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Interleukin-1β, interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis

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Introduction: Cytokines are of major importance in periodontal disease progression. Interleukin-12 (IL-12) stimulates interferon-γ production by T helper type 1 (Th1) cells while IL-18 induces Th1 responses when present with IL-12 but Th2 responses in the absence of IL-12. IL-1β has been correlated with periodontal disease destruction. This study determined the local concentrations of these cytokines in sites of gingivitis and periodontitis.
Methods: Gingival crevicular fluid was collected from two sites in each of 10 gingivitis patients and from two gingivitis sites and two periodontitis sites from each of 10 periodontitis patients. Serum samples were also collected. IL-1β, biologically active IL-12 p70, the IL-12 p40 subunit and IL-18 concentrations were determined by enzyme-linked immunoabsorbent assay.
Results: IL-1β and IL-18 concentrations were higher in the gingival crevicular fluid from periodontitis patients than in that from gingivitis patients; IL-18 concentrations were higher than those of IL-1β. Very little IL-12, either p40 or p70, was detected in the gingival crevicular fluid samples. In the serum, very low levels of cytokines were found. The level of serum IL-12 p40, however, was higher than in the fluid from periodontitis sites of periodontitis patients.
Conclusion: The local production of IL-1β and IL-18 in the gingival crevicular fluid increased with increasing inflammation and IL-18 was the predominant cytokine at both gingivitis and periodontitis sites. Very little IL-12 was detected with levels decreasing with increasing inflammation. These results suggest that there is an association between severity of periodontal disease and levels of IL-1, IL-12 and IL-18.

Key words: cytokines; gingival crevicular fluid; gingivitis; periodontitis

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Periodontal disease is the result of the inflammatory processes that occur in the tissues surrounding the teeth in response to bacterial accumulation (dental plaque) on the teeth, which is ultimately responsible for a progressive loss of collagen attachment of the tooth to the underlying bone (18). Periodontal pathogens elicit signals in resident gingival cells or in immune cells infiltrating the gingival tissues that result in immune responses; these responses lead to either the successful removal of the pathogens or to host-mediated destruction of the periodontal tissues. In this respect, cytokines in inflamed periodontal tissues, which have been the focus of numerous studies, have been cited as being of major importance in periodontal disease progression (25). The control of T helper type 1 (Th1) and/or Th2 expression is therefore fundamental in

understanding the immunoregulatory mechanisms in chronic periodontitis.

The cytokines interleukin-1 (IL-1), IL-12 and IL-18 play major roles in innate and adaptive immune responses. IL-1 is a mediator of the host inflammatory response to infections and other inflammatory stimuli (5). IL-1β has been found to be significantly increased in the periodontal tissues and gingival fluid from diseased sites, compared with healthy sites (13, 20, 34). IL-1β up-regulates matrix metalloproteinases and down-regulates tissue inhibitors of metalloproteinase production (24, 28) and is also a powerful and potent bone-resorbing cytokine (30, 32) which suggests that it has a role in degrading the extracellular matrix in periodontitis (30).

IL-12 is a heterodimer comprised of p35 and p40 subunits, which form the bioactive IL-12 (p70). However, the p40 subunit may antagonize the binding of the bioactive IL-12 heterodimer to the IL-12 receptor such that the ratio between IL-12 p70 and the p40 homodimer reflects the total activity of bioactive IL-12 (10). IL-12 is a principal mediator of the early innate immune response to intracellular microbes and is a key inducer of cell-mediated immunity. Its most important action is to stimulate interferon-γ (IFN-γ) production by T cells and natural killer cells and so promote Th1 responses (10, 21, 23, 27). IFN-γ also promotes Th1 development by stimulating the production of IL-12 by macrophages and the expression of functional IL-12 receptors on T cells (23). The importance of IL-12 is not limited to initiating an immune response, but may contribute to maintaining immunity because Th1 responses rapidly fade in the absence of IL-12 (29).

IL-18 is a proinflammatory cytokine that belongs to the IL-1 cytokine family. This cytokine was originally identified as an IFN-γ-inducing cytokine produced by natural killer and T cells. IL-18 acts synergistically with IL-12 to promote IFN-γ production and Th1 cell development but does not depend on IL-12 for its activity (2, 19, 22, 26). In the absence of IL-12, IL-18 induces the production of Th2 cytokines from T cells, natural killer cells and basophils/mast cells (22) suggesting that this cytokine can stimulate both Th1 and Th2 responses.

