Myofibroblasts in stroma of oral submucous fibrosis: An immunohistochemical study

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Abstract

Myofibroblasts play an important role in collagenization, and therefore, are a major component of the extracellular matrix in oral submucous fibrosis (OSMF), which is a pre-malignant disease. The present study is aimed at evaluating the presence of stromal myofibroblasts in OSMF and OSMF with dysplasia, and to compare the differences in the presence of these myofibroblasts amongst different grades of OSMF. A total of 30 OSMF specimens (formalin-fixed paraffin-embedded) were derived from the archives and were evaluated. 10 samples of normal oral mucosa as control group were taken. Myofibroblasts were identified immunohistochemically by discerning alpha smooth muscle actin (α-SMA) in the specimens. They were further categorized as zero, low, moderate and high, based on their positivity. When compared, differences were found in the presence of myofibroblasts amongst the specimens of OSMF and OSMF with dysplasia. Further analysis was done using the Mann-Whitney and Kruskal Wallis test. In the present study, normal oral mucosa showed complete absence of myofibroblasts. But, on the other hand, all the cases of OSMF showed the presence of stromal myofibroblasts, and when compared, they were statistically higher in cases of OSMF with dysplasia as compared to early and advanced OSMF (p<0.05). Myofibroblasts have a role in causing pathological fibrosis in OSMF. This was supported in the present study by the presence of increased number of α-SMA positive myofibroblasts, along with change in its distribution pattern amongst the cases of early, advanced and OSMF with dysplasia, respectively.

Key words: Immunohistochemistry, myofibroblasts, Oral submucous fibrosis, OSMF with dysplasia

Introduction

Oral submucous fibrosis (OSMF) is a potential malignant disorder of oral cavity; the characteristic feature of which is inflammation and juxta-epithelial fibrosis of oral soft tissues, that results in stiffening of an otherwise yielding mucosa. He called the condition as “atrophica idiopathica mucosa oris”. Later on in 1953, S G Joshi from Bombay termed it as oral submucous fibrosis. Areca nut is attributed to have a major role in causing the disease worldwide; however other etiological factors such as tobacco chewing, chillies, malnutrition, viruses have also been identified. Arecoline and arecaidine are the areca alkaloids

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which are considered as major stimulants in OSMF, and are thought to induce many inflammatory cytokines, growth factors and fibroblasts.\footnote{In OSMF, a major part of the stroma is formed by fibroblasts and its products (fibres and amorphous ground substance) which are thought to play a significant role in maintaining the cardinal probity of stromal connective tissue. There are fibroblasts exhibiting variation in the cytoskeletal proteins, with the positive appearance of alpha smooth muscle actin and other surface markers. Such modified fibroblasts are termed as myofibroblasts. Myofibroblasts are large stellate like spindle shaped cells which have long cytoplasmic extensions, amphophilic cytoplasm and indented nucleus. They are highly heterogeneous, exhibit different phenotypes and are thus considered as a transitional form between smooth muscle cells and fibroblasts.\footnote{Myofibroblasts have been found as effector cells in various fibrotic disorders like scleroderma, hepatic fibrosis, pulmonary fibrosis and pancreatic fibrosis. Thus, the present study aimed at evaluating the presence of myofibroblasts in different histological grades of OSMF and OSMF with dysplasia, using alpha-smooth muscle actin, as it is commonly used as a marker of myofibroblast formation. So far, only a few attempts have been made in this subject. So, with this study, we have tried to illuminate the expression of myofibroblasts in OSMF.}}

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Materials and methods

The present study was conducted at the Department of Oral Pathology and Microbiology and Forensic Odontology, Jaipur Dental College, Jaipur. 30 formalin-fixed, paraffin embedded sections of histopathologically diagnosed cases of OSMF were taken. Also, 10 tissue blocks of normal oral mucosa as control group were taken from the gingival and vestibular mucosa after extraction of impacted teeth. Clinical details were recorded from the patient files. All the OSMF cases were re-stained using hematoxylin and eosin stain (H and E), and subdivided into early, advanced and OSMF with dysplasia according to Pindborg and Sirsat criteria. The selected cases and controls were then subjected to immunohistochemistry to detect myofibroblasts with the help of expression of $\alpha$ – Smooth muscle actin (SMA).

**Immunohistochemistry:**

Supersensitive one step polymer-Horse Raddish Peroxidase (HRP) technique was performed for the immunohistochemical analysis of $\alpha$-SMA (Biogenix life sciences, San Ramon, CA, USA). Two slides were prepared; one slide was used for histopathological evaluation, which was stained with regular Hematoxylin and Eosin, and for the second slide, immunohistochemical staining was carried out with $\alpha$-SMA antibody to assess its expression.

