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Epidermal growth factor stimulates urokinase-type plasminogen activator expression in human gingival fibroblasts. Possible modulation by genistein and curcumin

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Background: Regulation of the extracellular matrix turnover is a crucial process in wound healing and the progress of periodontal disease. It has been proposed that urokinase-type plasminogen activator (uPA), under the control of growth factors or cytokines, provides the proteolytic potential to the accomplishment of these cellular events. Epidermal growth factor (EGF) is one of the growth factors that has been shown to be active in uPA regulation.

Methods: In this study, we have assessed the effect of EGF on uPA expression in primary cultures of human gingival fibroblasts. We also studied the signaling pathways involved in this process and the role of the dietary phytoestrogens curcumin and genistein as potential modulators of this response.

Results: Human gingival fibroblasts expressed a basal uPA activity, which was inhibited by genistein, but not by curcumin. After treatment with 10 ng/ml EGF, uPA production was strongly stimulated. Exposure to genistein and curcumin inhibited EGF-stimulated urokinase production, although only genistein showed a statistically significant inhibitory response. Using more specific inhibitors, we found that the mitogen-activated extracellular kinase and c-Jun N-terminal kinase (JNK) inhibitors PD98059 and SP600125 also blocked the EGF-dependent stimulatory effect. On the other hand, SB203580, inhibitor of the p38 member of mitogen-activated protein kinase family, did not alter this response. In accordance to these findings, EGF stimulated a potent activation of JNK and a mild activation of extracellular signal-regulated kinases 1/2. Finally, EGF stimulated the phosphorylation of its receptor and tyrphostin (AG1478), curcumin and genistein were able to inhibit this stimulatory effect.

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Conclusions: These results indicate that EGF constitutes a strong stimuli on uPA expression in human gingival fibroblasts. Our data also shows that EGF-stimulated uPA production involves the activation of the extracellular signal-regulated kinases 1/2 and JNK signaling pathways and might be modulated by the natural phytoestrogens curcumin and genistein.

Extracellular matrix molecules are actively remodeled during wound healing and chronic inflammatory diseases such as periodontitis (1). Fibroblasts constitute the predominant cell type in human gingival tissues and are thought to play a significant role in tissue remodeling through expression of proteolytic enzymes and synthesis of extracellular matrix glycoproteins and proteoglycans (2). The urokinase-type plasminogen activator (uPA) is a serine protease expressed by fibroblasts in gingival tissues during wound healing and chronic inflammation (3, 4). uPA is able to convert plasminogen into plasmin (5), another serine protease responsible for degradation of fibrin and conversion of latent matrix metalloproteinases (MMPs) into their active forms (6). During periods of active inflammation, fibroblasts are exposed to a wide range of cytokines and growth factors released by resident tissue cells or infiltrating leukocytes (7). Among this group of molecules, epidermal growth factor (EGF) has been identified in gingival tissue (8) and gingival crevicular fluid (9). EGF is thought to mediate several of the responses observed during wound healing and inflammation, such as stimulation of cell proliferation and extracellular matrix turnover (10). More specifically, EGF is able to stimulate the expression of several proteolytic enzymes in gingival fibroblasts including MMP-1, MMP-3 (11) and MMP-13 (12). Although factors regulating uPA expression are of critical importance to understand tissue remodeling of human gingiva, no studies have assessed the role of EGF on the expression of uPA in human gingival fibroblasts.

Several signaling pathways have been associated with uPA expression. Although the extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathway has been primarily associated with uPA expression in tumor cells (13), other studies have suggested that p38 (14) and c-Jun N-terminal kinase (JNK) (15–17) may also be implicated in this process. Considering that the signaling pathways that regulate uPA expression may play a key role in inflammation and cancer, previous studies have exploited the ability of dietary components to modulate or interrupt this response (18, 19). Curcumin and genistein are phytoestrogens abundant in Asian food that may have therapeutic potential due to their ability to inhibit these signaling pathways (20). Noteworthy, the signaling pathways regulating uPA expression in gingival fibroblasts are currently unknown.

The present study is an effort to evaluate the effect of EGF on uPA expression in human gingival fibroblasts, to unravel the signaling pathways involved in this process and to test the potential role of dietary components as possible modulators of this response.

