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Correlation between transforming growth factor-beta expression and mast cell count in different grades of oral submucous fibrosis

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Abstract

Background and Objectives: In India, oral submucous fibrosis (OSF) is a commonly encountered precancerous disorder. It is a disease with a protracted course and characteristic clinical features caused by the habit of consuming areca nut. Histologically, the main pathology is extensive fibrosis of the mucosa. OSF can be categorized into four grades based on histology. Inflammatory cells and keratocytes are stimulated by the areca nut alkaloids, and they release transforming growth factor-beta (TGF-β). It has been advocated to play a major role in OSF pathogenesis by increasing collagen synthesis and preventing collagen degradation. Mast cells (MCs) are the additional key players in the pathogenesis of OSF. There is confusion regarding the functional relationship of MC with TGF-β. Few studies have shown TGF-β can repress MC function, whereas other studies have established that TGF-β is a chemotaxin for MCs. However, there is dearth in literature regarding their interaction in OSF. Hence, this study attempted to show a relationship between TGF-β and MC in different grades of the disease, so as to further explicate the pathogenesis of OSF.

Materials and Methods: A total of 48 OSF cases were stained using TGF-β antibody. Its staining intensity and staining area were calculated. Sections from same blocks were also stain MCs using 1% toluidine blue. The number of MCs was counted for all the cases. Expression of TGF-β was correlated with MC count in all OSF grades.

Results: On correlating TGF-β and MC between the histologic grades, there was a strong negative correlation for Grade I, moderately positive for Grade II and no correlation for Grade III OSF.

Interpretation and Conclusions: Among the different grades of OSF, the correlation between MC and TGF-β varies, depending on the severity of inflammation, and the concentration of TGF-β and further investigation in this field may elucidate their role in determining the severity of OSF.

Keywords
Mast cells, oral submucous fibrosis, transforming growth factor-beta

Introduction

Oral submucous fibrosis (OSF) is a common premalignant condition in India. It is a disease with a prolonged course that presents with characteristic clinical features and is caused primarily by chewing areca nut. OSF has prevalence rates of 0.2-2% (average of 0.5%) in various parts of India. The reported incidence of epithelial dysplasia in OSF is 10-15%. Evidence of carcinoma has been reported in at least 5% biopsied cases of OSF.

Histologically, the main pathology appears to be extensive fibrosis, which secondarily leads to epithelial atrophy. OSF can be histologically grouped into Grade I (includes very early and early), Grade II (moderately advanced), and Grade III (advanced).

Development of OSF has been extensively explored, and the chief cause for the fibrosis has been identified as transforming growth factor-beta (TGF-β). This growth factor is secreted by keratinocytes, activated T lymphocytes, and macrophages.
When oral mucosa is exposed to ingredients of areca nut, there is production of insoluble collagen and reduced collagen degradation, where TGF-β as the strategic performer.[4]

On the other side of the platter, many research scholars have proved the presence of increased numbers of mast cells (MCs) in OSF and have deliberated their role in its development. In some studies, early grades of OSF were found to have more MCs, while no significant relationship has been established between MC count and the grade of OSF in other studies.[3] The MC in normal oral mucosa has been found to be 0-5 per microscopic field, whereas 2-9 MCs were reported in various grades of OSF.[3]

In addition, how MCs functionally relate to TGF-β is ambiguous. While TGF-β inhibited MCs in some studies, few others have shown it to be a chemotaxin for MCs.[6,7] Furthermore, there is scarcity in the literature about the interaction TGF-β with MCs in OSF. Henceforward, this attempt was made to connect TGF-β expression and MC in different OSF grades, so as to further comprehend the pathogenesis of this disease and thereby open new avenues for its management.

Materials and Methods

After obtaining the institutional ethical approval for the study, cases from archives of the department of oral and maxillofacial pathology, which were histologically confirmed as OSF, were considered for the study.

A total of 48 cases were selected; clinical information and histopathological slides were recovered from the department archives. Cases with other coexisting premalignant lesions/conditions and coexisting malignancy and cases with acute or chronic specific infections (other than pulp and periapical diseases, gingivitis, and periodontitis) were excluded from the study.

The study included three components: Comprising of grading of OSF, immunohistochemical staining using TGF-β, and calculating the number of MCs.

