Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) Levels in Peri-Implant Crevicular Fluid

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ABSTRACT

Background: Receptor activator of nuclear factor kappa B ligand (RANKL) is one of the key cytokines in the induction of osteoclastogenesis both in vitro and in vivo.Several reports indicated the presence of sRANKL in gingival crevicular fluid of patients with periodontal diseases. **Objective:** To determine the presence of RANKL in peri-implant crevicular fluid samples of implants with peri-implantitis, peri-implant mucositis and healthy controls. **Methods:** In this study, 40 implants were categorized as clinically healthy, peri-implant mucositis and peri-implantitis according to the clinical and radiographic findings. Filter paper strips were used to collect peri-implant crevicular fluid for 30 seconds in the base of the crevice/pocket.Peri-implant crevicular fluid (PICF) samples were obtained from buccal and lingual aspects of implants. Plaque index, probing depth, gingival index and bleeding on probing were recorded at six sites per implant. Enzyme-linked immunosorbent assay (ELISA) was performed to determine the PICF levels of sRANKL. **Results:** There were no statistically significant differences in sRANKL concentration between healthy group, peri-implant mucositis and periimplantitis ($p=0.12$). There were also no statistical correlation between the concentration of sRANKL and probing pocket depth ($R=0.051$, $p=0.65$), or any of the other clinical regarding ($p>0.05$). No differences between the mean sRANKL concentration in the buccal and lingual sites were found ($p=0.693$). **Conclusion:** Our results may suggest that peri-implant crevicular fluid analysis of sRANKL in conjunction with some other osteoclastogenic mediators could be further investigated in well-designed prospective longitudinal studies on a larger-scale sample size in the evaluation of dental implants.

Keywords: Crevicular Fluid, ELISA, RANKL

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INTRODUCTION

Retrospective reports have established osseointegration as a predictable form of therapy for the edentulous patients as well as partially dentate patients (1-4). Osseointegrated dental implants,by definition are in direct contact with the alveolar bone without intervening soft tissue (5). However predictability can become tainted when the criterion for success becomes confused with implant survival (6).

Studies in man and experiments in animals have documented that formation of a biofilm on the implant surface initiates a host response that involves the establishment of an inflammatory lesion in the peri-implant mucosa (peri-implant mucositis). This lesion is initially located in the connective tissue immediately lateral to the barrier epithelium and is, in many respects, similar to that which develops in the gingiva when plaque forms on adjacent root surfaces. In the continued presence of a submarginal biofilm, the lesion in the marginal mucosa around implants may occasionally spread in an apical direction to involve the hard tissue, compromise osseointegration, induce varying degrees of marginal bone loss (peri-implantitis), and eventually cause the loss of implant (7). Identification of the elusive osteoclast differentiating factor, known as receptor activator of nuclear factor-kappa B ligand (RANKL), led to many studies confirming its role as an essential factor in human osteoclastogenesis (8). RANKL is expressed predominantly as a membrane-bound ligand on osteoblasts, fibroblasts and activated T and B cells and its osteoclastogenic action can be blocked by its soluble decoy receptor osteoprotegerin (OPG) (9,10). Together with Macrophage-colony stimulating factor (M-CSF), RANKL is a key cytokine in the induction of osteoclastogenesis both in vitro and in vivo (11). It binds directly to RANK on the surface of preosteoclasts and osteoclasts, stimulating both the differentiation of osteoclast progenitors and the activity of mature osteoclasts (12,13,14). RANKL is present both as a cell membrane bound variant (mRANKL) and as a primary soluble or secreted form (sRANKL). The cellular distribution of RANKL was described in diseases of chronic inflammation and progressive bone loss such as rheumatoid arthritis (8), periodontitis (15) and loosening of orthopedic implants (16). Recent clinical studies have confirmed that RANKL can be detected in human gingival crevicular fluid (17). sRANKL was determined in gingival crevicular fluid from patients with periodontal disease, which resulted in increased total amount of RANKL in GCF (18). Peri-implant crevicular fluid (PICF) like gingival crevicular fluid can be obtained under defined conditions by the insertion of filter strips into the space between the implant and the surrounding soft tissue (19). PICF levels of sRANKL have also been described in some recent clinical studies. sRANKL was determined in peri-implant crevicular fluid of 19 implants with peri-implantitis by Monov et al. They reported no relationship between the total amount of RANKL and clinical parameters (20). Another study reported that the PICF level of sRANKL does not show a significant correlation with clinical parameters in 86 implants (21). A recent study of Duarte et al. has shown that anti-infective treatment of peri-implant mucositis and peri-implantitis has resulted in higher OPG/RANKL ratio in healthy implants than in untreated peri-implantitis (22). Understanding the pathophysiologic process of any disease is a prerequisite of the treatment strategies. Osteoclasts regulating factors are targets of some newly developed drugs that are used to prevent and treat peri-implantitis in experimental models. In addition, understanding how different biomaterials influence the expression of key osteoclastogenic factors may allow us to select materials for implantation that will last for longer periods. The aim of this analytical study was to

