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Immunohistochemical localization of tissue-type plasminogen activator and type I plasminogen activator inhibitor in radicular cysts

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BACKGROUND: The plasminogen/plasmin proteolytic system participates in a wide variety of extracellular matrix degradation. Detailed knowledge of plasminogen activators (PAs) and their inhibitors may be important for understanding the pathogenesis of radicular cysts. The purpose of this study was to investigate the *in situ* localization of tissue-type PA (t-PA) and type I PA inhibitor (PAI-1) in radicular cysts.

METHODS: Thirty formalin-fixed, paraffin-embedded specimens of radicular cysts were examined using immunohistochemistry. In addition, another section from each radicular cyst specimen was stained with hematoxylin and eosin to assess the presence of inflammatory infiltrates. Differences in t-PA and PAI-1 expression between tissues with low and high levels of inflammation were subsequently analyzed using Fisher's exact test.

RESULTS: Both t-PA- and PAI-1-positive cells were detected in the lining epithelium, connective tissue, inflammatory infiltrates, and endothelium. In addition, the t-PA signal was mainly expressed in epithelial cells. However, the PAI-1 signal was mainly expressed in fibroblasts. Moreover, significantly greater t-PA as well as PAI-1 expression was noted in radicular cysts with high levels of inflammation as compared to tissues with low levels of inflammatory cell infiltrates ($P < 0.05$).

CONCLUSIONS: The present study confirms earlier indications of local production of PA and its inhibitor in radicular cysts. In addition, this study further shows the tissue localization of the antigens for t-PA as well as PAI-1, and demonstrates that the expression of both t-PA and PAI-1 increases with the grade of inflammation in radicular cysts.

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Keywords: immunohistochemistry; inflammation; radicular cyst; tissue-type plasminogen activator; type I plasminogen activator inhibitor

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Introduction

Radicular cysts are the most common osteolytic lesions of maxillofacial skeleton (1). They are believed to result from inflammatory processes in the periapical tissues associated with bacterial infection and necrosis of the dental pulp in carious teeth (2). Histologically, the cyst lumen is lined by stratified squamous epithelium, which arises from the epithelial rests of Malassez. The cyst wall is composed of fibrous connective tissue containing a chronic inflammatory cell infiltrate. Radicular cysts grow within the periapical bone tissue; it is conceivable that intense bone resorption produced by activated osteoclasts may favor the intraosseous expansion of the lesion. However, very little is currently known about the precise mechanisms of growth of radicular cysts.

Tissue proteolysis is important for many pathological processes, such as inflammatory reactions and neoplastic growth. The broad-spectrum protease plasmin plays a central part in these reactions. Plasmin is formed from the ubiquitous pro-enzyme plasminogen, which is activated by plasminogen activators (PAs). Plasmin acts directly on connective tissue components, and also indirectly by activating the proforms of matrix metalloproteinases (MMPs; 3), and thereby has a central position in the regulation of connective tissue breakdown.

The plasminogen/plasmin system is composed of an inactive pro-enzyme, plasminogen, that can be converted to plasmin by either of the two PAs: tissue-type PA (t-PA) or urokinase-type PA (4). These PAs in normal plasma and in tissue are inactive and complexed to a PA inhibitor, of which type I PA inhibitor (PAI-1) is believed to be the most important (5).

Previously, Sugimura et al. (6) have demonstrated the presence of fibrinolytic activity in the homogenates of radicular cysts. This suggests that local fibrinolysis/proteolysis may play an important role in the pathogenesis of cystic

lesions in jaw bone. However, as this study was performed before the discovery of the specific PAI, the authors do not take into account the possibility of inhibition of PA. It also dose not reveal the type of PA responsible for the lytic activity or the cellular source of the plasminogen/plasmin system in radicular cysts. On the basis of these observations, the present work was undertaken to identify the *in situ* localization of t-PA and PAI-1 expression in radicular cysts, which have been associated with the fibrinolytic process, as a contribution to the knowledge of the pathogenesis of such lesions.

Materials and methods

Thirty formalin-fixed, paraffin-embedded specimens of radicular cysts were included in this study. Pathological diagnosis of radicular cyst was confirmed on the basis of clinical, radiographic, and histologic criteria. All patients had received cyst enucleation at the Department of Dentistry, Chung Shan Medical University Hospital.

