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Saliva concentrations of RANKL and osteoprotegerin in smoker *versus* non-smoker chronic periodontitis patients

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Abstract

Objectives: To compare the salivary receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) concentrations in smokers *versus* non-smokers with chronic periodontitis.

Material and Methods: Whole saliva samples were obtained from 67 untreated chronic periodontitis patients, of whom 34 were smokers, and from 44 maintenance patients, of whom 22 were smokers. Full-mouth clinical periodontal measurements were recorded. Saliva cotinine, sRANKL and OPG concentrations were determined by ELISA. Statistical analysis was performed using the Mann–Whitney *U* test, Bonferroni's correction for multiple comparisons and Spearman's correlations.

Results: Untreated smokers exhibited significantly higher values of clinical periodontal recordings than untreated non-smokers (all $p<0.05$). Salivary cotinine level correlated with clinical attachment level ($p=0.023$). Smoker *versus* non-smoker maintenance groups showed no significant differences in clinical parameters. There were significant differences in sRANKL and OPG concentrations between untreated and maintenance groups (all $p<0.01$). Salivary OPG concentration was significantly lower (all $p<0.01$) and the sRANKL/OPG ratio was higher (all $p<0.01$) in smokers than in non-smokers. OPG concentration correlated positively with probing depth, clinical attachment level and bleeding on probing (all $p<0.005$) and negatively with pack-year, and cotinine level ($p<0.05$).

Conclusion: Salivary RANKL and OPG concentrations are suggested to be affected by smoking as not only the untreated but also the treated smokers exhibited higher RANKL and lower OPG concentrations than non-smokers.

Key words: chronic periodontitis; cotinine; maintenance; osteoprotegerin; RANKL; saliva; smoking

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Periodontal diseases are a group of infectious/inflammatory diseases involving Gram-negative, anaerobic and microaerophilic bacteria that colonize the subgingival area and cause local and systemic elevations of pro-inflammatory prostaglandins and cytokines, resulting in tissue destruction. Risk factors including tobacco smoking modify the periodontal response to the microbial challenge. Smokers have been reported to be more susceptible to advanced and aggressive forms of periodontal disease than non-smokers (Haber et al. 1993, Ketabi & Hirsch 1997, Calsina et al. 2002). Smokers tend to respond less favourably to periodontal treatment procedures (Ah et al. 1994, Boström et al. 1998), although smoker and non-smoker patients exhibit more or less the same periodontal pathogens (Preber et al. 1992, Renvert et al. 1998, Buduneli et al. 2005a). Smoking influences angiogenesis (Rezavandi et al. 2002, Cooke & Bitterman 2004), adhesion molecule profiles and leucocyte recruitment (Rezavandi et al. 2002, Scott & Palmer 2002) and multiple aspects of leucocyte development and function (Seow et al. 1994, Barbour et al. 1997, van Eeden & Hogg 2000, Palmer et al.

Conflict of interest and source of funding statement

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2005). It has been suggested that smoking has an influence on host cytokine levels (Tappia et al. 1995, Boström et al. 1999, Rawlinson et al. 2003, Buduneli et al. 2005b, 2006); however, the exact mechanisms by which smoking exerts detrimental effects on the periodontal tissues remain unclear.

Interaction of three members of the tumour necrosis factor (TNF) superfamily, receptor activator of the NF-κB ligand (RANKL), RANK and osteoprotegerin (OPG) (Suda et al. 1999), is important in coordinating osteoclastogenesis and thereby alveolar bone resorption. RANKL is expressed by osteoblasts/stromal cells (Yasuda et al. 1998), fibroblasts (Takayanagi et al. 2000, Quinn et al. 2000) and activated T cells (Horwood et al. 1999, Kotake et al. 2001). 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 (Lacey et al. 1998, Hsu et al. 1999). RANKL can be found as a cell membrane-bound variant (mRANKL) as well as a primary soluble (secreted) form that has been described in activated T cells (Kong et al. 1999). It is well known that a chronic lesion of periodontal disease is predominated by T lymphocytes. On the other hand, OPG is a soluble molecule and a naturally occurring inhibitor of osteoclast differentiation. OPG binds to RANKL with a high affinity and blocks RANKL from interacting with RANK (Lacey et al. 1998). Periodontal ligament cells, gingival fibroblasts and epithelial cells have been reported to produce OPG (Sakata et al. 1999, Kanzaki et al. 2002).