Although IL-1 levels in periodontal disease have been studied extensively, few studies have reported on the presence of IL-12 and IL-18 in particular. We hypothesize that if local levels of IL-12 decrease with disease progression, or alternatively, if there are excess levels of the p40 subunit, which will prevent IL-12 binding to its receptor, then Th1 responses will be reduced in periodontitis lesions. As for IL-1, we hypothesize that IL-18 levels in the gingival crevicular fluid will increase with increased periodontal inflammation. The presence or absence of IL-12 will then determine whether a Th1 or Th2 response predominates with periodontal disease progression. The aim of this study therefore, was to assess the local cytokine response in relation to clinical periodontal status, by determining the concentrations of IL-1β, IL-12 and IL-18 in the gingival crevicular fluid obtained from gingivitis and periodontitis sites as well as in the serum samples of gingivitis and periodontitis patients.

Materials and methods

Subjects

Twenty healthy adult patients attending the Periodontal Clinic, School of Dentistry, University of Queensland for routine dental and radiographic examinations were invited to take part in the study. All subjects were in good general health and had not received previous periodontal therapy or taken antibiotics, immunomodulatory or anti-inflammatory drugs in the 6 months before the study. Pregnant women, smokers, patients requiring antibiotic prophylaxis for dental treatment, patients with any systemic disease, or those who were on long-term medication that could affect the expression of gingivitis or periodontitis were excluded from the study. A written explanation of the purpose of the study was provided for each subject and signed consent according to the Helsinki Declaration was obtained. Institutional ethics review committee approval for the study was obtained.

Full periodontal examinations were carried out on each patient and the participants were then divided into two groups of ten patients each. These groups were a gingivitis group and a periodontitis group and patients were assigned on the basis of the following clinical parameters. The gingival index (17), bleeding on probing index (33), probing depth and clinical attachment level were recorded using a conventional periodontal probe (Hu-Friedy, Chicago, IL). For the gingivitis group, only patients with gingival indices of 1 and/or 2 and no sites with probing depth or clinical attachment level greater than 4 mm were selected. For the periodontitis group, only patients with moderate-to-severe periodontal disease, as evidenced by at least four teeth with at least one site/tooth with a probing depth ≥6 mm, with positive bleeding on probing and with bone loss confirmed by radiography, were selected. The gingivitis and periodontitis groups each comprised five men and five women. The ages of the patients in the gingivitis group ranged between 27 and 57 years with an average of 40.2 years, whereas those of the periodontitis group were between 37 and 81 years old with an average age of 52.2 years.

Gingival crevicular fluid sampling

Gingival crevicular fluid was collected from two sites per patient in the gingivitis group. In the periodontitis group, gingival crevicular fluid was collected from two sites with gingivitis and from two sites with periodontitis per patient. Sites with profuse bleeding were avoided as test sites.

The identified test sites were isolated with cotton rolls and saliva ejector to avoid salivary contamination. The sites were gently air-dried and supragingival plaque was lightly removed with a sterile periodontal curette. A Millipore® filter strip was then inserted gently into the sulcus/pocket until mild resistance was felt, care being taken to avoid mechanical injury, and left in place for 30 s. This procedure was repeated three times per site. After collection of the gingival fluid, the strips were immediately placed in sterile Eppendorf tubes containing 10 mM NaH_2PO_4 and 150 mM NaCl, pH 7.2, followed by mixing and centrifugation at 800 **g**. The supernatants were collected and stored at −80°C until subsequent cytokine analysis.

Serum collection

At the time of the appointment 5-ml samples of venous blood were collected from each patient. The blood samples were centrifuged and the serum was collected and stored at −20°C.

Detection of IL-1β, IL-12 and IL-18

The presence of cytokines in the gingival crevicular fluid and serum samples was determined using commercial enzyme-linked immunosorbent assay kits. The kits detected human IL-1β, IL-12 p70 (the assay recognizes biologically active heterodimeric p70 which is composed of a 35-kDa light chain and a 40-kDa heavy chain, and does not cross-react with the p40 antagonist), IL-12 total (recognizes p40 in the monomeric, dimeric, or heterodimeric form) and IL-18 (Bender MedSystems, Vienna, Austria). The protocols

were followed and the optical densities of the wells were then read in a Bio-Rad microplate reader, model 3550 (Bio-Rad Laboratories, Regents Park, NSW, Australia) at an absorbance of 450 nm as the primary wavelength and 655 nm as the reference wavelength. Standard curves compiled using the recombinant human standards provided in the kits were used for each plate, allowing the concentrations of each cytokine (pg/ml) in the samples to be determined.

