

Original

Tumor necrosis factor α stimulates expression and secretion of urokinase plasminogen activator in human dental pulp cells

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Abstract: Plasminogen activator (PA) is the enzyme responsible for converting plasminogen to its active form, plasmin, which is involved in various physiological and pathological phenomena. PA exists in two forms: urokinase-type PA (uPA) and tissue-type PA (tPA). Here we investigated the effect of the inflammatory cytokine tumor necrosis factor α (TNF- α) on PA production and secretion in human dental pulp cells. When the cells were stimulated with TNF- α (10 ng/mL), PA activity in the medium clearly increased in a time-dependent manner, and this activity was reduced after immunoprecipitation with anti-uPA antibody, but not with anti-tPA antibody. In TNF- α -stimulated cells, the expression of uPA mRNA was enhanced, but was lower than that of tPA mRNA. The expression of uPA mRNA and PA secretion stimulated by TNF- α were reduced by the tyrosine kinase inhibitors herbimycin A and genistein, and by the NF κ B inhibitor pyrrolidine dithiocarbamate, but were augmented by the tyrosine phosphatase inhibitor sodium orthovanadate. In the presence of another inflammatory cytokine, interleukin 1 β (IL-1 β , 100 pg/mL), TNF- α -stimulated expression of uPA mRNA and secretion of uPA were enhanced. These observations suggest that TNF- α stimulates uPA production

and secretion, and that this effect is regulated via activation of NF κ B and tyrosine phosphorylation, apparently in conjunction with IL-1 β , during inflammation in human dental pulp. (J Oral Sci 54, 329-336, 2012)

Keywords: uPA; TNF- α ; tyrosine phosphorylation; NF κ B; dental pulp.

Introduction

It has been suggested that the progression of irreversible pulpitis is related to the morphological characteristics of human dental pulp, including the fact that it is almost completely surrounded by hard tissue and that few major vessels supply the dental pulp through the apical foramen. The process of inflammation in the pulp is essentially the same as that in connective tissue elsewhere in the body. Dental pulp infection is most commonly caused by extensive dental caries. Elastinolytic and collagenolytic activities are reportedly increased in diseased human dental pulp affected by suppurative pulpitis and necrosis (1), and cytokines have been reported to stimulate the production of elevated levels of matrix metalloproteinase (2).

Plasmin, a serine protease, is involved in the degradation of extracellular matrix and basement membranes, either directly or indirectly through activation of metalloproteinases and other zymogens (3,4). Plasminogen activators (PAs) act on plasminogen to yield plasmin (3). PAs and their inhibitors (PAIs) are thought to be key

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participants in the balance of proteolytic and antiproteolytic activities, and the plasminogen activation system is involved in physiological and pathological functions such as clotting, fibrinolysis, inflammation and tissue remodeling (5). Two types of PA, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), are expressed in numerous tissues. Both of these PAs activate plasminogen, but are considered to play different biological roles; tPA is primarily implicated in fibrinolysis because of its high affinity for fibrin and the extracellular matrix, whereas uPA promotes cell migration and proliferation via interaction with high-affinity cell surface receptors (uPAR) (6-8).

Tumor necrosis factor (TNF) and interleukin 1 (IL-1) are pro-inflammatory cytokines produced primarily by activated macrophages and T lymphocytes, contributing to several events that are essential for the initiation of an inflammatory response and, ultimately, tissue destruction (9-13). These cytokines can induce up-regulation of adhesion molecules on leukocytes and endothelial cells and stimulate the production of chemokines, both of which represent critical elements in the recruitment of circulating leukocytes (14,15). It has been reported that TNF and IL-1 are co-produced *in vivo* and can act synergistically to stimulate bone-resorptive activity (9). Thus IL-1 and TNF often, but not always, demonstrate overlapping biological properties. We have reported previously that IL-1 β stimulates the production of uPA, and that this may contribute to dental pulp inflammation (16). However, the effect of TNF- α on expression of PAs in dental pulp has not been well elucidated. In the present study, we examined the effect of TNF- α on uPA production and secretion in primary-cultured human dental pulp.

