Cytokine and metalloproteinases in gingival fluid from patients with chronic periodontitis

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Key words: cytokines; metalloproteinases; gingival crevicular fluid; periodontitis.

Abstract. The purpose of the present research was to determine the levels of IL-1α, IL-1β, TNF-α, IL-6, IL-6sR, IL-8, IL-10, MMP-3 and MMP-8 in gingival crevicular fluid (GCF) of subjects with chronic periodontitis. Clinical measurements were carried out in 20 patients with chronic periodontitis and 11 periodontally healthy controls. The clinical indexes evaluated were: gingival index (GI), plaque index (PI), bleeding on probing (BOP), probing depth (PD) and clinical attachment loss (CAL); the measurements were taken at six sites per tooth in all teeth in each subject. GCF samples were taken from one tooth per quadrant, and the levels of mediators were measured using an ELISA test. Statistically significant differences were observed between patients and control group in relation to all clinical parameters evaluated ($p<0.05$). The gingival concentrations, in pg/mL, of IL-1α (patients: 239.06 ± 65.5 vs control: 97.79 ± 15.81), IL-1β (patients: 157.19 ± 36.4 vs control: 63.44 ± 19.04), TNF-α (patients: 10.87 ± 1.7 vs control: 1.15 ± 0.84), IL-6 (patients: 3.77 ± 1.7 vs control: 0.43 ± 0.22), IL-6Sr (patients: 655.59 ± 185.8 vs control: 73.59 ± 23.18), IL-8 (patients: 496.3 ± 155.3 vs control: 206.13 ± 46.63), IL-10 (patients: 10.75 ± 3.6 vs control: 2.41 ± 0.57), MMP-3 (patients: 3531 ± 1558.2 vs control: 724.84 ± 289.51) and MMP-8 (patients: 8231.70 ± 1279.2 vs control: 1534.67 ± 814.90) were significantly greater in patients with periodontal disease than in the control group ($p<0.001$). The higher levels of the cytokines and metalloproteinases obtained in this study were significantly associated with the severity of the periodontal disease.

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Niveles de citocinas y metaloproteinasas en fluido gingival de pacientes con periodontitis crónica.

Resumen. El propósito de la presente investigación fue determinar los niveles de IL-1α, IL-1β, TNF-α, IL-6, IL-6sR, IL-8, IL-10, MMP-3 and MMP-8 en fluido gingival crevicular (FGC) de sujetos con periodontitis crónica. Se evaluaron los parámetros clínicos en 20 pacientes con periodontitis crónica y 11 controles periodontalmente sanos. Los índices clínicos evaluados fueron: indice gingival (IG), indice de placa dental (IP), sangramiento al sondaje (SS), profundidad del saco (PS) y nivel de inserción (NI). Las muestras de FGC fueron tomadas de un diente por cada cuadrante y los niveles de los mediadores fueron medidos utilizando la prueba de ELISA. Se observaron diferencias estadísticamente significativas entre los pacientes y el grupo control en relación a todos los parámetros clínicos evaluados (p<0,05). Las concentraciones en fluido gingival en pg/mL de IL-1α(pacientes: 239,06 ± 65,5 vs control: 97,79 ± 15,81), IL-1β (pacientes: 157,19 ± 36,4 vs control: 63,44 ± 19,04), TNF-α (pacientes: 10,87 ± 1,7 vs control: 1,15 ± 0,84), IL-6 (pacientes: 3,77 ±1,7 vs control: 0,43 ± 0,22), IL-6Sr (pacientes: 655,59 ± 185,8 vs control: 73,59 ± 23,18), IL-8 (pacientes: 496,3 ± 155,3 vs control: 206,13 ± 46,63), IL-10 (pacientes: 10,75 ± 3.6 vs control: 2,41 ± 0,57), MMP-3 (pacientes: 3531 ± 1558,2 vs control: 724,84 ± 289,51) and MMP-8 (pacientes: 8231,70± 1279,2 vs control: 1534,67± 814,90), estuvieron significativamente mayores en pacientes con enfermedad periodontal que en el grupo control. (p<0,001). Los niveles elevados de citocinas y metaloproteinasas obtenidos en este estudio estuvieron significativamente asociados con la severidad de la enfermedad periodontal.

