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Correlation of Mast Cells and Angiogenesis in Oral Squamous Cell Carcinoma and Verrucous Carcinoma

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ABSTRACT

Aim: Presence of Mast cells (MCs) near capillary sprouting sites suggests an association between MCs and anigogenesis. MCs are well recognized as being important in the generation of angiogenic response via the release of heparin and histamine and release and/or activation of extracellular matrix modified enzymes. Thus study was done to histologically evaluate and correlate the number of MCs and angiogenesis in different grades of Oral Squamous Cell Carcinoma (OSCC) and Verrucous Carcinoma (VC).

Materials and Methods: A retrospective study was conducted to elaborate upon the correlation between MCs and tumor angiogenesis. Microvessel density (MVD) was detected by immune-histochemical staining using anti CD34 monoclonal antibody and Mast cell density (MCD) was detected by toluidine blue staining.

Results: The results showed a significant correlation in the mean MVD and the mean MCD (**r= 0.75, p<0.001**) in Well Differentiated OSCC. However, in the other grades of carcinoma and normal tissue there was no significant correlation between the mean MVD and MCD.

Conclusion: MC accumulation in tumors is probably a part of response to tumor derived chemoattractants. From our study and previous literature, there is an evidence that MCs in tumors are potentially angiogenic, but at the same time are pro-inflammatory and immunoamplifying in action.

Keywords: Neovascularization, Mast cells, Squamous cell Carcinoma, Verrucous Carcinoma.

INTRODUCTION

OSCC is an epithelial malignancy characterized by dysplastic changes of various degrees within the oral epithelium along with invasion of dysplastic cells into the underlying connective tissue[1]. VC was first described by Lauren V Ackerman in 1948 as "a distinct variant of differentiated SCC with low grade malignancy, slow growth and no or only low metastatic potential". VC typically involves the oral cavity, larynx, genitalia and oesophagus. It manifests as a verrucous, exophytic or endophytic mass that typically develops at sites of chronic irritation and inflammation. It enlarges slowly but penetrates deeply into the skin, fascia and even bone[2].

MCs are cells of connective tissue which are scattered along the capillaries, containing numerous

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basophilic granules in their cytoplasm which may obscure the nucleus[3], MCs were first described by Paul Ehrlich in 1878 which he named as "mast zellen" meaning feeding cells in Greek. Currently they are considered as a part of the immune system[4]. These are round, oval or spindle shaped cells that measure about 12µ and are packed with 50 to 100 granules. These cells are said to be pro-inflammatory and immunoamplifying in action[5]. MCs release preformed secretory mediators such as heparin and histamine and release and/or activate extracellular matrix modified enzymes which have been implicated in promoting angiogenesis[6]. MCs are often found near blood vessels in the resting state and it is widely recognized that MCs associate with newly forming microvasculature. Thus, early accumulation of MCs locally at a tissue site appears to potentiate vessel growth[7].

Angiogenesis or neovascularisation is the process by which new blood vessels are produced in a host from the pre-existing vasculature. During embryogenesis, blood vessels develop via two processes: vasculogenesis, where by endothelial cells are formed from progenitor cell types and secondly, angiogenesis in which new capillaries sprout from existing vessels. In an adult, new vessels are produced only through angiogenesis[8].

The term angiogenesis was coined by Hertwig (1935) to describe the formation of new blood vessels in the placenta, though it is now used for both physiologic and pathologic conditions. Physiological angiogenesis is a highly regulated process with limited duration, perhaps for few days to weeks. Examples for physiologic angiogenesis include that which is seen during ovulation, embryogenesis, lactating breast and wound healing. Pathological angiogenesis is sustained, which results from perturbations in growth control which is part of the disease process. It can be found in conditions such as solid tumors, angiofibroma, corneal graft neovascularisation, psoriasis, pyogenic granuloma, juvenile periodontitis and rapidly progressing adult periodontitis etc[9]. Endothelial cells rarely divide with overall turnover rate of about 0.015 or in other words they divide approximately every 3 years. Yet, in response to appropriate stimuli, the quiescent vasculature can become activated to grow new capillaries[10].

