Brief Report

Quantitative and Qualitative Analysis of Mast Cells in Oral Lichen Planus and Its Effect on Basement Membrane Using Special Stains

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Abstract

Background: Oral lichen planus (OLP) is characterized histologically by epithelial basal cell destruction and a dense subepithelial lymphocytic infiltrate. Mast cells (MCs) play a role in the pathogenesis and progression of the disease causing changes in the basement membrane (BM). BM is seen as continuous or fragmented, distinct or indistinct, and afibrillar or fibrillar extensions. **Aims and Objectives:** This study was done to demonstrate the BM using acriflavine stain in addition to hematoxylin and eosin (H-E) stain. An attempt was also made to study MC using Azure A stain and assess the degree of changes in the thickness of BM associated with degranulated MC in patients with OLP. **Materials and Methods:** A total of 66 paraffin-embedded tissue sections which included 30 inflamed gingival mucosa (IGM) and 36 OLP were stained with H-E stain, Azure A, and fluorescent periodic acid–acriflavine stain. **Results:** MC density was higher in OLP when compared with MC in IGM. Degranulated MCs were found in abundance in OLP. Thickness of BM was significantly less in OLP when compared with IGM. Significant fragmentation was seen in OLP when compared with BM of IGM. **Conclusion:** Degranulated MC in OLP may or may not alter the quality of BM but definitely seems to influence the thickness of the BM both directly and indirectly.

Keywords: *Azure A, basement membrane, fluorescent microscope, fluorescent periodic acid–acriflavine, mast cells, oral lichen planus*

Introduction

Oral lichen planus (OLP) presents as white striations, plaques, erosions, or blisters affecting predominantly the buccal mucosa, tongue, and gingiva.[1] Basement membrane (BM) appears to be crucial in tumor invasion and metastasis and its loss has been associated with many types of carcinomas, and with tumor cells in lymph node and organ metastases.[2] Using electron microscopy and immunohistochemistry, discontinuities, duplication, thickening, and intensive and/or absent staining of the BM have been identified.[3,4]

In OLP, the basal cell layer shows a liquefactive degeneration with a narrow band of eosinophilic material in the position of BM. There is a well-defined zone of cellular infiltrate that is confined to the superficial part of the connective tissue, and the infiltrate consists mainly of lymphocytes except in the viscinity of erosion.[5]

Mast cell (MC) degranulation in OLP releases a range of proinflammatory mediators such as tumor necrosis factor (TNF), chymase, and tryptase which are implicated as having a role in causing basal cell apoptosis and epithelial BM disruption through various mechanisms.[1] MCs are characterized by cytoplasmic granules which are rich in heparin and are demonstrated metachromatically by the thiazin group of dyes. Apart from the routine toluidine blue for MC, Azure A are cationic dyes that typically stain tissues blue. Azure A exhibits a blue orthochromasia and a purple metachromasia. It identifies the charged mucin and proteoglycans thereby giving a marked contrast color for intact and degranulated MC.[6]

Special stain such as periodic acid–Schiff (PAS) is one of the common staining methods used to stain the BM. But for an enhanced color contrast and structure details, a not so common Fluorescent periodic acid–acriflavine staining can be used.[7]

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Hence, THIS study was performed to demonstrate the BM in OLP using acriflavine stain in addition to hematoxylin and eosin (H-E) stain. An attempt was also made to study MC using Azure A stain and assess the degree of changes in the thickness of BM associated with degranulated MCs in patients with OLP.

Materials and Methods

The study group included 30 cases of inflamed gingival mucosa (IGM) and 36 cases of OLP. IGM was histopathologically diagnosed as normal gingival mucosa with subepithelial inflammatory infiltrate. A total of 66 paraffin-embedded tissue blocks were retrieved from the archives for this study. Institutional ethical clearance was obtained before commencement of the study. Two sections of 4-µm-thick paraffin-embedded sections were taken and stained with Azure A and fluorescent periodic acid–acriflavine[8] which stained MCs purple and the BM a golden yellow, respectively [Figure 1].

