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Evaluation of Infiltration of Immunological cells (Tumour Associated Tissue Eosinophils and Mast cells) in Oral Squamous Cell Carcinoma by Using Special Stains

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Research Article

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ABSTRACT

Aim: Our study aimed to evaluate the infiltration of tissue eosinophils and mast cells in oral squamous cell carcinoma (OSCC) by using special stains.
Study Design: Comparative study.
Place of Study: Sharad Pawar Dental College, Sawangi (Meghe), Wardha, Maharashtra.
Methodology: The study was carried out with the sample size of 30 histopathologically diagnosed cases of oral squamous cell carcinoma and comparison of infiltration of these (tissue eosinophil and mast cells) inflammatory cells with control (normal) group of patients, was done by using special stains. Special stains are wonderful because they allow us to see which we cannot see with routine Haematoxylin and eosin stain. Special stains were used to demonstrate tissue eosinophils and mast cells. Carbol Chromotrope and congo red were used for tissue eosinophil and for mast cells staining toluidine blue and thionin were used.

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Results: The comparison of infiltration of tissue eosinophil and mast cell in OSCC with control group (normal) of patients shows significantly increased infiltration of these immunological cells in OSCC group of patients ($P<0.05$). We also found that among special stains carbol chromotrope is better than congo red for demonstration of tissue eosinophils and toluidine blue shows better staining intensity for mast cells than thionin.
Conclusion: We conclude that both inflammatory cells i.e. number of tissue eosinophils and mast cell infiltration is increased in OSCC. Special stains (carbol chromotrope and toluidine blue) are inexpensive and time saving rapid process for microscopic evaluation of infiltration of immunological cells (tissue eosinophil and mast cell) in tumour stroma.

Keywords: Tissue eosinophil; mast cell; carbol chromotrope; toluidine blue; OSCC.

1. INTRODUCTION

Tissue eosinophils and mast cells both are granulocytes which come under myeloid progenitor series of immune cells system (Stewart and Edward, 2001). Eosinophils were first described by Wharton Jones in 1846 as “coarse granule cells”. The eosinophils are 8µm in diameter and characterized by its bright red granules. Their nuclei are usually bilobed although three or more lobes are often observed. An eosinophil is a granular leukocyte which is normally found in the bloodstream and the gut lining (Lee et al., 1999). They contain proteins that help the body to fight infection from parasitic organisms, such as worms. But in certain diseases these proteins can damage the body (Weller and Goetzl, 1980). The term eosinophilia refers to conditions in which abnormally high amounts of eosinophils are found in either the blood or in body tissues (Saraswati et al., 2003).

Mast cells were first described by Paul Ehrlich in 1879. Some years later, Elie Metchnikoff in 1892 suggested that mast cells on account of their phagocytic capacity, might contribute to host defense. Mast cells are round or elongated in shape with characteristic metachromatically staining cytoplasmic granules. Mast cells can be appreciated as large cells with a diameter varying from 5-25 µm. Mast cells are derived from multipotential stem cells in bone marrow. The undifferentiated precursors are carried by the bloodstream to their final tissues of deposition (Castells, 1999). The main functions of mast cells are probably, regulation of vascular functions at the initiation of an inflammatory response and activation of other cells. Mast cells also initiate immediate hypersensitivity reactions, modulate allergic inflammation, and participate in the immune response to parasitic infections (Sharada et al., 2006). Mast cells, when stimulated, can exocytose a fraction of their secretory granules in to the surrounding tissue, a process called degranulation. These cells infiltration can be seen in mucocutaneous diseases, in periodontal diseases, oral submucous fibrosis and oral squamous cell carcinoma (Ankle et al., 2007). Eosinophils are thought to become active following the action of mast cells as mast cells secrete histamine and ECF (Eosinophils chemoattractant factor) which attract eosinophils in tissue (Culling et al., 1985).

