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Expression of TNF-α in oral lichen planus treated with fluocinolone acetonide 0.1%

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BACKGROUND: Oral lichen planus (OLP) is a common chronic inflammatory disease involving T cells-mediated immunity. Tumor necrosis factor (TNF)-α has been reported to be involved in the disease process. The purpose of this study was to investigate the effect of fluocinolone acetonide in orabase (FAO) 0.1% on the expression of TNF-α in patients with OLP.

METHODS: Eighteen Thai patients with atrophic or erosive OLP were recruited. Biopsy specimens were taken before and 1 month after treatment with FAO 0.1% and sent for histopathologic examination where they were immunohistochemically stained with antibody to TNF-α. Twenty normal mucosa specimens were identically processed. Oral squamous cell carcinoma tissue was used as a positive control for TNF-α expression, whereas OLP sections without primary antibody were served as negative control.

RESULTS: Sixteen of 18 cases (88.89%) of OLP exhibited positive staining for TNF-α. Most of the TNF-α was observed in the mononuclear cells. Ten cases (55.56%) of OLP demonstrated TNF-α expression in keratinocytes. The number of mononuclear cells positive for TNF-α before the treatment with FAO 0.1% in orabase was statistically higher than that after the treatment ($P = 0.000$) and in the normal mucosa ($P = 0.000$). Moreover, the number of mononuclear cells after treatment with FAO 0.1% in orabase was statistically lower than before the treatment ($P = 0.000$).

CONCLUSION: Our study exhibited that TNF-α may be associated with the immunopathogenesis of OLP in Thai patients and FAO 0.1% had an effect on the reduction of TNF-α expression.

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease of the skin and oral mucosa in which the cell-mediated cytotoxicity is regarded as a major mechanism of pathogenesis, as evidenced by T cells being the predominant cells in the inflammatory cell infiltrate in OLP (1). Various factors may precipitate the cell-mediated reaction resulting in OLP lesions such as mechanical trauma, systemic drugs, contact sensitivity, infective agents including some viruses (2). Although the specific antigen of OLP is still unclear, the combination of antigen presentation by basal keratinocytes and antigen-specific keratinocyte killing by $CD8^+$ cytotoxic T cells are thought to cause T-cell accumulation in the superficial lamina propria, basement membrane disruption, intraepithelial T-cell migration, and keratinocyte apoptosis in OLP (3). It has been reported that tumor necrosis factor (TNF)-α is the cytokine which is primarily involved in T cell-mediated immunologic reactions and plays an important role in regulation of cytokines in OLP lesions by recruitment of lymphocytes from local microvasculatures into subepithelial inflammatory infiltrate and subsequently migrated to the oral epithelium (4, 5).

The TNF-α is a small cytokine with a molecular weight of only 17 kDa secreted from inflammatory cells such as activated monocytes, macrophages, and many other cells including B cells, T cells, mast cells, and fibroblasts during infection or trauma (6, 7). TNF-α is a multifunctional cytokine that mediates inflammation, immune response, apoptosis and also has a significant role in normal development and homeostasis of several organs (8). Because TNF-α has a remarkable variety of functions and its action has not been clarified, the relationship of this cytokine in the immunopathogenesis

of OLP is interesting. Further study of this cytokine may shed more light on its pathogenesis in autoimmune and inflammatory diseases and improve the treatment of various immunologic disorders.

Due to the chronic nature of OLP, various treatments have been tried but complete cure of the lesion is very difficult to achieve. Topical steroids are widely used in the treatment of OLP to reduce pain and inflammation (9). Recently, there was a report about the treatment of OLP with potent topical steroid – fluocinolone acetonide in orabase (FAO) 0.1% and the results of this study showed its effectiveness in long-term follow up (10). However, the mechanism of FAO in the inflammatory process of OLP lesions is still unknown. It might inhibit one or several cytokines secreted into the OLP lesions. TNF-α is one of the cytokines that might be involved in the disease process. To our knowledge, the effect of topical steroids on the expression of TNF-α in OLP lesions has not previously been reported. Thus, the objective of this study was to investigate the effect of FAO on the expression of TNF-α in Thai patients with OLP.