For immunohistochemical staining, formalin-fixed paraffin embedded tissue blocks were cut into 4-5 microns thick sections and transferred onto Poly-L-lysine coated slides. As per the schedule, the sections were subjected to deparaffinization and rehydration through xylene and descending grades of alcohol. Commercial microwave antigen retrieval system was used for antigen retrieval, where the sections were placed in 10mM sodium citrate buffer. The sections were then rinsed with phosphate buffered saline (PBS). The primary blocking of endogenous peroxidase was done by treating the slide with peroxidase block for 15 minutes. Following this, non-specific antigenic sites were blocked using a power-block for 20 minutes. The sections were incubated with optimally pre-diluted antibody against $\alpha$-SMA (Biogenix, USA:clone 1A4) for 1 hour at room temperature. The sections were again washed with PBS, which were then incubated with one step polymer HRP reagent for 30 minutes. To visualize the immunoreactivity, sections were treated with diaminobenzidine. Counterstaining was done using hematoxylin, and the slides were then mounted with dibutylphthalate xylene (DPX). For each batch of staining, positive and negative controls were run simultaneously with the study specimens. To validate, normal salivary gland tissue and endothelial staining of blood vessels were used as internal positive control. Sections of Leiomyosarcoma served as positive control, and for negative control, primary antibody was replaced by non-immune mouse serum at same dilutions.

**Immunohistochemical analysis:**

The spindle shaped stromal cells which showed positive immunoreactivity to $\alpha$-SMA antibody
were identified as myofibroblasts, and it showed a cytoplasmic pattern of staining. To assess the immunostaining number, immunopositive cells were counted, and staining intensity was evaluated. The slides were scanned at 4x, 10x and at 40x. Ten random sites were captured showing high immunopositivity, and the immunopositive cells were counted in subepithelium and lamina propria. Scoring was done accordingly, and the sections were scored as 0 (no positive cells), 1 (2-20 positive cells), 2 (20-30 positive cells seen) and 3 (>30 cells seen). Staining intensity was observed under 40x magnification, and then graded as 0 for no staining, 1 for weak staining, 2 for moderate staining and 3 for strong staining. Staining index was calculated by multiplying the score of immunopositive cells with staining intensity. This index was further classified as zero (0), low (1 and 2), moderate (3 and 4) and high (6-9).

**Statistical analysis:**

The obtained data was statistically analyzed using Kruskal-Wallis test. The statistical significance was fixed at 0.05 and p-values at <0.05 were considered to be significant.

**Results**

In the current study, the 30 H and E stained sections of histologically proven cases of OSMF were graded as early, advanced and OSMF with dysplasia according to Pindborg J J and Sirsat S M criteria. For comparison, normal oral mucosa samples were stained. Endothelial cells within blood vessels and salivary gland tissues were taken as internal positive controls exhibiting α-SMA reactivity. It was important to use these internal controls for confirming that antigenic expression was correctly maintained in study sample. The presence of myofibroblasts was statistically significant when compared between normal, early and advanced OSMF and OSMF with dysplasia (Kruskal Wallis test; p <0.0001), and is demonstrated in Table 1. In addition, the statistical significance at p <0.001 was noted based on staining index and score of immunopositive cells between early, advanced and OSMF with dysplasia, but, it was non-significant on the basis of staining intensity as demonstrated in

<table>
<thead>
<tr>
<th>Score of immunopositive cells</th>
<th>Histopathological diagnosis</th>
<th>N</th>
<th>Mean rank</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early OSMF</td>
<td>10</td>
<td>10.30</td>
<td></td>
<td>0.001</td>
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<tr>
<td>Advanced OSMF</td>
<td>10</td>
<td>13.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSMF with dysplasia</td>
<td>10</td>
<td>22.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
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| Staining intensity            | Early OSMF                  | 10  | 12.60 | 0.083   |
| Advanced OSMF                 | 10                          | 14.90 |          |         |
| OSMF with dysplasia           | 10                          | 19.00 |          |         |
| Total                         | 30                          |       |           |         |

| Staining index                | Early OSMF                  | 10  | 9.50  | 0.001   |
| Advanced OSMF                 | 10                          | 13.60 |          |         |
| OSMF with dysplasia           | 10                          | 23.40 |          |         |
| Total                         | 30                          |       |           |         |

**Discussion**

OSMF is considered as a potentially malignant disorder that leads to marked limitation of mouth opening. Initially, the disease was classified as an idiopathic disorder. The pathology in OSMF develops in the connective tissue, and then the epithelium also shows changes. The fibro-elastic changes which are observed in the connective tissue are due to the abnormal accumulation of collagen, which finally leads to fibrosis. A key event in pathological tissue repair and in fibrosis is the activation of myofibroblasts, which are primary extra cellular matrix (ECM) secreting cells. By definition, myofibroblast is a spindle-cell or a cell with stellate morphology with pale eosinophilic cytoplasm and an abundant pericellular matrix, immunophenotypically characterized by positivity for alpha smooth muscle actin marker. ECM acts as a repository for various growth factors, and is multifunctional, providing mechanical stability, protection and guidance for cells. Remodelling of ECM by myofibroblasts causes disturbances in these functions, and thus has a great impact on the behaviour of other cells present in similar area.