INTRODUCTION

Periodontitis is a chronic inflammatory response to the subgingival bacteria, producing irreversible periodontal tissue destruction (1,2). The disease is clinically diagnosed by loss of connective tissue attachment and bone around the teeth in conjunction with the formation of periodontal pockets due to the apical migration of the junctional epithelium (3). Although bacteria are evidently the initiating agent in periodontitis, the complexity of the associated microflora and the critical role of the host in de-

termining the outcome of the bacterial challenge cause difficulties in defining specific disease markers in periodontal diseases (4). Bacterial virulence factors either result directly in degradation of host tissues or cause the release of biologic mediators from host tissue cells that lead to host tissue destruction. Mediators produced as a part of the host response that contribute to tissue destruction include proteinases, cytokines and prostaglandins (5).

Cytokines are soluble proteins that bind to specific receptors on target cells and initiate intracellular signaling cascades resulting in

phenotypic changes in the cell, via altered gene regulation (6,7). During the initiation of an inflammatory response in the periodontal connective tissue, numerous cytokines, such as IL-1β and IL-6 and TNF-α, are released from cells of the junctional epithelia, connective tissue fibroblasts and macrophages. Additionally, enzymes such as MMP-8 and -9, are produced by PMNs and osteoclasts, leading to the degradation of connective tissue collagen and alveolar bone (8).

IL-1 has been particularly studied as a critical determinant of bone and connective tissue destruction. Furthermore, some studies have reported that increased levels of both IL-1α and IL-1β in GCF correlate with the severity of the periodontal disease (9). IL-6 is important in T-cell activation and proliferation and acts synergistically with IL-1β, whereas IL-8 is the major chemoattractant for polymorphonuclear leukocytes, IL-1β and TNF-α are the major inducers of IL-6 and IL-8 and in this respect, IL-17 shows a synergistic effect with IL-1β and TNF-α (1,10).

Matrix metalloproteinases (MMPs) are enzymes that act in physiologic development and tissue remodelling and in pathologic tissue destruction (6). MMPs are important mediators of connective tissue destruction in periodontitis, including MMP-1, -3, -8, -9 (11-13).

MMP-3 has the capacity to activate pro-MMP-1, pro-MMP-8, and pro-MMP-9 in activation cascades. Increased mRNA expression for MMP-8, the main collagenase, in inflamed GCF has also been reported in gingival fibroblasts after stimulation with IL-1β and TNF-α (4,14).

IL-10 is another pleiotropic cytokine affecting a diverse range of cell types. It is produced by monocytes, macrophages, T and B-cells; inhibits the synthesis of the proinflammatory cytokines, suppresses macrophage activation and limits the duration and extent of the immune and inflammatory responses (2,15).

Analysis of an exudate originating from the gingival crevice may supply a non-invasive means of studying the host response by evaluation of the constituents of the fluid. GCF is derived from the periodontal tissues, and its analysis offers an early indication of biochemical changes in the tissues. GCF is an inflammatory exudate that seeps into gingival crevices or periodontal pockets around teeth with inflamed gingiva, contains a variety of constituents including leukocytes (mainly neutrophils), antibodies, complements proteins, enzymes, and cytokines (16). Another distinct advantage of using GCF analysis is the use of multiple sites in a mouth to be sampled and analysed (17).

The study of the host response in periodontal disease may provide a mechanism to monitor the progression of disease; the findings can help to explain the pathologic basis of chronic adult periodontitis, and to discuss specific mechanisms that can account for tissue destruction.

The purpose of the present research was to determine the levels of IL-1α, IL-1β, TNF-α, IL-6, IL-6sR, IL-8, IL-10, MMP-3 and MMP-8 related to clinical parameters in a sample of GCF in a group of Venezuelan patients with chronic periodontitis.

