Langerhans cell expression in oral submucous fibrosis: An immunohistochemical analysis

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Abstract

Introduction: Langerhans cells (LCs) are dendritic cells (DCs) of the epithelium which play a role in an array of oral lesions from gingivitis to oral cancer. Oral submucous fibrosis (OSMF), a potentially malignant disorder (PMD), is an insidious chronic disease with juxta-epithelial inflammatory changes leading to fibrosis. LCs may play a part in the ongoing inflammatory dysregulation of OSMF.

Objective: The study was aimed at elucidating the distribution of LCs in varying grades of OSMF.

Materials and Methods: A retrospective study using 18 cases of OSMF, graded using haematoxylin and eosin (H&E)-stained section. Immunohistochemistry was performed using polyclonal anti-CD1a antibodies to identify LCs in six cases of normal tissue and 18 samples of OSMF. The distribution of LCs among the various grades and normal mucosa analysed using Student's t-test.

Results: LC population in the OSMF was significantly higher when compared to the normal epithelium ($p < 0.001$). Within the grades, the advanced stage had more LCs than the other stages.

Conclusion: The increase in LCs might indicate the role of antigenic exposure in turn leading to cell-mediated immunity in OSMF. Thus, the fibrosis in OSMF might have a direct link to LCs.

Keywords: CD1a, cell-mediated immunity, dendritic cell

INTRODUCTION

The term 'dendritic cell' (DC) refers to a family of antigen-presenting cells, including Langerhans cells (LCs), which was coined by Steinman and Cohn.[1] They represent a large family of antigen-presenting cells that circulate through the bloodstream and are scattered in nearly all tissues of the body. DCs have a powerful capacity to activate immunologically naïve T cells in an antigen-specific way.[2] Three major DC subsets have been described in the peripheral tissues of humans, including two in the myeloid lineage—LCs and interstitial DCs (also known as dermal DCs), and the third being lymphoid or plasmacytoid DCs.[3]

The LCs were discovered by Paul Langerhans' in 1868, a medical practitioner in Berlin. He made these cells visible by means of gold chloride technique.[4]

LCs originate from bone marrow precursors, which upon circulation in the peripheral blood, populate in the skin.

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CD34+ haematopoietic progenitor cells have now been identified as the cells committed to the LC lineage.[5]

LCs *in situ* possess between 5 and 9 dendrites that extend out in the same horizontal plane and cover about 25% of the surface area of the skin and mucosa.[6]

Characteristic feature is the presence of Birbeck granules (100 nm to 1 μm in size), which appear either as rod-shaped bodies or, if the terminal vesicle is present, as the classic tennis-racket shape.[7] First described in LCs by Birbeck *et al*.[8] LCs are not evident in the epithelium by routine haematoxylin and eosin staining. The criteria for recognition and identification of LCs include a clear cytoplasm devoid of tonofilaments, desmosomes or melanosomes; a lobulated, frequently convoluted nucleus; and the presence of distinctive trilaminar cytoplasmic organelle, termed LC granule or Birbeck granule, thus commonly described as dendritic, non-keratinocytic clear cells.

Two types of LCs have been described based on their electron microscopic appearance: Type 1 is highly dendritic with an electronlucent cytoplasm, numerous granules and is usually found in the suprabasal layers; Type 2 shows fewer dendrites, a more electron-dense cytoplasm with fewer Birbeck granules and is usually located in the basal layer.[9]

Apart from occasional occurrences at extraepithelial sites (dermis, dermal lymphatics, aortic wall, lymph nodes, thymus), LCs are essentially confined to stratified squamous epithelia, ranging an average number of 160–550 cells/mm². Nonkeratinized mucosa had the highest counts, with a mean count of 508 ± 110 LCs/mm², whereas keratinized mucosa of the hard palate reported to have the lowest density. LCs constitute 2–4% of total epidermal cell population.[10] Considering marked variation in LCs distribution in the oral mucosa, it has been found that LCs in buccal mucosa were predominantly found in a suprabasal location, and in hard palate, the cells were predominantly found in the stratum spinosum.[11]

