VALUATION OF MAST CELLS IN ORAL EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMA STAINING WITH TOLUIDINE BLUE STAIN: A HISTOCHEMICAL STUDY

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ABSTRACT

INTRODUCTION

Mast cells present in the connective tissue stroma release pro inflammatory and mitogenic cytokines. The mast cell in tumor act as a host immunologic anti tumor response. More than 90% of oral cancers are squamous cell carcinomas with oral epithelial dysplasia being the most common potentially malignant disorder. Mast cells play an important role in tumourigenesis. In some studies, with different granules of mast cell is closely related with angiogenesis and tumor invasion. In this study we used toluidine blue for the staining because it reveals mast cells as large, purple, oval and granulated cells.

MATERIAL AND METHODS

30 cases of oral epithelial dysplasia, 30 cases of Oral squamous cell carcinoma (OSCC) and 10 cases of Normal oral mucosa (NOM) were studied for mast cell number using toluidine blue. Mast cells were counted using an Olympus CX41 microscope fitted with an Olympus oculometer grid. Counting was carried out at ×40 by two independent observers and was done in six non overlapping fields in each slide. Mast cells were identified on the basis of the purple color attained by the granules after toluidine blue staining, but the nucleus of these cells appeared blue.

RESULTS

Highly significant increase of mast cells in oral epithelial dysplasia on comparison with OSCC whereas there was only a significant increase in mast cells in OSCC on comparison with NOM.

CONCLUSION

Mast cells can be used an indicator of increased angiogenesis and can help in the prediction of carcinogenesis, its progression, and also in the prognosis of the malignant lesions

KEYWORDS

Mast cells, oral epithelial dysplasia, oral squamous cell carcinoma, toluidine blue

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INTRODUCTION

Mast cells are heterogeneous group of immune cells involved in multiple biological events. The multiple biologic functions of mast cell appear to be mediated by the variety of active molecules.[1] Mast cell was identified and named by Paul Ehrlich in 1878.[2] Mast cell are present in almost all of the major organs and tissues of the body.[3] The presence of mast cell in tumor has been explained as evidence of a host immunologic anti tumor response and if they are abundant the prognosis is good.[2] On the other hand, mast cells have also been implicated in the pathophysiology of many diseases, including allergy, asthma, anaphylaxis, gastrointestinal disorders, many types of malignancies, and cardiovascular diseases.[4] Oral leukoplakia, oral submucous fibrosis, and oral lichen planus are some potentially malignant lesions and conditions of oral cavity, whereas oral squamous cell carcinoma (OSCC)is a malignant lesion of the oral cavity.[5] The development of cancer in the oral mucosa occurs in two steps initiated by a potentially malignant disorder that is subsequently followed by oral cancer. Oral leukoplakia a well known potentially malignant disorder has a malignant transformation rate of 3.6 -17.5%.[6]

The surrounding stroma of the tumour is gaining importance because of its growth and diffusion, with the inflammatory cell infiltrate being actually responsible for cancer progression.[7] Mast cells, the local residents of the connective tissue, are associated with the release of certain kind of pro inflammatory and mitogenic cytokines. These substances when released from the mast cells may play a significant role in the pathogenesis of diseases.[5]

This is a characteristic trait of mast cell activation in chronic inflammatory settings, like cancer for instance and could aggravate the tumour growth. However, mast cells are also found to be helpful in tumour inhibition as the tumour-stroma microenvironment could alter the phenotypic behaviour of mast cells.[8]

Comprehending mast cells function in cancer progression cannot only improve prognosis but can also develop certain therapeutic methods that target mast cells. Therefore, the present study was undertaken to compare the mast cell count in normal oral mucosa, leukoplakia and OSCC and to evaluate the possible role of mast cells in carcinogenesis.

