Evaluation of mast cells in odontogenic cysts by toluidine blue & c-kit gene product (CD117)

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Abstract

Introduction: Odontogenic cysts are characterized by an expansile non–infiltrative growth through several mechanisms which include presence of mast cells in the capsule. The mast cells can be demonstrated histochemically as well as immunohistochemically.

Aim and Objectives: The primary objective of the study was to analyze the presence and distribution of mast cells in radicular cyst, dentigerous cyst and keratocystic odontogenic tumor histochemically and immunohistochemically, to intercompare their expression and to correlate it with the degree of inflammation.

Material and Methods: Tissue sections of 30 cases each of radicular cyst, dentigerous cyst and keratocystic odontogenic tumor were stained with toluidine blue and CD117. Quantitation and localization of mast cells was determined by dividing the section into subepithelial and deep zone. The mean number of mast cells was compared between toluidine blue and CD117 for each cyst, and also between two zones for each cyst, using ANOVA test. Correlation of mast cells with degree of inflammation was done using Pearson's correlation coefficient.

Results: The mean number of mast cells were maximum in keratocystic odontogenic tumor, both histochemically and immunohistochemically, with high localization in the subepithelial zone. Immunohistochemical staining detected higher number of mast cells than histochemistry (4.54 and 3.23 respectively). Statistically significant correlation of mast cells with degree of inflammation was observed.

Conclusion: Mast cells may play a role in the pathogenesis of odontogenic cysts as an elevated number of mast cells were found in the connective tissue capsule of all three odontogenic cysts.

Keywords: CD117; Keratocystic Odontogenic tumor; Mast cells; Radicular cyst; Toluidine blue.

Introduction

Odontogenic cysts, possibly the most common benign destructive lesions in human maxillofacial skeleton, are characterized by an expansile non–infiltrative growth.(1) The exact mechanism of growth and expansion associated with these lesions is still not clear, but it is known that several different cell types, including mast cells, can participate in these phenomena.(2)

Mast cells have often puzzled investigators from the time they were first identified and named by Ehrlich in 1879 as 'Mastung'/'Mastzellen', i.e. they exist to nourish the surrounding tissue.(3,4) They are divided into two subsets in rodents – connective tissue mast cells (CTMC) that are located in connective tissue, demonstrated by toluidine blue, and secrete enzymes tryptase and chymase; and mucosal mast cells (MMC) that are located within mucosa, demonstrated by Alcian blue at acidic pH, and secrete enzyme tryptase. The MMCs are predominantly found in the alveolar walls and small intestinal mucosa, whereas the CTMCs are ubiquitous.(5,6) Moreover, the visualisation of MMCs requires the application of appropriate fixative and staining procedures, as the glycosaminoglycans stored in their granules have relatively low molecular weight and though they are coupled with proteins, they can easily be dissolved in routinely used fixatives.(7) In humans, three types of mature mast cells are identified: MCt - mast cells containing only tryptase; MCtc - containing tryptase, chymase and carboxypeptidase; and the less frequent MCc - containing chymase.(8)

Mast cells participate in events associated with inflammation and bone resorption and interact with other cells of the immune system.(9) Their presence has also been recognized in odontogenic cysts in terms of total glycosaminoglycan content (heparin) of cyst capsular wall.(10)

Mast cells are not easily recognized by hematoxylin and eosin staining,(11) hence they are commonly stained by basic dyes like toluidine blue. The presence of sulphated mucopolysacchrides, within the granules gives the characteristic metachromatic staining reaction with toluidine blue.(12) However, such staining seems to be dependent on an intact number of mast cell granules, whereas the more sensitive immunohistochemical techniques are able to detect partially degranulated mast cells, which still contain enough tryptase.(9) Moreover, basophils also have the capacity to release tryptase in vivo, and so anti-tryptase antibodies cannot be used to distinguish these two cell types from one another; and therefore tryptase is not an exclusive marker of mast cells.(13)

Existing data revealed that mast cells have strong membrane reactivity for CD117 that may be useful in diagnosis of mast cell disorders.[14] Since literature review revealed very few studies where immunohistochemical evaluation of mast cells has been carried out using CD117 in periapical lesions and odontogenic cysts, the present study was planned with the aim of analyzing the presence and distribution of mast cells in radicular cyst (RC), dentigerous cyst (DC) and keratocystic odontogenic tumor (KCOT) histochemically and immunohistochemically; to intercompare their expression and to correlate it with degree of inflammation.