Gingival crevicular fluid (GCF) levels of RANKL were reported to be increased in periodontally diseased patients (Mogi et al. 2004, Vernal et al. 2004, Lu et al. 2006, Bostancı et al. 2007). Recently, we evaluated sRANKL and OPG levels in the peri-implant crevicular fluid (PICF) of clinically healthy endosseous dental implants and found that the OPG total amount correlated positively with PICF volume, gingival index and bleeding on probing (BOP), whereas a negative correlation was found between OPG amount and pack-years (Arıkan et al. 2008). Because these proteins are key regulators of bone metabolism, it is likely that they are involved in the regulation of alveolar bone destruction in chronic periodontitis. Possible alterations in the salivary composition may influence clinical periodontal status in smokers. Therefore, the aim of this study was twofold: (1) to evaluate the concentrations of sRANKL and OPG in saliva samples of smoker and non-smoker chronic periodontitis patients and (2) to investigate whether these salivary parameters are changed during the maintenance phase of periodontal treatment.

Material and Methods

Study population

A total of 111 subjects were included in the present study. Sixty-seven patients (34 females and 33 males) seeking dental treatment in the Periodontology Clinics, School of Dentistry, Ege University, İzmir and University of Glasgow, Dental School and Hospital were recruited. In addition, 44 patients (22 females and 22 males) discharged at least 3 years earlier, who had been receiving supportive therapy from their general practitioner, were invited back to the periodontology clinic for a reassessment of their clinical condition. They were then asked to take part in the study. Before the start of this study, ethical approval was obtained from the Ethics Committees at Ege University, İzmir and Glasgow Dental School and Hospital. Informed consent was obtained from each patient before clinical examination and saliva sampling. Patients were all in good health and had not received any antibiotic treatment during the last 3 months.

Saliva sampling

Saliva samples were obtained in the morning following an overnight fast, during which subjects were requested not to drink (except water) or chew gum. Whole saliva samples were obtained by expectoration into polypropylene tubes before clinical measurements. The saliva samples were weighed, immediately frozen and stored at -40°C until the sample collection period was completed.

Clinical examination

Subsequent to saliva sampling, clinical periodontal recordings, including dichotomous plaque index (+/−), probing depth (PD), clinical attachment level (CAL), gingival recession and dichotomous BOP (+/−) score as present or absent within 10 s after probing, were performed at six sites of each tooth present except the third molars. A Williams probe was used for clinical periodontal recordings. Furthermore, a radiographic examination was performed on periapical radiographs.

Enzyme immunoassay

Each saliva sample (500 μl) was pipetted into a clean microcap tube and clarified by centrifugation at 10,000 *g* for 5 min.; the supernatant transferred to clean microcap tubes and used immediately for an enzyme linked immunosorbent assay (ELISA). The human sRANKL (hsRANKL) ELISA development kit (Peprotech EC, London, UK), and the hOPG ELISA development kit (R&D systems, Abingdon, Oxfordshire, UK) were utilized to measure the amounts of these proteins in the saliva samples according to the manufacturers' guidelines. The results of sRANKL and OPG assays were expressed as pg/ml for concentrations. Saliva cotinine levels were also evaluated using the relevant EIA kit (Cozart, Oxford, UK), and smoking status was confirmed (smokers $\geqslant$100 ng/ml cotinine; non-smokers $<$10 ng/ml cotinine).

Statistical analysis

Statistical power calculations indicated that 68 patients (distributed between four roughly equal groups, 17 per group) would be required when the α was reduced to 0.0125, allowing four comparisons, to obtain $>$80% power. Statistical analysis was performed using the Mann–Whitney *U* test and Bonferroni's correction for multiple comparisons. Spearman's correlations were utilized to look at the relationship between salivary concentrations of sRANKL, OPG and the clinical parameters. The correlations were also corrected for the smoking and the treatment status of the patients.

