

Involvement of tryptase-positive mast cells and angiogenesis in the growth of inflammatory odontogenic cysts

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Abstract: Inflammatory periapical lesions are characterized by infiltration of different immune cell types, the functions of which depend on an effective vascular network. This study aimed to evaluate the mast cells density (MCD) in inflammatory odontogenic cysts capsules concerning microvascular density (MVD), microvascular area (MVA), and microvascular perimeter (MVP), and correlate such findings with the type of lesion, intensity of the inflammatory infiltrate, and thickness of the epithelial lining. Twenty inflammatory dentigerous cysts (IDCs), twenty radicular cysts (RCs), and twenty residual radicular cysts (RRCs) were submitted to immunohistochemical analysis using anti-tryptase and anti-CD34 antibodies. RCs exhibited the highest MCD, MVD, MVA, and MVP indexes ($p < 0.001$, $p = 0.008$, $p = 0.003$ and $p < 0.001$, respectively), and lesions with inflammatory infiltrate grade III showed the highest MVD ($p = 0.044$). Considering epithelial thickness, a higher MVP index was identified in lesions with hyperplastic epithelium ($p = 0.018$). In IDCs, RCs, and RRCs, a strong positive correlation was observed between MVA and MVP ($r = 0.950$ and $p < 0.001$; $r = 0.914$ and $p < 0.001$; $r = 0.713$ and $p < 0.001$, respectively). In IDCs, a moderate correlation was observed between MCD and both MVA and MVP ($r = 0.660$ and $p = 0.002$; $r = 0.634$ and $p = 0.003$, respectively). These results suggest that tryptase-positive mast cells might play an important role in the angiogenic activity of IDCs, while RCs had the highest indexes. Our findings also confirmed that the intensity of the inflammatory infiltrate and epithelial thickness influence angiogenesis.

Keywords: Microvascular Density; Antigens, CD34; Mast Cells; Tryptases; Odontogenic Cysts.

Introduction

Odontogenic cysts are characterized by bone destruction, and they develop from odontogenic epithelium components or epithelial remnants that remain trapped in bone or gingival tissue.¹ Radicular cysts (RCs) and dentigerous cysts (DCs) comprise the most frequent gnathic bone cystic lesions among odontogenic cysts.^{2,3}

RCs arise as a result of periradicular tissue inflammation caused by bacterial agents and consequent pulp necrosis.⁴ Eventually, the RC-affected tooth is extracted with little consideration to the periapical pathology,



which remains inside the maxillary bones as a residual radicular cyst (RRC).⁵ Differently, DCs are developmental odontogenic cysts associated with impacted teeth.⁶ However, it has been suggested that periapical inflammation of non-vital deciduous teeth near the follicles of their respective unerupted permanent successors may trigger cystic formations known as inflammatory dentigerous cysts (IDC).⁷

Inflammation and the subpopulations of inflammatory cells are crucial for the development of the aforementioned lesions.⁴ These cystic inflammatory infiltrate components include, among others, mast cells, which are granular cells of hematopoietic origin. These cells, when activated in response to microbial products, secrete numerous cytoplasmic granules and lipid mediators, such as chymase and tryptase, which stimulate inflammation and are associated with different biological processes, such as vascular proliferation and vasodilation, within the lesion microenvironment, as well as tissue repair, allergies, and host defense mechanisms.^{8,9}

Several studies have associated mast cells function to angiogenesis. Mast cells seem influence angiogenesis by the release of granular components after their degranulation.^{10,11,12} Angiogenesis is a complex, multi-stage process involving not only the formation of blood vessels, but also the proliferation and migration of endothelial cells.^{13,14} The use of immunohistochemical markers, such as Von Willebrand factor (vWF), CD31, CD105, and CD34, provides a measure of angiogenesis in a variety of pathological processes,¹⁵ which may be influenced by inflammation.¹⁶

The present study aimed to evaluate CD34 and tryptase expressions in RC, RRC, and IDC capsules, concerning density and microvascular pattern through immunohistochemical analyses, regarding type of lesion, intensity of the inflammatory infiltrate, and thickness of the epithelial lining.

