SUBMUCOSA PRECEDES LAMINA PROPRIA IN INITIATING FIBROSIS IN ORAL SUBMUCOUS FIBROSIS - EVIDENCE BASED ON COLLAGEN HISTOCHEMISTRY.

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Abstract

Oral submucous fibrosis is a chronic insidious disease and a well-recognized potentially malignant condition of the oral cavity. It is a collagen related disorder associated with betel quid chewing and characterized by progressive hyalinization of the lamina propria.

Objectives: It is traditionally held that in oral submucous fibrosis the hyalinization process starts from the lamina propria and progresses to involve the submucosal tissues. However reports of some investigators suggest that on the contrary, the fibrosis starts in the submucosa and not in the juxta epithelium as previously assumed.

Methods: A histochemical study comparing the pattern of collagen deposition and hyalinization in early and advanced cases of oral submucous fibrosis was done using special stain for collagen.

Result & Conclusion: The results suggest that in a certain percentage of cases submucosa precedes the lamina propria in initiating fibrosis in this disease. This could have implications on the differences in clinical presentation, biological progression, neoplastic transformation and responsiveness to treatment.

Introduction

Fibrosis, a conspicuous feature of chronically inflamed tissue is characterized by progressive and excessive accumulation of extracellular matrix collagen[1]. Oral submucous fibrosis (OSF) is an insidious chronic fibrotic disease and a well recognized premalignant condition that involves the oral mucosa and occasionally the pharynx and the upper portion of the esophagus[2,3]. The condition is characterized by mucosal rigidity of varied

intensity caused by fibrosis of the superficial connective tissue and leading to secondary atrophic or hypoplastic changes in the epithelium. Epidemiological studies have identified areca nut as the primary etiologic agent involved in this condition[4].

Varying and altered staining characteristics have been described in oral submucous fibrosis. Using standard connective tissue staining methods, Hamner et al demonstrated abnormal juxta-epithelial connective tissue, indicating a probable alteration in the collagen. As the disease progressed, the connective tissue lost its fibrillar staining pattern and became amorphous[5,14]. Sirsat and Pindborg described densely thickened collagen and uneven staining pattern when stained with Mallory stains and Weigert's resorcin fuchsin, especially in the deeper parts of the mucosa, but with no abrupt tinctorial change[6].

The traditional view is that the disease process starts from the connective tissue compartment close to the epithelium, viz. the lamina propria. Immunohistochemical studies of the distribution of procollagen type III, collagen type IV and tenascin indicated that fibrosis might start in the deeper subepithelial connective tissues and not close to the basement membrane (Reichart PA et al, 1994).[7] Despite extensive research into various aspects of this disease, the actual location of the initiation of fibrosis has eluded researchers and remains inconclusive. It is rather prudent to believe that at least in some cases of OSF, the plane of initiation of fibrosis is not at the lamina propria but further down in the submucosa. While the factors that determine this histological alteration in cases of OSF remain elusive, this seems to be a possible reason for some of the early manifestations of disease such as dryness of the mucosa and functional impairment.

The aim of this study is to determine where the fibrotic changes seen in oral submucous fibrosis initiate - in the connective tissue close to the epithelium or in the deeper submucosal tissue, by utilizing the tinctorial characteristics of collagen; and, to evaluate the usefulness of histochemistry in assessing incipient fibrotic change.

Material and Methods:

The study was conducted in the Department of Oral Pathology, Government Dental College, Thiruvananthapuram. A proforma was developed comprising of various parameters, broadly subdivided into demographic factors, clinical findings, histopathological findings and histochemistry

results. The study group comprised thirty patients with oral submucous fibrosis who were habitual chewers of areca nut. Twelve clinically diagnosed early OSF cases (mean age 35.6yrs; M:F=5:7) and eighteen clinically diagnosed advanced OSF cases (mean age 52.8yrs; M: F=7:11) comprised the study group. Ten healthy subjects without any areca quid habits were taken as the control group (mean age 32.5; M: F=5:5). The oral submucous fibrosis patients were grouped into early and advanced stages according to accepted clinical criteria such as burning sensation, mucosal blanching, mouth opening, tongue protrusion, tenderness on palpation of the sulcus and the presence of fibrotic bands[8,14].

