

Quantification of mast cells in oral reactive lesions - an immunohistochemical study

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Abstract. *Background and aim:* Reactive lesions (RLs) are the most common oral mucosal lesions that are benign in nature and are more likely to reoccur if the lesion or local irritants at the site are not completely removed. The histopathology is usually determined by the stage of the lesion, which includes neovascularization, inflammation, and fibrosis etc. To evaluate and compare mast cell counts in different reactive lesions with normal gingiva (NG) and to determine the correlation between mast cell count and inflammation, fibrosis, and angiogenesis using immunohistochemistry. *Materials & Methods:* 10 pyogenic granulomas (early and late), 10 irritational fibromas, 5 inflammatory fibrous hyperplasia, and 5 peripheral cemento-ossifying fibromas 5 normal gingiva were evaluated. Mast cell counts were compared. ANOVA and t-tests were used to analyze the data. Spearman correlation was used to compare the mast cell count to the inflammation, fibrosis, and vascular components. A p-value of 0.05 was considered statistically significant. *Results:* The mean number of mast cells were increased in oral reactive lesions when compared to NG. Although mast cells were significantly higher in IFH and IF, there was no correlation found among mast cells and fibrosis/inflammation/vascularity. *Conclusion:* Reactive process involves multiple interactions among mast cells, endothelial cells, fibroblasts, and other immune cells, among which the role of mast cells has been evaluated. Mast cell count increases in these reactive lesions, possibly reflecting an important role in microenvironment modification, but it is not the sole cause of these lesions' pathogenesis. (www.actabiomedica.it)

Key words: Reactive lesions, mast cell, tryptase, immunohistochemical analysis

Introduction

Reactive lesions (RLs) are most frequently encountered oral mucosal lesions which account for 10.3-12.4% of the routine histological oral biopsies. These swellings are non-neoplastic, but they are hyperplasia's caused by local irritants. Some of the local irritants in the oral cavity are dental plaque, calculus, impaction of food, sharp edges of carious teeth, ill-fitting or defective restorations and appliances etc (1-3). Inflammatory fibrous hyperplasia (IFH), irritational fibroma (IF), pyogenic granuloma (PG), peripheral cemento ossifying fibroma (PCOF) are some routinely seen oral reactive lesions.

PG is defined as a lesion that develops as an exaggerated localized connective tissue reaction to minor injury or irritation (4). Its response depends on the length of time it has been present, with early lesions displaying vascular proliferation resembling granulation tissue, and in the later stages the granulation tissue is replaced by fibrous tissue. Surface alterations such as ulceration, which can be observed as a thick fibrinopurulent membrane and acute inflammatory cells microscopically, can be noted in certain PGs (4,7). IFH, on the other hand, are the inflammatory lesions that develop because of chronic irritation. Exuberant tissue repair characterizes the response to tissue damage, with clinical and microscopic

appearances varying depending on development stage and intensity of mucosal irritation. Microscopically, it is characterized by variable collagen deposition and chronic inflammatory infiltrate (5). IFs are most common in buccal mucosa, clinically manifest an exophytic, firm and asymptomatic nodule with pink or flesh-colored and a smooth surface. Microscopically, IFs are described as a dense bundle of collagenized fibrous tissue. Unlike other oral reactive lesions, PCOF are limited to gingiva which supports histogenic derivative from periodontal ligament. Clinically, it appears as a pedunculated or sessile painless lump on the gingiva or alveolar mucosa. Lesions that are younger are uneven and red, whereas older lesions have a smooth pink surface. It is microscopically characterized by proliferation of fibroblasts along with pathological ossifications and or calcifications (6).

Clinical appearance of all these lesions is similar but histopathology is always confirmatory. A substantial overlap exists between various histological types of these lesions suggested that these are at different stages of development. For many years, it has been point of contention whether all of these reactive proliferations are distinct entities or stages in the evolution of a single lesion. They are highly vascular, reddish, and bleed easily at first, but later appear firm, pink, and relatively avascular. The key feature in all these lesions is hyperplastic reaction, this might be due to migration and proliferation of inflammatory cells, vascular endothelial cells, and fibroblasts (7).