Various laboratories do research in the areas of tumor vaccines, cancer immunotherapy, cellular therapy, and gene therapy. However, there are as yet no such licensed products in clinical use. The main barrier to successful product development is a limited understanding of the biology of tumors. So for the development of safe and effective cancer biotherapeutics, it is must to understand each and every aspect of tumour cell biology (Joshi

and Puri, 2009). Solid tumours do not consist of neoplastic cells in isolation rather they are composed of a range of inflammatory cell infiltrate. The chief inflammatory cells mounting the host response to tumourigenic cells include lymphocytes, macrophages, neutrophil, plasma cell mast cells and eosinophils. Stromal response to cancer is usually characterized by the intensity of lymphoplasmacytic infiltration surrounding the tumour. Histologically, dense lymphoplasmacytic invasion of the surrounding stroma is presumably indicative of good host immunologic response to the cancer (Stewart et al., 2001). Recently attention has been directed towards tumour associated tissue eosinophils and mast cells infiltration in OSCC (Goldsmith et al., 1987; Alkhabuli et al., 2007; Debta et al., 2011). So our study aimed to evaluate the infiltration of these cells (tissue eosinophils and mast cells) in OSCC by using special stains. Special stains are wonderful because they allow us to see which we can not see with routine Haematoxylin and eosin stain.

2. MATERIALS AND METHODS

The study was carried out with the sample size of 30 histopathologically diagnosed cases of oral squamous cell carcinoma and comparison of infiltration of these (tissue eosinophil and mast cells) inflammatory cells was done with control (normal)group (10) of patient. Inclusion criteria-1) Histopathologically diagnosed OSCC cases. 2) Surgically operated OSCC cases. 3) Intraoral primary tumour cases of OSCC. Exclusion criteria- 1) Patients who have been treated with chemotherapy or radiotherapy before surgery. 2) Tumours with extensive ulceration and/or necrosis. Two special stains; Toluidine blue & Thionin stains were used for mast cells staining and for tissue eosinophils staining Carbol Chromotrope & Congo red stains were used. High density areas of infiltration of these cells were selected randomly in section and cells were counted in high power fields. For evaluation of the inter/intra-examiner consistency slides were observed by two more examiner for counting of these cells.

Common steps for staining methods (Culling et al., 1985):

Study was performed on paraffin embedded tissue which was fixed in 10% neutral buffered formalin and routinely processed. Paraffin wax blocks were cut and the sections of 5µm were used for staining. All sections were dewaxed thoroughly in xylene and hydrated through descending grades (100%, 90%, 80% and 70%) of alcohol to water.

2.1 Eosinophils Staining (Culling et al., 1985; Bancraft et al. 2002; Alkhabuli et al., 2006)

Two special stains a) Carbol chromotrope and b) congo red special stains were used for tissue eosinophils staining.

a) Method of preparation of Carbol Chromotrope and staining method:

Procedure - Melt 1gm of phenol in a flask by immersing flask in hot water bath. Add 0.5 gm of Chromotrope 2R. Mix & dissolve the resultant sludge in 100ml of distilled water. Date the container. The prepared staining solution has shelf life of about 3 months. Sections were stained with Harries Haematoxylin for 5 minutes then differentiate with 1% acid alcohol. Then counterstaining with Carbol Chromotrope is done for 30 minutes. Result- Eosinophils granules- reddish to pink and Nuclei – blue (Figure 1).

b) Method of preparation of Congo red and staining method:

Add 100ml distilled water in 0.1 gm congo red. Mix it properly then filter the final solution to remove undissolved particles or dust from solution. Date the container as this prepared staining solution has shelf life of about six months. Final solution can be stored at room temperature. After preparation of 1% Congo red solution sections were stained in this solution for 5 minutes, then washed in water and dip in 2.5% KOH solution then counterstaining with haematoxylin was done. Result- Eosinophils granules- red and Nuclei – blue (Figure 2).

2.1.1 Mast cells staining (Culling et al., 1985; Bancraft et al., 2002)

Mast cells are not readily identified in haematoxylin and eosin stains because their metachromatic granules are refractile and do not take up the stain. This metachromasia is due to the high concentrations of the sulphated mucoploysaccharide heparin. Special stains a) Toluidine blue and b) Thionin were used for staining of mast cells.

a) Method of preparation of toluidine blue solution and staining method

Procedure - Add 100ml distilled water in 0.5 gm toluidine blue. Mix it properly then filter the final solution to remove undissolved particles or dust from solution. Date the container as this prepared staining solution has shelf life of about six months. Final solution can be stored at room temperature. After preparation of 0.5% toluidine blue solution sections were stained in this solution for 30 seconds. Result- Mast cell granules- red/purple and Tissue – Varying shades of blue (Figure 3).

b) Method of preparation of thionin solution and staining method

Add 100ml distilled water in 0.6 gm thionin. Mix it properly then filter the final solution to remove undissolved particles or dust from solution. Date the container as this prepared staining solution has shelf life of about three months & can be stored at the room temperature. After preparation of 0.6% aqueous thionin solution sections were stained in this solution for 30 minutes & then differentiate the washed section with 0.2% acetic acid (Figure 4). Result- Mast cell granules- red/purple and Tissue – Varying shades of blue.

