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ORIGINAL ARTICLE

Increased plasminogen activator inhibitor-1/tissue type plasminogen activator ratio in oral submucous fibrosis

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OBJECTIVE: Plasminogen activators and their inhibitors are thought to be key participants in the balance of proteolytic and antiproteolytic activities that regulate extracellular matrix (ECM) turnover. However, little is known about the expression of plasminogen/plasmin system at the site of oral submucous fibrosis (OSF).

METHODS: We compared the activities of tissue type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) between fibroblasts derived from normal buccal mucosa and OSF by using an enzyme-linked immunosorbent assay. Furthermore, arecoline, a major areca nut alkaloid, was challenged with normal buccal mucosal fibroblasts (BMFs) to elucidate whether the activities of t-PA and PAI-1 could be affected by arecoline.

RESULTS: Both t-PA and PAI-1 were found to be increased in OSF than in BMFs ($P < 0.01$). In addition, there was a statistically significant difference in PAI-1/t-PA ratio between OSF and BMF ($P < 0.01$). The addition of arecoline upregulated not only PAI-1, but also t-PA in BMFs ($P < 0.05$). In addition, the ratio between PAI-1 and t-PA was found to be significantly increased by a linear regression assay ($P < 0.01$).

CONCLUSION: These results suggest that OSF caused by areca quid chewing may be the result of an imbalance in the plasminogen/plasmin system, the net result of which is increased deposition of ECM.

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Keywords: arecoline; buccal mucosal fibroblasts; oral submucous fibrosis; tissue type plasminogen activator; plasminogen activator inhibitor-1

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Introduction

Oral submucous fibrosis (OSF) is regarded as a precancerous condition (Pindorg *et al*, 1984), and is a chronic insidious disease of the oral subepithelial connective tissue resulting in stiffness of the oral mucosa and inability to open the mouth. Although the exact etiology of OSF is not well known, there is strong epidemiological and experimental evidence suggesting areca quid chewing as a major etiologic factor (Sinor *et al*, 1990; Maber *et al*, 1994).

Tissue culture experiments using human fibroblasts obtained from normal buccal mucosa revealed that arecoline, a major areca nut alkaloid, was found to stimulate buccal mucosal fibroblast (BMF) proliferation (Harvey *et al*, 1986; Chang *et al*, 1998) and collagen synthesis (Harvey *et al*, 1986) *in vitro*. Stabilization of collagen and prevention of collagenase degradation in the oral mucosa (Kuo *et al*, 1995) and the attendant increase in lysyl oxidase activity (Ma *et al*, 1995; Trivedy *et al*, 1999) also contribute to abnormal deposition of collagen fibers in OSF. In addition, our recent studies have shown that the upregulation of tissue inhibitor of metalloproteinase-1 (Chang *et al*, 2002a), vimentin (Chang *et al*, 2002b), cyclooxygenase-2 (Tsai *et al*, 2003), and plasminogen activator inhibitor-1 (PAI-1) (Yang *et al*, 2003) may also contribute to the accumulation of extracellular components in OSF. However, little is known about the biochemical/molecular biology of areca quid-associated OSF.

The plasminogen/plasmin system represents a highly regulated enzymatic cascade with extracellular proteolysis, and is an important aspect in fibrinolysis, wound healing, and tissue remodeling. These enzymes convert plasminogen into plasmin, a trypsin-like serine protease, that is not only responsible for the degradation of fibrin, but also contribute to the degradation and turnover of the extracellular matrix (ECM). Plasminogen is activated by either tissue type plasminogen activator (t-PA) or urokinase type plasminogen activator (u-PA) (Kruithof *et al*, 1995). PAI-1 is the primary inhibitor of both t-PA

and u-PA, while α-antiplasmin is the physiologic inhibitor of plasmin (Vassall *et al*, 1991). Plasminogen activators and their inhibitors are thought to be key participants in the balance of proteolytic and antiproteolytic activities that regulate ECM turnover.

Fibrotic disease occurs when normal control of this process is compromised and excess fibrin material accumulates in the tissues. It is generally assumed that the persistence of fibrin in the matrix promotes fibrosis, and that the extent of fibrosis is limited by proteinases that remove the fibrin (i.e., the fibrinolytic system). To date, there have been relatively few studies addressing the expression of plasminogen/plasmin system at the site of OSF. In this study, we compared the activities of t-PA and PAI-1 between BMF and OSF by using an enzyme-linked immunosorbent assay (ELISA). To further explore the pathobiologic effects of area quid chewing on the human buccal mucosa, arecoline was used to evaluate the activity of t-PA and PAI-1 in BMF cultures.