Materials and methods

Eighteen Thai patients with atrophic or erosive OLP and 20 of normal mucosal donors were recruited for this study. All of the patients as well as the normal oral mucosa donors signed the consent forms and this study was approved by the Faculty of Medicine, Chulalongkorn University and Khon Kaen University ethical committee. The patient group consisted of 14 women and four men with a mean age of 39.11 ± 12.3 years, ranging from 17 to 67 years. All of the patients had oral symptoms and every case was diagnosed by clinical appearance and confirmed by histopathologic examination. The duration of the disease varied from 1 to 84 months with the mean duration of 18.86 ± 22.5 months. There were six cases of atrophic and 12 cases of erosive types of OLP, but none exhibited skin lesion. None of the patients had systemic diseases and they did not take any medication either topical or systemic. Moreover, patients with suspected restoration-related reaction were excluded from this study. Biopsy specimens of OLP were obtained from buccal mucosa ($n = 15$), gingiva ($n = 2$), mucobuccal fold ($n = 1$). Normal mucosae were obtained from 20 patients undergoing impacted teeth removal. All tissue specimens were immediately fixed in 10% formalin before sending for further tissue processing. FAO 0.1% was prescribed to the patients to apply at affected areas three times a day for 4 weeks, and then re-biopsies at the same areas of the first visit were carried out.

Criteria for the assessment of the OLP lesion

The OLP lesions were assessed after the treatment according to the criteria set by Thongprasom et al. (10).

Complete remission (CR): no symptoms or very mild symptoms, lesions disappear or only mild white striae.

Partial remission (PR): symptoms reduced, mild white striae and erythematous area.

No response (NR): symptoms persisted with no improvement or worsening of the lesions.

Immunohistochemical study

Immunohistochemical staining method in this study was modified from Sklavounou et al. (11). In brief, 5 μm tissue sections were deparaffinized. Antigen retrieval was performed by heating the tissue sections immersed in 0.01 M sodium citrate buffer pH 6.0 in a waterbath at 95°C for 50 min. The tissue sections were then immersed in 2% hydrogen peroxide in methanol for 10 min to eliminate endogenous peroxidase. The incubation of the tissue sections with goat antihuman TNF-α antibody (polyclonal, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was carried out in a moist chamber at 4°C overnight. Slides were washed with phosphate-buffered saline (PBS) and subsequently incubated with biotinylated secondary antibody in a moist chamber at room temperature for 60 min. After rinsing with PBS, tissue sections were incubated with avidin-biotinylated horseradish peroxidase (HRP) in a moist chamber at room temperature for 3 min. Peroxidase substrates were added to the tissue sections for 10 min and the tissue sections were counterstained with hematoxylin. Tissues of squamous cell carcinoma were used as positive controls, while negative controls were carried out by substituting primary antibody with PBS. Criteria for selecting areas for photography on the slides were as follows.

1 Three areas were selected for photography in each slide: one area each on the far left and the far right, the other in the middle of the slides.
2 Photographs were taken by the 40× objective lens with a final original magnification of 200×.

The immunohistochemical staining reaction for TNF-α in tissue sections was evaluated by counting cells in the photographs both before and after treatment with FAO 0.1% by two observers. Each observer independently counted both the positively stained and negatively stained mononuclear cells. The number of positively stained cells was calculated by averaging figures from two observers. A matched pairs *t*-test was used to compare positively stained cells before and after treatment with FAO 0.1%. An independent sample *t*-test was used to compare positively stained cells between normal and OLP groups. A *P*-value of < 0.05 was considered statistically significant.

