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Smoking modulates interleukin-6:interleukin-10 and RANKL:osteoprotegerin ratios in the periodontal tissues

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Background and Objective: This study evaluated the effect of smoking on the gene expression of interleukin-1α, -1ra, -6, -8 and -10, tumor necrosis factor-α, matrix metalloproteinase (MMP)-2 and -8, receptor activator of NF-κB ligand (RANKL) and osteoprotegerin, in sites with periodontitis.

Material and Methods: Gingival biopsies were divided into three groups: the healthy group (periodontally healthy subjects; $n = 10$); the periodontitis group [subjects with severe chronic periodontitis who never smoked (probing depth ≥ 7 mm) ($n = 25$)]; and the smoking group (subjects diagnosed with severe chronic periodontitis who smoked ≥ 1 pack per day for at least 10 years; $n = 25$). Gene and protein expressions were analyzed by quantitative polymerase chain reaction and enzyme-linked immunosorbent assay, respectively.

Results: Data analysis demonstrated that, except for MMP-8 and osteoprotegerin, the levels of all factors were increased by inflammation ($p < 0.001$). The levels of interleukin-1α, -1ra, -6 and -8, and RANKL, were higher in smokers with periodontitis compared with controls, whereas the levels of interleukin-10, MMP-8 and osteoprotegerin were lower ($p < 0.001$). Smoking lowered the levels of interleukin-1α, -8, -10, tumor necrosis factor-α, MMP-8 and osteoprotegerin, and increased the levels of interleukin-6 and -1ra in sites with a comparable type of periodontitis ($p < 0.001$).

Conclusion: In conclusion, smoking modulates gene expression in the periodontium, and the influence of smoking on periodontal disease may involve effects of interleukin-6:interleukin-10 and RANKL:osteoprotegerin ratios.

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The role of cytokines in periodontal disease has been evaluated in several studies (1,2). It was demonstrated that pro-inflammatory cytokines, such as interleukin-1, interleukin-6 and tumor necrosis factor-α, are synthesized in response to periodontopathogenic bacteria, hence inducing and maintaining an inflammatory response in the periodontium. Interleukin-1 is associated with the recruitment of inflammatory cells, facilitating neutrophil degranulation, increasing matrix metalloproteinase (MMP) production, inhibiting collagen synthesis and activating T and B cells (3). Tumor necrosis factor-α is involved in osteoclastogenesis, MMP secretion and

the promotion of interleukin-6 production (4). Both interleukin-1 and tumor necrosis factor-α were reported to induce osteoclast differentiation strongly by modulating receptor activator of NF-κB ligand (RANKL) expression (5). Finally, a number of actions, including osteoclastogenesis, are attributed to interleukin-6 in periodontitis (6). Concomitantly, other cytokines, including interleukin-10 and interleukin-1ra (interleukin-1 antagonist), with chiefly anti-inflammatory properties, are released that act to limit the duration and extent of the pro-inflammatory effect. Thus, the development and regulation of an immune response depends on a number of cytokines, which determine whether the response will be protective or non-protective.

Environmental risk factors, such as smoking, may modify the host response and hence modify disease progression, severity and outcome (7). Inflammatory components in the gingival crevicular fluid have been studied in relation to smoking in periodontal patients (8–11) and, in general, no conclusive results have been reported. Therefore, although significant, these studies collectively do not provide an accurate description of the underlying mechanisms by which smoking affects the periodontium. Thus, we tested the hypothesis that smoking affects periodontal destruction by modulating the expression of pro- and anti-inflammatory cytokines and pro- and antiresorptive agents in the gingival tissues.