Results

Clinical findings

Definitive statistics of clinical measurements are outlined in Table 1. The age, number of teeth present (median 24; range 19–27), number of PDs $>$5 mm, plaque index and gingival recession were all similar in the four study groups ($p>0.05$). To distinguish between the patient groups, the new patients have

Table 1. Clinical characteristics of the study groups

Group	Non-smoker maintenance median* (Q1–Q3)	Non-smoker untreated median (Q1–Q3)	Smoker maintenance median (Q1–Q3)	Smoker untreated median (Q1–Q3)
Age (years)	43.0 (41.3–45.0)	48.0 (43.0–51.0)	46.0 (40.3–51.8)	45.0 (40.0–50.8)
Number of teeth	24.0 (22.0–26.0)	24.0 (22.0–24.8)	23.0 (22.3–25.0)	24.0 (24.0–26.0)
Mean pocket depth (mm)	2.77 (2.63–2.96)	2.97 (2.65–3.37)	2.85 (2.69–3.00)	3.62†,‡ (2.86–4.38)
Clinical attachment loss (mm)	3.23 (2.95–3.63)	3.41 (3.17–4.52)	3.33 (3.11–3.78)	4.73†,‡ (3.87–5.61)
Proportion of sites with bleeding on probing	0.28 (0.21–0.37)	0.28 (0.15–0.86)	0.33 (0.27–0.39)	0.58 (0.22–1.00)
Plaque index	1.00§ (0.66–1.00)	1.00 (0.90–1.00)	1.00 (0.70–1.00)	1.00 (0.80–1.00)
Gingival recession (mm)	0.47 (0.24–0.76	0.59 (0.28–1.08)	0.61 (0.34–0.75)	0.89 (0.23–2.10)
Number of pockets >5 mm	8.5 (3.3–13.0)	8.0 (5.3–10.0)	10.0 (5.0–14.5)	11.0 (5.0–15.0)

*The results show the median and interquartile range (Q1–Q3) of each parameter.
†Untreated patients significantly different from supportive therapy (maintenance) patients.
‡Smokers significantly different from the non-smokers.
§Only 12 of the 44 patients in the maintenance groups had plaque indices recorded; five smokers and seven non-smokers.

been called the untreated group and the patients on supportive therapy have been called the maintenance group.

The untreated smoker chronic periodontitis group exhibited significantly higher values of PD and CAL compared with the untreated non-smoker group ($p<0.01$). Untreated smokers also exhibited significantly deeper periodontal pockets and clinical loss of attachment than smokers with periodontitis on maintenance (supportive therapy) or untreated non-smokers (all, $p<0.01$). BOP also appeared to be greater in the untreated smokers ($p=0.041$), but this difference was not statistically significant after a correction was made for multiple comparison. There were no statistically significant differences in the clinical measurements of the non-smoker groups other than an indication that BOP was higher in the untreated groups compared with the maintenance groups ($p=0.032$ and $p=0.032$), but not statistically significant when corrected for multiple comparison. Plaque indices were only recorded for a minority of the maintenance groups, but the results obtained were similar to those recorded for the untreated patient groups and in both smokers and non-smokers.

Biochemical analyses

The results of biochemical analysis are outlined in Fig. 1a–d. Untreated smokers had significantly lower OPG concentrations than untreated non-smokers with periodontitis ($p=0.001$). RANKL levels appeared to be greater in the smokers, but the difference was not statistically significant. When comparing patients on supportive therapy (maintenance), the smokers also had significantly lower OPG concentrations than the non-smokers ($p=0.003$).

Untreated non-smoker periodontitis patients have significantly greater concentrations of RANKL than the non-smokers on maintenance ($p=0.006$). Although there appeared to be a lower OPG concentration in the untreated non-smokers ($p=0.023$) than in the maintenance non-smokers, the difference was not statistically significant after Bonferroni's correction.

