Comparative immunohistochemical expression of RANK, RANKL and OPG in radicular and dentigerous cysts

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1. Introduction

Despite numerous investigations the precise mechanisms involved in the formation and enlargement of jaw cysts have not been completely established. Cyst formation is believed to be related to the proliferation of epithelial remnants that are activated by the release of cytokines and growth factors. The immunopathological events that trigger the proliferative activity of epithelial remnants concomitantly induce the secretion of bone resorption factors. However, how these events are involved in the formation of cystic cavities and resorption of adjacent bone continues to be a matter of numerous researches. In this respect, altered expression of bone metabolism-related factors may favour an increase in osteolytic activity and the consequent cystic expansion into adjacent bone tissue.

Odontogenic cysts are one of the most common osseous-destructive lesions affecting the jaws. They are classified traditionally into a developmental group, including dentigerous...
2. Materials and methods

2.1. Samples

The original haematoxylin and eosin (H&E)-stained slides and formalin-fixed paraffin-embedded specimen blocks of all RC and DC diagnosed between January 2000 and October 2009, were retrieved from the files of the Oral Pathology Service at UFRN, Natal, Brazil from the Department of Histopathology. The H&E slides were reviewed to confirm the diagnosis. The tissues removed were classified as cysts whenever a partial or total epithelium lining was present. The diagnosis of cysts was based mainly on radiographic and histopathologic examination. DC intensely inflamed and cysts with inadequate tissue samples were excluded. A total of 40 cysts were selected for the study (20 RC and 20 DC). Clinical and radiographic information, including age, gender, and anatomic site, were obtained from biopsy forms submitted by the clinicians.

2.2. Immunohistochemistry

For immunohistochemical analysis, 3 μm thick paraffin embedded tissue sections were placed on 3-aminopropyltriethoxysilane coated glass slides (Sigma Chemical Co., St Louis, MO, USA). The samples were deparaffinised with xylene, rehydrated in graded alcohols, and washed in deionised water and phosphate-buffered saline (PBS). Samples were then incubated with 3% hydrogen peroxide and immersed in a citrate buffer, pH 6.0 for 20 min. Sections were then blocked by incubation with 3% normal goat serum at room temperature for 20 min, and slides were incubated at 4 °C, overnight, in a humidified chamber with the following primary rabbit polyclonal antibodies: anti-OPG (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200; anti-RANK (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200; and anti-RANKL (N-19; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. After washing in TBS (tris-buffered saline), the sections were treated with a labelled streptavidin-biotin kit (LSAB; Dako, Glostrup, Denmark). Peroxidase activity was visualised by immersing tissue sections in 3,3’-diaminobenzidine (D5637; Sigma Chemical, St. Louis, MO) and counterstained with Mayer’s haematoxylin. A central giant cell granuloma was used as positive control. Negative controls were obtained by the omission of primary antibodies and substitution of primary antibodies by nonimmune rabbit serum (X0902; Dako).

2.3. Cell counting

Immunoreexpression of RANK, RANKL and OPG was evaluated in lining epithelium and fibrous capsule. The epithelial immunoreexpression was semiquantitatively evaluated by two observers, using 400× magnification and classified according to the scores: 0 or no staining (<10% of positive immunostaining cells), 1 or weak (11–25%), 2 or moderate (26–75%) and 3 or strong (>76%). In fibrous capsule, the analysis was quantitative and the number of positive cells was counted in 10 representative and consecutive microscopic high-power fields (1000×) over totally counted cells, irrespectively of cell type. Digital images were loaded on the software IMAGE J® (National Institutes of Health, Bethesda, Maryland, USA) to count the number of immunostained cells. Results are expressed as the mean percentage of observations per field, with the following modifications. Based on this mean percentage, gave up a score for each case, taking into account the standard scoring system used for the lining epithelium: score 0 (<10% immunostained cells), 1 or weak (11–25% of cells), 2 or moderate (26–75% of cells) and 3 or strong (more than 76% of cells). These counting procedures were performed for the three biomarkers in both lesions.

2.4. Statistical analysis

Comparative analysis of data was performed using the nonparametric Wilcoxon signed rank test and Mann–Whitney U test. Statistical significance was set at p ≤ 0.05.

3. Results

3.1. Clinical, radiographic and histological findings

In this study, there were 20 cases of RC and 20 cases of DC, with mean ages of 32.5 ± 13.67; 24.79 ± 12.35 years, respectively.
Female preponderance was found in RC cases and male preponderance in DC. RC was more commonly located in the anterior maxilla and DC in the posterior mandible. All samples were described as a well circumscribed unilocular radiolucency. Histological appearance of the cysts revealed the presence of a hyperplastic epithelium and an inflammatory infiltrate, which was moderate to intense in the most RC. DC showed an atrophic epithelium, quite hemorrhagic areas and scarce infiltrate in the most cases.