Material and methods

Study population

This study was approved by the Institutional Review Board of the University of Campinas School of Dentistry. All patients were, on an individual basis, informed about the nature of the proposed treatment, and informed consent forms were signed. All subjects were submitted to anamnesis and to clinical and periodontal examination (Table 1). Gingival tissue was obtained from 60 subjects undergoing periodontal surgery for disease-related or nondisease-related (e.g. esthetic) reasons. Exclusion criteria included any systemic disorder that would affect the periodontal condition (with the exception of smoking), patients who had received scaling and root planing within 6 mo prior to the study, and the use of systemic or subgingival antimicrobials or anti-inflammatory medication within 6 mo prior to baseline examination. Female patients were not pregnant, lactating or using any method of birth control. The smoking habit was recorded and patients who had consumed ≥1 pack per day (20 cigarettes) for at least 10 years were classified as smokers. Only patients who had never smoked were included in the nonsmoker group.

Experimental design

Gingival biopsies were harvested from single teeth (restricted to one or more sites around the tooth with probing depth > 7 mm) and divided into three groups, as follows: the healthy group (systemically and periodontally healthy subjects, with a high standard of oral hygiene, no bleeding on probing and probing depth ≤ 3 mm in the selected sites; $n = 10$); the periodontitis group [subjects who had never smoked, were systemically healthy and clinically diagnosed with severe chronic periodontitis (e.g. probing depth ≥7 mm and bleeding on probing); $n = 25$]; and the smoking group [systemically healthy subjects clinically diagnosed with severe chronic periodontitis (e.g. probing depth ≥7 mm and bleeding on probing) who had smoked ≥1 pack per day for at least 10 years; $n = 25$].

Table 1. Mean values and standard deviation for the clinical parameters in the selected sites, and demographic and behavioral data in smokers and nonsmokers

	Control $n = 10$	Non-smokers $n = 25$	Smokers $n = 25$
Age (in years)	40.57 ± 2.67a	49.50 ± 8.11a	45.33 ± 7.37a
(range)	(38–43)	(40–51)	(40–47)
Per cent men	35.75	35.71	55.55
Probing depth (mm)	1.50 ± 0.90b	8.35 ± 0.86a	8.51 ± 1.42a
Plaque index (%)	0b	100a	100a
Gingival index (%)	0b	100a	100a
Cigarettes/day	–	–	21.11 ± 1.38
Cigarette consumption (in years)	–	–	25.09 ± 5.70

Different letters, within each line, indicate statistical differences determined by one-way analysis of variance and the Bonferroni *t*-test ($\alpha = 0.05$).

Biopsies and RNA extraction

Gingival biopsies (≈ 100 mg) were obtained, rinsed with cold sterile saline solution and stored in a tube containing RNAlater® (Ambion Inc., Austin, TX, USA) at −70°C for checking the mRNA levels of interleukin-1α, -1ra, -6, -8 and -10, tumor necrosis factor-α, MMP-2 and -8, RANKL and osteoprotegerin. Total RNA was extracted using the TRIZOL reagent (Gibco BRL, Life Technologies, Rockville, MD, USA) following the manufacturer's recommendation.

Real-time polymerase chain reaction experiments

Reverse transcription — Total RNA was treated with DNase (Turbo DNA-free®; Ambion Inc.), and 1 μg was used for cDNA synthesis. The reaction was carried out using the first-strand cDNA synthesis kit (Roche Diagnostic Co., Indianapolis, IN, USA), following the manufacturer's recommendations.

Primer design — Primers were designed using the LIGHTCYCLER® probe design software (Roche Diagnostics GmbH, Mannheim, Germany) (Table 2). Experiments were run twice, with comparable results obtained on each occasion.

