Correlation of Mast Cell and Angiogenesis in Oral Squamous Cell Carcinoma: An Immunohistological Study

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Abstract:
Background: To evaluate the correlation between mast cell density (MCD) with angiogenesis and its role in progression of oral premalignancy and squamous cell carcinoma of oral cavity.

Materials and Methods: The present retrospective study was carried out on a total of 50 biopsy tissues retrieved from the archives of Oral and Maxillofacial Pathology Department, Career Dental College and Hospital, Lucknow, Uttar Pradesh, India. The study group included 30 cases of leukoplakia (13 non-dysplastic and 17 dysplastic), 15 cases of oral squamous cell carcinoma (OSCC) and 5 cases of normal buccal mucosa taken as control.

Results: The mean microvessel density (MVD) increased from non-dysplastic lesion (NDL) (122.1 ± 21.2/mm²) to dysplastic lesion (DL) (152.6 ± 18.8/mm²) to OSCC (215.7 ± 49.8/mm²) group, whereas in normal group the MVD (132.5 ± 24.4/mm²) was slightly higher than NDL (122.1 ± 21.2/mm²) group but was lower than DL and OSCC. Mean vessel density showed a moderate correlation with the area and mild correlation with the perimeter. While MCD showed no correlation between area and perimeter.

Conclusion: It can be concluded that mortgage credit directive 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.

Key Words: Angiogenesis, CD34, mast cell

Introduction
Oral squamous cell carcinoma (OSCC) remains a serious problem of oral health worldwide. Cancer of the oral cavity and oropharynx represent the sixth most common malignant neoplasm with an annual worldwide incidence of 500,000 cases.¹ Mast cell usually presents as round or elongated cell with a diameter ranging from 8 to 20.² 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.³

Mast cells act locally, as well as systemically, through the release of various mediators through degranulation. These mediators are usually stored in the cytoplasmic granules, and some of the mediators are produced when stimulation occurs. Mast cells secrete a variety of cytokines, the exact function of which is still not known but believed to play role in various pathological, as well as physiological conditions. Other mediators such as histamine, tumor necrosis factor alpha (TNF-α), tryptase, interleukin (IL)-4 can stimulate fibroblasts proliferation and also perform function of a chemotactic factor for polymorphonuclear leukocytes. Factors, such as heparin, vascular endothelial growth factor (VEGF), and fibroblasts growth factor, induce migration of endothelial cells and also stimulate new vessel formation.⁴

CD34 is basically an endothelial antigen used to highlight the microvessel density (MVD) and as a direct marker of neoangiogenesis; however, it can react with “newly forming” vessels and also with normal vessels which are trapped within a tumor tissue.

According to recent studies, the relevance of angiogenesis and mast cells in prognosis of squamous cell carcinomas, including those of oral cavity as well as in progression of premalignant lesions like leukoplakia has been correlated.⁵ ⁶

Materials and Methods
The present retrospective study was carried out on a total of 50 biopsy tissues retrieved from the archives of pathology labs in Lucknow, Uttar Pradesh, India. The study group included 30 cases of leukoplakia (13 non-dysplastic and 17 dysplastic), 15 cases of OSCC (Figure 1) and 5 cases of normal buccal mucosa taken as control.

Relevant information (e.g. age, sex, site of the lesion, clinical staging) were obtained from the medical records of the patient. The tissues had been fixed in 10% formalin, processed routinely and embedded in paraffin wax. The diagnosis and grading of dysplasia and carcinoma were reviewed under routine H and E stained sections of 4 μ thickness.
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 4 μm, 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 dewaxed in xylene and rehydrated in descending grades of alcohol. Sections were stained using 1% toluidine blue for one minute and washed in tap water to remove excess stain. Sections were dried, cleared in xylene, mounted with DPX, and were viewed under light microscope (Olympus BX 51) for assessing the immunohistochemical expression of CD-34.

The positive control was of paraffin embedded sections of pyogenic granuloma with known antigenic reactivity to CD34 (endothelial cells), and a negative control was performed without the step of primary antibody used during the staining procedure and resulted in decreased staining in all cases.

Results

The present study was carried out with an aim to evaluate the MVD and morphology, mast cell density (MCD) and their correlation in oral precancerous and cancerous lesions. For this purpose, a total of 45 biopsy tissues were obtained from the archives of the department, and 5 tissue samples of normal mucosa of the oral cavity were obtained from healthy individuals who came to the department of Oral Surgery for the extraction of impacted tooth.