Common steps after staining - Sections were washed in tap water then dehydrated through ascending grades (70%, 80%, 90%, and 100%) of alcohol, cleared and mounted in DPX (Distyrene plasticizer xylene). Slides were examined under microscope.

3. RESULTS

The data was collected from all cases and organized in a systemic manner. All data was formulated in table and graph derived from statistical analysis, for interpretation of results. Table 1 depict the age and sex wise distribution in OSCC patient and shows more number of male patient with age group more than 40 year age group.

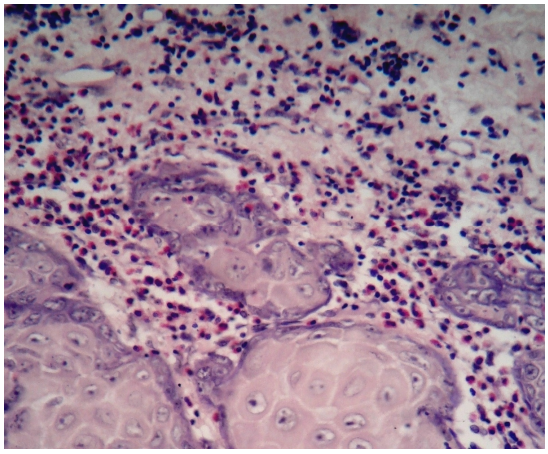


Figure 1. Carbol Chromotrope stained section showing tissue eosinophils

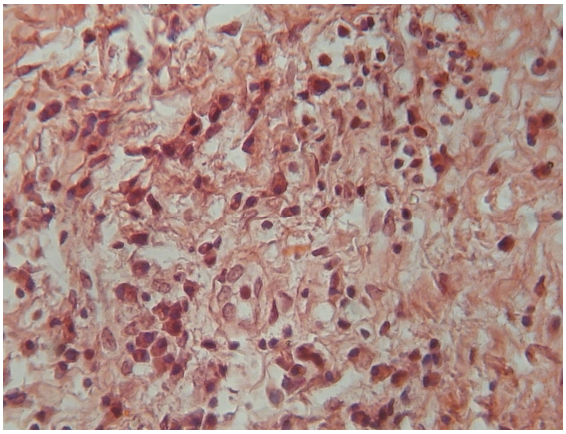


Figure 2. Congo red stained section showing tissue eosinophils

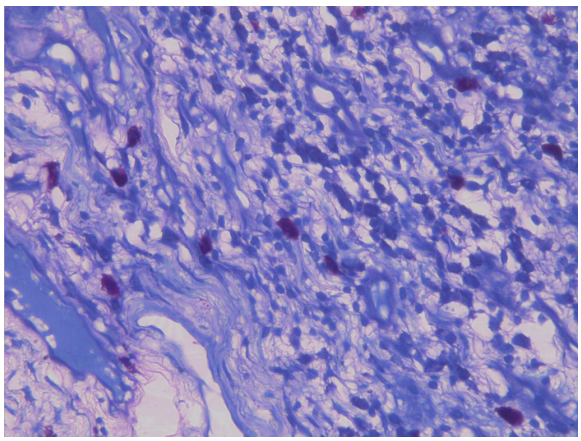


Figure 3. Toluidine blue stained section showing mast cells

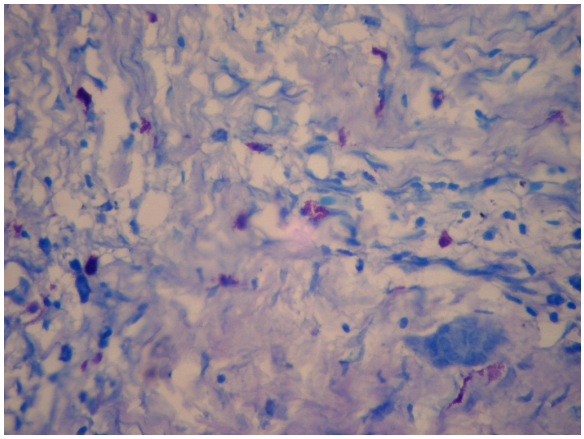


Figure 4. Thionin stained section showing mast cells

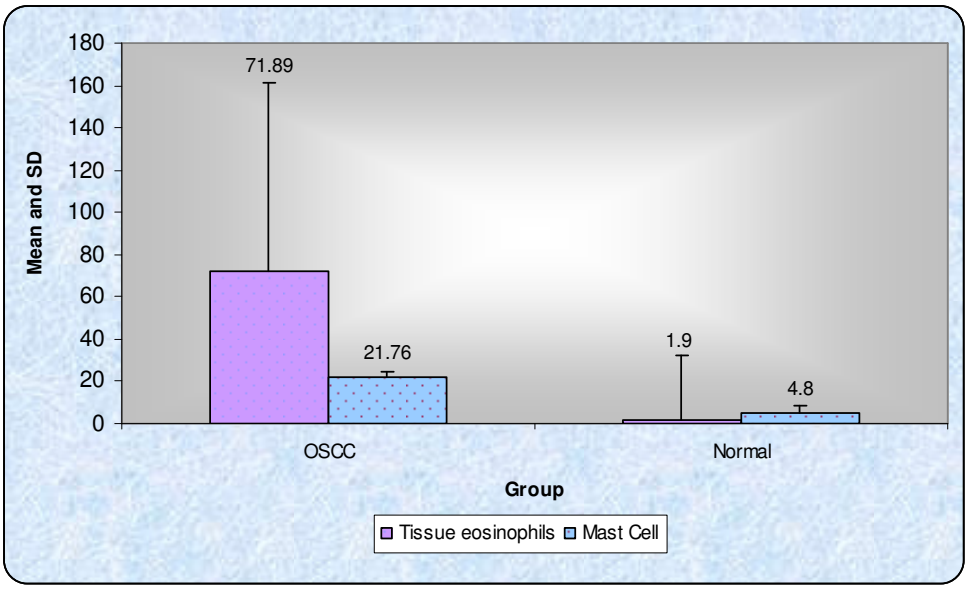
Table 1. Age wise and sex wise distribution of patients in OSCC Group

Age Group (Yrs.)	Male (%)	Female (%)	Total (%)
31-40	3 (14.28%)	0 (0.00%)	3 (10.00%)
41-50	6 (28.57%)	4 (44.44%)	10 (33.33%)
51-60	5 (23.80%)	2 (22.22%)	7 (23.33%)
61-70	3 (14.28%)	2 (22.22%)	5 (16.66%)
70-80	4 (19.04%)	1 (11.11%)	5 (16.66%)
Total	21 (70.00%)	9 (30.00%)	30 (100.00%)

