Examination of the signal transduction pathways leading to upregulation of tissue type plasminogen activator by *Porphyromonas endodontalis* in human pulp cells

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Abstract

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Aim To investigate the tissue type plasminogen activator (t-PA) activity in human pulp cells stimulated with *Porphyromonas endodontalis* (*P. endodontalis*) in the absence or presence of p38 inhibitor SB203580, mitogen-activated protein kinase kinase (MEK) inhibitor U0126 and phosphatidylinositaol 3-kinase (PI3K) inhibitor LY294002.

Methodology The supernatants of *P. endodontalis* were used to evaluate t-PA activity in human pulp cells using casein zymography and enzyme-linked immunosorbent assay (ELISA). Furthermore, to search for possible signal transduction pathways, SB203580, U0126 and LY294002 were added to test how they modulated the t-PA activity.

Results The main casein secreted by human pulp cells migrated at 70 kDa and represented t-PA. Secretion of t-PA was found to be stimulated with *P. endodontalis* during 2-day cultured period ($P < 0.05$). From the results of casein zymography and ELISA, SB203580 and U0126 significantly reduced the *P. endodontalis* stimulated t-PA production respectively ($P < 0.05$). However, LY294002 lacked the ability to change the *P. endodontalis* stimulated t-PA production ($P > 0.05$).

Conclusions *Porphyromonas endodontalis* enhances t-PA production in human pulp cells, and the signal transduction pathways p38 and MEK are involved in the inhibition of t-PA.

Keywords: casein zymography, *Porphyromonas endodontalis*, pulp cells, signal transduction pathway, tissue type plasminogen activator.

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Introduction

The dental pulp is a loose mesenchymal tissue enclosed within mineralized tissue. Caries may penetrate dentine, and allow the pulp to be challenged by bacteria or bacterial products and may result in pulpal inflammation. The black-pigmented *Bacteroides* species have gained special prominence in the search for aetiological organisms associated with endodontic infections (van Winkelhoff *et al.* 1988, Sundqvist *et al.* 1989). *Porphyromonas endodontalis* (*P. endodontalis*) is associated especially with endodontic infections and abscesses (van Winkelhoff *et al.* 1985a,b). The invasion of host tissue by *P. endodontalis* or its by-products frequently induces a variety of immunopathological reactions.

Plasminogen activators (PAs) is a group of serine proteolytic enzymes that specifically converts plasminogen to the active proteinase plasmin. The two major types of PA, tissue type PA (t-PA) and urokinase type PA (u-PA), differ with respect to

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distribution, catalytical, molecular and immunological properties (Sprengers & Kluft 1987). The u-PA is a 54-kDa enzyme composed of two disulphide-linked 24- and 30-KDa subunits. The native t-PA, in contrast, is a single 70-KDa polypeptide chain (Sawaya *et al.* 1991). At sites of inflammation, the plasminogen activating system is higher than other normal conditions (Huang *et al.* 2005, Tsai *et al.* 2004).

Recently, the roles of t-PA in pulpal inflammation have been highlighted. t-PA was found to be upregulated in human inflamed dental pulps using immunohistochemical methods (Huang *et al.* 2005). *P. endodontalis* was found to stimulate the production of t-PA in human pulps (Yang *et al.* 2003). However, the production and signal transduction pathways of t-PA in human pulp cells are poorly understood.

Expression of t-PA is controlled by many extracellular stimuli such as cytokines, bacterial products (Ueda & Matsushima 2001, Chang *et al.* 2003, Yang *et al.* 2003). A number of signal transduction proteins modulate the expression of PAs, such as p38, mitogen-activated protein kinase kinase (MEK) and phosphatidylinositaol 3-kinase (PI3K)-dependent signals (Ge *et al.* 2002, Spence *et al.* 2002, Tanaka *et al.* 2004). The aim of this study was, therefore, to investigate the production and the signal transduction pathways involved in the production of t-PA stimulated by *P. endodontalis* in human pulp cells by use of casein zymography and enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Chemicals and materials

All culture materials were obtained from GIBCO (Grand Island, NY, USA). SB203580 (p38 inhibitor), U0126 (MEK inhibitor) and LY294002 (PI3K inhibitor) were purchased from Promega (Madison, WI, USA). Interleukin-1α was directly dissolved in the culture medium. All kinase inhibitors were first dissolved in dimethyl sulphoxide 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 pulp cells as described previously (Huang *et al.* 2004). The final concentrations of SB203580, U0126 and LY294002 used in this study were 26, 23 and 163 μmol L^{-1} respectively.

