CORRELATION OF MAST CELL AND ANGIOGENESIS – AN IMMUNOHISTOLOGICAL STUDY

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ABSTRACT

Introduction: Mast cell contribution to neoangiogenesis during tumorigenesis in oral squamous cell carcinoma is not determined yet. objectives: to associate numerical mast cell density (mcd) to numerical microvessel density (mvd) during the progression of oral leukoplakia without dysplasia and leukoplakia with dysplasia to squamous cell carcinoma (oscc).

Materials and methods: Mvd was analysed immunohistochemically (mouse monoclonal anti-human cd34) in 50 paraffin-embedded specimens, 15 osccs, 30 leukoplakias and 5 normal oral tissues. toluidine blue counterstaining revealed mast cells. mcd and mvd were assessed at the same optical field.

Results: The mean microvessel density increased from NDL (122.1±21.2/sq mm) to DL(152.6±18.8/sq mm) to OSCC(215.7±49.8/sq mm) group, where as in NOM group the microvessel density(132.5±24.4/sq mm) was slightly higher than NDL(122.1±21.2/sq mm) group but was lower than DL and OSCC.

Conclusions: mast cells are attracted at the lesion site and may turn on an angiogenic switch during tumorigenesis in oscc.

KEYWORDS: Mast cell, Angiogenesis, Blood vessels, Leukoplakias, Squamous cell carcinoma

1. INTRODUCTION

Angiogenesis, the formation of new microvasculature, is an important component in many biological processes, both in physiological conditions, and in pathological conditions, such as rheumatoid arthritis, diabetic retinopathy, and neoplastic disease. It has been proposed that angiogenesis is needed for the growth of both primary and metastatic tumors for the demand of blood supply of lesion size beyond 1 or 2 mm. Relationship between mast cell and angiogenesis is evaluated.[1,2]

Mast cell usually presents as round or elongated cell with a diameter ranging between 8 to 20 microns, their single nucleus is round or oval in shape and the cytoplasm contains numerous secretory granules that metachromatically stain with thiazine dyes such as toluidine blue. Mast cells are found in almost all of the major organs and tissues of the body, particularly in association with connective tissue structures such as blood vessels, lymphatic vessels and nerves, and in proximity to surfaces that interface the external environment such as those of the respiratory and gastrointestinal system and the skin[3] with this background our aim was

To evaluate the micro vessel density in biopsy samples taken from normal oral mucosa, leukoplakia and oral squamous cell carcinoma by evaluation of immunohistochemical expression of CD34. To evaluate the numerical mast cell density in biopsy samples taken from normal oral mucosa, leukoplakia and oral squamous cell carcinoma with toluidine blue stain. To evaluate the correlation between mast cell density with angiogenesis. Lastly we evaluated the correlation between mast cell density with angiogenesis and its role in progression of oral premalignancy and oral squamous cell carcinoma.

2. MATERIALS & METHOD

The present retrospective study was carried out on a total of 50 biopsy tissues retrieved from the archives of Department of Oral and Maxillofacial Pathology, Saraswati Dental College & Hospital, Lucknow. The study group included 30 cases of leukoplakia (13 non dysplastic and 17 dysplastic), 15 cases of oral squamous cell carcinoma and 5 cases of normal buccal mucosa taken as control. The tissues had been fixed in 10% formalin, processed routinely and embedded in paraffin wax. The diagnosis and grading of dysplasia and carcinoma was reviewed under routine H&E stained sections of 4 µ thickness.

Grading of dysplasia

In the present study epithelial dysplasia group was subdivided as suggested by WHO grading system proposed by Warnakasuriya et al.[4] into two groups - Non dysplastic group (13 cases) and Dysplastic group (17 cases). For squamous cell carcinoma Anneroth's grading system was followed.