The mean MVD increased from non-dysplastic lesion (NDL) (122.1 ± 21.2/mm²) to dysplastic lesion (DL) (152.6 ± 18.8/mm²) to OSCC (215.7 ± 49.8/mm²) group, where as in normal oral mucosa (NOM) group the MVD (132.5 ± 24.4/mm²) was slightly higher than NDL (122.1 ± 21.2/mm²) group but was lower than DL and OSCC (Table 1 and Graph 1).

The mean difference was maximum between NDL and OSCC groups ($P < 0.001$). Statistically significant intergroup differences were observed between NOM and OSCC group ($P < 0.001$), NDL and OSCC ($P < 0.001$), and DL and OSCC groups ($P < 0.001$). None of the other differences (NOM vs. NDL, NOM vs. DL, and NDL vs. DL) were significant statistically ($P > 0.05$).

Mean microvascular area increased significantly from NOM (57.53 ± 0.9 µm²), to NDL (96.6 ± 68.1 µm²), to DL (149.6 ± 41.1 µm²) to OSCC (179.8 ± 66.1 µm²). The minimum microvascular area in a specimen was observed to be 35.19 µm² in a specimen from NDL group whereas maximum was observed as 303.88 µm² in a specimen from DL group (Table 2).

Graph 1: Mean vessel density.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean diff</th>
<th>SE</th>
<th>$P$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOM versus NDL</td>
<td>10.38</td>
<td>17.02</td>
<td>0.928</td>
<td>NS</td>
</tr>
<tr>
<td>NOM versus DL</td>
<td>−20.15</td>
<td>16.46</td>
<td>0.615</td>
<td>NS</td>
</tr>
<tr>
<td>NOM versus OSCC</td>
<td>−83.17</td>
<td>16.71</td>
<td>&lt;0.001</td>
<td>VHS</td>
</tr>
<tr>
<td>NDL versus DL</td>
<td>−30.53</td>
<td>11.92</td>
<td>0.064</td>
<td>NS</td>
</tr>
<tr>
<td>NDL versus OSCC</td>
<td>−93.55</td>
<td>12.26</td>
<td>&lt;0.001</td>
<td>VHS</td>
</tr>
<tr>
<td>DL versus OSCC</td>
<td>−63.02</td>
<td>11.46</td>
<td>&lt;0.001</td>
<td>VHS</td>
</tr>
</tbody>
</table>

NS: Not significant, S: Significant, HS: Highly significant, VHS: Very highly significant, NOM: Normal oral mucosa, NDL: Non-dysplastic lesion, DL: Dysplastic lesion, OSCC: Oral squamous cell carcinoma, MVD: Microvessel density, SD: Standard deviation

Table 3: Comparison of mean mast cell density among subjects in different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean MCD/mm²</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOM</td>
<td>5</td>
<td>5.5</td>
<td>5.4</td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>NDL</td>
<td>13</td>
<td>12.1</td>
<td>15.4</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>DL</td>
<td>17</td>
<td>12.8</td>
<td>15.5</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>OSCC</td>
<td>15</td>
<td>13.7</td>
<td>16.5</td>
<td>0</td>
<td>47.5</td>
</tr>
</tbody>
</table>

NOM: Normal oral mucosa, NDL: Non-dysplastic lesion, DL: Dysplastic lesion, OSCC: Oral squamous cell carcinoma, MCD: Mast cell density, SD: Standard deviation

Figure 1: Photograph showing H and E stained section in oral squamous cell carcinoma (×100).
The mean difference was maximum between NDL and OSCC groups ($P < 0.001$). Statistically significant intergroup differences were observed between NOM and OSCC group ($P < 0.001$), NDL and OSCC ($P < 0.001$), and DL and OSCC groups ($P < 0.001$). None of the other differences (NOM vs. NDL, NOM vs. DL, and NDL vs. DL group) were significant statistically ($P > 0.05$).

Mean microvascular area increased significantly from NOM (57.53 ± 0.9 $\mu$m$^2$), to NDL (96.6 ± 68.1 $\mu$m$^2$), to DL (149.6 ± 41.1 $\mu$m$^2$), to OSCC (179.8 ± 66.1 $\mu$m$^2$). Minimum microvascular area in a specimen was observed to be 35.19 $\mu$m$^2$ in a specimen from NDL group, whereas maximum was observed as 303.88 $\mu$m$^2$ in a specimen from DL group (Table 3 and Graph 2).