The tissue blocks were cut at 5 µm and subjected to the peroxidase-labeled streptavidin–biotin technique as described previously (7). The sections were immersed in 3% methanol–hydrogen peroxide (H_2O_2) solution for 10 min to block endogenous peroxidase activity and incubated with antit-PA antibody (goat antihuman, cat#AB774, lot. 19070912; Chemicon Internation Inc., CA, USA; 1 : 50 dilution), anti-PAI-1 antibody (rabbit antihuman, cat#-SC8979, lot. L120; Santa Cruz Biotechnology, CA, USA; 1 : 50 dilution), or leukocyte common antigen (LCA) (Dako, Carpinteria, USA; 1 : 50 dilution), a specific antibody for lymphocytes, for 18 h at 4°C with 1% bovine serum albumin. After washing in 20 mM Tris–HCl buffer (pH 7.4) containing 0.9% NaCl, the sections were (i) incubated at room temperature with biotinylated multilink swine antigoat, mouse and rabbit immunoglobulins (Dako), diluted 1 : 150 in Tris–HCl for 30 min; (ii) washed with Tris–HCl two times for 10 min; (iii) incubated for 30 min with horseradish peroxidase-conjugated streptavidin (Dako) diluted 1 : 50 in Tris–HCl; (iv) washed with Tris–HCl two times for 10 min; (v) incubated for 3 min with 0.01% diaminobenzidine tetrahydro-chloride (Sigma, St Louis, MO, USA) and 0.03% H_2O_2 in 20 mM Tris–HCl buffer (pH 7.4); and (vi) rinsed in distilled H_2O for 10 min and counterstained with 3-amino-9-ethylcarbazole (AEC; Dako). Buffer used for dilution was Dako antibody diluent with background-reducing components. Negative controls included serial sections from which either the primary or the secondary antibodies were excluded. Four biopsy specimens of inflamed gingiva were used as positive controls (8).

One section from each radicular cyst specimen was stained with hematoxylin and eosin to evaluate the magnitude of inflammation at the histologic level. Each specimen was graded at 200× magnification as: grade I, inflammatory cells less than 1/3 per field; grade II, inflammatory cells between 1/3 and 2/3 per field; and grade III, inflammatory cells higher than 2/3 per field. Grading of each specimen was based on the average inflammatory condition in three consecutive microscopic fields, starting from the epithelial–connective tissue border and proceeding gradually deeper into lamina propria.

The proportion of positively stained cells in radicular cyst was determined and was recorded as follows: +++, 67 to approximately 100%; ++, 66 to approximately 33%; and +, less than 33% of all cells were stained. The correlation between t-PA or PAI-1 expression, respectively, and the grade of inflammation were analyzed for statistical significance by Fisher's exact test. A *P*-value less than 0.05 was considered to be statistically significant.

Results

At light microscopic examination, all specimens revealed the typical morphology of a radicular cyst (Fig. 1a). The cyst lumen was partially or entirely lined by non-keratinized stratified squamous epithelium. The underlying fibrous connective tissue wall was inflamed with variable degrees of inflammatory cell infiltration. Many small blood vessels were scattered within the cystic wall, especially concentrated in the areas with inflammatory infiltrates. The infiltrate consisted mainly of lymphocytes which were labeled with LCA (Fig. 1b). Among 30 specimens, 5 cases (16.7%)

