Original Research
Oral Pathology

Immunohistochemical expression of RANKL in oral giant cell lesions is predictive of aggressiveness

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Abstract: The aim of this study was to evaluate the immunohistochemical expression of receptor activator of nuclear factor kappa-B ligand (RANKL) and of osteoprotegerin (OPG), important proteins correlated with osteoclastogenesis, in central giant cell lesions (CGCL) and peripheral giant cell lesions (PGCL) and to compare their expression with the histological and clinical parameters for quantification of multinucleated giant cells (MGC) and their nuclei, lesion size, and recurrences. Twenty cases of each lesion type were selected to quantify the number of MGCs and nuclei/mm^2 of connective tissue. The immunoreactivity of RANKL and OPG was expressed as a percentage of the marked area in the stroma. Clinical data were collected from pathoanatomical and medical reports. No statistical differences were found for the number of MGCs ($p = 0.24$) between PGCL and CGCL, but the number of nuclei within the MGCs was higher in CGCL ($p = 0.01$). RANKL expression was higher in CGCL than in PGCL ($p = 0.04$) and all recurrent lesions showed higher RANKL and OPG expressions than nonrecurrent lesions. We report higher RANKL expression and a greater number of nuclei in CGCL, which may explain the difference in clinical behaviour between these lesions and their pathogenesis.

Keywords: NF-kappa B; Osteoprotegerin; Immunohistochemistry.

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Introduction

Central and peripheral giant cell lesions are benign lesions that affect the gnathic bones, although the aetiology and pathogenesis remain unclear.[1] Peripheral giant cell lesion (PGCL) is described as a reactive lesion that only affects the gingiva and the alveolar ridge, manifesting as a red or purple nodule. This type of lesion can develop at any age; however, it is more common during the fifth and sixth decades of life. There is no sex predisposition and the lesions occur in both the maxilla and mandible.[2,3] It is still unclear whether PGCL is a discrete entity or a peripheral variant of a central giant cell lesion (CGCL).

CGCL is a benign intraosseous lesion that mainly affects women aged under 30 years. CGCL tends to involve the mandible more than the maxilla and it is more common in the anterior region.[4,5] The clinical behaviour ranges from a non-aggressive type, characterised by a slow-growing and painless lesion, to an aggressive type, characterised as a fast-growing lesion associated with pain, root resorption, and tooth displacement.[6]

Histologically, PGCL and CGCL are characterised by the presence of numerous multinucleated giant cells (MGCs) and mononuclear cells within a prominent and well-vascularised fibrous stroma, which may contain osteoid material.[3,7] Although these two lesions present the same histological features, their clinical behaviour is distinct. Several researchers have attempted to explain the difference in clinical behaviour through cytomorphometric evaluation of MGCs and their nuclei,[7] angiogenesis (CD34) and macrophage (CD68) markers,[8] and expression of p53 (tumour suppressor gene), proliferating cell nuclear antigen (PCNA), Ki-67 antigen (nuclear protein associated with cell proliferation), argyrophilic nucleolar organiser region (AgNOR),[9] receptor activator of nuclear factor kappa-B (RANK), glucocorticoid receptor alpha (GRa), and calcitonin receptor (CTR).[10]

Investigations focused on the RANK/RANKL/OPG system in osteoclastogenesis have greatly contributed to knowledge about bone turnover processes. RANK, expressed in osteoclast precursors, binds to receptor activator of nuclear factor kappa-B ligand (RANKL), expressed in the membrane of osteoblasts and stromal cells, and plays an important role in osteoclast differentiation and activation. Osteoprotegerin (OPG) inhibits this process by binding to RANKL. Overexpression of RANK and/or RANKL has been observed in several bone diseases, such as osteoporosis, skeletal metastasis, and odontogenic tumours,[11,12,13] and in conditions with higher osteolytic activity.[14]

The aim of this study was to evaluate the immunoexpression of RANKL and OPG in PGCL and CGCL and to determine the number of MGCs and their nuclei in order to further understand the clinical behaviour of these two lesions.