Reverse transcription–polymerase chain reaction — Quantitative polymerase chain reaction (PCR) was performed in the LightCycler® system (Roche

Table 2. Primer sequences for each gene, annealing temperature and amplicon length

Gene	Sequence (5′ → 3′)	Amplification profile [temperature (°C)/time (s)]	Amplicon (bp)
GAPDH	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	95/0; 51/9; 72/3	226
IL-1α	CTTCTTCGACACATGGGATAAC TTTGGGATCTACACTCTCCAGC	95/0; 52/12; 72/3	283
IL-6	CTAGAGTACCTCCAGAACAGATTTGA TCAGCAGGCTGGCATTT	95/0; 55/7; 72/20	154
IL-8	GCCAAGAGAATATCCGAACTTTAAT CTGGCTAGCAGACTAGGG	95/0; 55/7; 72/20	172
IL-10	GCCTACATGACAATGAAGATACGA CTATAAGAGAGGTACAATAAGGTTTCTCAA	95/0; 55/6; 72/20	151
IL-1ra	TCAGAAGGCGTCACAAGAAC ATCCTGAATGCAGAGGCGA	95/0; 55/6; 72/20	153
TNF-α	TCCACCCATGTACTCCTCAC CCTCCCAGATAGATGGGCTCATA	95/0; 55/6; 72/20	155
MMP-2	CGACCGCGACAAGAAGTA GCACACCACATCTTTCCGTCA	95/0; 55/6; 72/20	159
MMP-8	GAGAAGGCATCCTCAGCTAC TATCATAGCCACTCAGAGCCC	95/0; 55/6; 72/20	158
RANKL	CGACATCCCATCTGGTTCC GCTGGTTTTAGTGACGTACACC	95/0; 51/9; 72/3	199
OPG	CAAAGTAAACGCAGAGAGTGTAGA GAAGGTGAGGTTAGCATGTCC	95/0; 55/7; 72/20	187

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MMP, matrix metalloproteinase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TNF-α, tumor necrosis factor-α.

Diagnostics GmbH) using the Fast-Start DNA Master^{plus} SYBR Green kit (Roche Diagnostic Co.). For each run, water was the negative control. The reaction product was quantified with the Relative Quantification tool (LIGHTCYCLER® Software 4; Roche Diagnostics GmbH), with glyceraldehyde-3-phosphate dehydrogenase as the reference gene.

Enzyme-linked immunoabsorbent assay

The protocol for enzyme-linked immunoabsorbent assay (ELISA) followed that of a previous report (12). A portion of the same biopsies assayed for mRNA levels was stored at −20°C and homogenized in a solution of phosphate-buffered saline containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzotonic chloride, 10 mM EDTA and 20 Kl/ml of aprotinine (Sigma, St Louis, MO, USA). The samples were centrifuged at 12 350 ***g*** for 15 min at 4°C and the supernatant was used to evaluate the protein levels of interleukin-1β, -6 and -8 in the gingival tissues. Briefly, polystyrene high-binding 96-well microtiter plates (Nunc-Immuno Plate; Maxisorp, Nalge Nunc, Rochester, NY, USA) were coated with capture antibody, polyclonal rabbit anti-interleukin-1β (3 μg/ml; Pharmingen, San Diego, CA, USA), interleukin-6 (1 μg/ml; Pharmingen) and interleukin-8 (1 μg/ml; Pharmingen), diluted in phosphate-buffered saline coating buffer. After overnight incubation at 4°C, the plates were washed (as in subsequent steps) with phosphate-buffered saline containing 0.05% Tween 20 and 0.4 M NaCl, and then incubated, for 2 h at room temperature, with diluent buffer (phosphate-buffered saline containing 1.0% bovine serum albumin; 100 μl per well) to block nonspecific binding. After washing, the samples (100 μl per well) or the serially diluted standards of interleukin-1β (5000 pg/ml; Pharmingen), interleukin-6 (2000 pg/ml, Pharmingen) or interleukin-8 (2000 pg/ml, Pharmingen) were added to the plates, which were then incubated overnight at 4°C. After washing the plates, 100 μl of biotinylated sheep polyclonal antihuman interleukin-1β (0.2 μg/ml, Pharmingen), interleukin-6 (1 μg/ml; Pharmingen) or interleukin-8 (1 μg/ml; Pharmingen) was added to each well and the plates were incubated for 1 h at room temperature. Color was developed by the use of peroxidase-conjugated streptavidin (1 : 200; 100 μl per well) (DAKO Corp., Carpinteria, CA, USA) for 30 min and, after washing, the chromogen, *o*-fenilenodiamina-2HCL (Sigma) was added and incubation continued for 15 min. The reactions were quenched with 150 μl of 1.0 M sulphuric acid and the absorbance at 490 nm was measured.