Cell culture

Human pulp cells were cultured using an explant technique as described previously (Huang & Chang 2002a,b). Briefly, impacted third molars were obtained from healthy patients of the Oral Medicine Centre (Chung Shan Medical University Hospital, Taichung, Taiwan) with informed consent. Teeth were sectioned horizontally below the cemento-enamel junction with a number 330 high-speed bur with water spray. The pulp tissue was removed aseptically in lamina flow, rinsed with Hanks' buffered saline solution and placed in a 60-mm dish. Pulp tissue was minced with a blade into small fragments and grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} of fungizone). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid for 5 min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used in this study.

Bacteria culture and preparation of supernatants

P. endodontalis (ATCC 27067) were grown under anaerobic conditions and harvested at the end of the logarithmic phase of growth as described previously (Chang *et al.* 2002, Yang *et al.* 2004). Briefly, they were maintained in brain–heart infusion broth pre-reduced anaerobically, sterilized and supplemented with 5 mg L^{-1} haemin and 0.5 mg L^{-1} menadione. The density of the inoculum, prepared in brain–heart infusion broth, was adjusted to a turbidity of 2 McFarland standard (6×10^8 colony-forming units mL^{-1}). After centrifugation, supernatants were filter-sterilized using a 0.2-μm filter and stored at −80 °C until used. The supernatants of *P. endodontalis* were directly diluted in culture medium and the final dilution was 1 : 100.

Pharmacological agent treatments

Confluent pulp cells were trypsinized, counted and plated at a concentration of 1×10^5 cells in 60-mm-culture dish and allowed to attach for 24 h. The cells were then incubated with or without *P. endodontalis* in

the absence or presence of SB203580, U0126 and LY294002 for a 2-day cultured period. The conditioned medium samples were then 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 (Heussen & Dowdle 1980). Briefly, samples were prepared with standard sodium dodecyl sulphate (SDS) gel-loading buffer containing 0.01% SDS without β-mercaptoethanol and not boiled before loading. Then, prepared samples were subjected to electrophoresis with 8% SDS-polyacrylamide gels containing 2% casein and 20 μg mL^{-1} 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 SDS. The gel was then incubated in 50 mL reaction buffer (40 mmol L^{-1} Tris–HCl, pH 8.0, 10 mmol L^{-1} CaCl2, 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

Three replicates of each pharmacological agent were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was carried out by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analysed by Duncan's test and a value of $P < 0.05$ was considered statistically significant.

Results

The main casein secreted by human pulp cells migrated at 70 kDa and represented t-PA (Fig. 1). The effects of kinase inhibitors on the t-PA secretion in three different cell strains were similar, and their intracellular variations were limited.

P. endodontalis was found to upregulate t-PA production in human pulp cell cultures ($P < 0.05$). The conditioned medium samples from human pulp cells stimulated with *P. endodontalis* in the presence with different pharmacological agents during the 2-day culture period showed that the level of t-PA was found to be significantly inhibited by SB203580 and U0126 ($P < 0.05$) (Fig. 1). LY294002, however, did not affect the t-PA level compared with control. The quantitative measurement by the AlphaImager 2000 (Alpha Innotech) is shown in Fig. 2. The production of t-PA was enhanced by the *P. endodontalis* about 1.7-fold as compared with control ($P < 0.05$). In addition, the coincubation with U0126 and SB203580 were found to significantly reduce the t-PA production about 0.8- and 1.2-fold as compared with control respectively ($P < 0.05$). However, the level of t-PA by the coincubation with *P. endodontalis* and LY294002 was 1.7-fold. There was no statistically significant difference between *P. endodontalis* alone and *P. endodontalis* with LY294002 ($P > 0.05$).

Moreover, the results of casein zymography were confirmed by ELISA. A similar pattern was seen using casein zymography. The production of t-PA was enhanced by the *P. endodontalis* as compared with control ($P < 0.05$). In addition, the amount of t-PA protein were 75, 46, 63 and 73 ng 10^{-6} cells of *P. endodontalis* alone and *P. endodontalis* with U0126, SB203580 and LY294002 respectively. The addition of SB203580 and U0126 were found to significantly reduce the t-PA production stimulated by *P. endodontalis* ($P < 0.05$) (Fig. 3).

