated MT1-MMP production and proMMP-2 activation are downregulated after inhibition of the signaling activity of EGFR. Tumor necrosis factor-α may also stimulate EGFR phosphorylation and the expression of the EGFR ligands HB-EGF and TGF-α. These findings suggest that both signaling pathways may interact to control MT1-MMP production in GFs.

Our results show that MT1-MMP production is stimulated by the inflammatory cytokine TNF-α. Significant amounts of this cytokine have been detected in inflamed gingival tissues and gingival crevicular fluid (10,11,22). Therefore, it is possible that increased levels of TNF-α, such as those detected by previous studies of periodontal lesions, may stimulate GFs to produce MT1-MMP. These results are also consistent with other observations of skin and rheumatoid synovial fibroblasts, in which MT1-MMP production is also stimulated by TNF-α (12,13). Tumor necrosis factor-α is primarily produced as a type II transmembrane protein that is released via proteolytic cleavage by the metalloprotease TNF-converting enzyme (TACE; 23). Moreover, proTNF-α has been identified as one of the physiological substrates which can be processed by the proteolytic activity of MT1-MMP (24). Given the observation that in GFs, TNF-α may induce MT1-MMP expression, and that this proteolytic enzyme may also process TNF-α from the cell surface, it is tempting to speculate whether this system may constitute an autocrine feedback mechanism that positively regulates matrix remodeling in gingival tissues.

Mice deficient in EGFR develop a severe defect in lung morphogenesis and show a reduced expression of MT1-MMP in fibroblasts derived from this tissue (14). This defect in MT1-MMP production is also observed when $EGFR^{-/-}$ fibroblasts are stimulated with different growth factors, such as fibroblast growth factor-2, suggesting that EGFR may have a prominent role in the different signaling networks that regulate MT1-MMP production (14). In addition, EGFR also has a role in MT1-MMP expression in thyroid- (25) and glioma-derived cancer cells (26), suggesting that this may be a highly conserved mechanism present in different cell types. Moreover, recent studies have pointed out that, in mouse embryonal fibroblasts, EGFR is able to regulate the expression of several MMP members, including MT1-MMP, through an activator protein-1 (AP-1) mechanism (27). Our results show that when GFs are stimulated with TNF-α in the

presence of AG1478 (a selective inhibitor of the EGFR tyrosine kinase activity), both proMMP-2 activation and MT1-MMP production are downregulated, suggesting that EGFR is involved in MT1-MMP production in GFs.

Epidermal growth factor receptor is a member of the ERB tyrosine-kinase receptor family that binds a number of related peptide growth factors, which include EGF, TGF-α and HB-EGF (28). Cross-communication between distinct signaling systems is essential to integrate a variety of extracellular stimuli into a limited number of signaling pathways (29). Epidermal growth factor receptor has been identified as a key element in the complex signaling network that is transactivated by G-protein-coupled receptors, cytokine receptors, estrogen receptors, integrins, ion channels or stress-inducing agents (29–32). TNF-α exerts its biological functions via interaction with the TNF receptor (TNFR) family (9). Interaction between the TNFR and EGFR signaling pathways has been explored in previous studies. It has been observed that TNF-α sensitizes carcinoma cell lines for anti-EGFR therapy (33). In NIH3T3 cells, TNF-α-stimulated nuclear factor-kappa B (NF-κB) activation is regulated by EGFR signaling (34). In the present study, TNF-α was able to transactivate EGFR phosphorylation, clearly suggesting that interaction between these signaling pathways may occur in human GFs. Several mechanisms have been proposed to explain the interaction between the TNFR and EGFR signaling pathways; these include the activity of the enzyme TACE, which may release EGFR ligands from the cell surface (32,35). Other transactivation mechanisms, such as the Src family of kinases (32,36), are also involved. Clearly, future studies should explore whether some of these mechanisms may explain the nature of the interaction between these signaling pathways in gingival cells.

Our results show that after TNF-α stimulation, both TGF-α and HB-EGF mRNA expression levels were increased. Although the present results do not show that TGF-α or HB-EGF is involved in TNF-α-stimulated EGFR transactivation, both molecules may stimulate EGFR signaling activity and are considered to be significant physiological ligands for this receptor (37). We observed EGFR activation by TNF-α after 10 min of stimulation. This suggests that this is probably a non-transcriptional effect. In contrast, 12 h stimulation with TNF-α induced the expression of the genes for both TGF-α and HB-EGF. We envisage that in an *in vivo* scenario, the persistent exposure of GFs to TNF-α might chronically sustain a positive signal to sensitize the EGFR signaling system that modulates the prolonged production of MT1-MMP and their ability to remodel the extracellular matrix.

