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Signal transduction pathways involved in the stimulation of tissue type plasminogen activator by interleukin-1α and *Porphyromonas gingivalis* in human osteosarcoma cells

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Background: Recently, evidences have shown that tissue type plasminogen activator (t-PA) may play an important role in the pathogenesis of periodontal diseases. However, the mechanisms and signal transduction pathways involved in the production of t-PA in human osteosarcoma cells are not fully understood.

Objectives: The purpose of this study was to investigate the caseinolytic activity in human osteosarcoma cell line U2OS cells stimulated with interleukin-1α (IL-1α) or *Porphyromonas gingivalis* in the absence or presence of p38 inhibitor SB203580, mitogen-activated protein kinase kinase (MEK) inhibitor U0126, and phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002.

Methods: IL-1α and the supernatants of *P. gingivalis* were used to evaluate the caseinolytic activity in U2OS cells by using casein zymography and enzyme-linked immunosorbent assay (ELISA). Furthermore, to search possible signal transduction pathways, SB203580, U0126, and LY294002 were added to test how they modulated the caseinolytic activity.

Results: Casein zymography exhibited a caseinolytic band with a molecular weight of approximately 70 kDa, suggestive of the presence of t-PA. Secretion of t-PA was found to be stimulated with IL-1α and *P. gingivalis* during a 2-day culture period ($p < 0.05$). From the results of casein zymography and ELISA, SB203580, U0126, and LY294002 significantly reduced the IL-1α or *P. gingivalis*-stimulated t-PA production, respectively ($p < 0.05$).

Conclusions: Our findings demonstrated that IL-1α and *P. gingivalis* enhance t-PA production in human osteosarcoma cells, and that the signal transduction pathways p38, MEK, and PI3K are involved in the inhibition of t-PA. SB203580,

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U0126, and LY294002 suppress t-PA production and/or activity and may therefore be valuable therapeutics in t-PA-mediated periodontal destruction, and might be proved clinically useful agents, in combination with standard treatment modalities, in the treatment of periodontitis.

The plasminogen/plasmin system plays an important role in extracellular proteolysis and is involved in fibrinolysis, wound healing, and tissue remodeling. This may facilitate connective tissue breakdown and spread of inflammatory lesions. The system is activated by plasminogen activators (PAs), which are serine proteases catalyzing the conversion of the inactive proenzyme plasminogen into the active enzyme plasmin. Two types of human PAs can be distinguished by differences in molecular weight, affinity for fibrin, and immunoreactivity (1). The urokinase type plasminogen activator (u-PA) is thought to be involved in more generalized proteolysis and has been connected to tumor invasion (2). The tissue type plasminogen activator (t-PA) is activated by fibrin, which is though to be a key enzyme involved in fibrinolysis (3).

Previous studies have revealed differences in the localization of PAs between healthy and diseased gingival tissues, suggesting that PAs could play an important role in periodontal inflammatory processes (4, 5). The plasminogen activating system in gingival crevicular fluid has previously been characterized (6–8). The concentrations of t-PA in gingival crevicular fluid have been found to correlate with periodontal disease progression and can reflect tissue response to periodontal treatment (7–9). However, the precise mechanism of t-PA in the pathogenesis of periodontal breakdown needs to be further elucidated.

Interleukin-1 (IL-1), a proinflammatory cytokine, is enhanced in human gingival crevicular fluid from inflamed sites (10). It has been shown to stimulate bone resorption (11) and to up-regulate t-PA production by human gingival fibroblasts (12). *Porphyromonas gingivalis* is one of the predominant periodontal pathogens, and could stimulate *in vitro* bone resorption via enhanced IL-1 production by human peripheral blood mononuclear cells (13). *P. gingivalis* was also found to up-regulate t-PA production in human osteosarcoma cells (14) and gingival fibroblasts (15).

PA-generated plasmin is an activator of several matrix metalloproteinases (16, 17) and might participate indirectly in degradation of extracellular bone matrix. Plasminogen activator/plasmin pathway has been shown to participate in degradation of bone-like matrix (18, 19). A number of signal transduction proteins modulate the expression of plasminogen activators such as p38, mitogen-activated protein kinase kinase (MEK), and phosphatidylinositol 3-kinase (PI3K)-dependent signals (20–22). Understanding the inhibitory effects of pharmacological agents on t-PA production induced by IL-1α and *P. gingivalis* may lead to the development of new therapeutic strategies in periodontal therapy.