Results

In this study, there was 1 case with CR and 17 cases with PR after treatment with FAO 0.1% for 4 weeks. Sixteen of 18 cases (88.89%) showed positive staining for TNF-α before treatment and most of the TNF-α was observed in mononuclear cells. When compared clinically with before the treatment, TNF-α could not be detected after treatment with FAO 0.1% in one patient who demonstrated CR clinically (Fig. 1a,b). The expression of TNF-α in case of PR was illustrated in Fig. 2a,b. Ten of 18 cases (55.56%) with OLP expressed weak

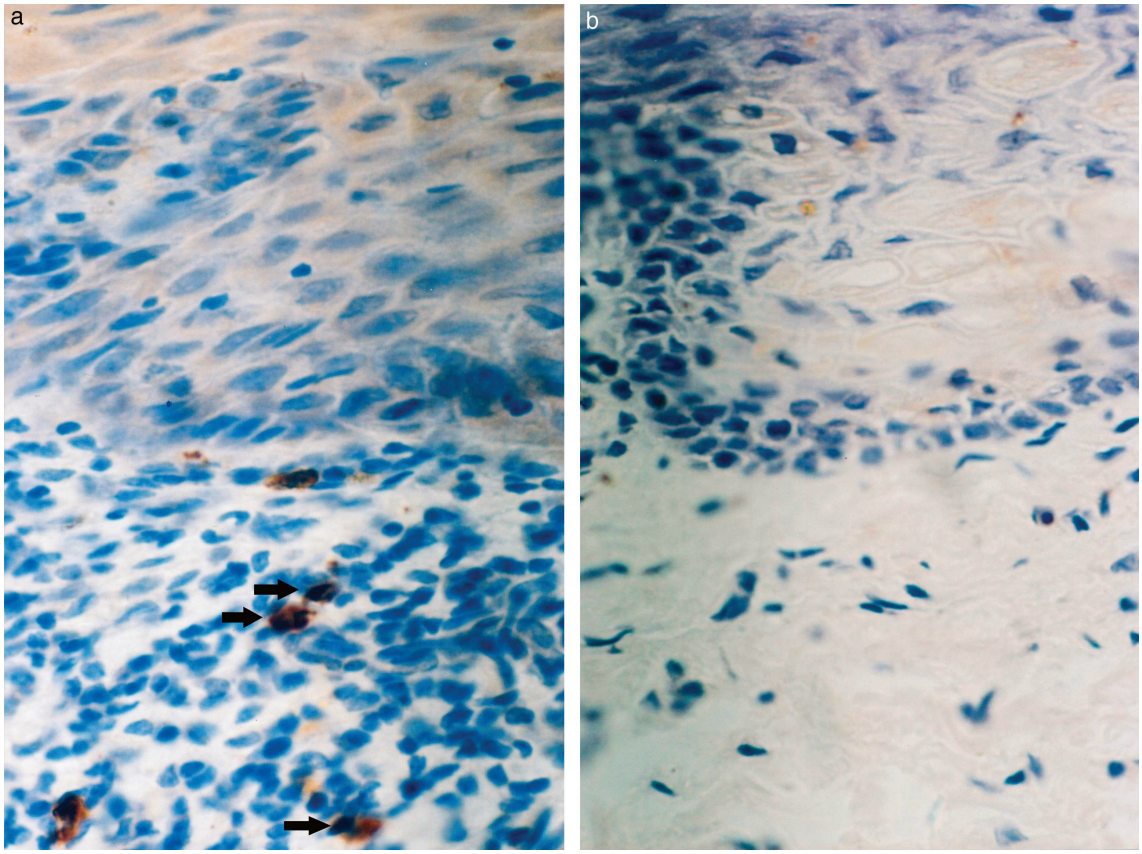

Figure 1 (a) The expression of tumor necrosis factor (TNF)-α by inflammatory mononuclear cells (arrows) in oral lichen planus (OLP) case 3 before treatment (original magnification 200×). (b) No expression of TNF-α with complete remission after treatment with fluocinolone acetonide in orabase (FAO) 0.1% for 1 month (original magnification 200×).

staining of TNF-α in keratinocytes. In normal mucosa, only two cases showed positive staining for TNF-α in mononuclear cells ranging from 0 to 2 cells.