Material and methods

Reagents

Recombinant human EGF, PD98059 and SB203580 were purchased from Calbiochem (San Diego, CA, USA). SP600125 was purchased from Biomol (Plymouth Meeting, PA, USA). Curcumin and genistein were obtained from Sigma Chemical (St Louis, MO, USA). The following antibodies were used: mouse monoclonal anti-pERK1/2, mouse monoclonal anti-ERK1/2 and rabbit polyclonal anti-pJNK were all purchased from Upstate Biotechnology (Lake Placid, NY, USA). Mouse monoclonal anti-JNK2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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 A. Gonzalez (Pontificia Universidad Católica de Chile) and have been previously characterized (21, 22).

Cell culture

Primary cultures of human gingival fibroblasts were established by the explant method (23). Tissue explants were obtained from the retromolar gingival tissue of five patients (one male, four females; mean age of 28 ± 15 years) undergoing extraction of third molars at a private dental practice in Santiago, Chile. No previous history of inflammation of the retromolar tissue was reported. All gingival biopsies were obtained with informed consent from each patient and the protocol for tissue obtainment was approved by the Ethical Committee of the Faculty of Dentistry of the University of Chile. No relevant pre-existing medical or drug histories were reported during the last 6 months. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (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.

Immunofluorescence studies

At passage 4, cells were grown on cover slips and fixed with 4% paraformaldehyde/phosphate-buffered saline. Fixed cells were permeabilized with 2% Triton X-100/1% bovine serum albumin/phosphate-buffered saline for 5 min and subsequently blocked with 5% bovine serum albumin/phosphate-buffered saline for 20 min. Cells were exposed to primary antibodies

against alpha-smooth muscle actin (Sigma), vimentin (Sigma), and human cytokeratin (Dako, Carpinteria CA, USA). As secondary antibody, anti-mouse immunoglobulin G coupled to fluorescence isothiocyanate (Sigma) was used.

Detection of uPA activity by casein zymography

uPA secreted activity of cell cultures was determined by caseinolytic zymography (24). Briefly, aliquots of conditioned serum-free medium, normalized for the same number of cells, were subjected to electrophoresis in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under non-reducing conditions. Sodium dodecyl sulfate was removed by extensive washing in 2.5% Triton X-100, the gels were placed on 1% agarose gels containing 0.5% casein and 2 μg/ml of plasminogen (Sigma), and incubated at 37°C for 24 h. Plasmin-dependent proteolysis was detected as a clear area in a white-blue field. Quantification of these areas was performed by densitometric analysis.

Immunodetection of epidermal growth factor receptor

Before each treatment, cells were starved in serum-free Dulbecco's modified Eagle's medium for 24 h. The EGFR was immunoprecipitated by the anti-C antibody as described (22) 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 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM sodium orthovanadate (all from Sigma) at 4°C. Immunocomplexes were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblot with antibodies AG10 for EGFR tyrosine phosphorylation and antibody EGFR984 for EGFR total mass (21). Immunoreactive bands were detected using the enhanced chemiluminiscence (ECL) detection kit (Amersham Corp., Arlington Heights, FL, USA).

Western Immunoblotting

Assay of mitogen-activated protein kinase (MAPK) activation — The activation of ERK1/2 and JNK was determined by western-blotting using antibodies specific for phosphorylated, activated forms of these MAPKs. Fibroblasts were treated with EGF in Dulbecco's modified Eagle's medium without serum at various time points and lysed in phosphate-buffered saline buffer containing 150 mM NaCl, 1% Triton X-100 pH 7.4 and 10 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, 2 mM *N*-ethylmaleimide, 1 mM orthovanadate and 1 mM NaF. Proteins were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride transfer membrane (PerkinElmer Life Sciences, USA). Membranes were exposed to primary antibodies, secondary antibodies coupled to horseradish peroxidase and finally developed with an ECL kit. As loading controls, western blots were also performed using antibodies against total ERK1/2 and JNK.