Materials and Methods

Materials

Fetal calf serum (FCS), α -minimal essential medium (α -MEM), fungizone and trypsin were obtained from Gibco BRL Life Technologies (Tokyo, Japan). Penicillin G and kanamycin were from Meiji Seika (Tokyo, Japan). Herbimycin A and pyrrolidine dithiocarbamate (PDTC) were from Seikagaku Corporation (Tokyo, Japan). Genistein was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium orthovanadate was from Sigma-Aldrich Japan (Tokyo, Japan). TNF- α and IL-1 β were obtained from Techne Corporation (Minneapolis, MN, USA). PA assay agents, fluorescent substrate S2251, plasminogen and tPA stimulator were obtained from Cromogenix (Milano, Italy). CNBr fibrinogen fragments were from American Diagnostica Inc. (Stamford, CT, USA). Urokinase (human urine) was from Calbiochem

(Darmstadt, Germany).

Cell culture

Human dental pulp cells were obtained under aseptic conditions from third molars extracted from a 20-year-old patient during orthodontic treatment. The patient gave informed consent before providing the samples. After the dental pulp had been extracted, the tissue was minced, placed on a 35-mm tissue culture dish, and covered with a sterilized glass coverslip. α -MEM supplemented with 10% FCS and antibiotics (100 μ g/mL penicillin G, 1 mg/mL kanamycin, 1 mg/mL fungizone) was used for culture. Once cell growth from the explants had reached confluence, the cells were detached with 0.05% trypsin in phosphate-buffered saline and subcultured in culture flasks. For the experiments, human dental pulp cells at 6th to 9th passage were plated at 2×10^5 cells/mL medium. This study was approved by the ethics committee of Nihon University School of Dentistry at Matsudo.

PA assay

Cells (1×10^5) were plated on 35-mm tissue culture dishes. The confluent cells were incubated for 24 h in medium containing 1% FCS, and then treated with 10 ng/mL TNF- α (17). PA activity in the cell lysate and conditioned medium was measured according to the method described previously (17). A mixture of 12 μ L plasminogen (0.75 mg/mL), 12 μ L CNBr fibrinogen (3.3 mg/mL) and 30 μ L Tris-HCl buffer (10 mM, pH 7.4) was added to a sample tube, and made up to a final volume of 90 μ L with distilled water. The assay was started by adding each sample, and incubating at 37°C for 45 min. Then 35 μ L of S2251 was added to the reaction mixture and further incubated for 15 min. The reaction was stopped by addition of 100 μ L citric acid (20 mg/mL). Color development was followed at 415 nm using a microplate reader (MTP-300 Crona Electric, Hitachinaka, Japan).

RT-PCR

Total cellular RNA was extracted from human dental pulp cells using an RNeasy mini kit (QIAGEN). The procedure for isolation of RNA was performed according to the protocol stipulated for the RNeasy mini kit. cDNA synthesis and amplification by RT-PCR were conducted using a One-Step RT-PCR kit. Specific primers were synthesized for uPA (forward: 5'-TTTGCGCCATCTACAGGAG-3', reverse: 5'-ACTGGGGATCGTTATACATC-3'), tPA (forward: 5'-CAGGAAATCCATGCCCGATT-3', reverse: 5'-GCTGATGAGTATGCCCCGCACAGGAACCG-3') and GAPDH as a control (forward:

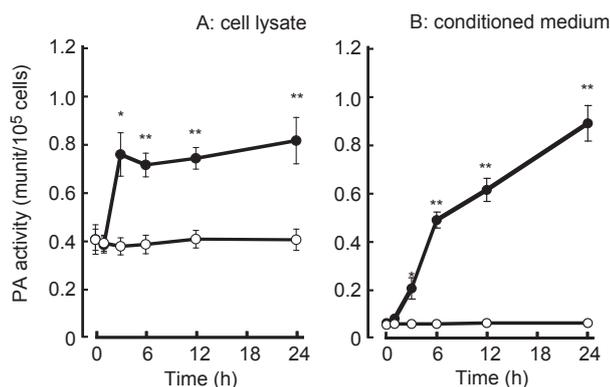


Fig. 1 PA activity in human dental pulp cells and conditioned medium after TNF- α stimulation. Human dental pulp cells were stimulated with (●) or without (○) 10 ng/mL TNF- α for the indicated times. PA activities in the cell lysate (A) and conditioned medium were then determined. Results are mean \pm SE ($n = 4$) * $P < 0.05$, ** $P < 0.01$, significantly different from the control.

