Immunohistochemical expression of CD34 for characterization and quantification of mucosal vasculature and its probable role in malignant transformation of atrophic epithelium in oral submucous fibrosis

Rajiv S. Desai a,*, G.S. Mamatha a, Musarrat J. Khatri a, Subraj J. Shetty b

a Department of Oral Pathology, Dr. D.Y. Patil Dental College and Hospital, Mahesh Nagar, Pimpri, Pune 411 018, India
b Department of Oral Pathology, Nair Hospital Dental College, Mumbai 400 008, India

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Summary

The concept of epithelial atrophy in oral submucous fibrosis (OSF) is based on the assumption of an ischemic epithelium consequent to poorly vascularized stroma, but the role of vascularity on the epithelial thickness in OSF is not clearly understood. This study aimed to investigate the immunohistochemical expression of CD34, for characterization and quantification of mucosal vasculature and its possible role in malignant transformation of atrophic epithelium in oral submucous fibrosis. After compilation of available data, the present study challenges the conventionally believed concept that epithelial atrophy in OSF is due to lack of perfusion, caused by decreased vascularity of subjacent connective tissue stroma. Characteristic pattern of juxtaepithelial loss of CD34 positive stromal cells and its role in malignant transformation in OSF deserves further attention.

Introduction

Oral submucous fibrosis (OSF) is a chronic, progressive, precancerous condition of the oral mucosa, which is associated with areca nut chewing habit prevalent in India and South East Asia. It is clinically characterized by burning sensation of the oral mucosa accompanied by pallor and progressive, irreversible fibrosis leading to difficulty in opening the mouth, speech and swallowing. Characteristic histopathological features of this disease include epithelial atrophy with loss of rete ridges, reduced vascularity, chronic inflammatory infiltrate and hyalinization of the submucosal tissue.

Although the epithelial atrophy in OSF is believed to be secondary to the connective tissue changes, the degree of vascularity of the diseased mucosa and its role on the epithelial thickness in OSF has always been a matter of debate.

Materials and methods

Tissue material

Thirty \((n = 30)\) previously untreated cases of OSF diagnosed on clinical grounds and confirmed histologically comprised the study group. Subjects having OSF with malignancy were excluded. Ten \((n = 10)\) age and sex matched healthy volunteers without habits were included in the control group. The staging of the disease was performed according to Lai DR et al.,\(^3\) based upon the degree of mouth opening, which was as follows. Stage 1: mouth opening greater than 35 mm; Stage 2: mouth opening between 30 and 35 mm; Stage 3: mouth opening between 20 and 30 mm and Stage 4: mouth opening less than 20 mm. The investigations were approved by the local research ethics committee.

Punch biopsies (5 mm) were performed on the test and control subjects from identical oral site (right buccal mucosa). The biopsy samples were immediately fixed in 10% neutral buffered formalin, routinely processed for histology and embedded in paraffin wax. Four-micrometer-thick sections were cut, deparaffinized, and stained with hematoxylin – eosin for histological examination.

Evaluation of epithelial changes in hematoxylin and eosin stained sections

Histopathological findings were mainly focused on the epithelial changes, which included (1) type of surface keratinization...
(parakeratinized, mixed, and orthokeratinized) (2) epithelial cell layer thickness (ET) (3) loss of rete ridges, and (4) dysplasia (mild, moderate and severe). For each section epithelial cell layers were counted at the thinnest and the thickest areas at X400 magnification and a range was established. Mean for the range was calculated accordingly and was distributed in the range of 1–5, 6–10, 11–15 and 16 and more epithelial cell layers.

There are no established criteria for epithelial atrophy in the literature, but most of the time decrease in ET and loss of rete ridges are considered as selection criteria for epithelial atrophy. Accordingly we included ET along with loss of rete ridges as criteria to label epithelium atrophic.

**Immuno histochemical analysis of angiogenesis**

For assessment of vascularity, we used 4-μm-thick sections placed on 3-[(triethyoxysilyl) propylamine coated slides stained with antibodies to CD34. Immuno histochemical staining procedure was done by using the streptavidine-biotin complex method.

