Upregulation of tissue-type plasminogen activator in inflamed human dental pulps

F.-M. Huang[1,2], C.-H. Tsai[3], Y.-J. Chen[4], C.-M. Liu[1], M.-Y. Chou[4] & Y.-C. Chang[1,5]

[1]Oral Medicine Center, Chung Shan Medical University Hospital; [2]Institute of Medicine, Chung Shan Medical University; [3]Department of Oral Pathology, Chung Shan Medical University; [4]Department of Oral Surgery, Chung Shan Medical University Hospital; and [5]Institute of Stomatology, College of Oral Medicine, Chung Shan Medical University, Taichung, Taiwan

Abstract

Huang F-M, Tsai C-H, Chen Y-J, Liu C-M, Chou M-Y, Chang Y-C. Upregulation of tissue-type plasminogen activator in inflamed human dental pulps. *International Endodontic Journal*, **38**, 328–333, 2005.

Aim To compare tissue-type plasminogen activator (t-PA) expression in normal human pulp and inflamed human pulp tissue specimens.

Methodology Thirty pulpal tissue specimens (13 normal and 17 inflamed pulps) were obtained from extracted third molars. The levels of t-PA between normal pulp and inflamed pulp tissues were compared using the quantitative reverse-transcriptase polymerase chain reaction analysis. In addition, immunohistochemistry was used to identify the *in situ* localization of t-PA expression in pulp specimens. Wilcoxon–Mann–Whitney rank sum test was applied for the statistical analysis of the results.

Results t-PA mRNA gene was found more in inflamed pulps when compared with normal pulp tissue ($P < 0.05$). The results from immunohistochemistry demonstrated that t-PA expression was significantly higher in the inflamed pulp ($P = 0.025$). t-PA stain was detected in the fibroblasts, inflammatory infiltrates and endothelial cells.

Conclusions t-PA expression was significantly higher in inflamed pulp tissue. t-PA may play an important role in the pathogenesis of pulpal inflammation.

Keywords: immunohistochemistry, inflammation, pulp, tissue-type plasminogen activator.

Received 13 September 2004; accepted 3 February 2005

Introduction

The human dental pulp consists of an odontoblast layer adjacent to the dentine and an immunocompetent tissue cover with nerves and vascular elements (Jontell *et al.* 1998). A common pulpal response to mechanical injury, bacterial invasion or bacterial products will always cause a pulpal inflammation once they have reached the dentine (Langeland 1987). Like any other inflammation, pulpal inflammation is associated with tissue degradation. At least three pathways have been recognized in soft-tissue breakdown: (i) the matrix metalloproteinase (MMP)-dependent pathway; (ii) the phagocytic pathway; and (iii) the plasminogen-dependent pathway (Birkedal-Hansen 1993).

Plasminogen activators belong to the serine protease family and catalyse the cleavage of plasminogen into active enzyme plasmin. Plasmin is an active protease capable of degrading many extracellular proteins as well as activating latent collagenase and MMP (Werb *et al.* 1977, Matrisian 1992). The plasminogen-activating system is involved in several physiological and pathological processes, such as local inflammatory reactions, neoplastic invasion and tissue remodelling (Collen 1999).

Plasmin can be formed locally at sites of inflammation and repaired by limited proteolysis of inactive precursor, plasminogen, which circulates in plasma and interstitial fluids (Deutsch & Mertz 1970). Plasminogen is activated by either urokinase-type plasminogen activator (u-PA) or tissue-type plasminogen activator (t-PA). These activating enzymes are produced by a wide range of

Correspondence: Dr Yu-Chao Chang, Oral Medicine Center, Chung Shan Medical University Hospital, 110, Sec. 1, Chien-Kuo N. Rd., Taichung, Taiwan (Tel.: 886 4 22015111, ext. 66268; fax: 886 4 24759065; e-mail: cyc@csmu.edu.tw).

mesenchymal, epithelial and endothelial cells in response to a variety of cytokines and stimuli (Vassall *et al.* 1991). Thus, at sites of inflammation, the potential for upregulation of the plasminogen-activating system is considered to be higher.