Histologic grading of OSF

Hematoxylin and eosin stained sections of the study sample were observed, and the diagnosis was reconfirmed. Each case was histologically graded using the criteria mentioned in Table 1.[8] After histological grading, the final sample included 17 cases of Grade I, 25 cases of Grade II, and 6 cases of Grade III OSF.

TGF-β expression in OSF

About 4 μm thick sections selected OSF cases (formalin-fixed; paraffin-embedded) were stained with antibody against TGF-β (clone TB 21 MAB1032). Super Sensitive™ Polymer-HRP IHC Detection System (BIOGENEX Life Sciences Pvt. Ltd.) was used. The sections were then counterstained with Harris Hematoxylin, dehydrated and mounted using Di-n butyl phthalate xylene. Sections of fibroadenoma of breast served as the positive control. Sections of OSF, incubated with the negative control serum supplied with the detection system, were used as negative control. Thus, stained sections were evaluated for staining area (SA) and staining intensity (SI) by 2 observers independently. In each section, the location of staining was documented. The SA was scored based on the approximate area stained throughout the section. The following criteria were used for scoring SA:[9]

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No staining</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1/3rd of tissue section stained</td>
</tr>
<tr>
<td>2</td>
<td>1/3rd to 2/3rd of tissue section stained</td>
</tr>
<tr>
<td>3</td>
<td>&gt;2/3rd of tissue section stained</td>
</tr>
</tbody>
</table>

In each section, 10 high power fields (randomly selected) were examined to calculate the SI as given below, and the mean SI was calculated for each section using the following scoring criteria:[5,9]

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No staining</td>
</tr>
<tr>
<td>1</td>
<td>Light yellow</td>
</tr>
<tr>
<td>2</td>
<td>Yellow to brown</td>
</tr>
<tr>
<td>3</td>
<td>Dark brown</td>
</tr>
</tbody>
</table>

Sections were considered negative or positive by adding the scores of SA and the mean SI for each case. A score of ≥3
was regarded as positive.\textsuperscript{[9]} The values obtained by both the observers were tabulated.

**MC count in OSF**

Tissue blocks of the OSF cases (forming-fixed; paraffin-embedded) were sectioned to 4 μm thickness. These sections, after staining with 1% toluidine blue, were examined using binocular brightfield microscope (Olympus CX 21 I). MCs were recognized by their purple metachromatic granules and sky blue nuclei. Intact as well as degranulated MCs were counted in each case, in 10 randomly selected high power fields under ×10 objective and ocular grid of 400 squares. MC was calculated in each case by two investigators independently, and a mean MC was obtained for each case.

**Statistical analysis**

Data attained was statistically analyzed for the following using Kruskal–Wallis test and Spearman’s Rho analysis for:

a. Correlation between TGF-β values and OSF grade
b. Correlation between MC and OSF grade
c. Correlation between TGF-β expression and MC in different OSF grades
d. Appraisal of correlation between MC and TGF-β in different OSF grades.

**Results**

Among the 48 cases, Grade II was the most common followed by Grade I and Grade III was the least common, with an age range of 21-70 years and a male to female ratio of 11:1.

TGF-β staining was observed in both the epithelium as well as connective tissue. In the epithelium, keratin and the basal cells were more frequently stained than the spinous cells and the basement membrane. For the cells, the stain was inside the cytoplasm (Figure 1). In the connective tissue, endothelium of the blood vessels (BV) and to lesser extent, the fibroblasts and MCs expressed TGF-β. Out of 48 cases, 12 cases (25%) did not stain with TGF-β. 4 cases in Grade I, 6 cases in Grade II, and 2 cases in Grade III were given score 0 due to lack of staining. The mean TGF-β expression was greater and was of higher intensity in Grades I/II than Grade III. Kruskal–Wallis test revealed no statistically significant difference (\(P = 0.149\)) in TGF-β among all the three grades of OSF [Table 2].

In all the cases, MCs were dispersed throughout the connective tissue including muscle and adipose tissue. In the majority of the cases, they were concentrated just below the epithelium and around the BV (Figure 2). The lowest mean MC was 1.0 and the highest was 20.2. In Grade I OSF cases, the lowest was 1.3 and highest counts were between 17 and 19. In Grade II cases, the lowest was 1 and the highest counts were between 19 and 21. The lowest MC in Grade II was 2.2 and the highest count was not more than 8.0. The mean MC was higher in Grade I/II in comparison with Grade III. In Grade I/II OSF, there was a wide range of MC compared to Grade III. A P value of 0.14 was obtained with Kruskal–Wallis test, indicating that the difference MC was not statistically significant among all the three grades of OSF [Table 3].