Immunodetection of secreted uPA — To evaluate at protein level the secretion of uPA, conditioned media (5 ml/treatment) from cell cultures was collected, lyophilized, and subsequently dissolved in 500 μl of distilled water. Proteins were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions and transferred to polyvinylidene

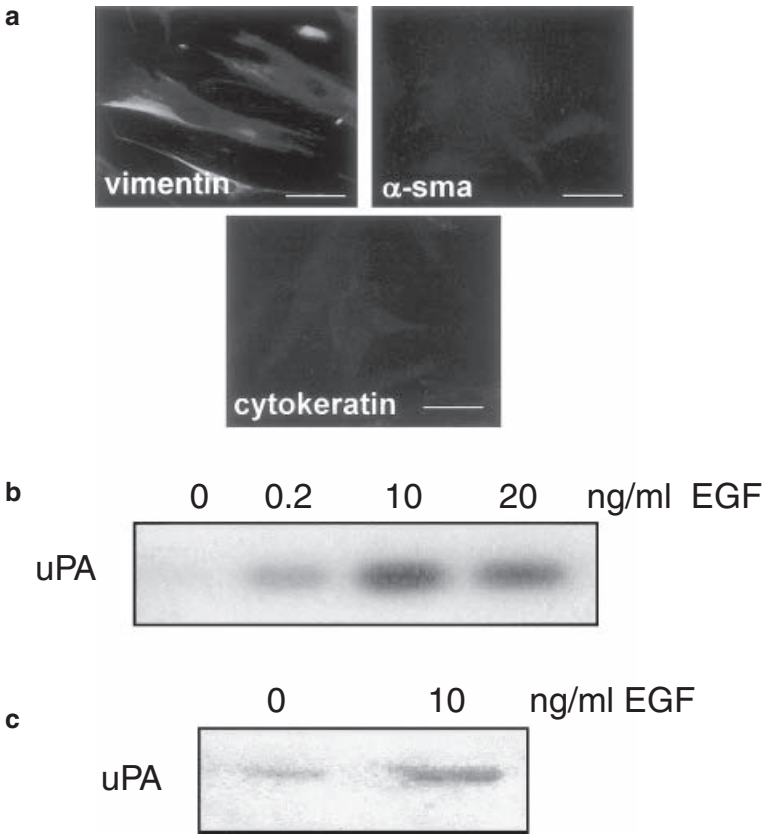

Fig. 1. Characterization of human gingival fibroblasts cultures and effect of epidermal growth factor (EGF) on urokinase-type plasminogen activator (uPA) production. (a) Human gingival fibroblasts at the fourth subculture were stained with antibodies recognizing vimentin, alpha-smooth muscle actin (α-sma) and pan-cytokeratins. Bar = 20 μm. (b) Human gingival fibroblasts derived from a healthy subject were stimulated with increasing concentrations of EGF during 48 h in Dulbecco's modified Eagle's medium without serum. Conditioned medium was analyzed through zymography. Results are representative of the observations in four independent samples. (c) Immunoblot for uPA in samples prepared as above.

difluoride transfer membrane. Membranes were exposed to primary antibodies, secondary antibodies coupled to horseradish peroxidase and finally developed with an ECL kit.

Statistical analysis

Differences between cellular uPA production after treatment with genistein or curcumin were assessed with a Mann–Whitney *U*-test.

Results

Phenotypic characterization of human gingival fibroblasts

Gingival fibroblast cultures established from gingival biopsies obtained as described in Material and Methods, expressed some phenotypic markers of the mesenchymal lineage. As shown in Fig. 1(a), at passage 4, cultured cells expressed vimentin, a mesenchymal marker, and were negative to alpha-smooth muscle actin. The lack of expression of this protein denotes the absence of myofibroblasts in culture and suggests the healthy condition of the samples from which these cells were obtained. The non-expression of cytokeratin indicates that cell cultures were not contaminated with epithelial cells.

Regulation of uPA expression by EGF

To analyze whether EGF was able to regulate uPA expression in gingival fibroblasts, cells were cultured in serum-free Dulbecco's modified Eagle's medium for 24 h and then stimulated with increasing concentrations of EGF during 48 h. As determined by casein zymography of conditioned medium, 10 ng/ml EGF exerted a potent stimulatory effect on uPA activity (Fig. 1b). This stimulus does not represent an activation phenomenon of a pre-existing uPA pool, as we confirmed by measuring the EGF stimulatory effect at the protein level by immunoblot. Figure 1(c) shows that concentrated conditioned medium expressed an augmented amount of immuno-reactive uPA when cells were stimulated with 10 ng/ml EGF.