Methodology

Study design and tissue samples

Sixty specimens (20 DCs, 20 RCs, and 20 RRCs), stored in the Oral Pathology Department of the Federal University of Rio Grande do Norte (UFRN),

were randomly selected. RCs showed a unilocular radiolucent area associated with a devitalized tooth without endodontic treatment. RRCs showed a radiolucent area at the site of an extracted tooth affected by a periapical lesion with histopathological diagnosis of RC. IDCs showed a unilocular radiolucent area associated with the crown of an unerupted vital tooth or attached to the tooth at the cemento-enamel junction. Pathological diagnoses were confirmed based on clinical, radiographic, and histological well-defined criteria. The histological criterion was the presence of a pathological cavity covered predominantly or entirely by non-keratinized stratified squamous epithelium of varying thickness with underlying fibrous capsule presenting different degrees of inflammation. The study was approved by the Ethics Committee of UFRN, Natal, Brazil (protocol number: 1.177.393).

Morphological analysis

For morphological analysis, 5- μ m-thick tissue sections were stained with hematoxylin and eosin. The intensity of inflammatory infiltrate was evaluated according to an adaptation of the method proposed by Tsai et al.⁴ Grading of each specimen was recorded on inflammatory condition in three consecutive microscopic fields, starting from the inner portion of the specimen and proceeding deeper into the connective tissue. Briefly, each specimen was graded at $\times 400$ magnification as: grade I, inflammatory cells restricted to the first microscopic field; grade II, inflammatory cells extending to the second microscopic field; and grade III, inflammatory cells in all three microscopic fields. The analysis of the epithelial lining thickness was performed considering all the cyst lining. Epithelial lining thickness was defined as atrophic (2–10 cell layers) or hyperplastic (> 10 cell layers), based on the predominant pattern in each case.¹⁷

Immunohistochemical methods

For the immunohistochemical study, tissue sections were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were then washed in phosphate-buffered saline (PBS). After treatment with normal serum, sections were incubated for 60 min and at 4°C overnight in a moist chamber with the

primary antibody anti-tryptase (Dako Corporation®, clone AA1, working dilution 1:200, Carpinteria, USA) and anti-CD34 (Cell Marque™, clone QBEnd 101, working dilution 1:200, Rocklin, USA), respectively. The sections were then washed twice in PBS and treated with streptavidin-biotin-peroxidase complex (LSAB; Dako, Glostrup, Denmark) at room temperature to bind the primary antibodies. Peroxidase activity was visualized by immersing the tissue sections in diaminobenzidine (D5637; Sigma Chemical, St. Louis, USA), resulting in a brown reaction product. Finally, tissue sections were counterstained with Mayer hematoxylin and coverslipped. Sections of neurofibroma and capillary hemangioma were used as positive control to tryptase and CD34, respectively. Negative control consisted of sections in which the primary antibody was omitted and replaced with either nonimmune murine IgG1 (X 0931; Dako) or 1% PBS-BSA.

Immunostaining assessment

Each specimen was analyzed under light microscopy (Olympus BX41, Olympus Japan Co., Tokyo, Japan) and, subsequently, the slides were scanned (Panoramic MIDI, 1:15 SPI, 3D HISTECH, Budapest, Hungary). First, histological sections from the same specimen, stained for respective anti-tryptase and anti-CD34 antibodies, were opened simultaneously in Panoramic Viewer 1.15.2 program (3D HISTECH, Budapest, Hungary), tiled horizontally, and synchronized by the slide window option to ensure the best approximation of same areas. Under 200x magnification (Panoramic MIDI, 1.15 SPI, 3D HISTECH, Budapest, Hungary), five representative and random fields were selected after identification of areas with the greatest immunoreactivity and images were analyzed using Imaging Processing and Analysis in Java (ImageJ®, National Institute of Mental Health, Bethesda, USA). Immunohistochemical expression of CD34 was determined to measure microvascular density (MVD), microvascular area (MVA), and microvascular perimeter (MVP) according to methodology adapted from the study by Lima et al.¹⁸ MVD was performed by counting positive blood vessels. For MVA and MVP, areas and perimeters of such structures were measured.

Individual endothelial cells or groups of endothelial cells, with or without a lumen, were considered as CD34-positive vessels when stained brown. Mast cell density (MCD) was assessed using anti-tryptase antibody and viewed under 200x magnification. For tryptase-positive mast cells identification, only cells with brownish intracytoplasmic staining were quantified. Subsequently, the mean number of marked cells in five photographed fields was determined.