Material and Methods

Patients: The study comprised of total 90 cases, 30 each of RC, DC and KCOT diagnosed on the basis of clinical and histopathological correlation. The specimens of those patients who presented with the history of chronic allergic reactions, inflammatory and parasitic infections were excluded. Also, the specimens that had undergone decalcification were excluded as mast cell granules could be masked by this technique. The study protocol was reviewed by the Ethical Committee of the institute and was granted ethical clearance. Formalin fixed paraffin embedded tissue blocks of these cases were retrieved from the archives of the Department of Oral and Maxillofacial Pathology of the institute.

Histochemical staining and evaluation: Serial sections of 4-5 µm thickness were cut from the paraffin wax blocks of 90 specimens. For histochemical evaluation, the sections were stained with freshly prepared 1% toluidine blue (adjusted at ph 2.0 – 2.5) for 2-3 minutes, using Toluidine blue staining protocol for mast cells.[15] Following dehydration and clearing, the sections were mounted with resinous mounting media [DPX (Rankem, RFCL Ltd)] and followed by mast cells counting. Round, oval, or fusiform cells containing purplish red metachromatic granules in their cytoplasm were considered to be mast cells (Fig. 1).

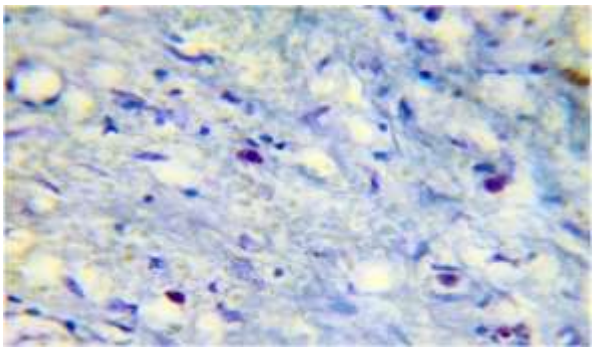

Fig. 1: **Photomicrograph showing mast cells under toluidine blue stain (100x magnification)**

Immunohistochemical staining and evaluation: For immunostain with CD117, the sections were mounted on super frost slides, dewaxed, and incubated with 0.3% hydrogen peroxide in methanol at room temperature for 10 min. Slides were then washed in running tap water for 15 min and treated with 0.1% trypsin (Sigma) mixed in 0.1% calcium chloride (pH 7.8) for 10 min at room temperature. Nonspecific binding of protein was blocked by incubation in normal rabbit serum diluted 1:5 in Tris-buffered saline (TBS; pH 7.6) for 15 min. the slides were then incubated for 18 h at 4^0C with the polyclonal rabbit anti-human antibody CD117 (anti-c kit antibody) DAKO ((A) 4502, Dako, Cambridge, UK), washed in TBS (Tris-buffered saline) for 7 min and then incubated for 30 min with biotinylated rabbit anti-mouse IgG (Amersham, UK). Later, the slides had been washed in TBS for 7 min; they were further incubated with streptavidin-biotin complex conjugated with HRP (horse-radish peroxidase) polymer for 30 min. The slides were developed in diaminobenzidine-hydrogen peroxide substrate (Sigma) for 10 min, rinsed in TBS and washed in tap water for 5 min. Sections were then counterstained with Harris' hematoxylin, dehydrated, cleared, and mounted in DPX (Rankem, RFCL Ltd). The controls for staining were obtained by omitting the primary antibody in the staining sequence which was replaced by normal mouse serum (Dako, Glostrup, Denmark).

The immunohistochemical results demonstrating strong membrane positivity in the round, oval, or fusiform cells, were considered to be mast cells (Fig. 2).

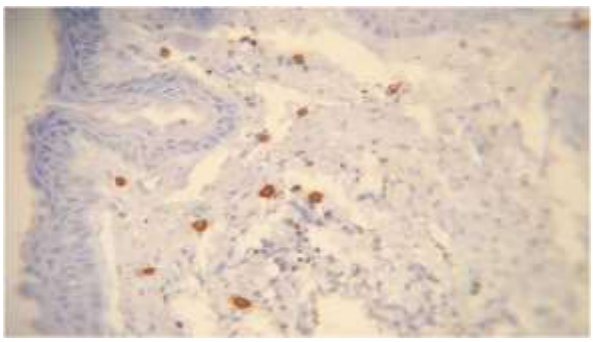

Fig. 2: **Photomicrograph showing mast cells under CD117 stain in Keratocystic Odontogenic Tumor (40x magnification)**

Counting of mast cells: For the quantitation and localization of mast cells by both toluidine blue and CD117, the section was divided into two zones: subepithelial and deep zone; using an ocular grid. For the subepithelial zone, the 1 cm^2 graticule was oriented along the connective tissue capsule at its junction with the epithelium and every alternate high power microscopic field was counted (The area encompassed by the graticule was taken as one microscopic field). The graticule was then moved two high power fields into the connective tissue and the procedure repeated for the deep zone. In this way, 10 fields were counted at each zone of the capsule in each specimen which makes

a total of 300 high power fields for each cyst (n=30 for each cyst).