MCs were analyzed quantitatively by counting the number of intact and degranulated MC. The Azure A–stained sections were first screened at low power (×10). Three high-density inflammatory areas were selected, and software grid (10 × 10) was created with an area of 0.04 mm^2 and was calibrated. The cells were counted throughout each of the tissue sections in three representative and consecutive grid fields (×40 magnification).

The mean of values was calculated and expressed as mean ± standard deviation (SD)/mm^2. The fields were studied in a step ladder fashion and care was taken to prevent the overlapping of fields.

Serial section was stained with acriflavine, and similar focus was viewed for the study of BM. Quantitative analysis for the thickness of BM and qualitative analysis were determined for continuity, contrast, and pattern. The use of fluorescent microscopy to identify the BM in fluorescent periodic acid–acriflavine-stained sections is not only responsible for its specificity but also increases the method's sensitivity and resolution. All the slides were scanned under ×40 for BM analysis. Stained sections were evaluated by three oral pathologists independently, and consensus was obtained when required. The slides were analyzed for BM in IGM and OLP, and the following parameters were considered:

1. Continuity: BM was continuous or fragmented
2. Contrast: BM was distinct or indistinct
3. Type: BM was fibrillar or afibrillar.

Descriptive and inferential statistical analyses were carried out in this study. Chi-square analysis was used to find the significance of study parameters on categorical scale. Student's *t*-test (two-tailed, unpaired) was used to find the significance of study parameters on continuous scale between two groups. Correlation coefficient was computed to measure correlation between thickness of BM with total MC, intact and degranulated MC, and total MC with continuity, contrast, and pattern of BM. The statistical software IBM SPSS statistics 20.0 (IBM Corporation, Armonk, NY, USA) was used for analyses of data.

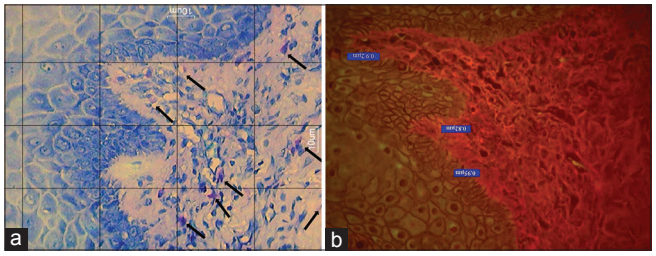

Figure 1: (a) Photomicrograph of oral lichen planus stained with Azure A showing numerous mast cells (×40). (b) Photomicrograph of oral lichen planus stained with fluorescent microscopy acriflavine stain showing reduced thickness of basement membrane (×40)

Results

In the study group, the mean total MC (8.72 ± 2.53) was higher when compared with MC in the control group (0.45 ± 0.30). The mean intact MC (0.68 ± 0.44) was higher when compared with intact MC in the control group (0.29 ± 0.18). In the study group, the mean degranulated MC (8.04 ± 2.49) was higher when compared with degranulated MC in the control group (0.16 ± 0.18). In the study group, the mean thickness of BM (0.71 ± 0.39) was lower when compared with thickness in the control group (2.28 ± 2.98).

A comparison of continuity of the BM between the groups showed that in the control group, BM was continuous in 100% of the samples. In the study group, BM was fragmented in 83.3% of the sample and continuous in 16.7% of the population. This difference was found to be statistically significant ($P < 0.001$) using Chi-square test [Table 1 and Figure 2].

Table 1: Comparison of status of basement membrane (continuous vs. fragmented) among the groups using Chi-square test

Group	Continuity		Total
	Fragmented	Continuous	
Control group			
Count	0	30	30
Percentage within group	0.0	100.0	100.0
Study group			
Count	30	6	36
Percentage within group	83.3	16.7	100.0
Total			
Count	30	36	66
Percentage within group	45.5	54.5	100.0
χ^2, *P*	45.833, <0.001**		

**$P<0.001$ – highly significant

In the control group, contrast of the BM was indistinct in 20% of the population and distinct in 80% of the population. In the study group, contrast of the BM was indistinct in 72.2% of the population and distinct in 27.8% of the population. This difference was found to be statistically significant ($P < 0.001$) using Chi-square test [Table 2 and Figure 3].

