The role of mast cells and angiogenesis in well-differentiated oral squamous cell carcinoma

ABSTRACT

Objective: Neo-vascularization is vital for an expansion and metastasis of a tumor and is influenced by a number of mediators. Mast cells are believed to release many pro-angiogenic mediators that may help in tumor expansion and invasion. This study describes the role of mast cells and angiogenesis in oral squamous cell carcinoma (OSCC).

Study Design: It was a prospective study in which 37 biopsies of well-differentiated OSCC were obtained. Micro-vessels were stained with cluster of differentiation (CD)-34 and mast cells were counted using the Toluidine blue stain.

Results: When compared to normal oral mucosal tissue, it was seen that micro-vessel density and mast cell density indeed increases significantly in squamous cell carcinoma; however, they are not correlated to each other.

Conclusion: This study reports that angiogenesis does increase in OSCC and mast cells also invade the peri-tumor tissue, but they are not directly correlated.

KEY WORDS: Angiogenesis, mast cells, oral cancer, squamous cell carcinoma

INTRODUCTION

More than 95% of the total intra-oral carcinomas in the developing countries are squamous cell carcinomas and are a leading cause of death.[1] Oral cancer is the sixth most common cancer world-wide and the regions characterized by the high incidence rates include South and South-east Asian countries (e.g., Sri Lanka, India, Pakistan, and Taiwan).[10] It is also the most common cancer in India forming 94% of all oral malignancies.[3] Hence, it remains a serious problem of oral health world-wide.

Mast cells are well-known to be involved in allergic, inflammatory, and immune reactions. In addition, they are also involved in pain, tissue damage as well as repair. However, in the recent past, mast cells have proven to be related to cancer and mediate their effect through release of many cytokines and chemokines, pro-angiogenic factors and via heparin release.[4,5] Therefore, mast cell function in developing tumors is being extensively investigated world-wide. The role of mast cells has already been studied in many pathological conditions and malignancies such as adenomatous polyps, colorectal cancers, malignant melanoma, oral squamous cell carcinoma (OSCC), skin dysplasias multiple myeloma, and many other tumors.[6-14] The increased number of mast cells alone cannot be correlated with the stage and prognosis of tumor. An increased mast cell count may be the result of tumor invasiveness; on the other hand, these mast cells may elicit tumor progression.[5]

Some studies suggest the pro-angiogenic and thus pro-tumourigenic role of mast cells in OSCC, whereas some studies do not support this theory.[8-12] This study shall evaluate the role of mast cells and angiogenesis in OSCC and the findings of this study can prove to be helpful in determining the prognosis and treatment modalities of OSCC in future.

MATERIALS AND METHODS

Sample size was calculated by using the P.A.S.S 2008 (Power analysis and sample size software) and 37 formalin-fixed oral biopsies of OSCC were obtained. After gross examination, paraffin embedded tissue sections were made. Following H and E based diagnosis and grading in each case, micro-vessel density (MVD) was assessed by using the monoclonal anti-human CD-34 class II antibody. The numerical mast cell density (MCD) was determined by staining the tissue slides with toluidine blue. All the primary tumors of histologically diagnosed OSCC were included from both sexes and all ages. However, patients those were immune-compromised or on radio/chemo-
therapy or undergoing any treatment that affects mast cells and metastatic tumors were excluded. Normal tissues were obtained from non-inflamed subgingival tissue of patients undergoing minor oral surgeries after patient’s consent.

The recorded clinical data included age, gender, and intra-oral location of tumor, clinical presentation and type of biopsy performed. Recorded histopathologic data included the grade of tumor, microscopic evidence of ulceration, blood vessel, lymphatic, and neural invasion.

From each paraffin block, three tissue sections each 4 µm thick were cut on three different slides for hematoxylin and eosin stain, CD-34 antibody and toluidine blue stain respectively.

**Determination of Micro-vessel Density**

**Immuno-histochemistry**

Immuno-histochemistry was carried out to determine the numerical MVD. A paraffin embedded tissue section of 3-5 µm thickness was taken from each block on super-frosted slides after coating them with Poly-L-lysine. These sectioned were reacted with mouse monoclonal anti-CD34 antibody (CD34, Mouse Monoclonal antibody, Clone QBend/10, Isotype Immunoglobulin type G-1 (IgG1), Localization membranous, provided by BioSB, Santa Barbara USA) using avidin-biotin-peroxidase complex method. Counter-staining with hematoxylin was carried out and slides were dehydrated and mounted with cover-slips using the DPX mounting medium.