Statistical analysis

Data were averaged and an intergroup comparison was performed by one-way analysis of variance ($\alpha = 0.05$). If statistical difference was detected, a pairwise multiple comparison test (Bonferroni *t*-test) was used to identify the difference among the groups. With respect to the comparisons of ratios, an intergroup analysis was performed by the Kruskal–Wallis test ($\alpha = 0.05$), followed by the Dunn's test in the event that differences were detected.

Results

Clinical observations

All clinical parameters were higher in the periodontally inflamed sites than in the healthy sites ($p < 0.05$). Regarding the periodontitis sites, there were no significant differences between smokers and nonsmokers in any of the parameters, including age, probing depth, plaque accumulation and bleeding on probing ($p > 0.05$) (Table 1).

Gene expression analysis

The results for the mRNA levels are presented relative to glyceraldehyde-3-phosphate dehydrogenase in the gingival tissues of smokers and nonsmokers (Figs 1 and 2). Data analysis demonstrated that interleukin-1α, -1ra, -6, -8 and -10, tumor necrosis factor-α, MMP-2 and RANKL mRNA levels were higher in the nonsmoking periodontitis group than in the healthy group ($p < 0.05$), while MMP-8 and osteoprotegerin were lower ($p < 0.05$). In addition, the levels of RANKL, and of interleukin-1α, -1ra, -6 and interleukin-8, were higher in the smoking periodontitis group compared with the healthy group ($p < 0.05$), whereas the levels of interleukin-10, MMP-8 and osteoprotegerin were lower ($p < 0.05$). No difference was observed for tumor necrosis factor-α levels between smoking periodontitis and healthy groups ($p > 0.05$). In the periodontitis sites, the levels of interleukin-1α, -8 and -10, tumor necrosis factor-α, MMP-8 and osteoprotegerin were lower in smokers than in nonsmokers ($p < 0.05$), whereas the levels of interleukin-6 and -1ra were higher ($p < 0.05$). Increased RANKL:osteoprotegerin and interleukin-6:interleukin-10 ratios were found in sites with periodontitis in smokers vs. nonsmokers, whereas in smokers the interleukin-1α:interleukin-1ra ratio was similar to that of the healthy group (Fig. 1B).

ELISA assays

To determine whether changes in the protein levels of some cytokines paralleled changes in gene expression, ELISA was performed. Data analysis showed that the protein levels of interleukin-1α, -6 and -8 presented a similar trend, as observed for their mRNA levels (Fig. 3). In nonsmokers, the inflamed sites presented significantly higher levels of interleukin-1α, -6 and -8 ($p < 0.05$), whereas smoking significantly increased interleukin-6 levels in the gingival tissues.

