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Differential patterns of RANKL/OPG expression in human periapical granulomas: possible association with progressive or stable nature of the lesions

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Abstract

RANKL and OPG are expressed in apical periodontitis, suggesting a role for these molecules during lesion development. However, the profiles of RANKL/OPG expression in periapical lesions remain unknown. In this study we investigated the patterns of RANKL and OPG mRNA expression by RealTime-PCR in human periapical granulomas (N=44), and compared them with sites presenting characteristic bone resorpting activity: healthy (n=14) and orthodontically stretched and compressed periodontal ligament (n=26), healthy gingiva (n=24), chronic gingivitis (n=32) and chronic periodontitis (n=34) samples. Both RANKL and OPG mRNA expression was higher in periapical granulomas when compared to healthy periodontal ligament. Distinct patterns of RANKL and OPG expression ratio were found in the granulomas and in different physiological and pathological conditions with characteristic bone resorption activity potentially being an indicative of the stable or progressive nature of the lesions. Lesions with radiographic image smaller than 5mm demonstrated higher RANKL/OPG expression than images greater than 5mm. Periapical granulomas presented heterogeneous patterns of RANKL and OPG expression, ranging from samples with RANKL/OPG ratio similar to that seen in sites with minimal or absent bone resorption, until samples with RANKL/OPG expression pattern comparable with active bone resorption sites.

Keywords

RANKL; OPG; periapical granulomas; bone resorption; Real Time-PCR

1. INTRODUCTION

Periapical inflammatory responses most commonly take the form of reactive granulomas and cysts, with the concomitant resorption of bone surrounding the roots of affected teeth (1). Bone resorption is a multi-step process where receptor activator NF κ B-ligand (RANKL) and osteoprotegerin (OPG) play important roles (2,3,4). RANKL binding to the receptor RANK present on the surface of preosteoclasts drives their maturation and activation, while OPG, the naturally occurring inhibitor of RANKL, is a decoy receptor that prevents RANK-RANKL engagement (2,3,4)(Fig. 1).

RANKL and OPG are expressed in apical periodontitis, suggesting a role for these molecules during lesion development (5,6,7,8). However, the detection of RANKL and OPG molecules does not characterize the actual lesion status, and these previous studies did not attempt to correlate RANKL/OPG expression with the actual bone resorption activity. Overall, the balance between RANKL and OPG expression is essential to determine the overall biological response in periodontal disease (9,10), orthodontic tooth movement (11,12) and in some osteolytic lesions in the facial skeleton (13)(Fig. 1). However, the profiles of RANKL/OPG expression in periapical lesions remain unknown.

Our goal was to evaluate the profile of RANKL and OPG transcript levels ratios in human periapical granulomas, and to correlate them with the patterns of RANKL/OPG ratios in sites with characteristic osteoclastic activity, attempting to recognize putative patterns that could reflect the progressive or stable nature of these periapical lesions.

2. MATERIAL AND METHODS

This study had IRB approval of Bauru Dental School, University of Sao Paulo, Ponta Grossa State University (UEPG) and University of Ribeirao Preto Dental School (UNAERP). Forty-four periapical granulomas were collected from previously selected patients aged 15–57 years old (average age 38.2) submitted to periapical surgery and curettage of the tissues as part of their clinical treatment. Radiographic examination, where clear bone loss and disappearance of the periodontal ligament space in the periapical region, diagnosed periapical lesion. Routine histological examination (HE staining of serially sectioned histological specimen) was performed, and granulomas represented a severe infiltration of inflammatory cells with no epithelial lining. Periapical cysts (fully developed cavities lined by stratified squamous epithelium and a fibrous capsule) were specifically excluded from the sample. Direct measure with an endodontic rule of lesions on periapical radiographs taken using an intra-oral film positioner determined the lesions diameter. RNA later (Ambion, Austin, TX, USA) conserved at least 1/3 of the removed tissues at -70° C until RealTime-PCR analysis.

The control group consisted of 14 subjects (8 males and 6 females, age 17–23) presenting good oral health from which biopsies of periodontal ligament were taken after tooth extractions realized for orthodontic reasons. Twenty-six patients aged 16–23 years-old and scheduled for tooth extraction due to orthodontic reasons comprised the orthodontic movement group. Periodontal ligament of 32 teeth exposed to orthodontic forces (7N) with Hyrax appliances for 7 days supplied compression and tension forces groups (12).