The objective of the present study was to compare and correlate the number of MCs and microvessels in normal oral mucosa and different histological grades of OSCC and VC.

MATERIALS AND METHODS

The present study was undertaken by retrieving previous records and paraffin embedded tissue blocks of diagnosed cases of different histological grades of OSCC and VC from the Department of Oral Pathology and Microbiology, Sri Sai College of Dental Surgery, Vikarabad, after obtaining the approval of the ethical committee. As control, normal oral mucosa specimens were collected from the Department of Oral and Maxillofacial Surgery, Vikarabad, from the patient's buccal flap raised during surgical removal of impacted mandibular third molars.

Forty cases of OSCC which included 20 cases of well differentiated squamous cell carcinoma (WDSCC), 10 cases of moderately differentiated squamous cell carcinoma (MDSCC) and 10 cases of poorly differentiated squamous cell carcinoma (PDSCC) were taken. In addition, 10 cases of VC and 10 of normal oral tissues making a total of 60 cases were included in this study. Tissue sections of 4µ thickness were taken and stained with toluidine blue for identification of MCs and immunostaining by anti CD34 monoclonal antibody to demonstrate endothelial cells.

The deparaffinised sections were placed in a coupling jar containing 0.01 M citrate buffer (pH – 6.0) and given three cycles of 5 minutes boiling in a microwave oven at 450^{O}C and allowed to cool to room temperature. The sections were then placed in a humid chamber, rinsed twice with distilled water and phosphate-buffered saline (PBS), covered with Peroxide block, incubated for 5-10 minutes and then drained and gently blotted. Then, the sections were covered with Power block and incubated for 10 minutes and drained and gently blotted. The slide was covered with primary antibody (Bio Genex ready to use super sensitive antibodies) and incubated for 60 minutes, then rinsed well with buffer and blotted around the sections. These sections were covered with poly

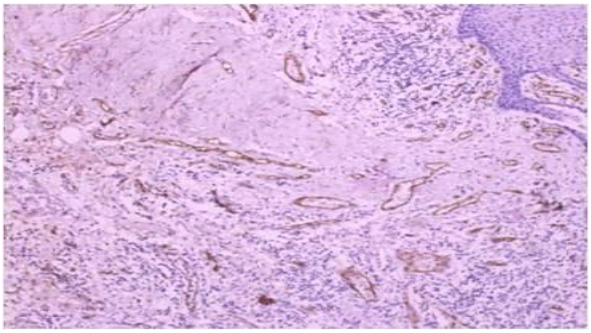


Fig 1: CD-34 immunostaining for angiogenesis in WDOSCC (10x).

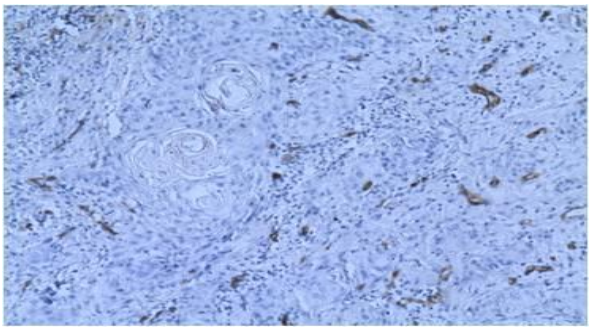


Fig 2: CD-34 immunostaining for angiogenesis in MDOSCC (10x).

