Receptor activator of nuclear factor kappa B ligand and osteoprotegerin levels in gingival crevicular fluid

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ABSTRACT

Background: Osteoclastogenesis is coordinated by the interaction of three members of the tumor necrosis factor (TNF) superfamily: Osteoprotegerin (OPG)/receptor activator of nuclear factor kappa B ligand (RANKL)/receptor activator of nuclear factor kappa B (RANK). The aim of this study was to investigate RANKL and OPG levels, and their relative ratio in gingival crevicular fluid (GCF) of patients with chronic and aggressive periodontitis, as well as healthy controls.

Materials and Methods: In this analytical study, GCF was obtained from healthy (n = 10), mild chronic periodontitis (n = 18), moderate chronic periodontitis (n = 18), severe chronic periodontitis (n = 20), and generalized aggressive periodontitis (n = 20) subjects. RANKL and OPG concentrations were measured by enzyme-linked immunosorbent assay. Statistical tests used were Kruskal–Wallis test, Mann–Whitney U rank sum test, and Spearman's rank correlation analysis. The level of statistical significance was set at P < 0.05.

Results: Mean RANKL concentration showed no statistically significant differences between groups (P = 0.58). There were also no significant differences between mean OPG concentration in the five groups (P = 0.056). Moreover, relative RANKL/OPG ratio did not reveal a significant difference between the three study group subjects: healthy, chronic periodontitis (mild, moderate, severe), and aggressive periodontitis (P = 0.41). There was statistically significant correlation between the concentration of sRANKL and Clinical Attachment Level (CAL) in moderate chronic periodontitis patients (R = 0.48, P = 0.04). There was also negative correlation between OPG concentration and CAL in moderate chronic periodontitis patients, although not significant (R = –0.13).

Conclusion: RANKL was prominent in periodontitis sites, especially in moderate periodontitis patients, whereas OPG was not detectable in some diseased sites with bleeding on probing, supporting the role of these two molecules in the bone loss developed in this disease.

Key Words: Enzyme-linked immunosorbent assay, gingival crevicular fluid, Osteoprotegerin, RANKL, Receptor activator of nuclear factor kappa B ligand

INTRODUCTION

Periodontitis is one of the most common chronic diseases throughout the world and destruction of the osseous support of the dentition is a hallmark of periodontitis.[1] An imbalance between the plaque biofilm and the host immune system results in the overexpression of proinflammatory cytokines and the subsequent destruction of alveolar bone.[2] The association of an increased volume of gingival crevicular fluid (GCF) with an increase in the severity of inflammation is well supported by evidence from the literature.[3–5] Therefore, GCF is regarded as a window for noninvasive analysis of periodontal conditions, including markers of connective tissue...
and bone destruction. However, the major attraction of GCF as a diagnostic marker is the site-specific nature of the sample. This fact allows laboratory investigations of GCF ingredients to be linked to clinical assessments at the site of sample collection.

Osteoclasts are bone resorbing cells. Osteoclastogenesis is coordinated by the interaction of three members of the tumor necrosis factor (TNF) superfamily: Osteoprotegerin (OPG)/receptor activator of nuclear factor kappa B ligand (RANKL)/receptor activator of nuclear factor kappa B (RANK).

Stromal cells, osteoblastic cells, fibroblasts, and activated T-cells express RANKL in a membrane-bound form which can be changed into a soluble form (sRANKL) by the TNF-α converting enzymes. RANKL binds directly to RANK on the surface of pre-osteoclasts, stimulating both the differentiation of osteoclasts progenitors and the activity of mature osteoclasts. OPG is a soluble decoy receptor for RANKL and prevents the interaction with RANK. Periodontal ligament cells, gingival fibroblasts, and epithelial cells have been reported to produce OPG. In the field of dentistry, some clinical studies have reported that both RANKL and OPG can be detected in human GCF during orthodontic tooth movement or in peri-implant crevicular fluid. sRANKL and OPG were also determined in crevicular fluid from patients with chronic periodontitis, which resulted in an increased total amount of sRANKL as well as sRANKL/OPG ratio. Bostanchi et al. reported that there were no differences in RANKL and OPG levels between chronic and generalized aggressive periodontitis groups. The aim of this study was to investigate RANKL and OPG levels as well as their relative ratio in GCF of patients with mild, moderate, severe chronic periodontitis, and aggressive periodontitis, as well as healthy controls.