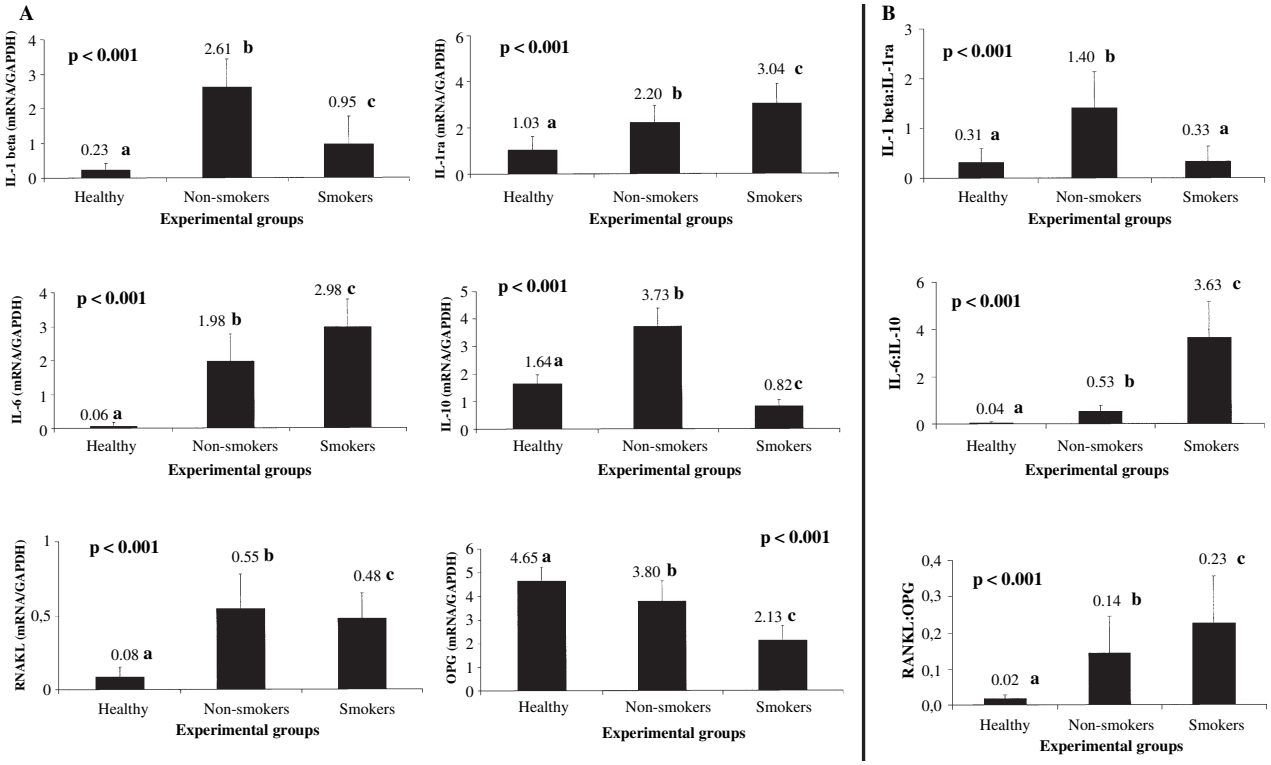

Fig. 1. (A) Mean and standard deviation mRNA levels (mRNA/glyceraldehyde-3-phosphate dehydrogenase) of the pro-inflammatory cytokines interleukin-1α and interleukin-6, the proresorptive factor receptor activator of nuclear factor-κB ligand (RANKL), the anti-inflammatory cytokines interleukin-1ra and interleukin-10, and the antiresorptive factors, in the gingival tissues harvested from sites with a healthy periodontal condition and from sites with with moderate to severe chronic periodontitis from nonsmokers and smokers. (B) Interleukin-1α:interleukin-1ra, interleukin-6:interleukin-10 and RANKL:osteoprotegerin mRNA level ratios. Mean values followed by different letters at the top of each bar indicate statistical differences determined by an intergroup analysis performed by one-way analysis of variance and the Bonferroni *t*-test ($\alpha = 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; OPG, osteoprotegerin.

