

# Levels of Cytokine Receptor Activator of Nuclear Factor $\kappa$ B Ligand in Gingival Crevicular Fluid in Untreated Chronic Periodontitis Patients

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**Background:** Receptor activator of nuclear factor  $\kappa$ B ligand (RANK-L) is a cytokine involved in the regulation of osteoclastogenesis in bone remodeling and inflammatory osteolysis. One of the major causes of tooth loss in humans is bone destruction. The aim of our study was to determine the presence of RANK-L in gingival crevicular fluid (GCF) samples from adult patients with untreated chronic periodontitis and in healthy controls. We also identified the RANK-L present in lesions undergoing episodic attachment loss from GCF.

**Methods:** GCF samples were collected from two periodontally affected sites (probing depth  $\geq 5$  mm, attachment loss  $\geq 3$  mm) in 20 patients (N = 40). After monitoring for 4 months, seven patients showed active periodontal disease, and GCF samples were collected from one active and one inactive site (N = 14 samples). The comparison with healthy controls was carried out by collecting GCF samples from 12 healthy volunteers (N = 24 samples). GCF was collected using a paper strip, and enzyme-linked immunosorbent assay (ELISA) was performed to determine the total amount of RANK-L.

**Results:** RANK-L was found in a higher proportion (85%) of samples from patients than from controls (46%). The total amount of RANK-L was significantly higher in patients ( $115.53 \pm 78.18$  picograms [pg]) than in healthy subjects ( $63.08 \pm 55.08$  pg) ( $P = 0.003$ ). Active sites, presumably associated with tissue destruction, had significantly higher levels of RANK-L than their inactive counterparts ( $125.95$  pg versus  $91.80$  pg,  $P = 0.007$ ).

**Conclusion:** GCF total amount of RANK-L is significantly increased in periodontal disease, supporting its role in the alveolar bone loss developed in this disease. *J Periodontol* 2004;75:1586-1591.

## KEY WORDS

Alveolar bone loss/diagnosis; gingival crevicular fluid/chemistry; receptor activator, nuclear factor kappa B/analysis.

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth caused by groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both.<sup>1</sup> Destruction of the osseous support of the dentition is a hallmark of chronic periodontitis.<sup>2</sup>

RANK-L (ligand to receptor activator of NF $\kappa$ B), also known as osteoprotegerin-ligand (OPG-L); tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE); osteoclast differentiation factor (ODF); and tumor necrosis factor superfamily (TNFSF)11 and its cell surface receptor RANK (TNFRSF11A) are key regulators of bone remodeling and essential for the development and activation of osteoclasts.<sup>3</sup> RANK-L, a member of the TNF family, was simultaneously cloned by four independent groups and encodes a molecule of 316 amino acids (38 kDa).<sup>4-7</sup> Three RANK-L subunits assemble to form the functional trimeric molecule which appears initially as a membrane-anchored molecule that can be released as a soluble molecule following proteolytic cleavage by the metalloprotease-disintegrin TNF $\alpha$  convertase (TACE).<sup>8</sup> Using culture systems in vitro, RANK-L both activates mature osteoclasts and mediates osteoclastogenesis in the presence of colony stimulating factor (CSF)-1.<sup>5,7</sup> RANK-L is produced by activated CD4+ and CD8+ T cells.<sup>6,9</sup>

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Tissues such as lymph nodes, spleen, immature CD4-CD8- thymocytes, and intestinal lymphoid patches also expressed RANK-L.<sup>4,5</sup>

RANK-L contributes to alveolar bone resorption and tooth loss; in an animal model that mimics human periodontal disease, the alveolar bone resorption around the teeth can be inhibited with osteoprotegerin.<sup>10</sup> RANK-L inhibition could, therefore, offer a rational approach to the treatment of periodontal disease. We measured the total amount and concentration of RANK-L in GCF samples from patients with untreated chronic periodontitis and from active sites where clear bone tissue destruction occurred and compared these with samples from healthy controls and inactive sites.

