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Short communication

Expression of cytokines in the gingival crevicular fluid and serum from patients with inflammatory bowel disease and untreated chronic periodontitis

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Background and Objective: Previous studies have reported an increased prevalence/severity of chronic periodontitis in patients with inflammatory bowel disease. However, the pathogenesis of periodontal lesions in such patients has not been characterized. The aim of this pilot study was to characterize the pattern of expression of cytokines in the gingival crevicular fluid and serum from patients with untreated chronic periodontitis and Crohn's disease, ulcerative colitis and systemically healthy controls.

Material and Methods: Fifteen patients with Crohn's disease, 15 patients with ulcerative colitis and 15 controls participated in the study. All subjects had been diagnosed with untreated chronic periodontitis. The clinical parameters evaluated were clinical attachment loss, bleeding on probing and percentage of plaque. The gingival crevicular fluid was sampled from four shallow and four deep periodontal sites of each patient. The concentrations of the cytokines interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, interferon-γ and tumor necrosis factor-α were measured using a commercially available Lincoplex kit and the concentration of IL-18 was measured using an ELISA.

Results: Multiple comparisons analysis showed that clinical attachment loss, bleeding on probing, percentage of plaque and volume of gingival crevicular fluid were similar across the groups. The concentration of IL-4 in the gingival crevicular fluid differed significantly between groups in shallow sites ($p = 0.046$), with higher values found for the controls. In serum, the concentration of IL-18 was also significantly different between groups, with lower values found for controls ($p = 0.018$).

Conclusion: This study showed a higher concentration of IL-18 in serum, but not in the gingival crevicular fluid, from periodontitis patients with Crohn's disease or ulcerative colitis compared with controls. The expression of cytokines was similar in the gingival crevicular fluid from patients with untreated chronic periodontitis

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who also had Crohn's disease or ulcerative colitis and in systemically healthy controls with untreated chronic periodontitis.

Periodontal disease occurs through a combination of factors, including the presence of periodontopathic bacteria and high levels of proinflammatory cytokines (1). Several cytokines involved in innate immunity have also been associated with periodontitis, such as interleukin (IL)-1β (2–4), tumor necrosis factor-α (TNF-α) (5,6) and IL-18 (7,8). However, the levels of IL-4, a T-helper type 2 (Th2) cytokine that inhibits interferon-γ (IFN-γ)-mediated activation of macrophages, decrease with increasing inflammation (9) and with collagen loss (10). The imbalance between cytokines of adaptive and innate immune systems might be crucial for the onset and progression of periodontal disease.

The literature is controversial about the prevalence and severity of periodontal disease in patients with inflammatory bowel disease (IBD). Flemmig *et al.* (11) reported that, compared with the assessment of oral health of United States adults, IBD patients had an 11.9% higher prevalence, but a lower severity, of periodontal disease. Grössner-Schreiber *et al.* (12) showed no distinct differences between IBD patients and controls regarding clinical attachment level. We, however, have previously shown that periodontal disease is significantly more pronounced in IBD patients than in systemically healthy controls (13). The reason for an increased prevalence of periodontitis is not clear. In this study we aimed to explore some aspects of the inflammation – oral and systemic – that may be of relevance for the initiation and progression of periodontitis.

Similarly to periodontal disease, the start and progression of IBD is related to an aberrant immune response to a normal microbiota (14). IBD is usually subdivided into Crohn's disease (CD) and ulcerative colitis (UC). They differ regarding their immunopathogenesis concerning $CD4^+$ T-cell differentiation. CD is considered to be a T-helper type 1 (Th1)-mediated disease, whereas UC exhibits a modified Th2 cytokine response (15). Proinflammatory cytokines, such as IL-6, TNF-α and IL-18, have been shown to exert an important role in IBD immunopathogenesis, which is clinically relevant for therapies and diagnostics in this field (16), while IL-4 and IL-10 are considered to have potential effects on the remission of IBD when administered systemically (17). Therefore, it is a matter of concern whether cytokines involved in the pathogenesis of IBD might also have an effect on the onset of periodontal disease. If differences in cytokine expression were shown that could explain an increased prevalence of periodontitis, this would have implications for periodontal pathogenesis in general.