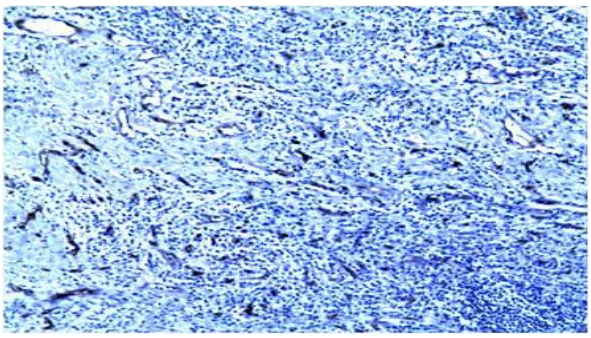


Fig 3: CD-34 immunostaining for angiogenesis in PDOSCC (10x).

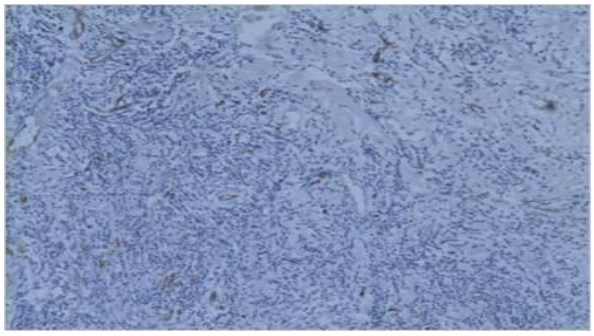


Fig 4: CD-34 immunostaining for angiogenesis in VC (10x).

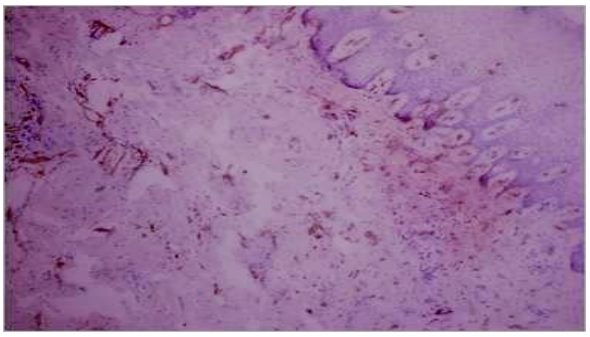


Fig.5 CD-34 immunostaining for angiogenesis in Normal Tissue (10x).

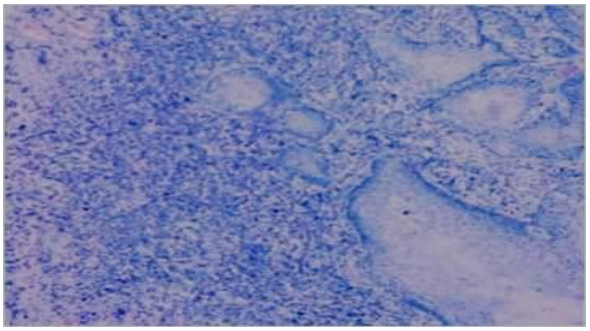


Fig 6: Toluidine blue staining for mast cells in WDOSCC(10x).

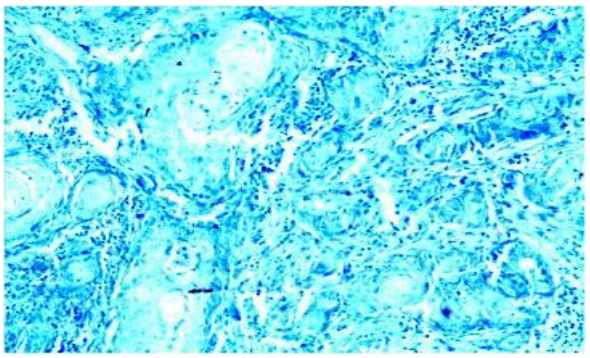


Fig 7: Toluidine blue staining for mast cells in MDOSCC(10x)

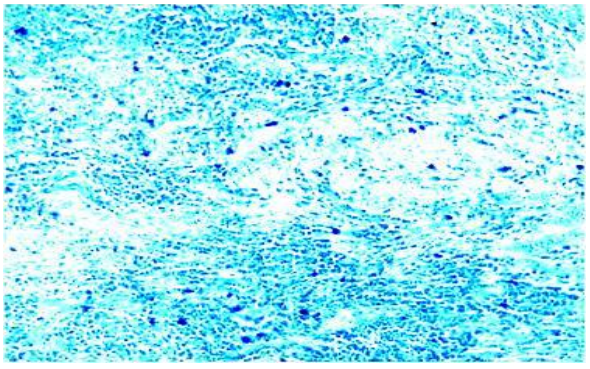


Fig 8: Toluidine blue staining for mast cells in PDOSCC(10x).