Protein concentrations (μg/ml) in all the gingival crevicular fluid and serum samples were determined using the BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The concentration of cytokines in each sample was then determined as ng/μg of protein.

Statistical analysis

Multivariate analysis of variance using the general linear model was used to test for differences between the concentrations of each cytokine in the gingival crevicular samples and the serum samples. Comparisons of each cytokine were made within each group (fluid and serum) and between gingival crevicular fluid and serum samples. Pairs of groups were then tested for significance using the Student's *t*-test. The Minitab statistical package (Minitab Inc., State College, PA) was used to perform the analyses.

Results

Gingival crevicular fluid

The mean concentration of IL-1 was significantly lower in the gingival crevicular fluid from gingivitis patients than in that from periodontitis sites from periodontitis patients ($P = 0.039$) and was almost significantly lower than in that from gingivitis sites in periodontitis patients ($P = 0.054$) (Fig. 1A). This was also true for IL-18, which was lower in the gingivitis subjects than in the gingivitis sites of periodontitis patients ($P = 0.005$); again there was a trend when compared with periodontitis sites ($P = 0.059$) from periodontitis patients (Fig. 1D).

There was very little IL-12, either p40 or p70, detected in any gingival crevicular fluid samples (Fig. 1B,C). The mean concentrations of IL-1β ($P = 0.000$) and of IL-18 ($P = 0.001$) in the gingival crevicular fluid of gingivitis patients were significantly higher than both IL-12 p70 and IL-12 p40. The same was true of both cytokines in the fluid from gingivitis sites ($P = 0.000$ for both IL-1β and IL-18) and periodontitis sites ($P = 0.000$ for IL-1β and $P = 0.001$ for IL-18) of periodontitis patients (Fig. 1A–D).

The mean concentration of IL-1 was significantly lower than that of IL-18 in the fluid from gingivitis sites in periodontitis patients ($P = 0.002$) and also from periodontitis sites in periodontitis patients ($P = 0.026$) (Fig. 1A,D).

Serum

There was no or very little IL-1, IL-12 p70 or IL-18 detected per μg of protein in the serum samples of both gingivitis and periodontitis patients (Fig. 1A,B,D). Although only low levels of IL-12 p40 were detected, the mean concentration in the serum of gingivitis and periodontitis patients was significantly higher than that of IL-1 ($P = 0.001$ and 0.000, respectively), IL-12 p70 ($P = 0.001$ and 0.000, respectively) and IL-18 ($P = 0.026$ and 0.000, respectively) (Fig. 1A–D).

Gingival crevicular fluid compared with serum

The mean concentration of IL-1 ($P = 0.001$) and IL-18 ($P = 0.015$) in the gingival fluid of gingivitis patients was significantly higher than in the serum of these subjects. The mean concentration of IL-1 and IL-18 in the gingival crevicular fluid from gingivitis sites ($P = 0.000$ for IL-1 and $P = 0.004$ for IL-18) and also from periodontitis sites ($P = 0.000$ for IL-1 and $P = 0.018$ for IL-18) of periodontitis patients was significantly higher than their respective concentrations in the serum of periodontitis patients (Fig. 1A,D).

The mean level of IL-12 p40 in the gingival crevicular fluid from periodontitis sites of periodontitis patients was significantly lower than in the serum samples of these patients ($P = 0.041$) (Fig. 1C).

Gingival crevicular fluid minus serum

To obtain an accurate measure of the level of cytokines produced locally in the gingival tissues, the concentration of each cytokine in the serum sample of each patient was subtracted from that found in the gingival crevicular fluid for that patient. The mean concentrations are listed in Table 1. As a result of the very low or zero concentrations of the cytokines in the serum samples, the local concentrations are the same or very similar to those found in the gingival crevicular fluid samples.