Degree of inflammation in the cyst wall was scored as per the criteria given by Netto et al,[2] Smith et al[10] and Debta P et al[16] by counting inflammatory cells in 10 random high-power fields and describing the mean number of cells per field according to the following scale: no inflammation (zero inflammatory cells), mild + (average of 15 inflammatory cells or fewer / field), moderate ++ (average of 16–50 inflammatory cells/ field), and severe inflammation +++ (average of more than 51 inflammatory cells / field).

Statistical analysis: The total number of mast cells per high power field for individual cyst was recorded in the two zones and the mean and standard deviation (SD) values were calculated. The mean number of mast cells was compared between toluidine blue and CD117 for each cyst, and also between the two zones for each cyst, individually for toluidine blue and CD117 using one way ANOVA (Analysis of Variance) test, where significance was established as $p<0.05$. The quantity of mast cells in each cyst was correlated with the degree of inflammation using Pearson's correlation coefficient. All the statistical analysis was carried out using SPSS software, version 11.5.

Results

Mast cells were evaluated in the connective tissue capsule of all the cyst specimens examined. On comparing the zone wise distribution of mast cells stained with toluidine blue, we found that the mean number of mast cells was higher in the subepithelial zone as compared to the deep zone in all the three cysts, with a statistically significant difference ($p<0.05$) between the observed zones. Also, this concentration was higher in KCOT (mean = 2.62) as compared to other cysts (Table 1).

Table 1: Comparison of distribution of mast cells between zones in the three cyst groups stained with Toluidine Blue

Cyst	Zone	Number of High Power Fields	Mean± Standard Deviation	p* Value
Radicular Cyst	Subepithelial	300	**2.13** ± 1.342	0.012
	Deep	300	1.55 ± 1.176	
Dentigerous Cyst	Subepithelial	300	**1.34** ± 1.087	0.017
	Deep	300	1.14 ± 1.086	
KCOT	Subepithelial	300	**2.62** ± 1.529	0.041
	Deep	300	2.17 ± 1.655	

* p value < 0.05 = statistically significant

Similarly, on comparing the zone wise distribution of mast cells stained with CD117, we found that the mean number of mast cells was higher in the subepithelial zone as compared to the deep zone in all the 3 cysts, with a statistically significant difference (p =0.001, 0.012, 0.009 for RC, DC and KCOT respectively) between the observed zones. Also this concentration was higher in KCOT (mean = 3.46) as compared to other cysts (Table 2).

Table 2: Comparison of distribution of mast cells between zones in the three cyst groups stained with CD 117

Cyst	Zone	Number of High Power Fields	Mean± Standard Deviation	p* Value
Radicular Cyst	Subepithelial	300	**2.89** ± 1.543	0.001
	Deep	300	2.19 ± 1.453	
Dentigerous Cyst	Subepithelial	300	**1.86** ± 1.134	0.012
	Deep	300	1.45 ± 1.563	
KCOT	Subepithelial	300	**3.46** ± 1.875	0.009
	Deep	300	2.45 ± 1.269	

*p value < 0.05 = statistically significant

Further intercomparison between toluidine blue and CD117 for the three cysts revealed a statistical significant difference ($p <0.05$), clearly indicating that the mean number of mast cells detected was higher in all cysts when evaluated with immunohistochemical marker CD117 (Table 3).

Table 3: Comparison of toluidine blue and CD117 stained mast cells in the three cyst groups

Cyst	Stain	Number of High Power Fields	Mean ± SD	p* Value
Radicular Cyst	Toluidine Blue	300	3.22 ± 1.653	0.005
	CD 117	300	**4.23 ± 1.542**	
Dentigerous Cyst	Toluidine Blue	300	2.43 ± 1.647	0.007
	CD 117	300	**3.65 ± 1.32**	
KCOT	Toluidine Blue	300	4.06 ± 2.064	0.001
	CD 117	300	**5.76 ± 3.786**	

*p value < 0.05 = statistically significant

Inflammation was maximum in the RC followed by KCOT and DC. The mean and standard deviation values for mast cells revealed a significantly positive correlation with the degree of inflammation in each of the cyst (Table 4).

