Relationship of fibrosis and the expression of TGF-β1, MMP-1, and TIMP-1 with epithelial dysplasia in oral submucous fibrosis

Rasika Pawiththra Illeperuma[1], Mi Heon Ryu[2], Ki Yeol Kim[1], Wanninayake Mudiyanselage Tilakaratne[3], Jin Kim[1]

[1]*Oral Cancer Research Institute, Department of Oral Pathology, Brain Korea 21 Project, Yonsei University College of Dentistry, Seoul, Korea*

[2]*Department of Oral Pathology, Pusan University College of Dentistry, Pusan, Korea*

[3]*Department of Oral Pathology, Faculty of Dental Sciences, University of Peradeniya, Sri Lanka*

Abstract: **Background: Oral submucous fibrosis (OSF) is a premalignant fibrotic oral disease. The aim of this study was to investigate the relationship between fibrosis and the expression of TGF-β1, MMP-1, and TIMP-1 and epithelial dysplasia in OSF. Methods: Forty-two tissue samples from the oral mucosa of OSF patients were histopathologically and immunohistochemically examined. The expressions of TGF-β1, TIMP-1, MMP-1 and PCNA were observed using immunohistochemistry. Results: Compared to normal oral mucosa (NOM), MMP-1 expression was attenuated in OSF while TGF-β1 expression was upregulated (*P*<0.05). TIMP-1 expression of OSF was slightly elevated or similar to NOM. Proliferating activity was correspondingly increased in dysplastic OSF and was found to be statistically significant (*P*<0.05). No significant relationship was found between epithelial dysplasia and expressions of TGF-β1, MMP-1, and TIMP-1 in OSF. Furthermore, the relationship between epithelial dysplasia and the degree of fibrosis failed to show any positive correlation in OSF. Conclusions: This study provides further evidence that the fibrotic process in OSF is not only due to excessive collagen deposition but also due to disequilibrium in the ECM remodeling process. Neither the ECM remodeling process nor tissue fibrosis of OSF directly shows any significant effect on epithelial dysplasia.**

[*Oral Med Pathol* 2010; 15: 21-28 doi: 10.3353/omp.15.21]

Key words: **epithelial dysplasia, MMP-1, oral submucous fibrosis, TGF-β1, TIMP-1**

Correspondence: Jin Kim, Oral Cancer Research Institute, Department of Oral Pathology, Yonsei University College of Dentistry, 134, Sinchon-Dong, Seodaemun-Ku, Seoul 120-752, Republic of Korea
Phone: +82-2-2228-3031, Fax: +82-2-392-2959, E-mail: jink@yuhs.ac

Introduction

Oral submucous fibrosis (OSF), a chronic, insidious, fibrotic disease of the oral cavity, was first reported in the early 1950s (1). There is strong epidemiological evidence to suggest the association of OSF with the habit of chewing areca quid (AQ) (2-3), which consists mainly of areca nut, betel leaves, slaked lime, and tobacco. Importantly, OSF has been proven as a premalignant condition (4). Although epithelial changes are variable in OSF, most cases show generalized and marked atrophy of the oral epithelium with the loss of rete processes (5-6). As a histological hallmark of premalignant lesions, epithelial dysplasia is evident in OSF lesions (6). Furthermore, a recent study showed that malignancy develops in about 7% -13% of OSF lesions (7).

The carcinogenesis of OSF into oral squamous cell carcinoma (OSCC) is still unclear, although a variety of approaches have been applied to analyze its carcinogenic process. Several hypotheses of pathogenesis have been postulated. Jeng *et al*. proposed that the various inflammatory cytokines released from oral keratinocytes induced by areca nut components lead to the pathogenesis of OSCC in OSF (8-9). Others have proposed that genotoxicity and cytotoxicity caused by the ingredients of AQ have an effect, directly or indirectly, on oral keratinocytes (10-12). Another study suggested that areca nut extract or components of AQ cause oral mucosal fibroblasts to activate matrix metalloproteinases (MMP)-2 or AKT signaling and then modulate cancerous transformation of oral epithelial cells (13). More recently, hypoxia has generated considerable interest for its potential role in the malignant transformation of OSF (14).

Fibrosis may occur as the healing process of inflammatory reaction and be modulated by various cytokines (15). TGF- β has been acknowledged to be one of the main growth factors implicated in the development of fibrotic lesions (16). Among TGF-β isoforms, TGF-β1 seems to be

the cytokine that plays a major role in wound repair and fibrosis (17). MMPs are a tightly regulated family of enzymes that degrade extracellular matrix (ECM) (18). MMPs activity is regulated by a group of specific tissue inhibitors of metalloproteinase (TIMP), of which TIMP-1 has been well identified (19). Thus, the final balance between the activity of MMPs and TIMPs is considered to be a key determinant of the degradation of the ECM. Accordingly, the imbalance between the activities of MMPs and TIMPs are associated with pathological conditions, namely proliferative scaring, keloid, submucosal fibrosis, gingival overgrowth, and plantar fibromatosis (20-24).