Discussion

Pulp cells are important for the homeostatic function of pulpal connective tissue and are responsible for the healing process when pulp tissues are insulted by mechanical, chemical injury, or microbial irritants. Normal diploid cells from primary culture have a more

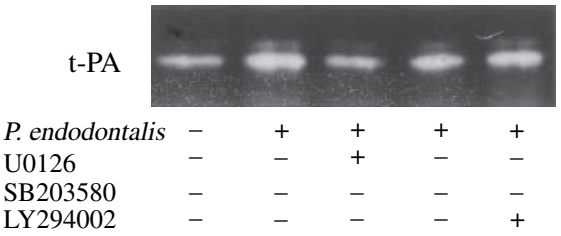

Figure 1 Casein zymogram of conditioned medium from human pulp cells treated with the *P. endodontalis* in the presence with different pharmacological agents during 2-day culture period.

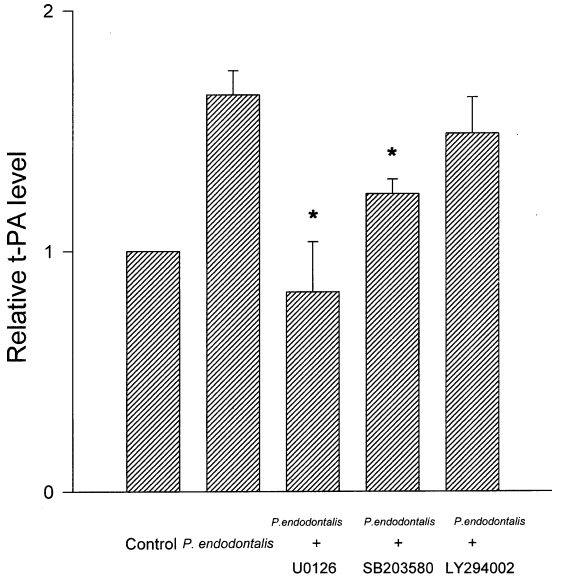

Figure 2 Levels of tissue type plasminogen activator (t-PA) from conditioned medium treated with the *P. endodontalis* in the presence with different pharmacological agents were calculated from their t-PA activity, as measured by AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA). Values are means and standard deviations of optical density from triplicate independent experiments. * Statistically significant between *P. endodontalis* alone and *P. endodontalis* added with different pharmacological agents, $P < 0.05$.

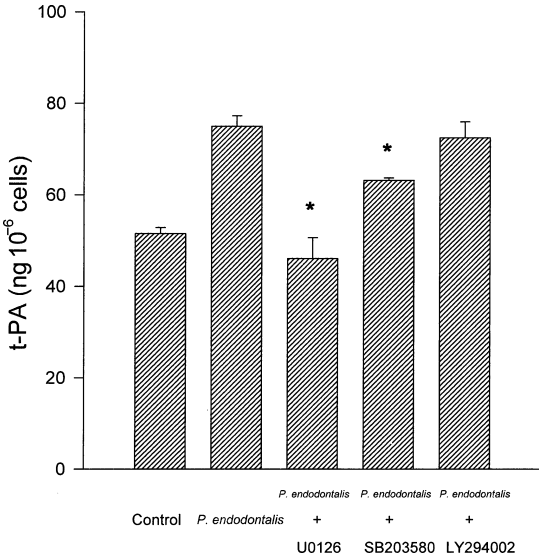

Figure 3 Expression the protein levels tissue type plasminogen activator (t-PA) of conditioned medium from human pulp cells treated with the interleukin-1α in the presence with different pharmacological agents during 2-day culture period. Values are means and standard deviations of optical density from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

normal phenotype and they correlate to the *in vivo* response more accurately (Chang *et al.* 1998, 2000). Human pulp cells were obtained as primary culture from explants of biopsy. The use of human pulp cells permits enhanced relevance.