Incisional biopsy was taken from the right buccal mucosa of both clinically early and advanced oral submucous fibrosis cases for histopathologic examination. Control cases were collected from the patients undergoing surgery for the removal of impacted molars, where some normal mucosa had to be resected and discarded for a better surgical closure. The biopsy specimens were fixed in 10% neutral buffered formalin, embedded in paraffin and 5μm sections were cut using a rotary microtome. Slides were prepared, incubated and stained with hematoxylin and eosin. Van Gieson technique (Van Gieson, 1889)[9] which stains collagen red and other tissues yellow was used for differential demonstration of collagen.

The tinctorial changes of the fibrous connective tissue in oral submucous fibrosis as seen in serial sections were qualitatively evaluated and graded depending on the staining intensity. The different patterns of response are: normal staining (+), mild staining (++), moderate staining (+++) and intense (++++). Two histopathologists made independent assessments of the staining and graded the slides. When the results were not in agreement, consensus was reached following discussion. Both the lamina propria and the submucosal pattern of staining was entered.

Statistical analysis of the results was done using the Mc - Nemar test to compare the number of early oral submucous fibrosis cases where greater stain intensity (which corresponds to fibrosis) was seen in the submucosa, in the lamina propia or in both. The significant difference is set as $p<0.05$. Relation between the regional variations in OSF and the location of fibrosis was also assessed.

COMPARISON OF COLLAGEN HISTOCHEMISTRY IN LAMINA PROPRIA AND SUBMUCOSA IN DIFFERENT STAGES OF ORAL SUBMUCOUS FIBROSIS.

Table I : Van Gieson Staining Intensity In Early OSF

Lamina propria	Submucosa			Total
	+	++	+++	
+	0	2	4	6
++	1	3	2	6
N	1	5	6	12

+ Normal staining ++ Mild staining +++ Moderate staining ++++ Intense staining

For the purpose of statistical analysis, the tables were merged:

Table II : Van Gieson Staining Intensity in Early OSF (merged table)

Lamina propria	Submucosa		N
	+	++/+++	
+	0	6	6
++	1	5	6
TOTAL	1	11	12

The Mc - Nemar test was applied for statistical analysis. This test is used for testing proprotions in a related sample. Mc - Nemar Chi square test: $X^2 = 2.29$. $p>0.05$, Not significant.

Table III: Staining Intensity in Advanced OSF

Lamina propria	Submucosa			
	+	++	+++	++++

+	0	0	0	0
++	0	0	0	0
+++	0	0	0	6
++++	0	0	0	12
N	18			

Table IV: Staining Intensity in Normal mucosa

Lamina propria	**Submucosa**			
	+	++	+++	++++
+	10	0	0	0
++	0	0	0	0
+++	0	0	0	0
++++	0	0	0	0
N	10			

Results

The tinctorial changes of collagen in oral submucous fibrosis as seen in serial sections were qualitatively evaluated and graded in sequence of severity of alteration. Tables I to IV give a comparison of collagen histochemistry in lamina propria and submucosa in different stages of oral submucous fibrosis. There was found to be a difference in the staining intensity in different zones of the connective tissue stroma in early oral submucous fibrosis, with a certain percentage of cases (8/12) showing more fibrosis in the submucosa while the same cases have normal staining in the lamina propria (Tables I, II). The Mc-Nemar test for statistically testing proportions in related samples was not significant. This is probably due to limited sample size of the present study. A difference in staining pattern was also observed between early OSF and advanced OSF groups as compared with the controls (Tables III, IV).

Discussion

Oral submucous fibrosis (OSF) is a form of pathological fibrosis affecting the oral mucosa and contiguous areas of the upper aero-digestive tract, having a relentless course of progression. Once initiated the disease is not amenable to reversal at any stage of the disease process even after cessation of the putative causative factor of areca nut chewing[3,8]. It differs from the other examples of pathological fibrosis (e.g. juvenile aggressive fibromatoses, abdominal desmoids) in that it harbors with it a definite tendency to induce the overlying epithelium to undergo neoplastic transformation at least in a small proportion of cases [10,11,12].

The conventional view is that the pathological alterations in oral submucous fibrosis begins in the lamina propria[5,7].We hypothesize that at least in some cases of OSF, the plane of initiation of fibrosis is not at the lamina propria but further down in the submucosa.