Unlike other fibrotic diseases, OSF is characterized to be a potentially malignant disease. Therefore, the present study aims to elucidate both the ECM remodeling process and fibrosis in OSF, as well as their relationships with epithelial dysplasia. For this study, we compared the expressions of TGF-β1, TIMP-1, MMP-1 in OSF and normal oral mucosa (NOM) by immunohistochemistry. In addition, we compared the proliferative activities of both OSF and NOM epithelium by proliferating cell nuclear antigen (PCNA) index. Finally, we evaluated the relationship of these expression patterns to epithelial dysplasia and tissue fibrosis.

Materials and methods

Patient population

Specimens from 50 adult patients were used for this study. Forty-two archival specimens in the form of paraffin blocks which had been histologically diagnosed as OSF were selected from the Department of Oral Pathology of Faculty of Dental Sciences, University of Peradeniya, Sri Lanka, together with eight NOM specimens from patients who have neither pathological mucosal lesions nor habits of smoking and/or betel chewing. NOM specimens were obtained from patients undergoing third molar disimpaction surgeries at the Dental Hospital, University of Peradeniya, Sri Lanka. Nineteen of the 42 OSF specimens were from patients diagnosed as having some degree of epithelial dysplasia according to the archival records. The clinical diagnosis of OSF was based on clinical features such as burning sensation of the mouth, presence of palpable vertical fibrous bands along the buccal mucosa, limited mouth opening and a given history of betel and areca nut consumption. The clinically diagnosed patients with OSF for this study were 36 men (86%) and 6 women (14%), ranging in ages from 21 to 65 years, with an average age of 40 years. Ethical approval and informed consent from patients were obtained.

Tissue specimens and histopathological diagnosis

All biopsy specimens were formalin-fixed and paraffin-embedded according to the standard histological techniques. Four μm sections submitted for routine hematoxylin and eosin (HE) staining were used for reevaluation of histopathological diagnosis. Two oral pathologists (WMT, JK) independently graded HE sections of OSF for epithelial dysplasia and diagnosed the stage of the disease based on the extent of fibrosis. Grading of epithelial dysplasia, ranging from mild epithelial dysplasia to severe epithelial dysplasia, was assigned according to the WHO criteria (25). Based on the extent of fibrosis, the disease was graded into one of three stages (early, intermediate, and advanced) according to the criteria described by Utsunomia *et al*. (26). There was complete congruence between the examiners regarding the final diagnosis of the specimens.

Immunohistochemistry

Formalin-fixed, paraffin-embedded serial tissue sections were cut to a thickness of 4 μm and mounted on silane coated slides (Muto Pure Chemicals Co Ltd, Japan). Sections were de-waxed and re-hydrated using xylene and varying concentrations of ethanol. Antigen retrieval for PCNA detection was achieved by autoclaving the sections for 20 min in 0.01 M citric acid buffer (pH 6) after which sections were allowed to cool; the sections were then placed into phosphate-buffered saline (PBS). Antigen retrieval for detection of TGF-β1, TIMP-1, and MMP-1 was achieved by using 0.4% trypsin solution (0.04 g trypsin in 10ml of distilled water for 20 minutes at 37°C). Endogenous peroxidase was blocked in the sections by incubating them for 10 min with freshly prepared 3% hydrogen peroxide solution. Before applying primary antibodies, sections were blocked with non-immunized goat serum (1:5 dilution in PBS) for 60 min at room temperature. Sections were then subjected to the streptavidin-biotin complex method. Immunostaining was achieved using four primary antibodies, namely mouse monoclonal PCNA antibody (Oncogene Research Products, San Diego, CA, USA; 1:100 dilution in PBS), rabbit polyclonal TGF-β1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:50 dilution in PBS), mouse monoclonal TIMP-1 antibody (Neomarkers Inc., Fremont, CA, USA; 1:50 dilution in PBS), and mouse monoclonal MMP-1 antibody (Oncogene; 1:100 dilution in PBS). The sections were visualized with 3, 3-diaminobenzidine tetrachloride (DAB, Vector Laboratories, Burlingame, CA, USA), counterstained with Mayer's hematoxylin, mounted, and then examined with an Olympus BH-2 light microscope (Olympus Corp., Tokyo, Japan). Positive controls were OSCC for PCNA, placental tissue for TGF-β1 and MMP-1, and breast cancer tissue for TIMP-1. In order to demonstrate the specificity of the staining, negative controls were included in which the primary antibody was replaced with PBS.

