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INTERLEUKIN 13 (IL-13) ACTS AS A PROFIBROTIC CYTOKINE FOR RECRUITMENT OF MYOFIBROBLASTS IN ORAL SUBMUCOUS FIBROSIS

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ABSTRACT

Oral submucous fibrosis (OSF) is a chronic inflammatory condition due to areca nut (*Areca catechu*) chewing which induces fibrosis leading to the difficulty in mouth opening. Various profibrotic cytokines and chemokines have been found involved in fibrosis. The present study was aimed to find the role of interleukin 13 (IL-13) in recruiting the myofibroblasts and as a causative factor for fibrosis in OSF. Twenty-five sections of OSF tissue and ten sections of normal mucosa were stained with IL-13, TGF-β1 and α-SMA antibodies using immunohistochemical method. Immunostaining was done to assess staining intensity and percentage of positive cells. The results were analyzed by SPSS software. The expression of all three markers IL-13, TGF-β1 and α-SMA showed significant difference between control and OSF ($p < 0.001$), control and early OSF ($p < 0.001$) control and advanced OSF ($p < 0.001$). No statistical difference was found between early OSF and advanced OSF. Significant expression of IL-13 and its correlation with TGF-β and α-SMA suggests its role in pathogenesis of OSF.

Keywords: Interleukin 13, myofibroblasts, oral submucous fibrosis, pathogenesis, transforming growth factor-β

INTRODUCTION

Oral submucous fibrosis (OSF), an oral precancerous condition, was first described by Pindborg and Sirsat in 1966 (Philip et al., 2014). OSF is a chronic debilitating disease mainly caused due to areca nut (*Areca catechu*) chewing. Other causative factors include the consumption of excessive chilli, iron and nutritional deficiencies, genetic abnormalities, autoimmunity, etc. OSF is characterized by chronic inflammation and a progressive fibrosis of lamina propria and deeper connective tissues leading to restricted mouth opening causing difficulty in food intake, chewing, swallowing and speech (Angadi *et al*., 2011; Sudarshan *et al*., 2012; Philip *et al.*, 2014; Singh *et al.*, 2017). Arecanut alkaloids initiate inflammation in response to the release of various cytokines. Myofibroblast cells positive for α-SMA are responsible for fibrosis induced through various mechanisms and signals from lymphocytes and macrophages, cytokines, chemokines, angiogenic factors, growth factors, etc. and have been recognized as important regulators of fibrosis (Wynn, 2008). In OSF, a pro-fibrotic cytokine TGF-β1 activates both pathways of collagen production and its degradation. It is reported to be responsible for differentiation of myofibroblasts in numerous fibrotic conditions including OSF (Rao *et al*., 2014; Mirza and Amanat, 2014).

IL-13 is a cytokine which is secreted by various cells like T helper type 2 (Th2) cells, mast cell, basophil cells, eosinophil cells, etc. and also regulates IgE synthesis, goblet cell hyperplasia, mucus hypersecretion, airway hyperresponsiveness, fibrosis and chitinase up-regulation (Rael and Lockey, 2011). In fibrosis, IL-13 helps in the differentiation of resident fibroblast and recruited fibrocytes to myofibroblast (Wynn and Ramalingam, 2012). It is one of the cytokine that have been studied in various organ fibrosis like liver cirrhosis, systemic sclerosis, pulmonary fibrosis etc. (Lee *et al*., 2001; Kaviratne *et al*., 2004). Role of IL-13 in recruiting myofibroblasts has been shown through both TGF dependent and TGF independent pathway (Lee *et al*., 2001; Wynn and Ramalingam, 2012). With this background the present study was carried out to find the role of IL-13 in recruiting myofibroblasts and as a causative factor for fibrosis in OSF.

MATERIALS AND METHODS

The present study was carried in Dr. D. Y. Patil Dental College and Hospital, Pimpri, Pune (India) from the year 2015 to 2018 after seeking proper approval from the institutional ethics committee (DYPDCH/IEC/1261/01/14; 5/12/2014). The study and control group comprised of 33 and 10 tissue sections of OSF and normal mucosa, respectively. The tissue blocks were retrieved from the archives of Department of Oral Pathology and Microbiology. Clinical staging of the patients was made depending on mouth opening by Lai *et al.* (Ranganathan and Mishra, 2006) as stage 1 (> 35 mm), stage 2 (30-35 mm), stage 3 (20-30 mm) and stage 4 (< 20 mm). The sections were stained for IL-13, TGF-B1 and α-SMA by immunohistochemical method (David, 2006).