The aim of this study was to investigate the production and signal transduction pathways involved in the production of t-PA in human osteosarcoma cell line U2OS cells by casein zymography and enzyme-linked immunosorbent assay (ELISA).

Material and methods

Chemicals and materials

IL-1α was purchased from Sigma Chemical Co. (St Louis, MO, USA). SB203580 (p38 inhibitor), U0126 [a specific inhibitor of MEK1/2 that inhibits extracellular signal-regulated protein kinase 1/2 (ERK1/2)], and LY294002 (phosphatidylinositol 3-kinase (PI3K) inhibitor) were obtained from Promega (Madison, WI, USA). All culture materials were obtained from Gibco (Grand Island, NY, USA). IL-1α was directly dissolved in the culture medium. Other kinase inhibitors were first dissolved in dimethyl sulfoxide and then diluted with the culture medium. The final concentration of solvent in the medium did not exceed 0.25% (v/v). At these concentrations the solvents used were not cytotoxic to human osteosarcoma cells. In this study, the final concentrations of IL-1α, SB203580, U0126, and LY294002 used were 10 ng/ml, 26 μM, 23 μM, and 163 μM, respectively, as described previously (23).

Bacterial strain and preparation of supernatant

P. gingivalis (ATCC 33277) was maintained in Brain Heart Infusion broth, prereduced anaerobically, sterilized, and supplemented with hemin (5 mg/l) and menadione (0.5 mg/l) for obligate anaerobes. The preparation of supernatants was according to our previous studies (14, 23). Briefly, the density of the inoculum, prepared in Brain Heart Infusion broth, was adjusted to turbidity of 2 McFarland standard (6×10^8 CFU/ml). After centrifugation, supernatants were filter-sterilized using a 0.2 μm filter and stored at −80°C until used. The supernatants of *P. gingivalis* were directly diluted in culture medium and the final concentration of dilution was 1:1000.

Cell culture

U2OS cells (ATTC HTB 96), derived from human osteogenic sarcoma, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml of streptomycin, 100 mg/ml of penicillin at 37°C in a humidified incubator under ambient pressure air atmosphere containing 5% CO_2 (24). Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every third day.

Drugs and bacterial supernatant treatment

Confluent cells were trypsinized, counted, and plated at a concentration of 5×10^4 cells in a 60-mm culture dish and allowed to achieve confluence. Cells were cultured for 48 h, at which time the medium was changed to a medium containing 10% fetal calf serum with or without the inflammatory mediators IL-1α or *P. gingivalis* in the absence or presence of SB203580, U0126, and LY294002 for a 2-day culture period. Then, the conditioned medium samples were collected and stored at −20°C until assayed.

Casein zymography

The activities of t-PA of the condition medium were measured by casein zymogram protease assays as described previously (25). Briefly, samples were prepared with standard sodium dodecyl sulphate-gel-loading buffer containing 0.01% sodium dodecyl sulfate without β-mercaptoethanol and not boiled before loading. Then, prepared samples were subjected to electrophoresis with 8% sodium dodecyl sulphate–polyacrylamide gels containing 2% casein and 20 μg/ml plasminogen. After electrophoresis, gels were washed twice with 100 ml distilled water containing 2% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove sodium dodecyl sulfate. The gel was then incubated in 50 ml reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.02% NaN_3) for 12 h at 37°C. The caseinolytic activities of samples were identified by their ability to clear substrate at their characteristic molecular weights and were visualized after staining with Coomassie Brilliant Blue R-250. The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Enzyme-linked immunosorbent assay

Levels of t-PA antigen were determined by ELISA (Biopool, Umea, Sweden). Briefly, 20 μl of conditioned media were directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer and t-PA levels were determined with a calibration curve using human t-PA as a standard. The amounts of t-PA were expressed as ng/10^6 cells protein. Each value was expressed as the mean ± SD.