Analysis of the staining

For each slide, 5 non-overlapping fields were randomly selected and photographed using a light microscope with an Olympus BX51T digital camera at magnification × 200. Sections were considered either negative or positive according to the absence or presence of brown staining in epithelial or stromal cells. The expression of TGF-β1, MMP-1, and TIMP-1 was evaluated in a semi-quantitative manner on 38 out of 42 slides. Due to unsatisfactory specimen status, 4 cases were not considered for evaluation of TGF-β1, MMP-1, and TIMP-1. The positive cases were

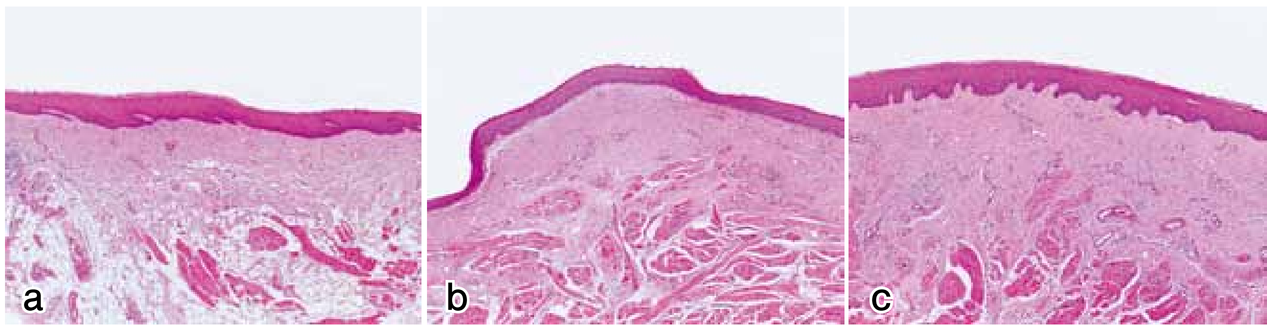

Fig. 1. Histopathological appearance of oral submucous fibrosis (OSF) according to the stage of fibrosis. (**a**) Early stage of OSF; (**b**) intermediate stage; (**c**) advanced stage. Hematoxylin and eosin (HE) stain, × 40. In the early stage, fibrosis was confined to the upper portion of the submucosa (a). In the intermediate stage, there was subepithelial hyalinization with fibrosis extending to deeper tissues (b).The advanced stage was demarcated with extensive full-thickness fibrosis of the submucosal tissue up to muscle layers together with hyaline changes (c).

graded into three easily reproducible subcategories depending on the frequency of positively stained cells: (a) positive expressions in less than 30% of cells, (b) positive expressions in 30-70% of cells, (c) positive expressions in more than 70% of cells. To evaluate proliferative index/ PCNA index similarly for each slide, 5 non-overlapping fields were randomly selected, the total number of epithelial cells were photographed, and the number of nuclei with positive PCNA expression were counted using Image-pro plus version 3.0 software (Media Cybernetics Inc., Bethesda, MD, USA). The proliferative index was calculated per specimen using the following formula:

$$\text{Proliferative Index (\%)} = \frac{\text{total number of positive cells}}{\text{total number of epithelial cells}} \times 100$$

Statistical Analysis

Results were computed and statistically analyzed using SPSS 13.0 version. Expressions of TGF-β1, MMP-1, and TIMP-1 in both OSF and NOM were statistically analyzed using the Chi-squared test. The mean values of PCNA index among different groups were statistically analyzed using the student's t- test. The relationship between the degree of fibrosis and the presence of epithelial dysplasia in OSF tissues, as well as the correlation among epithelial dysplasia, degree of fibrosis, prevalence of TGF-β1, MMP-1, and TIMP-1 expression, were also statistically analyzed using the Chi-squared test. P values < 0.05 were taken as significant.

Results

Histological features

Most of the OSF cases showed an atrophic epithelium while 5 cases showed epithelial hyperplasia. Of the 42 OSF cases, 19 (45.2%) showed epithelial dysplasia, whereas the other remaining 23 cases (54.8%) showed no dysplastic features. Of the 19 dysplastic cases, 6, 10 and 3 cases showed focal mild, mild and moderate epithelial dysplasia, respectively. With reference to the criteria described by Utsunomiya *et al*. (26), the stage of the disease was determined. The 42 cases were classified as follows: early stage, 8 cases (19%); intermediate stage, 17 cases (40.5%); and advanced stage, 17 cases (40.5%) (Fig. 1; Table 1).

Relationship between epithelial dysplasia and degree of fibrosis

The relationship between the degree of fibrosis and epithelial dysplasia was analyzed (Table 1). The different stages of fibrosis (early, intermediate and advanced) were compared with the three grades of dysplasia (focal mild, mild, and moderate). However, there was no statistically significant correlation among the groups based on the Chi-squared test (P>0.05).

Proliferating activity

PCNA positivity was determined by nuclear staining of epithelial cells. NOM exhibited proliferating cells in the basal and parabasal layers. The OSF showed positive PCNA reaction in the nuclei of basal, parabasal, and spinous cells of

Table 1. Relationship between epithelial dysplasia and fibrosis in 42 cases of oral submucous fibrosis

dysplasia	fibrosis			
	early	intermediate	advanced	total
without dysplasia	2	9	12	23 (54.8%)
with dysplasia	6	8	5	19 (45.2%)
focal mild	1	2	3	6
mild	3	6	1	10
moderate	2	0	1	3
total	8 (19%)	17 (40.5%)	17 (40.5%)	42 (100%)