Our results showed that when cells were seeded over a type I collagen film, both MMP-2 activation and MT1-MMP production were stimulated when compared to cells plated over plastic. This effect has previously been described in dermal fibroblasts (12,38). The NF-κB pathway has been implicated in MT1-MMP production in skin fibroblasts (12). Since signaling through NF-κB is stimulated when cells are cultured over different matrix substrates, such as collagen (39), it is possible that this pathway is involved in the enhanced production of MT1-MMP in gingival cells. It is interesting to mention that TNF-α is able to transactivate EGFR, leading to NF-κB signaling in NIH3T3 cells (34). Future studies should explore the role of integrin activation, NF-κB and EGFR in the production of MT1-MMP in gingival fibroblasts.

The findings of the present study help to unravel the molecular mechanisms that control MMP production in GFs and increase our understanding of the events that regulate connective tissue remodeling in gingival tissues. The finding that MT1-MMP induction by TNF-α is EGFR dependent reveals a novel molecular mechanism for matrix remodeling in gingival cells.

Acknowledgements

This work was totally financed by a FONDECYT grant (no. 1061065, to P.C.S.).

References

1. Bartold PM, Walsh LJ, Narayanan AS. Molecular and cell biology of the gingiva. *Periodontol 2000* 2000;**24:**28–55.
2. Zappa U, Reiking-Zappa M, Graf H, Case D. Cell populations associated with active probing attachment loss. *J Periodontol* 1992;**63:**748–752.
3. Birkedal-Hansen H, Moore WG, Bodden MK *et al*. Matrix metaloproteinases: a review. *Crit Rev Oral Biol Med* 1993;**4:**197–250.
4. Parks WC, Wilson CL, López-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* 2004;**4:**617–629.
5. Sato H, Takino T, Okada Y *et al*. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* 1994;**370:**61–65.
6. Holmbeck K, Bianco P, Caterina J *et al*. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 1999;**99:**81–92.
7. Beertsen WC, Holmbeck K, Niehof A *et al*. On the role of MT1-MMP, a matrix metalloproteinase essential to collagen remodeling, in murine molar eruption and root formation. *Eur J Oral Sci* 2002;**110:**445–451.
8. Fini ME, Cook JR, Mohan R, Brinckerhoff CE. Regulation of matrix metalloproteinase gene expression. In: Parks WC, Mecham RP, eds. *Matrix Metalloproteinases*. San Diego: Academic Press, 1998:299–356.
9. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003;**10:**45–65.
10. Rossomando EF, Kennedy JE, Hadjimichael J. Tumour necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. *Arch Oral Biol* 1990;**35:**431–434.
11. Roberts FA, McCaffery KA, Michalek SM. Profile of cytokine mRNA expression in chronic adult periodontitis. *J Dent Res* 1997;**76:**1833–1839.
12. Han Y-P, Tuan T-L, Huayang W, Hughes M, Garner WL. TNF-α stimulates activation of pro-MMP2 in human skin through NF-κB mediated induction of MT1-MMP. *J Cell Sci* 2001;**114:**131–139.
13. Migita K, Eguchi K, Kawabe Y *et al*. TNF-α-mediated expression of membrane-type matrix metalloproteinase in rheumatoid synovial fibroblasts. *Immunology* 1996;**89:**553–557.
14. Kheradmand F, Rishi K, Werb Z. Signaling through the EGF receptor controls lung morphogenesis in part by regulating MT1-MMP-mediated activation of gelatinase A/MMP2. *J Cell Sci* 2002; **115:**839–848.