Mast cells are described in the literature as bone marrow derived, oval to round shaped, uninucleate, large granular, multifunctional cells. They can secrete a wide variety of mediators and appear to play vital role in pathogenesis of various disease states like scleroderma, oral submucous fibrosis, gingival irritational fibromatosis, chronic hypertrophic dermatitis, arthritis, malignancies including oral squamous cell carcinoma etc. Degranulated mast cells secrete a variety of preformed and newly synthesized mediators into the surrounding environment (8). These mediators in turn either directly or indirectly act as a stimulant for mast cells, T lymphocytes and several other inflammatory cells. It has been demonstrated that these mediators in fact act as a mitogen on fibroblasts and epithelial cells. Mast cells can also contribute to break down connective tissue by activating procollagenases, causing inflammatory cells to infiltrate the tissue and facilitates angiogenesis (8, 9).

Mast cell identification has long been relied on metachromatic heparin staining with toluidine blue, although this is a less reliable method because it does not stain immature mast cells. Serine protease tryptase, a tetrameric protein, expression is the current gold standard for determining mast cell phenotype in various tissue components and by immunoperoxidase staining procedure with anti-tryptase monoclonal antibody is considered highly specific and sensitive method for its detection.

Oral reactive lesions histopathology is usually determined by the stage of the lesion, which is determined by the amount of vascularization, inflammation, and fibrosis. Cytokines are the key mediators in these processes and mast cells are well known to release a wide variety of these mediators. Therefore, to evaluate potential contributing factors in reactive lesions onset/progression, we analyzed mast cell population, fibrosis, inflammation, and angiogenesis in four types of oral reactive lesions. The purpose of this study was to use immunohistochemistry to examine the mast cell count in various reactive lesions and see if there is an association between mast cell count and inflammation, fibrosis, and angiogenesis.

Materials and Methods

In this cross-sectional study 35 cases diagnosed as PG (10), IF (10), PCOF (5), IFH (5), and 5 normal gingiva (NG) were retrieved from the archives of oral and maxillofacial pathology department, Vishnu dental college, Bhimavaram. Based on the vascular component and fibrosis, 10 cases of PG were divided into five early and five late lesions after reviewing the histological slides. Furthermore, in the current study IFH cases are all associated to denture irritation. Hematoxylin & Eosin-stained sections were used for the evaluation of inflammatory, fibrous, and vascular components and were graded as follows.

Inflammatory infiltrate:

Inflammatory component in these lesions is categorized according to intensity and localization as follows

- Absent - No inflammatory component
- Mild - Slight scattered inflammation

- Moderate - Predominant scattered inflammation
- Intense - Predominant diffuse inflammatory cells

Localization of inflammatory infiltrate was graded as Focal (areas of inflammatory infiltrate comprising up to 50% of the slide) or Diffuse (areas involving more than 50% of the slide). Predominant cell type was graded as acute, chronic, and mixed.

Fibrous component:

Fibrous component was graded as based on the distribution of fibers in the lesions and are categorized into thin and thick based on dense appearance of the fibers.

- + - < 25%
- ++ - 25-50%
- +++ - >50%

Vascular component:

Vascular component was also graded based on the distribution and the size of the vessels were designated as small, medium, and large sized vessels.

- + - < 25%
- ++ - 25-50%
- +++ - >50%

In all these lesions, to determine mast cell count connective tissue component was divided arbitrarily into two parts, juxta-epithelial connective tissue, and deeper connective tissue. Considering fibrinopurulent membrane is an ulcerated surface that recruits inflammatory cells, mast cells, and other components of chronic inflammation, 5 fields below the epithelium and 5 fields below fibrinopurulent membrane were investigated in the case of PG.