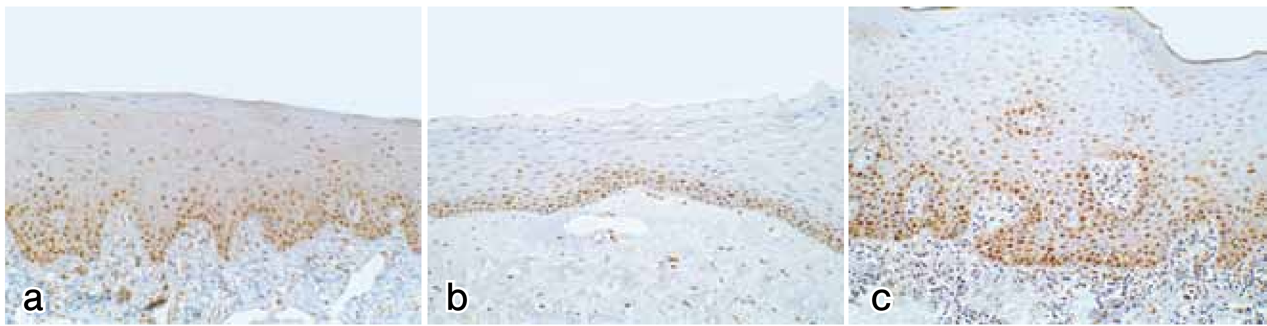

Fig. 2. Immunohistochemical staining for PCNA in normal oral mucosa (NOM) and OSF epithelium. (**a**) NOM epithelium; (**b**) OSF atrophic epithelium, (**c**) OSF dysplastic epithelium. Immunoperoxidase stain, hematoxylin counterstain, × 200. In NOM epithelium, PCNA-positive (+) cells were mainly located in the basal and parabasal layers and were few in the spinous layer (a). In atrophic epithelium of OSF, PCNA+ cells were in the basal and parabasal layers (b), while they were more spread over the basal to spinous layers in dysplastic epithelium (c).

the epithelium, while the dysplastic tissues showed positive cells in nearly all layers of the epithelium. Compared to NOM, the prevalence and intensity of PCNA staining was found to be increased in OSF (Fig. 2). This finding was especially noticed in the OSF tissues with dysplastic features. OSF had a PCNA index of 12.8 ± 3.1 compared to that of 6.0 ± 0.9 in NOM, which was statistically significant ($P<0.05$). The OSF with dysplasia had a PCNA index of 14.4 ± 2.2 compared to that of 10.3 ± 2.7 in the OSF without dysplasia. These results were also statistically significant ($P<0.05$) (Table 2).

Expression of TGF-β1, MMP-1, and TIMP-1

Prevalence of TGF-β1 expression was observed in the submucosal portion of OSF and NOM (Figs. 3a-3b). OSF showed over 30 % of TGF-β1 expression in 71 % of OSF cases, while only 33% of NOM showed over 30% expression (Table 3). This result was statistically significant ($P<0.05$). In the epithelial portion of both OSF and NOM, TGF-β1 expression was distributed throughout, except in the keratinized layer.

MMP-1 expression in OSF was attenuated compared to NOM. Only 21.1% of OSF cases showed over 70% MMP-1 expression compared to the 77.8% of NOM cases. There was a detectable difference in the prevalence and staining intensity of positive fibroblasts between NOM and OSF (Figs. 3c-3d). This difference of MMP-1 expression pattern between OSF and NOM was found to be statistically significant ($P<0.05$) (Table 3).

TIMP-1 expression between OSF and NOM showed no significant statistical difference in the connective tissue portion ($P>0.05$). However, staining intensity was observed