## MATERIALS AND METHODS

The 20 study patients were selected from those attending the Center of Diagnosis and Treatment of the Northern Metropolitan Health Services. Criteria included a minimum of 14 natural teeth, excluding third molars, with at least 10 posterior teeth (for the collection of gingival crevicular fluid, GCF). Chronic periodontitis was defined as having at least five teeth with probing depth  $\geq 5$  mm, attachment loss  $\geq 3$  mm, and extensive radiographically determined bone loss. Patients had not received any periodontal treatment when they entered the study; they did not have any systemic illness and had not received antibiotic or non-steroidal anti-inflammatory therapy in the 6 months prior to the study.

Twelve controls were selected from periodontally healthy volunteers who were either patients presenting for other dental treatment or University staff members with no evidence of periodontal disease (i.e., no clinical attachment loss, as determined by radiographs, or probing depth).

The study protocol was explained to all participants, and Institutional Review Board-approved informed consent forms were signed. Both controls and patients received a superficial prophylaxis at baseline to remove gross calculus.

### Clinical Measurements

The following clinical parameters were evaluated on all study teeth at baseline: probing depth (PD), clinical attachment loss (CAL) using an automated disk probe,<sup>§</sup> and dichotomous measurements of supragingival plaque (PI) and bleeding on probing (BOP) to the base of the crevice. Six sites were examined on each tooth: mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual. One calibrated examiner took all measurements and recorded the results.

Attachment level and probing depth measurements were taken again 7 days after the study began. Patients were monitored for the next 4 months, with clinical measurements repeated every 2 months. Patients were

entered into the treatment phase when two or more active sites, defined as those exhibiting  $\geq 2$  mm of attachment loss from baseline using the tolerance method,<sup>11</sup> were detected. Four patients exhibited disease activity during the first 2 months of the study and three during the following 2 months. GCF was collected from both active and inactive sites prior to therapy, which included scaling, root planing, and oral hygiene instruction.

### Collection of Gingival Crevicular Fluid

After isolating the tooth with a cotton roll, supragingival plaque was removed with a curet without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. GCF was collected using paper strips which were placed into the sulci/pocket until mild resistance was felt and left in place for 30 seconds. Strips contaminated by saliva or blood were excluded. Following GCF collection, the volume of the sample on the paper strips was measured<sup>||</sup> and converted to an actual volume ( $\mu$ l) by reference to the standard curve. After GCF collection, strips were placed in Eppendorf vials containing 100  $\mu$ l of phosphate buffered saline with 0.05% Tween-20. GCF was extracted by centrifugation<sup>¶</sup> at 10,000 g for 5 minutes at 4°C, the elution procedure was repeated twice,<sup>12</sup> and samples were stored at -20°C until further analysis.

Forty GCF samples were obtained from two periodontally affected sites (probing depth  $\geq 5$  mm, attachment loss  $\geq 3$  mm) from 20 patients with periodontal disease. Twenty-four GCF samples were obtained from two sites in the 12 healthy controls at the mesio-buccal gingival sulci at teeth 16 and 26.

GCF samples were taken again from the seven patients demonstrating disease activity as soon as that activity was detected, and cytokine levels from active and inactive sites were compared.

### Quantification of Cytokine RANK-L

Aliquots of each GCF sample were assayed by ELISA<sup>#</sup> to determine the level of RANK-L, according to the manufacturer's recommendations. Briefly, 100  $\mu$ l of standards and GCF samples were added to the respective wells in duplicate; 100  $\mu$ l detection antibody was added to all wells, except negative control, mixed gently, strips covered with plastic film, and incubated 16 to 24 hours at 4°C. Plates were washed five times and incubated with 200  $\mu$ l conjugate for 50 minutes at room temperature (18° to 26°C). The plates were then washed another five times and 200  $\mu$ l of substrate was

§ Florida Probe Corporation, Gainesville, FL.

|| Periotron 8000, ProFlow, Amityville, NY.