Therefore, the aim of this pilot study was to characterize the pattern of expression of cytokines in the gingival crevicular fluid and serum from patients with untreated periodontitis who also had CD or UC, and from patients with untreated periodontitis who were otherwise systemically healthy.

Material and methods

Subject selection

Thirty IBD patients – 15 with CD (seven women and eight men; mean age 38.2 ± 11.4 years), 15 with UC (eight women and seven men; mean age 45.0 ± 10.5 years) – and 15 systemically healthy controls (eight women and seven men; mean age 42.1 ± 7.8 years), all with chronic untreated periodontitis, participated in this study. These patients represent a subset sample population of a previous study (13). The patients previously diagnosed with periodontitis were invited to return for gingival crevicular fluid collection. Among the patients who returned, those who presented at least five inflamed sites with probing pocket depths of ≥ 5 mm and clinical attachment loss of ≥ 3 mm in different teeth were selected for the present study.

CD and UC patients were outpatients attending the IBD clinics at Pedro Ernesto University Hospital of the Rio de Janeiro State University and at the Clementino Fraga Filho University Hospital of Federal University of Rio de Janeiro, both in Rio de Janeiro, Brazil. The disease diagnosis was 7.7 years in the CD group and 7.8 years in the UC group. In the CD group, five patients had active disease and 10 were in remission. CD patients were taking immunomodulators ($n = 7$), aminosalicylates ($n = 4$) and immunomodulators + aminosalicylates ($n = 2$). Two CD patients were not taking any medication. In the UC group, the disease was active in three patients and was in remission in 12. In the UC group, patients were taking immunomodulators ($n = 1$), aminosalicylates ($n = 9$) and immunomodulators + aminosalicylates ($n = 5$). The control group was comprised by subjects who did not show any clinical signs of ongoing systemic disease. IBD and control patients had not taken antibiotics for at least 6 mo, or anti-inflammatories for at least 2 mo, before the start of the study. Subjects who were pregnant or had previously received periodontal therapy were excluded. Three CD patients, one UC patient and two controls were smokers. This study was approved by the Committee on Ethics and Research of the University Hospitals Pedro Ernesto and Clementino Fraga Filho, and by the Karolinska Institutet Stockholm, Sweden. All subjects signed an informed consent form.

Clinical examination

Clinical parameters were evaluated at six sites in all teeth, excluding third molars. The parameters included probing pocket depth, clinical attachment loss, presence of plaque and

presence of bleeding on probing. Probing pocket depth, clinical attachment loss and bleeding on probing were measured using a conventional periodontal probe (Hu-Friedy, Chicago, IL, USA). Chronic periodontitis was diagnosed according to criteria described by the American Academy of Periodontology (AAP) (18), whereby each patient should present at least five inflamed sites showing bleeding on probing, a probing pocket depth of ≥ 5 mm and clinical attachment loss of ≥ 3 mm in different teeth. After diagnosis of chronic periodontitis (13), the sites to be sampled were selected. In every subject, gingival crevicular fluid from four shallow sites (with clinical signs of inflammation, a probing pocket depth of ≤ 3 mm and clinical attachment loss of ≤ 1 mm) and four deep sites (with clinical signs of inflammation, a probing pocket depth of ≥ 5 mm and clinical attachment loss of ≥ 3 mm) were collected 1 wk after the clinical examination. The sites sampled were in different teeth. Two periodontists, involved in the examination and gingival crevicular fluid sampling, achieved substantial interexaminer reproducibility ($\kappa = 0.775$, $p < 0.001$) for all the variables analyzed.

Gingival crevicular fluid sampling

Gingival crevicular fluid was sampled with filter strips for 30 s (ProFlow Inc., Amityville, NY, USA) and the volume of gingival crevicular fluid absorbed by each strip was determined using a chairside located Periotron 8000® (ProFlow Inc.). Before sampling, supragingival plaque was gently removed, and the teeth were air-dried and isolated with cotton rolls. Strips with blood marks were discarded. All samples from the same category (deep or shallow) in each subject were pooled together in an Eppendorf tube containing 1 mL of phosphate-buffered saline. After elution for 40 min at room temperature without shaking, the samples were centrifuged at 3000 *g* for 5 min, and the supernatant was collected and immediately frozen at −20°C until analysis. The arbitrary number observed in the Periotron 8000® was converted into microliters using a standard curve obtained after calibration of the Periotron 8000®. We performed a recovery test of IL-1β from the strips using an ELISA, and it was above 90%. Therefore, the detection of IL-1β was used as the gold standard.

