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Short communication

Increased interleukin-18 in gingival crevicular fluid from periodontitis patients

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Introduction: This study aimed to measure the levels of interleukin-18 (IL-18) in inflamed shallow sites and inflamed deep sites in patients with periodontitis and to compare the data with results from inflamed shallow sites in patients with gingivitis. A secondary aim was to examine the composition of the subgingival microbiota in the sampled sites.
Methods: Gingival crevicular fluid was collected from five gingivitis sites and five periodontitis sites from 18 patients with chronic periodontitis, and from five gingivitis sites from 15 patients with gingivitis. Samples from each site category were pooled and IL-18 levels were measured using an enzyme-linked immunosorbent assay. The subgingival microbiota was analyzed by checkerboard DNA–DNA hybridization.
Results: All clinical parameters and gingival crevicular fluid volumes were higher in periodontitis sites compared with gingivitis sites from patients with periodontitis and gingivitis. The total amount of IL-18 was higher in periodontitis sites than gingivitis sites in both periodontitis ($P = 0.018$) and gingivitis ($P = 0.002$) patients and was higher in gingivitis sites from periodontitis patients than in those from gingivitis patients ($P = 0.015$). There were higher levels of *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* (red complex species) in periodontitis sites compared with gingivitis sites in both the periodontitis and gingivitis patients ($P < 0.001$).
Conclusion: Levels of IL-18 were higher in patients with chronic periodontitis compared with patients with gingivitis, even at sites with similar pocket depths. The presence of similar levels of red complex species in gingivitis sites from periodontitis patients and from gingivitis patients suggested that the higher levels of IL-18 were not associated with a different microbial challenge.

Key words: checkerboard DNA–DNA hybridization; interleukin-18; periodontitis; red complex

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Periodontal diseases result from an inflammatory response to bacteria located in dental biofilms. The response may be confined to the gingival tissues or may progress, leading to attachment loss. It has been suggested that disease progression is the result of a combination of factors, including the presence of periodontopathic bacteria and high levels of proinflammatory cytokines (4). Several cytokines have been associated with periodontitis, such as interleukin-1β (IL-1β) (3), tumor necrosis factor-α (TNF-α) (2), and more recently IL-18 (13).

IL-18, formerly called interferon-γ (IFN-γ)-inducing factor, is a proinflammatory cytokine related to the IL-1 family that is produced by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, osteoblasts, and adrenal cortex cells (1). IL-18 is produced as a 24-kDa inactive precursor and is cleaved by IL-1β-converting enzyme (ICE, caspase-1) to generate a biologically active, mature 18-kDa peptide (5). The primary functions of IL-18 include the induction of IFN-γ and TNF-α in T cells and natural killer cells (16) and the upregulation of T helper type 1 (Th1) cytokines including IL-2, granulocyte–

macrophage colony-stimulating factor, and IFN-γ (1). It plays an important role in the innate immunity and has been shown to induce not only Th1 cytokines but also Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13 (6). IL-18 is described as being upregulated in a range of chronic diseases, including type 1 diabetes, lupus erythematosus, and Crohn's disease (9). This cytokine has been implicated in the activation of neutrophils (11) and the modulation of IL-1β production (7). However, the role of IL-18 in the pathogenesis of periodontal disease has yet to be determined. Johnson & Serio (8) showed a highly significant correlation between IL-18 concentration and gingival sulcular depth, suggesting that IL-18 could be a useful target for either preventive or palliative periodontal therapy. Orozco et al. (13) showed that IL-18 was higher in gingivitis sites from periodontitis patients when compared with gingivitis sites from control patients. One possible explanation for these findings is the difference in the local microbiota. Therefore, the aim of this study was to measure the levels of IL-18 in inflamed shallow sites and inflamed deep sites in patients with periodontitis and to compare these with the levels present in inflamed shallow sites from patients with gingivitis. A secondary aim was to examine the composition of the subgingival microbiota at the different site categories using the high-throughput checkerboard DNA–DNA hybridization technique.

Material and methods

Subjects and sampling

The patient group comprised 18 patients with chronic periodontitis (mean age ± SD: 47.5 ± 7.7 years) with at least six pockets ≥5 mm and 15 control patients with gingivitis (mean age: 41.4 ± 10.9 years) who were attending the Dental School of Rio de Janeiro State University (UERJ), Rio de Janeiro, Brazil. The Ethics Committee of Pedro Ernesto University Hospital (UERJ, Rio de Janeiro, Brazil) approved this study. The participants had no ongoing systemic disease or infections and gave informed consent to participate.

