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Free soluble receptor activator of nuclear factor-κb ligand in gingival crevicular fluid correlates with distinct pathogens in periodontitis patients

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Abstract

Aim: The aim of the experiment was to investigate the levels of free soluble receptor activator of nuclear factor-κb ligand (sRANKL) in periodontal health and disease and their correlations to clinical parameters and important periodontal pathogens.

Material and Methods: Chronic periodontitis ($n = 35$) and periodontally healthy ($n = 38$) subjects participated in the present study. Pocket depth, recession and bleeding index were recorded at a total of 221 sites. Subgingival plaque samples from these sites were analysed for the levels of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* and *Treponema denticola*. Gingival crevicular fluid samples were analysed with ELISA for levels of free sRANKL. Comparisons between groups were performed by applying non-parametric tests (Mann–Whitney) and correlations among parameters were sought for with Spearman's *r* coefficient.

Results: Mean levels of free sRANKL were higher in periodontitis subjects and correlated significantly with mean counts of *T. denticola* on the subject level and *P. gingivalis*, *T. denticola* on the site level (Spearman's *r* coefficient, $p < 0.05$), but not with clinical parameters. No correlations were found between the levels of free sRANKL and investigated parameters in periodontally healthy individuals. No effect of smoking was found on investigated parameters and correlations (univariate analysis of variance and pairwise comparisons).

Conclusions: Findings from the present study suggest a correlation of levels of sRANKL with important pathogens in periodontitis patients.

Key words: crevicular fluid; free sRANKL; periodontitis

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Increasing evidence suggests the regulatory role of the receptor activator of nuclear factor-κb ligand (RANKL) and osteoprotegerin (OPG) system in bone destruction. This role had been previously shown in bone-related disease models (Jones et al. 2002, Schoppet et al. 2002) and more recently in periodontitis (Liu et al. 2003, Mogi et al. 2004, Vernal et al. 2004, Lu et al. 2006, Bostanci et al. 2007a, b, Nagasawa et al. 2007).

Studies related to periodontal disease have demonstrated in particular that an increase in RANKL expression or the RANKL/OPG ratio in gingival tissues and gingival crevicular fluid (GCF) is indicative of periodontal destruction (Liu et al. 2003, Mogi et al. 2004, Vernal et al. 2004, Lu et al. 2006, Bostanci et al. 2007a, b). It appears that in the periodontal environment, RANKL is produced mainly by T and B cells (Kawai et al. 2006, Han et al.

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2007) and OPG is known to derive from osteoblasts, and bone marrow cells (Schoppet et al. 2002) as well as dental mesenchymal cells (Sakata et al. 1999, Nagasawa et al. 2002). Although there are conflicting data in periodontal literature regarding the rate of RANKL-induced osteoclast formation in periodontitis patients, the presence of RANKL appears to be obligatory for bone resorption (Brunetti et al. 2005, Tjoa et al. 2008). It has been shown that RANKL has a membrane-bound (mRANKL) as well as a soluble form (sRANKL). The latter is antagonized by OPG for binding to the receptor RANK, therefore preventing an interaction that leads to osteoclastogenesis. Findings in the periodontal literature mainly apply to total soluble RANKL (both bound and unbound to OPG) and OPG can serve as an indirect index to distinguish between the two RANKL forms.

It is known that GCF is the most useful tool for the investigation of a number of molecules – markers of disease activity (Kinney et al. 2007). The recognition of on-going bone resorption by means of a non-invasive method is an attractive option, from the therapeutic point of view, and as such the free form of sRANKL appears a suitable candidate (Rodan & Martin 2000, Han et al. 2007, Kirkwood et al. 2007).

In addition, data suggest the importance of certain important periodontal pathogens, notably *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in upregulating RANKL production from osteoblasts, gingival fibroblasts and periodontal ligament cells (Okahashi et al. 2004, Belibasakis et al. 2005, 2007). Although these data are currently limited in literature, they strongly suggest that consensus pathogens can mediate their role in periodontal destruction through this molecule.

The aim of the present study was to investigate the levels of free sRANKL in periodontal health and disease and their correlations to clinical parameters and important periodontal pathogens.

Material and Methods

Study population

Patients participating in the present study ($n = 35$, mean age 47.09 ± 9.14) were recruited from the Clinic of the Department of Periodontology and Implant Biology, Dental School, Aristotle University of Thessaloniki, and provided an informed consent. These patients were diagnosed with generalized chronic periodontitis based on clinical and radiographic findings (Armitage 1999).