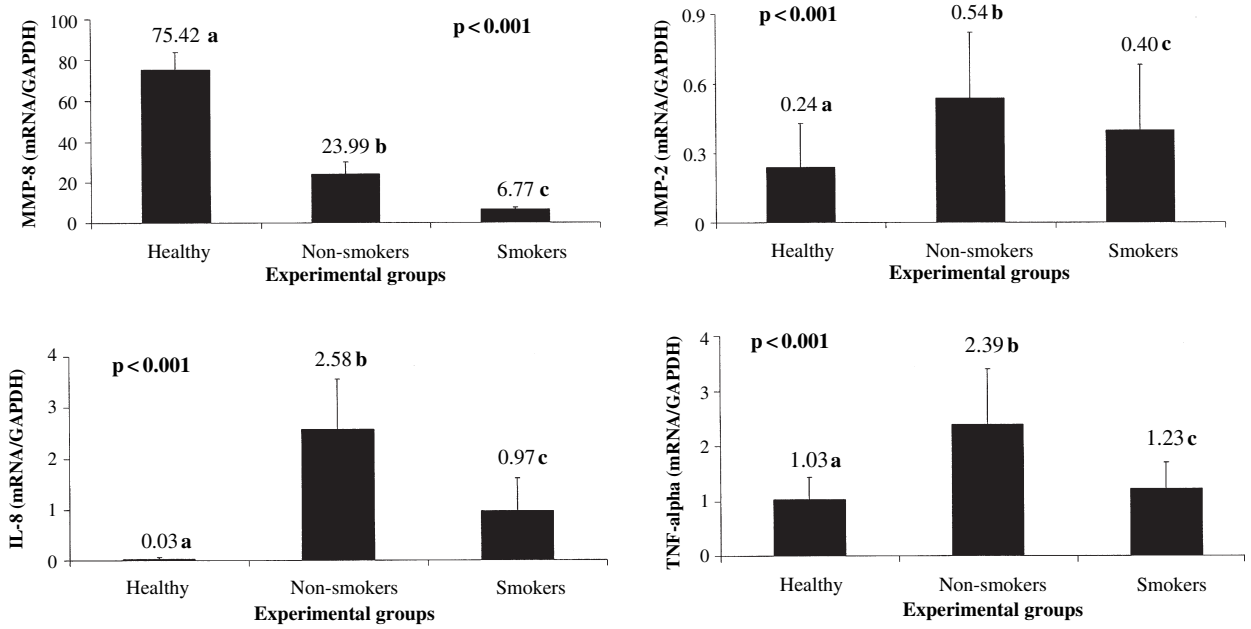

Fig. 2. Mean and standard deviation mRNA levels (mRNA/glyceraldehyde-3-phosphate dehydrogenase) of matrix metalloproteinase (MMP)-2 and -8, the chemokine interleukin-8 and the cytokine tumor necrosis factor-α in the gingival tissues harvested from sites with a healthy periodontal condition and from sites with moderate to severe chronic periodontitis, from nonsmokers and smokers. Mean values followed by different letters at the top of each bar indicate statistical differences determined by an intergroup analysis performed by one-way analysis of variance and the Bonferroni *t*-test ($\alpha = 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor-α.

Discussion

In the present study, the profile of a number of factors in the gingival tissues of smoking vs. nonsmoking individuals presenting severe chronic periodontitis was evaluated. Gingival biopsy was harvested and processed for monitoring the gene and protein expressions by real-time PCR and ELISA. Data analysis suggests that cigarette consumption may modulate gene expression in the periodontium and may favor bone resorption through increased ratios of interleukin-6:interleukin-10 and RANKL:osteoprotegerin.

Cytokine profiles are of considerable value when studying periodontal tissue destruction. It has been proposed that bacterial proliferation and/or bacterial products in periodontal pockets results in the recruitment and activation of the monocyte/T-lymphocyte axis. This, in turn, leads to the enhanced release of inflammatory cytokines, including interleukin-1α, interleukin-6 and tumor necrosis factor-α, associated with periodontal tissue destruction. Interleukin-8, secreted by monocytic cells and also from fibroblasts and endothelial cells, induces release of MMPs by neutrophils. Our findings therefore confirm a number of previous reports which indicated that elevated levels of pro-inflammatory cytokines, such as interleukin-1α, -6, -8 and tumor necrosis factor-α, plus the proresorptive factor, RANKL, and low levels of the antiresorptive factor, osteoprotegerin, are associated with sites showing periodontal destruction (1,13, 14). In addition, as also previously reported (15), higher mRNA levels for MMP-2, a potent collagenase that plays a critical role in depredating connective tissue, were found in inflamed vs. noninflamed sites. Intriguingly, in contrast to previous studies (16), in the present investigation mRNA levels for MMP-8 were found to be lower in diseased vs. clinically healthy sites. As it has been demonstrated that the levels of inflammatory components in the periodontium are highly influenced by the site profile (13,14), we believe that the inclusion/exclusion criteria for the selected sites may explain this apparent divergence between the present and previous studies.