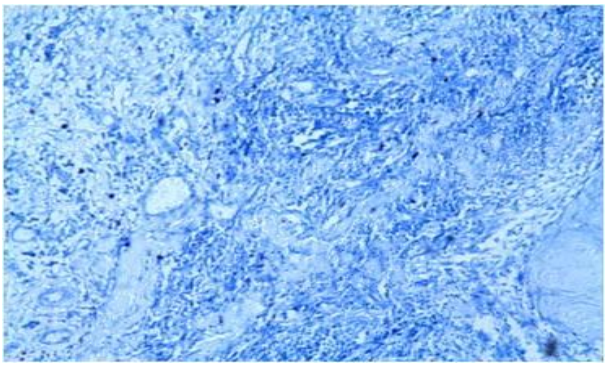


Fig 9: Toluidine blue staining for mast cells in VC (10x).

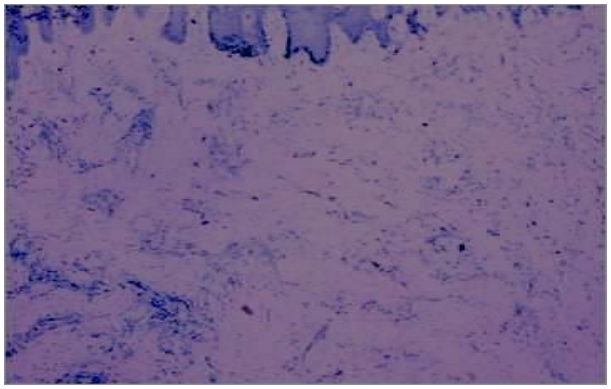
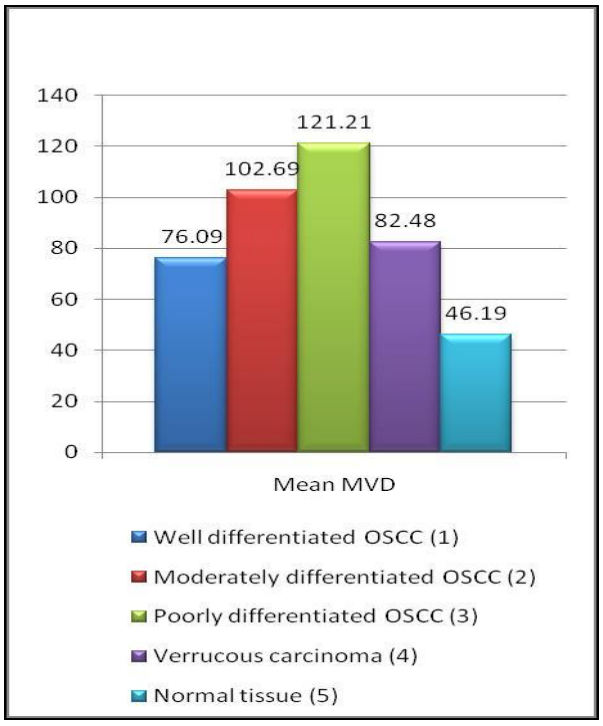
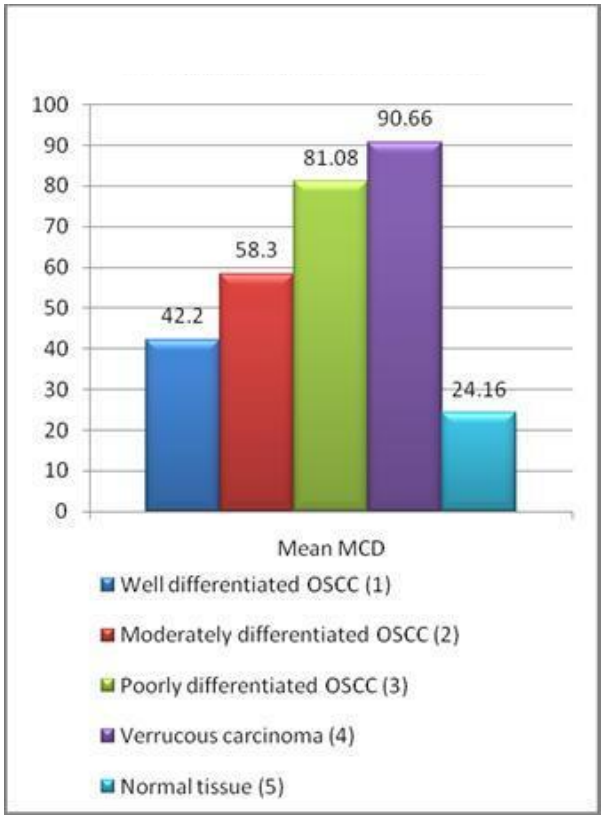