### 3.2. Qualitative and semi-quantitative analysis of lining epithelium

Immunohistochemical reactivity for RANK, RANKL and OPG was detected in the nuclei and cytoplasm of epithelial cells. Additionally, epithelial cells displaying a stellate shape exhibited positive cytoplasmic reactivity for RANK, RANKL and OPG (Fig. 1) likely indicating changes in cell–cell interactions such as the accumulation of extracellular fluid or even the loss of cell adhesion molecules. RANKL appears positive in the nuclei and cytoplasm of suprabasal epithelial cells in Fig. 2A. OPG appears positive in the nuclei and cytoplasm of basal and suprabasal epithelial cells in Fig. 2B. RANKL and OPG appears in the cytoplasm of epithelial cells in Fig. 2C and D, respectively.

The analyses of the immunoreactivity of RANK, RANKL and OPG according to percentage of the scores in the epithelium are shown in Fig. 3. No differences were observed in cell reactivity in the lining epithelium of the cysts \( (p > 0.05, \text{Table 1}) \). A similar expression of RANK, RANKL and OPG was observed.

In addition, significant differences were observed in the distribution of cases with respect to OPG and RANKL ranks of immunostaining scores in the lining epithelium. We observed that most of the cases of RC (55%) and DC (70%) exhibited a higher content of OPG than RANKL \( (p < 0.05, \text{Table 2}) \).

#### 3.3. Qualitative and quantitative analysis of fibrous capsule

With regard to reactivity for RANK, RANKL, and OPG in the stromal cells, the presence of positive fibroblasts-like, endothelial-like (Fig. 4A), polymorphonuclear neutrophil-like (Fig. 4B), plasmacyte-like, lymphocytes-like and macrophage-like cells (Fig. 4C and D) was observed. The immunoreactivity was predominantly in the cytoplasm. Additionally, the RANKL and OPG expression was observed in nests of odontogenic epithelial cells (Fig. 5).

**Table 1** summarises the quantitative analysis of lesions immunostained for RANK, RANKL and OPG in fibrous capsule. Statistically differences were observed in cell reactivity for RANK and RANKL between the cysts (Table 4). A mean percentage demonstrated an expression of RANK-positive and RANKL-positive cells higher in DC when compared with RC \( (p = 0.001 \text{ and } p = 0.005 \text{ for RANK and RANKL, respectively}) \).

**Fig. 6** summarises the distribution of cases of RC and DC according to percentage of the scores for RANK, RANKL and OPG in fibrous capsule. No differences were observed in the distribution of cases with respect to OPG and RANKL ranks of immunostaining scores \( (p > 0.05) \). Many cases of DC and the RC showed a tendency to present a similar pattern of expression for RANKL and OPG (Table 2). There was a predominance of moderate immunostaining for all cases.

No positive staining was observed when primary antibodies were omitted. Positive control samples showed strong reactivity.

### 4. Discussion

In the present study, we have examined the immunoeexpression to RANK, RANKL and OPG in radicular and dentigerous cysts. The main types of cells that expressed immunoreactivity were those showing characteristics of the monocyte–macrophage lineage, fibroblasts, and lymphocytes as also reported by other investigators.\(^9,12,23,25\) Additionally, we observed other types of immunostained cells exhibited microscopic features of endothelial cells, neutrophils and plasma cells in agreement with other studies.\(^9,14,16\)
Chuang et al.\textsuperscript{12} demonstrates the expression of RANK, RANKL and OPG in normal human oral mucosa. Strong cytoplasmic immunostaining of RANKL limited to epithelial cells of the basal layer has been noted. In contrast, there was a complete absence of immunostaining of RANK and OPG in all tissue of normal oral mucosa. In our study the epithelial lining of cysts exhibit immunostaining for RANK, RANKL and OPG in cells of the basal and suprabasal layer.

Cytoplasmic immunostaining for RANKL and OPG was also observed in epithelial cells in a stellate shape, similar to dendritic cells and in nests or strands of odontogenic epithelial cells scattered in the fibrous capsule of DC. Dendritic cells in the oral mucosa are antigen-presenting cells, which play a vital role in the regulation of adaptive immunity cell. Recently studies\textsuperscript{26,27} showed that human dendritic cells can transdifferentiate into osteoclasts in the

Table 2 – Distribution of cases in relation to OPG and RANKL ranks of immunostaining scores in epithelium and fibrous capsule.