Age-matched ($n = 39$, mean age 44.45 ± 12.29) personnel and patients from the department with no attachment loss and bleeding on probing (BOP) $< 10\%$ also volunteered to participate in the present study.

The criteria for inclusion were the following:

1. Absence of systemic diseases or bone metabolism conditions that might affect RANKL levels (self-reported).
2. No history of antibiotics within the past 6 months.
3. No history of periodontal treatment during the last 12 months.
4. Presence of at least 20 teeth.

Pregnant or lactating women were excluded from the present study. Smoking status (smoker, non-smoker), as reported by patients, was also recorded. The study was conducted according to the protocol outlined by the Research Committee, Aristotle University of Thessaloniki, Greece, and was approved by the Ethical Committee of the School of Dentistry.

Sampling sites

Periodontally healthy individuals contributed with two sampling sites with no attachment loss and probing depth (PD) > 3 mm. Periodontitis subjects contributed with up to 10 sampling sites with PD > 5 mm. The following parameters were recorded by the same calibrated examiner (D. S.) using a manual Williams probe (POW, Hu-Friedy, Chicago, IL, USA):

(a) PD
(b) recession (RE)
(c) BOP.

GCF samples

The GCF samples were obtained as follows. After isolation of the site with cotton rolls to prevent contamination with saliva, supragingival plaque was removed, the tooth air-dried and a 30-s GCF sample was collected on filter strips (Periopaper®, Interstate Drug Exchange, Amityville, NY, USA). Periopaper strips were gently inserted into the orifice of the periodontal pocket, 1–2 mm subgingivally. The samples (total = 223) were immediately placed in Eppendorf tubes containing 100 μl of elution buffer (PBS, pH 7.2) and stored at -80°C until assay. Samples visibly contaminated with blood were discarded. GCF samples were collected before the clinical measurements and microbial plaque sampling.

ELISA for free sRANKL

The GCF samples were processed as follows. On the day of the assay, samples were thawn, vortexed for 30 min., centrifuged for 10 min. at 8161,4g and analysed using the ampli-sRANKL commercial enzyme immunoassay kit for quantitative determination of free human sRANKL (Biomedica Medizinprodukte GmbH, Vienna, Austria) according to the manufacturer's instructions. The detection limit of the assay as reported by the manufacturer is 0.02 pmol/l (0.4 pg/ml) and the upper limit is 2 pmol/l (40 pg/ml). Standard curves were constructed from each run, and values from the samples were extrapolated by applying a polynomial fourth order analysis using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA). Values below the detection limit were excluded and levels of free sRANKL per site in picograms were calculated after transformation from picomoles, by accounting for dilution.

Microbiological examination

After GCF sampling, microbial plaque was collected from the same preselected pockets in both groups. Microbial plaque sampling proceeded clinical measurements. Subgingival samples were taken by means of a sterile Gracey curette, placed in 200 μl of TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 7.5) and stored, after treatment with an alkali solution (0.5 M NaOH), at -20°C. A total of 223 samples were processed for four bacterial species, using the ''checkerboard'' DNA–DNA hybridization technique as described in detail by Socransky et al. (1994, 1998). The subgingival species used for the development of digoxigenin-labelled whole genomic probes were *P. gingivalis* (FDC 381), *Tannerella forsythia* (FDC 338), *Treponema denticola* (TD1), and *A. actinomycetemcomitans* (Y4). Cell numbers were quantified by using software for array analysis (TotalLab

TL100 v 2005, NonLinear Dynamics Ltd., Newcastle Upon Tyne, UK).

Statistical analysis

The statistical analysis of the data was carried out with the statistic package SPSS, 14.0 version.

For clinical parameters, indicators of descriptive statistics were used, such as mean and standard deviation for each group, with the patient as the observational unit. Averaged bacterial numbers for each species under investigation and levels of free sRANKL (pg/site) from each subject were consequently averaged for each group as mean and standard error of the mean and compared. In order to identify specific differences between the two groups, the Mann–Whitney test was applied.

The effect of smoking on every investigated parameter was tested by applying univariate analysis of variance and setting as between-subjects factors the groups (periodontitis *versus* healthy), smoking (smokers *versus* non-smokers) and the interaction of both group and smoking. Pairwise comparisons with Bonferroni corrections for each variable under investigation were also performed in order to identify the effect of the group (periodontitis *versus* healthy) and smoking (smokers *versus* non-smokers) and adjust the observed significance level for the fact that multiple comparisons are made.

Correlations between all parameters investigated were sought for by calculating Spearman's r coefficient. Two statistical approaches were applied for this analysis, either with the patient or the site as the statistical unit.