The clinical measurements were taken at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) of every tooth present, except for the third molars, with a Williams probe (PCP10 Color Coded Probe – Hu-Friedy Co., Chicago, IL), by the same calibrated examiner. The parameters measured were: percentage of sites with visible plaque, percentage of sites with bleeding on probing, probing pocket depth, and clinical attachment loss.

IL-18 and microbiological assessment

In the periodontitis patients (n = 18), five sites with pocket depth ≥5 mm and bleeding on probing, and five sites with pocket depth ≤3 mm and bleeding on probing were sampled. In the control group (n = 15), five sites with pocket depth ≤3 mm with bleeding on probing, were sampled. The sampled sites were isolated with cotton rolls and dried gently with an air syringe. Supragingival plaque was carefully removed and gingival crevicular fluid was collected with Periopaper strips (IDE, Amityville, NY). The strip was inserted into the pocket until mild resistance was felt and kept in place for 30 s. Samples visibly contaminated with blood were discarded. Gingival crevicular fluid volume was measured using a calibrated gingival crevicular fluid meter (Periotron 8000; IDE). The strips from sites of the same category were pooled within a subject, resulting in 18 periodontitis sites and 18 gingivitis sites from patients with periodontitis and 15 samples from patients with gingivitis. The samples were diluted with phosphate-buffered saline up to 1 ml and, after elution for 30 min at room temperature and removal of the paper strips, were immediately centrifuged at 3000 ***g*** for 5 min. The supernatant was collected and frozen at −20°C pending analysis. IL-18 was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (IL-18; MBL, Nagoya, Japan), according to the manufacturer's instructions. IL-18 data were expressed as total amount in pg/site (total) and as concentration in pg/μl (conc.).

Pooled subgingival plaque samples were taken from a subset of three periodontitis sites and three gingivitis sites used for IL-18 sampling from the periodontitis patients (n = 18) and from a subset of three gingivitis sites used in the IL-18 sampling from gingivitis control patients (n = 9). After the removal of supragingival plaque, subgingival plaque samples were taken with individual sterile Gracey curettes. The samples from the same site category were placed in separate microcentrifuge tubes containing 0.15 ml TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 7.6), and 0.15 ml of 0.5 M NaOH. Samples were analyzed for their content of 40 bacterial species using the Checkerboard DNA–DNA hybridization technique (15). In brief, the samples were lyzed and the DNA was placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics), with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to 40 subgingival species were hybridized in the individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA hybrids were detected using an antibody against digoxigenin, conjugated with alkaline phosphatase, and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL) and were read using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA), a computer-linked instrument that reads the intensity of the fluorescence signals resulting from the probe-target hybridization. Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the Storm Fluorimager and were converted to absolute counts by comparison with standards on the same membrane. Failure to detect a signal was recorded as zero.

Statistical analysis

The significance of differences for IL-18 levels and for clinical and microbial parameters between patients with chronic periodontitis and patients with gingivitis was determined using the Mann–Whitney *U*-test and the significance of differences between samples from the same patient was determined using the Wilcoxon signed-rank test. Probability values >0.05 were considered not significant.

Microbiological data for each species were expressed as counts $\times 10^5$ at each site, averaged within each subject, within each site category, and then averaged across patients. Mean counts for *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola* (red complex species); *Campylobacter gracilis*, *Campylobacter rectus*, *Campylobacter showae*, *Eubacterium nodatum*, *Fusobacterium nucleatum* subsp. *nucleatum*, *F. n.* subsp. *polymorphum*, *F. n.* subsp. *vincentii*,

Table 1. Species comprising the red, orange, and yellow complexes

Red complex
Tannerella forsythia
Porphyromonas gingivalis
Treponema denticola
Orange complex
Campylobacter gracilis
Campylobacter rectus
Campylobacter showae
Eubacterium nodatum
Fusobacterium nucleatum subsp. *nucleatum*
Fusobacterium nucleatum subsp. *polymorphum*
Fusobacterium nucleatum subsp. *vincentii*
Fusobacterium periodonticum
Peptostreptococcus micros
Prevotella intermedia
Prevotella nigrescens
Streptococcus constellatus
Yellow complex
Streptococcus gordonii
Streptococcus intermedius
Streptococcus mitis
Streptococcus oralis
Streptococcus sanguinis

Fusobacterium periodonticum, *Peptostreptococcus micros*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Streptococcus constellatus* (orange complex species); and *Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguinis* (yellow complex species) (14) were determined by adding the mean levels of each species that comprised each complex. Significant differences between gingivitis sites from gingivitis patients and gingivitis sites from periodontitis patients and between gingivitis sites from gingivitis patients and periodontitis sites from periodontitis patients were determined using the Mann–Whitney test, whereas the significant differences between gingivitis sites from periodontitis patients and periodontitis sites from periodontitis patients were determined using Wilcoxon signed-rank test. Table 1 lists the species that composed each complex.