In the control group, the pattern of the BM was afibrillar in 93.3% of the population and fibrillar in 6.7% of the population. In the study group, the pattern of the BM was afibrillar in 19.4% of the population and fibrillar in 80.6% of the population. This difference was found to be statistically significant ($P < 0.001$) using Chi-square test [Table 3 and Figure 4].

Finally, when a correlation of total MC was done with continuity, contrast, and pattern of BM using Pearson's correlation coefficient, it was observed that there was a weak positive correlation between continuity of BM and total MC ($r = 0.182$; $P = 0.288$), contrast of BM and total MC ($r = 0.067$; $P = 0.699$), and a strong negative correlation between pattern of BM and total MC ($r = -0.580$; $P < 0.001$) [Table 4].

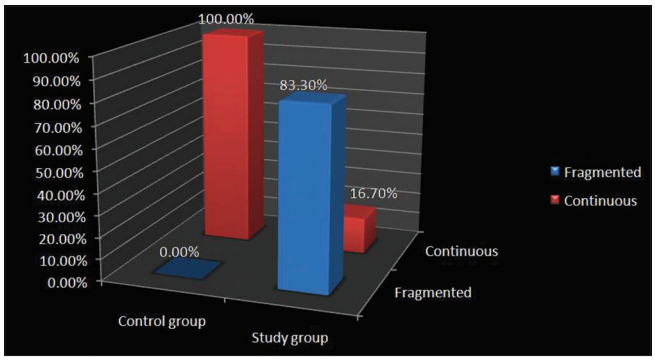

Figure 2: Graph 1 – comparison of continuity among both the groups using Chi-square test

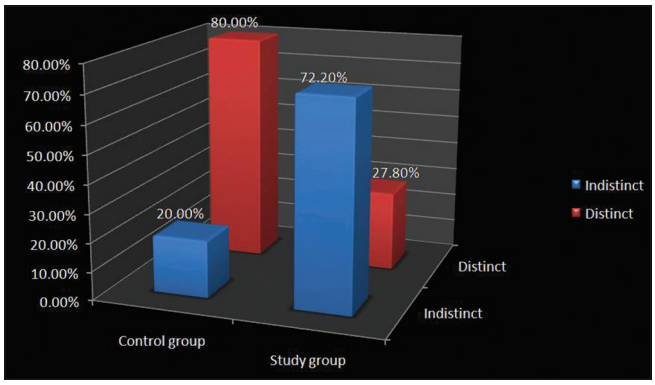

Figure 3: Graph 2 – comparison of contrast among both the groups using Chi-square test

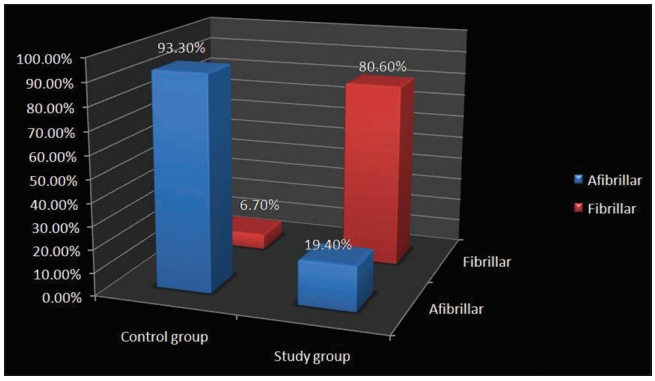

Figure 4: Graph 3 – comparison of pattern among both the groups using Chi-square test

Table 2: Comparison of contrast of basement membrane among the groups using Chi-square test