Materials and methods

Tissue samples

The archives from the Oral Pathology Laboratory at the Federal University of Santa Catarina for years 2006 to 2016 were retrospectively analysed. Twenty cases diagnosed with PGCL and 20 with CGCL were included in this study. Patients subjected to medical treatment or previous surgery were excluded. The data, including age, sex, size, anatomical site, and recurrences, were collected from pathoanatomical and medical reports.

This study was approved by the Human Research Ethics Committee of the Federal University of Santa Catarina (process # 42095715.1.0000.0121).

Quantification of giant cells and their nuclei

The sections (5-µm thick) were stained with haematoxylin and eosin and then used for the histological evaluation. The sections were blindly analysed by two calibrated evaluators using a light microscope (Axiostar Plus; Carl Zeiss, Oberkochen, Germany) equipped with a digital camera (Canon Powershot A620; Cannon, Lake Success, NY, USA). Five fields were captured from each slide (400x magnification), selected from the central area of the lesion with a higher prevalence of MGCs. Quantification of MGCs and their respective nuclei was performed using Image J 1.45s software (National Institutes of Health, MD, USA). The number of MGCs and of their nuclei, adapted from Peacock et al.,[15] was counted.

Immunohistochemistry

For immunostaining, paraffin-embedded tissues were sliced into 3-µm sections and mounted onto a series of glass slides containing 3-aminopropyltriethoxysilene (Sigma-Aldrich, St. Louis, USA). The sections were deparaffinised with xylene, followed by rehydration in decreasing concentrations of alcohol. Endogenous peroxidase activity was blocked by subsequently immersing slides in two baths containing 6% hydrogen peroxide diluted in methyl alcohol for 20 then 10 minutes at room temperature. Antigen retrieval was performed by immersing the slides in citrate buffer (pH 6.0) at 96°C for 40 minutes. The slides were incubated with anti-RANKL (N-19, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, USA) and anti-OPG (N-20, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, USA) antibodies for 18 hours at 4 to 8 °C in a humidified chamber. Thereafter, the slides were washed with phosphate-buffered saline (PBS) and incubated with 1:500 chicken anti-goat biotin conjugate antibody (Santa Cruz Biotechnology, Santa Cruz, USA). The reaction was visualised with a

Vectastain ABC kit (Vector Laboratories, Burlingame, USA) and developed with DAB (Dako Cytomation, Glostrup, Denmark). Negative and positive controls were included during immunohistochemical reactions according to the manufacturer's recommendations.

Immunohistochemical analysis

Immunoexpression of RANKL and OPG was analysed using ImageJ 1.45q software (National Institutes of Health, USA). The analysis was performed for five high-power fields (400x) for each sample, captured with a camera (Cannon A620, Ōita, Japan) attached to a light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany). Hot spots without artefacts or with a high hemosiderin concentration were selected for immunohistochemical evaluation. The immunoreactivity of RANKL and OPG was characterised by brown cytoplasmic staining and the values were expressed as a percentage of the marked area in the stroma for each case. The RANKL to OPG ratio was also measured for each group.

Statistical analysis

The number of MGCs and of their nuclei, clinical parameters, and immunoexpression were statistically analysed by Fisher's exact test and Mann-Whitney test using SPSS software version 18.0 (SPSS Inc., Chicago, USA). Correlations between the parameters were evaluated using the Spearman's test. The results were expressed as mean ± standard deviation (SD) and the level of statistical significance was 5% ($p < 0.05$).

Results

Clinical findings

Among the 20 cases of PGCL, 12 (60%) occurred in males, whereas 14 out of 20 (70%) cases of CGCL occurred in females. The mean age of patients with PGCL and CGCL was 37 ± 23.89 and 24 ± 17.67 years, respectively. CGCL were larger and had more recurrences than PGCL. The mandible was more affected than the maxilla in both groups (Table 1).

Histological findings

There was no statistical difference in the number of MGCs per square millimetre between PGCL and CGCL ($p > 0.05$). However, the number of MGC nuclei was clearly higher in CGCL ($p = 0.01$). The mean number of nuclei per MGC was 5.84 in PGCL and 7.21 in CGCL (Figures A and 1B, Table 1).