The significance level was set at 0.05 for all tests.

Results

Clinical and demographic data for participants are presented in Table 1.

No differences were observed between the mean age of subjects of the two groups (Table 1, Mann–Whitney test, $p = 0.54$).

One periodontally healthy female subject exhibited high levels of sRANKL in GCF (0.81 and 0.91 pg/site). Upon further questioning, she was recently diagnosed with seronegative spondyloarthritis. Her data were excluded and therefore the periodontally healthy group remained with 38 participants and total plaque and GCF samples, taken into statistical consideration, were 221.

All clinical and microbiological parameters under investigation were statistically different between the two groups (Tables 1 and 2, Mann–Whitney test, $p < 0.05$).

Mean levels of RANKL were statistically significant higher in the periodontitis group compared with controls (Table 2, Mann–Whitney test, $p < 0.05$).

In the present study, no effect of smoking was observed on investigated parameters. Univariate analysis of variance has shown the statistically significant effect of the group (periodontitis *versus* healthy), but no effect of smoking alone or in combination with the group ($p > 0.05$). Pairwise comparisons with Bonferroni adjustment for each investigated parameter have also shown no effect of smoking in the present sample ($p > 0.05$).

When seeking correlations of mean free sRANKL with clinical or microbiological parameters in both groups, a statistically significant correlation was found between the mean levels of free sRANKL and subgingival counts of *T. denticola* (Spearman's r correlation, $p < 0.05$) in the periodontitis group. A plot of correlations between investigated species and levels of sRANKL for the two groups is shown in Fig. 1. No correlations were found between mean levels of free sRANKL and counts of investigated species (Fig. 1) or clinical parameters in the periodontally healthy subjects (Spearman's r correlation, $p > 0.05$).

When correlating the levels of free sRANKL, with the site as the observational unit, with other investigated parameters, a statistically significant correlation was found between the mean levels of free sRANKL and subgingival counts of *T. denticola* and *P. gingivalis* (Spearman's r correlation, $p < 0.05$) in the periodontitis group.

Table 1. Demographic and clinical parameters of participants (mean ± SD)

	Healthy	Periodontitis
No. of participants	38	35
Smokers	24	10
Age	44.45 ± 12.29	47.09 ± 9.14
PD (mm)	2.15 ± 0.33	6.36 ± 0.62*
RE (mm)	0.01 ± 0.08	1.41 ± 0.82*
BI	0.06 ± 0.17	0.92 ± 0.18*

*Significant difference between groups (Mann–Whitney U test, $p < 0.05$) after correction for smoking (univariate analysis of variance, pairwise comparisons with Bonferroni corrections $p > 0.05$).
PD, probing depth; RE, recession; BI, bleeding index.

Discussion

After the hallmark discovery of the involvement of the OPG and receptor for activation of NF-κB ligand (RANKL) in osteoclastogenesis (Simonet et al. 1997, Lacey et al. 1998, Teitelbaum 2000, 2007), a number of studies have demonstrated the importance of these molecules in aetiopathogenesis of bone diseases and the possibility of new therapeutic targets for osteoclast inhibition (Rodan & Martin 2000).

Periodontal diseases are among the most interesting models for bone destruction and therapeutically important due to their wide prevalence. Several recent studies have focused on the relation of these molecules or their ratio with periodontal disease. These studies have investigated both gingival tissues and crevicular fluid. Combined findings from these studies suggest an increase of the levels of RANKL as well as the RANKL/OPG ratio in inflammed periodontal tissues and GCF.

Table 2. Microbial counts (mean ± SEM × 10^5) and levels of free sRANKL

	Healthy (N samples = 76)	Periodontitis (N samples = 145)
Porphyromonas gingivalis	0.82 ± 0.18	3.92 ± 0.33*
Aggregatibacter actinomycetemcomitans	0.92 ± 0.17	2.83 ± 0.41*
Tannerella forsythia	0.85 ± 0.24	3.32 ± 0.31*
Treponema denticola	0.72 ± 0.26	3.41 ± 0.33*
Free sRANKL (pg/site)	0.07 ± 0.17	0.19 ± 0.04*

*Significant difference between groups (Mann–Whitney U test, $p < 0.05$) after correction for smoking (univariate analysis of variance, pairwise comparisons with Bonferroni corrections, $p > 0.05$).
sRANKL, soluble receptor activator of nuclear factor-κb ligand.