Effect of curcumin and genistein on EGFR phosphorylation and EGF-stimulated uPA expression in human gingival fibroblasts

To assess the effect of curcumin and genistein on growth factor-stimulated uPA production in gingival fibroblasts, cells derived from 3 healthy individuals were exposed to these agents 15 min before a 48-h treatment with EGF. Secreted uPA activity was evaluated by casein zymography. As shown in Fig. 2, genistein, but not curcumin, was able to inhibit the non-stimulated uPA production. Treatment of fibroblasts with genistein induced a statistically significant decrease in EGF-stimulated uPA production (Mann–Whitney *U*-test, $p = 0.049$). Addition of curcumin also decreased the amount of EGF-stimulated uPA production although this effect was not statistically significant (Mann–Whitney *U*-test, $p = 0.82$). EGF exerts its cellular activities after binding to its surface receptor, EGFR (25). Following ligand binding, EGFR undergoes homo or heterodimerization and a further auto-phosphorylation of specific

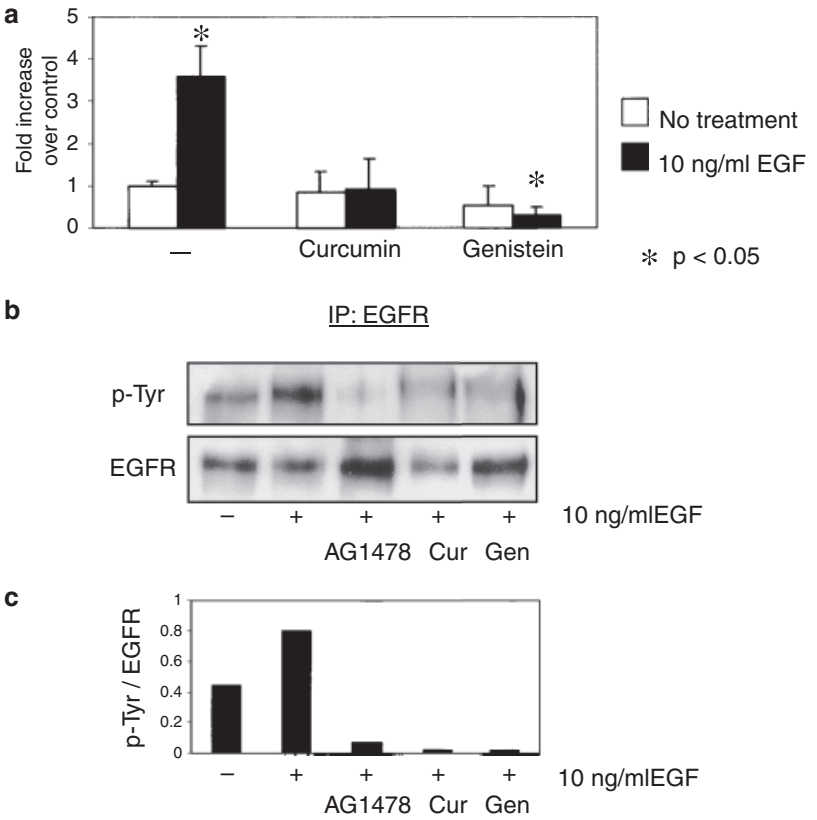

Fig. 2. Effect of curcumin and genistein on urokinase-type plasminogen activator (uPA) production and epidermal growth factor receptor (EGFR) activation in human gingival fibroblasts. (a) Gingival fibroblasts from three healthy individuals, stimulated or not with 10 ng/ml of epidermal growth factor (EGF), were exposed to 10 μM curcumin or 20 μM genistein for 15 min prior to a 48-h incubation time in Dulbecco's modified Eagle's medium without serum. uPA secreted activity into conditioned medium was detected by casein zymography as in Fig. 1 and the bands of casein digestion were analyzed by densitometry in three individual samples. Bars represent means of three determinations ± SD. Asterisks indicates statistically significant differences. (b) Serum-starved gingival fibroblasts were exposed to 20 μM curcumin (Cur), 10 μM genistein (Gen) or 1 μM AG1478 for 30 min. After this, cells were incubated during 15 min in the absence or presence of 10 ng/ml EGF. The EGFR was immunoprecipitated and the immune-complexes analyzed by immunoblotting with an antibody against phosphorylated tyrosines and an anti-EGFR to determine total mass of receptor. (c) Normalized levels of tyrosine phosphorylation, relative to total mass of EGFR obtained after band digitalization from (b) are represented.