Immunohistochemical analysis

RANKL and OPG immunoreactivity was detected in MGC cytoplasm and membrane and in mononuclear cells, such as lymphocytes and endothelial cells. Immunohistochemical reactivity was observed in mononuclear cells in all cases; however, not all MGCs stained positive for RANK or OPG.

RANKL expression in CGCL was higher than in PGCL ($p = 0.04$). There was no statistical difference between the two types of lesions for OPG expression ($P = 0.09$). Recurrent CGCL and PGCL had higher RANKL and OPG expressions than nonrecurrent lesions (Table 2). The RANKL to OPG ratio was not statistically different between the two groups (Figures C–F).

Table 1. Clinical, microscopic, and immunohistochemical findings in patients with central and peripheral giant cell lesions.

Variable	PGCL (n = 20)	CGCL (n = 20)	p-value
Age (years ± SD)	37 ± 23.89	24 ± 17.67	0.168
Sex			
Female	8	14	0.057
Male	12	6	
Anatomic site			
Mandible	14	15	0.004*
Maxilla	6	5	
Size (mm)	17.4 ± 10.1	28.5 ± 20.3	0.037**
Recurrence (%)	5	15	0.302
MGCs/mm^2	39.3 ± 19.1	46.7 ± 20.2	0.245
MGC nuclei/mm^2	230.3 ± 106.1	336.7 ± 144.3	0.010**
RANKL (%/area)	4.9 ± 3.1	10.2 ± 8.3	0.042**
OPG (%/area)	4.6 ± 2.7	9.1 ± 7.9	0.091
RANKL to OPG ratio	1.16	1.76	0.330

PGCL: peripheral giant cell lesion; CGCL: central giant cell lesion; SD: standard deviation; MGCs: Multinucleated giant cells; RANKL: Nuclear factor kappa-B ligand; OPG: Osteoprotegerin; *Fisher's exact test; **Mann-Whitney test.