The role of the plasminogen-activating system has not been well clarified in human pulp tissue. Recently, reports have shown that t-PA was upregulated by proinflammatory cytokines and black-pigmented *Bacteroides* in human pulp cells *in vitro* (Hosoya *et al.* 1998, Ueda & Matsushima 2001, Chang *et al.* 2003, Yang *et al.* 2003). These findings imply that t-PA may play an important role in the pathogenesis of pulpal disease. However, these studies do not reveal the cellular source of t-PA or compare the t-PA levels in both clinically healthy and inflamed human dental pulps.

It was hypothesized that the levels of t-PA would increase in inflamed dental pulps. Therefore, the aim of the present investigation was to quantify the levels of t-PA in both clinically healthy and inflamed human dental pulps by using the reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. In addition, immunohistochemistry was used to identify the *in situ* localization of t-PA expression in dental pulps.

Materials and methods

Sample collection

Thirty pulpal tissue specimens (13 normal and 17 inflamed pulps) were obtained from extracted third molars (28 patients) at the Department of Oral Surgery, Chung Shan Medical University Hospital, Taichung, Taiwan. Patients selected in this study were in good health. In addition, they had not taken any long-term anti-inflammatory medication or antibiotics. The inflamed pulps were obtained from teeth with carious pulp exposure, and showing spontaneous pain and/or lingering pain in response to cold and/or heat stimulus. Pulps from non-carious teeth that had no radiographic evidence of caries or periapical pathology were used as normal controls.

Preparation of pulpal tissue specimens

Immediately after extraction, the teeth were longitudinally grooved with a fissure bur and then split in half. The dental pulp was then extracted carefully using a spoon excavator. For RT-PCR assay, pulp tissue samples were manually homogenized in saline in individual Eppendorf tubes. For immunohistochemistry, tissue specimens were fixed with 10% buffered formalin overnight, the specimens were dehydrated in an ascending series of graded alcohols and embedded in paraffin.

Reverse-transcriptase polymerase chain reaction

Total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA), following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μL reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco Laboratories). The reaction mixture was diluted with 20 μL of water, and 3 μL of the diluted reaction mixture was used for the polymerase chain reaction (PCR). PCR reaction mixture contained 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 30 cycles for t-PA in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 57 °C, and 1 min of extension at 72 °C. The sequences of primers employed are listed in Table 1. The PCR products were analysed by agarose gel electrophoresis.

When the samples were probed for t-PA mRNA production by RT-PCR, a 500-bp band for t-PA was noted. These bands were consistent with the size as designed by primers. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Immunohistochemistry

The tissue blocks were cut at 5 μm and subjected to the peroxidase-labelled streptavidin–biotin technique

Table 1 Nucleotide sequence and size of the expected PCR products for oligonucleotide primers used for RT-PCR

Gene	Sequence	PCR product (bp)
GAPDH	5′-TCCTCTGACTTCAACAGCGACACC-3′	207
	5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′	
t-PA	5′-AGGCTCATGTCAGACTGTACC-3′	500
	5′-CCTGAAATCAGACCAAGTCC-3′	