Tobacco smoking, mostly in the form of cigarette smoking, is recognized as the most important environmental risk factor in periodontitis and as a major risk factor that contributes to the pathogenesis of periodontal disease. It would seem logical to expect that factors which are associated with tissue destruction should be higher in smokers than in nonsmokers. In agreement with this assumption, there are some data showing that cytokine expression may, in fact, be up-regulated by smoking and/or its compounds. In an *in vitro* study, nicotine, in association or not with lipopolysaccharide from periodontopathogenic bacteria, was shown to increase interleukin-6 and interleukin-8 protein production by human gingival fibroblasts (17). In a recent study, a microarray analysis demonstrated that when peripheral blood mononuclear cells were exposed for 5 min to tobacco smoke, elevated expression of 20 genes, previously reported to be associated with periodontal pathogenesis, occurred (18). Higher levels of tumor

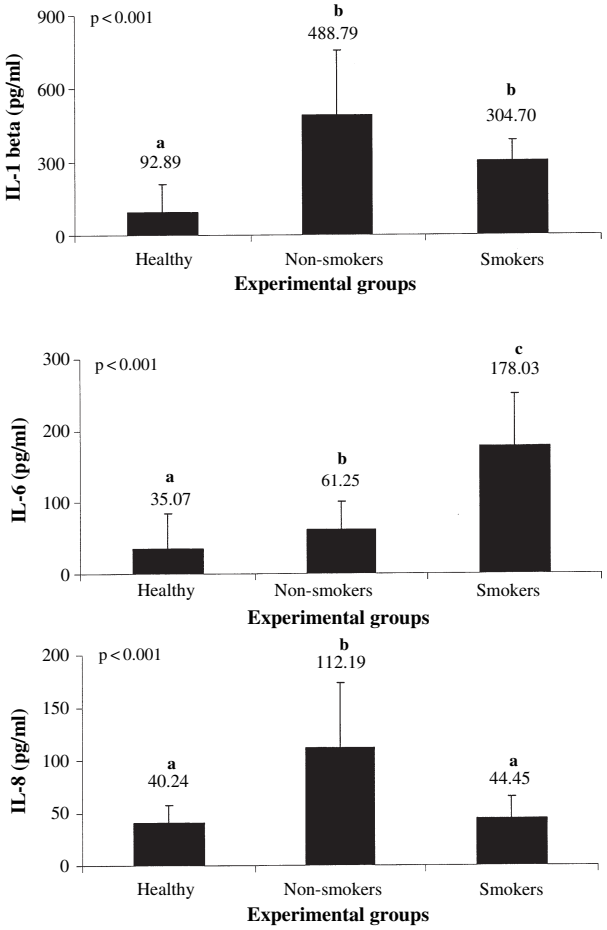

Fig. 3. Mean and standard deviation protein levels of interleukin-1α, interleukin-6 and interleukin-8 (pg/ml), as evaluated by enzyme-linked immunosorbent assay (ELISA), of gingival biopsies harvested from sites with a healthy periodontal condition and from sites with with moderate to severe chronic periodontitis (probing depth ≥7 mm) from nonsmokers, and from sites with comparable types of periodontitis from smokers. Mean values followed by different letters at the top of each bar indicate statistical differences determined by an intergroup analysis performed by the one-way analysis of variance and the Bonferroni *t*-test ($\alpha = 0.05$).