15. Faúndez V, Krausse R, Holuigue L, Garrido J, González A. Epidermal growth factor receptor in synaptic fractions of the rat central nervous system. *J Biol Chem* 1992;**267:**20363–20370.
16. Salazar G, González A. Novel mechanism for regulation of epidermal growth factor receptor endocytosis revealed by protein kinase A inhibition. *Mol Biol Cell* 2002;**13:**1677–1693.
17. Larjava H, Heino J, Kähäri V-M, Krusius T, Vuorio E. Characterization of one phenotype of human granulation-tissue fibroblasts. *J Dent Res* 1989;**68:**20–25.
18. Birkedal-Hansen H. Catabolism and turnover of collagens: collagenases. *Methods Enzymol* 1987;**144:**140–171.
19. Kwon YJ, Perez P, Aguilera S *et al.* Involvement of specific laminins and nidogens in the active remodeling of the basal lamina of labial salivary glands from patients with Sjogren's syndrome. *Arthritis Rheum* 2006;**54:**3465–3475.
20. Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transduction. *Oncogene* 2001;**20:**1594–1600.
21. Harris RC, Chung E, Coffey RJ. EGF receptor ligands. *Exp Cell Res* 2003;**284:** 2–13.
22. Heasman PA, Collins JG, Offenbacher S. Changes in crevicular fluid level of Interleukin-1β, leukotriene B4, prostaglandin E_2, and tumour necrosis factor α in experimental gingivitis in man. *J Periodontal Res* 1993;**28:**241–247.
23. Black RA, Rauch CT, Kozlosky CJ *et al.* A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* 1997;**385:**729–733.
24. Tam EM, Morrison CJ, Wu YI, Stack MS, Overall CM. Membrane protease proteomics: isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. *Proc Natl Acad Sci U S A* 2004;**101:**6917–6922.
25. Yeh MW, Rougier JP, Park JW *et al.* Differentiated thyroid cancer cell invasion is regulated through epidermal growth factor receptor-dependent activation of matrix metalloproteinase (MMP)-2/gelatinase A. *Endocr Relat Cancer* 2006; **13:**1173–1183.
26. Van Meter TE, Broaddus WC, Rooprai HK, Pilkington GJ, Fillmore HL. Induction of membrane-type-1 matrix metalloproteinase by epidermal growth factor-mediated signalling in gliomas. *Neuro Oncol* 2004;**6:**188–199.
27. Kajanne R, Miettinen P, Mehlem A *et al.* EGF-R regulates MMP function in fibroblasts through MAPK and AP-1 pathways. *J Cell Physiol* 2006;**212:**489–497.
28. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;**2:**127–137.
29. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr Relat Cancer* 2001;**8:**11–31.
30. Carpenter G, Cohen S. Epidermal growth factor. *J Biol Chem* 1990;**265:**7709–7712.
31. Biscardi JS, Ishizawar RC, Silva CM, Parsons SJ. Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res* 2000;**2:**203–210.
32. Guerrero J, Santibañez JF, González A, Martínez J. EGF receptor transactivation by urokinase receptor stimulus through a mechanism involving Src and matrix metalloproteinases. *Exp Cell Res* 2004; **292:**201–208.
33. Hambek M, Solbach C, Schnuerch H-G *et al.* Tumor necrosis factor-α sensitizes low epidermal growth factor receptor (EGFR)-expressing carcinomas for anti-EGFR therapy. *Cancer Res* 2001;**61:**1045–1049.
34. Hirota K, Murata M, Itoh T, Yodoi J, Fukuda K. Redox-sensitive transactivation of epidermal growth factor receptor by tumor necrosis factor confers the NF-κB activation. *J Biol Chem* 2001; **276:**25953–25958.
35. Argast GM, Campbell JS, Brooling JT, Fausto N. Epidermal growth factor receptor transactivation mediates tumor necrosis factor-induced hepatocyte replication. *J Biol Chem* 2004;**279:**34530–34536.
36. Wu W, Graves LM, Gill GN, Parsons SJ, Samet JM. Src-dependent phosphorylation of the epidermal growth factor receptor on tyrosine 845 is required for zinc-induced Ras activation. *J Biol Chem* 2002;**277:**24252–24257.
37. Citri A, Yarden Y. EGF-ERBB signaling: towards the systems level. *Nat Rev Mol Cell Biol* 2006;**7:**505–515.
38. Zigrino P, Drescher C, Mauch C. Collagen-induced proMMP-2 activation by MT1-MMP in human dermal fibroblasts and the possible role of α2β1 integrins. *Eur J Cell Biol* 2001;**80:**68–77.
39. Ferri N, Garton KJ, Raines EW. An NF-κB-dependent transcriptional program is required for collagen remodeling by human smooth muscle cells. *J Biol Chem* 2003;**278:**19757–19764.