Table 2 and graph1 depict the comparison of infiltration of Tissue eosinophil and Mast cell in OSCC with control group (normal) of patients by using Mann Whitney U test and showing significantly increased infiltration of these cells in OSCC group of patients ($P<0.05$). We also found that among special stains carbol chromotrope is better for demonstration of tissue eosinophil than congo red (Figures 1 and 2) and toluidine blue shows better staining intensity for mast cells than thionin in OSCC tissue section (Figures 3 and 4).

Table 2. Comparison of Tissue eosinophil and Mast cell in OSCC group and Normal group: Mann Whitney U test (Descriptive Statistics)

Inflammatory cell	Group	N	Mean	Std. Deviation	Std. Error Mean	Z-value	p-value
Tissue eosinophil	OSCC	30	71.89	89.78	16.67	3.94	0.00 S, p<0.05
	Normal	10	1.90	2.55	0.80		
Mast Cell	OSCC	30	21.76	30.74	5.61	1.66	0.04 S, p<0.05
	Normal	10	4.80	3.58	1.13		



Graph 1. Comparison of Tissue eosinophil and Mast cell in OSCC and Normal group of patients

4. DISCUSSION

Tumour stroma consists of various inflammatory cells like lymphocytes, macrophages, neutrophils, plasma cells, mast cells and eosinophils (Saraswati et al., 2003, Sharada et al., 2006). Cells of immune system comprised of lymphoid series and myeloid progenitor series cells. Mast cells and tissue eosinophils both are granulocytes which come under myeloid progenitor series of immune cells system (Stewart and Edward, 2001).