Patients and methods

Cell culture

Twelve healthy individuals without areca quid chewing habits were selected from the Oral Medicine Center (Chung Shan Medical University Hospital, Taichung, Taiwan) with the informed consent for this study. Biopsy specimens were derived from histologically normal areas of the surgical third molar extraction from patients. The OSF specimens were obtained from the buccal mucosa from 26 patients with areca quid chewing habits during surgical biopsy. Clinical diagnosis was confirmed by histopathologic examination of the biopsy specimens. Fibroblast cultures were grown and maintained according to procedures described previously (Chang *et al*, 2000, 2001). The tissues were minced using sterile techniques and washed twice in phosphate-buffered saline supplemented with antibiotics (100 U ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 0.25 $\mu g\ ml^{-1}$ fungizone). Explants were placed in 60 mm dishes and maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics as described above. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached by treatment with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured with the same medium.

Assessment of t-PA and PAI-1 activity in OSF and BMF

Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in a 60-mm culture dish and the conditioned medium samples were collected after a 2-day culture period. Levels of t-PA and PAI-1 antigen (Biopool, Umea, Sweden) were determined by ELISA. Briefly, 20 μl of conditioned media was 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 and PAI-1 levels were determined with a calibration curve using human t-PA and PAI-1 as the standard.

Effect of arecoline on t-PA and PAI-1 activity in BMF

The BMF used in this study were derived from six different strains. Briefly, cells were seeded 1×10^5 cells per well in a 6-cm culture dish and incubated for 24 h. The medium was then changed to a medium containing 10% heated-activated FCS and various concentrations of arecoline (Sigma, St Louis, MO, USA) (0–80 $\mu g\ ml^{-1}$). The conditioned medium samples were collected after 24 h as described above.

Statistical analysis

For testing the differences in the level of t-PA and PAI-1 between BMF and OSF, the Wilcoxon–Mann–Whitney rank sum test was applied. The linear regression analysis was employed for determining the correlation between the PAI-1/t-PA ratio and various concentrations of arecoline. Separate triplicate experiments were performed throughout the study. The significance of the results obtained from the control and treated groups was statistically analyzed by using the Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

The individual values of t-PA and PAI-1 from BMF and OSF cultures are shown in Tables 1 and 2, respectively. The amount of t-PA protein in BMF was about 33.58 ng/10^6 cells. The amount of PAI-1 protein in BMF was about 34.61 ng/10^6 cells. In addition, the PAI-1/t-PA ratio was about 1.07 in BMF. The amount of t-PA protein in OSF was about 52.15 ng/10^6 cells. The amount of PAI-1 protein in OSF was about 64.65 ng/10^6 cells. In addition, the PAI-1/t-PA ratio was about 1.38 in OSF.

Table 1 Level of t-PA, PAI-1, and PAI-1/t-PA ratio from BMF by ELISA

Subject no.	*t-PA (ng/10^6 cells)*	*PAI-1 (ng/10^6 cells)*	*PAI-1/t-PA ratio*
1	21.90	29.38	1.34
2	36.22	33.19	0.91
3	39.33	36.59	0.93
4	28.98	34.41	1.18
5	38.12	33.60	0.88
6	41.74	34.14	0.81
7	22.08	35.09	1.58
8	33.81	34.41	1.01
9	23.80	30.74	1.29
10	36.39	38.63	1.06
11	38.81	35.77	0.92
12	41.74	39.31	0.94
Mean ± SD	33.58 ± 7.47	34.61 ± 2.85	1.07 ± 0.23

t-PA, tissue type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; BMF, buccal mucosal fibroblast; SD, standard deviation.

Table 2 Level of t-PA, PAI-1, and PAI/t-PA ratio from OSF by ELISA

Subject no.	*t-PA (ng/10^6 cells)*	*PAI-1 (ng/10^6 cells)*	*PAI-1/t-PA ratio*
1	27.94	61.49	2.20
2	28.11	61.28	2.17
3	29.67	55.42	1.86
4	32.08	55.84	1.74
5	32.94	54.16	1.64
6	36.74	64.62	1.75
7	40.36	62.95	1.55
8	42.26	62.11	1.46
9	42.60	61.28	1.43
10	42.61	59.39	1.39
11	42.63	68.81	1.61
12	44.16	67.76	1.53
13	44.67	66.30	1.48
14	45.36	72.36	1.59
15	50.54	69.85	1.38
16	53.82	69.85	1.29
17	59.51	56.05	0.94
18	59.85	74.45	1.24
19	61.41	94.53	1.53
20	68.13	45.17	0.66
21	77.97	67.97	0.87
22	78.14	55.84	0.71
23	91.94	72.15	0.78
24	118.16	65.25	0.55
25	50.23	60.44	1.20
26	54.06	75.71	1.40
Mean ± SD	52.15 ± 21.07	64.65 ± 9.41	1.38 ± 0.42

t-PA, tissue type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; OSF, oral submucous fibrosis; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