5'-ATCACCATCTTCCAGGAG-3', reverse: 5'-ATGGA CTGTGGTCATGAG-3'). These primers (500 nM) and RNA (250 ng) were used for PCR. A GeneAmp 9600 PCR system (Perkin-Elmer) was programmed for cDNA synthesis and pre-denaturation for 30 min at 50°C and 15 min at 95°C, followed by 22 thermal cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final extension for 10 min at 72°C.

Immunoprecipitation

After the cells had been treated with TNF- α for 12 h, the conditioned medium was isolated, then incubated with protein G-Sepharose 4FF conjugated with normal mouse IgG at 4°C for 1 h. After centrifugation 15,000 $\times g$ for 10 min, the supernatant was incubated overnight with mouse anti-mouse-IgG, anti-mouse-tPA or anti-mouse-uPA antibodies conjugated with protein G-Sepharose 4FF at 4°C. The supernatant and protein G-Sepharose were then separated by centrifugation, and the supernatant was subjected to PA assay.

Protein assay

Protein concentrations were determined by the method of Bradford (18) with bovine serum albumin as the standard.

Statistical analysis

Results are presented as means \pm standard error (S.E.) for the numbers of experiments indicated. Tukey test was performed for statistical analysis.

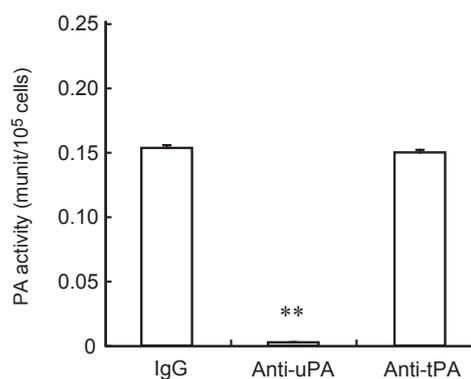


Fig. 2 Disappearance of PA activity secreted from TNF- α -stimulated cells upon treatment with anti-uPA antibody. The conditioned medium in which human dental pulp cells had been stimulated with TNF- α (10 ng/mL) for 12 h was isolated, and then incubated overnight with protein Sepharose A/G conjugated with mouse-IgG, anti-mouse-uPA or anti-mouse-tPA at 4°C. After removing the protein Sepharose by centrifugation, PA activity in the conditioned medium was determined. Values are means \pm SE from four independent experiments. ** $P < 0.01$, significantly different from the control (IgG).

Results

TNF- α stimulates PA secretion in human dental pulp cells

We first examined the effect of TNF- α on PA activity in human dental pulp cells. TNF- α at 10 ng/mL stimulated PA activity in the cell lysate and conditioned medium in a time-dependent manner (Fig. 1). PA activity in the cell lysate increased 2-fold and reached a plateau at 3 h (Fig. 1A), whereas that in the conditioned medium increased gradually up to 24 h (Fig. 1B).

TNF- α -stimulated uPA secretion

Two types of PA, uPA and tPA, have been identified. Here we examined the type of PA secreted from TNF- α -stimulated human dental pulp cells. After the cells had been incubated with TNF- α (10 ng/mL) for 12 h, the medium was isolated. As shown in Fig. 2, when the medium was treated with protein G-Sepharose conjugated with anti-uPA antibody and centrifuged, PA activity in the medium disappeared. On the other hand, when anti-tPA antibody or normal IgG was used instead of anti-uPA antibody, PA activity remained in the medium. These results suggested that PA was immunoprecipitated with the anti-uPA antibody, and that therefore the PA secreted from TNF- α -stimulated dental pulp cells is the urokinase type.

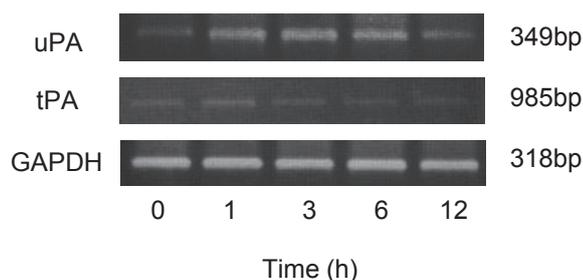


Fig. 3 Effect of TNF- α on expression of uPA mRNA in human dental pulp cells. Human dental pulp cells were incubated with 10 ng/mL TNF- α for the indicated times. Levels of mRNA for uPA, tPA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assessed by RT-PCR. PCR fragments were electrophoresed on 2% agarose gel and subsequently stained with ethidium bromide.