MATERIALS AND METHODS

Experimental Group

Twenty patients diagnosed with chronic periodontitis with 40.1 ± 15.1 and 11 controls with 40.09 ± 10.9 age averages, respectively, were selected for this study. Demographic data show similar distribution between groups respect to gender and age.

Patients were attended at the Clinical of Periodontal Post-graduated Student, from Facultad de Odontología , Universidad Central de Venezuela, in Caracas, Venezuela. The periodontal diagnosis was established on the basis of clinical and radiographic criteria defined by the 1999 International World Workshop for a Classification

of Periodontal Diseases and Conditions (18), under the following inclusion criteria: diagnosis of chronic periodontitis of moderate to severe, with depths to probing $\geq$ 4mm and insertion loss $\geq$ 5mm in more than eight teeth, regardless of age or gender, no history of systemic diseases or intake of drugs, six months prior to the study, in addition to having given a consent to be included in the research. Pregnant or lactating women were not allowed to participate in the study.

Individuals in the control group were systemically and periodontally healthy, according a medical clinical history. Clinical measurements, including probing depth (PD) and clinical attachment loss (CAL) were carried out in patients diagnosed with chronic periodontitis (15 females and 5 males) and periodontal healthy controls (8 females and 3 males). The clinical indexes evaluated were: gingival index (GI) as previously described by Löe (19) and plaque index (PI), according to Silness and Löe (20). The measurements were taken at six sites per tooth (mesio-bucccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual) for all teeth excluding third molars in each subject.

GCF sampling and processing

GCF samples were taken from one tooth per quadrant, and the mediators levels were measured using an ELISA assay. The teeth were gently washed with water, and the sites under study were isolated with cotton rolls (to minimize saliva contamination) and gently dried with an air syringe. Four paper points were carefully inserted 1 mm into the gingival crevice of each teeth selected to sampling, and allowed to remain there for 30 seconds. Care was taken to avoid mechanical injury. Paper points from individual sites were stored at -30ºC until further processing.

Prior to evaluation, the paper points were placed individually in 100 µL of buffer A (12 mM Tris-HCl pH 7.2, 0.1 M NaCl and 0.05 per cent Tween 20) and then were vortexed for 30 minutes by repeating the procedure three times. The paper points were then removed and the eluated sample was centrifuged for 5 min at x 3000g, the supernatants were separated and frozen at -30ºC for later use.

Enzyme immunoassay

The presence and levels of immflamatory mediators in GCF were measured using a commercial ELISA kit (Quantikine, R&D Systems Inc., Minneapolis, Minnesota, USA). The results were reported as the total amount of cytokines and MMPs expressed in pg/mL.

Statistical Analysis

Clinical parameters and cytokines levels were computed for each participant, averaged within a participant and then averaged across participants. Mean and standard deviation were calculated. Data were first examined for normality, in this case the Shapiro-Wilks non-parametric test was used, and the data that achieved normality were analyzed using the Student t test to compare the study group and control, and the Pearson's correlation coefficient to establish relationships among clinical parameters and mediators levels in GCF. Statistical significance was considered for p-value < 0.05.

RESULTS

The clinical parameters and level of inflammatory mediators were measured in GCF in both groups. The periodontal measurements of participants recruited for this study are summarized in Table I. The data show statistically significant differences in all periodontal parameters evaluated when compared between groups (p<0.001). The mean values observed in the periodontitis group on the basis of the amount of clinical attachment loss show moderate to severe destruction of the periodontal tissue.