Table 3 Summary of t-PA, PAI-1, and PAI-1/t-PA ratio from BMF and OSF

	BMF (n = 12)		*OSF (n = 26)*	
Subjects	*Media*	*Range*	*Media*	*Range*
t-PA	36.31	21.9–41.74	45.02*	27.45–118.16
PAI-1	34.41	29.38–39.31	63.79*	45.17–94.53
PAI-1/t-PA ratio	0.94	0.81–1.58	1.43*	0.55–2.2

t-PA, tissue type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; BMF, buccal mucosal fibroblast; OSF, oral submucous fibrosis.
*Statistically significant between BMF and OSF, $P < 0.01$.

As shown in Table 3, both t-PA and PAI-1 were found to be increased in OSF than in BMF ($P < 0.01$). In addition, there was a statistically significant difference in PAI-1/t-PA ratios between OSF and BMF as analyzed by the Wilcoxon–Mann–Whitney rank sum test ($P < 0.01$).

To further explore the pathobiologic effects of area quid chewing on the human buccal mucosa, arecoline was used to evaluate the activity of t-PA and PAI-1 in BMF cultures. As shown in Figure 1, arecoline was found to upregulate not only PAI-1, but also t-PA in BMF cultures ($P < 0.05$). In addition, the ratio between PAI-1 and t-PA was found to be significantly

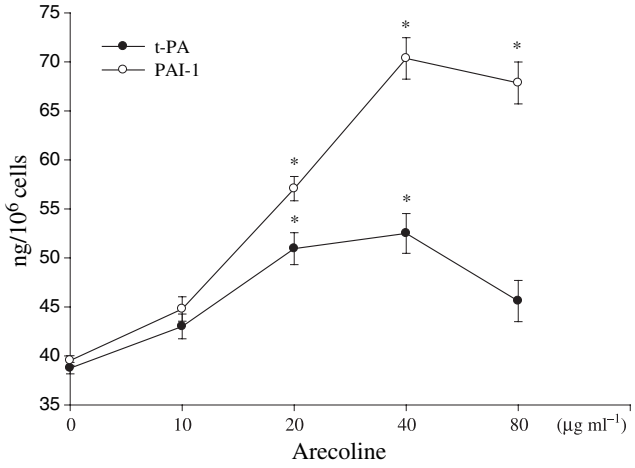

Figure 1 Protein level expression of plasminogen activator inhibitor-1 (PAI-1) and tissue type plasminogen activator (t-PA) in arecoline-treated human buccal mucosal fibroblasts. *Significant differences from control values with $P < 0.05$

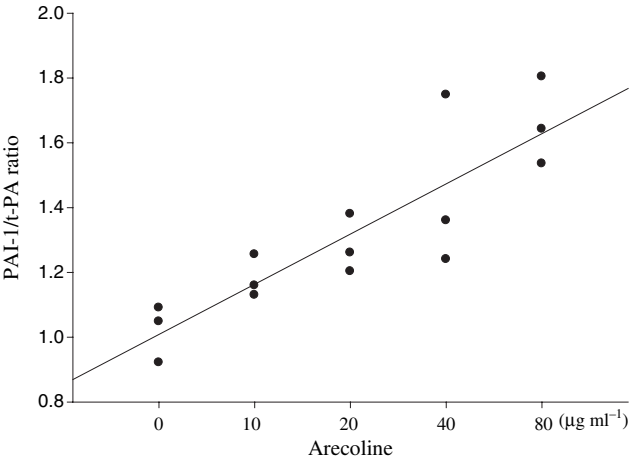

Figure 2 Linear regression analysis showing a significant direct correlation between arecoline concentration and plasminogen activator inhibitor-1 (PAI-1)/tissue type plasminogen activator (t-PA) ratio in arecoline-treated buccal mucosal fibroblast cultures ($Y = -97.163 + 96.433X$, $R^2 = 0.734$, $P < 0.001$)

increased by linear regression assay ($P < 0.01$) (Figure 2).