tyrosine residues in its cytoplasmic domain. This covalent modification generates docking sites for proteins containing SH2 domains that allows the EGF-dependent intracellular signaling to start (26). To test if curcumin and genistein interfered with the EGFR activation, we assayed the tyrosine phosphorylation of EGFR in the presence of these two agents. As shown in Fig. 2(b), both curcumin and genistein provoked a blockage of EGF-dependent EGFR activation that is comparable in magnitude with the inhibition induced by tyrphostin (AG1478), a specific inhibitor of the EGFR intrinsic tyrosine kinase activation.

Signaling pathways involved in uPA expression stimulated by EGF in gingival fibroblasts

Once we demonstrated the potent stimulus of EGF on uPA expression in gingival fibroblasts, we evaluated the potential signaling pathway involved in this stimulus. The Ras-MAPK pathway leading to ERK, JNK or p38 activation is a well-identified pathway that is triggered after EGF binds to its receptor. We utilized chemical inhibitors designed to inhibit these specific routes to block the EGF-stimulated uPA production. We tested whether the EGF stimulus on uPA production was sensitive to PD98059 (40 μM), a specific inhibitor of MEK1/2 (upstream activator of ERK1/2) to the JNK inhibitor SP600125(10 μM) and to the selective p38 inhibitor SB203580(10 μM). As Fig. 3(a) shows, both PD98059 and SP600125, completely abrogated the stimuli of EGF on uPA production. Meanwhile, SB203580 did not alter this response. To test if both signaling pathways are indeed activated by EGF, we analyzed the kinetic of activation of both routes by EGF. Figure 3(b) shows that EGF was able to activate JNK with a maximum effect at approximately 15 min. After that, the activation signal decayed significantly. EGF also activated ERK1/2 in gingival fibroblasts but to a lower level as shown in Fig. 3(c).

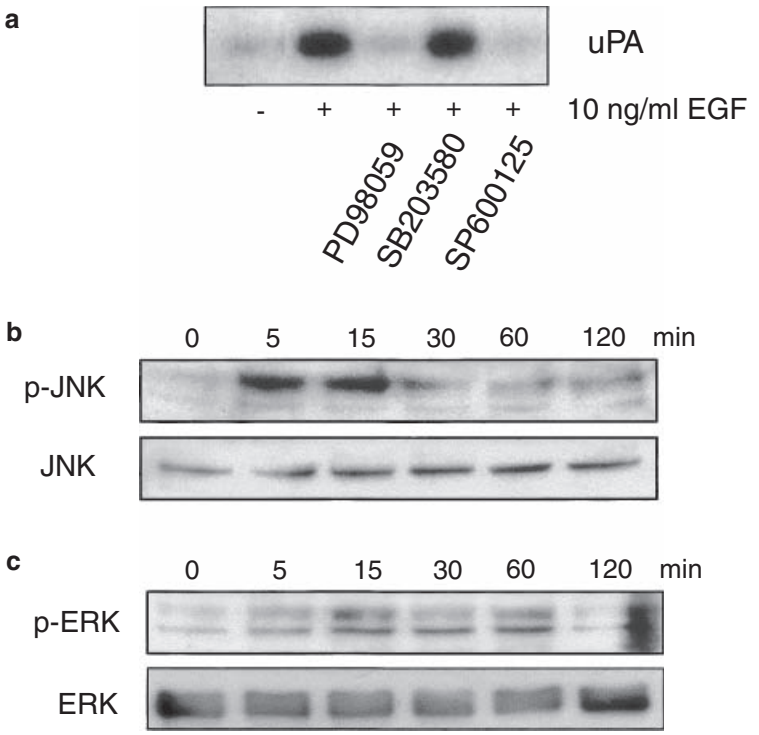

Fig. 3. Signal transduction pathways involved in epidermal growth factor (EGF)-stimulated urokinase-type plasminogen activator (uPA) activity. (a) Human gingival fibroblasts were incubated for 15 min in the presence of specific mitogen-activated protein kinase inhibitors: 40 μM PD98059, 10 μM SB203580 or 10 μM SP600125 in serum-free Dulbecco's modified Eagle's medium. After this, cells were treated with 10 ng/ml EGF for 48 h. Secreted uPA activity was evaluated by casein zymography as described. Results are representative of experiments performed in fibroblasts from two healthy individuals. Human gingival fibroblasts were stimulated with 10 ng/ml EGF and the activation kinetics of c-Jun N-terminal kinase (JNK) (b) and extracellular signal-regulated kinase (ERK) (c) were determined through immunoblot of the phosphorylated and non-phosphorylated forms of ERK and JNK as described in Material and Methods.