The mean number of the TNF-α-positive mononuclear cells count in lamina propria before the treatment was 7.06 ± 5.01 cells, whereas that after the treatment with FAO 0.1% was 1.44 ± 1.76 cells. The mean number of the TNF-α-positive keratinocytes count before the treatment with FAO 0.1% was 1.11 ± 1.75 cells and after treatment was 0.72 ± 1.87 cells (Table 1). There was no statistical difference between the number of TNF-α-positive keratinocytes before and after the treatment with FAO 0.1% ($P = 0.218$). The amount of TNF-α in virtually all patients was reduced after 1-month treatment with FAO. The number of positive mononuclear cells for TNF-α before the treatment with FAO 0.1% in orabase was statistically higher than that after the treatment ($P = 0.000$; Fig. 3). Two normal mucosa specimens demonstrated TNF-α expression and there was a statistical difference between TNF-α expression in mononuclear cells of OLP lesion before the treatment and the normal mucosa ($P = 0.000$). Moreover, the number of mononuclear cells after the treatment with FAO 0.1% (93.56 ± 42.96) was significantly lower than before the treatment (188.06 ± 50.98) in all cases ($P = 0.000$; Fig. 4).

Table 1 Immunohistochemical staining for TNF-α in oral lichen planus and the normal mucosa

					Mononuclear (cells)[a]			*Keratinocyte (cells)*[a]	
Group	*Type**	*n*	*Clinical evaluation*	*Mononuclear, +ve case (%)*	*Before treatment*	*After treatment*	*Keratinocyte, +ve case (%)*	*Before treatment*	*After treatment*
OLP	E-LP	12	PR = 12	10 (55.56%)	6.5 ± 5.3 (0–16)	1.67 ± 1.97 (0–6)	6 (33.33%)	1.08 ± 1.78 (0–6)	0.33 ± 1.16 (0–4)
	A-LP	6	CR:PR = 1:5	6 (33.33%)	8.17 ± 44.62 (4–17)	1 ± 1.27 (0–3)	4 (22.22%)	1.17 ± 1.84 (0–4)	1.5 ± 2.81 (0–7)
Total		18	CR:PR = 1:17	16 (88.89%)	7.06 ± 5.01 (0–17)	1.44 ± 1.44 (0–6)	10 (55.56%)	1.11 ± 1.75 (0–6)	0.72 ± 1.87 (0–7)
Control	Normal mucosa	20	–	2 (10%)	0.05 ± 0.15 (0–2)	–	–	–	–

[a]Mean ± SD of positive cells (range).
E-LP, erosive lichen planus; A-LP, atrophic lichen planus; CR, complete remission; PR, partial remission.