Three-micron slices were cut from each formalin-fixed, paraffin-embedded sample. Mast cell tryptase was used to detect the presence of mast cells. Sections were collected on positively charged glass slides (Fisher-Scientific, Biogenix), deparaffinized with xylene, and rehydrated using a series of alcohols. Antigen retrieval was accomplished by submerging the slides in citrate buffer, pH 6.0 (Tris-citrate buffer in distilled water and pH was adjusted by adding 1N HCl) in microwave oven for about 15 min (3 cycles-5min each). The slides were then incubated with primary antibody against tryptase for 1hr (monoclonal mouse anti human mast cell tryptase, clone G3, Biogenix) and washed in Tris-buffered saline. Following

that, the sections were cultured. After around 20 minutes with the super enhancer, the slides were incubated with a secondary antibody for about 30 minutes. A solution containing a drop of 3,3'diaminobenzidine in 1ml of diluent (DABchromogen, Biogenix) for 2mins acts as a chromogen. The slides were then mounted after being counterstained with Harris hematoxylin. Neurofibroma served as a positive control, while primary antibodies were omitted in the negative controls. The results were based on the stain's brown color distribution.

Using a digital camera DP71 and a visual monitor screen coupled to an Olympus BX51 research microscope, each slide was carefully evaluated by three observers at the same time until consensus was achieved. The mean number of mast cells, as well as their distribution in superficial and deeper connective tissue, were analyzed in ten high power fields (40X). Mast cells were evaluated independently as intact and degranulated, yielding a total number of mast cells when totaled. Mast cells were classified as ovoid, spindle, or irregular based on their shape.

Statistical Analysis

The aim was to compare the mean number of mast cells in distinct oral RLs with NG. Mast cells from superficial and deeper connective tissue, as well as degranulated and granulated cells, were compared. The tests that were used are ANOVA and the t-test. Spearman correlation was used to compare the mast cell count with inflammatory component, fibrosis, and vascularity. Results were considered statistically significant when the p-value was <0.05.

Results

Mast cell count was detected in all the (PG, IFH, IF, PCOF, and NG) cases. The morphological parameters such as form and characteristics of inflammatory infiltration, fibrosis, and vascularity are shown in Table 1.

When compared to NG, the mean number of mast cells increased significantly in oral reactive lesions, and the mast cell count was slightly higher

Table 1. Characteristics of the inflammatory infiltrate, fibrosis, and vascular component

MORPHOLOGICAL CHARACTERISTICS	PARAMETERS	PG(n=10)	IF(n=10)	IFH(n=5)	PCOF(n=5)	NG(n=5)
SHAPE	OVOID	8	5	1	2	0
	IRREGULAR	2	5	4	3	5
	SPINDLE	0	0	0	0	0
INFLAMMATORY CELL DENSITY	NO	0	3	0	0	4
	MILD	1	6	3	0	1
	MODERATE	5	1	2	5	0
	SEVERE	4	0	0	0	0
INFLAMMATORY CELL DISTRIBUTION	NO	0	3	0	0	4
	FOCAL	6	6	5	5	1
	DIFFUSE	4	1	0	0	0
FIBRE DENSITY	THIN	10	2	5	3	0
	MODERATE	0	4	0	0	0
	THICK	0	4	0	2	5
	THICK					
FIBRE DISTRIBUTION	+	6	0	0	0	0
	++	4	0	1	5	5
	+++	0	10	4	0	0
MICROVESSEL DENSITY	MILD	0	1	1	0	5
	MODERATE	6	7	3	5	0
	SEVERE	4	2	1	0	0
VESSEL LUMEN	SMALL	6	10	5	5	5
	LARGE	4	0	0	0	0

beneath the epithelium, but the difference was not statistically significant ($p=0.4$)

In comparison to all the lesions that are evaluated, IFH had a higher percentage of degranulated mast cells. In IFH and IF, the mean percent of degranulated cells were much higher and the results are statistically significant (p -value - 0.001). Granulated mast cells are significantly higher in pyogenic granuloma and less in IFH in deeper connective tissue (p -value - 0.001) (Table 2).