¶ Hermle Labortech Nik., Z-233 MK-2, Wehingen, Germany.

# American Laboratory Products Company, ALPCO Diagnostics, Windham, NH.

added and incubated for 30 minutes at room temperature (18° to 26°C) in the dark. The reaction was stopped by the addition of 50 µl stop solution, and color was measured in an automated microplate spectrophotometer.\*\* The amount of RANK-L was determined in picograms (pg). Cytokine levels in the GCF were calculated with a standard curve (3.9 to 500 pg) obtained with recombinant cytokine. Values lower than the detection limit (3.9 pg) in our assay were considered undetectable. Cytokine concentration (pg/µl) was calculated from the volume of GCF estimated from the calibration unit reading according to the following formula:

$$\text{cytokine conc (pg/}\mu\text{l)} = \frac{\text{total cytokine (pg)}}{\text{volume GCF (}\mu\text{l)}}$$

### Data Analysis

Clinical parameters as well as the total amount and concentration of cytokine RANK-L in healthy and diseased sites were expressed as subject means  $\pm$  standard deviation. The unpaired Student *t* test and Mann-Whitney *U* test were used to analyze differences in clinical and biochemical parameters between the patient and control groups. The significance ( $P < 0.05$ ) of differences was assessed using the Tukey test.

### RESULTS

The clinical characteristics of patients and controls are given in Table 1. There were no statistically significant age or gender differences between the groups.

Variations in total amounts and concentrations of cytokine RANK-L in the periodontitis and control groups are shown in Table 2. In the patient group, mean values of  $115.53 \pm 78.18$  pg and an estimated concentration of  $116.99 \pm 83.04$  pg/µl were obtained. The corresponding values for controls were  $63.08 \pm 55.08$  pg and  $188.07 \pm 103.40$  pg/µl. Significantly lower GCF volume was obtained from control subjects as compared with periodontitis patients ( $P = 0.0001$ ).

In the patient group, 85% of the sites had detectable levels of RANK-L compared with 46% of the controls. Total amount of cytokine RANK-L was significantly higher in the periodontitis group than in the control group ( $P = 0.003$ ).

Table 3 shows that active sites, identified by an increase in CAL and associated with tissue destruction, contained higher levels of RANK-L than inactive sites, measured as total amount or as concentration ( $125.95$  pg versus  $91.80$  pg,  $P$  value = 0.007;  $131.17$  pg/µl versus  $108.80$  pg/µl,  $P > 0.05$ , respectively).

**Table 1.**

### Clinical Characteristics of Patient and Control Groups

	Patients (N=20)	Controls (N=12)
Age (mean $\pm$ SD)	46.60 $\pm$ 8.33	40.50 $\pm$ 8.42
Range	35-65	35-57
N females	12	9
PD (mm, mean $\pm$ SD)	3.48 $\pm$ 0.56*	1.93 $\pm$ 0.52*
CAL (mm, mean $\pm$ SD)	3.57 $\pm$ 0.48†	0.165 $\pm$ 0.33†
% sites with plaque	74.06‡	35.60‡
% sites with BOP	57.93§	1.58§

\* Mean probing depth in periodontitis versus control groups:  $P = 0.0001$ .

† Mean attachment level in periodontitis versus control groups:  $P = 0.0001$ .

‡ Percentage of sites with plaque in periodontitis versus control groups:  $P = 0.001$ .

§ Percentage of sites with bleeding on probing in periodontitis versus control groups:  $P = 0.0001$ .

**Table 2.**

### Cytokine RANK-L in GCF From Patient and Control Groups

	Patients (N=20; 40 samples)		Controls (N=12; 24 samples)	
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
RANK-L (pg)*	115.53 $\pm$ 78.18‡	13.20-249.00	63.08 $\pm$ 55.08‡	12.33-213.70
RANK-L (pg/µl)†	116.99 $\pm$ 83.04	14.04-283.90	188.07 $\pm$ 103.40	72.52-381.60
GCF (µl)	0.94 $\pm$ 0.21§	0.65-1.41	0.22 $\pm$ 0.13§	0.10-0.56

\* Total amount of RANK-L.