Both TGF-β expression and MC were found to be higher in Grades I and II when compared to Grade III OSF, although the difference was not significant statistically. Hence, the correlation of MC with TGF-β was analyzed using Spearman’s correlation coefficient in different grades [Table 3]. In Grade I OSF, it was found that there was a strong negative correlation, which was significant statistically (\(P = 0.004\)) with a value of −0.658. Hence, we can infer that in Grade I OSF, higher the TGF-β, lower the MC. In Grade II, it was found that there was a statistically significant moderate positive correlation (\(P = 0.04\)) with a value of 0.407. Hence, in Grade II OSF, we can infer that higher the expression of TGF-β, higher the MC. In Grade III, it was found that there was a weak negative correlation with a Spearman’s Rho coefficient of −0.029, which was not significant statistically.

**Discussion**

OSF is a common precancerous condition in Southeast Asia, including India.\textsuperscript{[5]} Epidemiological studies in India show a female predilection of OSF by some authors. Conversely, few.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade of OSF</th>
<th>TGF-β expression</th>
<th>Summary</th>
<th>Value of (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>3.17±2.24</td>
<td>0.14</td>
<td>TGF-β expression greater in Grades I and II than Grade III; Not statistically significant</td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>3.09±2.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade III</td>
<td>1.48±1.23</td>
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<thead>
<tr>
<th>TGF-β expression</th>
<th>Grade of OSF</th>
<th>Grade</th>
<th>P value</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.09±2.04</td>
<td>Grade II</td>
<td>0.14</td>
<td></td>
<td>TGF-β expression greater in Grades I and II than Grade III; Not statistically significant</td>
</tr>
<tr>
<td>1.48±1.23</td>
<td>Grade III</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>MC count</th>
<th>Grade of OSF</th>
<th>TGF-β expression</th>
<th>Summary</th>
<th>Value of (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>9.04±4.64</td>
<td>0.14</td>
<td>Wide range of MCC in Grade III; Not statistically significant</td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>8.58±4.83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade III</td>
<td>5.04±1.99</td>
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</table>

<table>
<thead>
<tr>
<th>MC count</th>
<th>Grade of OSF</th>
<th>TGF-β expression</th>
<th>Summary</th>
<th>Value of (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>−0.658</td>
<td>0.004</td>
<td>Strong negative correlation; statistically significant at 0.01 level (2-tailed)</td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>0.407</td>
<td>0.04</td>
<td>Moderate positive correlation; statistically significant at 0.05 level (2-tailed)</td>
<td></td>
</tr>
<tr>
<td>Grade III</td>
<td>−0.029</td>
<td>0.957</td>
<td>Weak negative correlation; not statistically significant</td>
<td></td>
</tr>
</tbody>
</table>

| Table 3: Correlation between TGF-β expression and mast cell count in different grades of OSF using Spearman’s Rho analysis |
|------------------|-------|---------|----------------|
| Grade of OSF     | Correlation Coefficient | P value | Interpretation |
| Grade I          | −0.658| 0.004  | Strong negative correlation; statistically significant at 0.01 level (2-tailed) |
| Grade II         | 0.407 | 0.04   | Moderate positive correlation; statistically significant at 0.05 level (2-tailed) |
| Grade III        | −0.029| 0.957  | Weak negative correlation; not statistically significant |

| TGF-β: Transforming growth factor-beta, OSF: Oral submucous fibrosis, MCC: Microcrystalline cellulose |

**Table 2:** TGF-β expression and mast cell count in different grades of OSF using Kruskal–Wallis test

**Table 3:** Correlation between TGF-β expression and mast cell count in different grades of OSF using Spearman’s Rho analysis

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125
TGF-β and mast cell count in oral submucous fibrosis