Effect of curcumin and genistein on ERK and JNK phosphorylation

Given that the ERK and JNK pathways were identified as important signaling routes implicated in the stimulatory effect of EGF on uPA production and that curcumin and genistein were able to block the EGFR activation, we analyzed the inhibitory potential of these agents in the activation of ERK1/2 and JNK. As Fig. 4 shows, stimulation of gingival fibroblasts with EGF induced a modest increase in ERK activation at 15 min that was blocked by the EGFR inhibitor AG1478 and more strongly by curcumin and genistein. As we showed previously, treatment of gingival fibroblasts with EGF induced a potent stimulus on JNK phosphorylation that was completely blocked by AG1478. Treatment with curcumin and genistein also diminished JNK phosphorylation, albeit at a lesser extent when compared to AG1478.

Discussion

Regulation of uPA expression is a critical step in the turnover of extracellular matrix proteins during wound healing and chronic inflammation (27). In these conditions, uPA may stimulate activation of plasminogen into plasmin (5), a serine-proteinase with a wide range of specificity that includes degradation of fibrin and activation of MMPs through proteolytic processing of the NH_2-terminal fragment of the enzyme (6).

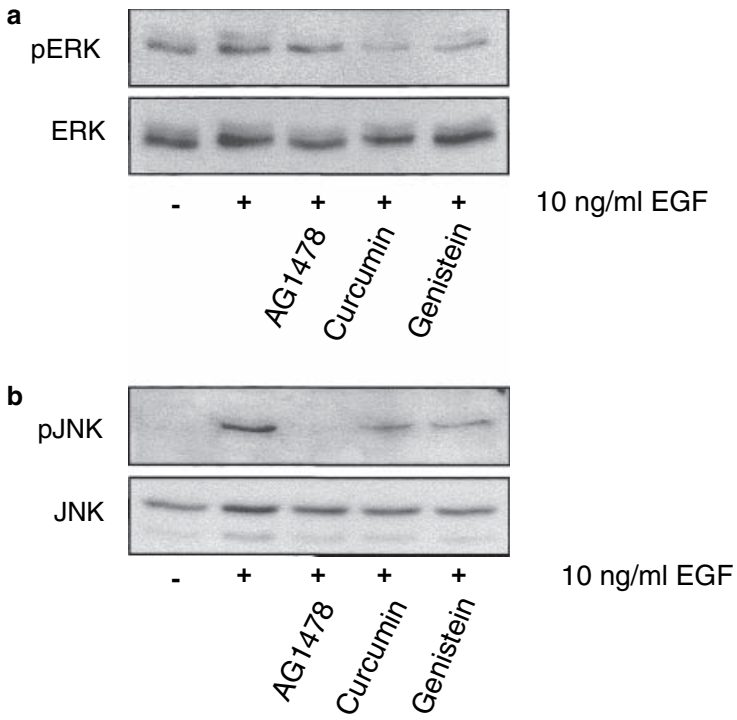

Fig. 4. Effect of curcumin, genistein and AG1478 on the activation of extracellular signal-regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK). Serum-starved gingival fibroblasts were exposed to 20 μM curcumin or 10 μM genistein or 1 μM AG1478 for 30 min prior to a 15-min incubation with EGF and ERK (a) or JNK activation (b) were analyzed as in Fig. 3.