Statistical analysis

Triplicate or more separate experiments were performed throughout this study. The significance of the results obtained from control and treated groups was statistically analyzed by the paired Student's *t*-test. A *p*-value of < 0.05 was considered to be statistically significant.

Results

Casein zymography exhibited a caseinolytic band with a molecular weight of approximately 70 kDa, suggesting the presence of t-PA (Fig. 1). This result was also measured immunologically by ELISA, represented as t-PA.

IL-1α was found to up-regulate t-PA production in U2OS cells ($p < 0.05$). The conditioned medium samples from U2OS cells stimulated with IL-1α in the presence of different pharmacological agents during a 2-day culture period showed that the level of t-PA was significantly inhibited by U0126, SB203580, and LY294002 (Fig. 1). The quantitative measurement by the 2000 is shown in Fig. 2. The production of t-PA was enhanced by IL-1α about 1.5-fold, as compared with the control ($p < 0.05$). In addition, the co-incubation of IL-1α with U0126, SB203580, and LY294002 significantly reduced the t-PA production as compared with control by about 0.6, 1.3, and 1.1-fold, respectively ($p < 0.05$). The inhibitory effect depended on the pharmacological agents tested.

Moreover, the results of casein zymography were confirmed by ELISA. A similar pattern was seen by casein zymography. The production of t-PA was enhanced by IL-1α as compared with the control ($p < 0.05$). In addition, the amount of t-PA protein was 77, 23, 63, and 35 ng/10^6 cells for IL-1α alone and IL-1α with U0126, SB203580, and LY294002, respectively. The addition of U0126, SB203580, and LY294002 significantly reduced the t-PA production stimulated by IL-1α ($p < 0.05$) (Fig. 3).

P. gingivalis up-regulated t-PA production in U2OS cells ($p < 0.05$). The conditioned medium samples from U2OS cells stimulated with *P. gingivalis* in the presence of different pharmacological agents during a 2-day culture period showed that the level of t-PA was significantly inhibited by U0126, SB203580, and LY294002

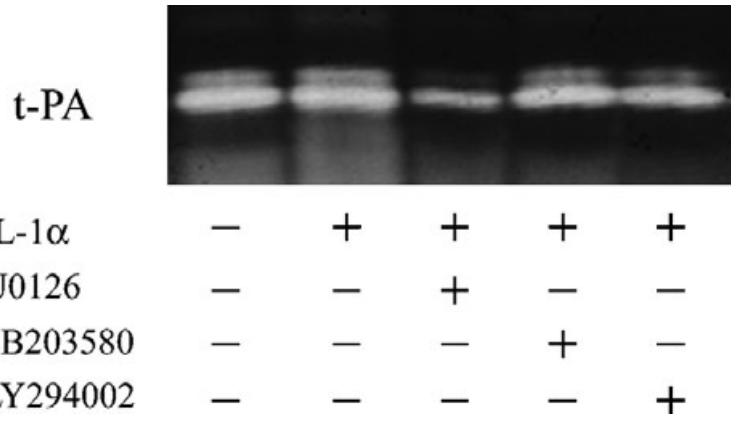

Fig. 1. Casein zymogram of conditioned medium from U2OS cells treated with interleukin-1α (IL-1α) in the presence of different pharmacological agents during a 2-day culture period. t-PA, tissue type plasminogen activator.