Fig 10: Toluidine blue staining for mast cells in normal tissue (10x).



Graph 1: Showing mean MVD.



Graph 2: Showing mean MCD.

HRP reagent and incubated for 30 minutes and then rinsed thoroughly with buffer. After wiping off excess of buffer, the sections were incubated with DAB (Di amino Benzedrine tetra hydrochloride) substrate solution for 10 minutes and gently rinsed with distilled water. The sections were immersed in a Harris haemotoxylin stain for 30sec, washed gently under running tap water, dedifferentiated by dipping in 1% acid alcohol, dehydrated by dipping in xylene and mounted in DPX, a non aqueous permanent mounting medium. The anti CD34 antibody highlighted the micro vessels by staining endothelial cell membrane. In each case, the slide demonstrating the vessels better was taken for morphometric measurements in image analysis. (Figures 1-5). The acidified toluidine blue technique was used here as it gives rapid crisp staining of MCs. The tissue sections were dewaxed in xylene and rehydrated in descending grades of alcohol and transferred to potassium permanganate solution for 2 minutes. The sections were then rinsed in distilled water and transferred to potassium meta-bisulphate solution for 1 minute or until section appeared white. Later, the sections were washed in

Table 1: Spearman's rho Correlation Coefficient.

Group			MCD
		Spearman's rho Correlation Coefficient	0.75
		Sig. (2-tailed)	<0.001
WDOSCC (1)	MVD	N	20.00
		Spearman's rho Correlation Coefficient	-0.18
		Sig. (2-tailed)	0.64
MDOSCC (2)	MVD	N	10.00
		Spearman's rho Correlation Coefficient	-0.33
		Sig. (2-tailed)	0.42
PD OSCC (3)	MVD	N	10.00
		Spearman's rho Correlation Coefficient	0.11
		Sig. (2-tailed)	0.82
VC (4)	MVD	N	10.00
		Spearman's rho Correlation Coefficient	0.29
		Sig. (2-tailed)	0.49
Normal tissue (5)	MVD	N	10.00

tap water for 3 minutes, rinsed in distilled water and placed in acidified toluidine blue for 5 minutes. Subsequently, they were again rinsed in distilled water, cleared in xylene and mounted. MCs stained purple and the nuclei blue. Both intact and degranulated MCs were identified by the structure of purple colored granules. These were scattered throughout the connective tissue, with some MCs near to or adhered to the vessels. Only those MCs found in the hot spot areas were counted (Figures 6-10).