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Ranks</th>
<th>OPG &lt; RANKL</th>
<th>OPG &gt; RANKL</th>
<th>OPG = RANKL</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC (n = 20)</td>
<td>Epithelium</td>
<td>1 (5%)</td>
<td>11 (55%)</td>
<td>8 (40%)</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Capsule</td>
<td>4 (20%)</td>
<td>6 (30%)</td>
<td>10 (50%)</td>
<td>0.527</td>
</tr>
<tr>
<td>DC (n = 20)</td>
<td>Epithelium</td>
<td>2 (10%)</td>
<td>14 (70%)</td>
<td>4 (20%)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Capsule</td>
<td>2 (10%)</td>
<td>5 (25%)</td>
<td>13 (65%)</td>
<td>0.257</td>
</tr>
</tbody>
</table>

RC, radicular cyst; DC, dentigerous cyst; OPG, osteoprotectorin; RANKL, receptor nuclear factor-κB ligand.

* Non-parametric Wilcoxon signed rank test.
presence of M-CSF and RANKL in vitro, suggesting that dendritic cells may directly contribute to osteoclastogenesis. Loser et al.28 demonstrated that RANKL expression is inducible on keratinocytes and that this is a molecular pathway that couples the epidermis to local and systemic immunosuppression. Moreover, RANKL expression is induced on activated T cells, and RANK expression can be found on dendritic cells, in accordance our results. The finding of immunoreactivity in nests of odontogenic epithelial cells agrees with the results of Silva et al.16 The expression in the nests of odontogenic epithelial cells suggests that the odontogenic epithelium may actually induce and initiate the resorption process, perhaps through synthesising and secreting RANKL and OPG.

Fig. 3 – Distribution of cases of RC and DC according to percentage of the scores for RANK, RANKL and OPG in the epithelium.

Fig. 4 – A Endothelial-like cells (arrows-head) stained with anti-OPG in radicular cyst (1000×), (B) polymorphonuclear neutrophil-like cells (arrow) stained with anti-RANK in fibrous capsule of dentigerous cyst (1000×), (C) weak reactivity for RANKL in fibrous capsule of radicular cyst (1000×), and (D) strong reactivity for OPG in fibrous capsule of radicular cyst. Lymphocytes-like and macrophage-like cells (arrow-head) stained with anti-OPG. Predominance of mononuclear cells stained (1000×).
Fig. 5 – Nests of odontogenic epithelium (arrow-head) stained with anti-RANKL in fibrous capsule of dentigerous cyst (1000×).

Analysis of the distribution of cases according to immunostaining in the lining epithelium of RC and DC revealed larger scores for OPG than RANKL in most specimens, a finding could be indicating minimal osteoclast activity. In vitro studies demonstrate that the effects of OPG include inhibition of differentiation, survival and osteoclast fusion, as well as stimulation of apoptosis of osteoclasts, thereby reducing the ability of bone resorption. Moreover, the overexpression of OPG in mice or administration of OPG to normal rodents inhibits osteoclastogenesis, osteoclast activation and bone resorption, resulting in an osteopetrotic phenotype.22,25,29

In cystic lesions, this finding (OPG > RANKL) may suggest a role of epithelial cells as a barrier in an attempt to restrict invasion into underlying bone and thus to prevent cystic expansion. In RC, this higher immunoeexpression of OPG in the epithelium might be related to the abscess theory30 where inflammatory cells inside granulation tissue secrete RANKL and surrounding epithelial cells release OPG in response to this increase in an attempt to restrict cystic expansion. Vernal et al.31 showed high RANKL levels in granulation tissue of periapical granulomas. One may suppose a role of these OPG-positive epithelial cells in the reestablishment of periapical tissue considering that in most cases of RC endodontic treatment is sufficient3 to permit regression of the cystic lesion.

Hofbauer22 and Baud’huin et al.31 conducted in vivo experiments that suggested RANKL to be a pro-resorption factor. According to these authors, an increased expression of RANKL would be related to increased osteoclast activity, thus favouring resorption. Although Menezes et al.15 demonstrated a higher secretion of RANKL under inflammatory conditions, this increased secretion would not always guide the bone resorption process since the presence of the inhibitory receptor OPG would decisively influence the process of bone expansion and, furthermore, other bone metabolism-related factors may also be involved in this complex process.

Comparison of the positive cells immunostaining for RANK, RANKL and OPG in the capsule of RC and DC showed a larger number of RANK- and RANKL-positive cells in the capsule of DC compared to the capsule of RC. One may speculate that this higher immunoeexpression observed in the capsule of DC is related to a greater expansive potential of these cysts by indicating the presence of a larger number of osteoclast precursors expressing RANK that are able to interact with its specific receptors (RANKL), leading to osteoclast differentiation and maturation. The expression in nests of odontogenic epithelial cell also may have contributed to this greatest expression in fibrous capsule of DC.

