ELISA Evaluation of RANKL Levels in Gingival Fluid in Patients with Periodontitis and Occlusal Trauma

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The aim of this study was to evaluate the differences in RANKL levels in crevicular fluid (GCF) in patients with chronic periodontitis, with or without chronic occlusal trauma. The study group consisted of 40 patients from whom 72 samples of crevicular fluid were collected. RANKL levels were analyzed by ELISA. We noticed significantly higher differences in RANKL levels for the study group (occlusive trauma patients) than for systemic healthy patients (p = 8.008). Research has shown that secondary occlusal trauma associated with periodontal disease is characterized by significantly higher RANKL levels in patients with chronic occlusal trauma. This partially clarifies the molecular mechanisms that underlie more severe tissue destruction in patients with occlusal trauma.

Keywords: RANKL, chronic periodontitis, occlusal trauma

Periodontitis is a complex, multifactorial process, affected by bacterial plaque components and host defence mechanisms [1]. Inflammation of the periodontium can lead to the destruction of the ligament and the alveolar bone. Although the host response is the body's way of fighting bacterial infection associated with periodontitis, this response can lead to significantly increased levels of inflammatory cytokines such as IL-1, IL-6 and TNF-α in periodontal tissues [2]. As a result of an exaggerated host response, these inflammatory cytokines lead to osteoclast activation and stimulate bone resorption in periodontium [3, 4].

The periodontium tries to adapt to the forces exerted on the crown. This ability to adapt varies from one person to another and even to the same person at different times. The effect of occlusal forces on periodontium is influenced by the size, direction, duration and frequency of the forces. If the intensity of the occlusal forces is increased, the periodontium responds with a widening of the desmodontal space, an increase in the number and width of the periodontal ligament fibres and an increase in the density of the alveolar bone [5,6].

Changing the direction of occlusive forces causes a reorientation of strains and deformations in periodontium. The main fibres of the periodontal ligaments are arranged so as to best adapt to the occlusal forces in the long axis of the tooth. Side (horizontal) and rotation forces are more likely to injure periodontitis. The response of the alveolar bone is also affected by the duration and frequency of occlusal forces [7]. Constant bone pressure is more damaging than intermittent forces. Frequent application to intermittent force is more detrimental to periodontal tissues.

In the literature, tissue damage associated with occlusion trauma is often classified as primary and secondary. The primary form includes a tissue reaction that is caused around a tooth with a periodontium of normal height, while the secondary form is related to situations where the occlusal forces cause damage in periodontium of low height.

The distinction between a primary form and a secondary form of primary and secondary occlusal trauma does not serve any significant purpose, since the changes occurring in periodontitis as a consequence of occlusion trauma are similar and independent of the height of the target tissue, i.e. the periodontium. It is, however, important to understand that the symptoms of occlusal trauma can develop only in situations where the magnitude of the occlusion load is so great that the periodontium of the exposed tooth cannot properly resist nor distribute the resulting force with a position unaltered and stability of the tooth involved. This means that in cases of severely reduced periodontal height even relatively small forces can cause traumatic lesions or adaptive changes of periodontal tissues [8-10].

In teeth with bacterial periodontal disease, occlusal trauma may, under certain conditions, increase the disease progression rate, that is, act as a cofactor in the destructive process. From the clinical point of view, this aspect strengthens the importance that the aetiological therapy has in the parodontopathic patient approach [11]. This treatment will stop the destruction of periodontal tissues, even if the occlusal trauma persists. Treatment only for trauma (occlusal adjustment, selective grinding) can reduce the mobility of the traumatized teeth and even favour some bone regeneration but will not stop the loss of periodontal support caused by the bacterial plaque.

Pro-inflammatory cytokines, such as interleukins (IL-1, IL-6, TNF-α), have elevated levels in tissues affected by periodontal disease [12]. An interaction between these cytokines and the activated B lymphocyte ligand activated receptor (RANKL) / NF-KB (RANK) / Osteoprotegrin (OPG) receptor activator receptor activator system has been described. High amounts of IL-1 and TNFα cause elevated levels of RANKL, stimulating osteoclastic genesis [13]. RANK activation by RANKL (a member of the TNF family) is unhealthy for osteoclast formation, differentiation and activity. RANK is expressed on the surface of osteoclast precursors and RANK activation initiates differentiation and maturation. This activation can be counteracted by soluble osteoprotegrin. OPG acts as a receptor eludator, linking to RANKL and inhibiting the interaction between RANKL and RANK [14].