IHC staining for CD-34

Immunohistochemical detection of CD-34 was performed using Biogenex Super sensitive Polymer Horse Raddish Peroxide IHC Detection Kit. For immunohistochemical staining the sections were cut at approximately 4µm, floated on to Poly-L-Lysine coated slides and incubated at 37°C for one day and further incubated at 58°C for overnight. Later the sections were de waxed in xylene and rehydrated in descending grades of alcohol.

Positive control consisted of paraffin embedded sections of pyogenic granuloma with known antigenic reactivity to CD34 (endothelial cells) and a negative control was performed by omitting the step of primary antibody during the staining procedure which resulted in lack of staining in all cases.

COUNTERSTAINING [5]

TOLUIDINE BLUE

Toluidine Blue Stock Solution:

Toluidine blue O (Sigma) -------- 1 g

70% alcohol ---------- 100 ml

Mix to dissolve.

Sodium Chloride (1%):

Sodium chloride ---------- 0.5 g

Distilled water ---------- 50 ml

Mix to dissolve (make this solution fresh each time). Adjust pH to 2.0~2.5 using glacial acetic acid or HCl.

Toluidine Blue Working Solution (pH 2.0~2.5):

Toluidine blue stock solution ---------- 5 ml

1% Sodium chloride, pH 2.3 ---------- 45 ml

Mix well. The pH should be around 2.3 and less than 2.5

Make this solution fresh and discard after use. pH higher than 2.5 will make staining less contrast.

Results:

Mast cells ------ violet/red purple.

Background --- blue.

Quantification of Microvascular Density: Generally accepted criteria for determining a vessel profile (Tae et al, 1991)[6] were used, including any stained endothelial cell or endothelial-cell cluster that was separate from adjacent microvessels. Vessel lumens were not required for identifying a structure as a microvessel. Microvessel counting was performed by identifying the vascular '**hot-spot**' area by scanning the section under scanner view (x40)(Fig 1a). The area of highest vascularisation was identified and four high power (x400) fields were selected using a OLYMPUS BX51 microscope. The expression of CD34 positively stained microvessels were assesed on four non overlapping fields at x400 magnification and photographs were taken using OLYMPUS E-330 camera. The photographs were analysed using IMAGE PRO EXPRESS 6.0 for WINDOWS (media cybernetics). The area of the field was calculated using the software and total number of positive stained microvessels was counted in a same field using point selection tool(Fig 1b). The micro vessel density was expressed as no. of vessels per Sq mm.

Quantification of Mast Cell Density: In the same corresponding photographs, the number of toluidine blue positive mast cells was counted and results were expressed as average number of mast cells per Sq.mm

3. RESULTS

Patients and Tissue Characteristics

This retrospective study was carried out on 50 biopsy tissues comprising of 30 cases of oral leukoplakia (13 NDL & 17 DL) and 15 cases of OSCC and 5 cases of NOM. In our study, among all cases, the buccal mucosa was the single most common site of involvement (27cases) followed by alveolar mucosa (7), labial mucosa (6), others (3).In our study group all patients with either leukoplakia or OSCC had a habit of using tobacco. Higher number of patients used chewable

form of tobacco (64.4%). Also almost two thirds of the study group had a history of consuming tobacco for more than 5 times per day (68.8%) and for more than 5 years (64.3%).

Most of the previous reports had measured mast cells and microvessels in different optical fields.[7,8,9,10] Measuring mast cells and microvessels together, at the same field, seemed a more appropriate approach. In the current study we therefore used immunohistochemical staining with a pan-endothelial marker CD 34 and counterstaing with toludine blue to stain mast cells in order to evaluate microvessel density and mast cell density in the same optical field.[11,12](Fig 1a-f)

Numerical Microvessel Density

In normal oral epithelium, leukoplakia without dysplasia and leukoplakia with dysplasia lesions, microvessels are mainly located just underneath the epithelium and were stained as brown spots, lines or lumens. MVD was counted in every tissue.(Fig 1c,d,e,f. MVs in red arrow)