Moreover, the presence of hemorrhagic areas in the capsule of DC could be explained by increased vascular permeability which in turn may reflect the increased expression of vascular endothelial growth factor (VEGF), which in previous studies31–33 were also overexpressed in the lining

Table 3 – Distribution of immunostained cells for RANK, RANKL and OPG according to each type of lesion in the fibrous capsule.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lesions</th>
<th>RC</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>DC</th>
<th>Mean ± SD</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANK</td>
<td>34.17 ± 24.51</td>
<td>26.81</td>
<td>62.01 ± 22.54</td>
<td>64.67</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RANKL</td>
<td>35.73 ± 18.38</td>
<td>28.10</td>
<td>58.20 ± 24.08</td>
<td>63.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG</td>
<td>44.72 ± 22.24</td>
<td>38.04</td>
<td>58.20 ± 24.43</td>
<td>63.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RC, radicular cyst; DC, dentigerous cyst.

Table 4 – Parameters used for calculation of the Mann–Whitney U test for the evaluation of positive cells immunostained by RANK, RANKL, OPG in the fibrous capsule, according to the lesion type.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lesion type</th>
<th>n</th>
<th>Median of ranks</th>
<th>U</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANK</td>
<td>RC</td>
<td>20</td>
<td>26.81</td>
<td>14.45</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>20</td>
<td>64.67</td>
<td>26.55</td>
<td></td>
</tr>
<tr>
<td>RANKL</td>
<td>RC</td>
<td>20</td>
<td>28.10</td>
<td>15.3</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>20</td>
<td>63.05</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td>OPG</td>
<td>RC</td>
<td>20</td>
<td>38.04</td>
<td>17.35</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>20</td>
<td>63.24</td>
<td>23.65</td>
<td></td>
</tr>
</tbody>
</table>

RC, radicular cyst; DC, dentigerous cyst.
epithelium and in fibrous capsule. Min et al. showed that VEGF up-regulates expression of RANK and increases angiogenic responses of endothelial cells to RANKL. In addition, studies demonstrated that VEGF could substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. VEGF was shown to induce osteoclast differentiation and enhance survival of mature osteoclasts. We observed that factors like the type and intensity of inflammation and the vascularity should be evaluated in future studies.

The lack of a significant correlation between RANKL and OPG in the fibrous capsule of cysts suggests that different expression patterns of these markers are associated with different stages of disease progression. Although no significant correlation was observed in the present study, there were cases indicating homeostasis (OPG = RANKL) and cases indicating minimal osteoclast activity (OPG > RANKL). Evaluation of gene expression kinetics as done by Kawashima et al. would be interesting for the analysis of the RANKL/OPG ratio since it outlines changes in the expression of these markers during development of the lesion. In this respect, determination of mean ratios might be inaccurate since the results obtained only reflect a point in time when the lesions are already established in the patient.

Although most studies reported an elevated immunoreactivity to RANKL compared to OPG in osteolytic lesions, we believe that this RANKL/OPG imbalance may occur during the early phase of formation of the cystic cavity, which is difficult to be demonstrated in vivo. Although involvement of the OPG/RANKL/RANK system is likely to occur at some point, no imbalance between these markers that would favour bone-resorptive activity was observed in the present study.

Although an increased RANKL activity associated with a reduced regulatory activity of OPG has been reported to play a role in different diseases such as osteoporosis, arthritis, periodontal disease, odontogenic cysts and tumours and, more recently, squamous cell carcinoma, the present results obtained for the epithelium and capsule of RC and DC are not compatible with these findings. As mentioned earlier, although a higher RANKL reactivity compared to OPG is expected in osteolytic lesions, some studies have demonstrated higher OPG immunoreactivity in these lesions. In agreement with these results, higher or similar OPG expression when compared to RANKL was observed in most cystic lesions studied here. Since bone is a dynamic tissue, the relationships established between these receptors that culminate in the differentiation and maturation of osteoclasts occur throughout the development of alterations in the expression levels of these markers, i.e., throughout cyst formation.

The identification of these biomarkers may indicate their relationship with the process of osteoclast activation and bone loss in cyst lesions. Furthermore, increased expression of OPG compared to RANKL in the lining epithelium may act as a barrier against the expansion of these lesions in the underlying bone. Moreover, molecular biology studies evaluating the levels of these markers and their expression kinetics in these lesions are necessary not only to demonstrate the presence of these proteins but also to quantify the transcripts in each lesion. Further studies are also needed to investigate whether the OPG/RANKL/RANK system is involved in the development of cystic lesions in order to better understand the underlying mechanism and to establish new therapeutic strategies for the treatment of these lesions that are often highly destructive.

REFERENCES