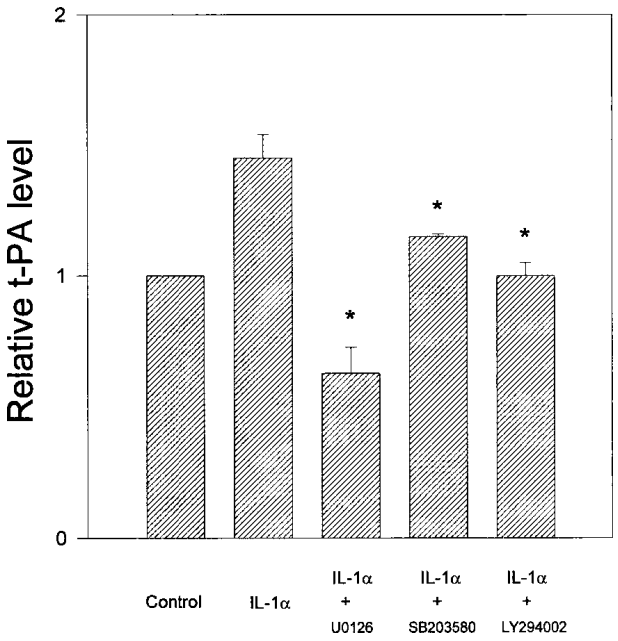

Fig. 2. Levels of tissue type plasminogen activator (t-PA) from conditioned medium treated with interleukin-1α (IL-1α) in the presence of different pharmacological agents were calculated from their t-PA activity, as measured by AlphaImager 2000. Values are means and standard deviations of optical density from triplicate independent experiments. *Statistically significant between IL-1α alone and IL-1α with different pharmacological agents, $p < 0.05$.

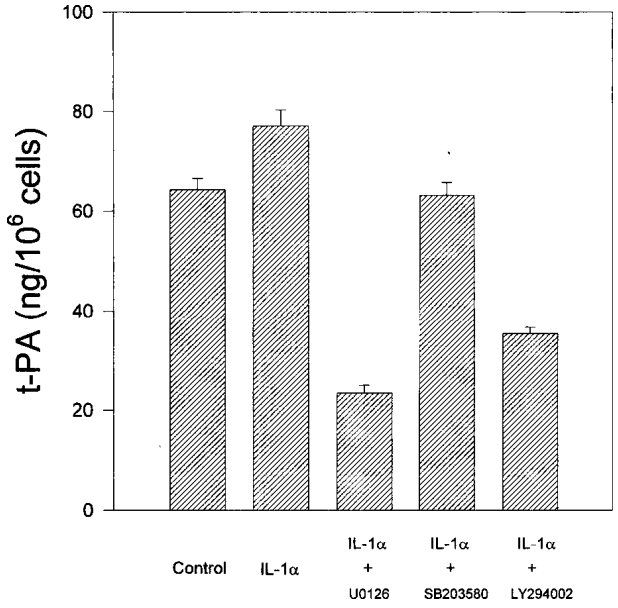

Fig. 3. Expression of tissue type plasminogen activator (t-PA) protein levels of conditioned medium from U2OS cells treated with interleukin-1α (IL-1α) in the presence of different pharmacological agents during a 2-day culture period. Values are means and standard deviations of optical density from triplicate independent experiments. *Statistically significant between IL-1α alone and IL-1α with different pharmacological agents, $p < 0.05$.

(Fig. 4). The quantitative measurement by the AlphaImager 2000 is shown in Fig. 5. The production of t-PA was enhanced by *P. gingivalis* about 2.0-fold as compared with the control ($p < 0.05$). In addition, co-incubation with U0126, SB203580, and LY294002 significantly reduced the t-PA production by about 0.9, 1.7, and 0.9-fold as compared with control, respectively ($p < 0.05$).

Moreover, the results of casein zymography were confirmed by ELISA. A similar pattern was seen by casein zymography. The production of t-PA was enhanced by *P. gingivalis* as compared with the control ($p < 0.05$). In addition, the amount of t-PA protein was 86, 47, 69, and 41 ng/10^6 cells for *P. gingivalis* alone and *P. gingivalis* with U0126, SB203580, and LY294002, respectively. The addition of U0126, SB203580, and LY294002 significantly reduced the t-PA production stimulated by *P. gingivalis* ($p < 0.05$) (Fig. 6).

Discussion

A major problem in periodontitis is the loss of connective tissue attachment arising from the degradation of collagen fibers that exist between the root cementum and alveolar bone. The plasminogen activating system has received considerable attention because of its participation in a wide variety of biological activities and in pathological conditions involving tissue breakdown.

Osteoblasts are the principal cells responsible for the alveolar bone. Thus, the human osteosarcoma cell line U2OS cells used in this study has been used as a model for osteoblasts because these cells express the osteoblast phenotype (26). Recently, the U2OS cell line was found to play an important role in the recruitment of immune cells and to contribute to the bone breakdown (14, 23). This is the reason why we chose U2OS cells in this study.