Mast cell counts in the juxtraepithelial connective tissue are significantly higher in early PG when compared to mast cell counts below the fibrinopurulent membrane (p -value - 0.001) (Table 3)

Mast cell counts were also correlated with inflammatory component, fibrosis, and vascular component using the Spearman correlation coefficient test, but the results were not statistically significant. The chi-square test implies that there was an increase in inflammatory component in IFH, increase in fibrous component in IF, and increase in blood vessel density in early cases

of PG. The images below demonstrate immunohistochemically stained mast cells in various oral reactive lesions (Fig: 1).

Discussion

Mast cell counts in PG, IFH, IF, PCOF, and NG were compared in this study. Mast cell counts are highest in IFH, followed by IF, PCOF, and PG, according to the findings of this study. When compared to normal gingiva, all these lesions have an increased number of mast cells. The previous literature revealed varying results in different studies, with the difference most likely due to the method used in their studies. Similar findings have been seen in other studies, such as an increase in mast cell count in IFH patients (5, 10). This increased number of mast cells could be explained by the fact that all these lesions are inflammatory. Mast cells, along with other inflammatory cells, have increased in number. In the presence of a

Table 2. Mean and standard deviation of mast cell count in different oral reactive lesions compared to normal gingiva.

Lesions	(Mean±SD) Mast cells				
	Total Cells	Juxtaepithelial CT	Deeper CT	Degranulated	Granulated
Pyogenic Granuloma	45.64±23.45	26.46±13.65	19.18±10.90	23.71±13.75	22.02±10.80
Inflammatory Fibrous Hyperplasia	64.90±9.57	29.84±8.80	27.61±12.83	58.66±10.55	6.22±4.24
Irritational Fibroma	50.75±22.57	26.13±13.32	24.62±10.66	33.22±14.71	17.33±12.62
Peripheral Ossifying Fibroma	51.56±13.85	32.28±19.63	19.28±9.55	37.5±11.93	14.12±16.72
Normal Gingiva	18.6±5.44	8.84±2.01	9.76±3.46	7.74±3.02	10.86±2.43

Table 3. Mean and standard deviation of mast cell count in pyogenic granuloma

Pyogenic Granuloma (PG)	Early PG	Late PG
Below Fibrinopurulent membrane	7.12±3.6	24.44±25.91
Juxtaepithelial CT	27.86±14.32	27.36±7.87
Deeper CT	19.18±10.90	18.88±10.90

condensed fibrotic matrix, the significant increase in PCOF and IF could indicate that the presence of mast cells in these lesions is most likely a marker of inflammation that leads to fibrosis. Several studies have been revealed that mast cells play a crucial role in fibroproliferative disorders, owing to the secretion of several chemical mediators such as tryptase and it interacts with the protease activation receptor (PAR-2) that leads to fibroblast proliferation and fibrosis (17).

Present study also separately evaluated mast cell count between the juxta epithelium and the deeper connective tissue, where mast cells were slightly higher below the epithelium in all the lesions. The possible explanation could be the mast cell growth factor (MGF) which is a key mediator that influences mast cell migration. MGF is synthesized by endothelial cells and epidermal keratinocytes and is thought to direct the homing of mast cell precursors beneath the epithelial tissues (11). This could also be due to the release of interleukin-6 (IL-6) and IL-8 by epithelial cells, which aid in the recruitment of further mast cells. Mast cells in PGs were evaluated separately beneath the fibrinopurulent membrane and the epithelium. Mast cells were significantly higher beneath the epithelium in early cases of PG. In this case, as well, the above reason applies, as the epithelium aids in the migration and recruitment of mast cells.