RESULTS

Micro vessel density (MVD): (micro vessels/mm²)

When mean MVD was compared in normal (46.19±9.54), WDOSCC (76.09 ±18.87), MDOSCC (102.69±21.54), PDOSCC (121.21±23.50) and VC (82.48± 9.54), increase in mean MVD from normal to WDOSCC to VC to MDOSCC to PDOSCC was observed (Graph1).

When one way ANOVA was applied for MVD, all the groups showed highly statistically significant differences ($p<0.001$). ANOVA was followed by Bonferroni correction for multiple comparisons and statistically significant differences were found between the groups. The number of microvessels were found to be increased significantly between normal oral mucosa and WDOSCC ($p<0.001$), MDOSCC ($p<0.001$) and PDOSCC OSCC ($p=0.001$) and also between normal oral mucosa and VC ($p=0.011$).

Mast cell density (MCD): (cells/mm²)

When mean MCD was compared in normal (24.16 ± 3.02), WDOSCC (42.20±6.37), MDOSCC (58.30±6.08), PDOSCC (81.80±10.15) and VC (90.665±12.64), there was increase in mean MCD from normal to PDOSCC progressively and also to VC (Graph 2). When one way ANOVA was applied for MCD, all the groups showed highly statistically significant differences ($p<0.001$).

ANOVA followed by Bonferroni correction for multiple comparisons was done and statistically significant differences were found between the groups. The number of MCs were found to be increased significantly between normal oral mucosa and WDOSCC ($p<0.001$), MDOSCC ($p<0.001$) and PDOSCC ($p<0.001$) and also normal oral mucosa and VC ($p<0.001$).

Correlation between MVD and MCD

Only in the WDOSCC the mean MVD and the mean MCD were significantly correlated ($r= 0.75$ $p<0.001$). In the other grades of carcinoma and

normal tissue there was no significant correlation between the MVD and MCD (Table 1).

DISCUSSION

OSCC and VC are malignant tumors of the epithelial tissue origin. OSCC is the most common cancer and despite various attempts and approaches to intervene the disease, it remains a serious problem of oral health worldwide. However, VC which is a highly differentiated variety of SCC, shows a low degree of malignancy. Oral cavity is the most common site of occurrence of VC where it represents 2-12% of all oral cancers[2].

Angiogenesis or neovascularisation has long been known to aid in progression and metastasis of malignant tumors. It is the propelling force for tumour growth and metastasis by providing nutrients and oxygen for metabolism and removal of resultant waste products. Angiogenesis is thought to be initiated by an increase in the level of angiogenic stimuli and a concomitant decrease in the level of angiogenic inhibitors. These factors are produced by tumor cells, stromal cells and inflammatory cells such as MCs and macrophages[8].

MCs are normal residents of connective tissue and usually accumulate near sites of new capillary formation, at the periphery of the tumor site and highly vascularised areas of certain tumours[11]. MCs release preformed secretory mediators and have been implicated in promoting angiogenesis[5]. Angiogenic factors including VEGF, βFGF produced by tumor cells stimulate MC migration to tumor site[7,12]. MCs play a significant role in promoting tumor angiogenesis, by secreting several potent angiogenic factors including histamine, heparin, VEGF, βFGF and tryptase. Tryptase directly induces endothelial proliferation and MCs act at the site of new vessel formation by secreting tryptase[13,14]. Recent studies in malignant tumors suggested that MCs may have a role in tumor angiogenesis and MCD appeared to be a reliable prognostic marker[2,15,16].

In the present study, the results suggested that MVD and MCD increase with increasing histological grades of OSCC, but in VC, MCD increased more than MVD. In most of the previous studies that investigated angiogenesis in OSCC by means of MVD showed an increase in MVD with disease progression and lymph node metastasis thus suggesting that MVD could be used as independent prognostic indicator for tumor progression and metastasis[17-21]. However, some authors detected inverse correlation between MVD and disease progression[22-24].