MATERIALS AND METHODS

Study population and clinical examination

A total of 86 samples were included in this study. The healthy group consisted of 5 females and 5 males with a mean age of 25 ± 1.41 years. The generalized aggressive periodontitis group consisted of 9 females and 11 males with mean age of 29.3 ± 2.51 years. The chronic periodontitis group consisted of 10 females and 8 males with mild chronic periodontitis (mean age of 36.8 ± 9.8 years), 7 females and 11 males with moderate chronic periodontitis (mean age of 44.8 ± 12.8 years), and 8 females and 12 males with severe chronic periodontitis (mean age of 47.6 ± 13.07 years). All subjects were recruited from Department of Periodontics, Dental center, Islamic Azad University, Tehran, Iran. Written and informed consent was obtained from each subject before enrollment in the study. Complete medical and dental histories were taken from all subjects. They did not have any systemic illness and none of the subjects had taken medications such as antibiotics or non-steroidal anti-inflammatory therapy for at least 3 months before the study.

In addition, no patient had received any periodontal treatment within the 3 months preceding the study. All the procedures were performed in accordance with the Helsinki protocol (version 2002) and the study protocol was reviewed and approved by the ethical and research committee of Dental Center, Islamic Azad University. All subjects had a clinical examination including the measurement of Probing Depth (PD), Clinical Attachment Level (CAL), Bleeding on Probing (BOP), and Plaque Index (PI).

Before GCF sampling, the plaque index (Silness and Loe 1964) was recorded. Then, the teeth surfaces were cleared of supragingival plaque, isolated with cotton rolls, and dried with a gentle stream of air to prevent saliva contamination. A sterile periopaper strip (Periopaper, Oraflow Inc., NewYork, USA) was gently inserted into the crevice of teeth and left in place for 30 s. Mechanical irritation was avoided and strips contaminated with blood were discarded. Samples were obtained from the mesiobuccal aspect of one single-rooted tooth in each patient. After GCF collection, strips were placed in vials, lyophilized, and stored at −70°C before laboratory analysis.

Then, PD was recorded for all teeth at four sites around each tooth (mesiobuccal, midbuccal, distobuccal, and midlingual) using Williams probe (Hu Friedy®). The probe was inserted into each sulcus using gentle pressure until it could not be advanced further apically. No pain should be caused by probing. CAL was measured as the distance between the cementoenamel junction and the most apical penetration of the probe, for all teeth at the four mentioned sites. Dichotomous BOP scores were recorded as present or absent per tooth according to Ainamo and Bay.
Criteria for classification of periodontal diseases
Chronic periodontitis was diagnosed if the CAL was commensurate with the amount of local factors in the patients. The severity of chronic periodontitis is determined as slight: 1-2 mm, moderate: 3-4 mm, or severe: ≥5 mm, based on the amount of CAL. The aggressive periodontitis group demonstrated CAL ≥ 4 mm on eight or more teeth; at least three of those were other than central incisors or first molars, showed severe periodontal destruction and loss of periodontal support inconsistent with age and plaque. The healthy group had no clinical signs of gingival inflammation (no BOP), CAL = 0, PPD < 3 mm, and no radiographic evidence of alveolar bone loss.[28] They consisted of university staff and students who were informed of the study and volunteered to give their GCF samples to be used as reference samples.

Quantification of RANKL and OPG in GCF
For analysis, 100 µl of phosphate-buffered saline (PBS, pH 7.2) was used to re-elute the samples. The tubes were shaken gently for 1 min and then centrifuged at 1800 × g for 30 min at 4°C before being processed on the enzyme-linked immunosorbent assay (ELISA) plate. The contents of RANKL and OPG in the samples were determined by commercial ELISA kits in accordance with the manufacturer’s instructions (total sRANKL ELISA kit: BioVendor, Modrice, Czech Republic; and Human OPG EISA kit: Bender Medsystems, Vienna, Austria). The minimum detection limit was 0.2 pmol/l for sRANKL and 2.5 pg/ml for OPG.

The 96-well microtiter plates were coated with appropriate antibodies. One hundred microliters of standards and samples were added to wells and the plates were incubated for 18 h. Then, the wells were washed five times with wash solution (0.35 ml per well). Afterward, the detection antibody (biotin-labeled antibody) was added into each well and incubated for 60 min at room temperature (18ºC-25ºC). Then, the wells were washed again five times. One hundred microliters of streptavidin–HRP conjugate was incubated at room temperature for 1 h and the wells were washed again. Then, the wells were developed with 100 µl tetramethylbenzadine (TMB) for 10 min at room temperature in the dark. The plate was covered with aluminum foil. Then, the reaction was stopped by the addition of 100 µl stop solution and color was measured in an automated microplate spectrophotometer set to 450 nm, and the samples were compared with the standards. Data were then calculated and obtained by methods of interpolation of a predetermined standard curve. Values of total amounts are expressed as picomoles/liter for concentrations of RANKL and as picograms/liter for OPG, when adjusted for GCF volume.