compare the levels of sRANKL in PICF of three groups of endosseous dental implants: 1-Healthy implants 2-Implants with peri-implant mucositis 3-Implants with peri-implantitis, and to compare these findngs with clinical parameters.

MATERIALS AND METHODS

Study Population and Clinical Examination. The 40 study implants were selected from patients attending the maintenance programme after placement of dental implants at the Department of Periodontics, Dental Branch, Islamic Azad University, Tehran, Iran. The implants had been placed and loaded at least 10 and 6 months, respectively, before the examination. Written and informed consent was obtained from each subject before enrolment 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. All the patients were non-smokers.

In addition, no patient had received any peri-implant 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 the Dental Branch, Islamic Azad University, Tehran, Iran. All subjects had a clinical examination including the measurement of probing depth (PD), bleeding on probing (BOP), plaque Index (PI) and gingival index (GI). All data of the study were recorded by the same examiner and the examiner was trained in standardized procedures for making the required measurements and was also calibrated prior to the study. Before PICF sampling, the plaque index (Silness & Loe 1964) (23) was recorded. Then the implant 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., NY, USA) was gently inserted into the crevice of implants and left in place for 30 seconds. Mechanical irritation was avoided and strips contaminated with blood were discarded. Samples were obtained from buccal and lingual aspects of implants. After PICF collection, strips were placed in vials, lyophilized and stored at -70^0 C before laboratory analysis.

Then probing depth was recorded for all implants at six sites around each implant (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual & distolingual) using Hawe perio probe 1370.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. Dichotomous bleeding on probing (BOP) scores was recorded as present or absent per implant according to Ainamo and Bay (24). The severity of peri-imlant mucositis and its location in the six mentioned sites per implant was determined by the gingival index as described by Loe and Silness in 1963(25). Radiographic examination was also performed on periapical radiographs.

Criteria for Classification of Peri-Implant Diseases. All implants were categorized as clinically healthy, peri-implant mucositis and peri-implantitis according to the clinical and radiographic findings. Implants without plaque and calculus and surrounded by healthy peri-implant tissues, as evidenced by absence of BOP, absence of suppuration, probing depth of usually not exceeding 3 mm and no sign of bone loss in the radiographs were considered clinically healthy. Implants with BOP but no suppuration or radiographic evidence of bone loss and no PD deeper than 5 mm were

considered implants with peri-implant mucositis and implants with probing depth deeper than 5 mm in at least one site, exhibiting BOP and/or suppuration, and also having radiographic evidence of crestal bone loss in at least one site were considered implants with peri-implantitis.

Quantification of RANKL in PICF. 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 minute and then centrifuged at 1800×g for 30 minutes at 4 ^{0} C before being processed on the enzyme-linked immunosorbent assay (ELISA) plates. The contents of RANKL in the samples were determined by commercial ELISA kits in accordance with the manufacturer's instructions (total sRANKL ELISA kit: BioVendor, Modrice, Czech Republic). The minimum detection limit was 0.2 pmol/l for sRANKL. 96-well microtiter plates were coated with appropriate antibodies. 100 µl of standards and samples were added to the wells and the plates were incubated for 18 hours. Then the wells were washed 5 times with the wash solution (0.35 ml per well). Afterwards the detection antibody (Biotin labeled Antibody) was added into each well and incubated for 60 min at room temperature (18 to 25º C). Then the wells were washed again five times. 100 µl Streptavidin-HRP conjugate was added to each plate fallowed by incubation at room temperature for 1 hour and washing the wells again. Then the wells were developed with 100 µl tetramethylbenzadine (TMB) for 10 min at room temperature in the dark. The plates were covered with aluminium foil. The reaction was then stopped by the addition of 100µl stop solution and the color developed was measured in an automated microplate spectrophotometer set to 450nm, 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 pmol/l for concentrations when adjusted for PICF 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, cytokine levels were compared among groups using the Kruskal-Wallis one-way analysis of variance and between each pair of groups using independents samples t-test. A paired t-test was used to compare the cytokines within the buccal and lingual sites of the implants. Spearman's correlation analysis was used to determine correlation between PICF levels of sRANKL and the clinical parameters of the implants. The level of statistical significance was assigned at $p<0.05$.