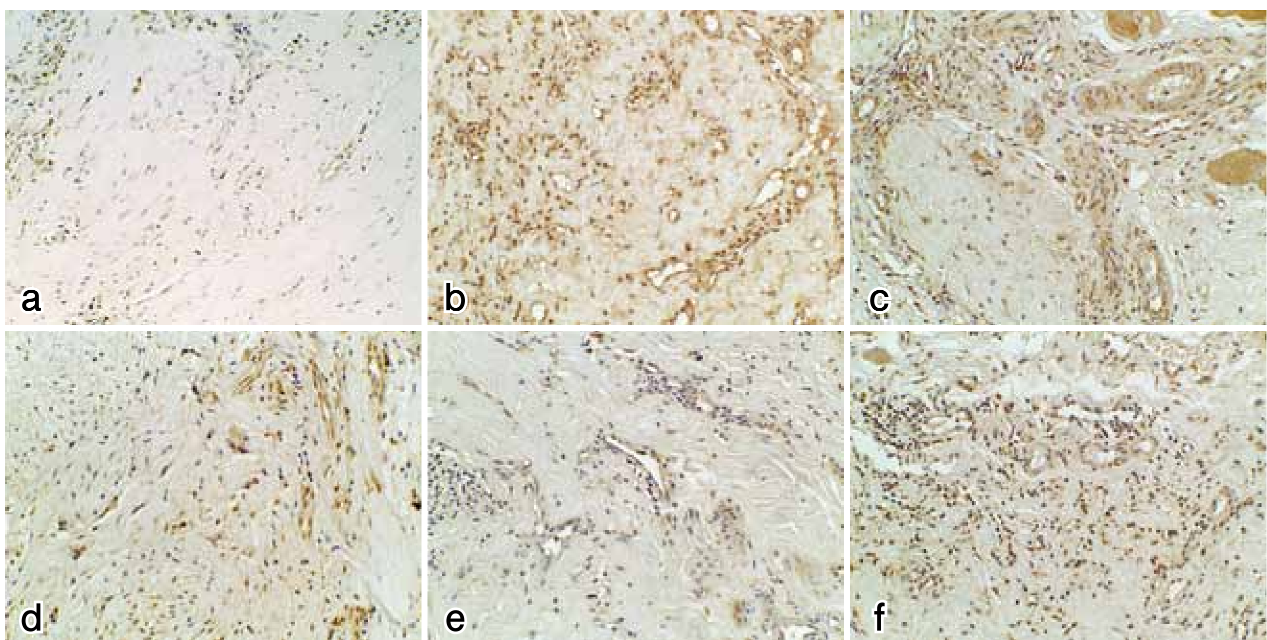

Fig. 3. Immunohistochemical staining for TGF-β1, MMP-1, and TIMP-1 in normal oral mucosa and OSF epithelium. **(a, c, e)** NOM; **(b, d, f)** OSF. Immunoperoxidase stain for TGF-β1 **(a, b)**, MMP-1 **(c, d)**, and TIMP-1 **(e, f)**, hematoxylin counterstain, × 200. In NOM, TGF-β1 was focally positive in fibroblasts in the submucosal layer (a), while it was markedly positive in fibroblasts in OSF (b). MMP-1 was diffusely positive among fibroblasts of the NOM submucosal layer (c). Although OSF fibroblasts showed positive stainings for MMP-1, their distribution was not as diffuse as that of NOM fibroblasts (d). TIMP-1 staining of the NOM submucosa showed focal positive staining in the fibroblasts (e), while in OSF submucosa TIMP-1 staining showed diffuse positive staining in the fibroblasts (f).

Table 2. PCNA index of the epithelium in normal oral mucosa (NOM) and oral submucous fibrosis (OSF)

	NOM	OSF	
		without dysplasia	*with dysplasia*
PCNA Index (%)		10.3 ± 2.7*	14.4 ± 2.2*
average	6.0 ± 0.9	12.8 ± 3.1*	

* Statistically significant compared with *P*-value of the NOM by student t-test ($P<0.05$)

Table 3. TGF β-1, MMP-1, and TIMP-1 expression in the submucosa of NOM & OSF

prevalence (%)	NOM			OSF		
	<30%	30 - 70%	>70%	<30%	30 - 70%	>70%
cytokine						
TGFβ-1*	66.7	22.2	11.1	29.0	57.9	13.1
MMP-1*	11.1	11.1	77.8	18.4	60.5	21.1
TIMP-1	55.6	33.3	11.1	26.3	65.8	7.9

* Difference in the prevalence between OSF and NOM is statistically significant by Chi- squared test ($P<0.05$)

Table 4. Relationship between epithelial dysplasia and expression of TGF β1, MMP-1 and TIMP-1 in OSF

prevalence (%)	TGFβ-1			MMP-1			TIMP-1		
	<30%	30 - 70%	>70%	<30%	30 - 70%	>70%	<30%	30 - 70%	>70%
dysplasia									
without dysplasia (20)	4 (20.0)	13 (65.0)	3 (15.0)	5 (25.0)	13 (65.0)	2 (10.0)	7 (35.0)	11 (55.0)	2 (10.0)
with dysplasia (18)	7 (38.0)	9 (50.0)	2 (11.1)	2 (11.1)	10 (55.6)	6 (33.3)	3 (16.7)	14 (77.8)	1 (5.5)
total (38)	11 (29.0)	22 (57.9)	5 (13.1)	7 (18.4)	23 (60.5)	8 (21.1)	10 (26.3)	25 (65.8)	3 (7.9)

Table 5. Relationship between degree of fibrosis and expression of TGF β1, MMP-1 and TIMP-1 in OSF

prevalence (%)	TGFβ-1			MMP-1			TIMP-1		
	<30%	30 - 70%	>70%	<30%	30 - 70%	>70%	<30%	30 - 70%	>70%
fibrosis									
early (7)	2 (28.6)	5 (71.4)	0 (0.0)	0 (0.0)	5 (71.4)	2 (28.6)	3 (42.9)	4 (57.1)	0 (0.0)
intermediate (14)	4 (28.5)	8 (57.0)	2 (14.2)	4 (28.6)	7 (50.0)	3 (21.4)	3 (21.4)	9 (64.3)	2 (14.3)
advanced (17)	5 (29.4)	9 (53.0)	3 (17.6)	3 (17.7)	11 (64.7)	3 (17.7)	4 (23.5)	12 (70.6)	1 (5.9)
total (38)	11 (29.0)	22 (57.9)	5 (13.1)	7 (18.4)	23 (60.5)	8 (21.1)	10 (26.3)	25 (65.8)	3 (7.9)

to be stronger in the fibroblasts of OSF than in those of NOM (Figs. 3e-3f) (Table 3).