Statistical analysis
All data analyses were performed using the SPSS 16.0 software. Normality of the distributions for the variables was measured by the Smirnov–Kolmogorov test. Therefore, comparisons between all groups were performed using the Kruskal–Wallis test. Cytokine levels were compared between each pair of groups using Mann–Whitney U rank sum test. Spearman’s rank correlation analysis was used to determine correlation between gingival crevicular fluid levels of sRANKL, OPG, and periodontal parameters. The level of statistical significance was set at P < 0.05.

RESULTS
The demographic and clinical data are outlined in Table 1. There were statistically significant differences between mean CAL, PD, and PI in five groups.
Variations in concentrations of cytokines (RANKL and OPG) in the study groups are shown in Table 2. As shown in Table 2, mean RANKL concentration for 60 min at room temperature (18ºC-25ºC). Then, the wells were washed again five times. One hundred microliters of streptavidin–HRP conjugate was incubated at room temperature for 1 h and the wells were washed again. Then, the wells were developed with 100 µl tetramethylbenzadine (TMB) for 10 min at room temperature in the dark. The plate was covered with aluminum foil. Then, the reaction was stopped by the addition of 100 µl stop solution and color was measured in an automated microplate spectrophotometer set to 450 nm, and the samples were compared with the standards. Data were then calculated and obtained by methods of interpolation of a predetermined standard curve. Values of total amounts are expressed as picomoles/liter for concentrations of RANKL and as picograms/liter for OPG, when adjusted for GCF volume.

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| Table 1: Demographic and clinical characteristics of the study groups (mean±SD) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Variable        | Healthy (n=10)  | Mild chronic periodontitis (n=18) | Moderate chronic periodontitis (n=18) | Severe chronic periodontitis (n=20) | Generalized aggressive periodontitis (n=20) |
| Gender F:M      | 5:5             | 10:8             | 7:11             | 8:12             | 11:9             |
| Age             | 25±1.41         | 36.8±9.8         | 44.8±12.8        | 47.6±13.07       | 29.3±2.51        |
| PD              | 1.05±0.53       | 3.22±0.55        | 4.05±0.94        | 6.00±1.89        | 6.80±1.36        |
| CAL             | 0.00±0.00       | 1.67±0.48        | 3.56±0.51        | 6.65±1.78        | 6.00±1.12        |
| PI (%)          | 25.00±7.90      | 62.66±18.15      | 75.16±16.39      | 90.83±11.14      | 38.33±28.43      |
| BOP Positive (%)| 0 (0)           | 18 (100)         | 18 (100)         | 20 (100)         | 20 (100)         |
| BOP Negative (%)| 10 (100)        | 0 (0)            | 0 (0)            | 0 (0)            | 0 (0)            |

PD: Probing depth; CAL: Clinical attachment level; PI: Plaque index; BOP: Bleeding on probing.
showed no statistically significant differences between groups ($P = 0.58$). There were also no significant differences between mean OPG concentration in the five groups ($P = 0.56$). Moreover, RANKL/OPG ratio was found to be slightly elevated in aggressive periodontitis compared to chronic periodontitis and healthy specimens, but this finding did not reveal a significant difference between the three study groups: Healthy, chronic periodontitis (mild, moderate, severe), and aggressive periodontitis subjects ($P = 0.41$). In aggressive periodontitis patients group, 50% of the sites had detectable levels of RANKL, compared with 46.4% of chronic periodontitis and 40% of healthy controls. OPG was present in 60% of sites without BOP and 27.6% of sites with BOP, and the difference was statistically significant ($P = 0.04$). As shown in Figure 1, total OPG concentration was higher in healthy subjects (154.71 ± 20.77) compared to chronic periodontitis (150.54 ± 16.33) and aggressive periodontitis patients (145.00 ± 14.92), but the difference was not statistically significant ($P = 0.36$). Correlations between periodontal variables (PD, CAL, PI, and BOP) and cytokine (RANKL and OPG) levels were analyzed and the most important results are shown in Table 3. Table 3 demonstrates that there was statistically significant correlation between the concentration of sRANKL and CAL in moderate chronic periodontitis patients ($R = 0.48, P = 0.04$). There was also negative correlation between OPG concentration and CAL in moderate chronic periodontitis patients, although it was not significant ($R = -0.13$).