TNF- α -induced expression of uPA mRNA

Using RT-PCR, we then examined the effect of TNF- α on expression of uPA and tPA mRNAs in human dental pulp cells. As shown in Fig. 3, 349-bp and 985-bp bands, corresponding to the uPA and tPA mRNAs, respectively, were detected in human dental pulp cells, although the uPA band was much clearer than the tPA band. When the cells were stimulated with TNF- α (10 ng/mL), expression of uPA mRNA was enhanced by 3 h and then subsequently declined. On the other hand, the effect of TNF- α on expression of tPA mRNA was less marked (Fig. 3). These results suggested that TNF- α stimulated uPA production and secretion in human dental pulp cells.

Contribution of tyrosine phosphorylation to TNF- α -induced uPA expression

Because the effect of TNF- α is well known to be coupled to tyrosine phosphorylation (16,19), we examined the contribution of tyrosine phosphorylation to TNF- α -induced uPA secretion in human dental pulp cells. When the cells were incubated in the presence of either of the tyrosine kinase inhibitors herbimycin A (1 μ M) and genistein (50 μ M), TNF- α -induced uPA secretion (PA activity in the conditioned medium) was reduced (Fig. 4A). On the other hand, TNF- α -induced uPA secretion was enhanced from cells treated with sodium orthovanadate (100 μ M), a tyrosine phosphatase inhibitor (Fig. 4A). These observations suggested that tyrosine phosphorylation contributes to TNF- α -induced uPA secretion.

We then examined the effects of the tyrosine kinase and phosphatase inhibitors on TNF- α -induced expression

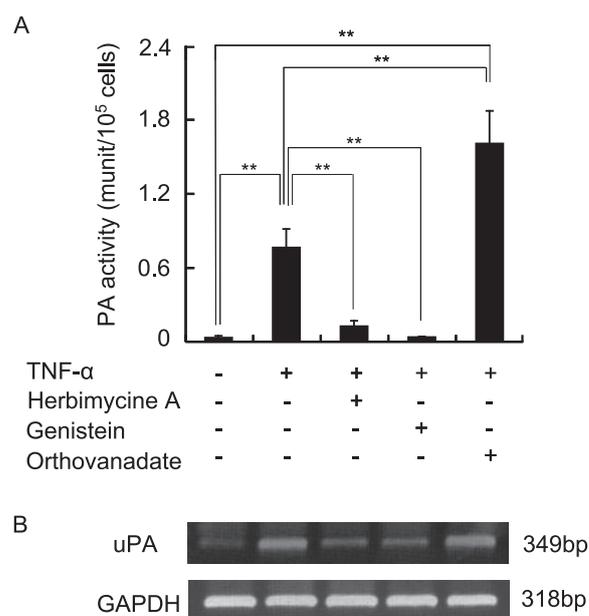


Fig. 4 Effects of inhibitors of tyrosine kinase and tyrosine phosphatase on TNF- α -induced secretion of uPA and expression of its mRNA. Human dental pulp cells preincubated in the absence or presence of 50 μ M genistein, 1 μ M herbimycin, or 100 μ M sodium orthovanadate for 30 min were stimulated with 10 ng/mL TNF- α , or unstimulated. (A) PA activity secreted from the cells after incubation for 12 h was then determined. Results are mean \pm SE ($n = 4$) * $P < 0.05$, ** $P < 0.01$, significantly different from the control. (B) The levels of mRNAs for uPA or GAPDH in the cells stimulated for 1 h were assessed by RT-PCR. PCR fragments were electrophoresed on 2% agarose gel and subsequently stained with ethidium bromide.

of uPA mRNA. As summarized in Fig. 4B, TNF- α -stimulated expression of uPA mRNA was reduced in the presence of herbimycin A or genistein, and was slightly enhanced in the presence of sodium orthovanadate. These results suggested that TNF- α -induced uPA expression is coupled to tyrosine phosphorylation.

Involvement of NF κ B in TNF- α -induced expression of uPA

NF κ B is a critical factor involved in the transcriptional control of several inflammatory-related genes (16). Therefore, we investigated whether NF κ B is involved in TNF- α -induced uPA expression and secretion in human dental pulp cells. In the presence of the NF κ B inhibitor PDCT (50 μ M), TNF- α -induced uPA secretion and mRNA expression were partially inhibited (Fig. 5A and 5B, respectively), suggesting that TNF- α induces expression of uPA mRNA via NF κ B activation.