Immunohistochemistry

The tissue sections of 4 µm thickness were deparaffinised and rehydrated in a graded series of alcohol. The sections were kept in pressure cooker at 90°C for 10 min in citrate phosphate buffer (pH - 6.0) for antigen retrieval and then washed with distilled water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 30 min. IL-13 (polyclonal antibody, biorbyt dilution 1:20), TGFβ1 (polyclonal antibody, biorbyt dilution 1:250) and α-SMA was applied as primary antibody for 45 min at 37°C. After 2 washes with phosphate buffered saline, secondary antibody was applied to the sections and then incubated for 30 min. at 37°C. After wash with phosphate buffered saline diaminobenzidine was applied for 10 min. then the sections counterstained with Mayer's hematoxylin, dehydrated and mount.

IL-13 was expressed in lymphocytes, TGF –B1 was expressed by epithelial cells and stromal cells (fibroblasts) and α-SMA by stromal cells (fibroblasts). The above cells for all the stains showed the expressions as brown cytoplasmic staining. The stained slides were interpreted as described by Soini *et al.* (2001). Scoring was based on a) the intensity of immunostaining in lining epithelial cells (0 = absent, 1 = weak, 2 = moderate, 3 = strong, and 4 = very strong) and b) the percentage of positive cells (0 = 0% positive cells, 1 = < 25% positive cells, 2 = 25-50% positive cells, 3 = 50-75% positive cells and 4 = >75% positive cells). The final immunostaining score was determined summing the scores of a) and b). Final scores ranged from 0 to 8 (0 = absent, 1-4 = weak, and 5-8 = strong).

Statistical analysis

The differences in the expressions of IL13, TGFβ1 and α-SMA in different groups were analyzed using chi-square and Fisher's exact test. The correlation between IL13, TGFβ1 and α–SMA expression was calculated using Spearman's correlation coefficient. The data were statistically analyzed using SPSS, version 17.0 for Windows and P <0.05wasconsidered as statistically significant.

RESULTS AND DISCUSSION

The alkaloids and flavonoids from the betel quid cause both chemical and mechanical irritation leading to juxta-epithelial inflammatory response which facilitates release of various profibrotic mediators. TGF-β1 remains as the key growth factor for pathways of fibrosis. (Rajalalitha and Vali, 2005). Apart from TGF-β1, various cytokines and chemokines like IL-13, IL-21, MCP-1, MIP-1*β*, VEGF, PDGF etc., have been studied as distinct fibrotic regulators and also investigated as potential targets for antifibrotic drugs (Wynn, 2008). The present study included 33 OSF cases and 10 controls. The data showed male predominance (M:F = 28:5) and controls showed female predominance (M:F = 4:6). Age group of OSF cases ranged from 16-45 yrs with mean of 34.04 yr. Oral habits used by cases were either gutka alone or a combination of areca nut and tobacco. Most of the cases used gutka alone and belong to stage 3 & 4. There were 4 cases in stage 1, 8 in stage 2, 10 in stage 3 and 11 in stage 4. Analysis was done as early (stage 1 and 2) and advanced (stage 3 and 4) stages of OSF.

The present study evaluated the profibrotic cytokines IL-13 and TGF-β1in myofibroblastic differentiation. α-SMA was used as a myofibroblastic marker. Through several experimental models, IL-13 was identified as the dominant effector cytokine of fibrosis (Chiaramonte *et al*., 1999; Blease *et al*., 2001; Kolodsick *et al*., 2004; Aliprantis *et al*., 2007; Keane *et al*., 2007). The sources of IL-13 were Th2 cells, mast cells, and basophils T cells (Saw *et al*., 2009). In present study, lymphocytes showed IL-13 expression (Fig. 1). The expression of IL 13 in OSF cases was significant when compared to normal. No significant difference was found between early and advanced OSF (Table 1).