as described previously (Tsai *et al.* 2002, 2003). The sections were immersed in 3% methanol-hydrogen peroxide solution for 10 min to block endogenous peroxidase activity and incubated with an anti-t-PA antibody (goat anti-human, cat #AB774, lot. 19070912; Chemicon International Inc., Temecula, CA, USA; dilution 1 : 50), or with an anti-LCA antibody (DAKO, Carpinteria, CA, USA; dilution 1 : 50), specific for lymphocytes, for 18 h at 4 °C with 1% bovine serum albumin. After washing in 20 mmol L^{-1} Tris–HCl buffer (pH 7.4) containing 0.9% NaCl, the sections were incubated at room temperature with biotinylated multilink swine anti-goat, mouse and rabbit immunoglobulins (DAKO) diluted 1 : 150 in Tris–HCl for 30 min. The sections were then washed with Tris–HCl twice for 10 min and incubated for 30 min with horseradish peroxidase-conjugated streptavidin (DAKO) diluted 1 : 50 in Tris–HCl. After washing with Tris–HCl twice for 10 min, incubation for 3 min with 0.01% diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA) and 0.03% H_2O_2 in 20 mmol L^{-1} Tris–HCl buffer at pH 7.4 was performed. A final rinse in distilled H_2O for 10 min was performed before counterstaining with AEC (3-amino-9-ethylcarbazole) (DAKO). The buffer used for dilution was DAKO antibody diluent with background-reducing components. Negative controls included serial sections from which either the primary or secondary antibodies were excluded. Three biopsy specimens of radicular cysts were used as positive controls (Tsai *et al.* 2004).

Statistical analysis

For testing of differences in the t-PA between the clinically healthy and inflamed human dental pulps, the Wilcoxon–Mann–Whitney rank sum test was applied. A *P*-value of <0.05 was considered to be statistically significant.

Results

As shown in Fig. 1, inflamed pulp specimens exhibited significantly higher t-PA mRNA gene expression than normal pulp tissues. From the AlphaImager 2000, the intensity of t-PA mRNA from inflamed pulp was elevated about 3.5-fold as compared with normal pulp ($P < 0.05$).

The t-PA staining in pulp tissue was stronger in inflamed pulps than in normal pulp. t-PA staining was scarcely detected in normal pulp specimens (Fig. 2). All inflamed pulps showed the presence of t-PA positive cells. t-PA expression was observed mainly in the cytoplasm of fibroblasts, endothelial cells and inflammatory infiltrates (Fig. 3). The infiltrate consisted mainly of lymphocytes which were labelled with LCA (Fig. 4).

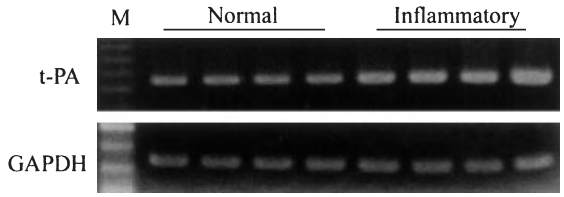

Figure 1 Comparison of the t-PA mRNA gene level from normal pulps and inflamed pulps using RT-PCR assay. GAPDH gene was performed in order to monitor equal RNA loading. Inflamed pulp specimens exhibited significantly higher t-PA mRNA gene expression than normal pulp tissues.

In the positive controls (Fig. 5), t-PA stain was detected in the epithelium, inflammatory infiltrates and endothelium.

Discussion

Pulpal inflammation is characterized by extensive dental caries or bacterial infection that may lead to pulpal and periapical tissue breakdown. This process could be mediated by neutral proteases. 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. Regulation of plasminogen activator is a key element in controlling proteolytic events in the extracellular matrix and is mediated by specific plasminogen inhibitors. At sites of inflammation, the plasmin-dependent pathway for activation of MMP is considered to be a significant mechanism for the induction of matrix degradation (Birkedal-Hansen 1993).

Two types of human plasminogens, identified in previous studies, u-PA and t-PA, can be distinguished by differences in molecular weight, affinity for fibrin and immunoreactivity (de Vries *et al.* 1988). In contrast to u-PA, which is thought to be involved in more generalized proteolysis and has been connected to in tumour invasion (Mignatti *et al.* 1986), t-PA, which is activated by fibrin, is thought to be a key enzyme involved in fibrinolysis (Saksela 1985).