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OPG is expressed at the level of gingival fibroblasts, periodontal ligament cells or epithelial cells. In periodontitis cases, the RANKL / OPG ratio is favorable for bone resorption. Elevated levels of RANKL are an indicator for a site with active disease [15]. Results of studies in mice indicate that the level of RANKL is correlated with the level of pro-inflammatory cytokines (IL-1β, TNFα, interferon-gamma) during periods of active bone loss [16].

The aim of this study was to evaluate the differences in RANKL levels in crevicular fluid (GCF) in patients with chronic chronic periodontitis, with or without chronic occlusal trauma.

Experimental part

The study group consisted of 40 patients, investigated at the Clinic of Periodontology of the Faculty of Dental Medicine of UMF Grigore T. Popa, Iasi.

In total, 72 samples were collected from disease sites. Subjects received instructions not to eat, drink or brush their teeth for 1h before sampling the GCF. Prior to sampling, the tooth was isolated with cotton rolls, the supragingival plaque was carefully removed and the site was gently dry with the air spray. A sterile paper cone was inserted into each selected periodontal bag, left in place for 30 s and then immediately inserted into sterile Eppendorf tubes which were stored at 20°C until further analysis. In case of visible contamination with blood, the paper cone has been removed and a new site has been selected.

For the determination of RANKL, the paper cones were thawed, cut at 1 cm in length and thawed with 50 μL 1X buffer [13 mM Na2HPO4, 7 mM NaHPO4, 100 mM NaCl (*p*H 7.0)] at 4°C; afterwards, the paper cones were centrifuged at 13000 x g for 10 min at 4°C.

RANKL concentrations were determined using the ELISA assay (MyBioSource, San Diego, CA, USA) in a Luminex 200 ™ analyzer (Luminex Corporation, Austin, TX, USA). Measurements were performed according to manufacturer's instructions and standards and the samples were measured in duplicate. The detectable minimum concentrations were 0.4μg / mL for RANKL. Samples with concentrations below the detection limit were marked with 0.

The statistical analysis was performed using SPSS 20.0 (IBM, Armonk, NY, USA) and $p < 0.05$ was considered to indicate a statistically significant difference. Continuous variables with a normal distribution are expressed as mean ± standard deviations of the mean and were analyzed using parametric tests (T or P independent test). Because the RANKL levels were not normally distributed, data is expressed as median (minimum and maximum) and analyzed using non-parametric tests (Mann Whitney U test for unrelated samples or Wilcoxon test for related samples). Fisher and McNemar tests were used to compare frequencies between affiliated or independent samples.

Results and discussions

We examined a total of 40 patients with chronic periodontitis, divided into two groups: the study group - patients with occlusal trauma (n = 20) and the control group - patients without occlusal trauma (n = 20).

We noticed significantly higher differences in RANKL levels for the study group (occlusive trauma patients) than for systemic healthy patients ($p < 0.05$) (table 1).

Despite advances in research methodology and laboratory tests, in order to identify the factors associated with chronic periodontal disease, it is still unclear how to predict the progression of periodontal disease [17-19]. Periodontitis was clinically characterized by episodes of destruction followed by rest periods and stability. The elusive nature of the disease is complicated by the fact that different teeth as well as different sites of the same tooth can show different degrees of severity of the disease.

The occurrence and evolution of periodontitis is related to multiple factors. The disease is of polymicrobial etiology, because different types of bacteria are the initiators of the inflammatory process. Non-specific immunity is the host's first line of defence [20-21]. It operates through TLRs, which recognize the conserved molecular patterns on pathogenic bacteria. A secreted cytokine network leads to lymphocyte activation, but the progression of periodontal lesions is caused by the deregulation of released molecules [22-23]. The specific cellular activity of these secreted factors is implicated in bone regulation, and their imbalance leads to modified periodontal bone remodelling. Osteoclastic activity is intensified, with poor bone turnover, leading to decreased alveolar bone level [24].