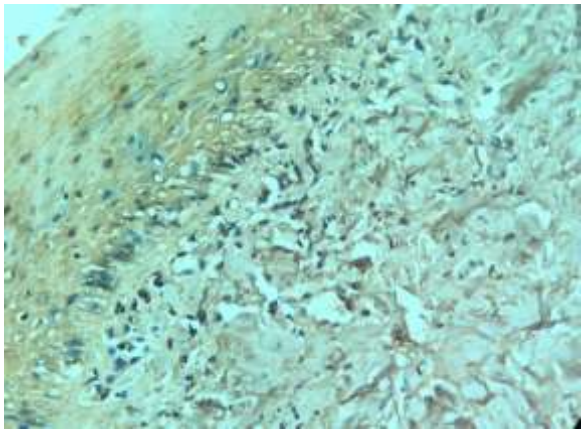

Fig. 1: Photomicrograph showing strong expression of IL-13 in OSF. [immune-histochemistry; total magnification 400x]

Similar results of increase in IL-13 expression have been reported in systemic sclerosis and lung fibrosis by Fuschiotti *et al.* (2013)and Lee *et al.* (2001), respectively. Kaviratne *et al.* (2004) demonstrated up-regulation of genes related to collagens, MMPs, and TIMP-1 in TGF-B1$^{-/-}$mice by IL-13 confirming that fibrogenesis is IL-13 dependent but TGF-β1independent indicating the importance of targeting IL-13 directly in the treatment of infection-induced fibrosis. Hence IL-13 inducing fibrosis can be through both TGF-β1dependant /independent pathway. This is the first study to demonstrate IL-13 in OSF, an oral fibrotic condition and its significant expression indicates IL-13 can play a role in inducing fibrosis.

Table 1: Comparison of IL-13 staining between control and different grades of OSF

Markers	Groups	Number (n)	IHC staining			p-value
			Absent	Weak	Strong	
IL-13	Control	10	07	03	0	p< 0.001*
	OSF	33	0	18	15	
	Control	10	07	03	0	p< 0.001*
	Early OSF	12	0	7	5	
	Control	10	07	03	0	p< 0.001*
	Advanced OSF	21	0	11	10	
	Early OSF	12	0	7	5	0.3
	Advanced OSF	21	0	11	10	

*Significant at p<0.001

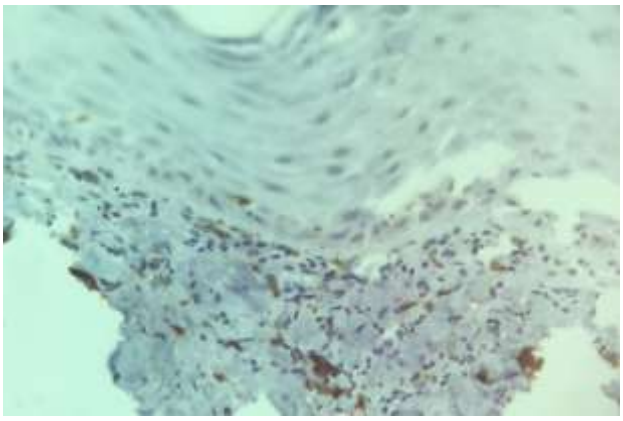

Fig. 2: Photomicrograph showing strong expression of TGF-β1 in OSF [Immunohisto-chemistry; total magnification 400x]

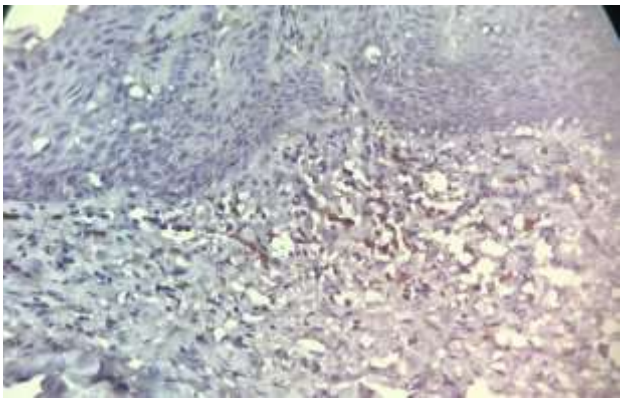

Fig. 3: Photomicrograph showing strong expression of α-SMA in OSF [Immunohisto-chemistry, total magnification 400x]

TGF-β1 is a key regulator of extra-cellular matrix (ECM) assembly and remodeling in OSF. The expression of TGF-β1 was demonstrated in 48% epithelial cells and in stromal cells (Fig. 2). Our results showed significant expression of TGF-β1 in OSF cases than in controls (Table 2). Kale *et al*. (2013) also reported that epithelial cells, fibroblasts, macrophages and inflammatory cells depicted intense staining of TGF β in early cases than advanced cases. Kamath *et al*. (2015) reported that TGF-β1 is the most prominent cytokine in fibrotic pathway of OSF than TGF-β2. The present study did not show any statistical significance between early and advanced OSF (Table 2).