Discussion

The relationship between ECM degradation and fibrogenesis is complex, as abnormal ECM accumulation is often preceded or combined with an increased expression of ECM-degrading enzymes (Stetler-Stevenson, 1996). This increased proteolytic activity is presumably required for the degradation of normal ECM by infiltrating inflammatory and fibroblastic cells and its replacement by abnormal ECM. The plasminogen/plasmin system, one of the main proteolytic systems, is known to play an important role in the fibrotic process (Rondeau *et al*, 1995). Understanding the association between plasminogen activators and their inhibitors in

the pathophysiology of OSF might provide additional information about the mechanisms underlying the host responses and point to new therapeutic approaches.

The present investigation was designed to study the extracellular concentrations of t-PA and PAI-1 from OSF and BMF cultures by ELISA. Both t-PA and PAI-1 were found to be increased in OSF than in BMF cultures. Similar results were found in our recent report that demonstrated the upregulation of PAI-1 mRNA and protein in OSF (Yang *et al*, 2003). Our data are also in agreement with those of Yang *et al* (2002) who demonstrated that the deficiency of t-PA protects the kidney from developing fibrotic lesions. Buduneli *et al* (2004) reported that the levels of t-PA and type II plasminogen activator inhibitor in the gingival crevicular fluid were significantly higher in cyclosporin A-induced gingival overgrowth in renal transplant patients compared with healthy control subjects. These results imply that both plasminogen activators and their inhibitors may play an important role in the pathogenesis of tissue fibrosis.

The balance between plasminogen activators and their inhibitors reflects the net plasminogen-activating capacity and can be expressed as a numerical ratio. In the present study, the ratio of PAI-1/t-PA was used to reflect the net plasminogen activity, this being a functional entity. The ratio of PAI-1/t-PA was nearly 1:1 in BMF cultures in this study. These results demonstrate that a fine balance exists in the expression of components of the plasminogen/plasmin system in order to maintain tissue homeostasis in the normal buccal mucosa. However, the ratio of PAI-1/t-PA was much higher in OSF compared with BMF. Thus, OSF may be the result of imbalance between enhanced matrix synthesis and diminished breakdown of connective tissue proteins, the net result of which is increased deposition of ECM.

One way in which areca quid causes OSF is through activation of resident cells and the system to produce and/or release enzymes and other molecules associated with tissue fibrosis (Ma *et al*, 1995; Trivedy *et al*, 1999; Chang *et al*, 2002a; Yang *et al*, 2003). Arecoline is considered as one of the most important factors related to the pathogenesis of OSF. In the present study, arecoline was found to upregulate not only PAI-1, but also t-PA in BMF cultures. In addition, the ratio between PAI-1 and t-PA was found to be significantly increased by a linear regression assay. This suggests that one of the pathogenic mechanisms of OSF may be the imbalance between PAI and t-PA by resident cells in responses to areca quid stimulation.

The mechanism responsible for the PAI-1/t-PA expression by arecoline may be explained as follows. Recently, we showed that arecoline can stimulate PAI-1 mRNA and protein expression in BMF (Yang *et al*, 2003). Similarly, the present data also show that in BMF cultures, arecoline increased the production of extracellular PAI-1 by ELISA. The increase in the production of t-PA by BMF exposed to arecoline could indicate a role for this protein in the protection of the tissue from fibrosis stress, and by activation of plasminogen activator-dependent pericellular proteolytic processes. In addition, extracellular plasminogen activator inhibitors may contribute to the regulation of plasminogen activators activity on the cell surface and in the pericellular space (Kruithof *et al*, 1995). As a result, this may insure a decrease in cell surface-associated proteolytic activity by areca quid chewing and finally lead to OSF.

In conclusion, the plasminogen/plasmin system has received considerable attention because of its participation in a wide variety of biologic activities in ECM turnover. Regulation of plasminogen activation is a key element in controlling proteolytic events in the ECM and this regulation is achieved through the action of specific plasminogen activators and inhibitors. The present study has shown that OSF exhibited higher PAI-1 and t-PA expression than BMF and that the ratio was significantly higher in OSF. Taken together, areca quid chewing activates the plasminogen activator/inhibitor system in the normal buccal mucosa through an unbalanced regulation of t-PA and PAI-1, which may result in an uncontrolled accumulation of ECM in the connective tissue and a rapid progress in areca quid-associated OSF.

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