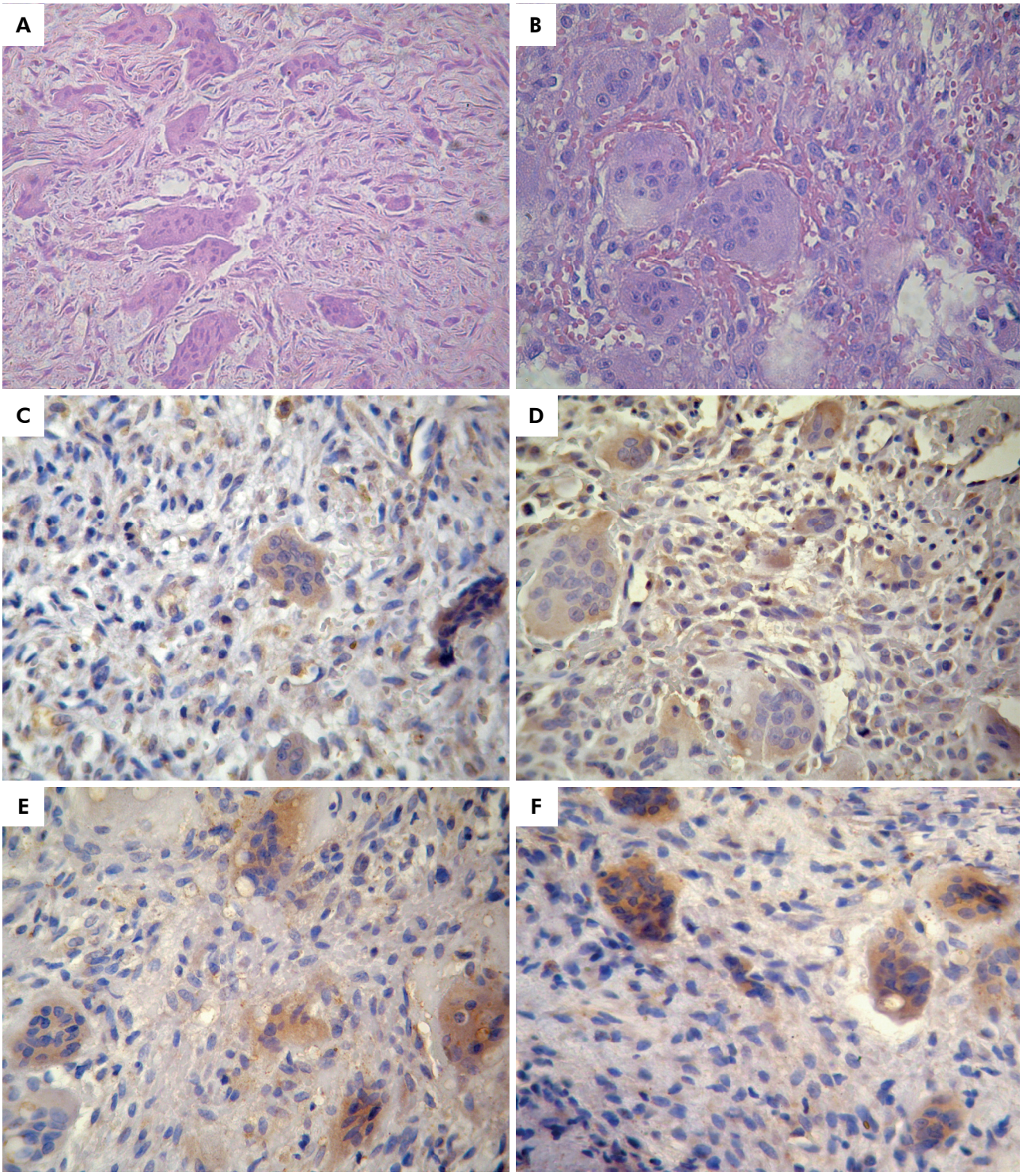

Figure. A. Photomicrograph showing high proliferation of multinucleated giant cells (MGCs) in peripheral giant cell lesion (Haematoxylin and eosin, original magnification 400x); B: Photomicrograph showing high proliferation of multinucleated giant cells (MGCs) in central giant cell lesion (Haematoxylin and eosin, original magnification 400x); C: Immunohistochemical expression of RANKL in MGCs and in mononuclear cells of peripheral giant cell lesion (Original magnification 400x); D: Immunohistochemical expression of RANKL in MGCs and in mononuclear cells of central giant cell lesion (Original magnification 400x); E: Immunohistochemical expression of osteoprotegerin in MGCs and in mononuclear cells of peripheral giant cell lesion (Original magnification 400x); F: Immunohistochemical expression of osteoprotegerin in MGCs and in mononuclear cells of central giant cell lesion (Original magnification 400x).

Table 2. Comparison of multinucleated giant cells and immunoexpression of nuclear factor kappa-B ligand and osteoprotegerin in central and peripheral giant cell lesions according to recurrences.

Variable	Recurrent (n = 04)	Nonrecurrent (n = 36)	p-value
MGCs/mm²	68.75 ± 20.27	40.18 ± 18.32	0.013*
MGC nuclei/mm²	399.75 ± 84.88	270.62 ± 135.39	0.032*
RANKL (%/area)	8.12 ± 5.83	7.54 ± 6.96	0.680
OPG (%/area)	7.38 ± 1.75	6.85 ± 6.61	0.191
RANKL to OPG ratio	1.17 ± 1.00	1.49 ± 2.00	0.394

MGCs: Multinucleated giant cells; RANKL: Nuclear factor kappa-B ligand; OPG: Osteoprotegerin; *Mann-Whitney test.

Discussion

In the present study, we evaluated central and peripheral giant cell lesions. Both are benign lesions that affect gnathic bones and, despite presenting the same histological features, they have a different clinical behaviour. PGCL is more common than CGCL[1] and both can occur at any age. In our study, we observed a higher prevalence of both lesions in younger patients, consistent with other studies in the literature. [2,7,16]

Regarding sex distribution, PGCL, unlike CGCL, was more prevalent in males, which is in agreement with other studies.[2,5,7,16] A higher prevalence of PGCL and CGCL was found in the mandible, as described by some authors.[1,2,16,17] However, other studies have reported a higher prevalence of PGCL and CGCL in the maxilla.[7,18]

Microscopically, we found a higher number of MGC nuclei in CGCL when compared to PGCL, consistent with other studies.[1,7] Although these lesions have similar microscopic features, they exhibit a different clinical behaviour. Many researchers have tried to explain these differences by cytomorphometric evaluation of the number of MGCs and of the number of nuclei per MGCs. Aghbali et al.[7] analysed the microscopic aspects of these lesions, observing a mean number of nuclei per MGC of 9.54 in CGCL and of 8.58 in PGCL, while Florez-Moreno et al.[1] found a higher number of nuclei within MGCs in CGCL than in PGCL.