Blood collection

Eight milliliters of venous blood was collected from each patient. The tubes containing blood were stored at 4°C for 30 min, centrifuged, and the serum samples thus obtained were aliquoted into Eppendorf tubes and stored at −20°C until analysis.

ELISA for IL-18

One-hundred microlitres of the gingival crevicular fluid sample was analyzed for IL-18 using a commercially available ELISA (ELISA MBL, Nagoya, Japan), in duplicate, according to the manufacturer's protocol. For analyzing the concentration of IL-18 in serum, a twofold dilution was performed by adding 50 μL of the calibrator diluent to 50 μL of serum per well. The absorbance was read at 450 nm using a spectrophotometer (Labsystems Multiscan, Vantaa, Finland). The level of IL-18 was determined as the total amount (in pg) per site.

Luminex assay

A 50-μL sample was analyzed for IL-1β, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α and IFN-γ using a commercially available Lincoplex kit (Millipore, St Charles, MO, USA) within a Luminex analyzer, according to the manufacturer's instructions. The results were calculated through the Bio-Plex Manager Software (Bio-Rad Laboratories, Hercules, CA, USA), and the cytokine levels were determined in pg. The concentration in gingival crevicular fluid was expressed in pg/μL and in plasma as pg/mL.

Statistical analysis

The groups were tested for normality using the Shapiro–Wilk test. The significance of differences among groups was calculated using analysis of variance (ANOVA) for clinical data and the Kruskal–Wallis test for immunological data. Univariate comparisons between a given group and the control were performed using the Mann–Whitney *U*-test with the significance value of < 0.05. The correlations were calculated using Pearson's correlation test with the significance value of < 0.01. Analyses were performed using the software package STATISTICA (StatSoft, Inc. 2005, Tulsa, OK, USA) version 7.1.

Results

Multiple comparisons analysis showed that age, clinical attachment loss, bleeding on probing, percentage of plaque and gingival crevicular fluid volume did not differ significantly between the groups (Table 1). The total amount of IL-4 in the gingival crevicular fluid differed significantly between groups in shallow sites ($p = 0.046$), with higher values observed for the controls. In serum, IL-18 also showed a significant difference between groups, with lower values observed for controls ($p = 0.018$) (Table 3).

Univariate comparisons showed that CD patients had a significantly lower total amount of IL-18 in the gingival crevicular fluid obtained from shallow sites, and a significantly lower total amount of IL-4 in the gingival crevicular fluid obtained from deep sites, when compared with controls (Table 2). UC patients were found to have a significantly lower total amount of IL-4 in the gingival crevicular fluid obtained from shallow sites, and a significantly higher total amount of IL-6 in the gingival crevicular fluid obtained from deep sites, when compared with controls (Table 2). No significant difference was observed between CD and UC groups. The total amounts of IL-10, IL-12p40, IL-12p70 and TNF-α were below the levels of detection in gingival crevicular fluid. Regarding the concentration of cytokines, there was no significant difference between the groups (Table 2).

Both the CD and UC groups had significantly higher serum levels of IL-18 than controls (Table 3). There was no significant difference regarding the other cytokines analyzed. The

Table 1. Age, probing pocket depth (PPD), clinical attachment loss (CAL), percentage of sites with plaque (% Plaque) and volume of gingival crevicular fluid (Volume) of 15 Crohn's disease (CD) patients and 15 ulcerative colitis (UC) patients compared with 15 healthy controls (Control)

	Shallow pockets				Deep pockets			
	CD	UC	Control	ANOVA	CD	UC	C	ANOVA
Age (years)	38.2 (11.4)	45.0 (10.5)	42.1 (7.8)	0.192				
PPD (mm)	2.6 (0.3)	2.5 (0.4)	2.8 (0.2)	0.065	5.2 (1.4)	5.3 (1.0)	5.3 (1.5)	0.785
CAL (mm)	0.6 (0.3)	0.7 (0.3)	0.9 (0.1)	0.243	4.3 (2.3)	3.9 (1.3)	3.7 (1.7)	0.569
% Plaque	47.3 (43.7)	56.1 (37.6)	73.3 (35.8)	0.175	63.8 (35.0)	53.9 (36.9)	64.3 (39.6)	0.653
Volume (μL)	1.9 (0.9)	1.8 (1.2)	1.8 (0.8)	0.878	2.9 (1.0)	3.0 (1.5)	2.6 (1.1)	0.679

Data for CD, UC and Control patients are expressed as mean (SD).