Maintenance smokers, i.e., smokers on supportive therapy, had significantly lower OPG levels ($p<0.001$) and increased RANKL/OPG ratios ($p<0.001$) than the non-smokers on maintenance.

In the untreated patient groups, the OPG concentration in saliva showed a statistically significant positive correlation with PD, CAL and BOP ($p<0.001$, $p=0.001$). In the untreated smokers, the pack-year correlated with PD, CAL and gingival recession ($p<0.05$). In all patient groups, there were negative correlations between OPG concentration and pack-year and also saliva cotinine level ($p<0.005$). In the maintenance groups, the salivary sRANKL concentration and the sRANKL/OPG ratio were also highly statistically significantly positively correlated with the cotinine concentration and the number of pack-years (all $p<0.001$). These correlations remained statistically significant after controlling for age and gender.

Discussion

In the present study, we analysed saliva levels of sRANKL and OPG as well as the clinical condition in 111 systemically healthy patients with the diagnosis of chronic periodontitis. The study compares 44 patients who have undergone periodontal therapy and are currently under a maintenance regime with 67 similarly aged patients who had been recently diagnosed with chronic periodontitis. To our knowledge, this is the first study to investigate the role of smoking in the saliva concentrations of sRANKL and OPG in smoker *versus* non-smoker chronic periodontitis patients. Self-reporting by the patients of their smoking status turned out to be accurate; there was a 100% match with the cotinine test results and we can claim that there was a very clear discrimination between the smoker and non-smoker groups, enabling us to evaluate the possible effects of smoking.

Several methods of saliva collection are available, including the collection of unstimulated whole saliva; whole saliva stimulated with, typically, paraffin wax,