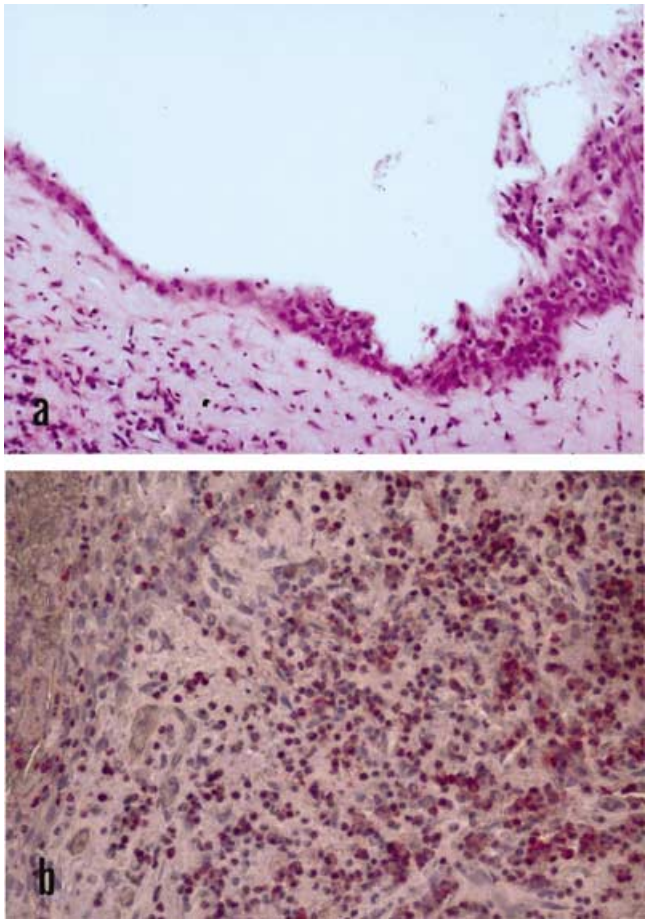

Figure 1 (a) The typical morphology of a radicular cyst. The cyst lumen was partially or entirely lined by non-keratinized stratified squamous epithelium. The underlying fibrous connective tissue wall was inflamed with variable degrees of inflammatory cell infiltration (H&E 200×). (b) Photograph showing lymphocytes labeled by LCA in inflamed connective tissue wall of a radicular cyst by a peroxidase-labeled streptavidin–biotin technique (200×).

Table 1 The condition of inflammation, t-PA expression, and PAI-1 expression in 30 radicular cyst specimens

Specimen	*Inflammation*	*t-PA*	*PAI-1*
1	I	+	+
2	III	++	+
3	II	+++	++
4	I	++	++
5	II	++	++
6	II	++	+++
7	II	++	+++
8	I	+	+
9	III	++	+++
10	III	++	+++
11	III	++	+++
12	II	+++	++
13	I	++	+
14	III	+++	+++
15	III	++	+++
16	III	+++	+++
17	III	+++	++
18	III	++	+++
19	III	++	++
20	III	++	+++
21	II	+	++
22	III	++	++
23	II	+++	++
24	II	+++	+
25	II	++	+
26	III	+++	+++
27	I	+	+
28	III	+++	++
29	III	++	+++
30	III	++	+++

Grade I, inflammatory cells less than 1/3 per field; grade II, inflammatory cells between 1/3 and 2/3 per field; grade III, inflammatory cells higher than 2/3 per field.
+, t-PA or PAI-1 positively stained cells (less than 33%); ++, t-PA or PAI-1 positively stained cells (66 to approximately 33%); +++, t-PA or PAI-1 positively stained cells (67 to approximately 100%).

exhibited slight inflammation (grade I); 9 cases (30%) exhibited moderate inflammation (grade II); and 16 cases (53.3%) exhibited severe inflammation (grade III; Table 1).

As shown in Fig. 2(a), t-PA stain was detected in the lining epithelium, connective tissue, inflammatory infiltrates, and endothelium. The rank orders with respect to t-PA positively stained cells were found as follows: lining epithelium (30/30) > inflammatory cells (23/30) > fibroblasts (14/30; Fig. 2b; Table 2). t-PA expression in radicular cysts with different levels of inflammation is listed in Table 3. Differences in t-PA expression among different levels of inflammation were subsequently analyzed using Fisher's exact test. t-PA expression was related to the degree of inflammation ($P = 0.02$).

As shown in Fig. 3(a), PAI-1 staining was also detected in the lining epithelium, connective tissue, inflammatory infiltrates, and endothelium. However, the PAI-1 signal was mainly expressed in fibroblasts (23/30) followed by inflammatory cells (16/30) and lining epithelium (7/30; Fig. 3b; Table 2). PAI-1 expression in radicular cysts with different levels of inflammation is listed in Table 4. Differences in PAI-1 expression among different levels of inflammation were subsequently analyzed using Fisher's exact test. PAI-1 expression was related to the degree of inflammation ($P = 0.003$).