Statistical analysis

The results were submitted to statistical analysis using the Statistical Package for the Social Sciences (version 20.0; SPSS Inc., Chicago, USA). After analysis for normality and variance of the data, the non-parametric Mann-Whitney and Kruskal-Wallis tests were applied. Spearman's correlation test was performed to determine possible correlations. For all tests, significance level was set at 5% ($p < 0.05$).

Results

Morphological analysis

Analysis of inflammatory infiltrate in IDCs revealed that 13 cases (65.0%) exhibited an inflammatory infiltrate grade III, and 7 cases (35.0%), grade II (Figure 1A). In RCs, 19 cases (95.0%) showed an inflammatory infiltrate grade III, and 1 case (5.0%), grade II (Figure 1C). In RRCs, 7 cases (35.0%) showed an inflammatory infiltrate grade III, 10 cases (50.0%), grade II, and 3 cases (15.0%), grade I (Figure 1B). Due to the scarcity of cysts with mild inflammation, cases with inflammatory infiltrates grades I and II were grouped.

Morphological analysis of epithelial thickness in IDCs and RCs revealed the presence of a hyperplastic epithelium in 13 cases (65.0%) and an atrophic epithelium in 7 cases (35.0%). In RRCs, 8 cases (40.0%) presented a hyperplastic epithelium and 12 cases (60.0%) showed an atrophic epithelium.

Immunohistochemical analysis

Mast cells density

Immunoexpression of tryptase was positive for all evaluated cases of IDCs, RRCs, and RCs (Figures D, E and F, respectively). The mean MCD was 8.54 in

IDCs, 18.05 in RCs, and 13.84 in RRCs. A significantly higher median number of tryptase-positive mast cells was identified in RRCs when compared to RCs and IDCs ($p < 0.001$) (Table 1).

Considering the intensity of the inflammatory infiltrate, the mean MCD was 15.02 in lesions with inflammatory infiltrate grade I / grade II, and 12.65 in

lesions with grade III. No significant difference was observed between the groups ($p = 0.381$).

In relation to epithelial thickness, the mean MCD was 15.73 in lesions with hyperplastic epithelium and 20.65 in lesions with atrophic epithelium. No significant difference was verified between the groups ($p = 0.163$).

Table 1. Analysis of MCD, MVD, MVA, and MVP according to type of lesion, intensity of inflammatory infiltrate, and epithelial thickness.

Variable	n	Mast cells density		Microvascular density		Microvascular area		Microvascular perimeter	
		Mean \pm SD (n)	p-value	Mean \pm SD (n)	p-value	Mean \pm SD (μm^2)	p-value	Mean \pm SD (μm)	p-value
Type of lesion									
Inflamed dentigerous cyst	20	8.54 \pm 8.25	< 0.001*	24.00 \pm 12.42	0.008*	2989.89 \pm 3030.44	0.003*	333.84 \pm 146.69	< 0.001*
Radicular cyst	20	18.05 \pm 10.28		34.99 \pm 15.54		3202.25 \pm 1974.46		400.00 \pm 145.25	
Residual radicular cyst	20	13.84 \pm 3.45		23.95 \pm 5.09		1488.54 \pm 817.17		225.64 \pm 79.58	
Inflammatory infiltrate									
Grade I / Grade II	21	15.02 \pm 10.15	0.381	22.36 \pm 5.02	0.013*	2159.00 \pm 2244.29	0.099	269.30 \pm 97.61	0.093
Grade III	39	12.65 \pm 7.77		30.49 \pm 14.71		2776.29 \pm 2237.31		347.03 \pm 159.51	
Epithelial thickness									
Hyperplastic epithelium	34	15.73 \pm 10.21	0.163	29.96 \pm 14.57	0.061	2900.64 \pm 2347.46	0.062	362.86 \pm 164.78	0.018*
Atrophic epithelium	26	20.65 \pm 12.93		24.61 \pm 9.38		2115.08 \pm 2051.44		263.56 \pm 89.11	

* $p < 0.05$.