## DISCUSSION

In the present study, we analyzed the levels of RANKL and OPG as well as their relative ratio in GCF from healthy and periodontally diseased subjects. There was a statistically significant direct relationship between periodontal attachment loss and GCF level of RANKL in moderate periodontitis subjects, while there was an inverse relationship between periodontal attachment loss and GCF level of OPG in this study group, but neither RANKL nor the OPG concentration showed significant differences between groups.

In a similar study, Vernal et al. reported that RANKL was found in a higher proportion (85%) of samples from patients than from controls (46%). Their patients were adult patients with untreated chronic periodontitis. However, they did not determine any severity classification for the 20 study patients. In our study, in aggressive periodontitis patients group, 50% of the sites had detectable levels of RANKL, compared with 46.4% of chronic periodontitis and 40% of healthy controls, although it was not statistically significant. With respect to the fact that mean CAL of the patients in their study was 3.57 mm, it can be interpreted that most of their patients had moderate periodontitis while our chronic periodontitis patients consisted of mild, moderate, and severe periodontitis. They also monitored their patients for 4 months with clinical measurements repeated every 2 months. Then, GCF was collected from both active and inactive sites. Active sites were
defined as those exhibiting ≥2 mm of attachment loss from baseline. We did not detect attachment loss from baseline in our study. We have measured BOP. Gingival BOP indicates an inflammatory lesion both in the epithelium and in the connective tissue that exhibits specific histological differences compared with healthy gingiva.[29] Even though gingival BOP may not be a good diagnostic indication for CAL, its absence is an excellent negative predictor of future attachment loss. Therefore, the absence of BOP is desirable and implies a lower risk of future CAL.[30] Our results showed that OPG was present in 60% of sites without BOP and 27.6% of sites with BOP, and the difference was statistically significant.

In another similar study, Bostanci et al. reported that RANKL/OPG ratio was similar in all three periodontitis groups (chronic periodontitis, generalized aggressive periodontitis, and chronic periodontitis subjects under immunosuppressant therapy).[29] They did not determine any severity classification for the 28 chronic periodontitis patients of their study too. Moreover, the mean CAL of their chronic periodontitis patients was 7.8 mm.

Lu et al. divided the sampling sites of 20 patients with generalized chronic periodontitis into four groups (healthy, mildly diseased, moderately diseased, and severely diseased) like in our study.[23] They too did not detect attachment loss from baseline in their study. They reported that in diseased subjects, OPG showed no significant differences on intergroup comparisons, while RANKL was markedly increased in moderately and severely diseased sites compared with healthy sites. They also failed to detect OPG in healthy subjects.

Chronic periodontitis is generally slowly progressive with some patients having increased susceptibility to bone loss and pocketing. World Workshop in Periodontics (1996) reported that sufficient evidence exists to consider three microorganisms as etiologic agents: *Aggregatibacter actinomycetemcomitans* (formally *Actinobacillus actinomycetemcomitans*), *Porphyromonas gingivalis*, and *Tannerella forsythia* (formally *Bacteroides forsythus*). Of these bacteria, *P. gingivalis* and *T. forsythia* often are found in chronic periodontitis, whereas *A. actinomycetemcomitans* often is found in cases of aggressive periodontitis.[31] Microbial stimulation with *A. actinomycetemcomitans* induced RANKL expression on the surface of CD4 + cells and in vivo inhibition of RANKL function; using the decoy receptor OPG diminished alveolar bone destruction and reduced the periodontal osteoclasts after microbial challenge.[32] Furthermore, Muelman et al. reported that gene-expression analysis demonstrated that the presence of *P. gingivalis* in the dentogingival area significantly decreased the RANKL/OPG ratio, compared with ligature alone.[33] Although these results show that RANKL contributes to alveolar bone loss in periodontitis and tooth loss, recent findings have suggested that the increased RANKL/OPG ratio may serve as a biomarker that denotes the occurrence of periodontitis, but may not necessarily predict ongoing disease activity. Its steadily elevated concentration post treatment may indicate that the molecular mechanisms of bone resorption are still active.[34] Bostanci et al. also reported that although the RANKL/OPG ratio has a potential diagnostic value for untreated periodontitis, it may not be a suitable predictor of clinically successful treatment outcome. It has also been reported that RANKL/OPG ratio in untreated and treated sites may be negatively influenced by poor glycemic control in subjects with type 2 diabetes.[35]

**CONCLUSION**

In conclusion, in this cross-sectional study of GCF in periodontal disease, the RANKL was prominent in periodontitis sites especially in moderate periodontitis patients, whereas OPG was not detectable in some diseased sites with BOP.

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