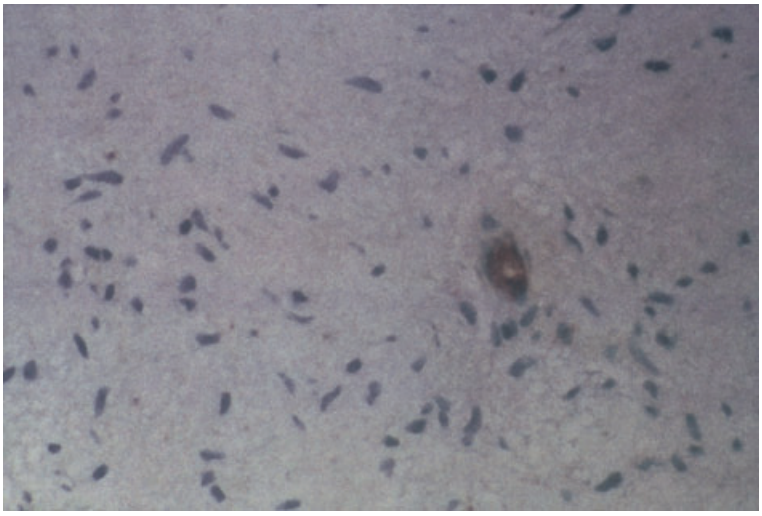

Figure 2 Very faint immunoreactivity of t-PA was observed in normal human pulp and almost totally limited to the endothelium (200×).

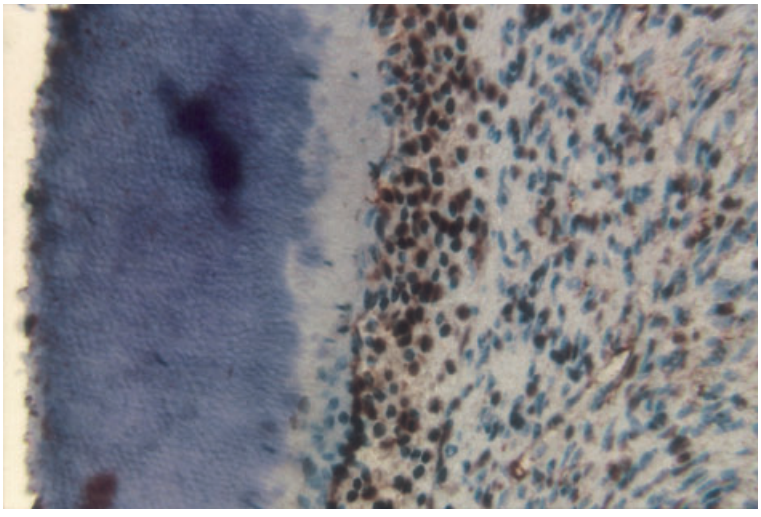

Figure 3 Immunolocalization of t-PA in an inflamed pulp specimen. t-PA stain was detected in the fibroblasts, inflammatory infiltrates and endothelial cells (×400).

In the present study, it was noticed that t-PA mRNA was upregulated in inflamed pulp tissues compared with healthy counterparts. Surprisingly, there have been relatively few studies addressing the *in situ* location of t-PA in human pulp tissues. In the present study, the t-PA staining in pulp tissue was stronger in inflamed pulps compared with normal pulp. t-PA stain was detected in the fibroblasts, inflammatory infiltrates and endothelial cells. These results were in agreement with recent studies showing that t-PA is found to be upregulated in inflamed gingival tissues (Kinnby *et al.* 1999) and radicular cysts (Tsai *et al.* 2004). Taken together, upregulation of t-PA expression may play an important role in the pathogenesis of pulpal inflammation.