Quantifying the MGCs present in these lesions has also been used as a tool for predicting their clinical behaviour, as performed in a study by Ficarra et al.[19] These authors evaluated 32 cases of CGCL which were divided into two groups, non-aggressive and aggressive, and they observed a higher mean number of nuclei per MGC in the aggressive group. Clinical and radiographic criteria have been used to classify these lesions as aggressive or non-aggressive and to predict prognosis.[7,15]

Only few studies in the literature have evaluated the expression of RANKL and OPG in PGCL and CGCL. In our study, we observed higher expression of RANKL in CGCL than in PGCL as well as higher expression of RANKL and OPG in recurrent lesions. In a previous study, RANKL overexpression was higher in CGCL when compared to fibrous dysplasia (FO) and simple bone cysts (SBC). In the same study, FO and SBC presented a higher expression of OPG for the samples analysed.[20] Another study evaluated the expression of RANKL and c-fos, a transcription factor that activates protein complex 1, essential for osteoclastogenesis, in CGCL,[21] and both markers showed strong immunoreactivity. Fanourakis et al.[22] evaluated the expression of RANKL and OPG in 22 cases of PGCL and observed high expression of these two markers.

Another report described higher expression of RANKL than of OPG in epithelial and mesenchymal cells of ameloblastic fibroma when compared to other odontogenic tumours with better clinical behaviour and fewer recurrences.[11] A higher expression of RANKL was also described in bone metastasis from breast cancer and giant cell tumour, a locally aggressive benign osteolytic tumour with a high potential of recurrence.[23,24] Investigations correlating RANKL expression with aggressiveness and recurrence potential in CGCL are scarce, but some case reports on CGCL with difficult management treated with denosumab, a RANKL inhibitor, demonstrated good results.[25]

The cytoplasmic and membrane-bound immunoreactivity detected in our samples was in accordance with that reported by other studies.[11,13,20,26,27] RANKL and OPG are expressed by different cell types, including the monocyte/macrophage lineage, osteoclastic precursors, B and T cells, dendritic cells, and fibroblasts.[11,27,28,29,30] Some reports on giant cell tumour have stated that RANKL overexpression in mononuclear cells could be the true neoplastic component of these lesions. [24,31,32]

Because CGCL is an osteolytic lesion, the samples were expected to have different OPG immunoexpression; however, no differences were observed between CGCL and PGCL. Furthermore, we observed a similar positive correlation between the RANKL to OPG ratio in both CGCL and PGCL. Differences in OPG functionality may depend on cell types. Osteoclasts undergo rapid apoptosis in the absence of trophic factors, such as M-CSF and RANKL. Extracellularly secreted OPG can indirectly induce apoptosis of the osteoclast itself by limiting the availability of RANKL that can be bound to RANK.[33] Controversially, OPG secreted during osteoclastogenesis can bind to TRAIL, a member of the tumour necrosis factor family of cytokines, and inhibit cell death by apoptosis, which is opposite to the effects of the binding of OPG to RANKL.[34,35]

As limitations of this study, there were difficulties in the immunohistochemical evaluation of some cases due to the large deposition of hemosiderin. In addition, some patient clinical records were incomplete, meaning that correlations between some clinical and histological aspects could not be made.

Information on RANKL and OPG expression in bone pathologies has contributed to the better understanding of the mechanisms that regulate osteoclastogenesis in physiological and pathological states. Our findings showed high RANKL expression, which may explain the difference in clinical behaviour between CGCL and PGCL and their pathogenesis. However, further investigation is necessary to clarify and confirm these findings.

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