The reasons for these mixed results could be the different antibodies (CD31, factor VIII etc) used to define endothelium, different methodologies in the assessment of MVD and interobserver variation. Finally, it could be due to variation in the degree of vascularisation in different sites of oral mucosa, which may lead to variations in MVD in the tumors arising from different locations of oral mucosa.

Angiogenesis has been shown to play an important role in transition of normal tissue to preneoplastic state and eventually to full blown cancer. Most of the previous studies found a positive correlation between the severity of oral lesions and all the blood vessel parameters[18-20].

However, our observations were in sharp contrast to those of Y. Jin et al (1955) who showed that the MVD was more in WDOSCC as compared to MDOSCC and PDOSCC in their study[21]. Even Juma.O.Alkhabuli (2007) observed decreased number of blood vessels in MDOSCC when compared to PDOSCC and WDOSCC[25]. But in their study, it was found that the lumina of vessels were thinner and smaller in MDOSCC and PDOSCC when compared with WDOSCC which was also observed in our study. The present study showed a positive correlation between MVD and MCD in WDOSCC and VC but a negative correlation in MDOSCC and PDOSCC.

The number of MCs in SCC affecting the oesophagus, lip, tongue, lungs and oral cavity showed that there is a significant increase in MCs in SCC which is in direct correlation with tumor angiogenesis and that the progression of tumors was mainly due to an increase in the number of the MCs through promotion of angiogenesis[14,15,26-30]. These findings were consistent with our results.

However, Juma O.Alkhabuli (2007) in his study had shown that there was an increase in the number of MCs in WDOSCC as compared to their low number in PDOSCC. Further, it was also found

that those patients with increased MCs had prolonged survival as compared to when their number was less suggesting that MCs have inhibitory effect on tumor progression[25]. Farmoush et al (1983) investigated the MCs population during experimentally induced carcinogenesis in the skin of Swiss Webster mice and observed a dense foci of MCs subepithelially, particularly beneath the regions of hyperplastic epithelium which showed that MCs may play a role in abnormal epithelial proliferation[31].

In our study, the MCD was high in VC as compared to any of the grades of OSCC and normal mucosa. This could be explained in two different ways as follows: MCs are of two types namely MC_T and MC_{TC}. While MC_T helps in neoangiogenesis, MC_{TC} helps in degradation of extracellular matrix and tumor progression at the invasion front. Since in VC most of the MCs were confined to the subepithelial zone, probably these MCs could be MC_{TC} helping in progression at epithelial-connective tissue junction which is normally seen in VC. However, to come to such a conclusion, immunohistochemical staining to identify the subset of MCs would be helpful. Alternatively, Juma O Alkhabuli (2007) has suggested that the increase in the number of MCs may be related to prolonged survival of the patient and that the MCs have inhibiting effect on tumor progression[25].

VC is considered to be mild form of SCC and an increase in number of MCs observed could be attributed to body's inflammatory response which is high in the initial stages. As suggested by Tomita M et al (1999), the MCs infiltrate the connective tissue underlying a developing carcinoma and suppress the tumor activity[6]. Probably similar phenomenon occurs in VC also. However, the drop in number of MCs in WDOSCC followed by increase in MDOSCC to PDOSCC could be explained by the role played by proangiogenic and antiangiogenic factors along with various cytokines that in turn influence the number of MCs.

CONCLUSION

From our study and previous literature, there is sufficient evidence that MCs in tumors are potentially angiogenic, but at the same time they are also pro-inflammatory and immunoamplifying in action. MCs are also known to play a part in inhibiting tumor progression as well. The mediators in MCs are known to vary with the variation in microenvironment in various diseases. Further investigations into MCs derived angiogenic factors induced by tumor cells might provide a better understanding of the interaction of MCs and tumor cells during angiogenesis and help to predict their response. Hence, studies on a much larger sample and using antibodies to identify the subtypes of MCs need to be carried out to establish the exact role of MCs and their role in tumor progression.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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