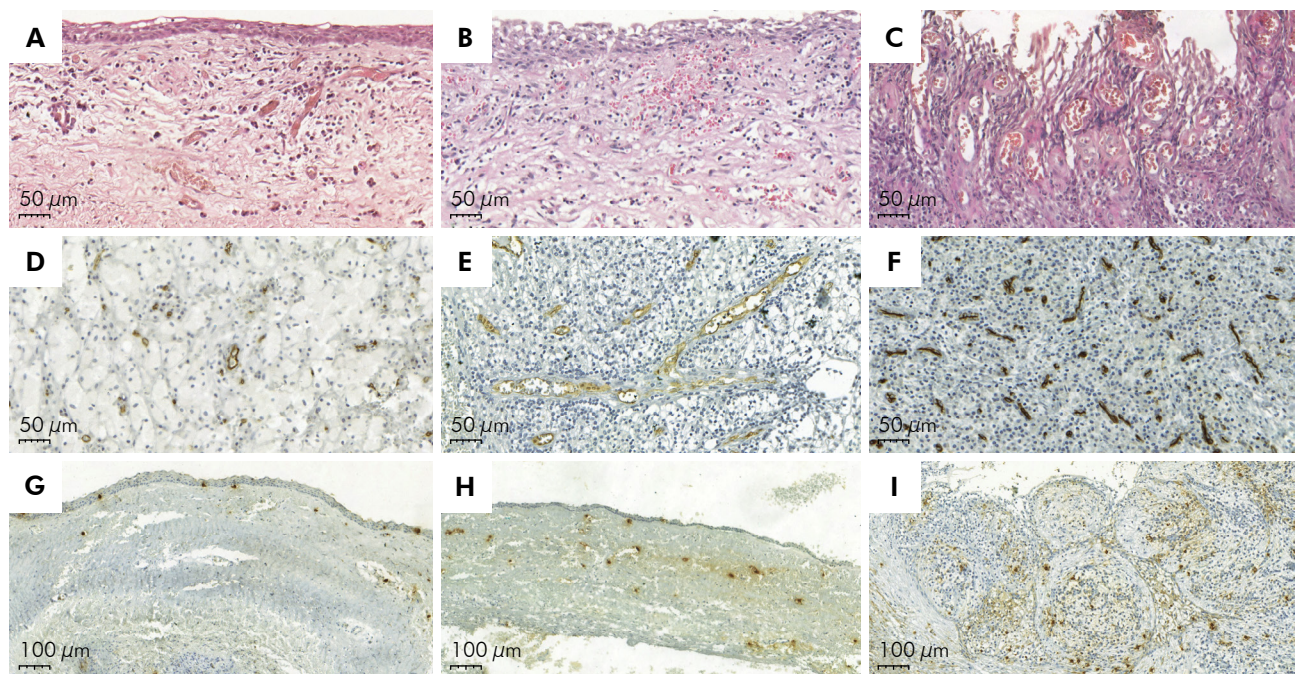


Figure. (A, B, C) Histopathological aspects of IDC, RRC, and RC, respectively. (D, E, F) Immunoexpression of tryptase in IDC, RRC, and RC, respectively. (G, H, I) Immunoexpression of CD34 in IDC, RRC and RC, respectively.

Microvascular density

Analysis of immunoexpression showed CD34 positivity for all evaluated cases of IDCs, RRCs, and RCs (Figures 1G, H, I, respectively). The mean MVD was 24.00 in IDCs, 34.99 in RCs, and 23.95 in RRCs. A statistically significant higher median number of CD34-positive blood vessels was identified in RCs when compared to RRCs and IDCs ($p = 0.008$) (Table 1).

Regarding the intensity of the inflammatory infiltrate, the mean MVD was 22.36 in lesions with inflammatory infiltrate grade I / grade II, and 30.49 in lesions with grade III. A statistically significant higher median number of CD34-positive blood vessels was identified in lesions with grade III when compared with those with grade I and II ($p = 0.013$).

Considering the epithelial thickness, the mean MVD was 29.96 in lesions with hyperplastic epithelium and 24.61 in lesions with atrophic epithelium. No statistically significant difference was verified between the groups ($p = 0.061$).

Microvascular area

The mean MVA was 2989.89 μm^2 in IDCs, 3202.25 μm^2 in RCs, and 1488.54 μm^2 in RRCs. A statistically significant higher median number of MVA was identified in IDCs and RCs when compared to RRCs ($p = 0.003$) (Table 1).

With respect to the intensity of the inflammatory infiltrate, the mean MVA was 2159.00 μm^2 in lesions with inflammatory infiltrate grade I / grade II, and 2776.29 μm^2 in lesions with grade III. No statistically significant difference was verified between the groups ($p = 0.159$).

Considering the epithelial thickness, the mean MVA was 2900.64 μm^2 in lesions with hyperplastic epithelium and 2115.08 μm^2 in lesions with atrophic epithelium. No statistically significant difference was verified between the groups ($p = 0.062$).