Recent studies have highlighted mucosal LCs as important determinants of mucosal immunity, in response to antigen of microbial or tumour origin, but also of tolerance to self-antigen and commensal microbes.[3]

Inflammation, infection or injury provokes extensive migration of LCs from the epidermis, generating an empty niche that is repopulated. LCs are able to migrate to draining lymph nodes. Keratinocytes that are stressed during inflammation, rapidly upregulate ligands for the lymphocyte activation receptor natural killer group 2D (NKG2D), resulting in migration of LC populations out of the epidermis.[12] In the oral cavity, they are associated with the immunopathogenesis of various lesions such as gingivitis and periodontitis, oral lichen planus (OLP), contact hypersensitivity, recurrent aphthous Stomatitis and a plausible role in oral cancer has also been demonstrated.[13]

Oral submucous fibrosis (OSMF) is a premalignant disorder associated with the chewing of areca nut (betel nut). The habit is prevalent in South Asian populations but has been recognized nowadays also in Europe and North America. OSMF causes significant morbidity.[14]

OSMF is invariably associated with an inflammatory process which causes the release of fibrogenic cytokines such as TGF-β, IL-6, TNF and IF-α. This is primarily due to the presence of activated T lymphocytes.[15]

Haque *et al*.[16] provided direct evidence for an ongoing cell-mediated immunity in OSMF.

Increased DCs also noted in the epithelium of OSMF patients. Chiang *et al*.[17] further established the role of cell-mediated immunity in the pathogenesis of OSMF by demonstrating an increased number of T cells over B cells and also found that CD4+ cells were significantly higher than the CD8+ cells.

Bharghavi Narayanan and Malathi Narasimhan in their study showed a definitive increase in LCs expression in the OSMF.[18]

The increased DCs and cytokines suggest that LCs might recognize the unknown antigen in OSMF which migrates with the support of chemokines expression through the lymphatics and promote CMI.

Therefore, we aimed at evaluating the distribution of LCs in various stages of histopathologically diagnosed OSMF as LCs are involved in stimulating T cell reaction.

Principle of the study: T cells on stimulation release various cytokines such as IL-6, TNF and IF-α and growth factors like PDGF and TGF-β[19,20] leading to fibrosis.[21] This will aid in the etiopathogenesis and thus can help in the diagnosis of OSMF.

MATERIALS AND METHODS

Eighteen patients previously clinically and histopathologically diagnosed as OSMF and six patients with normal mucosa

diagnosed histopathologically were retrieved randomly from the archives of the Department of Oral Pathology, after obtaining ethical clearance from Institutional Ethical Committee—Indira Gandhi Government Dental College, Jammu, UT of J&K, India. The control samples were collected from apparently normal buccal mucosa and histopathologically confirmed for the absence of any immunological reaction. The OSMF cases were graded according to Pindborg JJ and Sirsat SM staging[22] as, very early stage, early stage, moderately advanced stage and advanced stage using haematoxylin and eosin-stained sections. Very early and early stages were grouped together as early stage and moderately advanced stage and advanced stage were grouped together as advanced stage. Case of histiocytosis was selected as the immunohistochemistry protocol control.

Immunohistochemistry

(Sample size was statically calculated using GPOWER software)

Paraffin-embedded specimens were sectioned of 4µm thickness on charged slides, placed in warmer at 60°c for 5 minutes, followed by deparaffinization in two changes of xylene for five minutes each. After this three changes of alcohol, each for five minutes was performed. Heat-induced antigen retrieval was done. The staining procedure was according to manufacturer's protocol as described below. Tris buffer wash was performed thrice for five minutes each; it was followed by peroxide block for five minutes. After Tris buffer washes, superblock was applied for fifteen minutes which was followed by incubation of primary antibody, polyclonal anti-CD1a antibody for thirty minutes. Anti-polyvalent HRP polymer (secondary antibody) incubated for one hour followed by three Tris buffer washes for five minutes each followed for incubation with DAB substrate and DAB mixture for one minute. It was counterstained by Harris Haematoxylin. Case of histiocytosis was selected as the immunohistochemistry protocol control.