Role of TGF-β in OSF

TGF-β has an important role in modulation of most cell functions such as cell growth, division, differentiation, and movement.\(^5\) It is believed to be the key factor responsible for tissue fibrosis.\(^5\) TGF-β super family includes TGF-β1, TGF-β2, TGF-β3, activins, and bone morphogenic proteins. Among these, the prototype is TGF-β1. It is synthesized and secreted by most types of tissues and cells.\(^6,9\) TGF-β induces connective tissue growth factor, and this, in turn, mediates the stimulatory action of TGF-β on extracellular matrix (ECM) synthesis. Transient presence of TGF-β1 is useful in repair and regeneration of tissue, whereas its persistent presence can result in excessive fibrosis.\(^5\) The latter process has been implicated as being crucial in the development of OSF, wherein TGF-β increases collagen production, causes cross-linking of mature collagen which is resistant to lysis and inhibits degradation mechanisms, as detailed by Rajalalitha and Vali.\(^4\)

In the present study, TGF-β was found to be higher in Grades I and II and later decreased in Grade III OSF cases. However, when a correlation test was done between TGF-β and OSF grade, it was not statistically significant.

In our study, TGF- β was expressed more in basal layer than upper epithelial layers. Intensity and area of staining were more in Grades I/II OSF than Grade III cases. This is in accordance with the conclusions by Kale et al.\(^3\) They correlated TGF-β with lipodystrophy in OSF and concluded that TGF-β inhibited adipogenesis and induced lipodystrophy. Gao et al.\(^2\) too found that TGF-β1 mRNA was expressed by keratinocytes in Grades I and II OSF.\(^12\) Studies have shown that areca nut stimulates keratinocytes to secrete cytokines such as endothelin and TGF-β.\(^3\) This would explain the reason for its higher expressivity in early grades.

Kale et al. also noted that the stromal cells expressed more TGF-β in early OSF than in advanced grades.\(^3\) This finding too was similar to our study, where we observed more TGF-β in Grade I and II cases. In the stroma, TGF-β was expressed by inflammatory cells, endothelial cells as well as fibroblasts.

In early OSF, external stimuli and inflammation cause increased secretion of TGF-β when widespread fibrosis can be detected clinically. As the disease progresses, vertical fibrous bands can be felt and cheeks become sunken. In this later stage, TGF-β decreases as the tissue is already fibrosed and the adipose tissue is lost.\(^6\)

Role of MCs in OSF

Paul Ehrlich, in 1877, first described an MC as “Mastzellan” – a well-fed cell.\(^9\) They are derived from the bone marrow and are mobile.\(^7\) MCs exit the bone marrow in an immature form and mature at the tissue site.\(^13\) They can be easily identified using toluidine blue stain due to metachromasia exhibited by the granules.\(^14,15\) The mast granules contain heparin, histamine, leukotrienes (B4, C4, D4, E4), tumor necrosis factor-alpha, interleukin-1,3, 5,10, chondroitin sulfate, proteoglycan and...
numerous enzymes including collagenase and tryptase. They participate in remodeling the ECM during wound healing. The role of MCs in several oral diseases, such as periapical lesions, reactive lesions (pyogenic granuloma in particular), OSF, oral lichen planus, orofacial granulomatosis, odontogenic cysts, gingivitis, and oral squamous cell carcinoma, has been investigated. In OSF, the mediators from their granules are responsible for many of the clinicopathological features.

Ankles et al. reported an MC of 25.5 cells/mm² in the normal oral mucous membrane, as against 48.25 cells/mm² in OSF. In our study, we found a wide range of MC in different areas in each section. When MC was correlated with the grade of OSF, there was no significant difference. However, MCC was higher in Grades I/II than Grade III OSF. Many other researchers have also obtained a similar result. The probable reasons could be as follows:

- In Grades I and II OSF, degranulation of MCs occurs continuously, depleting their contents. These flattened cells, though they are present may not be easily visible in light microscopy, even with toluidine blue. This postulation stems from Khatri et al. study, where they found more MCs with c-kit in advanced OSF cases.
- The lifespan of MCs is weeks to months. It takes a long time for progression of OSF, and by this time, the MCs recruited in early phases may have undergone programmed cell death.
- With increase in severity of OSF, complete fibrosis of connective tissue takes place. This dense fibrosis and compressed BV may impede inflammatory cell movement (including MCs) in advanced cases.