MATERIAL AND METHODS

The tissue specimens for the present study included 70 formalin fixed paraffin embedded tissue blocks comprising 30 cases of OSCC, 30 cases of oral epithelial dysplasia, and 10 cases of normal oral mucosa (NOM), histopathologically diagnosed (using hematoxylin and eosin), retrieved from the archives of the Department of Oral and maxillofacial Pathology, Universal college of medical and dental sciences, Bhairahawa, Nepal from September 2017 to October 2020. Mast cells were counted by two observers using an oculometer grid in six non over lapping grid field at a magnification of 40X. Mast cells were expressed as the number of mast cells per grid field. Statistical analysis was done using SPSS (Statistical Package for Social Sciences) version 16.0 and Epi-info version 3.0 and the findings obtained by the two observers were subjected to paired t-test. The p-value thus obtained was found to be non-significant in all the three groups i.e., oral epithelial dysplasia, OSCC and normal oral mucosa. Thus mast cell counts by only one observer were considered for further statistical analysis. This retrospective cross-sectional study was approved by the institutional review committee (IRC). Ethical approval letter no. UCMS/IRC/086/21. Period of study was from 9th November 2022 to 15th March 2023.

Toluidine blue staining:

- 5 µm sections were cut using the microtome and lifted on to the adhesive coated glass slides
- Slides were placed on the slide warmer to melt the wax and for the adhesion of the section onto the slide
- The slides were then transferred to a Coplin jar containing xylene. Three changes of xylene were used for 5 min each
- The slides were further passed through decreasing grades of alcohol (100%, 90%, 70%, and 50%) for 10 dips each
- The sections were washed in distilled water for 10 dips
- The sections were covered with toluidine blue solution for 10 s followed by washing with running tap water
- Differentiation in 100% alcohol was done for 2 min
- Finally, after clearing was done in xylene, the sections were mount in DPX and covered by cover slips.

Procedure:

Mast cells were counted using an Olympus CX41 microscope fitted with an Olympus oculometer grid. Counting was carried out at ×40 by two independent observers and was done in six non overlapping fields in each slide. Mast cells were identified on the basis of the purple color attained by the granules after toluidine blue staining, but the nucleus of these cells appeared blue. All the other components of the sections were seen in different shades of blue as shown in Figures 1 to 4.

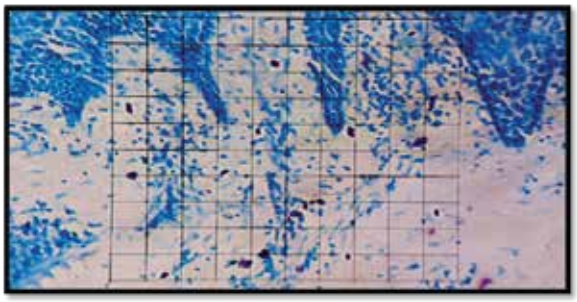

Figure 1. Grid field for counting of mast cells in toluidine blue stained section (×40)

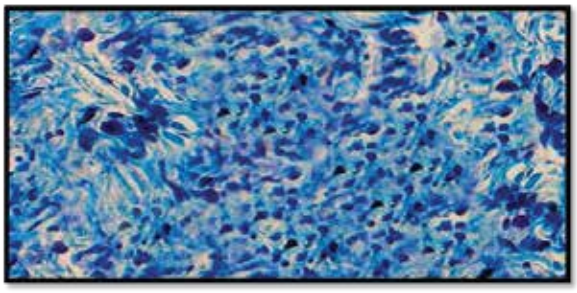

Figure 2. Mast cells in toluidine blue stained section of oral epithelial dysplasia seen intermixed with other chronic inflammatory cells (×40)

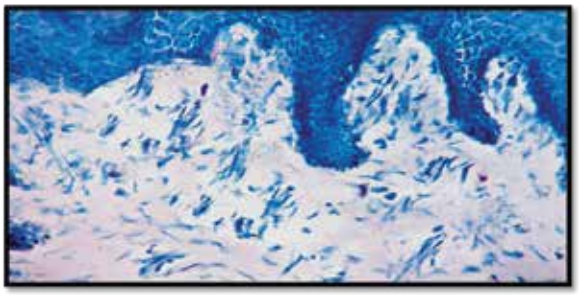

Figure 3. Mast cells in toluidine blue stained section of normal oral mucosa (×40)