Evaluation

The stained slides were viewed by two investigators and analysed under light microscope to eliminate inter-observer bias. Using a five-header microscope, the disagreements were resolved. Brown surface-stained cell was taken as positive for CD1a as CD1a is a surface marker. The number of LCs per high-power field (400x) was counted from six fields in the varying layers of epithelium, and the average LCs in a high-power field were calculated for each of the sections. The presence of LCs if present in the connective tissue was also noted.

Statistical analysis

Statistical analysis was performed using Student's t-test to determine the difference in the number of LCs between normal epithelium and OSMF tissues and for comparison between early and advanced stages, respectively.

RESULTS

The OSMF sections were graded with H and E sections, by two observers to overcome inter-observer variation, out of which 9 were early and 9 advanced stage of OSMF. The protocol control– LCH tissue showed positivity in the positive control [Figure 1a and b]. In the normal epithelium (study control), the LCs stained by CD1a antibody were found to be restricted to the suprabasal layer with an average cell count of 1 per high-power field [Figure 2a and b]. In OSMF tissues, there was a considerable increase in LCs with an average cell count of 10 cells per high-power field in the 18 cases. There was a significant difference in the number of LCs between normal and OSMF tissues (p value < 0.001) [Table 1 and Figures 2a and b, 3a and b, 4a and b]. The cells were distributed suprabasally, in spinous layer and few in the superficial layers. Morphological alterations were the presence of a prominent dendritic morphology of the cells appeared in OSMF tissues when compared to normal tissues. The cell count distribution in the various stages of OSMF is tabulated [Table 2 and Figures 2a and b, 3a and b, 4a and b].

Few early and mostly the advanced stage of OSMF showed the highest number of LCs. In the advanced cases, the CD1a positive cells were also found in the connective tissue which showed characteristic dendritic morphology. These cells were predominantly present in the juxta- epithelium [Figure 5a and b]. When the LCs expression was compared between two groups, early with advanced grade was significant with *P* value <0.001 using Student's *t*-test.

DISCUSSION

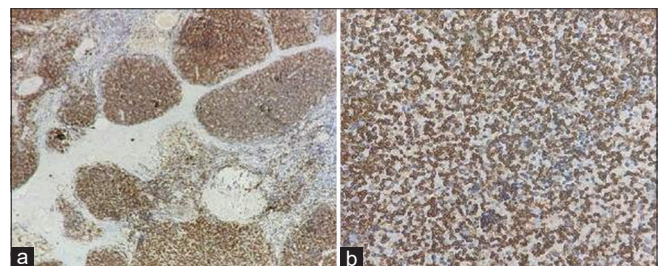Oral submucous fibrosis (OSMF) is a potentially malignant

Figure 1: (a and b) Positive control CD1a, 10X and 40X

disorder (PMD) which was described by Schwartz in 1952 as 'Atropica idiopathica mucosae oris' and later by Jens J. Pindborg in 1966 as 'an insidious chronic disease affecting any part of the oral cavity and sometimes pharynx. It is associated with juxta-epithelial inflammatory reaction followed by fibroelastic changes in the lamina propria layer, along with epithelial atrophy which leads to rigidity of the oral mucosa proceeding to trismus and difficulty in mouth opening.[23]

OSMF is multifactorial in origin with etiological factors are areca nut, capsaicin in chilies, micronutrient deficiencies of iron, zinc and essential vitamins. Autoimmune etiological basis of disease with the demonstration of various autoantibodies with a strong association with specific human leukocyte antigen (HLA) antigens has also been suggested.[24]

Table 1: Expression of CD1a in normal mucosa and OSMF tissues

Group	Mean	Standard deviation (SD)	Variance	't'	P
Normal (*n*=6)	5.16	±1.940	3.76	0.84	0.219
OSMF (*n*=18)	58.21	±33.19	1102.18	5.510	0.000023**