In this study, U2OS cells have been demonstrated to produce primarily t-PA. This has clearly shown that t-PA, whether assessed by an activity assay or measured immunologically, is presented in U2OS cells. It is now evident that lytic bands in the 70-kDa zone represented t-PA, as documented by the strong correlation between this lysis area and the ELISA. A similar result was found by Yang *et al.* (14),

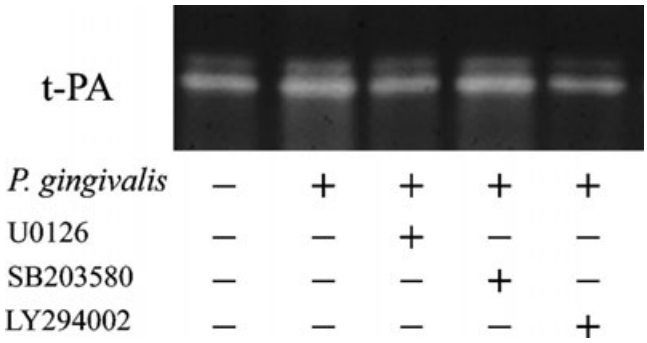

Fig. 4. Casein zymogram of conditioned medium from U2OS cells treated with *Porphyromonas gingivalis* in the presence of different pharmacological agents during a 2-day culture period. t-PA, tissue type plasminogen activator.

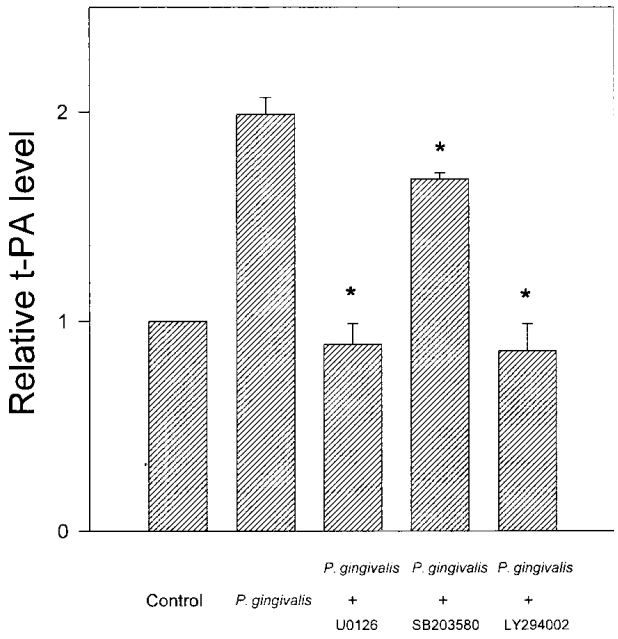

Fig. 5. Levels of tissue type plasminogen activator (t-PA) from conditioned medium treated with *Porphyromonas gingivalis* in the presence of different pharmacological agents were calculated from their t-PA activity, as measured by AlphaImager 2000. Values are means and standard deviations of optical density from triplicate independent experiments. *Statistically significant between *P. gingivalis* alone and *P. gingivalis* with different pharmacological agents, $p < 0.05$.

who reported that U2OS cells can express t-PA mRNA at the transcriptional level. Furthermore, we found that the production of t-PA was up-regulated by IL-1α and *P. gingivalis* in U2OS cells during a 2-day culture period. These results were in agreement with those of previous studies, in which t-PA was stimulated by IL-1α in human pulp cells (12) and gingival fibroblasts (12, 27). These results were also in agreement with Yang *et al*. (14), who found that t-PA mRNA was stimulated by *P. gingivalis* in U2OS cells.

As far as we know, the mechanisms and signal transduction pathways involved in the production of t-PA in U2OS cells are not fully understood. Mitogen-activated protein kinases (MAPKs) are members of a serine/threonine kinase family that link receptor activation in the cell nucleus (28). MAPK family members are extracellular signal-regulated protein kinases (ERK), Jun NH_2-terminal kinase (JNK) and p38 MAPK. The MAPK families regulate a number of transcription factors, with subsequent activation of PAs and cytokine expression. Our data demonstrated here that MAPK inhibitors SB203580 and U0126 reduced the IL-1α and *P. gingivalis* up-regulated t-PA production in this study. These results are in line with Spence *et al*. (20), who demonstrated that U0126 inhibited t-PA mRNA expression in human lung carcinoma cell line Calu-1, and Ge *et al*. (21) who found that U0126 and SB203580 decreased u-PA secretion in human A375 melanoma cells. In contrast, p38 inhibitor SB202190 did not alter t-PA mRNA level in Calu-1cells (20) and u-PA mRNA and protein in human ovarian cancer cell line HRA (22). The discrepancy between the above results may be explained by differences in the cell system used, and further studies are required to clarify the mechanisms involved in the IL-1α and *P. gingivalis*-mediated t-PA expression.