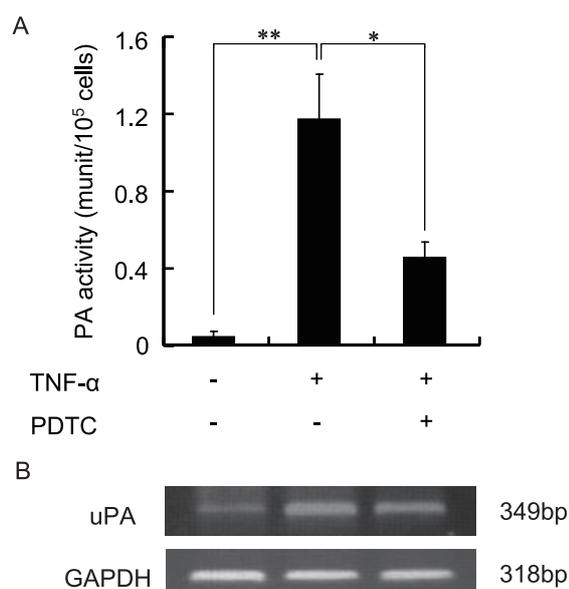


Fig. 5 Effect of NF- κ B inhibitor on TNF- α -induced secretion of uPA and expression of its mRNA. Human dental pulp cells preincubated in the absence or presence of 50 μ M PDTC for 30 min were stimulated with 10 ng/mL TNF- α , or unstimulated. (A) PA activity secreted from the cells after incubation for 12 h was then determined. Results are mean \pm SE ($n = 4$) * $P < 0.05$, ** $P < 0.01$, significantly different from the control. (B) The levels of mRNAs for uPA or GAPDH in the cells stimulated for 1 h were assessed by RT-PCR. PCR fragments were electrophoresed on 2% agarose gel and subsequently stained with ethidium bromide.

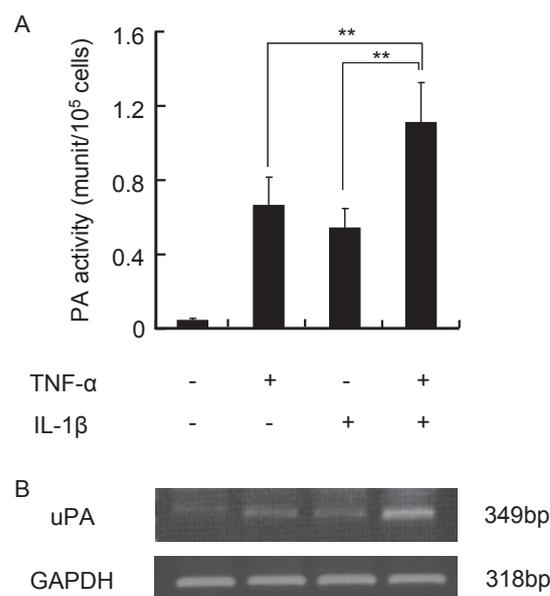


Fig. 6 Enhancement of TNF- α -stimulated uPA secretion and uPA mRNA expression in the presence of IL-1 β . Human dental pulp cells were stimulated with TNF- α (10 ng/mL), IL-1 β (100 pg/mL) or TNF- α (10 ng/mL)/IL-1 β (100 pg/mL). (A) PA activity secreted from the cells after incubation for 12 h was then determined. Results are mean \pm SE ($n = 4$) * $P < 0.05$, ** $P < 0.01$, significantly different from the control. (B) The levels of mRNAs for uPA or GAPDH in the cells stimulated for 1 h were assessed by RT-PCR. PCR fragments were electrophoresed on 2% agarose gel and subsequently stained with ethidium bromide.