**Interpretations of Immunohistochemistry**

Five microscopic high power fields (HPFs) at ×400 with the largest number of micro-vessels adjacent to the invading tumor margins were observed in each case. The microvascular density (angiogenesis) was determined by counting the mean numbers of small blood vessels lined by endothelial cells, individual endothelial cells or their clusters separated from neighboring micro-vessel immunoreactive to CD34 antibody per HPF.

**Determination of MCD**

**Toluidine blue staining**

After following the same initial steps as for Hematoxylin and Eosin staining, the sections were immersed in toluidine blue working solution for 2-3 min and washed in distilled water, for three changes. They were then dehydrated quickly through 90% and two changes of 100% alcohol (10 dips each since stain fades quickly in alcohol). Sections were then cleared in xylene, two changes, and 3 min each and mounted with DPX mounting medium.

As the mast cell cytoplasm contains granules (metachromatic) composed of heparin and histamine. Toluidine blue stains mast cells red-purple (metachromatic staining) and the background blue (orthochromatic staining).

**Interpretation of toluidine blue staining**

Three HPFs containing the largest number of mast cells adjacent to tumor invasive margins were selected and mast cells were counted and their measure that is MCD was given as the number of mast cells seen per HPF (at ×400).

**RESULTS**

Mean age of patients of OSCC was 54.30 ± 1.561 (95% confidence interval [CI]: 51.13-57.46) in a range of 32-80 years. Out of the 37 cases of OSCC, 20 (54.1%) were males while 17 (45.9%) were females. Mean age of male patients was 55.05 ± 2.336 (95% CI: 50.16-59.94) and female patients was 53.41 ± 2.053 (95% CI: 49.06-57.76). The most frequent location of the tumor in the oral cavity was buccal mucosa (32.4%), followed by tongue (21.6%), palate (10.8%) and lower lip (10.8%) as shown in Figure 1.

OSCC presented in various clinical forms such as ulcer, induration leukoplakia, erythroplakia, erythro-leukoplakia, and fungating growth. The most common clinical presentation of OSCC was as non-healing indurated ulcer (51.4%) and it was significantly common on the buccal mucosa (P = 0.001).

On microscopic examination, all 37 cases were diagnosed as well-differentiated squamous cell carcinomas. 45.9% cases showed microscopic evidence of ulceration and 10.8% showed vascular invasion. Vascular invasion was insignificantly associated with gender of patients (P = 0.367), location of the tumor (P = 0.156), clinical type of lesion (P = 0.529) or with microscopic evidence of ulceration (P = 0.242).

Mean MVD in 5 normal tissues was found to be 9.20 ± 0.860/HPF, whereas mean MVD in 37 cases of OSCC was 25.84 ± 1.525/HPF as shown in Figure 2. The number of micro-vessels was significantly increased in OSCC (P = 0.0001). Mean MCD in normal tissues was 1.00 ± 0.316/HPF, whereas in 37 cases of OSCC it was 10.08 ± 0.909/HPF as shown in Figure 3. The number of mast cells also increased significantly in OSCC (P = 0.001) as shown in Table 1. However, by applying Pearson correlation, the MVD was found to be insignificantly correlated.
with the MCD ($r = -0.053, P = 0.754$). Similarly, by applying the power curve fit model, no significance was found ($P = 0.828$) as shown in Figure 4.

**DISCUSSION**

Mast cells exert their tumorigenic effect through four mechanisms, i.e., (1) immunosuppression (2) angiogenesis (3) degradation of extracellular matrix and (4) mitogenesis. Among these, angiogenesis plays a vital role in tumor growth. In order to outgrow the size of 2 cubic mm, solid tumors need oxygen supply and angiogenesis is necessary to remove waste products and to provide nutrition and immune cells to the growing tumor. Angiogenesis is an early event in tumorigenesis and is found in many pre-malignant conditions such as gastric dysplasia, carcinoma in situ of breast, atypical adenoma of colon, and oral leukoplakia with dysplasia.