Microbial-induced tissue destruction may activate one or more of five distinct host degradative pathways (plasminogen-dependent pathway, matrix metalloproteinase pathway, phagocytic pathway, polymorphonulcear-serine proteinase pathway and osteoclastic bone resorption) or by direct cleavage of extracellular matrix constituents by microbial proteinases (Birkedal-Hansen 1993). The activation of endogenous destructive pathways may be mediated by immune response, resulting in the expression of degradative cellular phenotypes amongst both immigrant and resident cell populations. Expression of degradative phenotypes may be triggered by direct influences on host cells of microbial products such as lipopolysaccharide, toxins and proteolytic enzymes.

This study has clearly shown that t-PA, whether assessed by an activity assay or measured immunologically, is produced in human pulp cell cultures. It is now evident that lytic band is the 70-kDa zone represented t-PA as documented by ELISA. Similar results have demonstrated that human pulp cells are known to synthesize and secrete t-PA by Western blot and reverse-transcriptase polymerase chain reaction assays (Ueda & Matsushima 2001, Chang *et al.* 2003, Yang *et al.* 2003). Furthermore, it was found that the production of t-PA was upregulated by the *P. endodontalis* in pulp cell cultures during 2-day cultured period.

These results were in agreement with those of previous studies, in which t-PA was stimulated by *P. endodontalis* in human pulp cells (Chang *et al.* 2003) and osteoblastic-like cells (Yang *et al.* 2003).

The mechanisms and signal transduction pathways involved in the production of t-PA in pulp 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 (Chang & Karin 2001). MAPK family member includes extracellular signal-regulated protein kinases, Jun NH2-terminal kinase and p38 MAPK. The MAPK family regulates a number of transcription factors, with subsequent activation of PAs and cytokine expression. The present data demonstrated that MAPK inhibitors SB203580 and U0126 inhibited the *P. endodontalis* upregulated t-PA production. These results are in agreement with those of Spence *et al.* (2002) who demonstrated that U0126 was found to inhibit t-PA mRNA expression in human lung carcinoma cell line Calu-1, and Ge *et al.* (2002) who found that U0126 and SB203580 inhibited u-PA secretion in human A375 melanoma cells. In contrast, p38 inhibitor SB202190 did not alter t-PA mRNA level in Calu-1 cells (Spence *et al.* 2002) and u-PA mRNA and protein in human ovarian cancer cell line HRA (Tanaka *et al.* 2004). The discrepancy between the above results may be explained by the difference in the cell systems used, and further studies are required to clarify the mechanisms involved in the *P. endodontalis* mediated t-PA expression.

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 (Cantley 2002). In this study, LY294002 lacked the ability to change the *P. endodontalis* upregulated t-PA production. Previously, LY294002 was found to inhibit u-PA mRNA and protein expression in HRA cells (Tanaka *et al.* 2004). The reason is not clear but might be explained by differences in the cell systems used. However, further studies are required to clarify the mechanisms involved in the *P. endodontalis* mediated t-PA expression through the PI3K signal transduction pathway.

This study demonstrated that human pulp cells can produce t-PA, and that t-PA production is enhanced by *P. endodontalis*. Furthermore, t-PA was found to be inhibited by SB203580 and U0126. Pharmacological agents that target p38 and MEK, pathway may contribute to the pathogenesis of pulpal inflammation. However, the detailed mechanism of activation of PAs by other endodontic pathogens *in vivo* remains to be further defined.

Conclusions

The plasminogen/plasmin system can modulate pulpal homeostasis by participating in many aspects of the pathological reactions associated with pulpal diseases. In the present study, the main casein secreted by human pulp cells migrated at 70 kDa and represented t-PA by casein zymography. These findings demonstrated that *P. endodontalis* enhanced t-PA production in human pulp cells, and the signal transduction pathways p38 and MEK are involved in the inhibition of t-PA.

References

Birkedal-Hansen H (1993) Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research* **28**, 500–10.

Cantley LC (2002) The phosphoinositide 3-kinase pathway. *Science* **296**, 1655–7.

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**, 37–40.

Chang YC, Huang FM, Cheng MH, Chou LSS, Chou MY (1998) *In vitro* evaluation of the cytotoxicity and genotoxicity of root canal medicines on human pulp fibroblasts. *Journal of Endodontics* **24**, 604–6.

Chang YC, Tai KW, Huang FM, Huang MF (2000) Cytotoxic and nongenotoxic effects of phenolic compounds in human pulp cell cultures. *Journal of Endodontics* **26**, 440–3.