Mast cell degranulation releases preformed granules containing mediators such as histamine, tumor necrosis factor, serotonin, and numerous proteases, which are responsible for the majority of mast cell-dependent functional responses (12). Therefore, determining whether mast cell is intact or degranulated may be good indicator to assess whether mast cells are involved in specific biological process (7, 13). Thus, immunohistochemistry was used in this study to demonstrate intact mast cells and degranulated mast cells.

Degranulated mast cells were significantly higher in IFH, followed by PCOF and IF in the present study. The degranulated mast cell-derived mediators could influence adjacent cells, resulting in a wide range of stimuli. It creates a pathway that leads to inflammation. In vitro, mast cell degranulation promotes the release of cytokines such as IL-8, IL-5, Tumor necrosis factor (TNF), tryptase and heparin, which leads to T-lymphocyte recruitment, which releases chemokines such as Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) and aids in further mast cell degranulation. Thus, it helps in chronic inflammation (8,15).

Although mast cells play a role in the initiation and progression of inflammatory processes, studies have also suggested that mast cell activation in disease

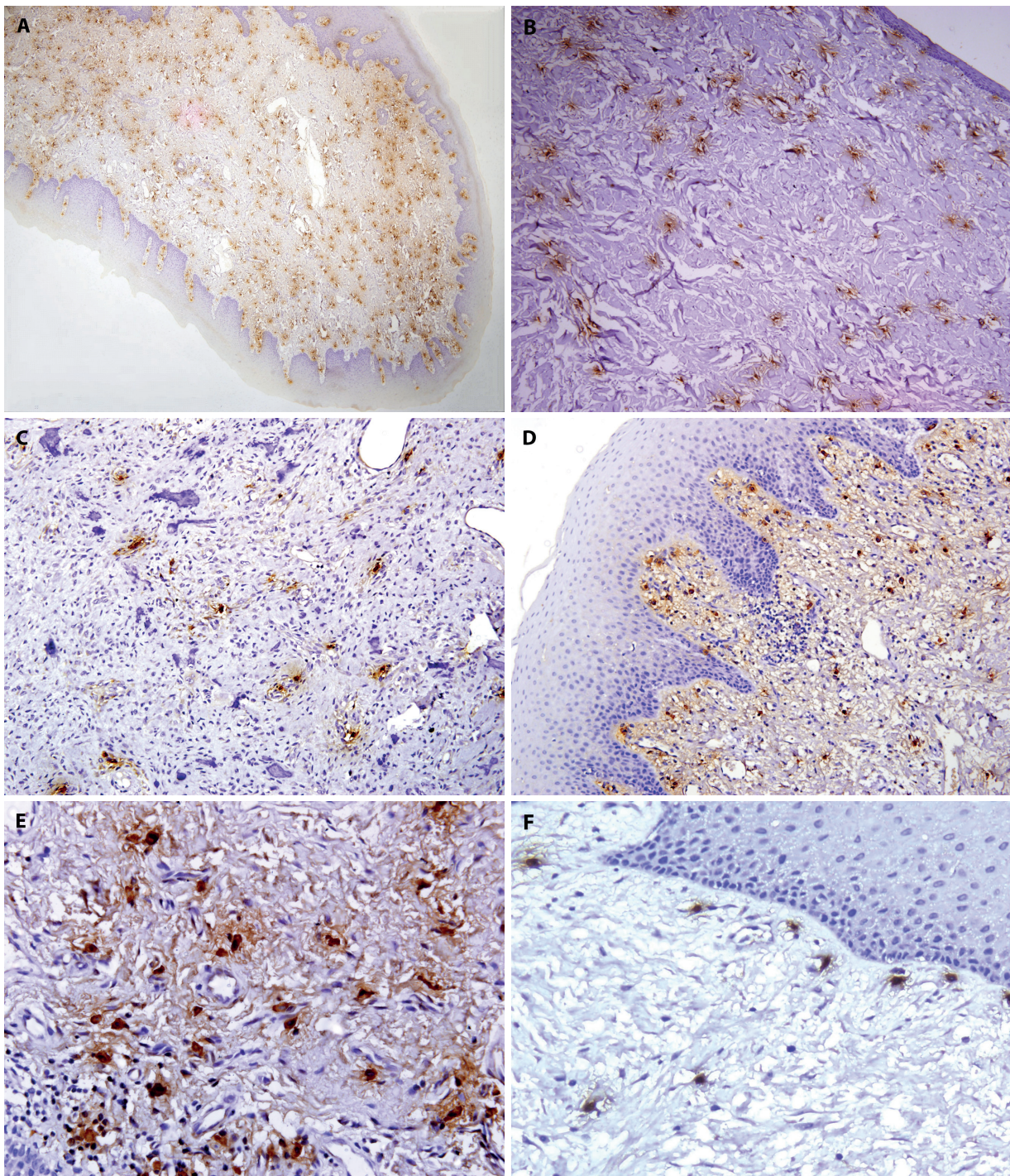


Figure 1. Immunohistochemically stained mast cells in different oral reactive lesions: a) 4X magnification showing diffuse distribution of mast cells in IF b) 10X magnification in IFH c) 20X magnification showing mast cell distribution in the connective tissue of PCOF d) 20X magnification showing more number of mast cells near epithelium in PG e) 40X magnification showing distribution of mast cells in the connective tissue of PG f) 20X magnification showing granulated mast cells in normal gingiva

states such as scleroderma and fibrotic lung disease suggests that mast cell interaction with fibroblasts includes the process that leads to fibrosis (12, 14). Previous studies have demonstrated increase no of mast cells in PG, IF, PCOF, Oral submucous fibrosis (10). The association of these cells with intensity of fibrosis has been reported in salivary gland tissue in patients with Sjogren's syndrome. In addition to oral lesions presence of mast cells has been shown in several conditions including skin fibrosis, scleroderma, fibrosis in appendix, hyperplastic and connective tissue tumors of skin, breast carcinoma, renal fibrosis, and others (15-17). Mast cells influence fibroblast function and fibrosis by releasing prefabricated mediators like histamine, prostaglandins (PG), matrix metalloproteinases (MMPs), and a variety of other mediators. Fibroblasts may be stimulated as a result of mast cell activation in PCOF and IFH lesions, culminating to fibrosis.