Table 4: Correlation of mast cells with degree of inflammation in odontogenic cysts

	Inflammation	RC	DC	KCOT
Pearson Correlation	1	.680(*)	.589(*)	.697(*)
Sig. (1-tailed)		.017	.034	.011
N	90	30	30	30

*Correlation is significant at the 0.05 level (1-tailed)

Discussion

Most physicians and immunologists, if they think of the mast cell at all, regard it as something of a 'pariah'. Other cells of the hematopoietic origin, such as neutrophils, macrophages, and platelets, are clearly important in host defense, hemostasis, or both. In contrast, the contributions of mast cell to pathologic conditions come to mind much more readily than any role it may have in the maintenance of health.[17]

Mast cells contribute to a broad spectrum of physiologic, immunologic and pathologic processes of inflammation.[18] Degranulation, i.e. the extracellular release of mast cell mediators, releases both preformed [histamine, proteoglycans (heparin, chondroitin sulphates) and neutral proteases (tryptase) stored in secretory granules] and newly synthesized mediators [arachidonic acid metabolites such as leukotrienes and prostaglandins, cytokines, TNF and interleukins (IL)-4, IL-5 and IL-6]. Under normal circumstances, these mediators help to orchestrate the development of a defensive acute inflammatory reaction and immediate allergic reactions initiated by immunoglobulin IgE.[5] This action is significant in the pathogenesis of different lesions like lichen planus, early periodontal diseases, ulcerative colitis, pulmonary fibrosis, inflammatory bowel, systemic mastocytosis and odontogenic cysts.[19]

Odontogenic cysts can destroy bone and undergo expansive growth in the jaw as a consequence of breakdown of the extracellular matrix, buildup of osmotic pressure in cystic fluid, and/ or perilesional bone resorption.[2] Chatterjee[1] formulated the mechanism of cystic expansion and proposed that the degranulating mast cells release products that contribute to cystic enlargement in different ways. The release of heparin and other hydrolytic enzymes facilitates the breakdown of glycosaminoglycans and proteoglycans present in the connective tissue capsule of odontogenic cysts. These released components then largely diffuse into the luminal fluid owing to the poor lymphatic drainage in the cyst wall, thereby raising the internal hydrostatic pressure.[16] Cyst expansion is also affected by the rate in which the surrounding bone is destroyed particularly at the cyst-bone interface. Teronen et al[20] stated that activated mast cells stimulate the production of prostaglandins, interleukin-1α, TIMP and other collagenases, which are said to be important in bone resorption.

The present study endeavors to compare the number, distribution, and location of mast cells in these cysts, both histochemically and immunohistochemically, and strives to elucidate their potential association with the processes related to cyst growth. Here, we observed that the number of detected mast cells was higher when stained with CD117 (mean = 4.54 mast cells/field) in comparison with toluidine blue staining (mean = 3.23 mast cells/field) in all the three cysts. Thus, in accordance with the results of Netto et al.[2] We suggest that immunohistochemistry should be employed whenever possible. Also, on comparing the three cysts using toluidine blue and CD117, we observed that the total mast cell count was higher in KCOT followed by RC and then DC. These results were in accordance with the results of Smith et al.[8] and Chatterjee et al.[1]

Chatterjee et al.[1] stated that the greater concentration of mast cells in KCOT than dentigerous and radicular cyst suggests an increased breakdown of capsular matrix. KCOT epithelium has been shown to be nonkeratinized at places, which causes a transport of breakdown matrix products into the cystic lumen, and consequently can determine an elevated osmolality of the cystic fluid, which partly explains the greater aggressiveness of KCOT compared to other odontogenic cysts.

A few studies focusing on the presence of mast cells in periapical lesions using different histochemical and immunohistochemical techniques have been published. Rodini et al.[21] and Mahita et al.[22] through toluidine blue staining, found more mast cells in periapical cysts in comparison with periapical granulomas. Similarly, but through immunohistochemical membranous detection of CD117 protein, Drazic et al.[9] found that mast cells were more common in cysts than in granulomas and suggested that this difference could be associated with the longer duration of cysts as well as with the presence of the fibrous connective tissue wall. In contrast to these studies, Shojaei S et al.[23] observed more numerous mast cells in periapical granulomas compared to periapical cysts suggesting that the fibrotic complications in periapical lesions are related to mast cells functions. But these results cannot be directly compared with the present study as these authors have not included other odontogenic cysts in their studies.