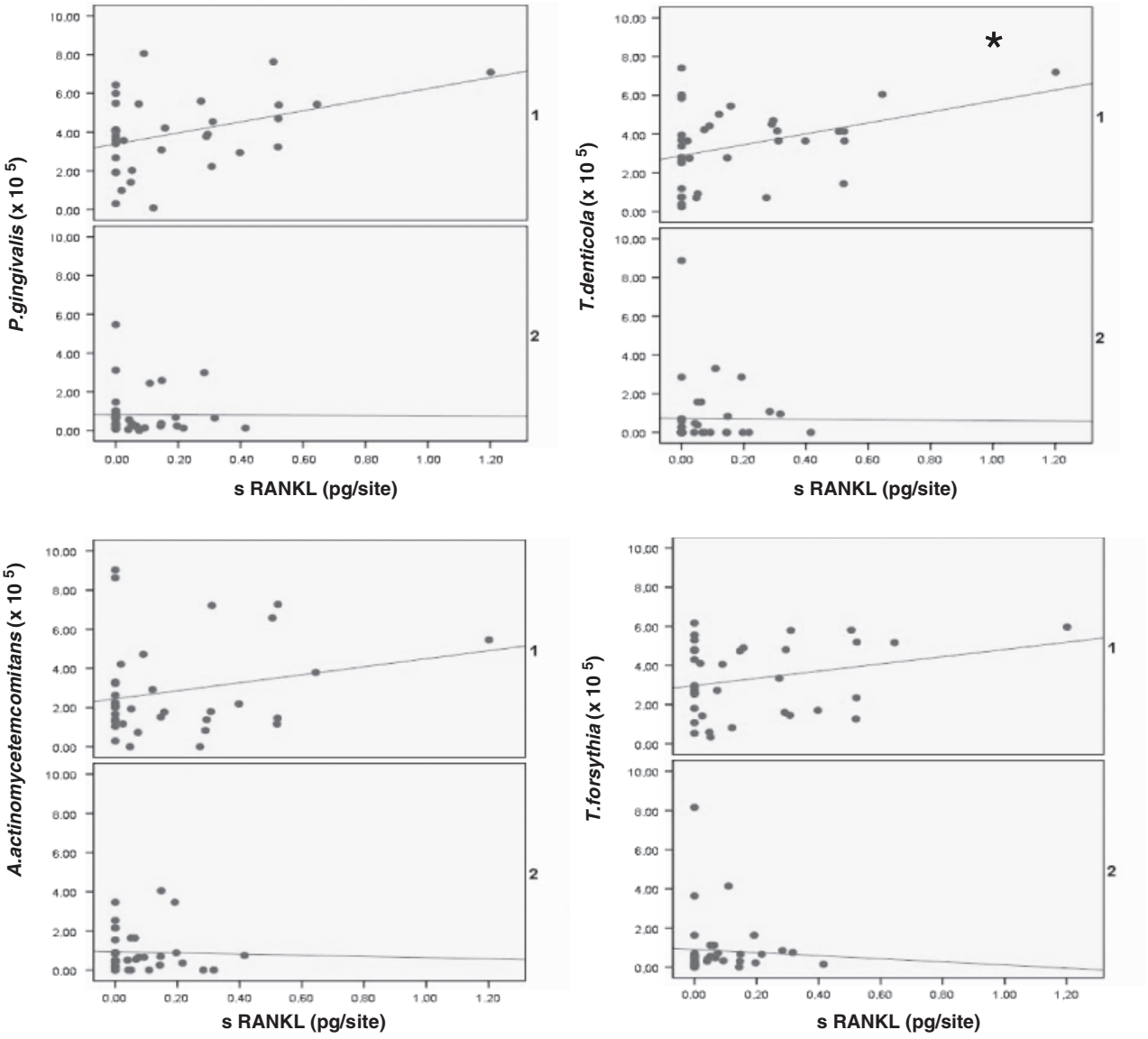

Fig. 1. Correlations between subgingival counts of investigated species and levels of soluble receptor activator of nuclear factor-κb ligand (sRANKL) for the two groups. A significant correlation was observed between *Treponema denticola* and sRANKL in the periodontitis group. No correlation was observed in periodontally healthy individuals. 1, periodontitis subjects. 2, periodontally healthy individuals. *Statistically significant correlation (Spearman's r coefficient, $p < 0.05$).

It is known that RANKL can be found either bound on cells (mRANKL) or as a soluble form (sRANKL). sRANKL, which is considered more potent in initiating osteoclastic function (Taubman et al. 2007), can either be bound to OPG or remain in a free form.