**P < 0.05 Significant

Table 2: Distribution of Langerhans cells (LCs) in varying grades of oral submucous fibrosis (OSMF)

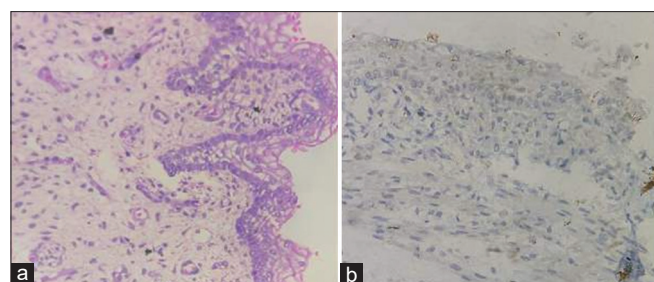| Group	Mean	Standard deviation (SD)	Variance	't'	P
Early OSMF (*n*=9)	32.1	±7.007	49.1	5.510	0.000024**
Advanced OSMF (*n*=9)	84.33	±27.54	7.59		

**P < 0.05 Significant

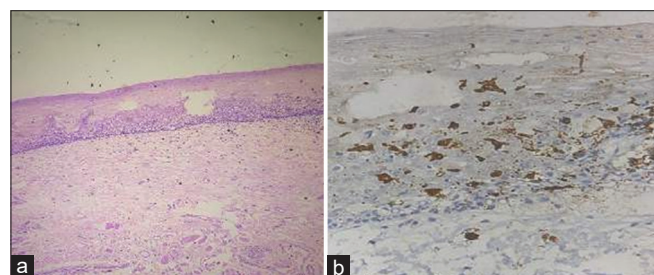**Figure 2:** (a and b) Normal oral mucosa, H&E and CD1a

Figure 4: (a and b) Advanced stage of OSMF, H&E(10X) and CD1a(40X)

OSMF bears an ongoing immunological process which leads to altered release of various cytokines effectively causing dysregulation in the collagen formation and degradation. In this study, we have aimed to establish the role of antigen-presenting cells which plays a pivotal role in initiating and modulating immunity.[18]

Haque *et al.*[16] demonstrated the predominance of CD3 and CD4 positive cells. A striking finding was the presence of HLA-DR DCs in the epithelium and lamina. These dendrites appeared to face the epithelium simulating a picture of an underlying antigenic response. Our study showed increase in the LCs in the OSMF epithelium when compared to normal epithelium with active dendritic morphology in OSMF tissues which further emphasizes the role of T cell immunity in OSMF.

Chiang *et al.*[17] demonstrated increase in T cell density in moderately advanced cases than the advanced cases which was comparable to the normal tissue in the connective tissue. In our study, there was the presence of interstitial LCs in the connective tissue indicating an ongoing immune response in the advanced cases and a role of an unknown antigen eliciting the response that can aid to explain the increase in density of T cells in this stage.

On the contrary, their study showed similar density of T cells in both the stages in the epithelium. Our study showed an increase in LCs in the advanced stage than the early stage which might be attributed to the continuous antigenic
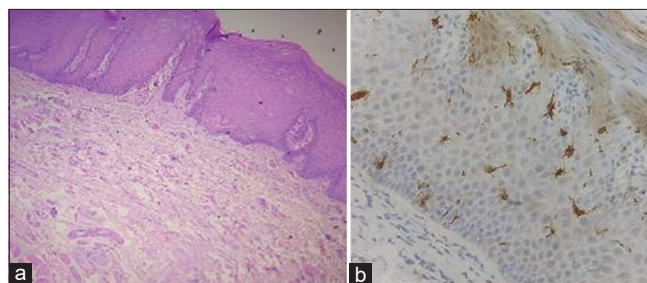