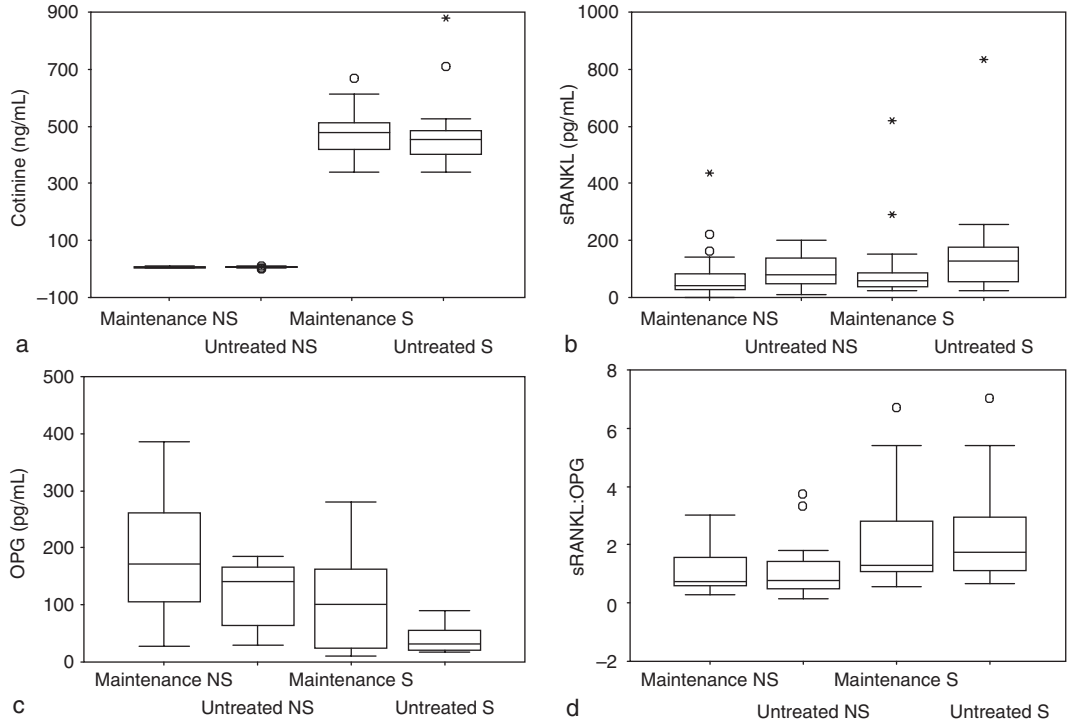

Fig. 1. Box plots showing salivary concentrations of (a) cotinine (ng/ml), (b) soluble receptor activator of nuclear factor κ B (sRANKL; pg/ml), (c) osteoprotegerin (OPG; pg/ml) and D the ratio of soluble receptor activator of nuclear factor κ B to the osteoprotegerin concentrations (sRANKL/OPG) in chronic periodontitis patients consisting of 22 non-smokers receiving supportive therapy (maintenance NS), 33 non-smokers who were new consults to the periodontology clinic (untreated NS), 22 smokers receiving supportive therapy (maintenance S) and 34 smokers who were new consults to the periodontology clinic (untreated S). Outliers and extreme values are indicated by the open circles and the asterisks, respectively.

Statistically significant differences were observed between the smoker groups and the equivalent non-smoker groups for the following parameters: Cotinine, OPG and sRANKL/OPG (all $p<0.01$). In addition maintenance non-smokers had statistically significantly lower sRANKL levels than untreated non-smokers and maintenance smokers (both $p<0.01$).

gum base or citric acid; or the collection of saliva from specific salivary glands. Whole saliva contains GCF, immune cells and tissue metabolites (Navazesh 1993, Kaufman & Lamster 2000) and reflects the predominant intraoral condition most closely (Edgar 1992). Stimulation, on the other hand, may increase the flow of GCF and this may result in false increases in the concentration of evaluated biomarkers in the saliva (Chapple et al. 1997). Accordingly, we used unstimulated whole saliva samples for the present study.

Abundant cross-sectional data support the relationship between smoking and periodontal diseases. Comprehensive reviews on this subject have been published recently (Kinane & Chestnutt 2000, Johnson & Hill 2004). A strong dose–response relationship between the amount smoked and the severity of periodontal destruction has also been shown, further supporting the role of smoking as a risk factor for periodontitis (Grossi et al. 1994, 1995, Calsina et al. 2002). Smokers are almost four times more likely to have severe periodontitis than non-smokers (Haber et al. 1993). However, the precise mechanisms by which smoking exerts its deleterious effects on periodontium remain unclear. Appropriate treatment may reduce the impact of smoking on the progression of the disease because; a recent study (Fisher et al. 2008) has shown that over a 3-year period, periodontal disease appears to progress no faster in smokers than in non-smokers on maintenance therapy. Although the investigation was performed on a relatively small cohort of patients, it supports our decision to use both patients on maintenance therapy and the new referrals to the periodontal clinic in the current study.

In vitro binding of RANK with its cognate RANKL results in osteoclastogenesis by monocyte/macrophage progenitor differentiation to osteoclasts and the activation of mature osteoclasts (Hsu et al. 1999). OPG, on the other hand, competes with RANK for RANKL binding and is therefore, an effective inhibitor of osteoclast maturation and osteoclast activation in vitro and in vivo (Simonet et al. 1997, Kong et al. 1999). As discussed before (Lappin et al. 2007), one possible pitfall of the ELISAs is that they are able to detect both free and complexed RANKL and OPG. OPG was effective in inhibiting alveolar bone resorption in an animal model mimicking human periodontal disease (Teng et al. 2000). An increased RANKL/OPG ratio was reported in periodontitis sites compared with gingivitis/healthy sites (Lu et al. 2006). Crotti et al. (2003) reported significantly higher levels of RANKL protein in periodontally diseased tissue, whereas OPG was significantly lower than that in the healthy control group. Furthermore, Mogi et al. (2004) showed that the ratio of the GCF concentration of RANKL to OPG significantly increases in periodontal disease patients. Vernal et al. (2004) reported a higher detection

rate of GCF RANKL levels in untreated chronic periodontitis patients (85%) than healthy controls (46%). Moreover, the total amount of RANKL was significantly higher in patients than controls, whereas active sites revealed significantly higher levels than their inactive counterparts. Recently, Bostancı et al. (2007) reported a positive correlation between the GCF RANKL/OPG ratio and PD in periodontitis patients. Considering the new data on RANKL and periodontal tissue destruction, Taubman et al. (2007) suggested that new therapeutic strategies aimed at RANKL inhibition may be helpful in periodontal treatment. Very recently, Silva et al. (2008) reported a higher RANKL total amount in GCF of active sites than that of inactive sites, providing further support for the hypothesis that local levels of RANKL may be an indicator of clastogenesis during the episodes of connective tissue loss seen in periodontal disease progression.