The periodontal disease groups comprised control subjects (C), chronic gingivitis (CG) and chronic periodontitis (CP) patients selected from subjects scheduled for treatment at UNAERP (14). CG patients (n=32) were characterized as old-adults (age 51–62) with poor plaque control/oral hygiene, high scores of bleeding on probing sites (>70%), absent or minimal (at most 1 tooth per sextant attachment loss =1mm) bone loss, and no history of tooth loss resulting from periodontitis, whose biopsies were obtained during surgical procedures due to prosthetic reasons. CP patients (n=34, age 40–57) presented moderate to advanced PD (at least 1 tooth

per sextant with probing depth >6mm and attachment loss >3mm). CP patients that exhibited no improvement in clinical condition and persistent bleeding on probing 4 weeks after the basic periodontal therapy supplied gingival tissues. Biopsies of gingival tissue of clinically healthy periodontium of subjects submitted to wisdom tooth removal comprised the control group (n=24, age 17–31). Exclusion criteria comprised patients with medical history indicating use of systemic modifiers of bone metabolism; systemic antibiotic, anti-inflammatory, hormonal or other assisted drug therapy in the last 6 months prior to the study, with primary or secondary acute periodontitis, pregnant or lactating females.

PrimerExpress2.0 software (Applied Biosystems, Foster City, California, USA) designed primer sequences (Table 1) for gene amplification in Real-TimePCR experiments performed in ABI-5700 system (Applied Biosystems)(15). SYBRgreen quantified the products formed during the reaction, and the Ct method determined the relative expression of a target gene in comparison to the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase), HPRT (hypoxanthine-guanine phosphoribosyltransferase), β -actin and BCRP (breast cancer resistance protein), as previously described (15,16,17).

The differences of means between independent samples were calculated using Unpaired sample t-test. Based on quartiles analysis, the samples were separated in 3 groups for further analysis: RANKL expression at least 2X higher than OPG expression (RANKL>OPG), OPG expression at least 2X higher than RANKL expression (RANKL<OPG), and a third group, the ratio of RANKL and OPG expression was lower than 2X (RANKL $\cong$ OPG). Fisher exact test analyzed the frequencies of the different samples in groups with distinct RANKL/OPG expression.

3. RESULTS

Periapical granulomas showed higher RANKL and OPG mRNA expression when compared to healthy periodontal ligaments ($p<0.0001$), high variability among the values for RANKL and OPG transcript levels and no significant correlation between the levels of RANKL and OPG (Fig. 2). In the analysis of RANKL/OPG ratios periapical granuloma samples presented similar distribution between RANKL>OPG, RANKL $\cong$ OPG and RANKL<OPG groups, while control samples were preferentially found in the RANKL $\cong$ OPG group ($p<0.0001$, controls vs granulomas, Fisher exact test)(Fig. 3).

Since clinical assessments do not allow for determination if the periapical samples were stable or active lesions, we compared the profiles of RANKL/OPG expression in our periapical lesions to the expression profile seen in sites where bone resorption activity is already established, such as the compression and tension sides of teeth submitted to orthodontic forces, and sites of chronic gingivitis and periodontitis (Fig. 3). RANKL>OPG group predominates in the compression sides (where bone resorption is a feature) while RANKL<OPG group predominates in the tension sides ($p<0.0001$, compression vs tension). Healthy gingiva and chronic gingivitis samples predominantly showed RANKL $\cong$ OPG and RANKL<OPG expression in opposition to CP samples ($p<0.0001$, periodontitis vs healthy and vs gingivitis). Considering RANKL and OPG expression and radiographic size of the lesions (smaller or greater than 5mm in diameter), our results demonstrate a trend towards higher RANKL and lower OPG expression in smaller lesions (Fig. 4) and a higher frequency of RANKL>OPG samples in opposition to predominance of RANKL $\cong$ OPG and RANKL<OPG groups in lesions >5mm ($p=0.0006$).

4. DISCUSSION

It has been proposed that proinflammatory cytokines play a fundamental role in periapical bone destruction through the induction of RANKL, while OPG synthesis is supposed to attenuate

lesion expansion (13,14,15,16). RANKL and OPG detection in previous studies did not attempt to characterize the actual stable or progressive status of the periapical lesions, and its contribution to the development and progression of periapical granulomas remains unknown.