Mean mast cell count increased from NOM group (5.5 ± 5.4/mm$^2$), to NDL (12.1 ± 15.4/mm$^2$), to DL (12.8 ± 15.5/mm$^2$), to OSCC group (13.7 ± 16.5/mm$^2$). Mean MCD was minimum in normal group and maximum in OSCC group. In all the groups, minimum count was observed as 0. Maximum count was observed as 50 in dysplastic leukoplakia group (Table 4 and Graph 3).

MVD showed a moderate correlation with area and mild correlation with perimeter while MCD showed no correlation between area and perimeter. A strong correlation was observed between area and perimeter.

**Discussion**

Various previous studies had demonstrated that carcinogenesis is usually associated with the formation of new blood vessels and that solid tumor mass need increased vascular network to reach a size of clinical evidence and also to get the power to metastasize. Tumors cannot enlarge beyond 1-2 mm in diameter or thickness unless they are vascularized. Beyond certain size, the tumor does not enlarge without vascularization because of hypoxia-induced cell death.\(^9\)\(^-\)\(^11\)

Mast cells are believed to be an important source of a variety of pro-angiogenic and angiogenic factors, such as heparin, VEGF, chymase, basic fibroblast growth factor (bFGF), 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. The CD34 molecule is a cell membrane glycoprotein found on certain lymphoid and myeloid hematopoietic progenitor cells and vascular endothelium. An endothelial antigen CD34 has been used to highlight microvessels, and its role as an angiogenesis marker has been demonstrated in past.\(^12\)\(^-\)\(^14\)

In our study, the MVD increased in a stepwise manner from NDL to DL to OSCC, but the difference was significant only for OSCC. 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. Furthermore, the counts in leukoplakia group markedly overlapped with those from NOM group. In fact, the mean MVD for NOM (132.5 ± 24.4/mm$^2$) was slightly higher than NDL (122.1 ± 21.2/mm$^2$) but the difference was not statistically significant. Our findings are similar to findings of Tae et al., who showed that MVD was higher in NOM 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.\(^15\) Kalra et al. 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.\(^16\)\(^,\)\(^17\)
Samples from a healthy, uninflamed mucosa would have formed an ideal control group, but we were unable to obtain them because of ethical issues.\textsuperscript{16}

The lack of difference in angiogenesis between normal mucosa and leukoplakia has also been explained by the fact that the method employed for assessing neoangiogenesis is an indirect one where all vessels in the tissue are marked and counted using an endothelial marker, and this whole vascularity is thought to represent the angiogenic status of the tissue. This can lead to conflicting results as it is known that endothelial cells consist of a quiescent population in adult humans, and a pan-endothelial marker like CD34 (as used in the present study) cannot distinguish between active and resting angiogenic vessels (Figure 2). It is suggested that specific markers for angiogenic vessels will be of greater value in studying tumor angiogenesis.\textsuperscript{17}

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.\textsuperscript{18,19}

The MCs might also directly influence the growth of the cancer cells by releasing a high number of bioactive mediators, such as TNF-$\alpha$, IL-1, IL-4, IL-5, IL-6, IL-8, IL-13, NGF, beta-FGF, and TGF-beta, histamine and serine proteases, tryptase, and chymase. The mast cells may directly increase the tumor cells was established by many authors.\textsuperscript{20-22}

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.

There was a significant rise in MCD in OSCC (13.7±/mm$^2$) when compared to DL (12.8±/mm$^2$). Rooney \textit{et al.} suggested that heparin from the mast cells caused vaso proliferation and increased the half-life of bFGF which was a potent angiogenic substance, thereby promoting tumor angiogenesis and facilitating local tumor invasion.\textsuperscript{23}

Based on the findings of our study it can be concluded that we found that the densities of MCs and MVs increased from NOM 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, and DL and OSCC. Therefore, it can be concluded that there is an increased angiogenesis and MVD in OSCC as compared to NOM, NDL, DL also the microvessel area and perimeter are significantly increased in the case of OSCC. When MCD was calculated; there was an increase from NOM to NDL to DL to OSCC group, but there was no statistically significant difference found. There was a poor correlation between MVD and MCD.

\textbf{Conclusion}

From the present, 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.

\textbf{References}

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