In our present study, we investigated any possible relations between free sRANKL, clinical parameters and important periodontal pathogens in health and disease. Data in the literature referring to levels of free sRANKL have established a relationship between this factor, bone and haematological disorders (Terpos et al. 2006, Angelopoulos et al. 2007, Martini et al. 2007). The biological importance of free soluble RANKL is not well described, although it could be assumed that, as it is unbound to OPG, it can exert biological properties and induce osteoclastogenesis.

Findings from the present study enhance those from previous studies that demonstrated a correlation of levels of sRANKL with periodontal disease, although our data are expressed differently, as absolute amounts (pg/site) and not as concentration in GCF. All investigated parameters differed statistically between the two groups (Tables 1 and 2). Although no statistical correlation was found between the levels of free sRANKL and clinical parameters of periodontal disease, these findings are anticipated if this molecule, when detected free, is indicative of on-going osteoclastogenesis and not of past periodontal destruction like clinical indices. No effect of smoking on investigated parameters and correlations was observed in the present study. However, although our data were corrected for smoking, these findings have to be interpreted with caution, due to the fact that in the present study no matching was performed according to smoking status and the number of samples per group was uneven (Table 1). Current data in the literature, support the suppresive effect of smoking on serum levels of

OPG but not of RANKL in supportive therapy periodontitis patients, therefore implicating another possible mechanism of bone loss in smokers (Lappin et al. 2007).

In the present study, we have sought any possible correlation between free sRANKL and levels of important periodontal pathogens (Fig. 1). A correlation was found between mean levels of free sRANKL and mean counts of *T. denticola*, but not for the remaining pathogens, only in the periodontitis group. We have included the site-based statistical approach as more appropriate for investigating subtle biological correlations and according to this analysis, a strong correlation was observed between the levels of free sRANKL and counts of *T. denticola* and *P. gingivalis* in periodontitis subjects.

These findings corroborate previous ones in the literature which investigated virulence factors and pathways involved in bone destruction by periodontal pathogens. It has already been shown that *P. gingivalis* culture supernatants can induce RANKL and reduce OPG mRNA expression by periodontal ligament cells and gingival fibroblasts (Belibasakis et al. 2007). The same authors reported that the molecule responsible for RANKL induction may be regulated by Arg-gingipains (Belibasakis et al. 2007), while cysteine proteases from the same microorganism have been implicated in RANKL induction from mouse osteoblasts (Okahashi et al. 2004). In addition, *P. gingivalis*-stimulated human microvascular endothelial cells have been shown to produce OPG, which might be partly degraded by the microorganism, thus creating an OPG–RANKL imbalance that can contribute to bone destruction (Kobayashi et al. 2004). According to the above-mentioned findings, the correlation of the levels of free sRANKL with counts of *P. gingivalis* in periodontitis subjects observed in the present study are also indicative of the potential of this microorganism to induce production of RANKL in the periodontal environment. To our knowledge, no data exist in literature concerning mechanisms of *Treponema*–RANKL association, but it is known that *Treponema* spp. possess osteoclastogenic potential (Ellen & Galimanas 2005).

No correlation was observed, in the present study, between free sRANKL and counts of *A. actinomycetemcomitans* in periodontitis patients. It has been shown that *A. actinomycetemcomitans* extracts induce RANKL production from periodontal connective tissue cells, possibly through the cytolethal distending toxin (Cdt) (Belibasakis et al. 2005) and also that *A. actinomycetemcomitans*-responsive B lymphocytes had greater levels of RANKL expression and induced a significantly higher level of osteoclast differentiation in rats (Han et al. 2006). Therefore, a correlation between free sRANKL and levels of *A. actinomycetemcomitans* was anticipated in the present study. Two factors might have accounted for this finding. Small subject sample and, most importantly, the fact that the DNA probe used in the present study targets *A. actinomycetemcomitans* strain Y4, which might not possess the same properties like strains employed in the above-mentioned studies.

Findings from the present study indicate the correlation of levels of important periodontal pathogens with free sRANKL in deep periodontal pockets. We intend to further investigate, in a larger sample, whether sites exhibiting both high subgingival counts and high levels of free sRANKL undergo bone destruction, by investigating for other markers of osteoclastic activity.

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Clinical Relevance

Scientific rationale for the study: Levels and correlation of free sRANKL with clinical and microbiological parameters were investigated in the present study.

Principal findings: Mean levels of free sRANKL were higher in periodontitis subjects and correlated significantly with mean counts of *T. denticola* on the subject level and *P. gingivalis*, *T. denticola* on the site level, but not with clinical parameters.

Practical implications: Free sRANKL is a suitable candidate as a marker of osteoclastogenesis in the periodontal environment, by means of a non-invasive procedure, such as GCF collection.