Results

Mean pocket depth, attachment loss, % of sites with bleeding on probing, and gingival crevicular fluid volume were significantly higher in periodontitis sites compared with gingivitis sites from both periodontitis and gingivitis patients. However, there were no significant differences between gingivitis sites from gingivitis patients and gingivitis sites from periodontitis patients for these clinical parameters (Table 2).

Table 2. Mean values (±SD) for pocket depth (PD), attachment loss (AL), bleeding on probing (BOP), and gingival crevicular fluid (GCF), and total content (total) and concentration (conc.) of IL-18 in gingivitis sites from gingivitis patients (GG), gingivitis sites from periodontitis patients (GP) and periodontitis sites from periodontitis patients (PP)

	GG (n = 15)	$P1$	GP (n = 18)	$P2$	PP (n = 18)	$P3$
PD	2.34 (±0.27)	ns	2.32 (±0.23)	<0.0001	6.75 (±1.27)	<0.0001
AL	2.44 (±0.17)	ns	2.41 (±0.25)	<0.0001	7.22 (±1.51)	<0.0001
BOP	81%	ns	88%	0.014	100%	0.002
GCF	1.09 (±1.02)	ns	0.63 (±0.41)	<0.0001	1.50 (±0.59)	0.013
IL-18 total	15.15 (±11.24)	0.015	26.64 (±17.57)	0.018	33.51 (±22.52)	0.002
IL-18 conc.	23.37 (±25.18)	0.004	51.70 (±39.13)	0.001	27.43 (±21.86)	ns

$P1$, difference between GG and GP calculated with Mann–Whitney test; $P2$, difference between GP and PP calculated with Wilcoxon signed-rank test; $P3$, difference between GG and PP calculated with Mann–Whitney test. ns, not significant.

The total amount of IL-18 in gingival crevicular fluid was significantly higher in periodontitis sites when compared with gingivitis sites from periodontitis patients (P = 0.018) and gingivitis patients (P = 0.002). Gingivitis sites from periodontitis patients exhibited significantly higher levels of IL-18 compared with the levels in patients with gingivitis (P = 0.015). They also showed a significantly higher concentration of IL-18 when compared with periodontitis sites from periodontitis patients (P = 0.001) and gingivitis patients (P = 0.004). No significant difference was observed between gingivitis patients and periodontitis sites from periodontitis patients (Table 2).

There were significantly higher mean levels of the red complex species in samples from periodontitis sites compared with gingivitis sites from both periodontitis and gingivitis patients (P < 0.001) (Fig. 1). The levels of orange complex species were also significantly higher in periodontitis sites when compared with gingivitis sites from periodontitis patients. No significant difference was observed between gingivitis sites from gingivitis patients and gingivitis sites from periodontitis patients for any of the three microbial complexes described in Table 1 (Fig. 1).

Discussion

Higher gingival crevicular fluid levels of IL-18 were found in inflamed sites from periodontitis patients regardless of severity of disease when compared with patients with gingivitis only. Moreover, shallow pockets in periodontitis patients had a significantly higher total level and concentration of IL-18 when compared with shallow pockets in patients with gingivitis only. Gingivitis sites from periodontitis patients and gingivitis sites from gingivitis patients presented similar levels of the red, orange, and yellow complex species. The red and orange complex species were chosen for the analyses because of their association with periodontal disease initiation and progression, while the yellow complex represented host-compatible species. To our knowledge, this is the first paper to show that a similar microbiota can