The mean in chemokine levels in the GCF samples of patients and control groups are pre-

TABLE I
CLINICAL CHARACTERISTICS OF THE STUDY GROUPS

Clinical parameters	CP group ±SD	Control group ±SD	p value
PI	1.7 ± 0.61	0.62 ± 0.51	p<0.001
GI	1.8 ± 0.54	0.45 ± 0.45	p<0.001
PD (mm)	4.2 ± 1.14	1.8 ± 0.42	p<0.001
CAL (mm)	5.6 ± 2.4	1.8 ± 0.53	p<0.001

CP=Chronic Periodontitis; PI= Plaque Index; GI= Gingival Index
PD= Pocket Depth; CAL= Clinical Attachment Loss
SD=Standard Deviation

TABLE II
GINGIVAL CREVICULAR FLUID MEDIATORS (pg/mL) OF STUDY
AND CONTROL GROUPS

	CP group ± SD	Control group ± SD	p value
IL-1β	157.19 ± 36.4	63.44 ± 19.04	$p < 0.05$
IL-1α	239.06 ± 65.5	97.79 ± 15.81	$p < 0.05$
TNF-α	10.87 ± 1.7	1.15 ± 0.84	$p < 0.05$
MMP-3	3531 ± 1558.2	724.84 ± 289.51	$p < 0.05$
MMP-8	8231.70 ± 1279.2	1534.67 ± 814.90	$p < 0.05$
IL-6	3.77 ± 1.7	0.43 ± 0.22	$p < 0.05$
IL-6sr	655.59 ± 185.8	73.59 ± 23.18	$p < 0.05$
IL-8	496.3 ± 155.3	206.13 ± 46.63	$p < 0.05$
IL-10	10.75 ± 3.6	2.41 ± 0.57	$p < 0.05$

CP=Chronic Periodontitis
SD=Standard Deviation

sented in Table II. All the cytokines evaluated were detected in both groups; however, the concentration levels in the GCF samples were higher in the patients diagnosed with chronic periodontitis when compared with the healthy group, showing statistically significant differences. However, in crescent order IL-1β, IL-1α and IL-8 levels were 2.4 fold higher in the periodontitis group, IL-10 was 4.4 fold more elevated, MMP-3 increased 4.8 fold, MMP-8 augmented 5.3 fold, with the highest increase in IL-6, IL-6sr and TNF-α showing increasing 8.7, 8.9 and 9.53 fold respectively.

The correlation between GCF mediators and clinical parameters was analyzed. A strong significant correlation was observed between PD with CAL (R=0.8, p<0.01); in the CP group moderate correlation was observed between IP and MMP-8 and IL-10 (R=0.5, p<0.05). In the control group a significant positive correlation was found among PI with GI (R= 0.6, p<0.05), and PD with CAL (R=0.8, p<0.01). IL-10 levels were positiveli correlated with PI (R=0.8, p<0.01).

DISCUSSION

Chronic periodontitis is an inflammatory disease affecting connective tissues of the teeth. Actually the understanding of the pathophysiology and the interaction between various components of the host response specially related to the function of cytokines network, is an important issue that can help in the management of periodontal disease (21).

In the present study we evaluated the GCF levels of IL-1α, IL-1β, TNF-α, IL-6, IL-6sR, IL-8, IL-10, MMP-3 and MMP-8 and analyzed the inflammatory response in subjects with chronic periodontitis and correlated these with periodontal clinical parameters.

These results are in agreement with other studies (22-25). Hyun et al.(22) found that IL-1β levels were significantly higher at deep PPD sites than at shallow PPD sites; Noh et al.(23), employing human gingival tissue samples, found that IL-6, IL-8 and TNF-α were detected in all the samples, with particularly high levels of IL 8 compared with those of the other two cytokines. Becerik et al.(24) detected significantly higher IL-1β and IL-6 in GCF from patients with chronic periodontitis and aggressive periodontitis compared with the healthy group. Rescala et al. (25) observed that the mean clinical parameters and GCF volumes were higher in patients with chronic periodontitis and aggressive periodontitis compared to the gingivitis group. However, other studies using the same methodology, found that IL-1β, TNF-α, IL-4 and IL-10 concentrations in a chronic periodontitis group were statistically lower than those of the controls (26). Goutoudi et al.(1) reported increased levels of IL-6 and IL-8 in non-disease sites.