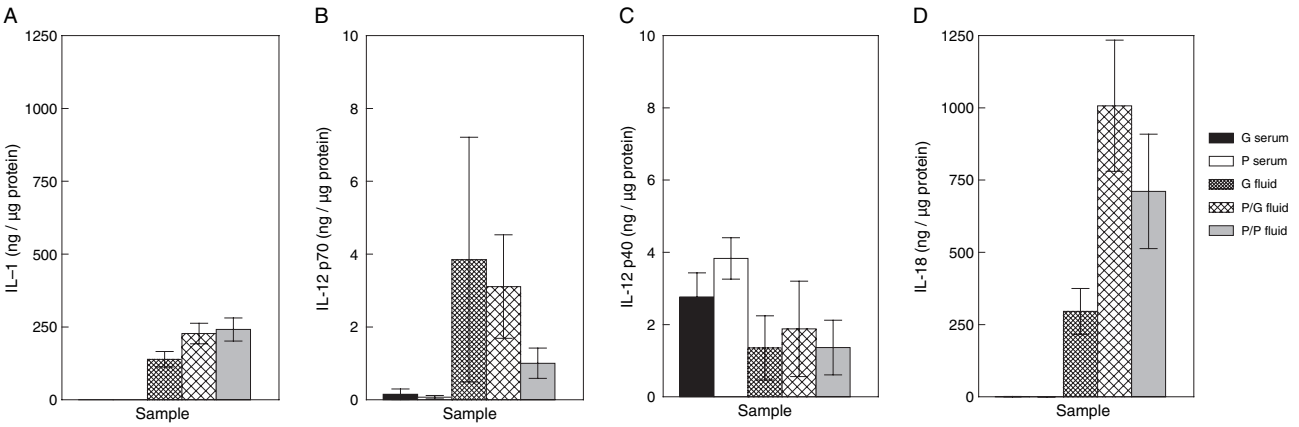

Fig. 1. Mean concentrations ± standard error of the mean of IL-1 (A), IL-12 p70 (B), IL-12 p40 (C) and IL-18 (D) in the gingival crevicular fluid from gingivitis sites of gingivitis patients (G fluid) and from gingivitis sites (P/G fluid) and periodontitis sites (P/P fluid) of periodontitis patients. The cytokine levels were also determined in the serum samples of the gingivitis (G serum) and periodontitis (P serum) patients.

Table 1. Mean cytokine levels (ng/μg protein) ± SE in the gingival crevicular fluid of gingivitis and periodontitis patients

	Cytokines			
Group	IL-1	IL-12 p70	IL-12 p40	IL-18
Gingivitis – G	139.10 ± 26.90	3.77 ± 3.36	1.02 ± 0.71	294.90 ± 79.40
Periodontitis – G	227.40 ± 35.30	3.08 ± 1.40	1.56 ± 1.17	1006.00 ± 227.00
Periodontitis – P	241.40 ± 39.50	0.93 ± 0.40	0.70 ± 0.40	710.00 ± 198.00

G, gingivitis sites; P, periodontitis sites; SE, standard error of the mean.

Discussion

The results of the present study have shown that the local production of IL-1 in the gingival crevicular fluid increased with increasing inflammation. Numerous studies have examined IL-1 in periodontal disease and demonstrated increases in the periodontal tissues and gingival crevicular fluid from diseased sites compared with healthy sites (13, 20, 34). Recently, IL-1α, IL-1β and IL-1 receptor antagonist were all found to be higher in the gingival crevicular fluid from diseased sites and their levels were associated with periodontal status. IL-1 and IL-1 receptor antagonist levels were significantly reduced after the treatment of diseased pockets (9, 12). IL-1 is a powerful and potent bone-resorbing cytokine (30) that also increases collagenase production by periodontal ligament fibroblasts (24). In this respect, IL-1 levels have been determined to be correlated with the alveolar bone loss score (14). It is also possible that gingival B cells may be a major source of IL-1 in periodontitis (11). Periodontopathic bacteria have been shown to be polyclonal B-cell activators and it has been speculated that periodontal tissue destruction may be a result of the production of large amounts of IL-1 by polyclonally activated B cells (31).

At low concentrations, IL-1 functions as a mediator of local inflammation. However, when secreted in larger quantities, IL-1 enters the bloodstream and exerts endocrine effects. These include the ability to cause fever, to induce the synthesis of acute-phase plasma proteins by the liver and to initiate metabolic wasting (1). The present study demonstrated zero concentrations of IL-1β in the serum samples of both gingivitis and periodontitis patients. This is not surprising however, because the patients chosen to be in the study were all in good general health and had not taken antibiotics, immunomodulatory or anti-inflammatory drugs within 6 months of the study commencing. Also not included in the study were those individuals who were smokers, or who required antibiotic prophylaxis for dental treatment, or who had any systemic disease, or were on long-term medication. These strict guidelines for choosing patients would also account for the fact that very low or no levels of all cytokines were detected in the serum samples of both gingivitis and periodontitis patients.