Table 2. Cytokine levels in shallow pockets and deep pockets of 15 Crohn's disease (CD) patients and 15 ulcerative colitis (UC) patients compared with 15 healthy controls (Control)

		Shallow pockets				Deep pockets			
		CD	UC	Control	Kruskal– Wallis	CD	UC	Control	Kruskal–Wallis
Total amount	IL-18	38.0 (11.7)*	43.9 (23.4)	48.7 (42.8)	0.065	31.1 (21.5)	35.4 (22.2)	28.4 (32.4)	0.492
	IL-1β	0.4 (1.3)	0.7 (1.3)	1.2 (2.0)	0.55	2.5 (3.4)	2.1 (5.6)	1.3 (1.6)	0.554
	IL-4	0.7 (1.5)	0 (0.8)*	1.2 (2.1)	0.046	0.9 (1.0)*	1.0 (1.7)	1.9 (2.6)	0.070
	IL-6	0 (0.0)	0 (0.7)	0 (0.0)	0.283	0 (0.9)	0.3 (0.6)*	0 (0.3)	0.068
	IFN-γ	0 (0.2)	0 (0.2)	0 (0.0)	0.518	0 (0.2)	0 (0.2)	0 (0.0)	0.326
Concentration	IL-18	24.0 (28.6)	38.5 (65.5)	29.6 (31.3)	0.219	7.7 (10.2)	13.4 (17.9)	12.0 (14.9)	0.351
	IL-1β	0.2 (0.9)	0.2 (0.6)	0.6 (0.9)	0.406	1.0 (1.4)	0.6 (2.0)	0.5 (0.8)	0.691
	IL-4	0.2 (1.0)	0 (0.5)	0.9 (1.7)	0.129	0.4 (0.5)	0.3 (0.5)	0.7 (1.6)	0.125
	IL-6	0 (0.0)	0 (0.2)	0 (0.0)	0.359	0 (0.3)	0.1 (0.1)*	0 (0.1)	0.110
	IFN-γ	0 (0.1)	0 (0.1)	0 (0.2)	0.340	0.1 (0.2)	0.1 (0.3)	0.1 (0.2)	0.314

*Univariate significant difference compared with controls ($p < 0.05$).
The cytokine levels are expressed in pg and in pg/μL as median and quartile range.
IFN-γ, interferon-γ; IL, interleukin.

concentrations of IL-1β and IL-12p40 were below the level of detection.

The correlations between gingival crevicular fluid and serum were tested for each group separately. CD patients showed a strong, significantly positive correlation between IL-6 in gingival crevicular fluid and IFN-γ in serum ($R = 0.948$, $p < 0.001$). UC patients also showed a significant, positive correlation between IL-1β in gingival crevicular fluid and IL-18 in serum ($R = 0.636$, $p = 0.01$). In the control group, no correlation was observed between cytokine levels in the gingival crevicular fluid and serum.

Discussion

The results of this exploratory study showed that the expression pattern of cytokines was very similar in the gingival crevicular fluid from CD, UC and control patients, all with untreated chronic periodontitis. These findings might indicate that bowel disease has a relatively small effect on the local expression of the cytokines measured in this study.

We also demonstrated that the amounts of IL-4 differed significantly between groups in shallow sites, being higher value for systemically healthy individuals with untreated chronic periodontitis. The possibility that low levels of IL-4 in the gingival crevicular fluid are associated with increased periodontal deterioration has been previously shown (19). Bozkurt *et al.* (20) also found lower levels of IL-4 in gingival crevicular fluid samples from patients with rheumatoid arthritis and in systemically healthy subjects with chronic periodontitis. Considering the anti-inflammatory (9) and anti-apoptotic properties (21) of IL-4, decreased levels of this cytokine could be associ-