For CD34 immunostaining, the sections were deparaffinized in two changes of xylene for 10 min each, and then were rehydrated through graded alcohols. Endogenous peroxidase activity was quenched with freshly prepared 3% H2O2 in methanol for 30 min at room temperature. Sections were then washed in phosphate-buffered saline (PBS). Antigen retrieval was performed by incubating sections immersed in 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave oven (600 W) at 5 min intervals for 10 min at 100 ºC. The sections were cooled for 20 min at room temperature and incubated with monoclonal mouse anti-human CD34 (Dako, clone QB End 10, 1:50 dilution, Glostrup, Denmark) antibody for 60 min at room temperature. Biotinylated antimmonoglobulin was used as a secondary antibody for 30 min. After being rinsed in PBS, the slides were incubated with the peroxidase conjugated streptavidine label for 30 min at room temperature. The sections were again rinsed in PBS and incubated with diaminobenzidine for 7 min. After chromogen development, slides were washed in two changes of water and counterstained with hematoxylin, washed in water, dehydrated, cleared and mounted. A negative control was performed in all cases by omitting the primary antibody. Oral squamous cell carcinoma tissue sections were used as positive control.

All morphological structures with a lumen surrounded by CD34-positive endothelial cells were considered as microvessels. The assessment was carried out at the level of endothelial cells lining the blood vessels by their brown cytoplasmic staining. Microvessel density was assessed in areas showing the highest density of staining (hot spots) as determined by an initial scan at X100 magnification.

Microvessels were counted at X400 magnification by means of computer assisted image analysis using Image Pro Software (Leica QWin V3, Leica Microsystems – Leica DM 2500, Germany) and the individual microvessel profiles were circled to prevent the duplication or omission of microvessel count.

According to Lee et al., the vessels count was recorded from the 3 most vascular fields (X400 magnification) next to the epithelium; images were captured and quantified by means of computer assisted image analyser for mean vascular density (MVD), mean vascular luminal diameter (MVLD) and mean vascular area percentage (MVAP).

**Statistical analysis**

The relationship between angiogenesis and epithelial atrophy in various groups of OSF and control was statistically analyzed. ANOVA and unpaired t-test were used for the statistical analysis and p values <0.05 were considered statistically significant.

**Results**

The present study consisted of 30 OSF (study group) and 10 healthy subjects (controls). Clinical staging of the patients was made depending upon the mouth opening as suggested by Lai et al. There were 4 cases in stage 2, 17 cases in stage 3 and 9 cases in stage 4. There was not a single case in stage 1 of the disease.

**Histological evaluation**

**Type of surface keratinization**

All 10 healthy controls were parakeratinized, while 20 of the cases showed parakeratinized, 6 showed mixed and 4 cases showed orthokeratinized surface epithelium.

**Epithelial cell layer thickness (ET)**

ET in healthy controls were in the range of 14–36 with mean ET of 26. OSF cases showed mean ET of 8.6. Thus OSF cases had statistically significantly reduced ET when compared to controls (Table 1). In stage 2, there were 2 cases with mean ET in the range of 6–10 and 2 cases in the range of 11–15. In stage 3, there were 2 cases with mean ET in the range of 1–5, 10 cases in the range of 6–10 and 5 cases in the range of 11–15. In stage 4, there was 1 case with mean ET in the range of 1–5, 5 cases in the range of 6–10 and 3 cases in the range of 11–15 (Table 2). When comparisons were made between the three stages of OSF no significant difference was noticed (Table 3).

**Establishment of criteria for atrophic epithelium**

Since mean ET of controls was 26, we decided to take half of this mean value as cut off value to label epithelium atrophic. As all the controls showed presence of rete ridges, loss of rete ridges was considered as a additional criteria for atrophic epithelium.

**Table 1**

<table>
<thead>
<tr>
<th>Total</th>
<th>Mean ET</th>
<th>SD</th>
<th>Unpaired t-test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>26</td>
<td>4.39</td>
</tr>
<tr>
<td>OSF</td>
<td>30</td>
<td>8.6</td>
<td>2.9</td>
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Abbreviations: ET, epithelial cell layer thickness; OSF, oral submucous fibrosis; SD, standard deviation.

**Table 2**

<table>
<thead>
<tr>
<th>Total</th>
<th>Mean ET</th>
<th>SD</th>
<th>ANOVA (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>26</td>
<td>4.39</td>
</tr>
<tr>
<td>Stage 2 OSF</td>
<td>4</td>
<td>10</td>
<td>4.2</td>
</tr>
<tr>
<td>Stage 3 OSF</td>
<td>17</td>
<td>8</td>
<td>2.8</td>
</tr>
<tr>
<td>Stage 4 OSF</td>
<td>9</td>
<td>9.2</td>
<td>2.5</td>
</tr>
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</table>

Abbreviations: ET, epithelial cell layer thickness; OSF, oral submucous fibrosis; SD, standard deviation.