RESULTS

The demographic and clinical characteristics of all three groups of implants are shown in Table 1. The mean age of the patients was 51.85 ± 1.14 years (age range, 18 to 72years). Mean PPD was 2.51± 0.92 in the healthy implants and 3.59 ± 0.94 and 4.79 ± 1.86 in the peri-implant mucositis and peri-implantitis groups, respectively and this showed statistically significant differences ($p<0.00$). There were also significant differences between mean GI in healthy implants (0.73 ± 0.45) and the other two diseased groups (2 ± 0.00, $p<0.000$). An estimate of plaque accumulation has also revealed a significant difference between the three groups of implants. PI was 1.11 ± 0.76 in the healthy implants, 1.71 ± 0.89 in peri-implant mucositis group and 1.03 ± 0.72 in peri-implantitis group ($p=0.011$).

Table 1. Demographic parameters of the patients and the clinical characteristics of the implants.

Variables		Healthy sites n=26	Peri-implant Mucositis sites n=28	Peri-implantitis sites n=26
Gender F:M		15:11	20:8	19:7
Age		49.92 ± 1	52.21 ± 1.22	53.38 ± 1.2
PPD*		2.51 ± 0.92	3.59 ± 0.94	4.79 ± 1.86
GI**		0.73 ± 0.45	2 ± 0.00	2 ± 0.00
PI***		1.11 ± 0.76	1.71 ± 0.89	1.03 ± 0.72
BOP****	Positive	0(0%)	28(100%)	26(100%)
	Negative	26(100%)	0(0%)	0(0%)

* Probing pocket depth;** Gingival index; ***Plaque index, ****Bleeding on probing

As shown in table 2, mean RANKL concentration was 0.2892 ± 0.01 pmol/l in healthy group, 0.2851 ± 0.006 pmol/l in peri-implant mucositis and 0.2876 ± 0.006 pmol/l in peri-implantitis and they showed no statistically significant differences (p=0.12). There were also no statistically significant correlation between the concentration of sRANKL and probing pocket depth (R=0.051, p=0.65), or any of the other clinical recordings (p>0.05). No difference between the mean sRANKL concentration in the buccal (0.287 ± 0.008) and lingual (0.286 ± 0.006) sites was also observed (p=0.693).

Table 2. sRANKL levels in PICF samples.

Variable	Healthy sites n=26	Peri-implant Mucositis sites n=28	Peri-implantitis sites n=26	P-value
sRANKL* concentration (pmol/l)	0.2892 ± 0.01	0.2851 ± 0.006	0.2876 ± 0.006	0.12

* sRANKL, soluble receptor activator of nuclear factor-kB ligand

DISCUSSION

Our data demonstrated no correlation between sRANKL levels of PICF and clinical parameters of peri-implantitis. In the present study the level of RANKL in PICF from healthy implants, implants with peri-implant mucositis and implants with peri-implantitis were evaluated. The major attraction of crevicular fluid as a diagnostic marker is the site-specific nature of the sample. This allows laboratory investigation of crevicular fluid constituents to be linked to clinical assessments at the site of sample collection (19). Our results are in accordance with those of Arikan et al. and Monov et al. in that no significant correlations were found between the sRANKL levels of PICF

and the clinical parameters of the implants (20, 21). However Monov et al. did not give any information about the health status of the study implants in term of clinically healthy, peri-implant mucositis and peri-implantitis. They have also performed statistical analysis only for those 29 samples exhibiting sRANKL levels above the minimum detection limit of the assay kit and excluded the rest of the PICF samples. Arikan et al. have included 86 implants in their study. Furthermore, their study population had an unbalanced distribution of samples into three health categories. They have investigated 79 healthy implants, 4 implants with peri-implant mucositis and only 3 implants with peri-implantitis. We tried to include approximately equal numbers of samples in the three groups. We evaluated 26 healthy sites, 28 peri-implant mucositis and 26 peri-implantitis sites. Age may be a factor that influences the level of bone remodeling systemically. In our study there were no statistically significant differences among the mean ages of the healthy group of implants (49.92 ± 1 years), the implants with peri-implant mucositis (52.21 ± 1.22 years) and those with peri-implantitis (53.38 ± 1.2 years).