Regarding role of tissue eosinophil in the tumour stroma various studies has been conducted by Lowe and Fletcher in 1984, Gold Smith et al. (1987), Gold Smith et al. (1992), Gao et al. (1997), Dorta et al. (2002), and Debta et al. in 2011. All these studies favours that increased number of tissue eosinophil associated with antitumoural role and shows good prognosis. But the studies done by Wong DTW et al. (1990), Horiuchi et al. (1993), Van Driel et al. (1996) and Wong et al. (1999) suggested that tissue eosinophils play a tumor promoting role in oral squamous cell carcinoma. Oliveira et al. (2009) found that tumour associated tissue eosinophilia showed no prognostic value in OSCC and suggest that intense tumour associated tissue eosinophil seems to reflect the stromal invasion of the OSCCs that occur in advanced clinical stage.

Regarding role of mast cells in tumour stroma various studies has been done by Tanooka et al. (1982), Sand et al. (2002), Samoszuk et al. (2005), Ch'ng et al. (2006), Alkhabuli et al. (2007), Sinnamon et al. (2008), Ueda et al. (2008) and Debta et al. (2011) suggestive of antitumoural role of mast cells and its correlation with good prognosis. Theoharides et al., in 2004 suggested that Mast cells produce TNF-alpha, is directly cytotoxic to tumour cells. But studies done by Imada et al. (2000), Elpek et al. (2001), Nam et al. (2002), Iamaroon et al. (2003) and Rojas et al. (2005) suggested that mast cells play tumour promoting role.

The main barrier to successful product development is a limited understanding of the biology of tumors. So for the development of safe and effective cancer biotherapeutics, it is must to understand each and every aspect of tumour cell biology. As there is still controversy regarding role of tissue eosinophil and mast cells in tumour stroma. As some studies have shown that infiltration of these inflammatory cells (tissue eosinophils and/or mast cells) suggestive of a favourable prognosis but other studies however, suggest tumour promoting role.

So to address these controversy, as a first step our study aimed to evaluate the infiltration of both immunological cells (tissue eosinophils and mast cells) in oral squamous cell carcinoma by using special stains. Out of thirty cases of OSCC, 70% were males and 30% were female. Numerous studies have highlighted that females have a much lower annual incidence rate than males and shows male to female gender ratio of 3:1(Neville et al., 2005). In our study the ratio is 2.33:1 (Table 1). The male predominance may be due to predominant habit of tobacco chewing & smoking habit in male. The majority of study cases were above the age group of 40 years (Table-1). This finding reinforces the concept that oral cancer is a disease of increasing age as approximately 95% of oral cancer occurs in patients older than 40 years (Lynch et al., 1994). We also found that there is significant increase infiltration of tissue eosinophils and mast cells in OSCC group in comparison to control group (Table 2). The increase in number of tissue eosinophil was also reflected with an increase of mast cell, as mast cell secrets ECF (Eosinophil chemoattractant factor) which attract tissue eosinophils (Culling et al., 1985).

To prepare special stains for staining of immunological cells, care should be taken, for measurement (weighing) of chemicals and it should be done with accuracy. Inaccurate percentage of preparation of special stains can cause under or over staining of cells and along with it proper uniform cutting of tissue section is must that should be done with a good microtome (Culling et al., 1985; Bancraft et al., 2002).

The special stains (i.e. carbol chromotrope and congo red) were used for demonstration of presence of tissue eosinophils in tumour stroma we found that carbol chromotrope stain is better than Congo red (Figures 1 and 2). Toluidine blue shows better staining intensity for mast cells than thionin in OSCC tissue section (Figures 3 and 4). The most striking morphological features of mast cells are the larger number of strongly metachromatic granules present in the cytoplasm. Mast cell granules often cover the cell nucleus (Culling et al., 1985).

5. CONCLUSION

We conclude that both immunological cells i.e., number of tissue eosinophils and mast cell infiltration is increased in oral squamous cell carcinoma (OSCC). Thus quantitative assessment of eosinophils and mast cells are the most important aspects of the microscopic evaluation of oral squamous cell carcinoma. For proper evaluation of these immunological cells in OSCC, special stains are one of the important tools that are inexpensive and give a good rapid result.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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