Chang YC, Lai CC, Yang SF, Chan Y, Hsieh YS (2002) Stimulation of matrix metalloproteinases by black-pigmented *Bacteroides* in human pulp and periodontal ligament cell cultures. *Journal of Endodontics* **28**, 90–3.

Chang YC, Yang SF, Huang FM, Tai KW, Hsieh YS (2003) Induction of tissue plasminogen activator gene expression by proinflammatory cytokines in human pulp and gingival fibroblasts. *Journal of Endodontics* **29**, 118–20.

Ge X, Fu YM, Meadows GG (2002) U0126, a mitogen-activated protein kinase kinase inhibitor, inhibits the invasion of human A375 melanoma cells. *Cancer Letters* **179**, 133–40.

Heussen C, Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical Biochemistry* **102**, 196–202.

Huang FM, Chang YC (2002a) Cytotoxicity of resin-based restorative materials on human pulp cell cultures. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **94**, 361–5.

Huang FM, Chang YC (2002b) Cytotoxicity of dentine-bonding agents on human pulp cells *in vitro*. *International Endodontic Journal* **35**, 905–9.

Huang FM, Yang SF, Hsieh YS, Liu CM, Yang LC, Chang YC (2004) Examination of the signal transduction pathways involved in matrix metalloproteinases-2 in human pulp cells. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **97**, 398–403.

Huang FM, Tsai CH, Chen YJ, Liu CM, Chou MY, Chang YC (2005) Upregulation of tissue type plasminogen activator in inflamed human dental pulps. *International Endodontic Journal* **38**, 328–33.

Sawaya R, Ramo OJ, Shi ML, Mandybur G (1991) Biological significance of tissue plasminogen activator convert in brain tumors. *Journal of Neurosurgery* **74**, 480–6.

Spence MJ, Streiff R, Day D, Ma Y (2002) Oncostatin M induces tissue-type plasminogen activator and plasminogen activator inhibitor-1 in Calu-1 lung carcinoma cells. *Cytokines* **18**, 26–34.

Sprengers ED, Kluft C (1987) Plasminogen activator inhibitors. *Blood* **69**, 381–7.

Sundqvist G, Johansson E, Sjogren U (1989) Prevalence of black-pigmented *Bacteroides* species in root canal infections. *Journal of Endodontics* **15**, 13–9.

Tanaka Y, Kobayashi H, Suzuki M, Kanayama N, Terao T (2004) Transforming growth factor-b1-dependent urokinase up-regulation and promotion of invasion are involved in Src-MAPK-dependent signaling in human ovarian cancer cells. *Journal of Biological Chemistry* **279**, 8567–76.

Tsai CH, Weng SF, Yang LC, Huang FM, Chen YR, Chang YC (2004) Immunohistochemical localization of tissue type plasminogen activator and type I plasminogen activator inhibitor in radicular cysts. *Journal of Oral Pathology & Medicine* **33**, 156–61.

Ueda I, Matsushima K (2001) Stimulation of plasminogen activator activity and matrix metalloproteinases of human dental pulps derived cells by tumor necrosis factor-α. *Journal of Endodontics* **27**, 175–9.

van Winkelhoff AJ, Carlee AW, de Graaff J (1985a) *Bacteroides endodontalis* and other black-pigmented *Bacteroides* species in odontogenic abscesses. *Infection and Immunity* **49**, 494–7.

van Winkelhoff AJ, van Steenbergen TJM, Kippuw N, De Graaff J (1985b) Further characterization of *Bacteroides endodontalis*, an asaccharolytic black-pigmented *Bacteroides* species from the oral cavity. *Journal of Clinical Microbiology* **22**, 75–9.

van Winkelhoff AJ, van Steenbergen TJ, de Graaff J (1988) The role of black-pigmented *Bacteroides* in human oral infections. *Journal of Clinical Periodontology* **15**, 145–55.

Yang SF, Hsieh YS, Huang FM, Yang LC, Chang YC (2003) Effect of black-pigmented bacteria on the plasminogen/plasmin system in human pulp and osteoblastic cells. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **95**, 621–5.

Yang LC, Tsai CH, Huang FM et al. (2004) Induction of vascular endothelial growth factor expression in human pulp fibroblasts stimulated with black-pigmented *Bacteroides*. *International Endodontic Journal* **37**, 588–92.