Microbially induced tissue destruction may activate the plasminogen-activating system or may act by direct cleavage of extracellular matrix constituted 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 between both immigrant and residual cell populations. In this study, it was revealed that t-PA could be immunolocalized in inflamed dental pulps and t-PA was found to be upregulated in mRNA level. Recently, studies have also shown that black-pigmented *Bacteroides* (Yang *et al.* 2003) and proinflammatory cytokines (Ueda & Matsushima 2001, Chang *et al.* 2003) can induce t-PA in human pulp cells, thereby initiating tissue degradation. Therefore, t-PA

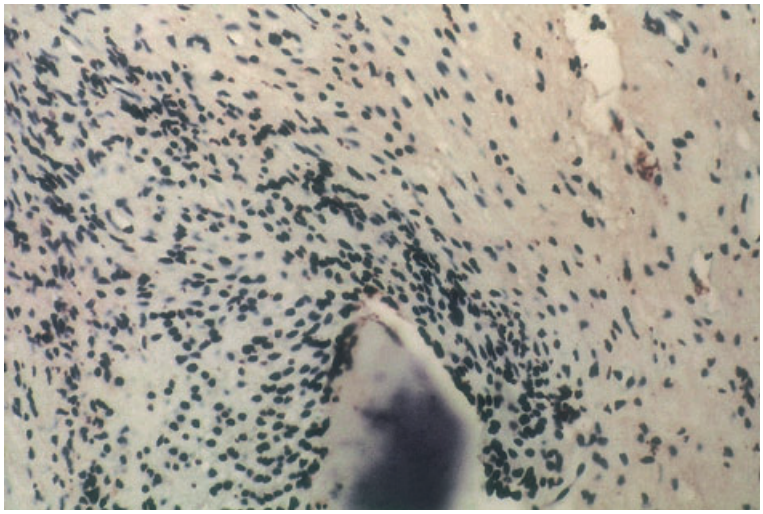

Figure 4 Photograph showing lymphocytes labelled by LCA in the inflammatory infiltrates of an inflamed pulp specimen (×200).

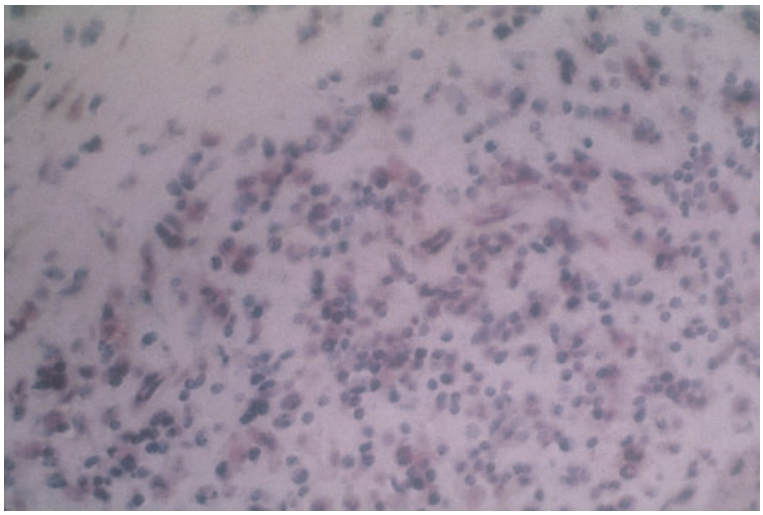

Figure 5 Radicular cyst specimen was used as positive control. t-PA stain was detected in the lining epithelium, connective tissue, inflammatory infiltrates and endothelium (×100).

expression in the inflamed dental pulp may be induced either directly by bacteria or indirectly by inflammatory cytokines generated by resident cells. Thus, these cells may play an important role in the pathogenesis of pulpal inflammation by controlling the synthesis of proinflammatory cytokines and t-PA.