† Concentration of RANK-L.

‡ Total amount of RANK-L in periodontitis versus control groups:  $P = 0.003$ .

§ Gingival crevicular fluid volume in periodontitis versus control groups:  $P = 0.0001$ .

**Table 3.**

### Cytokine RANK-L in GCF in Active and Inactive Sites (mean $\pm$ SD)

Sites	Total Amount (pg)	Concentration (pg/µl)	GCF (µl)
Active (N=14)	125.95 $\pm$ 22.2*	131.17 $\pm$ 35.1	0.99 $\pm$ 0.15
Inactive (N=14)	91.80 $\pm$ 10.7*	108.80 $\pm$ 18.9	0.86 $\pm$ 0.15

\* Total amount of RANK-L in active versus inactive sites:  $P = 0.007$ .

### DISCUSSION

Our data demonstrate that cytokine RANK-L is present in the GCF of adult patients with chronic periodontitis

\*\* Bio-Tek Instruments, Inc., Highland Park, IL.

in a significantly higher amount than in healthy subjects, which might correlate this osteoclastogenic protein with alveolar bone loss and teeth loss occurring. RANK-L is a recently discovered transmembrane molecule of the tumor necrosis factor (TNF) ligand superfamily that is highly expressed in lymphoid tissues and trabecular bone, particularly in areas associated with active bone remodeling or inflammatory osteolysis.<sup>4-7</sup> RANK-L is the essential and final common signal required both in vitro and in vivo for full osteoclast differentiation from multipotential hematopoietic precursor cells into mature multinucleated bone-resorptive osteoclasts in the presence of the permissive factor macrophage colony-stimulating factor (M-CSF).<sup>7</sup>

RANK-L expressed on the surface of osteoblasts or bone marrow stromal cells interacts with a cell surface receptor, RANK, present on pre-osteoclasts (induced by M-CSF) and mature osteoclasts to stimulate their fusion, development, bone resorption, and cell survival.<sup>13</sup> Three variants of RANK-L exist: 1) a cell membrane-bound variant produced by the majority of tissues;<sup>5</sup> 2) a secondary soluble ectodomain form generated from the cellular form by post-translational processing by TNF- $\alpha$ -converting enzyme-like protease (TACE) that is limited to TACE-producing tissues and cell types;<sup>8</sup> and 3) a primary soluble (secreted) form that has been described in activated T lymphocytes.<sup>9</sup> RANK-L is produced in several tissues and cell types and is most abundant in bone and lymphoid tissues which are rich in T cells.<sup>4,14</sup> The infiltrate present in periodontal disease contains mononuclear cells, mainly transmigrated mononuclear phagocytes and lymphocytes, whereas T lymphocytes predominate in the chronic lesion.<sup>15</sup> Therefore, the high levels of RANK-L found in GCF samples from patients suggest its role in the osseous tissue destruction and remodeling and in the pathogenesis of periodontal disease.

Our findings demonstrate that total amount of RANK-L is significantly higher in active versus inactive sites ( $P < 0.05$ ), considered as a marker of activity progression. Disease activity is generally accepted to be loss of soft or hard tissue attachment to the tooth; a change in clinical attachment level may represent a true change in the attachment level.<sup>16</sup> Episodic periodontal attachment loss may be associated with variations in the supracrestal inflammatory cell populations where significantly more mast cells, monocytes/macrophages, and plasma cells are present in activity sites compared with inactive sites.<sup>17</sup> The presence of RANK-L in GCF of sites with episodic loss of connective tissues implies a potential role in the mechanisms of tissue destruction associated with periodontitis. There are two molecules considered essential to support osteoclastogenesis: macrophage colony-stimulating factor and RANK-L. The process of bone resorption is initiated with a re-

sorptive stimulus.<sup>18</sup> These stimulators typically affect bone resorption through the activation of M-CSF or RANK-L.<sup>19</sup>