The presence of myofibroblasts was seen in all grades of OSMF as well as in OSMF with dysplasia;
but the score on analyzing statistically showed a highly significant difference between the number of immunopositive cells in early, advanced and OSMF with dysplasia (p value <0.001) (Figures 1 - 4). These finding are in concordance with the findings of Li X et al, who found that myofibroblasts number increased from moderately to advanced stages of OSMF when compared with early OSMF.

With the increasing grades of OSMF, the number of myofibroblasts increase, and this can be explained based on the fact that in that initial stages few myofibroblasts are present; however, in later stages, they are seen in large numbers as demonstrated by Desmouliere et al. During the initial stages of wound granulation, type III collagen is deposited by myofibroblasts. But, once granulation tissue is resorbed and physiological repair begins, myofibroblasts undergo programed cell death, and they start depositing the type I collagen which is
more rigid. This can be identified biochemically. Under pathological conditions, type III collagen appears to be increased. Abundant Type I and Type III collagen can be demonstrated in densely fibrotic tissues of OSMF. When wound healing is towards completion, various apoptotic gene programs are expressed within myofibroblasts, and they result in a scar which has relatively less number of cells. The present evidences suggest that in pathological fibrosis, there is a failure to initiate programmed cell death of myofibroblasts which causes tenacity of these cells in the fibrous tissues. Hence, the number of myofibroblasts increases with the severity of pathology.

Another reason for the increased number of myofibroblasts in the case of OSMF could be due to enhanced stimulation of fibroblasts associated with the release of numerous cytokines, including PDGF (platelet-derived growth factor), IL-1 and 4 (interleukins), IGF (insulin-like growth factor II) and TGF-β1 (transforming growth factor). TGF-β is solely responsible for creating imbalance between the collagen deposition and collagen degradation that is seen in OSMF. This is caused by the action of arecoline. Moutasim et al demonstrated the increased presence of αvβ6 integrin in OSMF, and found it to be responsible for the formation of myofibroblasts by the activation of TGF-β.

This study showed that the expression of myofibroblasts was significant in the different stages of OSMF and OSMF with dysplasia. During the dysplastic transformation of the epithelium of OSMF, myofibroblast expression was noted to be high with score of 6-9, and minimal presence of myofibroblasts was observed in normal tissue. The myofibroblast immunoreexpression in OSMF with dysplasia is not reported, and most studies are focused on oral epithelial dysplasia, verrucous carcinoma and OSCC. Although some molecules have been identified in the pathogenesis of OSMF, such as endothelin-1 and human telomerase reverse transcriptase, the role of myofibroblasts in OSMF with dysplasia is not very clear. Myofibroblasts have a key role in the process of oral carcinogenesis by facilitating tumour progression. The malignant transformation in OSMF has been recently indicated to be caused by the interaction of epithelium and stroma.

Myofibroblasts are also known to secrete Stromal cell Derived Factor (SDF)-1, which enhances the proliferating potential of epithelium, and also promotes expansion of stromal cells. This is well supported by Shibata W et al., and in their study of SDF-1, they found that the transgenic mice showed SDF-1, which when overexpressed resulted in dysplasia of gastric mucosa. So, it was concluded that the deeper movement of proliferating epithelial cells during preneoplastic process contributes to SDF expression by myofibroblasts. Therefore, expansion of α-SMA positive myofibroblasts in OSMF can lead to secretion of SDF-1.

**Conclusion**

Not many studies have been done accounting the role of myofibroblasts in oral submucous fibrosis and OSMF exhibiting dysplasia. Hence, the present study stressed upon the importance and the role of myofibroblasts in different grades of OSMF and OSMF with dysplasia. In conclusion, the observations made in the present study showed an increase in the count of α-SMA positive myofibroblasts, and change in its distribution pattern from early OSMF to advanced OSMF and also in OSMF exhibiting dysplasia. This expression of myofibroblasts in OSMF might suggest its role in pathological fibrosis. The findings could suggest that myofibroblasts increase in number because of their ability to overcome the apoptotic process, and they act as collagen secreting cells, thus, leading to fibrosis. It also shows diffuse arrangement of myofibroblasts in OSMF with dysplasia; the finding represented a possible role of myofibroblasts in promoting epithelial dysplasia. The study had certain limitations, as the sample size was small and also there was very little literature available on the role of myofibroblasts in OSMF and OSMF with dysplasia. In future, to attain better results, the study should be done by taking a higher number of cases of OSMF and OSMF with dysplasia, and evaluation should also be done to find the connection between myofibroblasts and SDF-1 in cases of OSMF with dysplasia.
References