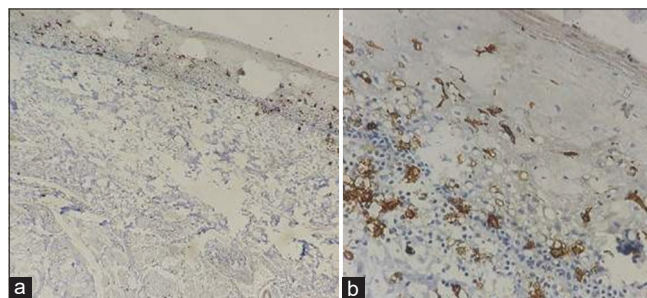**Figure 3:** (a and b) Early stage of OSMF, H&E(10X) and CD1a(40X)

Figure 5: (a and b) Advanced stage of OSMF, CD1a (10X) and CD1a(40X)

exposure. Though LSs release IL-1α leading to increase in collagenase,[25] the simultaneous activation of T cells topples the effect by the release of fibrogenic cytokines.

Haque *et al*.[16] verified that OSMF tissues showed high localization of IL-6, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and IL-1. An altered cytokine profile in peripheral blood of OSMF patients where an increased IL-6, IL-8, IL-1β and TNF-α than in normal individuals was also demonstrated[20], thereby suggesting their role in altered collagen metabolism.

Niessen *et al*.[26] demonstrated an abnormal increase of LCs in the hypertrophic scars, and hypertrophic scars remaining hypertrophic even after 12 months of follow-up suggesting their role in scar tissue formation by altered matrix production. In a similar way, an aberrant epithelial regulation indicated by the marked raise in the LCs in OSMF as seen in our study, either through the keratinocytes or directly on the fibroblasts, has a pivotal role in fibrosis.

Yong Xie *et al*.[27] demonstrated increased active LCs expression in cases of localized scleroderma suggesting that LCs might play a paramount role in this autoimmune disorder. An autoimmune aetiology has also been suggested in OSMF as autoantibodies in the sera of patients have been reported.[15]

Silva LC *et al*.[28] investigated the distribution of immature DCs, LCs and plasmacytoid DCs in OSMF. OSMF is associated with oral squamous cell carcinoma (OSMF-OSCC), oral leukoplakia (OL) and oral squamous cell carcinoma (OSCC). They demonstrated decrease in the number of CD1a+ and CD207+ cells may be associated with the development of oral OSCC, and in OPMDs and might be indicators of malignant transformation.

On analysing the results of the study, it can be concluded that LCs may play a role in the fibrosis in OSMF directly or indirectly. The limitations of the study include lack of information on the duration and constituents of the deleterious habits and usage of a purely histopathological staging for the analysis. Role of LCs has been discussed in state of dysplasia and OSCC where there is increase in LCs expression in the submucosa, whereas negative correlation has been noted in the higher grades of OSCC. This illustrates that LCs play a role in presenting the altered antigens in dysplastic cells and the decrease in the immune status in OSCC patients in turn diminishes the Langerhans activity.[29-31]

Neoplastic transformation of epidermal LCs caused by dysfunctional immune activation leads to the pathogenesis of Langerhans cell histiocytosis (LCH).[32]

CD1a+and CD207+ cells LC also appear to be essential in immunopathogenesis oral lichen planus (OLP) and oral lichenoid lesions (OLL).[33]

Due to the malignant potential of OSMF, observation of changes of LCs in these lesions may throw an insight in the malignant transformation but there is no literature evidence to directly suggest the LCs expression in OSCC transformed from an OSMF.

CONCLUSION

Our study showed increase in LCs expression in the OSMF which can either suggest a role LCs to alter the epithelial characteristics and alter the submucosal matrix deposition or act as only as APC cells to unknown antigens or have any direct influence on the fibroblasts, but the mechanism still requires clarification. LCs have a significant immunological role in most of the oral lesions. LCs act as immune mediator cells, tumour cells, vectors of infected cells and phagocytic cells. This wide range of functions of LCs creates immense scope for further research to ascertain the precise role of LCs in various oral lesions in the coming years.

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Conflicts of interest
There are no conflicts of interest.

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