Clinical measures, such as probing depth (PD), clinical attachment (CAL), or bleeding on probing (BOP) have limitations to provide a real-time assessment of disease status. Furthermore, these clinical measures are inaccurate predictors of evolution [25]. An ideal diagnostic tool would not only identify the presence and severity of the disease, but could also predict the subsequent clinical development of the infection.

In our study, we demonstrated that secondary occlusal trauma in periodontal subjects is characterized by significantly higher RANKL levels in gingival crevicular fluid. This partially clarifies the molecular mechanisms that underlie more severe tissue destruction in patients with occlusal trauma.

The network of cytokine interactions that regulate bone loss is complex, involving synergic effects, sequential pathways and redundant systems. Thus, many of these cytokines not only regulate osteoblasts and osteoclasts directly but also alter the response of immune cells including B lymphocytes, T lymphocytes, and dendritic cells. The RANK / RANKL / OPG system is essential in this process; however, additional co-stimulatory pathways may alter the net response.

RANKL is overregulated in cytokine osteoblasts such as IL-1β, IL-11, TNF-α, and PGE2 as well as hormones such as dexamethasone, 1,25 (OH) 2D3, and PTH, and inhibited by TGF- β. The biological actions of RANKL are dose-dependent and OPG-inhibited. RANKL increases the differentiation and activity of osteoclasts and inhibits their apoptosis. Importantly, previous observations suggest that soluble and membrane-bound forms of RANKL require cell-to-cell contact in some models and a soluble form in other models using conditioned media from osteoblasts or stromal cells. The soluble form is cleaved from the

RANKL (pg/site)	Study group (occlusal trauma)	Control group	*p* Value
	0.79 (0.00-146.75)	0.15 (0.00-75.20)	0.008
GCF=gingival crevicular fluid; $p<0.05$ indicates statistical significance. The results are expressed as median (minimum-maximum interval).			

Table 1
RANKL LEVELS IN GCF SAMPLES

metalloproteinase membrane; however, it is still unclear whether the relationship between these two forms or the regulation of this process is biologically significant [26].

RANKL injection in vivo promotes osteoclast formation in the bone, but not in other tissues, suggesting a requirement for additional co-stimulators in the bone microenvironment, such as matrix proteins or adhesion molecules.

RANK, the receptor for OPG and RANKL, is expressed on osteoclasts, T lymphocytes and stromal cells and has 40% homology with CD40. On stromal cells, RANK expression is stimulated by CD40 ligand, and on T lymphocytes it is stimulated by IL-4 and TGF-β.

Activation of RANKL by RANKL triggers a complex intracellular signalling cascade in osteoclasts. Antibodies that bind to the extracellular domain of receptor-specific binding determine increased osteoclastogenesis and mimic the effect of RANKL, a process that is blocked by Fab fragments of the polyclonal antibody or soluble RANKL [27-28].

Immunofluorescence and confocal microscopy studies in periodontal patients have shown abundant expression of RANKL on LyT CD3 + and LyB CD20 +, less or no expression in CD14 + monocytes [29]. More than 50% of LyT and 90% of LyB expressed RANKL in the affected gingival tissue, while the percentage for healthy tissues was <20%. Therefore, LyT and LyB present in inflammatory sites play an essential role in triggering bone resorption through RANKL-dependent mechanisms. Lymphocytes, particularly LyT subsets, are involved in RANKL-mediated bone resorption stimulation or inhibition; T helper type 1 cells will favor bone resorption, while LyT regulators will suppress LyTh1-mediated bone loss.

Recent studies pointed out a link between the alloys used for manufacturing fixed dental restoration and the periodontal status.[30]

Conclusions

In the context of the presence of chronic periodontitis, even in a superficial form, occlusal trauma is accompanied by much higher levels of RANKL in crevicular fluid, with more severe and faster tissue destruction.

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