We observed a significantly higher expression of RANKL and OPG mRNAs in human periapical granulomas when compared to healthy periapical tissues reinforcing their active role in lesion progression and expansion. Our results demonstrate high individual variation, up to 10 times, in the RANKL and OPG levels, but without correlations between RANKL and OPG levels in the lesions, suggesting that distinct expression patterns could be associated with different stages of disease progression. Indeed, the samples were similarly distributed in the three distinct groups regarding RANKL/OPG expression patterns. These patterns are significantly different from the ones observed in healthy periodontal ligament samples, where the majority of the samples (80%) presented a balanced RANKL/OPG expression. In addition, our results showed higher ($\cong 70\%$) RANKL expression (RANKL>OPG group) in samples from periodontal ligament under compressive orthodontic force, whereas samples from tension sides presented higher OPG expression ($\cong 75\%$), confirming previous data (12). Correlating these findings with the clinical patterns of tooth movement (12,18), we can assume that samples from the RANKL>OPG group are associated with active bone resorption, while the RANKL<OPG group indicates minimal resorption activity. In addition, based on the analysis of control samples, we can suggest that similar levels of RANKL/OPG (RANKL $\cong$ OPG group) would be associated with homeostatic tissue turnover, where bone formation and bone resorption are thought to be in equilibrium.

One may argue that orthodontic tooth movement is a force-induced bone resorption process and not an inflammatory disease to be compared to periapical lesions. Nevertheless, chronic inflammatory periodontal diseases share some of the features of periapical lesions (19). Our results demonstrate that RANKL<OPG (56%) and RANKL $\cong$ OPG (35%) patterns predominate in chronic gingivitis lesions, while the RANKL>OPG predominates (70%) in chronic periodontitis lesions. Gingivitis and periodontitis are considered to be a continuity of the same inflammatory disease, but the bone resorption is absent in chronic gingivitis patients (20).

Therefore, based on the patterns of RANKL/OPG expression in different physiological and pathological conditions (9,10,11,12,21), we may suggest that sites with active bone resorption present a differential pattern of RANKL/OPG expression when compared to sites where bone resorption is absent or minimal. Comparing these characteristic patterns of RANKL/OPG expression to that seen in periapical lesions, we can suggest that samples where expression of RANKL predominates (RANKL>OPG group, n=18/44, 38,63%) are putative progressive lesions, whereas samples where expression of RANKL and OPG is similar (RANKL $\cong$ OPG, n=15/44, 34,05%), or where OPG is prevalent (RANKL<OPG (N=11/44, 24,97%) groups are potentially stable lesions. It is also important to consider that RANKL/OPG balance, and consequently the outcome of periapical lesions, can be modulated by systemic factors, since severe periapical bone resorption induced by estrogen deficiency is mediated by RANKL overexpression (22).

We also showed that lesions greater than 5mm in diameter presented higher expression of OPG mRNA in opposition to smaller lesions, accordingly to previous studies reporting RANKL up regulation at the initial stages of periapical lesion expansion (8) and experimental periodontitis (9). Higher OPG levels may reflect a homeostatic mechanism of the skeleton to refrain the increased bone resorption (9,14,21,22). Interestingly, at the early stage of periapical lesions development in ovariectomized rats, the osteoblastogenesis was accelerated, also suggesting a kind of reactive protective response in order to minimize the bone resorption (22)..

Taken together, our results suggest that the differential RANKL/OPG expression could account for different stages of the periapical lesions. At a certain timepoint during lesion development, equilibrium of RANKL and OPG and reduction of osteoclast activation tend to occur further resulting in a stable lesion. However, irrespective of the numerical values of relative expression and the limits in using mRNA expression relative measurement, the clear association of RANKL/OPG patterns with different bone remodeling sites, in accordance with several previous studies (9,10,11,12,21,22,23), is a strong indicative of their biological relevance. Additional factors such as anatomic variations and complexity, the load, type and virulence of microorganisms involved in the infection, as well as the uncertain time of onset and duration of the pathological process can also be important modifiers of disease progression (24,25,26, 27,28,29), and must be considered in further studies regarding periapical lesions development.