Furthermore, on comparing the zone wise distribution of mast cells, we found that the number of mast cells was more in the subepithelial zone in all the three odontogenic cysts, both with toluidine blue and CD117, with a statistically significant difference between the observed zones. Also this concentration was higher in KCOT as compared to other two cysts. These results were in accordance with the results of Smith et al.,[10] Chatterjee et al.[1] and Shylaja et al.[19]

Smith et al[10] and Shylaja et al[19] stated that the subepithelial collection of mast cells in odontogenic cysts could be ascribed to their chemotactic stimulus, attracting them to the epithelial lining or luminal fluid contents. The nature of such stimulus is ambiguous, but the secretory matrix proteins of the normal odontogenic epithelium have been reported to be chemotactic to mast cells. Although odontogenic cysts are not known to secrete enamel matrix proteins, the epithelial lining stains positively for keratins and has been shown to share common antigenic determinants with enamel matrix proteins.

Our results were in contrast to that obtained by Netto et al.[2] and Teronen et al.[20] who used immunohistochemical staining of mast cell tryptase. Netto et al.[2] observed a higher frequency of mast cells in the deepest region of the connective tissue wall in all three studied groups. This can indicate higher activity of these cells in the most external layer of the cystic wall, in close proximity to perilesional bone, suggesting that these cells may be associated with the phenomenon of bone resorption. Similarly, Teronen et al.[20] noted that the density of intact mast cells decreased outwards from the cyst lumen. Degranulated mast cells were highest at the periphery of the cysts, at their border with bones, indicating higher activity of mast cells in this area.

This difference could be attributed to the mast cell anti-tryptase antibody that has been used as a marker for mast cell activation; which also stains degranulated mast cells, but this is not a property of the marker used in our study - CD117. CD117 is an important cell surface marker, which is a transmembrane tyrosine kinase receptor protein encoded by the proto-oncogene c-kit that maps to chromosome 4 (4q11-12).[24] When this receptor binds to its ligand known as SCF (a substance that causes certain types of cells to grow), also known as "steel factor" or "c-kit ligand",[25] it forms a dimer that activates its intrinsic tyrosine kinase activity, that in turn phosphorylates and activates signal transduction molecules that propagate the signal in the cell.[26] Moreover, CD117 is a proto-oncogene, meaning that overexpression or mutations of this protein can lead to cancer, which could partly explain the aggressive behavior and neoplastic nature of KCOT, as increased number of mast cells were found in it as compared to other to cysts.

Further, the infiltration of mast cells in the present study revealed significant positive correlation with degree of inflammation in all three cysts; with maximum correlation with RC, followed closely by KCOT and DC. Smith et al[10] observed that the association of mast cells with increasing inflammation was apparent in non-keratinizing cysts, but not in odontogenic keratocyst. They also noted that mast cells were not found within areas of dense inflammatory infiltrate but rather more adjacent to them. While Debta et al.[16] found that the mast cell infiltration did not necessarily correlate with the degree of inflammation in the three cyst types. Netto et al[2] and Teronen et al[20] found that mast cells were especially localized in the connective tissue wall and also adjacent to inflammatory cells and speculated that mast cell participation in odontogenic lesions was at least partially associated with the presence of inflammation.

The treatment protocol often used for these odontogenic cysts is surgical enucleation, but in extreme cases where surgical intervention is not possible, one might consider the role of the inhibitors of mast cells or that of mast cell tryptase. Substances directed to mast cell membrane targets, such as the humanized monoclonal antibody omalizumab, directed to the receptor binding domain of circulation IgE (blocking its attachment to FceRI on inflammatory cells), or to extracellular targets, such as tryptase inhibitors, should inhibit cystic growth, although there is no available confirmatory evidence. As mast cells

could also interact with other inflammatory cells, modulating antibody responses to specific antigens should promote other additional effects.[27-29]

Conclusion

Based upon the present study and previous similar investigations, it can be concluded that mast cells are imperative in the pathogenesis of odontogenic cysts as an elevated number of mast cells was found in the connective tissue capsule of all three odontogenic cysts. Also, the number of detected mast cells was observed to be higher with CD117 than with toluidine blue staining which suggests that immunohistochemistry should be employed whenever possible. Further studies examining the influence of mast cell antagonists on cyst expansion may help unravel the precise role of mast cells in the pathogenesis of odontogenic cysts.

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