Myofibroblasts are the specialized fibroblasts with contractile property which express α-SMA protein play a role in tissue repair during wound healing, but myofibroblasts have a pathological role in tissue fibrosis by disproportionate secretion of ECM proteins and contraction. Different pathways have been studied and reported to understand the myofibroblastic differentiation in OSF (Moutasim *et al*., 2011; Chang *et al*., 2014). α-SMA was expressed by stromal cells (Fig. 3). Our results showed significant difference between OSF and controls (Table 3) which were similar to those of Rao *et al*. (2014), Sarode *et al*. (2017) Angadi *et al*. (2011), Philip *et al*. (2014), Singh *et al*. (2017) and Gandhi *et al.* (2017). These previous immunohistochemical studies showed the expression of alpha smooth muscle actin (α-SMA) increases significantly in OSF cases but our study showed no significant difference between early and advanced OSF. The finding of increased number of myofibroblasts in OSF showed failed wound healing due to the lack of apoptosis of myofibroblasts as mature myofibroblasts disappear through apoptosis or by dedifferentiation after wound healing.

Table 2: Comparison of TGF-β1 staining between control and different grades of OSF

Markers	Groups	Number (n)	IHC Staining			p-value
			Absent	Weak	Strong	
TGF- β1	Control	10	6	3	1	p< 0.001*
	OSF	33	0	15	18	
	Control	10	6	3	1	p< 0.001*
	Early OSF	12	0	4	8	
	Control	10	6	3	1	p< 0.001*
	Advanced OSF	21	0	11	10	
	Early OSF	12	0	4	8	0.2
	Advanced OSF	21	0	11	10	

*Significant at p<0.001

Table 3: Comparison of α-SMA staining between control and different grades of OSF

Markers	Groups	Number (n)	IHC Staining			p-value
			Absent	Weak	Strong	
α-SMA	Control	10	6	3	1	p< 0.001*
	OSF	33	0	19	14	
	Control	10	6	3	1	p< 0.001*
	Early OSF	12	0	6	6	
	Control	10	6	3	1	p< 0.001*
	Advanced OSF	21	0	13	8	
	Early OSF	12	0	6	6	0.3
	Advanced OSF	21	0	13	8	

*Significant at p<0.001

In the present study, significant difference was found when expression of IL-13 was correlated with TGF-β1 and α-SMA with r value of 0.46666 and 0.447, respectively. Hashimoto *et al.* (2001) studied different pathways and reported that JNK (Janus kinase) pathway regulates the phenotypic differentiation of lung fibroblast to myofibroblasts through IL13 and IL4 than p38 MAP (mitogen-activated protein) kinase and Erk (extracellular-signal-regulated kinase) pathways. Guo *et al*. (2015) demonstrated that IL-13 is a potent stimulator and activator of lung fibroblasts through AKT-mediated YY1 activation in in a dose and time-dependent manner, resulting in increased α-SMA. Saito *et al*. (2003) investigated the effect of two Th2 cytokines IL-4 and IL 13 in differentiating fibroblasts to myofibroblasts and showed they would stimulate myofibroblastic differentiation of lung fibroblasts in asthma and this response is attenuated by IFN-gamma. Although the expression of markers increased in advanced stage, there was no statistical significance between early and advanced stages of OSF. Hence, based on study results we suggest the role of each marker in the pathway of fibrinogenesis in OSF but cannot comment on the progression of the disease.

Conclusion: Significant expression of IL 13 and its correlation with TGF beta and alpha SMA suggests its role in pathogenesis of OSF. The study has the limitation of not using positive controls for the study markers. These results can be considered as baseline data for further specific study where therapy will be directed towards the cytokine IL-13 and TGF-β1antagonists for the treatment of OSF. Further studies need to be focused on exploring the role of various other cytokines and chemokines to understand SMF pathogenesis better for therapeutic interventions.

Conflict of Interest: There is no potential conflict of interest.

Ethical statement: The present study was undertaken after seeking proper approval from the Institutional Ethical Committee vide their communication No. DYPDCH/IEC/1261/01/14 dated 5 December, 2014.

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