Similar to periodontal disease, the absence of bleeding on probing (BOP) may reflect a stable condition, while repeated BOP may be indicative of a higher chance to develop tissue disintegration (26). Disease activity around each implant is generally accepted as increasing probing depth and /or increasing recession and signs of continuous loss of tissue or osseointegration; a change in the probing attachment level as described by Buser et al. represent a true change in the attachment level (27). Episodic peri-implant attachment loss is associated with variations in the supracrestal inflammation as well as inflammatory cell populations where more monocytes/macrophages, plasma cells and mast cells can be seen in active sites compared with inactive sites (28). We did not detect attachment loss from baseline around the implants. All the implants that showed signs of inflammation were quickly entered into the treatment phase after the clinical examination and PICF sampling. As it seems to be easier to provoke bleeding after probing at implants compared to teeth (29), it can be interpreted that in our study as well as the other two previous studies, PICF samples were collected from both active and inactive sites prior to therapy. Furthermore inconsistencies have been reported between tissue expression and crevicular fluid levels of this cytokine. This point highlights the complexity of clinical data interpretation, and may relate to a combination of factors such as variation in disease progression stage. In addition, it has been reported that there could be a lag time between the production of these molecules in the tissue and their subsequent release into the periodontal pocket microenvironment (30). In the study of Monov et al. 35% of the samples revealed detectable levels of sRANKL, whereas Arikan et al. have detected sRANKL in 12% of the samples. We detected sRANKL in all of the samples. It can be explained by the health status of the implants studied or as explained by Bostanci et al. That such difference may account for different levels of sub-clinical inflammation among healthy subjects or for differences in the sensitivity of various ELISA kits employed in each study (30). In a study on chronic periodontitis patients, Vernal et al. have reported that the total amount of RANKL level in GCF was significantly higher in patients than controls whereas active sites revealed significantly higher levels than their inactive counterparts (28). Other researchers have reported that the expression of RANKL showed no correlation with disease severity (17,18). So controversies of this type can also be seen in studies on chronic periodontitis. Activated osteoclasts initiate bone destruction. The activation signaling is modulated by RANKL, RANK and osteoprotegerin (OPG), three novel members of the TNF ligand and

receptor superfamilies. However three variants of RANKL exist: 1) A cell membrane-bound variant produced by the majority of tissues; 2) A secondary soluble form generated from the cellular form by post-translational processing by TNF-α- converting enzyme-linked protease (TACE) that is limited to TACE-producing tissues and cell types; 3) A primary soluble (secreted) form that has been described in activated T lymphocytes (27). Therefore inflammatory cytokines can also induce the expression of membrane-bound RANKL by osteoblasts, which are not released into the crevicular fluid and fail to be detected by immunoassay. Nakashima et al. have reported that membrane-bound RANKL may work more efficiently as a potent ligand for osteoclastogenesis than sRANKL (31). Some other investigators concluded that mRANKL may be more relevant as an inducer of bone resorption in peri-implantitis (20).

RANKL alone may not be sufficient for osteoclast formation from precursor cells as RANKL may be active in the presence of M-CSF which acts as a survival factor for both mature osteoclasts and their precursors (11). Sabokbar et al. have reported that osteoclasts may form by a RANKL independent mechanism (31,32) and others have shown that TNF-α and other factors may stimulate osteoclast formation in the absence of RANKL activity (33). Although our study has some strong points, it has also great limitations. We have pooled two PICF samples from one implant taken from buccal and lingual sites of each implant. We have also excluded the smokers in our study but the major limitation of our study is its analytical design. We did not separate initial peri-implantitis from severe peri-implantitis sites. The other point is that, levels of RANKL, OPG, M- CSF and other mediators of osteoclast formation in vitro are also modulated in response to various metal particles. These differences may reflect the osteoclastogenic potential of different chemical composition of biomaterials or their wear debris and our study implants were not manufactured by one single company. In view of our findings, further well-designed longitudinal studies on a larger-scale sample size, would be desirable to evaluate this cytokine during the active/inactive periods, to compare the expression of membrane-bound RANKL with sRANKL in peri-implantitis and to define the RANKL/Osteoprotegerin (OPG) concentration ratio in PICF.

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