Relationship between epithelial dysplasia or degree of fibrosis and expression of TGF-β1, MMP-1, and TIMP-1 in OSF

Statistical analysis revealed that there is no significant correlation between epithelial dysplasia and expression of TGF-β1, MMP-1, and TIMP-1, respectively (Table 4). P values were found to be beyond the significant level ($P>0.05$).

When we compared the expression of TGF-β1, MMP-1, and TIMP-1 with the degree of fibrosis, TGF-β1 expression appeared to be increased along the advanced stage of fibrosis, evidenced by the increased incidence of cases showing more than 70% expression in intermediate and advanced stages (Table 5).

Likewise, TIMP-1 expression was also increased following the severity of fibrosis. Correspondingly, the cases showing more than 70% of MMP-1 expression seemed to be reduced following the severity of fibrosis. However, the relationship between the degree of tissue fibrosis and expression of TGF-β1, MMP-1, and TIMP-1 revealed no statistical significance by Chi-squared test. This result could be due to the small sample size.

Discussion

The pathogenesis of OSF and its malignant transformation has been a subject of debate for decades without definite scientific conclusion. Multiple aspects of tumorigenesis may have contributed to this confusion. The role of cytokines secreted from fibroblasts has been suggested by some investigators (7, 13), whereas direct or indirect damage to oral keratinocytes causing genetic changes has also been discussed by others (10-12).

Considering the histological characteristics of OSF showing fibrosis and epithelial atrophy, we hypothesized that fibroblasts or their products may have an active role in epithelial transformation in OSF. The main question addressed by the present study was whether the epithelial dysplasia in OSF may be related to the factors provoking fibrosis. In this preliminary study, we attempted to evaluate whether tissue fibrosis and/or the expression of TGF-β1, MMP-1, and TIMP-1 may be related to epithelial dysplasia.

TGF-β is a known potent mediator which stimulates collagen and other ECM production (27). As expected in this study, we found significantly increased TGF-β1 expression in the connective tissue portion of OSF compared to NOM, resulting in increased tissue fibrosis in OSF. The results of this study corroborate the findings of several previous studies that found the levels of TGF-β in OSF to be higher than in NOM (28-29).

Excessive collagen deposition due to improper regulation of the proteolytic equilibrium is a common basis for pathological fibrosis. There have been several studies which clearly showed the involvement of MMPs and TIMPs in wound healing-related diseases where the remodeling mechanisms are disrupted (30-31). In cirrhotic liver tissue, decreased MMP-1 synthesis and increased TIMP-1 and TIMP-2 functions promoted fibrosis (32). Regarding OSF, Chang *et al*. cultured buccal mucosal fibroblasts which were arecoline-treated and found the decrease of MMP-2 synthesis and its activities as well as increased TIMP-1 synthesis (33). Rajendran *et al*. described the increased expression of TIMP-1, TIMP-2, and MMPs in OSF by an immunohistochemical study. However, in the same study they found reduced MMP activity in OSF tissue by gelatin zymography (34).

Because MMP-1 is the main human enzyme that degrades fibrillar collagen and is also related to the remodeling process in wound healing (18), the present study examined MMP-1 expression in OSF. As expected, MMP-1 levels in the OSF connective tissue were attenuated compared to NOM. This shows that collagen degradation caused by MMP-1 is down regulated in the OSF, causing accumulation of ECM in the connective tissue. On the other hand, no statistically significant difference in the fraction of TIMP-1 positive cells was found between OSF and NOM, even though TIMP-1 showed stronger intensity in fibroblasts of OSF than in NOM. Taken together, the equilibrium between MMP-1 and TIMP-1 tends to be disturbed in OSF. Therefore, our results help to elucidate the fact that excessive fibrosis in OSF is not only due to excessive deposition but also due to decreased collagen degradation. This concept is collectively confirmed by previous studies (33-35).

Our study further showed that neither the degree of fibrosis nor the grade of epithelial dysplasia is dependent on each other when evaluating their relationship. This finding corroborates with some previous studies, as well (14). Interestingly, we also found that the tissue expressions of TGF-β1, MMP-1, and TIMP-1 have no correlation with the tissue expression of epithelial dysplasia in OSF. Based on these results, it appears that the deregulation of the collagen remodeling process itself may not result in epithelial dysplasia.

PCNA is a useful immunohistochemical marker of cell proliferation because its expression and distribution correlates with cellular proliferation rates and DNA synthesis (36-37). The increase of PCNA expressions has been observed as gradual progression from normal epithelium to hyperplasia, dysplasia, and head and neck squamous cell carcinoma (38-39). In this study, proliferative activity (PCNA index) of the OSF epithelium was found to be higher than that of NOM. When comparing the proliferating activities of the dysplastic OSF with the non-dysplastic group, the PCNA index of the dysplastic group showed a significant increase, as expected. Our results are supported by previous studies which have shown that the PCNA index of the epithelium is significantly increased in OSF compared to NOM (39-41). Considering the PCNA index of this study together with already published data (39-43), the increased PCNA index can correlate with the increased malignant transformation potential.