The results of the present study also showed that the local production of IL-18 in the gingival crevicular fluid was increased in periodontitis patients compared with gingivitis patients. A similar result in a recent study demonstrated higher IL-18 concentrations in gingival biopsies adjacent to sites where the probing depth is >6 mm than from healthy sites (15). The present study also demonstrated that IL-18 concentrations were significantly higher than IL-1 at both gingivitis and periodontitis sites, inferring a high significance for this cytokine in periodontal disease. In this respect, a report has shown that in collaboration with IL-12, IL-18 promotes IFN-γ production in T cells and Th1 cell development. However, IL-18 alone can induce IL-4, IL-5, IL-10 and IL-13 from both T cells and natural killer cells and also induces prostaglandin E_2 production from activated macrophages (16). IL-18 therefore enhances IL-12-induced Th1 responses and can also stimulate Th2 responses in the absence of IL-12 (30). The results of the present study found very little locally produced IL-12 in gingivitis and periodontitis patients, either as the bioactive form or as the p40 homodimer. This strongly suggests that in the gingival tissues, the high levels of IL-18 in the absence of IL-12 may steer T-cell responses towards a Th2 phenotype.

Interestingly, the in present study, the levels of the p40 subunit of IL-12 in the serum were higher than the levels of bioactive p70 heterodimer and further, serum IL-12 p40 concentrations were higher than in the gingival crevicular fluid of periodontitis sites of periodontitis patients. This suggests that any IL-12 present in the serum samples would not be bioactive. Furthermore, while IL-12 p40 levels were similar in the gingival fluid from gingivitis or periodontitis sites, there was a trend towards a decrease in IL-12 p70 concentrations in the periodontitis sites. In support of the results of this study, IL-12 levels in biopsies adjacent to the >6-mm depth gingival sulcus have been reported to be decreased compared to gingival tissues adjacent to ≤3 and 4–6 mm depths (6). An *in vitro* study of periodontitis patients has shown a significant down-regulation of IL-12 p70 in whole blood cell cultures stimulated with *Escherichia coli* lipopolysaccharide compared with cells from control subjects. Furthermore, IL-12 p70 levels were greatly increased after the patients underwent periodontal therapy (7). No differences between the subject groups were seen with respect to IL-12 p40 levels (8). Another study showed that when peripheral blood mononuclear cells from healthy subjects were treated with sonicated extracts of *Porphyromonas gingivalis*, B cells produced a large amount of IL-10 but no IL-12, although the major producing cells were monocytes (3). However, no increase in macrophage numbers and little evidence of macrophage activation have been demonstrated in advanced periodontitis compared with minimally inflamed tissues (4), suggesting that in the gingival tissues, macrophages may not produce significant quantities of IL-12.

Contrary to the results of these studies, another report found that the total amount of IL-12 was significantly higher in gingival crevicular samples from chronic periodontitis and gingivitis sites than from healthy sites (35). Yamazaki et al. (36) demonstrated that while there was no difference in the mean expression of IL-12 p35 mRNA in the gingival tissues of patients with chronic periodontitis and their peripheral blood mononuclear cells, there was a higher expression of IL-12 p40 in the gingival tissues in six out of 16 samples. This increase in the IL-12 antagonist suggests a decrease in IL-12 bioactivity in periodontitis tissues.

P. gingivalis is a major periodontopathogen and the cysteine proteinases (gingipains) produced by this microorganism have been associated with virulence in destructive periodontitis. These gingipains are able to hydrolyse IL-12 and reduce the IL-12-induced IFN-γ production from $CD4^+$ T cells. The inactivation of IL-12 by the gingipains could disrupt the cytokine balance and favor Th2 activities in periodontitis lesions (37). Also, *P. gingivalis* lipopolysaccharide has been found to establish an activation loop with IL-12 and IFN-γ where stimulated monocytes primed with IFN-γ released IL-12, resulting in

enhanced IFN-γ production by T cells. Furthermore, while *P. gingivalis* gingipains reduced IFN-γ accumulation in the presence of IL-12, when added in the presence of *P. gingivalis* lipopolysaccharide, IFN-γ levels were partially restored (37, 38). These authors suggested that the release of gingipains independently of lipopolysaccharide could lead to the down-regulation of Th1 responses, while gingipains together with lipopolysaccharide could have a net stimulatory effect (38).

In conclusion the results of the present study have shown that the local production of IL-1 and IL-18 in the gingival crevicular fluid increased with increasing inflammation and IL-18 was the predominant cytokine at both gingivitis and periodontitis sites. There was very little IL-12 detected, either as the bioactive form with levels decreasing with increasing inflammation or as the p40 subunit. These results suggest that there is an association between severity of periodontal disease and levels of IL-1, IL-12 and IL-18.

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