Mast cells are highly granulated bone marrow derived cells and secrete many pro-angiogenic factors such as angiopoietin-1, vascular endothelial growth factor (VEGF), basic Fibroblast Growth Factor (bFGF), Monocyte Chemoattractant Protein-4 (MCP-4) (chymase) and histamine and there is impressive evidence of pro-angiogenic and thus pro-tumor role of mast cells. VEGF has been shown to be significantly increased in premalignant and invasive oral lesions. However, in some tumors such as breast cancer, mast cells seem to play anti-tumor effects and represent a favorable prognosis, whereas, in some tumors such as non-small cell lung carcinoma the role of mast cells is still controversial.

To evaluate the role of mast cells in OSCC, 37 biopsies were collected. Mean age was found to be 54.30 ± 1.561 (95% CI: 51.13-57.46), which is in consistence with other studies conducted in Punjab, Pakistan in which the mean age of patients with OSCC was shown to be 51 and 53 years. However, two other studies conducted in Sindh, Pakistan showed a comparatively younger age group showing average age of 49.47 ± 9.28 and 46.28. This may be due to increase the prevalence of tobacco, pan and betel nuts in Sindh. Other studies world-wide have shown mean age to be around 60 years.

Our study shows almost equal incidence of OSCC among males and females, which is the same as shown by Bhurgri and Keski-Santti et al. Some studies have also shown a male predominance. The most common sub-site of OSCC in oral cavity is buccal mucosa according to this study, which is also consistent with some other studies in Pakistan. While different studies world-wide have shown different
most commonly involved intra-oral sites such as mandibular alveolus and lip vermilion, the incidence of oral cancer of buccal mucosa seems to be increasing in our region, which is a cause of concern because of its worst prognosis as shown by Lin et al.\textsuperscript{[10,31]}

In our study, the average MCD in 37 cases of OSCC was 10.08 ± 0.909/HPF, which is significantly higher than mean MCD 1.00 ± 0.316/HPF found in normal oral mucosa obtained from gingiva and alveolus (P = 0.001). This finding is very close to the findings of a Greek study in which the average MCD in 30 cases of OSCC was 9.33 ± 6.26/HPF.\textsuperscript{[10]} Similarly, in our study the mean micro-vessel density in OSCC was 25.84 ± 1.525/HPF, which is again significantly higher than mean MVD found in normal oral mucosa 9.20 ± 0.860/HPF with P value = 0.0001. These findings are also very similar to the findings by Michailiduo et al., in which the mean MCD in 30 cases of OSCC was 9.33 ± 6.26/HPF and mean MVD was 24.78 ± 6.1/HPF with similar significance values.\textsuperscript{[10]} A recently conducted Indian study found the mean MCD in well- differentiated OSCC to be 12.85, which is slightly higher than our findings and the mean micro-vessel density in their study was 51.55 in well-differentiated, 90.15 in moderate and 164.25 in poorly differentiated OSCC, which are quite higher than the mean MVD found in our study.\textsuperscript{[11]} This difference may be explained by the difference of tumor markers used, which was a mouse monoclonal anti-human CD34 class II antibody in our study and study by Michailiduo et al., whereas, anti-collagen type IV antibody was used by Kalra et al.

Our study found that the mean MVD and mean MCD were insignificantly correlated with each other despite their significant rise is OSCC. Interestingly, this finding is in contrast with the study by Michailiduo et al., in which their correlation was found to be significant, whereas, the study in India by Kalra et al., found the correlation to be inverse, which stresses the need for further research on the role of mast cells and angiogenesis in oral cancer. Moreover, most studies show that the micro-vessels and mast cells do rise significantly in malignant oral mucosa, which can define the future therapeutic approach toward its treatment and also might play a role toward the prognosis of pre-malignant lesions. The difference in results of various studies could be attributed to the difference of immunomarkers and stains used. Tryptase immunostaining is believed to be the precise staining for human mast cell detection, but most studies including our study have used toluidine blue stain. Another factor could be the degranulation of mast cells at a given point of time. However, our study shows that the process of angiogenesis has no correlation with MCD that is the pro-angiogenic factors are not necessarily released by mast cells only. Increased mast cells in these tumors can be the result of increased angiogenesis and further research with larger sample sizes is needed to conclude their role in OSCC.

CONCLUSION

According to this study, the MCD and micro-vessel density are significantly increased in OSCC, but are not correlated to each other.

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