Microvascular perimeter

The mean MVP was 333.84 μm in IDCs, 400.00 μm in RCs, and 225.64 μm in RRCs. A statistically significant higher median number of MVP was identified in IDCs and RCs when compared to RRCs ($p < 0.001$) (Table 1).

With respect to intensity of inflammatory infiltrate, the mean MVP was 269.30 μm in lesions

with inflammatory infiltrate grade I / grade II, and 347.03 μm in lesions with grade III. No statistically significant difference was verified between the groups ($p = 0.174$).

Considering the epithelial thickness, the mean MVP was 362.86 μm in lesions with hyperplastic epithelium and 263.56 μm in lesions with atrophic epithelium. A statistically significant higher median number of MVP was identified in lesions with hyperplastic epithelium when compared with those with atrophic epithelium ($p = 0.018$).

Correlation between MCD, MVD, MVA, and MVP

In IDCs, Spearman's correlation test showed strong positive correlation between MVA and MVP ($r = 0.950$; $p < 0.001$), and moderate positive correlation between MCD and MVA ($r = 0.660$; $p = 0.002$) and between MCD and MVP ($r = 0.634$; $p = 0.003$). In RCs and RRCs, a strong positive correlation was observed between MVA and MVP ($r = 0.914$; $p < 0.001$ and $r = 0.713$; $p < 0.001$, respectively) (Table 2).

Table 2. Spearman's correlation test between MCD, MVD, MVA, and MVP, according to type of lesion.

Type of lesion	n	r	p-value
Inflamed dentigerous cyst			
MCD x MVD	20	0.129	0.587
MCD x MVA	20	0.660	0.002*
MCD x MVP	20	0.634	0.003*
MVD x MVA	20	-0.102	0.670
MVD x MVP	20	-0.067	0.779
MVA x MVP	20	0.950	< 0.001*
Radicular cyst			
MCD x MVD	20	0.187	0.429
MCD x MVA	20	0.179	0.450
MCD x MVP	20	0.209	0.376
MVD x MVA	20	-0.165	0.488
MVD x MVP	20	0.006	0.980
MVA x MVP	20	0.914	< 0.001*
Residual radicular cyst			
MCD x MVD	20	-0.018	0.941
MCD x MVA	20	-0.306	0.190
MCD x MVP	20	-0.079	0.740
MVD x MVA	20	0.057	0.810
MVD x MVP	20	0.134	0.573
MVA x MVP	20	0.713	< 0.001*

* $p < 0.05$.

Discussion

Despite their high prevalence, the etiopathogenesis and the exact growth and expansion mechanisms of inflammatory odontogenic cysts remain poorly understood. However, it is known that several different types of cells, including mast cells, can participate in such phenomena.¹⁹ Mast cells are immunoregulatory cells with several cytoplasmic granules and associated with immediate and late immune responses that have been linked to the development, growth, and expansion of periapical inflammatory lesions.^{18,20,21,22} Antibodies against the constituents of these protein granules (such as tryptase or chymase) or against their cell surface receptors (such as CD117-c-KIT) are used for the immunohistochemical detection of tissue mast cells.²³

In the present study, the MCD index progressively increased from the IDC to RRC and RC. These results corroborate those evidenced by other authors who demonstrated greater immunolabeling in RC compared to IDC and in IDC in relation to their non-inflammatory counterpart.^{6,19} According to Netto et al.,¹⁹ the higher concentration of mast cells observed in inflammatory lesions could indicate their participation in the inflammatory response associated with odontogenic lesions. The higher MCD in RCs seems to be due to the active presence of a constant source of antigenic stimulation caused by bacterial toxins involving a greater variety of immunological cell types, including mast cells.²⁴ Considering the findings obtained with RRCs, we believe in the persistence of other antigenic stimuli in the RC area, which maintain the inflammatory infiltrate and facilitate the cystic growth process.²⁵

In disagreement with other studies, tryptase-positive mast cells were detected mostly in lesions with a mild/moderate inflammatory infiltrate.^{19,24,26} It is known that mast cells synthesize and release certain prostaglandins and leukotrienes that together contribute to the immediate inflammatory response. Because of mast cell activation, other inflammatory cells are recruited and activated, and a cascade of inflammatory mediator production and release is activated²⁷. In this perspective, we consider that lesions with inflammatory infiltrate grade III present

different cell types due to the chronicity of the inflammatory process, consequently triggering an adaptive immune response.