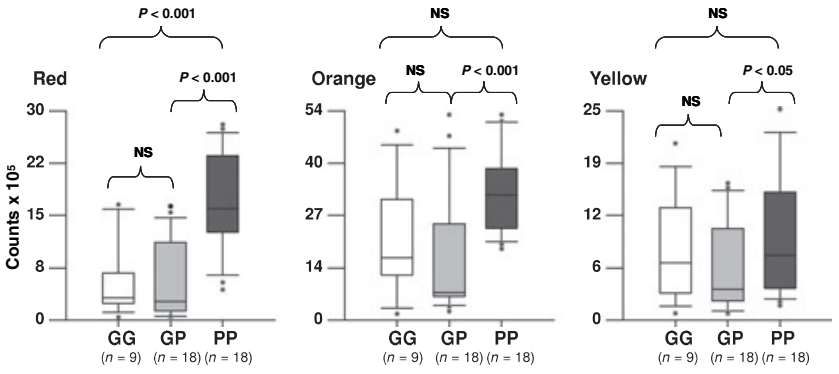

Fig. 1. Box plots of the mean levels (×10^5) of red, orange, and yellow complex species for gingivitis sites from gingivitis patients (GG) (white boxes), gingivitis sites from periodontitis patients (GP) (light gray boxes), and periodontitis sites from periodontitis patients (PP) (dark gray boxes). Significant differences between GG and GP sites and between GG and PP sites were determined using the Mann–Whitney test, whereas the significant differences between GP and PP sites were determined using Wilcoxon signed-rank test. ns, not significant.

elicit a different cytokine response in shallow pockets. However, only a subset of the microbiota was evaluated and both the gingival crevicular fluid and the subgingival biofilm samples were pooled in the different site categories. Thus, associations at individual sites could not be determined.

Understanding the progression of gingivitis to periodontitis has been a matter of intense investigation for several decades. A search for possible key cytokines involved in this process might clarify the mechanisms involved in the activation of the collagen breakdown, and consequently the loss of attachment. We previously showed that IL-1β might function as a distinguishing factor between gingivitis in periodontitis patients and gingivitis in otherwise healthy patients (3). The present results also suggest that IL-18 might function in the same way. Moreover, IL-18 seems to promote a priming effect on neutrophils, which could upregulate the production of IL-1β (7). Our group showed that the plasma levels of IL-18 might be involved in the process of periodontal destruction in juvenile patients with rheumatoid arthritis (12). Johnson & Serio (8) were the first to show the association between IL-18 and the degree of local periodontal destruction. They demonstrated that IL-18 concentration was higher in gingival biopsies adjacent to sites where the probing depth was >6 mm when compared to healthy sites. Orozco et al. (13) found IL-18 to be increased in shallow inflamed sites in periodontitis patients when compared with gingivitis sites in control patients. Our results are in accord with the findings of the Orozco group, but we have also investigated the microbiota. The microbiological findings showed that the red complex of species was significantly increased in the periodontitis sites from periodontitis patients when compared to gingivitis sites from both gingivitis and periodontitis patients, which has been previously reported (17, 18). Our findings suggest an association between the presence of red complex species and higher levels of IL-18 in patients with periodontitis, while the higher levels of IL-18 in shallow sites in these patients compared to patients with gingivitis alone could not be explained using differences in the microbiota. In addition, we have demonstrated that shallow sites in patients with chronic periodontitis expressed higher levels of IL-18 than shallow sites in gingivitis patients, irrespective of the presence of a similar microbial challenge.

The concentration of IL-18 was higher in gingivitis sites from periodontitis patients than in gingivitis sites from gingivitis patients and in periodontitis sites from periodontitis patients. The reason for that is unknown. We believed that it was the result of the smaller volume of gingival crevicular fluid collected on these samples. The higher leakage of plasma from the microvessels, measured as gingival crevicular fluid volume, might be responsible for the dilution of IL-18 in gingivitis sites from gingivitis patients and periodontitis sites from periodontitis patients. According to Lamster (10) the total amount of the mediator and not concentration of the mediator, in the gingival crevicular fluid sample can be reported when timed samples are collected. In the present study we choose to present both results.

There were certain limitations to the current study including the limited numbers of patients and of samples evaluated. In addition, there was pooling of gingival crevicular fluid and subgingival samples, which may have obscured individual site associations. However, the results of the present study demonstrated that IL-18 was increased in sites from periodontitis patients regardless of the degree of tissue destruction. The similarity in the levels of red, orange, and yellow complex species between shallow pockets in gingivitis patients and shallow pockets in periodontitis patients suggested that, within the scope of the study, the higher levels of IL-18 in shallow pockets in periodontitis patients were not associated with the microbial challenge.

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