**Table 3**

<table>
<thead>
<tr>
<th>Total</th>
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<th>SD</th>
<th>ANOVA (p value)</th>
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<td>Stage 2 OSF</td>
<td>4</td>
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Abbreviations: ET, epithelial cell layer thickness; OSF, oral submucous fibrosis; SD, standard deviation.
Present study showed 17 cases of mean ET of less than 13 without rete ridges, 11 cases of mean ET of less than 13 with rete ridges, and 2 cases of mean ET of more than 13 with rete ridges.

**Epithelial dysplasia**

We did not find a single case of OSF with dysplastic features.

**Quantitative and qualitative results of angiogenesis**

The number and size of blood vessel profiles was demonstrated by the endothelial cell marker CD34 (Figs. 1–4). The MVD in stage 2, stage 3, stage 4 and control was 8.15, 7.18, 7.69, respectively. The difference of MVD between cases and controls was statistically not significant (Table 4). MVLD in stage 2, stage 3, stage 4 and control was 150.22, 130.98, 127.16 and 105.7 μm, respectively. The MVLD of cases was more when compared to controls but the difference was not statistically significant (Table 5).

MVAP in stage 2, stage 3, stage 4 and control was 3.21, 2.62, 2.46 and 2.06, respectively. Thus MVAP was more in cases when compared to normal controls but the difference was statistically insignificant (Table 6).

Although it was not our aim to study the number and distribution of CD34-positive spindle shaped stromal cells, we observed characteristic pattern of CD34-positive stromal cells in OSF and controls. We observed uniform distribution of CD34-positive stromal cells in normal mucosa (Fig. 5). In OSF, juxtaepithelial region characteristically showed significantly decreased or complete loss of CD34-positive stromal cells compared to controls (Figs. 6–8).

**Discussion**

OSF is a unique chronic debilitating premalignant condition, which predominantly occurs in Indians and South East Asians. It is characterized by a mucosal rigidity of varying intensity due to
the fibroelastic changes of the juxtaepithelial layer, resulting in a progressive inability to open the mouth.1

Characteristic histopathologic features of OSF include submucous fibrosis associated with atrophic epithelium which is vulnerable to the effects of oral carcinogens.7 Although epithelium of OSF has been described as atrophic, no study has been conducted so far in the English literature to measure ET in OSF to label it as atrophic epithelium. We made a sincere attempt to measure ET in OSF, probably for the first time in the English literature. Histopathologically all the OSF cases showed reduced ET when compared to controls. Thus present study scientifically proves OSF epithelium as atrophic.

Till today no definite explanation has been given for the atrophic epithelium in OSF. It is mainly based on assumptions and logical deductions. The atrophic epithelium in OSF is explained to be the aftermath of the stromal changes, which undergo progressive hyalinization, decrease in vascularity and cellularity with resultant tissue ischemia. Thus the “atrophy” of the overlying epithelium was described as “ischemic atrophy”.8

The degree of vascularity of the diseased mucosa in OSF has always been a matter of dispute. Although angiogenesis cannot be measured directly, it can be inferred by quantification of vasculature, usually in the form of MVD, thus providing an index of angiogenesis.9–11

Microvascular density is the mean value of microvessel count, obtained using a specific objective magnification with known field diameter on a selected microscope in a limited number of fields (three or four), subjectively selected from the vascularized areas (hot spots). The generally acceptable criteria for a microvessel profile is an endothelial marker-stained cell or cluster, that is separate from adjacent microvessel profiles and present within the tumor but not in necrotic or sclerotic zones.12

CD34 (human hematopoietic progenitor cell antigen) is a 110-kDa transmembrane surface glycoprotein of unknown functions. It is expressed on hematopoietic stem cells, endothelium, the interstitial cells of Cajal and dendritic cells present in the dermis, around blood vessels, and in the nerve sheath. CD34 is also considered to be an important marker for tissue vascularization and represents microvascular density in the tissue.13 Since the expression of CD34-positive endothelial cells has shown an important role in understanding the process of angiogenesis in oral cancer and precancer,13 present study has characterized and quantified mucosal vasculature in OSF as MVD, MVLD and MVAP using immunohistochemical marker CD34 for the demonstration of endothelial cells and vascularity.

Our study showed increase in MVD in OSF cases (8.3 vessels) compared to normal (7.69 vessels), which was statistically insignificant. Thus present study did not show significantly decreased
vascularity of the underlying connective tissue in OSF compared to controls. In fact the amount of MVD for atrophic epithelium in OSF was much more than MVD for ET in controls. Thus it does not sound prudent to believe that epithelial atrophy in OSF is due to the result of poorly vascularized stroma, the aftermath of tissue ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy. Our findings are in accordance with Rajendran et al. who demonstrated more or less same ischemia and therefore ischemic atrophy.