Taken together, the results of the present study indicate that the increased expression of TGF-β1, followed by fibroblast proliferation and collagen synthesis, are contributing factors that cause fibrosis in OSF. Also, the imbalance between TIMP-1 and MMP-1 enhances the ECM accumulation which further affects the tissue architecture irreversibly in OSF. Its malignant transformation potential is also evidenced by an increased PCNA index in OSF and dysplastic OSF. However, there is no correlation between deregulation of the collagen remodeling process and epithelial dysplasia, suggesting that the deregulated collagen remodeling may not directly affect the cancerous transformation of OSF. Further study about whether any fibrosis-inducing factors may cause epithelial dysplasia will provide insight in elucidating the mechanisms of cancer transformation in OSF.

Acknowledgment

This study was supported partly by the Priority Research Centers Program through the National Research Foundation of Korea (NRF) (2009-0094028) and partly by the Basic Science Research Program through NRF (R13-2003-013-04001-0) funded by the Ministry of Education, Science and Technology, Korea. The authors express sincere gratitude to Prof. Linda Kilpatrick-Lee, Dept. of English Language and Literature at Yonsei University, for critically reading the manuscript.

References

1. Joshi SG. Submucous fibrosis of palate and pillars. *Ind J Otolaryngol* 1953; **4**: 1-4.
2. Sinor PN, Gupta PC, Murti PR, *et al*. A case-control study of oral submucous fibrosis with special reference to the etiologic role of areca nut. *J Oral Pathol Med* 1990; **19**: 94-8.
3. Maher R, Lee AJ, Warnakulasuriya KA, Lewis JA, Johnson

NW. Role of areca nut in the causation of oral submucous fibrosis: a case-control study in Pakistan. *J Oral Pathol Med* 1994; **23**: 65-9.

4. Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Pindborg JJ, Mehta FS. Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing. *J Oral Pathol Med* 1995; **24**: 145-52.
5. Pindborg JJ, Sirsat SM. Oral submucous fibrosis. *Oral Surg Oral Med Oral Pathol* 1966; **22**: 764-79.
6. Pindborg JJ, Mehta FS, Daftary DK. Occurrence of epithelial atypia in 51 Indian villagers with oral submucous fibrosis. *Br J Cancer* 1970; **24**: 253-7.
7. Tilakaratne WM, Klinikowski MF, Saku T, Peters TJ, Warnakulasuriya S. Oral submucous fibrosis: review on aetiology and pathogenesis. *Oral Oncol* 2006; **42**: 561-8.
8. Jeng JH, Ho YS, Chan CP, *et al*. Areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes. *Carcinogenesis* 2000; **21**: 1365-70.
9. Jeng JH, Wang YJ, Chiang BL, *et al*. Roles of keratinocyte inflammation in oral cancer: regulating the prostaglandin E2, interleukin-6 and TNF-alpha production of oral epithelial cells by areca nut extract and arecoline. *Carcinogenesis* 2003; **24**: 1301-15.
10. Jeng JH, Hahn LJ, Lin BR, Hsieh CC, Chan CP, Chang MC. Effects of areca nut, inflorescence piper betle extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. *J Oral Pathol Med* 1999; **28**: 64-71.
11. Chang MC, Ho YS, Lee JJ, Kok SH, Hahn LJ, Jeng JH. Prevention of the areca nut extract-induced unscheduled DNA synthesis of gingival keratinocytes by vitamin C and thiol compounds. *Oral Oncol* 2002; **38**: 258-65.
12. Tsai YS, Lee KW, Huang JL, *et al*. Arecoline, a major alkaloid of areca nut, inhibits p53, represses DNA repair, and triggers DNA damage response in human epithelial cells. *Toxicology* 2008; **249**: 230-7.
13. Lu HH, Liu CJ, Liu TY, Kao SY, Lin SC, Chang KW. Areca-treated fibroblasts enhance tumorigenesis of oral epithelial cells. *J Dent Res* 2008; **87**: 1069-74.
14. Tilakaratne WM, Iqbal Z, Teh MT, *et al*. Upregulation of HIF-1alpha in malignant transformation of oral submucous fibrosis. *J Oral Pathol Med* 2008; **37**: 372-7.
15. Kovacs EJ. Fibrogenic cytokines: the role of immune mediators in the development of scar tissue. *Immunol Today* 1991; **12**: 17-23.
16. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994; **331**: 1286-92.
17. Sporn MB, Roberts AB. TGF-beta: problems and prospects. *Cell Regul* 1990; **1**: 875-82.
18. Pilcher BK, Sudbeck BD, Dumin JA, Welgus HG, Parks WC. Collagenase-1 and collagen in epidermal repair. *Arch Dermatol Res* 1998; **290 Suppl**: S37-46.
19. Woessner JF, Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991; **5**: 2145-54.
20. Zamora RL, Heights R, Kraemer BA, Erlich HP, Groner JP. Presence of growth factors in palmar and plantar fibromatoses. *J Hand Surg Am* 1994; **19**: 435-41.
21. Shaffer JJ, Taylor SC, Cook-Bolden F. Keloidal scars: a review with a critical look at therapeutic options. *J Am Acad Dermatol* 2002; **46**: S63-97.
22. Robson MC. Proliferative scarring. *Surg Clin North Am* 2003; **83**: 557-69.
23. Falanga V. The chronic wound: impaired healing and solutions in the context of wound bed preparation. *Blood Cells Mol Dis* 2004; **32**: 88-94.
24. Nahm DS, Kim HJ, Mah J, Baek SH. In vitro expression of matrix metalloproteinase-1, tissue inhibitor of metalloproteinase-1 and transforming growth factor-beta1 in human periodontal ligament fibroblasts. *Eur J Orthod* 2004; **26**: 129-35.
25. Warnakulasuriya S, Reibel J, Bouquot J, Dabelsteen E. Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. *J Oral Pathol Med* 2008; **37**: 127-33.
26. Utsunomiya H, Tilakaratne WM, Oshiro K, *et al*. Extracellular matrix remodeling in oral submucous fibrosis: its stage-specific modes revealed by immunohistochemistry and in situ hybridization. *J Oral Pathol Med* 2005; **34**: 498-507.
27. Chiu CJ, Chang ML, Chiang CP, Hahn LJ, Hsieh LL, Chen CJ. Interaction of collagen-related genes and susceptibility to betel quid-induced oral submucous fibrosis. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 646-53.
28. Haque MF, Harris M, Meghji S, Barrett AW. Immunolocalization of cytokines and growth factors in oral submucous fibrosis. *Cytokine* 1998; **10**: 713-9.
29. Rajalalitha P, Vali S. Molecular pathogenesis of oral submucous fibrosis--a collagen metabolic disorder. *J Oral Pathol Med* 2005; **34**: 321-8.
30. Murphy G, Docherty AJ. The matrix metalloproteinases and their inhibitors. *Am J Respir Cell Mol Biol* 1992; **7**: 120-5.
31. Birkedal-Hansen H, Moore WG, Bodden MK, *et al*. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 1993; **4**: 197-250.
32. Arthur MJ. Fibrosis and altered matrix degradation. *Digestion* 1998; **59**: 376-80.
33. Chang YC, Yang SF, Tai KW, Chou MY, Hsieh YS. Increased tissue inhibitor of metalloproteinase-1 expression and inhibition of gelatinase A activity in buccal mucosal fibroblasts by arecoline as possible mechanisms for oral submucous fibrosis. *Oral Oncol* 2002; **38**: 195-200.
34. Rajendran R, Rajeesh MP, Shaikh S, Shanthi, Pillai MR. Expression of matrix metalloproteinases (MMP-1, MMP-2 and MMP-9) and their inhibitors (TIMP-1 and TIMP-2) in oral submucous fibrosis. *Indian J Dent Res* 2006; **17**: 161-6.
35. Schiller M, Javelaud D, Mauviel A. TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci* 2004; **35**: 83-92.
36. Jaskulski D, deRiel JK, Mercer WE, Calabretta B, Baserga R. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. *Science* 1988; **240**: 1544-6.
37. Zuber M, Yasui W, Tan EM, Ryoji M. Quantitation and subcellular localization of proliferating cell nuclear antigen (PCNA/cyclin) in oocytes and eggs of Xenopus laevis. *Exp*

Cell Res 1989; **182**: 384-93.

38. Shin DM, Voravud N, Ro JY, Lee JS, Hong WK, Hittelman WN. Sequential increases in proliferating cell nuclear antigen expression in head and neck tumorigenesis: a potential biomarker. *J Natl Cancer Inst* 1993; **85**: 971-8.
39. Chiang CP, Lang MJ, Liu BY, *et al*. Expression of proliferating cell nuclear antigen (PCNA) in oral submucous fibrosis, oral epithelial hyperkeratosis and oral epithelial dysplasia in Taiwan. *Oral Oncol* 2000; **36**: 353-9.
40. Cox SC, Walker DM. Epithelial growth fraction and expression of p53 tumour suppressor gene in oral submucous fibrosis. *Aust Dent J* 1996; **41**: 91-6.
41. Srinivasan M, Jewell SD. Quantitative estimation of PCNA, c-myc, EGFR and TGF-alpha in oral submucous fibrosis--an immunohistochemical study. *Oral Oncol* 2001; **37**: 461-7.
42. Tsuji T, Shrestha P, Yamada K, *et al*. Proliferating cell nuclear antigen in malignant and pre-malignant lesions of epithelial origin in the oral cavity and the skin: an immunohistochemical study. *Virchows Arch A Pathol Anat Histopathol* 1992; **420**: 377-83.
43. Kobayashi I, Matsuo K, Ozeki S, Ohishi M, Ishibashi Y, Sakai H. The proliferative activity in oral epithelial dysplasia analyzed by proliferating cell nuclear antigen immunostaining and argyrophilic nucleolar organizer region staining. *Hum Pathol* 1995; **26**: 907-13.

Received April 22, 2009   Accepted July 1, 2010