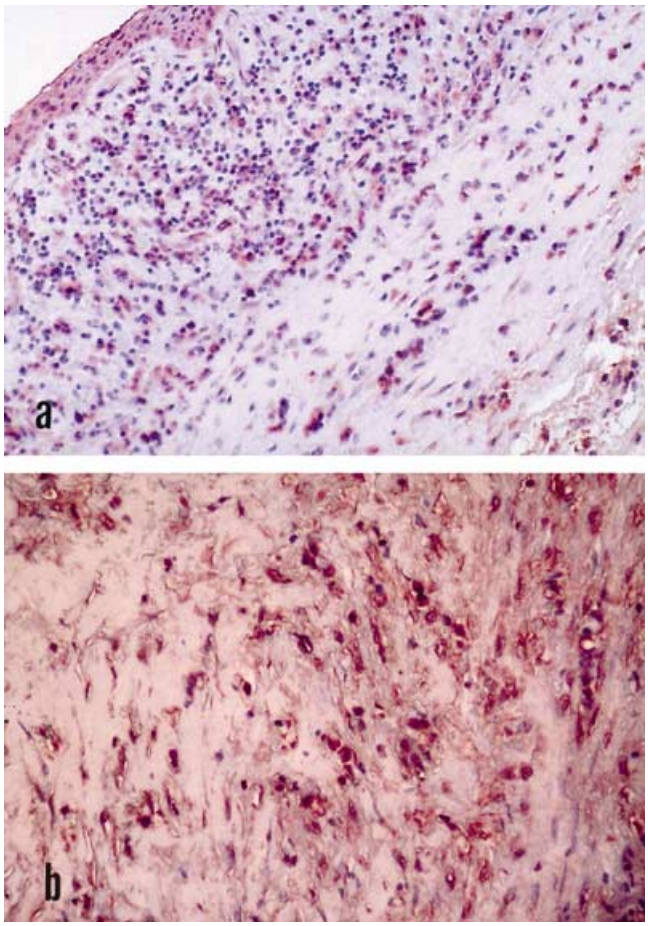

Figure 2 Immunolocalization of t-PA in radicular cysts by a peroxidase-labeled streptavidin–biotin technique. (a) t-PA stain was detected in the lining epithelium, connective tissue, inflammatory infiltrates, and endothelium (100×). (b) The high t-PA expression in the cytoplasm of inflammatory cells (200×).

Table 2 Localization and number of t-PA and PAI-1 expression in 30 radicular cyst specimens

	Epithelial cell (%)	*Lymphocyte (%)*	*Fibroblast (%)*
t-PA	30 (100)	23 (76.7)	14 (46.7)
PAI-1	6 (20)	16 (53.3)	23 (76.7)

Table 3 The results of t-PA expression and the grade of inflammation in radicular cysts analyzed in this study

	t-PA		
Inflammation	+	++	+++
I	3	2	0
II	1	4	4
III	0	11	5

A significantly greater t-PA expression was noted in radicular cysts with high levels of inflammation as compared to tissues with low levels of inflammatory cell infiltrates by Fisher's exact test ($P = 0.02$).

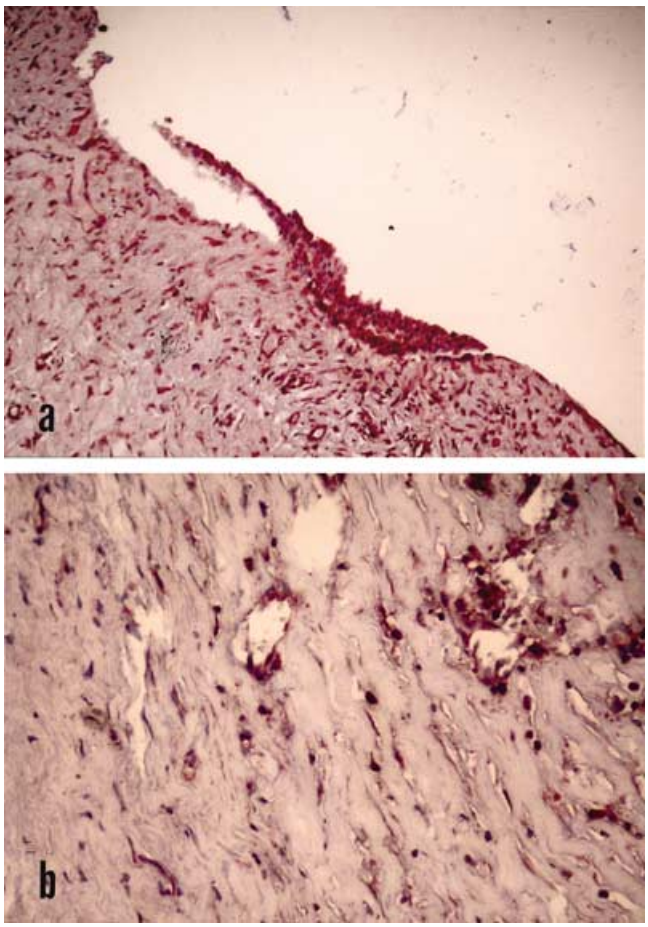

Figure 3 Immunolocalization of PAI-1 in radicular cysts by a peroxidase-labeled streptavidin–biotin technique. (a) PAI-1 stain was detected in the lining epithelium, connective tissue, inflammatory infiltrates, and endothelium (100×). (b) The high PAI-1 expression in the cytoplasm of fibroblasts and endothelial cells (200×).