Mogi et al. (2004) and Lu et al. (2006) failed to report significant correlations between GCF concentrations of RANKL and/or OPG and clinical measurements of disease severity in terms of pocket depth, attachment level and of inflammation in terms of BOP in chronic periodontitis patients. Tanaka et al. (2006) evaluated the effect of nicotine and lipopolysaccharide on the expression of macrophage colony-stimulating factor (M-CSF), OPG and prostaglandin E_2 (PGE_2) in osteoblasts, and the indirect effect of nicotine and LPS on the formation of osteoclast-like cells. OPG expression was increased in the initial stages of culture with nicotine and LPS but decreased in the later stages of culture.

Lappin et al. (2007) reported decreased serum OPG levels and greater sRANKL/OPG ratios in smoker patients in the maintenance programme than the non-smoker counterparts. The results of this study confirm these earlier findings and show that the untreated smokers also had lower levels of OPG than the non-smokers. Furthermore, the results indicated that the maintenance non-smoker patients had lower sRANKL levels than the untreated non-smokers. This could be an indication that the inflammatory process was more active in the untreated patients. Whether reduced sRANKL is related to the efficacy of the maintenance regime and indicates a lower level of disease progression in the patients on supportive therapy is yet to be determined, but it appears to be a possibility. Smoking seems to suppress OPG levels and might contribute towards the increased bone destruction often seen in smokers. Although the median RANKL/OPG ratio was not significantly different, the untreated smokers had the lowest median OPG concentration and the highest median sRANKL concentration, which might indicate that they were possibly at more risk of bone loss than the smoker maintenance therapy group. The fact that the untreated smoker patients had the greatest median clinical attachment loss and the greatest median pocket depth would tend to support this idea. It has been known for some time that smoking reduces the rate of bone formation as indicated by serum osteocalcin levels (Laroche et al. 1994), and also appears to increase the rate of bone destruction as measured by serum collagen telopeptide (ICTP) levels in post menopausal women (de Valk-de Roo et al. 1997) and that periodontal therapy has a greater impact in reducing the levels of this marker in non-smokers (Al-Shammari et al. 2001).

Furthermore, the reduction of the levels of sRANKL and increased OPG may be indicative of a reduction in the inflammatory process in the maintenance groups and shows that a clinical intervention to remove the inflammatory stimulus and maintain a level of plaque control has a beneficial effect. The precise interaction between sRANKL and OPG levels and the rate of alveolar bone loss in the periodontitis patient is unclear and may be complicated by compensatory mechanisms. It clearly takes place over a very long time and may take many years before a difference is manifest. Clearly, measurement of markers of bone formation and bone destruction, such as osteocalcin and ICTP, respectively, may help to clarify the situation and are the subjects for further investigation.

As a conclusion, the findings of this study suggest that OPG concentrations in the saliva are affected by smoking and the significantly increased sRANKL/OPG ratio in smokers provides further support for the hypothesis that smoking increases the risk for periodontitis.

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Clinical Relevance

Scientific Rationale for the study: Smoking may affect salivary RANKL and OPG levels in untreated and treated chronic periodontitis patients.

Principal findings: The salivary OPG concentration was significantly lower and the sRANKL/OPG ratio was higher in smokers than in non-smokers. OPG concentration correlated positively with PD, CAL and BOP, whereas there was a negative correlation between OPG concentration and pack-year and cotinine level.

Practical implications: Smoking seems to decrease salivary OPG levels and increase the RANKL/OPG ratio. This may at least partially explain the greater potential for bone loss in smokers.