In summary, our results demonstrate that periapical granulomas presented heterogeneous patterns of RANKL and OPG expression, ranging from samples with RANKL/OPG ratio similar to that seen in sites with minimal or absent bone resorption, until samples with RANKL/OPG expression pattern comparable with active bone resorption sites. Therefore, it is possible that RANKL/OPG balance may be associated with the stable or progressive nature of periapical granulomas, and such knowledge may provide important insights to the development of diagnostic, preventive and therapeutical strategies to improve the clinical management of periapical lesions.

5. CONCLUSIONS

The patterns of RANKL and OPG expression may be associated with the stable or progressive nature of periapical granulomas.

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Abbreviations

RANK	Receptor activator of NFκB
RANKL	Receptor activator of NFκB-ligand
OPG	osteoprotegerin

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Figure 1. The role of RANK, RANKL and OPG in osteoclastogenesis in homeostatic and inflammatory conditions

A schematic representation of RANK/RANKL/OPG networks in homeostatic and inflammatory conditions. The osteoclast differentiation and activation is driven by the interaction of RANK (receptor activator of nuclear factor- κ B) with its ligand RANKL, an event regulated by OPG, a decoy receptor of RANKL that inhibits RANK-RANKL engagement. In homeostatic conditions (left side), RANKL and OPG levels are thought to be in a dynamic balance, which allows a controlled generation of osteoclasts responsible for the homeostatic bone resorption. Conversely, the overexpression of RANKL in inflammatory conditions (right side), mainly due the presence of inflammatory cells, result in an imbalance of RANKL/OPG levels leading to excessive osteoclast activity, and consequently to pathologic bone resorption.

Figure 2. Quantitative expression of RANKL and OPG in periapical granulomas

Total RNA was extracted from periapical granulomas (G, N=44) and periodontal ligament control samples (C, N=14), and levels of (A) RANKL and (B) OPG mRNA were measured quantitatively by RealTimePCR SYBR-Green system. The results are presented as expression of the individual mRNAs, with normalization to housekeeping genes using the Ct method. Linear regression analysis (C) was used to test the correlations between the levels of RANKL and OPG expression. Values of p and r^2 are identified in the graphs.

Figure 3. Patterns of RANKL and OPG in periapical granulomas and their comparison with sites with characteristic bone resorption activity

Total RNA was extracted from periapical granulomas (N=44)(A), periodontal ligament control samples (N=14)(B), periodontal ligament from compression (C) and tension (D) sides of tooth submitted to orthodontic forces (N=18), healthy gingivae (N=24)(E), chronic gingivitis (N=32) (F) and chronic periodontitis (n=34)(G), and levels of RANKL and OPG mRNA were measured quantitatively by RealTimePCR SYBR-Green system. The samples were then categorized regarding the pattern of RANKL/OPG expression in three groups: RANKL>OPG, RANKL~OPG and RANKL<OPG, as described in Material and Methods. The N and % of the samples in each group is indicated in the graphs.

Figure 4. Patterns of RANKL and OPG in periapical granulomas with radiographic diameter > or < than 5mm

Total RNA was extracted from periapical granulomas (N=44), and levels of RANKL and OPG mRNA were measured quantitatively by RealTimePCR SYBR-Green System. The samples were then categorized regarding the radiographic diameter of the lesions (D< or D> than 5mm), and the individual levels (A) and the patterns (B) of RANKL/OPG were evaluated. The N and % of the samples in each group is indicated in the graphs.

Table 1

Sequence of primers used in this study for the amplification of the respective genes

Gene	Primer sequence
human <i>RANKL</i>	F: 5' ACATATCGTTGGATCACAGCACAT 3' R: 5' CAAAAGGCTGAGCTTCAAGCTT 3'
human <i>OPG</i>	F: 5' CAAAAGGCTGAGCTTCAAGCTT 3' R: 5' AATGCCTCCTCACACAGGGTAA 3'
human <i>βACTIN</i>	F: 5' GGCACCCAGCACAATGAAG 3' R: 5' CCGATCCACACGGAGTACTTG 3'
human <i>BCRP</i>	F: 5' CCTTCGACGTCAATAACAAGGAT 3' R: 5' CCTGCGATGGCGTTCAC 3'
human <i>GAPDH</i>	F: 5' ACCCACTCCTCCACCTTTGA 3' R: 5' CTGTTGCTGTAGCCAAATTCGT 3'
human <i>HPRT</i>	F: 5' GAACGTCTTGCTCGAGATGTGA 3' R: 5' TCCAGCAGGTCAGCAAAGAAT 3'