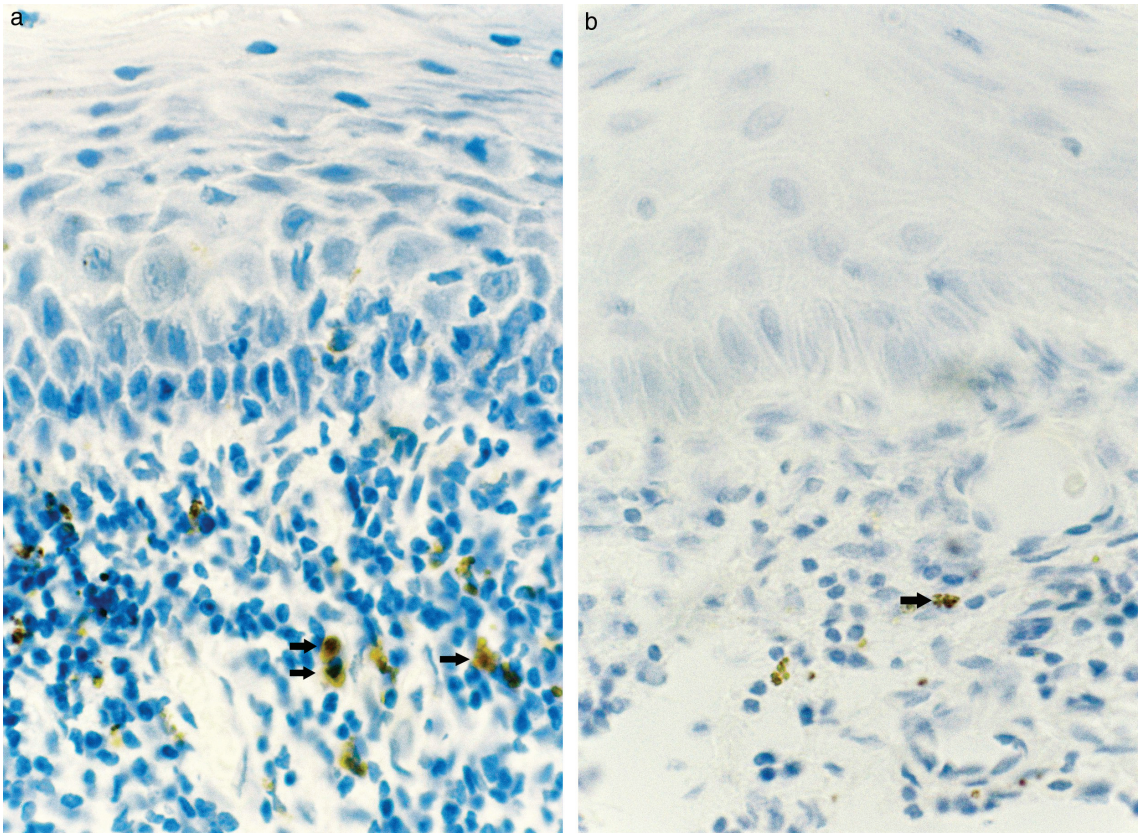

Figure 2 (a) The expression of tumor necrosis factor (TNF)-α by inflammatory mononuclear cells (arrows) in oral lichen planus (OLP) case 11 before treatment (original magnification 200×). (b) The expression of TNF-α with partial remission in case 11 after treatment with fluocinolone acetonide in orabase (FAO) 0.1% for 1 month (original magnification 200×).

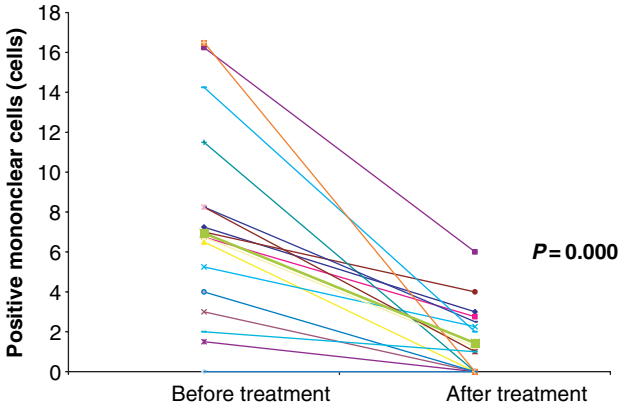

Figure 3 Graph showing the average number of tumor necrosis factor (TNF)-α positive before and after the treatment with fluocinolone acetonide in orabase (FAO) 0.1%.

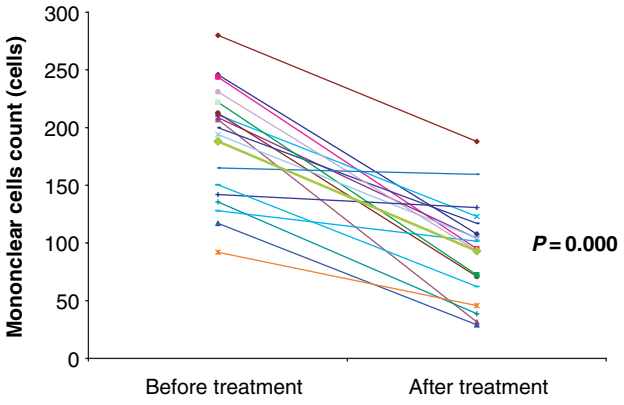

Figure 4 Graph showing the average number of mononuclear cell before and after treatment with fluocinolone acetonide in orabase (FAO) 0.1%.