These results are in accordance with Offenbacher´s et al.(17) research in patients with chronic gingivitis after evaluating 33 cytokines, and they observed three different groups related to the concentration. The group integrated by cytokines with constitutively "low" expression levels (IL-6, IL-10, TNF-α); an intermediate cluster (IL-1β), and the highest basal secretion levels band (IL-1α, IL-8 and IL-1ra). In this study, the concentration values observed, allow us to group in a similar way the mediators evaluated in three groups: low expression (TNF-α, IL-6, and IL-10), intermediate levels (IL-1β, IL-1α, IL-6sr e IL-8), and highest levels (MMP-3, MMP-8).

The concentration values of the cytokines and MMPs obtained in this work were higher than the reported by other authors (1, 7, 9, 24), this could be explained by the methodology used to collect the GCF samples. Another factor to consider is the individual variation in cytokine expression and profiles due to genetic, environmental, epigenetic and microbiota heterogeneity. This could impact on the susceptibility,

severity and outcome of the disease (3). Furthermore, we observed in growing order that IL-1β, IL-1α and IL-8 levels were 2.4 fold higher in the study group, IL-10 was 4.4 fold more elevated, MMP-3 amplified 4.8 fold, MMP-8 augmented 5.3 fold, with the highest increase in IL-6, IL-6sr and TNF-α that rising 8.7, 8.9 and 9.53 fold, respectively.

Periodontal inflammation is regulated by an orchestrated cytokine and chemokine network. Although the exact role of each cytokine is not completely clear, cytokines are considered the major regulators of the host's immune reaction at different stages of inflammation (27). Evidence for cytokines functioning in network are reported for interleukin IL-1 β, IL-6 and more recent a pro-inflammatory role for IL-10 in periodontitis in addition to its anti-inflammatory function (3). The highest amplification seen in this study for TNF- α, IL-6, and IL-6sR, must be due to the sampling method used in this study and the clinical characteristics of the study group. In this case the samples were collected from sites with active chronic periodontal disease and moderate levels of biofilm. When the disease is in active period, the innate response cells (monocytes, fibroblasts, mast cells, endothelial and epithelial cells) are stimulated and is probable that exists an upregulated cytokine secretion, which promotes a higher secretion of IL-6 and TNF-α (28). TNF-α acts in the cell migration process at multiple levels inducing the up-regulation of adhesion molecules and the production of chemokines, further to up-regulate the production of other cytokines, such as IL-1β an IL-6 (1, 29-33). TNF-α, a potent inducer of IL-6, has been demonstrated to stimulate osteoclast formation and bone resorption. It can synergistically act with other proinflamatory cytokines, such as IL-1β, to up regulate the production of MMPs (27).

Interleukin-6, one of the most studied inflammatory markers of periodontal disease, was found in elevated levels in the inflamed gingival tissues and gingival crevicular fluid (GCF) of patients with gingivitis and periodontitis (27, 34-36). Additionally, we observed that the concentration levels of IL-6sR were higher than IL-6 levels. This is likely, since the reading of the concentrations of IL-6sR includes the total amount of the soluble receptor present in samples (the total amount of free receptor plus the total amount of receptor bound to IL-6). Sikora, et al. (36) evaluated the involvement of proinflammatory cytokines (IL-6, IL-8), cytokine inhibitors (IL-6sR, TNFRII), and anti-inflammatory cytokines (IL-10, IL-13) in the progress child sepsis. They found increased levels of proinflamatory cytokines and suggested that in response to these increases, adaptation mechanisms associated with the release of cytokine inhibitors, (IL-6sR, sTNFRII) and anti-inflammatory cytokines (IL-10, IL-13) are induced. These results could also explain why the IL-6sR concentration levels were higher than IL-6.

MMP-8 is part of collagenases and MMP-3 of the stromelysins. Upon bacterial presence, triggered leucocytes migrate to the site of inflammation and release MMP-8 and MMP-9, which are locally activated. MMP-3 has the capacity to activate pro-MMP-1, pro MMP-8 and MMP-9 in activation cascades (6,37). MMP-8 and -9 are believed to mediate, the matrix-destroying events during the stages of periodontal disease. The results from our investigation are in agreement with the overall findings that MMP-8 and -9 seem to be key biomarkers that are elevated in the oral fluids of periodontal patients (38-42). Of the several biomarkers that have been studied, one of the strongest potential candidates in point-of-care (POC) tests is MMP-8, the most prevalent MMP in diseased periodontal tissue and saliva (43).