TNF- α and IL-1 β synergistically stimulate uPA expression and secretion in human dental pulp cells

TNF- α and IL-1 β contribute cooperatively to the initiation of several inflammatory events related to tissue destruction. We have previously demonstrated that IL-1 β at 100 pg/mL stimulates uPA expression and secretion in human dental pulp cells (16). Therefore, we next compared the effects of IL-1 β and TNF- α on expression of uPA mRNA and uPA secretion. As shown in Fig. 6, uPA secretion and expression of uPA mRNA induced by TNF- α (10 ng/mL) did not differ significantly from those induced by IL-1 β (100 pg/mL). However, when the cells were stimulated simultaneously with both TNF- α (10 ng/mL) and IL-1 β (100 pg/mL), uPA secretion and mRNA expression were enhanced about 2-fold, suggesting that TNF- α and IL-1 β cooperatively induce uPA production and secretion in human dental pulp cells.

Discussion

TNF- α is a well-known inflammatory cytokine. Here we demonstrated that TNF- α -induced both the expression of uPA mRNA and uPA secretion in primary-cultured human dental pulp cells. TNF- α has been reported to enhance the expression of both uPA mRNA and uPA receptor (uPAR) mRNA in gastric fibroblasts (20). In gingival fibroblasts, uPA production stimulated by epidermal growth factor has been considered a critical step in inflammation and wound-healing (21,22). In inflamed dental pulp and periapical lesions, high levels of both TNF- α and another inflammatory cytokine, IL-1 β , have been detected (23). Therefore, it is conceivable that TNF- α -induced uPA expression and secretion could contribute to the process of acute dental pulp inflammation, such as that occurring in pulpitis. A previous study found that TNF- α did not stimulate expression of uPA mRNA in human dental pulp cells (24). At the present time, the reason for the difference between the findings

of that study and the present one remains unclear.

We also demonstrated here that the effect of TNF- α on uPA mRNA expression was completely inhibited by the tyrosine kinase inhibitors herbimycin A and genistein, and activated by the tyrosine phosphatase inhibitor orthovanadate (25,26). These observations suggest that tyrosine phosphorylation is crucial for stimulation of uPA production by TNF- α . Secretion of uPA induced by TNF- α was also inhibited by these tyrosine kinase inhibitors and enhanced by the tyrosine phosphatase inhibitor, suggesting that secretion of uPA is coupled to uPA production.

We further investigated the relationship of an inducible transcription factor, NF κ B, to uPA expression and secretion. NF κ B controls a number of genes involved in immuno-inflammatory responses. NF κ B resides in the cytoplasm as an inactive heterotrimer consisting of p50, p65 and I κ B α subunits, the latter undergoing phosphorylation and degradation, and the p50-p65 heterotrimer being released for translocation to the nucleus upon activation of the complex (27,28). PDTC is an effective inhibitor of NF κ B activation, presumably working through inhibition of I κ B degradation (29-31). In human dental pulp cells, PDTC inhibited both the expression of uPA mRNA and uPA secretion. Previous studies have demonstrated that the inhibitory effect of genistein on NF κ B activation is likely caused by inhibition of tyrosine kinase activity, and that herbimycin A chemically modifies the p50 subunit of NF κ B, consequently preventing it from binding to the DNA (25). Therefore, we consider that NF κ B plays a crucial role in protein tyrosine phosphorylation of the signaling pathway responsible for expression of uPA mRNA and uPA secretion. It is expected that tyrosine kinase inhibitors contributing to uPA production will become potential targets for the design of therapeutic drugs for pathological conditions such as pulpitis induced by pro-inflammatory cytokines.

We have previously demonstrated that IL-1 β stimulates the expression of uPA mRNA via tyrosine phosphorylation and NF κ B activation in human dental pulp cells, as was the case for TNF- α demonstrated in the present study (16). However, the effect of TNF- α on expression of uPA mRNA and uPA secretion was maximal in the presence of IL-1 β (100 pg/mL). This observation suggests that, after activation of their respective receptors, the signaling pathway for uPA mRNA expression and uPA secretion induced by TNF- α is distinct from that induced by IL-1 β .

uPA is a multifunctional molecule that acts as either a proteolytic enzyme or a signal-inducing ligand. In order for uPA to function, binding to its specific surface receptor (uPAR) is necessary. uPA and uPAR provide

a cell surface-integrated multimolecular complex that regulates pleiotropic functions including the inflammatory response (31-33). In fact, inflammatory cytokines such as TNF- α and IL-1 β enhance the expression of uPAR (34,35). Therefore, further studies are necessary to clarify the regulation of uPAR expression in the uPA/uPAR system in human dental pulp cells.

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