MVD provides a quantitative measure of angiogenesis, which has been previously determined in a variety of pathological processes. Pan-endothelial immunohistochemical markers, such as CD34, are applied to obtain MVD, which stain preexisting and newly formed blood vessels.¹⁸ CD105 (endoglin) is predominantly expressed in endothelial cells present in reactive and neoplastic tissues, in such a way that all are submitted to active angiogenesis.²⁸ Because CD105 has specificity for labeling newly formed vessels and the present study aimed to evaluate the general vascular pattern of the lesions, CD34 was chosen as a marker. In addition, Jamshidi et al.²⁹ demonstrated poor CD105 immunoblotting in DCs, probably due to their indolent biological behavior and low recurrence rates.

Similar to the results reported by Lima et al.,¹⁸ the RC cases analyzed in the present study exhibited the highest MCD and MVA indexes. Such findings suggest that mast cells are also involved in vascular compartment maintenance in these cystic lesions, corroborating previous studies that attribute angiogenic properties to mast cell tryptase.

Considering the intensity of the inflammatory infiltrate, higher MVD were observed in specimens classified as grade III (intense) compared to the other two categories. These results are similar to those reported by Zizzi et al.,¹⁶ who suggested that the inflammatory infiltrate plays an important role in neoangiogenesis and, consequently, in RC development and growth. The proangiogenic role of mast cells was shown in a study carried out by de Souza Júnior et al.³⁰ where they interacted directly with endothelial cells, resulting in the release of angiogenic factors, which then accelerated *in vitro* vascular neof ormation. In addition, the authors demonstrated that the conditioned medium of a co-culture of these two cell types contains the necessary factors to stimulate the angiogenesis process.

Higher scores for MVA and MVP were detected in RC specimens. In a previous study carried out by Lima et al.,¹⁸ most blood vessels marked with CD105 displayed greater vascular lumen, were

generally associated with cellular areas, and contained a loose perivascular connective tissue. On the other hand, numerous tiny vessels were also immunolabelled with CD34 within the fibrous stroma of both RC and periapical granulomas.

Intermediate scores for MVA and MVP were observed for IDCs in comparison to the other cysts evaluated. However, both parameters correlated with MCD in this type of cyst. Published studies with IDCs are quite scarce especially those that address the vascular events present in this type of cyst. El-Labban and Aghabeigi³¹ demonstrated that DC blood vessels have endothelial lining ruptures and the presence of cell membrane fragments in the space between endothelial cells. However, the authors do not report the diameter of such structures. The positive correlations observed between MCD and both MVA and MVP suggest that mast cells also play an important role in angiogenesis of IDCs, contributing to their growth and expansion. Such results are essential to better understand these cysts, since published studies are quite rare.

In addition, the three assessed lesions displayed a positive correlation between MVA and MVP. In general, this result was expected, since these morphological parameters are related and complement each other concerning vascular structure.

No differences were found between IDCs, RCs, and RRCs in thickness of the epithelial lining. Previous studies also did not detect differences in epithelial lining thickness among types of cystic.^{25,32,33} Although

inflammation is a common feature of the studied lesions, we believe that these results are due to biological behavior variations and exposure to antigenic stimuli, which influence the mitogenic potential of the cystic epithelium.

On the other hand, a higher MVP was identified in lesions lined by hyperplastic epithelium. The state of the cystic epithelium has been suggested as a reliable histological parameter of biological activity and/or inactivity for the growth of such lesions.¹⁷ Since keratinocyte division must occur during cystic growth, it is assumed that lesions with hyperplastic epithelium would be more active than those lined with atrophic epithelium.³⁴ Thus, we suggest that the vascular dilatation observed in the studied cases was due to the increase in tissue metabolism and nutrition requirement of the proliferating epithelium. Considering that mast cells can release cytokines, such as IL-1, which induces epithelial proliferation,³⁵ our results also indicate that these cells are involved in cystic growth stage.

Conclusions

Taken together, the results of the present study suggest that tryptase-positive mast cells might play an important role in the angiogenic activity of IDCs, while RCs presented the highest indexes, probably due to the active and constant presence of an antigenic stimulation source. Our findings also confirmed that the intensity of the inflammatory infiltrate and epithelial thickness influence angiogenesis.

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