Immunohistochemical study on the distribution of procollagen type III, collagen type VI by Reichart and van Wyk showed that in oral submucous fibrosis these were expressed in a specific pattern which allows a clear differentiation between fibrotic areas and adjacent normal connective tissue stroma and that the process of fibrosis starts in the deeper subepithelial connective tissue and not close to the subepithelial basement membrane.[6]

Our study was designed to locate the site of commencement of initial fibrosis in oral submucous fibrosis using histochemical method. For the study, we compared the tinctorial alterations of collagen in the lamina propria and submucosa of early OSF and normal cases. The early cases were selected according to the criteria such as diffuse blanching of the mucosa, occurrence of hyperpigmented areas adjacent to zones with loss of pigment, burning sensation, dryness of the mucosa or hypersalivation and tenderness on palpation of the buccal sulcus but without palpable fibrous bands.[7] Controls were non-OSF patients. In advanced oral submucous fibrosis cases due to dense fibrosis throughout the connective tissue, it would be difficult to assess the area of early fibrotic change and such a comparison would be inconclusive.

Van Gieson stain was chosen for the selective demonstration of collagen fibers, muscle and fibrin. This is a simple stain which gives varying shades of red depending on the density of collagen fibers. It can correlate as the amount of fibrosis and indicate the area of initial fibrotic change in OSF.

One of the limitations of this study include small sample size of the study group. As the symptoms and clinical changes in early OSF are mild with minimal restriction of mouth opening, patient compliance for a biopsy procedure is difficult. Moreover, van Gieson stain is not specific for fibrosis and lacks the high specificity of immunohistochemical techniques. We have adjusted for the possible technique errors by cutting sections of the same thickness (5μm), standardization of the staining procedure, and staining the slides at the same time. Two histopathologists independently graded the slides to minimize inter observer variations.

The present study showed a difference in Van Gieson staining intensity between the controls and OSF groups, with more intensity (fibrosis) noted in the OSF group. It implies that the collagen content in OSF is greater than in normal mucosa. This finding is in keeping with previous studies using direct tissue extracts[8,13]. In addition, we found that the initial fibrotic change in early OSF group as assessed by stain intensity was greater in the submucosa, with 67 percentage of cases showing more fibrosis in the submucosa. Statistically, however this was not significant, probably due to the limited sample size of this study. Alternatively, there can be cases where the fibrotic process initiates both in lamina propria and/or submucosa.

The progression of fibrosis from the submucosa to the lamina propria could explain the xerostomia seen clinically in early OSF [2,7]. The submucosa contains muscle tissue and minor salivary acini. Involvement of the submucosa early in the fibrotic process could affect these structures because dense fibrous tissue may act as a barrier for diffusion of nutrients to the adjacent muscle and salivary tissues. This can cause early degenerative changes in the muscle fibers and as a result functional impairment may be high even in early stages of OSF. This will warrant early therapy in cases where the submucosa precedes the lamina propria in initiating fibrosis. Fibrosis around the salivary acini could produce the dryness of mucosa which is an early clinical manifestation. Xerostomia moreover can result in secondary colonization of candida which is known to promote epithelial dysplasia by releasing nitrosamines. This may aggravate the pre-malignant potential of OSF. This provides impetus to the need of localizing the site of initiation of fibrosis.

Conclusion

In the present study an attempt has been made to elucidate the site of initiation of fibrotic alteration in oral submucous fibrosis and to study the effect of the plane of accumulation of fibrosis on the clinical presentations of OSF.

We found that the initial fibrotic change in early OSF group as assessed by stain intensity was greater in the submucosa, with a certain percentage of cases showing more fibrosis in the submucosa. This was found to be clinically significant although statistical significance was not seen.

It is rather prudent to believe that at least in some cases of OSF, the plane of initiation of fibrosis is not at the lamina propria as previously believed but further down in the submucosa and from there extends more superficially. The factors that determine this histological alteration in cases of OSF remain elusive. Being an archetype of pathological fibrosis having a relentless course of progression and irreversible nature, it is important to investigate every aspect which could have treatment implications to help reduce the morbidity of this obscure fibrotic condition.

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