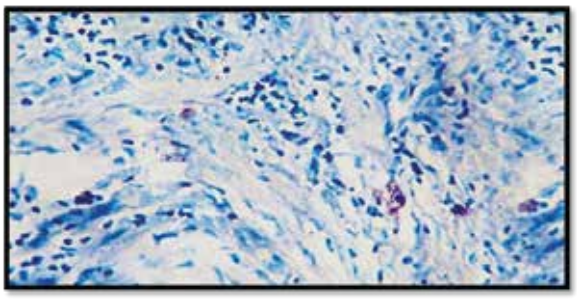

Figure 4. Mast cells in toluidine blue stained section of oral squamous cell carcinoma (×40)

RESULTS

The patients in cases of oral epithelial dysplasia were in range of 23-65 yrs with the mean age being 43.87 yrs and majority patients were males. Whereas in cases of OSCC the age range was 20-80 yrs with mean being 49.16 and majority were also males (Table 1).

The findings obtained by the two observers were subjected to paired t-test (Table 2).

In the present study when toluidine blue stained sections of normal oral mucosa and oral epithelial dysplasia were compared and the *p*-values obtained on using unpaired t-test were found to be very highly significant ($p = 0.001$) [Table 3]. Similar comparision between toluidine blue stained sections of normal oral mucosa and OSCC yielded significant *p* value ($p = 0.049$) on applying unpaired t- test [Table 4]. Further comparision of the toluidine blue stained sections of the counts of mast cells in both oral epithelial dysplasia and OSCC, yielded a very highly significant *p*-value (0.000) on applying unpaired t-test (Table 5).

Table 1. Intergroup comparison of age (years)

Groups	Number of cases	Mean	SD
NOM	10	38.50	14.89
OED	30	43.87	11.51
OSCC	30	49.16	13.51

SD: Standard deviation, OED: Oral epithelial dysplasia, OSCC: Oral squamous cell carcinoma, NOM: Normal oral mucosa

Table 2. Inter observers variability (paired t test)

Groups	Observer 1		Observer 2		*p*	Significance
	Mean	SD	Mean	SD		
NOM	0.67	0.50	0.62	0.46	0.279	NS
OED	3.41	2.00	3.37	2.01	0.115	NS
OSCC	2.04	1.74	2.17	1.76	0.168	NS

NS: Nonsignificant, SD: Standard deviation, OED: Oral epithelial dysplasia, OSCC: Oral squamous cell carcinoma, NOM: Normal oral mucosa

Table 3. Comparison between normal oral mucosa and oral epithelial dysplasia (unpaired t test)

Groups	Observer 1			*p*
	N	Mean	SD	
NOM	10	0.67	0.50	0.0001*
OED	30	3.41	2.00	

SD: Standard deviation, OED: Oral epithelial dysplasia, NOM: Normal oral mucosa

Table 4. Comparison between normal oral mucosa and oral squamous cell carcinoma (unpaired t test)

Groups	Observer 1			*p*
	N	Mean	SD	
NOM	10	0.67	0.50	0.049*
OSCC	45	2.04	1.74	

SD: Standard deviation, OSCC: Oral squamous cell carcinoma, NOM: Normal oral mucosa

Table 5. Comparison between oral epithelial dysplasia and oral squamous cell carcinoma (unpaired t test)

Groups	Observer 1			*p*
	N	Mean	SD	
NOM	45	3.41	2.00	0.0001*
OSCC	45	2.04	1.74	

SD: Standard deviation, OED: Oral epithelial dysplasia, OSCC: Oral squamous cell carcinoma