Discussion

The TNF-α has been implicated in the pathogenesis of many autoimmune and inflammatory diseases and it can be secreted by many inducers such as endotoxin, certain viruses, immune complexes, the neuropeptide substance P, interleukin (IL)-1 or even TNF-α itself (4). Moreover, TNF-α mediates the growth of the oral epithelium and its activity can recruit the inflammatory cells into the OLP lesion. It can also induce the synthesis and secretion of other cytokines, but the factors of releasing TNF-α are still unknown even though the degranulation in the mast cells from some drugs may be involved (12). Although the exact antigen is still unknown, mast cell degranulation in OLP releases many proinflammatory cytokines such as

TNF-α, chymase, and tryptase (2). On the contrary, matrix metalloproteinases (MMP9) are also induced by T cell-secreted TNF-α and excessive MMP9 activity is associated with non-healing chronic wounds. It may also cause basal keratinocytes to be detached from the basement membrane that can be damaging and destructive of the epithelium (13). Hence, basement membrane disruption in OLP may be mediated by mast cell proteases directly or indirectly via activation of TNF-α and T cell-secreted MMP9.

In the present study, 16 of 18 cases (88.89%) of Thai patients with OLP expressed TNF-α in mononuclear cells including macrophages and lymphocytes. In 10 cases (55.56%), TNF-α was detected in keratinocytes and unexpectedly, two cases of OLP did not express TNF-α in the lesions either in mononuclear cell or keratinocyte even though we repeated the experiment twice. The plausible reason why TNF-α could not be detected in every case of OLP is that the two specimens may be obtained when the level of this cytokine dropped. TNF-α is produced at maximal level after stimulation with antigen and significantly decreased after stimulation (14). Other supporting evidence is that TNF-α could not be detected during the chronic stage in chronic diseases such as rheumatoid arthritis (15). In contrast, this cytokine was found throughout the epithelia in OLP in one study that was different from our study (16). Our findings may be explained by the assumption that not only TNF-α is involved in the disease process, but also other cytokines may be involved in the immunopathogenesis of Thai patients with OLP as well. However, the activated mononuclear cells infiltrating the lesions produced TNF-α in most cases, but the source of antigens that triggered the inflammatory process is not known. It is possible that the mononuclear cells may secrete TNF-α and then TNF-α triggers keratinocyte apoptosis in OLP. The present study suggests that TNF-α is involved in the inflammatory process and immunopathogenesis in Thai patients with erosive or atrophic OLP. However, there was no statistical difference in the expression of TNF-α between these two types of lesions.

In addition, the number of TNF-α-positive mononuclear cells after the treatment with FAO 0.1% was significantly lower than before the treatment ($P = 0.000$). This potent steroid can inhibit the inflammatory process of OLP. The amount of TNF-α in virtually all patients was reduced after 1-month treatment with FAO 0.1%. Furthermore, TNF-α could not be detected in one patient who demonstrated CR clinically. The effect of this steroid on TNF-α synthesis advocates the fact that TNF-α plays an important role in the inflammatory process of OLP in Thai patients.

Recently, numerous cytokines including TNF-α have been detected at increased levels in oral fluids and serum of OLP patients. These results indicate that TNF-α is involved in the inflammatory process of this disease (17, 18). Moreover, the presence of salivary TNF-α levels showed positive correlation with clinical forms of OLP, being significantly higher in the erosive/atrophic type than in the reticular type of OLP (19). In conclusion, TNF-α may be associated with the immunopathogenesis of OLP in Thai patients and FAO 0.1% had an effect on reduction of TNF-α expression indirectly and parallel with the healing process. To our knowledge, this is the first report of the potent topical steroid affecting the expression of TNF-α in OLP. Consequently, the development of anti-TNF-α therapy has been proposed for the treatment of many inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (20). TNF-α production by the immune system is a double-edged sword. It has both a beneficial effect by initiation of the inflammatory response and promoting the reparative process as well as a devastating effect to the body. For example, excessive production of this cytokine during wound healing can result in tissue damage, cachexia, shock, and even death (7, 21). Because TNF-α has many functions, the anti-TNF-α treatments can cause side effects in clinical trials such as the development of antinuclear antibodies (ANA) and double-stranded DNA (dsDNA) antibodies, as well as the infrequent development of systemic lupus erythematosus (SLE)-like disease (22, 23). Hence, we should consider modulating the level of TNF-α and other related cytokines in the treatment of oral lesions by weighing the good it will do against the risk it may cause. Thus, understanding the biologic effects of TNF-α is important for dermatologists and oral medicine clinicians because the clinical application of this cytokine in the treatment of oral immunologic disorders will be safer and more effective.

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