Our results showed that mean microvessel density (MVD) increased from NDL (122.1±21.2/sq mm) to DL (152.6±18.8/sq mm) to OSCC (215.7±49.8/sq mm) but the difference was significant only for OSCC. In fact the mean MVD for NOM (132.5±24.4/sq mm) was slightly higher than NDL (122.1±21.2/sq mm)(Table 1a) but the difference was not statistically significant. In our study a statistically significant difference ($P<0.001$) was found between OSCC and other study groups (NOM, NDL, and DL).(table 1b)

Numerical Mast Cell Density

Toluidine blue staining revealed mast cells as large, purple,oval and highly granulated cells. Mast cells were observed at the lamina propria highly populating areas around the tumor margins. Mast cells were also observed at the tumor periphery and at the stem of the lesion when this existed. Increased numbers of mast cells were also found in areas of high vascularization (namely the 'hot spots')(Fig 1a). MCD was counted in every tissue.(Fig 1c,d,e,f MCs in green arrow)

Our study showed increased mean MCD from NOM (5.5±5.4/mm^2) to NDL (12.1±/mm^2) to DL (12.8±/mm^2) and OSCC (13.7±/mm^2). There was a significant rise in MCD in OSCC (13.7±/mm^2) when compared to DL (12.8±/mm^2)(Table 2a). But there was no statistical significant difference within the study groups ($P=0.768$).(Table 2b)

Correlation Between MCD and MVD - Previous studies have shown that the number of microvessels was found to correlate significantly with the number of mast cells.[8, 16,17] In contrast our study showed no statistically significant linear correlation between MCD & MVD(Table 3).

4. DISCUSSION

It has been demonstrated in many experimental models that carcinogenesis is associated with new vessel formation and that solid tumor need a rich vascular network in order to reach a clinically evident size and also to acquire the ability to metastasize. Tumors cannot enlarge beyond 1 to 2mm in diameter or thickness unless they are vascularized.[12,18,19] Mast cells are an important source of several pro angiogenic and angiogenic factors, such as heparin, chymase, basic fibroblast growth factor bFGF, vascular endothelial growth factor (VEGF), and transforming growth factor-beta (TGF- β).Several studies have implicated host immune cells particularly mast cells to have a role in tumor progression by promoting angiogenesis.[7,8,11,16]

The CD34 molecule is a cell membrane glycoprotein found on certain lymphoid and myeloid hematopoietic progenitor cells and vascular endothelium .The CD34 is an endothelial antigen that has been used to highlight microvessels and its role as an angiogenesis marker has been demonstrated in past.[20,21,22]

In our study the microvessel density increased in a stepwise manner from NDL to DL to OSCC(Table 1a) but the difference was significant only for OSCC(Table 1b). Although DL showed a higher MVD than NDL there was a substantial overlap between the counts of both the group and hence the difference was not statistically significant. Also the counts in leukoplakia group markedly overlapped with those from NOM group. In fact the mean MVD for NOM (132.5±24.4/sq mm) was slightly higher than NDL (122.1±21.2/sq mm)(Table 1a) but the difference was not statistically significant(Table-1b). Our findings are similar to findings of Tae et al.[6] who showed that MVD was higher in normal oral mucosa group than mild dysplasia. The reason for this finding may be that the samples of NOM taken in this study were obtained from retromolar area during the third molar extraction. These tissues usually show varying amounts of inflammation which in turn could have caused increased vascularity. Kalra et al.[10] have emphasized on the role of inflammation in angiogenesis and studies have shown that expression of endothelial cell markers significantly correlated with the presence of inflammatory cells.[23]

In our study a statistically significant difference ($P<0.001$)(Table 1b) was found between OSCC and other study groups (NOM, NDL, and DL), which is similar to the findings of Shivamallappa et al.[24] who also showed statistically significant increased MVD in OSCC as compared to normal mucosa and leukoplakia. They did not find any significant difference in MVD of normal mucosa and various grades leukoplakia which is similar to our observations.