Table 3. Serum cytokine levels in patients with Crohn's disease (CD) and ulcerative colitis, (UC) and in systemically healthy controls (C)

Cytokine	CD ($n = 14$)	UC ($n = 15$)	C ($n = 15$)	Kruskal–Wallis
IL-18	156.0 (117.5)*	159.5 (107.1)*	107.1 (73.2)	0.018
IL-4	0 (5.1)	0 (10.7)	0 (10.6)	0.793
IL-6	3.2 (5.4)	3.8 (4.4)	2.8 (20.6)	0.261
IL-10	1.2 (3.0)	4.3 (28.5)	4.6 (20.9)	0.152
TNF-α	4.3 (3.1)	3.3 (2.3)	4.6 (4.7)	0.106
IL-12p70	0 (0.0)	0 (0.0)	0 (3.3)	0.349
IFN-γ	0 (0.2)	0 (0.0)	0 (0.4)	0.335

*Significant difference compared with controls ($p < 0.05$).
The serum cytokine levels are expressed (in pg/mL) as median and quartile range.
IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

ated with an increased degree of initiation and progression of periodontitis. Periodontal destruction is based on the degradation of collagen and apical migration of the junctional epithelium. IL-4 has been shown to exert a profibrotic effect through the activation of cultured human intra-hepatic (myo) fibroblasts, to induce collagen production and secretion (22). Although we cannot disregard the fact that our findings on IL-4 might be a statistical artifact caused by a weak *p*-value, it is possible that the lack of IL-4 might influence negatively the balance between cytokines in inflamed shallow sites, therefore contributing to the shift from gingivitis to periodontitis. Our univariate analysis indicates that this effect might be stronger in UC patients.

The serum levels of IL-18 differed significantly between groups and were significantly higher in CD and UC when compared with controls. This is in agreement with other studies demonstrating elevated levels of IL-18 in CD patients (23–25), and with the study of Wiercinska-Drapalo *et al*. (26) who showed higher concentrations of IL-18 in the serum of UC patients compared with controls. Naftali *et al*. (27) demonstrated that the levels of IL-18 are higher in patients with IBD than in healthy individuals, and this rise in IL-18 is similar to that observed for patients with sepsis (28), rheumatoid arthritis (29) and chronic hepatitis (30). Moreover, we found that the serum levels of IL-18 in UC patients might influence positively the gingival crevicular fluid levels of IL-1β. This correlation seems to have singular importance in the association of both diseases because IL-1β is a key cytokine in the pathogenesis of periodontal disease. Some of the biological effects of IL-1β include stimulation of T-lymphocytes, fibroblast proliferation, release of metalloproteinases, promotion of osteoclast formation and bone resorption (31–33).

In our study, 18 patients with IBD were taking immunosuppressive drugs. Such patients were found to have significantly lower concentrations of IL-4 and IFN-γ in the gingival crevicular fluid when compared with controls (data not shown). For the other cytokines, no significant differences were observed. One could expect to find more significant differences in the cytokine levels when subjects taking immunosuppressive drugs were compared with controls. Switalla *et al*. (34) found a significant reduction of granulocyte–macrophage colony-stimulating factor, IL-1β and IFN-γ after using dexamethasone as an immunosuppressive drug for anti-inflammatory therapy in lung disease. However, it is unknown whether the medications used by these patients have any effect on the peridontium.

Comparison between subjects with active disease and those in remission was not possible because this distribution was skewed. Moreover, because of the symptoms of IBD, most patients with active disease were unable to attend the dental clinic to allow the gingival crevicular fluid to be collected. Comparisons between smokers and nonsmokers could not be performed because of the small number of smokers. Although smoking is a known risk factor for periodontitis, there is insufficient evidence currently available to determine the immune-inflammatory mechanisms underlying the clinical response in smoker and former-smoker subjects (35). These issues should be investigated in more detail in future studies.

Our univariate analysis demonstrated only slight differences between patients with CD and UC, and healthy controls. Except for IL-4, discussed above, we could not determine any pathological implication for these variances. In conclusion, this study showed a higher concentration of IL-18 in serum, but not in gingival crevicular fluid, from periodontitis patients with CD or UC compared with controls. The expression of cytokines was similar in gingival crevicular fluid from untreated chronic periodontitis patients with CD, UC and healthy controls.

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