DISCUSSION

First described by Paul Ehrlich in his doctoral thesis, mast cells are bone marrow derived tissue homing leukocytes.[9] They tend to concentrate around blood vessels in inflammatory and neoplastic foci and later accumulate near tumors before the onset of tumor associated angiogenesis.[10] They also play an important role in the regulation of physiological and pathological neovascularization, mostly on the basis of histological observations.[11] Mast cells are a prime source of angiogenic factors. Under physiological conditions, they are particularly prominent near capillaries and lymphatic channels. In many inflammatory disorders characterized by profound vascular remodeling, the infiltrate exhibits numerous mast cells which show structural features of degranulating elements. In various tumor models, mast cells appear at the edges of invasive tumors to facilitate angiogenesis by releasing preformed mediators or by triggering proteolytic release of extracellular matrix bound angiogenic compounds.[12] Algire and Chalkley were the first to suggest that tumor growth is closely related to the development of an intrinsic vascular network.[12] Angiogenesis is necessary to provide oxygen, nutrients and immune cells to the tumor microenvironment and also removes its waste products.[13] In

the early phase of hyperplasia and dysplasia, infiltrating mast cells degranulate and activate dermal fibroblasts which intensify angiogenesis. They also activate progelatinase B (matrixmetalloproteinase family) which is involved in extracellular remodeling and angiogenic regulation. MCs activate and progressively intensify angiogenesis by releasing sequestered angiogenic activators.[14] As the neoplastic sequence progresses, angiogenic growth factor gene expression is upregulated in cancer cells. This is the progression to the second cancer phase where tumor cells directly control their angiogenic phenotype instead of manipulating inflammatory cells to indirectly affect neovascularization.[14]

The numerous cytoplasmic granules in the mast cells bind to basic dyes such as toluidine blue and show metachromatic staining properties.[15] The pharmacologically active agents in the mast cell granules most likely contribute to the inflammatory reaction seen in epithelial dysplasia.[16] The mast cell degranulation releases IL-1 which may cause increased epithelial proliferation and increased lymphocytic and plasma cell infiltration as seen in leukoplakia.[17] Histamine which is released causes increased mucosal permeability and allows the antigens to reach into the underlying connective tissue. Heparin further causes endothelial cell proliferation and migration which results in increased vascularity of the stroma and in epithelial ulceration.[18] A study by Rakesh et al[15] found that MCs and their regulatory role in angiogenesis and inflammation by the release of mediators may play an important role in tumor progression, facilitating the transformation of oral leukoplakia into invasive carcinoma reinforcing the observation of this study. Another supporting study by Ankle MR et al.[19,20] found a significant correlation between MCD and MVD in OSCC, concluding that MCs may promote tumor progression by regulating angiogenesis.

We saw a highly significant increase of mast cells in the case of oral epithelial dysplasia as compared to NOM, and these results are in accordance with the study done by Ankle et al.[20] This though is opposite to the study done by Jandinski et al.[21] There was a highly significant increase of mast cells in oral epithelial dysplasia on comparison with OSCC whereas there was only a significant increase in mast cells in OSCC on comparison with NOM. These findings can be supported by the study done by Oliveira Neto et al.[22] in which the authors have stated that the decrease of mast cells in OSCC may reflect an important modification in the microenvironment during squamous tumorigenesis and maybe related to the failure in migration and/or a local depletion of these cells. In contrast, study done by Michailidou et al.[18] have shown a measurable increase in mast cell counts in both oral epithelial dysplasia and OSCC as compared to NOM, with OSCC having a higher count as compared to oral epithelial dysplasia. In the current study, it was observed that using metachromatic dyes such as toluidine blue, the visual detection of mast cells become clearer and hence this could prove to be a reliable method to be used for the same. The reliability of this technique is strengthened by the non significant inter observer variability [Table 2]. However, the findings of the present study should be further consolidated by studies using larger and more varied sample sizes to enhance the acceptability of the technique used in this study.

CONCLUSION

The metachromatic property of the mast cells using toluidine blue stain helps in to provide a better and accurate visible distinction of the mast cells in tissue sections. The literature has proven that mast cells can be an indicator of increased angiogenesis and hence can help in the prediction of carcinogenesis, its progression, and prognosis of the malignant lesions. The distinct metachromasia of mast cells and their role in the pathogenesis of cancer can together be used for patient welfare.

CONFLICT OF INTEREST

None

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