They suggested that in the development of OSCC angiogenic phenotypic change occurs in carcinomatous stage rather than pre cancerous stage. The authors explained the lack of difference in the MVD between normal mucosa and leukoplakia by stating that majority of cases of leukoplakia exhibited only hyperkeratosis or mild dysplasia and were hence not expected to have increased angiogenesis as compared to lesions exhibiting greater degree of dysplasia like carcinoma in situ and other pre-invasive lesions.

The lack of difference in angiogenesis between normal mucosa and leukoplakia has also been explained by the fact that normal vascular endothelial cells constitute a quiescent population in adult humans and a pan-endothelial marker like CD34 (as used in the present study) cannot distinguish between resting and active angiogenic vessels. It is suggested that specific markers for angiogenic vessels will be of greater value in studying tumor angiogenesis.[6] Molecules like $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been identified as potential candidates for assessing neoangiogenesis and may be more helpful in future studies.[25,26]From these observations, it might be suggested that MVD is a useful marker to identify those patients with a more aggressive tumor, for whom a more adequate therapeutic approach could be taken into consideration.[2, 27,28] Our findings are similar to the findings of Biviji AT et al, [29] Ankle MR et al, [30] Michailidou et al, [16] who showed an increase in the MVD from NOM (5.5±5.4/mm^2) to NDL (12.1±/mm^2) to DL (12.8±/mm^2)(Table 1a). The reason might be that biologically and pharmacologically active agents in the mast cells might contribute to inflammatory reactions seen in leukoplakia. These stimulated mast cells may release IL-1, which caused increased epithelial proliferation that is seen in leukoplakia. Histamine may cause increased mucosal permeability, which could facilitate increased access for the antigen to the connective tissue.

Our study showed increased mean MCD from NOM (5.5±5.4/mm^2) to NDL (12.1±/mm^2) to DL (12.8±/mm^2) and OSCC (13.7±/mm^2).(Table 2a) Our finding are concurrent with the findings of Iamaroon A et al,[22] Mohtasham N et al,[110] Ankle MR et al,[112] Michailidou et al,[23] Elpek GO et al.[20]

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seen in leukoplakia. Histamine may cause increased mucosal permeability, which could facilitate increased access for the antigen to the connective tissue.

There was a significant rise in MCD in OSCC (13.7±/mm^2) when compared to DL (12.8±/mm^2)(Table 2a). Rooney et al[31] suggested that heparin from the mast cells caused vasoproliferation and increased the half life of basic fibroblast growth factor(bfgf) which was a potent angiogenic substance, there by promoting tumor angiogenesis and facilitating local tumor invasion.

But there was no statistical significant difference within the study groups (P=0.768)(Table 2b). Also when the analysis of variance was calculated between the study groups the difference was non significant. Our results were similar to the study of Mohseni MG et al[32] and Jahanshahi G et al, [33] Grizzi F et al.[34] and Jandinski JJ et al.[35] which also showed no statistical significance between study groups.

According to **Jandinski JJ et al.**[35] showed an increase in the number of mast cells from normal to benign hyperkeratotic and dyskeratotic tissues, but this increase was not significant while the mast cell number increased significantly from normal tissue to low grade carcinoma. He suggested that this increase in number of mast cells decreased in medium – high grade carcinomas, and attributed this to unfavourable cellular environment.

Our study showed no statistically significant linear correlation between MCD & MV(Table 3) which was concurrent to the study of Jahanshahi et al[33] who found no correlation between angiogenesis and mast cell density in normal mucosa and OSCC. Porzionato et al[36 and] Glimelius [37] in his study also showed poor correlation between MCD & MVD in hodgkins lymphoma. He proposed that the lack of correlation might be due to different pathways for the proliferation of microvessel in HL compared to other lymphomas.