Sabarinath et al. correlated the density of MCs (MCD) and microvascular density (MVD) in the normal oral mucous membrane and various grades of OSF. They found that MCD and MVD were more in OSF when compared to normal mucosa. As MCD increased, MVD increased and this was most prominent in moderately advanced cases. Thus, they concluded that MCs are vital for the angiogenesis seen during early stages of OSF.

**The association among TGF-β and MCs**

A literature survey about the functional relationship of TGF-β and MCs revealed contrasting relationships between the two. MCs have serine/threonine types I and II receptors for TGF-β, showing that TGF-β responses are elicited via signaling through TβR-I/TβR-II complexes. These membrane receptors cause signal transduction and hence, it can be deduced that TGF-β influences MC activity. TGF-β can impede functioning of MC, or acts as a chemotaxin for MCs depending on its concentration. These effects of TGF-β on function of MCs depend on the concentration of TGF-β. This behavior illustrates the autocrine property of MCs. In addition, there is dearth in the literature regarding TGF-β/MCs relation; though it is believed to be chemotactic for endothelial cells and also causes growth of fibrous tissue. This study thus aimed at finding the existence of an association between TGF-β with MC in all the histologic grades of OSF.

We found a strong negative correlation concerning TGF-β and MC in Grade I. Lower the expression of TGF-β higher the MC. This is in accordance with the findings of Olsson et al., wherein TGF-β in low concentration acted as chemotaxin for MCs. In early OSF, TGF-β is expressed less in keratinocytes and inflammatory cells when compared to advanced stages of OSF. In Grade II, a moderately positive correlation of TGF-β with MCC was observed; this means higher the TGF-β, higher the MC. This is in accordance with the notion that MC concentrate at a site of inflammation. In early grades, TGF-β has acted as a chemotaxin, and MC has migrated to the affected site.

In our study, Grade III OSF did not show any statistically significant association between TGF-β and MC, though there was a weak negative correlation. The possible reasons for this could be that with progressive fibrosis of the connective tissue; there was less recruitment of MCs. Moreover, the MCs that were already present may have undergone programmed cell death. Furthermore, in higher concentrations, since TGF-β constrains anti-apoptotic mechanisms of MCs, they may be dying earlier than their expected lifespan.

Therefore, MCs and TGF-β values individually did not show any significant difference across the histologic grades of OSF. On correlating TGF-β values with MC, it is clear that TGF-β does have an influence on functioning of MC, both positively and negatively, and this influence seems concentration dependent. Nevertheless, no conclusive inferences can be made. Hence, further research is required to understand this complex relationship because, in addition to TGF-β, various other factors such as RANTES, stem cell factor, IL-8, C3a and C5a, activins, serum amyloid, and BMP have been implicated as MC chemotaxin, and many more mediators from keratinocytes and inflammatory cells regulate the function of MCs. Our results may have been confounded by this too.

Our study sample is small without normal distribution of cases, and definitive conclusion cannot be established. Larger study sample is required to arrive at a proper result and to establish the exact relationship between MC and TGF-β.

**Conclusion**

This study correlated TGF-β expression and MC in various grades of OSF. The following conclusions ensue from the results obtained of our study:

1. TGF-β was expressed more in Grades I/II OSF, but it was found to decrease in Grade III, though the relation was not statistically significant.
2. MC was greater in Grade I/II OSF, when compared to Grade III, though the relation was not statistically significant.
3. On correlation between TGF-β expression and MC, it was found that:
   - In Grade I, there was a strong negative correlation
   - In Grade II, there was a moderate positive correlation
   - In Grade III, there was a weak negative correlation (not significant).
Consequently, in Grades I/II of OSF, TGF-β presumably acts as a chemotaxin for MCs. This can be confirmed by further research using cell culture. In Grade III, although there is a weak negative correlation, no conclusions can be drawn. Abridged, this study has brought into limelight that there is a high possibility of interaction between MCs and TGF-β in OSF. To further elucidate this relationship, research is required with a larger sample size and use of more advanced techniques such as cell culture. Since both TGF-β and MCs are key players in the pathogenesis of OSF, understanding their functional relationship in OSF might help in innovation of new treatment modalities for the disease.

Clinical Significance

Understanding the functional relations amid MCs and TGF-β may help in further elucidating the pathogenesis. This could provide a new management strategy for OSF by controlling this interaction and thereby controlling the fibrosis.

References