Table 4 The results of PAI-1 expression and the grade of inflammation in radicular cysts analyzed in this study

	PAI-1		
Inflammation	+	++	+++
I	4	1	0
II	2	5	2
III	1	4	11

A significantly greater PAI-1 expression was noted in radicular cysts with high levels of inflammation as compared to tissues with low levels of inflammatory cell infiltrates by Fisher's exact test ($P = 0.003$).

Inflamed gingivae were used as positive controls in this study. As shown in Fig. 4(a), t-PA stain was detected in the epithelium, inflammatory infiltrates, and endothelium. PAI-1 stain was detected in fibroblast and endothelial cells (Fig. 4b).

Discussion

The plasminogen/plasmin proteolytic system has been the subject of a great deal of research interest because of its participation in a wide variety of biologic activities and pathological conditions involved in tissue destruction, as well as bone turnover. This system can modulate pulpal/periapical homeostasis by participating in many aspects of the pathological reactions associated with pulpal and periapical diseases (9–11). An understanding of the roles of PAs and PAIs in the pathophysiology of radicular cysts will give rise to additional understanding of the mechanisms underlying the host responses.

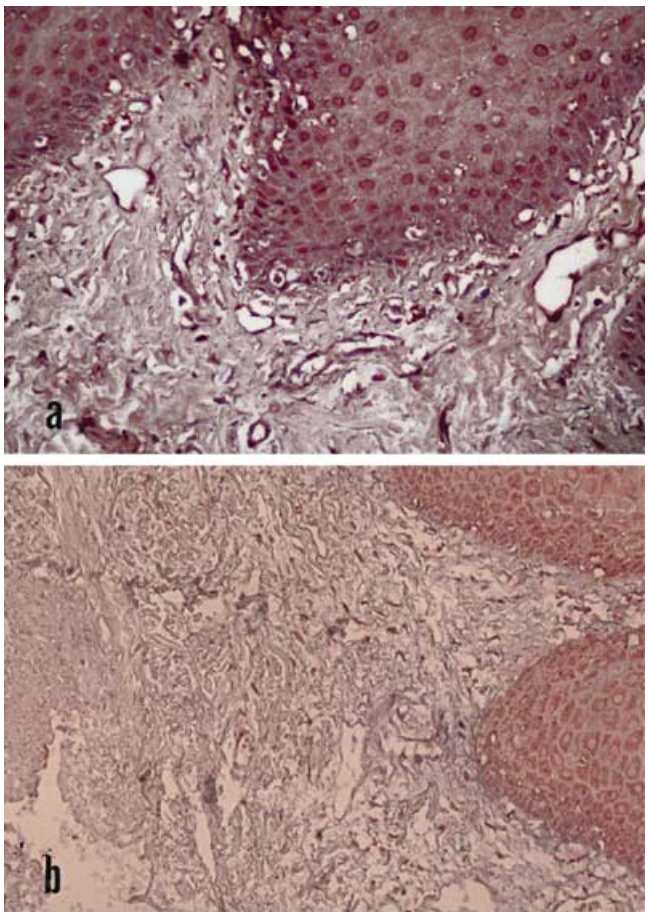

Figure 4 Photomicrograph showing staining for t-PA (a) or PAI-1 (b) in inflamed gingival tissues as positive controls by a peroxidase-labeled streptavidin–biotin technique (a and b, 400×).

To the best of our knowledge, in this study, t-PA and PAI-1 expression was first found in radicular cysts by immunohistochemistry. Positive t-PA staining was detected in the epithelial cells, subepithelial fibroblasts, inflammatory cells, and endothelial cells. In addition, t-PA was mainly higher expressed in epithelial cells. PAI-1 reactivity was also found in all radicular cysts, but it was more frequently expressed in fibroblasts. These findings confirmed a previous biochemical study showing that radicular cysts contain PA and the inhibitor antiplasmin (6). Our results were also in agreement with recent studies showing that PAs and PAIs immunolocalized to inflamed gingival tissues (8, 12, 13). Taken together, co-expression of PAs and PAIs may play an important role in the pathogenesis of radicular cysts.