Mast cells are rich in MMPs such as heparin, tryptase, and plasminogen activators, which contribute to the degradation of extracellular matrix, which is the first step in neo-angiogenesis and tumor invasion (18). Mast cells are thus regarded as an angiogenic switch during the early stages of the development of an inflammatory lesion (19). Mast cells may also act directly by stimulating the migration and proliferation of endothelial cells by degrading the connective tissue matrix and by activating collagenases, thus providing space for the formation of neurovascular sprouts, which helps in revascularization, collagen deposition, and matrix remodeling, all of which have a reparative role (20). Thus, mast cells produce remodeling through neovasculogenesis in early lesions and reparative processes through collagen deposition in later lesions. According to the current study, their significant increase may contribute to micro environmental modification in these specific lesions.

Conclusion:

Reactive process involves multiple interactions among mast cells, endothelial cells, fibroblasts, and

other immune cells, among which the role of mast cells has been evaluated. According to the results of our study, mast cell count increased in all the reactive lesions compared to normal gingiva, possibly reflecting an important role in microenvironment modification during initiation and progression of these oral reactive lesions. Despite the fact there was no relation between mast cell count and inflammation, fibrosis, or angiogenesis, we believe that mast cells have complex interactions with surrounding environment. Mast cells, however, are not the sole cause of these lesions' pathogenesis. As a result, more studies with a larger sample size are required to obtain a reliable relationship. The current study's limitation is small sample size, but further research into mast cells may improve our understanding of their role in the pathogenesis of various oral reactive lesions.

Conflict of interest: Each author declares that he or she has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

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