The lack of correlation between high MVD and MCD(Table 3) in our study might indicate a different pathway for the proliferation of microvessels [18]. Another factor that has been considered is the role of macrophages in tumor angiogenesis. Macrophages, constituents of tumor stroma, are members of the mononuclear phagocyte system of inflammatory cells. It is thought that peripheral blood monocytes are recruited to the tumor microenvironment as a result of the secretion of various chemotactic cytokines such as colony-stimulating factor, granulocyte macrophage colony-stimulating factor, VEGF, TGFβ-1, and a number of angiogenic factors including bFGF, VEGF, tumor necrosis factor alpha, and interleukin-8 (IL- 8). In head and

neck SCCs, tumor cells attract monocytes and activate them to secrete angiogenic factors. In addition, macrophages produce cytokines that act on tumor cells and stimulate them to produce increased levels of IL-8 and VEGF.[33]

Rajendran R et al.[38] and Clamon N H et al.[39] explained the discordant result with mast cell density in OSCC, could be probably because mast cells may have degranulated as disease progresses. Lack of mast cell granules in advanced disease states may have resulted in negative staining with toluidine blue.

Reed et al.[40] found that the simultaneous expression of several substances providing mast cell chemotaxis in tumor cells could influence the degree of mast cell response and could define the presence of different types of mast cells containing specific receptors against these chemoattractants.

As reported previously, the number of mast cells stained in formalin-fixed specimens is consistently less than in specimens fixed in Carnoy's solution. In the present study, we used formalin-fixed specimens because we could not obtain specimens fixed in other solutions. Therefore, the possibility remains that our results would be different if our specimens had been preserved in Carnoy's solution.[41]

It is also instructive to look at the other side of the coin. The presence of mast cells around the tumoral cells may hamper tumor growth by producing IL-1, 4, 6 and TNF. [42]

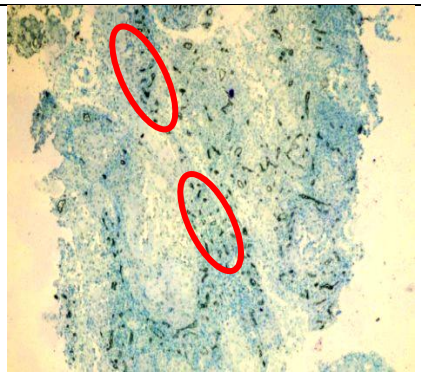

Figure 1 a Photograph showing HOT SPOT areas for micro

Figure 1 Photographs showing CD34 positive

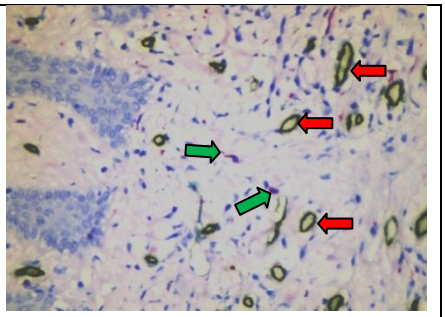

Figure 1 c Photo showing Toluidine blue positive mast

vessel	microvessels were counted using point selection tool (x400)	cells (green arrow) in a section of normal oral mucosa(x400)(MVs in red arrow)

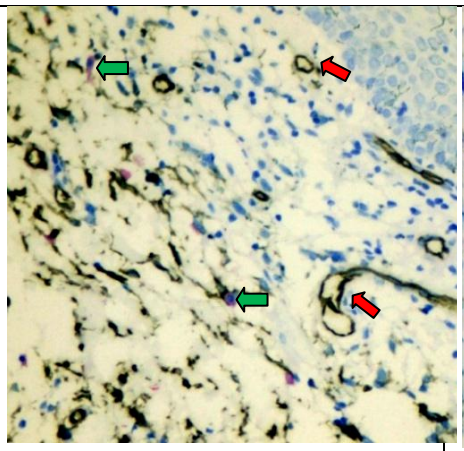

Figure 1 d Photograph showing Toluidine blue positive Mast Cells (green arrow) in a section of non dysplastic leukoplakia (x400) (MVs in red