Several studies have suggested that EGF may regulate cell behavior during wound healing and chronic inflammation (28, 29); however, the cellular mechanisms involved in these responses are still unclear. In spite of the fact that the role of EGF on uPA expression has been studied by many groups, at present there is not a single proposed mechanism for its action. Although some authors have observed that EGF enhances uPA expression (30), others have demonstrated that this stimulus is restricted to uPA receptor (31) and others have even proposed that the growth factor induces an inhibition of uPA expression (32). Those discrepant results suggest that the effect of EGF on uPA activity is strictly dependent of the cellular context. Our findings show that in gingival fibroblasts, EGF potently stimulates uPA expression at the protein level. This clearly suggests that under conditions in which this growth factor is up-regulated (i.e. periodontal disease) this stimulus enhances the tissue proteolytic potential. This observation is also in line with previous reports that demonstrated that EGF also stimulates MMPs expression in human gingival fibroblasts (11, 12).

Urokinase expression is tightly regulated at the transcriptional level. The uPA promoter contains an AP1-enhancer element, located at −2.4 kb, that is known to respond to several extracellular stimuli (33). One of the major targets of EGF signaling is the activation of the AP-1 complex. Experiments that identified EGF as an important stimulus on uPA production also determined that ERK1/2 was the prototypical pathway involved in this stimulation (13). Other studies, however, pointed out that both p38 and JNK pathways are also involved in the stimulation of uPA expression by primary agents that are more associated with cellular stress than cell proliferation, such as UV irradiation, alkylating agents and phorbol 12-myristate 13-acetate (14–17). From our results we can state that the routes of ERK and JNK are involved in the production of uPA after the stimulus of EGF, a growth factor mainly associated with cell proliferation. This stimulus on uPA expression may depend on the adequate production and assembly of c-Fos and c-Jun (molecular components of AP-1), a process in which JNK plays an important role through the activation of c-Jun. It has also been demonstrated that JNK can activate the transcription factor ATF-2, which after association with c-Jun stimulates uPA expression (34).

EGF receptors (EGFR) are transmembrane proteins with an intrinsic tyrosine kinase activity, which is stimulated after binding to EGF (26). Binding of EGFR to its ligand induces dimerization of this receptor and subsequently triggers autophosphorylation of the cytoplasmic tyrosine residues, creating docking sites for various membrane-targeted signaling proteins (25). It has been shown that EGFR may activate the mitogen-activated protein kinase kinase-1 (MEKK1) in various cell types (35–37). Catalytically inactive inhibitory mutants of MEKK1 block EGFR activation of the JNK and ERK pathways (38). Interestingly, MEKK1 deficient mice display a greatly reduced uPA expression after stimulation with either fibroblast growth factor-2 or phorbol 12-myristate 13-acetate (17).

Previous studies have identified EGF as one of the growth factors involved in the gingival responses during wound healing and inflammation (9, 39). Periodontitis is a highly prevalent disease characterized by a high rate of extracellular matrix turnover (1, 40). This process depends on the regulated expression of proteolytic enzymes by cytokines and growth factors (1). Chemoprevention of periodontal disease constitutes an attractive alternative to avoid tissue damage and to improve periodontal health. In the present work we chose to test the potential of dietary components (curcumin and genistein) to block uPA production as a means to preserve the

integrity of the gingival matrix. Curcumin is a major component of turmeric, the dried rhizome of *Curcuma longa L*, which is commonly used as a flavoring agent in Asian food. Previous studies have reported that curcumin may exert anti-carcinogenic (41) and anti-inflammatory activities (42). Genistein is an isoflavone compound present in soy, which is an important nutritional component in eastern countries. This agent may prevent tumor formation (43) and exert a beneficial effect on inflammatory lesions (19). In our study, genistein treatment of gingival fibroblasts potently inhibited EGF-induced tyrosine phosphorylation of EGF receptors and the activation of ERK and JNK pathways. These effects resulted in the inhibition of uPA production. Curcumin has been described as a more specific inhibitor of cell signaling. Specifically, curcumin may inhibit the activation of the JNK signaling pathway by various agonists (44) and may also inhibit the EGFR intrinsic kinase activity and EGF-induced tyrosine phosphorylation of EGF receptors in human epidermoid carcinoma cells (45). In gingival fibroblasts, curcumin was able to inhibit the EGF-induced EGFR-phosphorylation, the activation of ERK and JNK, and the factor-dependent uPA production. To the best of our knowledge, this is the first report that demonstrates that curcumin may inhibit EGFR phosphorylation in non-tranformed cells. On the basis of these results, we propose that these natural compounds could be considered as putative preventive/therapeutic agents for the control of periodontal inflammation.

Acknowledgements

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