Furthermore, pulp cells can be stimulated by proinflammatory cytokines (Chang *et al.* 2001) and endodontic pathogens (Chang *et al.* 2002) to secrete increased amounts of MMP into culture medium. Indeed, the plasmin-dependent pathway is understood to be a significant alternate pathway for the initiation of extracellular matrix degradation by MMPs (Birkedal-Hansen 1993). Thus, the interaction between t-PA and MMP in the pathogenesis of pulpal inflammation needs further investigation.

t-PA could be detected in areas of the inflamed pulp tissue, suggesting that t-PA, stored in the cytosol of lymphocytes and fibroblasts, may represent a reservoir of t-PA activity that can be released at certain stages of the inflammatory reaction. It appears that intracellular storage permitted the immunohistochemical detection of t-PA in lymphocytes and fibroblasts within inflamed pulp tissue. This study has shown that there is evidence to suggest that plasminogen activator system plays a significant role in the tissue destruction associated with pulpal inflammation.

Conclusion

t-PA mRNA gene was found to be upregulated in inflamed pulps as compared with normal pulps. t-PA

expression was significantly higher in the inflamed pulp and t-PA stain was detected in the fibroblasts, inflammatory infiltrates and endothelial cells. Taken together, t-PA expression may play an important role in the pathogenesis of pulpal inflammation.

Acknowledgements

This study was supported by a research grant from the Chung Shan Medical University (CSMU 93-OM-B-003).

References

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

Chang YC, Yang SF, Hsieh YS (2001) Regulation of matrix metalloproteinases-2 production by cytokines and pharmacological agents in human pulp cell cultures. *Journal of Endodontics* **27**, 679–82.

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.

Collen D (1999) The plasminogen (fibrinolytic) systems. *Thrombosis and Haemostasis* **82**, 259–70.

Deutsch DG, Mertz ET (1970) Plasminogen: purification from human plasma by affinity chromatography. *Science* **170**, 1095–6.

Hosoya S, Ohbayashi E, Matsushima K et al. (1998) Stimulatory effect of interleukin-6 on plasminogen activator activity from human dental pulp cells. *Journal of Endodontics* **24**, 331–4.

Jontell M, Okiji T, Dahlgren V, Bergenholtz G (1998) Immune defense mechanisms of the dental pulp. *Critical Reviews in Oral Biology and Medicine* **9**, 179–200.

Kinnby B, Lindberg P, Lecander I, Matsson L (1999) Localization of plasmingen activators and plasminogen activator inhibitors in human gingival tissues demonstrated by immunohistochemistry and in situ hybridization. *Archives of Oral Biology* **44**, 1027–34.

Langeland K (1987) Tissue response to dental caries. *Endodontics and Dental Traumatology* **3**, 149–71.

Matrisian LM (1992) The matrix-degrading metalloproteinases. *Bioassays* **14**, 455–63.

Mignatti P, Robbins E, Rifkin DB (1986) Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. *Cell* **47**, 487–98.

Saksela O (1985) Plasminogen activation and regulation of pericellular proteolysis. *Biochimica et Biopphysica Acta* **823**, 35–65.

Tsai CH, Huang FM, Yang LC, Chou MY, Chang YC (2002) Immunohistochemical localization of cyclooxygenase-2 in radicular cysts. *International Endodontic Journal* **35**, 854–8.

Tsai CH, Hsieh YS, Yang SF, Chou MY, Chang YC (2003) Matrix metalloproteinase 2 and 9 expression in human oral squamous cell carcinoma and the effect of protein kinase C inhibitors: preliminary observations. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics* **95**, 710–6.

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 and 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.

Vassall JD, Sappino AP, Belin D (1991) The plasminogen activator/plasmin system. *Journal of Clinical Investigations* **88**, 1067–72.

de Vries C, Veerman H, Blasi F, Pannekoek H (1988) Artificial exon shuffling between tissue-type plasminogen activator (t-PA) and urokinase (u-PA). A comparative study on the fibrinolytic properties of t-PA/u-PA hybrid proteins. *Biochemistry* **27**, 2565–72.

Werb Z, Mainardi C, Vater CA, Harris ED (1977) Endogenous activation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. *New England Journal of Medicine* **296**, 1017–23.

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.