The anti-inflammatory mechanisms associated with the control of the immune response, elicited to maintain homeostasis, involve the release of anti-inflammatory cytokines (IL-4, IL-10, IL-13), the decrease expression of cytokine receptors, and the release of soluble cytokine

receptors or receptors antagonists (36).

IL-10 is a pleiotropic cytokine affecting a diverse range of cell types. It is produced by monocytes, macrophages, and T- and B-cells; suppress macrophage activation; and limits the duration and extent of the immune and inflammation responses. Interleukin-10, an anti-inflammatory cytokine, plays a role in periodontitis by inhibiting synthesis of proinflammatory cytokines such as IL-1, -2, -6, and -8, TNF-α, IFN-γ, and stimulating protective antibody production (44). Offenbacher et al. (17) found that IL-10 fall into concentration groups of constitutively "low" expression levels. Hirose et al. (48) showed IL-10 levels to be lower in diseased compared to healthy gingival tissues, we obtained higher levels in group with chronic periodontitis.

The lower expression of IL-6, IL-10, TNF-α was similar to the results reported by Offenbacher (17), although in the present study, a greater variation between periodontitis group and the healthy group was found. These differences can be attributed to the fact that they used a gingival experimental model and we included patients with chronic periodontitis and moderate to severe inflammation.

In this study, IL-1β, IL-1α and IL-8 were the cytokines with less amplification response compared to the other cytokines evaluated. It has been reported that IL-6 can regulate the expression the IL-1 through the stimulation of tissue inhibitor of MMPs and the induction of IL-1 receptor antagonist (IL-1Ra), diminishing IL-1 expression (1,33-35). In the present study group the IL-6 response was amplified 8.7 fold, therefore their action capacity to regulate IL-1 expression can be increased. Interleukin 1 is a central mediator of innate immunity and inflammation, which is induced by bacterial infections, and has been correlated with the destruction of periodontal tissue in the chronic lesion by stimulating matrix metalloproteinase secretion, connective tissue degradation, and inflammatory

bone resorption (29,44). IL-1α and IL-1β are the principal inflammation-induced cytokines stimulating bone resorption in periodontitis (45). Sakai et al. (16) found that IL-1α was expressed at low levels and was not significantly different between healthy and diseases sites. We observed higher levels in diseased subjects.

With respect to IL-8 a potent chemokine that function in the recruitment and activation of human granulocytes, it can be secreted from many different host cells including monocytes/macrophages, lymphocytes, endothelial and epithelial cells. Other inducers of IL-8 are IL-1, TNF-α and immune complex (46). IL-8 is normally present in health, is released by periodontal pocket ephitelial cells to maintain the cleansing efflux of neutrophils into the gingival sulcus, to keep the low levels of bacteria, by restricting them to the sulcular environment (16,46,47). In periodontal patients, IL-8 has been reported in both GCF and periodontal tissues. McGee et al. (48) found that IL-8 concentrations were significantly higher in gingival tissue adjacent to probing pocket depth ≤ 3 mm and lowest adjacent to >6 mm sulci. According to Mathur et al. (49) the total amount of Il-8 was significantly higher in diseased compared to healthy sites, while in other study (50) no significant difference in GCF IL-8 levels between localized juvenile periodontitis and healthy subjects was shown.

The new evidence developed in several reviews set clearly that the host response to the microbial biofilm in periodontal disease is highly complex and considerable individual variation is present in all aspects related to innate, inflammatory an immune response to the periodontal microbial biofilm.

The higher levels of the cytoquines and metalloproteinases obtained in this study were significantly associated with the severity of periodontal disease. A more quantitative analysis is required to address the mechanism of periodontitis.

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