Group	Contrast		Total
	Indistinct	Distinct	
Control group			
Count	6	24	30
Percentage within group	20.0	80.0	100.0
Study group			
Count	26	10	36
Percentage within group	72.2	27.8	100.0
Total			
Count	32	34	66
Percentage within group	48.5	51.5	100.0
χ^2, P	17.867, <0.001**		

**P<0.001 – highly significant

Table 3: Comparison of pattern of staining of basement membrane (fibrillar vs. afibrillar) among the groups using Chi-square test

Group	Pattern		Total
	Afibrillar	Fibrillar	
Control group			
Count	28	2	30
Percentage within group	93.3	6.7	100.0
Study group			
Count	7	29	36
Percentage within group	19.4	80.6	100.0
Total			
Count	35	31	66
Percentage within group	53.0	47.0	100.0
χ^2, P	17.867, <0.001**		

**P<0.001 – highly significant

Table 4: Correlation of total mast cells with continuity, contrast, and pattern of basement membrane using Pearson's correlation coefficient

Basement membrane	Total mast cells	
	R (correlation coefficient)	P
Continuity	0.182	0.288
Contrast	0.067	0.699
Pattern	−0.580	<0.001**

**P<0.001 – highly significant

Discussion

OLP is considered to be a disease of unknown etiology that affects the oral mucosa and is characterized by periods of exacerbation and remission. A complex series of events are purported to cause the initiation and perpetuation of this condition.[9]

MC products which are part of nonspecific mechanisms proposed in the development of OLP have been suggested to bring about structural changes in the epithelium and connective tissue in lesions of lichen planus, and the close association of these cells with T-lymphocytes has added impetus to the concept that these cells could be responsible for the chronicity of these lesions. In this study, an attempt has been made to evaluate the MC and its effect on BM. A morphometric analysis was done, as it is able to determine the parameters used in quantitative histopathological diagnosis, as a complement to basic qualitative diagnosis, and thus offering impartial assessment.

It has been substantiated that MC and T-lymphocytes behave in a bidirectional manner, thus influencing each other in various aspects.[10] A predominance of connective tissue MC has been found in OLP by various investigators who suggested that they could be involved in the pathogenesis of lichen planus.[11,12]

Following degranulation, MC mediators are deposited in large quantities in the extracellular environment, where they exert effects on endothelial cells and other cell types. MC may subsequently synthesize and secrete additional mediators that are not preformed in their granules. Key mediators that are preformed in MC are serine proteases tryptase, chymase, cathepsin G, histamine, heparin, serotonin acid hydrolases, and cytokine TNF-α and interleukin-16.[10]

When activated, MCs may either undergo explosive degranulation and then resynthesize their granules, or they may release solitary granules into their environment on an ongoing basis, a process termed "piecemeal degranulation" that has been observed in both the oral mucosa and skin.[11,12] An important interaction between MC and other cell types is that of antigen presentation to T-lymphocytes. While MCs are not "professional" antigen-presenting cells, antigen presentation and costimulatory signals delivered by MC may contribute to the development of a specific T-lymphocyte response in the induction phase of inflammation, in conditions such as OLP.[13] Walsh *et al*. suggested that cytokines released by tissue MC may be the trigger for induction of vascular adhesion molecules to allow the entry of MC to extravascular compartment.[12]

MCs in normal buccal mucosa have been reported to be distributed preferentially. Many studies have indicated a significantly low count of MC in normal oral mucosa. Lagdive *et al*. also proposed an increase in the number of MC in IGM when compared with periodontally healthy gingiva.[14] In this study, IGM was taken as the control group because of the presence of increased amount of MC when compared with normal healthy gingiva, and there is no change in the thickness of BM along with continuity, contrast, and pattern.

Zhao *et al*. in 2001 conducted a study of MC in OPL and concluded that approximately 60% of MCs were degranulated in OLP, compared with 20% in normal buccal mucosa.[15]

In this study, the total MC was higher in the study group when compared with MC in the control group and this was found to be statistically significant. A count of the number of intact MC and degranulated MC was also found to be highly significant which is consistent with other studies till date.