In OSF, it is prudent to believe that the stromal alteration is ever present and is progressive as the disease advances. Various studies in OSF have demonstrated possible role of inducible nitric oxide synthetase (iNOS), basic fibroblast growth factor (b-FGF), transforming growth factor-\( \beta \) (TGF-\( \beta \)), platelet derived growth factor (PDGF), and hypoxia inducible factor – 1\( \alpha \) (HIF-1\( \alpha \)), all of which are known endogenous angiogenic promoters. All these growth factors may be playing an important role in maintaining the vascularity of underlying connective tissue in OSF, as demonstrated by CD34-positive endothelial lined blood vessels in the present study.

As such there is no definite explanation given for the atrophic epithelium in OSF, but recent study by Rajendran et al. has proposed the possible role of iNOS in atrophic epithelium of OSF by demonstrating the enhanced expression of iNOS in OSF mucosa. According to their study iNOS being a powerful cytotoxic and genotoxic agent probably exerts effect on epithelial keratinocytes and stromal cells leading to progressive cellular hypoplasia with lack of cellularity noticed in the stroma of advanced OSF.

Rajendran et al. has also demonstrated no morphologic evidence of increased cell death (apoptosis/necrosis) associated with atrophy in OSF epithelium, when compared to site equivalent healthy normal mucosa. This prompted them to consider an alternative hypothesis in favour of a reduced proliferation index of the adult stem cell compartment of the oral epithelium. They proposed a hypothesis of epithelial hypoproliferation, rather than atrophy, which causes thinning of surface epithelium in clinically advanced OSF.

Present study supports the view put forward by Rajendran et al. that as the stroma becomes more and more hyalinized due to progressive deposition and cross-linkage of mature collagen bundles, the tissue suffers resultant ischemia/hypoxia due to physical and biochemical effects of the process. Pursuing further the pathological mechanism, the tissue tries to cope up with hypoxia by actively promoting neo-vascularization as an adaptive response on the part of the mucosa in survival of the atrophic epithelium in OSF.

Tilakaratne et al. have hypothesized that dense fibrosis and less vascularity of the corium, in the presence of an altered cytokine activity creates a unique environment for carcinogens from both tobacco and areca nut to act on the epithelium. They assumed that carcinogens from areca nut accumulate over a long period of time either on or immediately below the epithelium allowing the carcinogens to act for a longer duration before it diffuses into deeper tissue. According to them less vascularity may deny the quick absorption of carcinogens into the systemic circulation. Our findings do not support their view of reduced vascularity in OSF. In fact presence of vascularity in OSF demonstrated by the presence of CD34-positive blood vessels may play an important role in tumor proliferation, once the malignant transformation takes place in OSF (Fig. 9).

In normal oral mucosa, CD34 is present in endothelial cells, in perivascular/interstitial dermis, and in spindle-shaped cells around the skeletal muscle fibres. Studies proposed that invasive carcinomas of oropharyngeal and laryngeal areas induce stromal remodeling characterized by loss of CD34-positive fibrocytes and subsequent gain of \( \alpha \)-SMA and TGF-\( \beta \)\( 1 \) positive myofibroblasts. Although it was not part of our study to observe the number and distribution of CD34-positive spindle shaped stromal cells, we observed characteristically decreased or complete absence of CD34-positive stromal cells, especially in the juxtaepithelial location in OSF, against the uniform distribution of CD34-positive stromal cells in normal mucosa. Probably, so far, no such observation has been documented in the literature.

\textbf{Figure 9} Schematic presentation of possible role of vascularity in proliferation of malignantly transformed OSF.
Some reports hypothesized that CD34-positive stromal cells may have role as antigen presently cells and multipotent mesenchymal cells. Some researchers confirmed distinctive association between increased density of stromal myofibroblasts and the development of carcinoma in human hypopharynx and in a rat 4NQO-induced tongue rat carcinogenesis model. Defining the mechanism of juxtaepithelial suppression of CD34-positive stromal cells in OSF is expected to broaden our knowledge on the microenvironment events occurring in OSF and during the malignant transformation of OSF.

In conclusion, the conventionally believed concept that epithelial atrophy in OSF is due to lack of perfusion, caused by decreased vascularity of subjacent connective tissue stroma does not hold true against findings of the present study. Characteristic pattern of juxtaepithelial loss of CD34-positive stromal cells in OSF deserves further attention.

Conflict of interest statement

None Declared.

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