Microbially induced tissue destruction may activate the plasminogen/plasmin proteolytic system, or may act by

direct cleavage of extracellular matrix constituted by microbial proteinases (14). 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, we first revealed that t-PA could be immunolocalized in radicular cysts. Positive t-PA staining was detected in the epithelial cells, fibroblasts, inflammatory cells, and endothelial cells. The more intense expression of t-PA in the epithelium of inflamed radicular cysts is consistent with earlier fibrin-overlay studies on gingiva, which showed fibrinolytic activity localized to cells in epithelium in inflamed gingiva (15). Recently, our studies have also shown that endodontic pathogens, IL-1α and TNF-α, can induce t-PA in human pulp fibroblasts, gingival fibroblasts, and osteoblastic cells (10, 11), thereby initiating tissue degradation. Previous studies have also shown that inflammatory cytokines IL-1α, IL-6, TNF-α, and cyclooxygenase-2 were found in radicular cyst specimens (1, 16–18). Therefore, t-PA expression in the radicular cyst may be induced either directly by bacteria from necrotic pulps or indirectly by inflammatory cytokines generated by resident cells. Thus, these cells may play an important role in the pathogenesis of radicular cysts by controlling the synthesis of pro-inflammatory cytokines and t-PA.

Plasmin might participate indirectly in collagen breakdown through the activation of latent MMPs (19). Previous studies have shown that MMP-1 (20, 21), MMP-2 (20), and MMP-9 (22) expression was found in radicular cyst. However, the roles of MMP and PA expression in the pathogenesis of radicular cysts are worthy of further investigation. Radicular cysts are believed to result from continuous antigenic stimulation from inflamed or necrotic root canals. As radicular cysts grow within the jaw bone, it is conceivable that expansion of a cyst is accompanied by the growth of the cyst epithelium and is aided by the rate at which the surrounding bone is destroyed. Then, one of the possible mechanisms of radicular cyst expansion *in vivo* may be the result of t-PA activation.

PAI-1 is thought to be the major physiologic inhibitor of t-PA in human plasma (23). In addition to the ability to bind and inhibit t-PA, it is also associated with virtronectin in blood and in the extracellular matrix. The modulation of PA activity by PAI-1 production is an important regulation of the net effect of the plasminogen/plasmin system. In this study, PAI-1 was associated with blood vessels, and the ratio in epithelium was only 20%. These distributions are similar to that found in normal epidermis (24). In highly inflamed radicular cyst specimens, PAI-1 was seen in several cellular components in connective tissue, particularly, inflammatory cells and fibroblasts. These results generally agree with those reported by Lindberg et al. (13) and Yang et al. (25). Furthermore, PAI-1 levels have been shown to be elevated by endodontic pathogens in pulp fibroblasts and osteoblasts *in vitro* (10). In addition, our data showed that the expression of PAI-1 increases with the grade of inflammation in the surrounding connective tissue in radicular cysts. Thus, PAI-1 may also play an important role in the pathogenesis during inflammatory processes.

Furthermore, the results of this study indicated not only t-PA but also PAI-1 expression in radicular cysts. The physiologic role of the plasminogen/plasmin system in radicular cyst is, however, not yet fully understood. The proximity to bacteria and the high permeability of the non-keratinized epithelium facilitate inflammatory responses, with signal substances activating the various cellular population in the region, and leading to high activity of both activator and inhibitor. The increase in PAI-1 might be a physiologic reaction to increased production of the activator. A fine balance exists in the expression of components of the plasminogen/plasmin system, whereby tissue homeostasis is maintained. Thus, this might partly explain the relatively slow growth of a radicular cyst.

The plasminogen/plasmin proteolytic system has received considerable attention because of its participation in a wide variety of biologic activities in many inflammatory diseases. Regulation of plasminogen activation is a key element in controlling proteolytic events in the extracellular matrix, and this result is achieved through the action of specific PAs and inhibitors. Our data showed that t-PA and PAI-1 were immunolocalized in epithelial cells, inflammatory cells, fibroblasts, and endothelial cells. Moreover, the expression of both t-PA and PAI-1 also increases with the grade of inflammation in radicular cysts. Further studies are required in order to fully elucidate the roles of the plasminogen/plasmin system in the pathogenesis of radicular cyst.

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