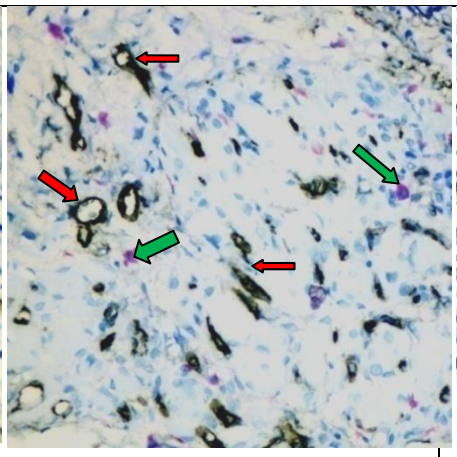

Figure 1 e Photograph showing Toluidine blue positive Mast Cells (green arrow) in a section of dysplastic leukoplakia (x400) (MVs in red arrow)

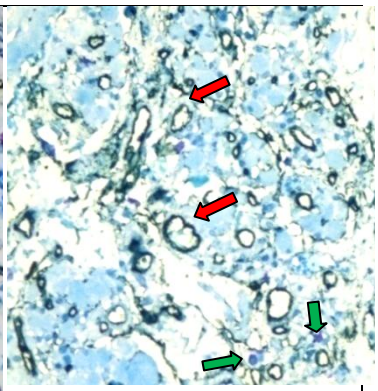

Figure 1 f Photograph showing Toluidine blue positive Mast Cells (green arrow) in a section of oscc (x400) (MVs in red arrow)

Group	N	Mean MVD /sq mm	SD	Min	Max
NOM	5	132.5	24.4	115	175
DL	13	122.1	21.2	95	165
DL	17	152.6	18.8	112.5	187.5
OSCC	15	215.7	49.8	140	340

a

Comparison	Mean Diff	SE	"p"	Significance
NOM vs NDL	10.38	17.02	0.928	NS
NOM vs DL	-20.15	16.46	0.615	NS
NOM vs OSCC	-83.17	16.71	‹0.001	VHS
NDL vs DL	-30.53	11.92	0.064	NS
NDL vs OSCC	-93.55	12.26	‹0.001	VHS
DL vs OSCC	-63.02	11.46	‹0.001	VHS

NS=Not significant; S=Significant; HS=Highly Significant; VHS=Very highly significant

b

Table 1: shows comparison of mean Microvessel density among subjects in different groups:

Group	N	Mean MCD/sq mm	SD	Min	Max
NOM	5	5.5	5.4	0	12.5
NDL	13	12.1	15.4	0	40
DL	17	12.8	15.5	0	50
OSCC	15	13.7	16.5	0	47.5

a

Comparison	Mean Diff	SE	"p"	Significance
NOM vs NDL	-6.62	7.98	0.841	NS
NOM vs DL	-7.29	7.72	0.781	NS
NOM vs OSCC	-8.17	7.83	0.726	NS
NDL vs DL	-0.68	5.59	0.999	NS
NDL vs OSCC	-1.55	5.75	0.993	NS
DL vs OSCC	-0.87	5.37	0.998	NS

NS=Not significant; S=Significant; HS=Highly Significant; VHS=Very highly significant

b

Table:2 Shows comparison of Mean Mast Cell Density among Subjects In Different Groups:

Correlation between MCD and MVD

"r" value	0.160
"p" value	0.268

CONCLUSION

Based on the findings of our study it can be concluded that the densities of MCs & MVs increased from NDL to DL to OSCC with the exception that the densities of MVs was slightly higher in NOM group than NDL but this difference was not statistically significant. There was a statistically significant difference between NOM and OSCC, NDL and OSCC, DL and OSCC, therefore it can be concluded that there is an increased angiogenesis and MVD in OSCC as compared to NOM, NDL, DL. There was a poor correlation between MVD and MCD. Therefore it can be concluded that MCD is not a contributing factor for angiogenesis, rather a number of other factors play a role in neoangiogenesis thereby leading to tumor progression from premalignant lesions.

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