Hall WB reported the lining of MC along the BM in cases of OLP. This lining of MC along the BM has been thought to be a response to external agents or antigenic stimuli, to release histamine. In this study, MCs were evaluated quantitatively and seen juxtraepithelially except in cases of severe inflammation.[5] El-Labban NG, in their ultrastructural study, found that the lamina densa appeared intact and is of a normal thickness, but often defects were seen of variable sizes. Also free fragments of lamina densa were seen in the lamina propria.[16] Jahanshahi *et al*. did a comparison of OLP with lichenoid reactions and found an inverse relationship in the ratio of degranulated MC with mean of BM thickness in OLP group. As this ratio increased, BM thickness decreased. Discontinuities of BM in OLP have been associated mainly with matrix metalloproteinases (MMPs) secreted by T cells and macrophages and are further enhanced by MC chymase and tryptase together with T-cell-derived MMPs that degrade BM structural proteins.[17] In this study, the thickness of BM was less in the study group when compared with the control group and the difference was statistically significant. In addition, fluorescent acriflavin staining demonstrated BM areas with clarity. OLP group showed a continuous thin, linear band of BM in some areas, while other areas showed fragmented numerous strands extending into the connective tissue. This study was consistent with the study done by Zhou *et al*. and Jahanshahi *et al*.[15,17]

Jose *et al*.[18] stated that MCs that migrated from blood vessels in the deeper connective tissue to the extravascular compartment subsequently moved toward the subepithelial zone, where they exerted their biologic effect on the blood vessels and helped in recruitment of inflammatory cells to the lesional area. Thus, MC has two types of action on BM. A direct effect on BM by the release of chymase and an indirect biological effect on blood vessel by recruiting inflammatory cells and activating MMP-9 secreted by OLP lesional T-cells.

In this study, it was observed that when there is a severe inflammatory infiltrate, degranulated MCs seen below the BM are less in number and they are seen away from the inflammatory band namely, more toward the blood vessels in deeper tissues.

Pujar *et al*.[7] compared the efficacy of H-E, PAS, and fluorescent PAS–acriflavine techniques for demonstration of BM in OLP. When they evaluated contrast of BM stained with acriflavine, it was seen that a better contrast of BM was seen in acriflavine (70%) followed by PAS (60%). When evaluated for contrast with control group, OLP (80% of cases) showed better contrast of BM. In this study, contrast of the BM in the study group was indistinct in 72.2% of the population, and in control group contrast of the BM was distinct in 80% of the population. These were found to be statistically significant.

Sime *et al*.[19] stated that MCs adhere to and migrate on BM laminins (LM-332 and LM-511 through $\alpha3\beta1$ integrin). These laminin isoforms may contribute to migration and localization of MCs within tissues. Interaction of MC ($\alpha3\beta1$ integrin) with laminins ($\alpha3$ and/or $\alpha5$) may partially explain the characteristic tissue distribution of MCs, particularly their intimate association with BM of epithelia (skin and mucosa). Accordingly, MC binding to laminins through $\alpha3\beta1$ integrin, and MC secretion of laminin-511, may not only contribute to wound healing and host defense against infections but also to allergic, autoimmune, or other inflammatory disorders, and even malignancy.

In this study, correlation of total MCs with continuity, contrast, and pattern of BM was also evaluated using Pearson's correlation coefficient in both study group and control group.

When continuity and contrast were correlated with total MC in the study group, an insignificant result was obtained. However, a strong positive correlation and a highly significant *P* value were obtained between pattern and total MCs. MCs have been previously regarded as having a role as a mediator during the inflammatory process which takes place in OLP.[20]

This study found that degranulated MCs in OLP may or may not alter the quality of the BM but definitely seem to influence the thickness of BM both directly and indirectly.

The role of MC in OLP and its effect on BM was well understood from this study, but the depth and severity of inflammation should have also been assessed to correlate its effect on BM and also on MCs.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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