The observation that the RANK-L total amount increased with CAL loss is clear; however, the explanation is not as obvious. Th1 and Th2 cells define two forms of the specific CD4+Th cell-mediated immune response based on their differential cytokine secretion.<sup>20</sup> Th1 or Th2 cell cytokines have been detected in periodontal diseases.<sup>21,22</sup> One possibility may be that increasing Th1 expression is required before progression to periodontal destruction, while certain Th2 anti-inflammatory cytokines are involved in protection from and repair of tissue loss.<sup>23,24</sup> Quantitative polymerase chain reaction analysis clearly demonstrates a predominantly mixed Th1 and Th2 expression profile associated with pathogen-specific cell-mediated immunity via RANK-L-mediated alveolar bone destruction in vivo.<sup>25</sup> Thus, a recent study<sup>26</sup> suggests the existence of dynamic programs in the differentiation/activation process of human Th1 and Th2 cells, and the relationship of these findings with disease progression remains to be established.

Gingival crevicular fluid contains a rich array of cellular and biochemical mediators that reflect the metabolic status of periodontal tissues.<sup>27</sup> As GCF transverses the inflamed tissue, it carries molecules involved in the destructive process, and therefore offers great potential as a source for factors that may be associated with osteoclastic activity with the potential of being detected in advance of irreversible bone loss. GCF is close to the site of destruction and thus may provide more information than markers in the serum or urine.<sup>28</sup> Several materials have been analyzed in GCF, including plasma proteins, enzymes with collagenolytic activity, other microbial and host cell enzymes, and inflammatory mediators, in attempts to identify factors to facilitate the diagnosis of active periodontal disease.<sup>29-31</sup> In the present study, we demonstrated that the total amounts of RANK-L present in the GCF of periodontitis patients were higher than those in healthy controls. There are few studies relating RANK-L and its potential role in periodontal tissue. Recently, RANK and RANK-L have been found in dental tissues and cells in human deciduous teeth,<sup>32</sup> and RANK-L also has been associated with alveolar bone tissue destruction during periodontal infection in an animal model study.<sup>10</sup> Furthermore, microbial stimulation with *A. actinomycetemcomitans* induced RANK-L expression on the surface of CD4+ cells and in vivo inhibition of RANK-L function; using the decoy receptor osteoprotegerin (OPG) diminished alveolar bone destruction and reduced the periodontal osteoclasts after microbial challenge.<sup>10</sup> These results indicate that the alveolar bone destruction observed in periodontitis is due, at least in part, to the action of osteoclasts and is mediated by RANK-L.



Genetic and functional experiments by different groups indicate that the balance between RANK-L-RANK signaling and the levels of biologically active OPG regulate development and activation of osteoclasts and bone metabolism.<sup>3</sup> Semiquantitative image analysis has demonstrated that significantly higher levels of RANK-L protein were expressed in periodontally diseased tissue.<sup>33</sup> Conversely, OPG protein was significantly lower in periodontal tissues than that in the healthy group.<sup>33</sup> Clearly, the level of OPG mRNA in both advanced and moderate periodontitis was lower than that in the healthy group.<sup>34</sup> It appears that the ratio of RANK-L to OPG mRNA in periodontitis has increased.<sup>34</sup> It has recently been shown in vivo that the ratio of the concentration of RANK-L to that of OPG in the GCF was significantly higher for periodontal disease patients than for healthy subjects.<sup>35</sup> Thus, OPG and/or modulation of RANK-L-RANK function via small molecules are promising avenues to prevent periodontal diseases.

Together with its receptor RANK and the decoy receptor OPG, RANK-L is a key regulator for osteoclast development and the activation of mature osteoclasts.<sup>3</sup> The identification of the RANK-L-secreting cells present in the inflammatory periodontal tissues and its regulation during the active/inactive periods are preliminary approaches which will help to determine the function and relevance of this cytokine during the pathogenesis and progression of the periodontal disease.

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