Recently, the PI3K signal transduction pathway has emerged as one of the main signal routes that coordinate complex events leading to changes in cell metabolism, cell growth, cell movement, and cell survival (29). In this study, LY294002 was found to reduce the IL-1α and *P. gingivalis* up-regulated t-PA production. A similar result has shown that LY294002 inhibits u-PA mRNA and protein expression in HRA cells (22). These studies suggest that the regulation of PAs activity in these cells may be mediated through the PI3K signal transduction pathway.

At sites of inflammation, the plasmin-dependent pathway for activation of matrix metalloproteinases (MMPs) is considered to be a significant mechanism for the induction of matrix degradation (30). Recently, our previous studies have demonstrated that MMP-2 was up-regulated by the IL-1α and *P. gingivalis* in human pulp cells (31, 32) and periodontal ligament fibroblasts (32) and that MMP-9 was stimulated by the IL-1α and *P. gingivalis* in U2OS cells (23). Plasmin degrades fibrin and several extracellular

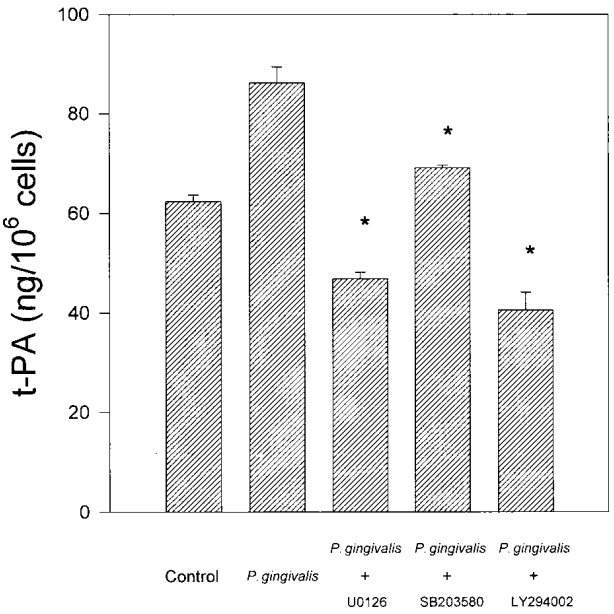

Fig. 6. Expression of tissue type plasminogen activator (t-PA) protein levels of conditioned medium from U2OS cells treated with *Porphyromonas gingivalis* in the presence of different pharmacological agents during a 2-day culture period. Values are means and standard deviations of optical density from triplicate independent experiments. *Statistically significant between *P. gingivalis* alone and *P. gingivalis* with different pharmacological agents, $p < 0.05$.

matrix proteins and adhesion proteinases and, by activation of procollagenases, may contribute to collagen degradation (33). Indeed, the plasmin-dependent pathway is understood to be a significant alternative pathway for the initiation of extracellular matrix degradation by MMPs (34). The interaction between MMPs and t-PA is worthy of further investigation.

This study demonstrated that U2OS cells can produce t-PA extracellularly, and that t-PA production is enhanced by IL-1α and *P. gingivalis*. Furthermore, t-PA was inhibited by U0126, SB203580, and LY294002. Understanding the inhibitory effects of pharmacological agents on t-PA production induced by IL-1α and *P. gingivalis* in U2OS cells may lead to development of new therapeutic strategies. Pharmacological agents that target p38, MEK, and PI3K pathway may contribute to the pathogenesis of alveolar bone inflammation. However, the detailed mechanism of activation of PAs by inflammatory cytokines or periodontal pathogen *in vivo* remains to be further defined.

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