necrosis factor-α and interleukin-8 in the gingival crevicular fluid of smokers, compared with nonsmokers, has additionally been reported (10,19). In contrast, however, the levels of pro- and anti-inflammatory cytokines have been reported to be lower in association with smoking and its compounds. It seems that cigarette smoke contains potent inhibitors of both gene expression and protein production, at least for interleukin-1α, -8, -2 and tumor necrosis factor-α (20,21). Corroborating the data from *in vitro* studies, the production of interleukin-1α, -1ra, -1α and -4 has been reported to be significantly lower in the gingival crevicular fluid of smokers from periodontally diseased sites compared with nonsmokers (10,22). In addition, although not statistically significant, other studies have shown a tendency towards reduced levels of interleukin-1α and tumor necrosis factor-α in the gingival crevicular fluid of smoker subjects with moderate to severe periodontal disease (8,9). Thus, the findings of the present investigation are in line with previous studies showing that a pro-inflammatory cytokine (interleukin-1α), a chemokine (interleukin-8) and a protease (MMP-8) were lower in smokers than nonsmokers with comparable types of periodontal disease. It is interesting, however, that the negative effect of smoking on the production of cytokines such as interleukin-1α, interleukin-8, tumor necrosis factor-α and MMP-8, may be transient, with significant recovery occurring 8 wk after smoking cessation for MMP-8 (20).

Additionally, the results in the present study suggest that increased interleukin-6:interleukin-10 and RAN KL: osteoprotegerin ratios may be mechanisms involved in the modulation of periodontal disease by smoking. Interleukin-6 is known to stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage (23); however, the main activity of interleukin-6 in bone is its effect on osteoclastogenesis and bone resorption (24). Interleukin-6 has been shown to stimulate bone resorption, either by increasing RANKL or by directly inducing osteoclast formation, via a RANKL-independent mechanism (25). Interleukin-6 production was recently shown to be positively correlated with severity of diseased sites and therefore it has been suggested that interleukin-6 may play a role in modulating the inflammatory cascade of chronic periodontitis (26). Interleukin-10 has been reported to be a strong inhibitor of interleukin-6 production by *Porphyromonas gingivalis* lipopolysaccharide-stimulated human gingival fibroblasts and, as a result, to have a significant impact on modulating bone resorption in periodontal disease (27). Therefore, in the present study, data analysis suggests that by inhibiting mRNA levels of interleukin-10, smoking favors osteoclastogenesis modulated by an increased production of interleukin-6. Nevertheless, additional studies are necessary to investigate such a hypothesis in greater detail. With regard to the pro- and antiresorptive factors, similar levels of RANKL gene expression in the periodontitis sites in smokers and nonsmokers were observed in the present investigation and both presented higher levels than in healthy control sites. In contrast, mRNA levels of osteoprotegerin were higher for healthy sites than diseased sites, regardless of the smoking status, and

smoking resulted in the lowest osteoprotegerin expression. Therefore, an increase in the RANKL:osteoprotegerin ratio, favoring bone resorption, was promoted by smoking. To the authors' knowledge, this is the first study that shows a possible modulation by smoking of the mRNA levels of RANKL and osteoprotegerin in the periodontium. Evidence has emerged demonstrating that osteoclast formation from precursor cells, as well as osteoclast activation, requires the molecule RANKL (28). Osteoprotegerin, a secreted glycoprotein, is a decoy for RANKL that inhibits osteoclast formation and differentiation (29). In periodontal disease, a recent study in an animal model suggests the involvement of these two molecules in the pathogenesis of periodontitis (30). Subsequently, an increased concentration of RANKL and decreased concentration of osteoprotegerin were detected in the gingival crevicular fluid and periodontal tissues from patients with periodontitis, suggesting that these two molecules contribute to osteoclastic bone destruction in periodontal disease (14). Taken together, the results of the present study indicate that smoking modulation of bone destruction in periodontal disease may involve reduced levels of anti-inflammatory and anti-resorptive factors, such as interleukin-10 and osteoprotegerin, respectively, and may also involve high levels of pro-inflammatory cytokines, such as interleukin-6.

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