Interleukin-6 and Interferon-α Levels in Gingival Crevicular Fluid in HIV-1 Patients with Chronic Periodontitis

Niveles de Interleukin-6 e Interferon-α de Fluido Crevicular Gingival en Pacientes VIH-1 con Periodontitis Crónica

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ABSTRACT: The influence of cytokine on the progression of chronic periodontitis in human immunodeficiency virus (HIV) patients is still controversial and poorly investigated. This study aimed to analyze and compare IL-6 and IFN-α levels in the gingival crevicular fluid of HIV-1-positive and HIV-1-negative patients with chronic periodontitis and different grades of tissue destruction and inflammation. Samples from the gingival crevicular sulcus were obtained from 35 HIV-1-positive individuals with chronic periodontitis and 35 seronegative patients with chronic periodontitis. Probing depth and clinical attachment level, as well as the results of the Enzyme-Linked Immunosorbent Assay for confirmation of patient diagnostics, were evaluated. Statistical analyses were performed using Student t, Mann-Whitney and Spearman tests. IL-6 levels were significantly lower, while IFN-α levels were significantly higher in HIV-1 patients. Clinical attachment level was directly associated with IFN-α levels in HIV-1 carriers, connected to probing depth in these patients. Clinical data in association with gingival crevicular fluid cytokine levels may reveal a localized immunological response pattern, which may contribute to the understanding of periodontitis pathogenesis in HIV-1 carriers.

KEY WORDS: HIV-AIDS; periodontal disease; cytokine.

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is characterized by an advanced state of immunosuppression, and is considered a serious public health issue worldwide, despite the reduction in morbidity and mortality in patients using antiretroviral therapy (ART) (Mataftsi et al., 2011). Worldwide prevalence of individuals infected with HIV has been estimated at around 34 million (UNAIDS - Joint United Nations Programme on HIV/AIDS, 2012).

The Human Immunodeficiency Virus (HIV-1) replicates in cells that possess CD4 receptors on their surface, such as CD4+ T lymphocytes (CD4+ T cells), monocytes, macrophages and Langerhans cells, which are essential for the host immunological response (Ryder et al., 2012). A decrease in the levels of CD4+ T cells causes the individual to become more susceptible to opportunistic infections and increases the probability of developing systemic manifestations, such as lymphadenopathy, pharyngitis, myalgia, arthralgia, as well as oral conditions, including candidiasis, parotitis, angular stomatitis, linear gingival erythema and periodontal disease, among others (Peeters et al., 2013).

Periodontal conditions have been widely associated with immunosuppression induced by HIV. They may be one of the first clinical signs of HIV

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infection and are attenuated with the use of ART (San
to et al., 2010; Ponnam et al., 2012). The initial
symptoms of periodontal disease in individuals with HIV
are gingival inflammation and bleeding on probing
(Doshi et al., 2008). The presence of gingivitis and the
severity of periodontitis, indicated by bone loss and
increased probing depth, are commonly seen in
individuals with HIV (Aichelmann-Reidy et al., 2010;
Andrukhov et al., 2011; Khammissa et al., 2012).

Susceptibility and extent of tissue destruction
seem to be determined by the complex balance of
cytokine production and by the presence of multiple
associations between periodontal microorganisms.
When host response is exacerbated, it may lead to tissue
damage and loss of periodontal support (Aichelmann-
Reidy et al.; Andrukhov et al.; Nussbaum & Shapira,
2011). The association between periodontitis progression
and factors such as presence of periodontal pathogens,
high levels of pro-inflammatory cytokines, and low levels
of cytokines that inhibit the inflammatory process are
well described (Andrukhov et al.; Nussbaum & Shapira;
Ford et al., 2010).

In individuals infected with HIV, immune
mediators play a complex role in viral replication. A
direct impact on virus production was observed
(Goutoudi et al., 2012; John et al., 2013) controlled by
the balance between the cytokines produced by the
Th1 and Th2 cells of the immune system (Rempel et
al., 2008). Therefore, periodontal health depends on
the local balance between cells of the immune system,
cytokines and chemical inflammatory mediators
(Andrukhov et al.).

Interleukin-6 (IL-6) is an important cytokine,
produced by a number of cells, including monocytes,
fibroblasts, osteoblasts and vascular endothelial cells,
which helps to regulate infection and inflammatory
response (Wilson et al., 1996). IL-6 plays an important
role in B cell differentiation and T cell proliferation, as
well as in inducing bone resorption (Ryder et al.). On
the other hand, Interferon-α (INF-α) is an important
mediator in viral infections, binding with specific
receptors present on non-infected neighboring cells,
and exerting an antiviral effect. In addition to activating
Natural Killer (NK) cells, stimulating apoptosis in
infected cells and inhibiting further infection, INF-α also
plays an essential role in the defense against
intracellular viruses and pathogens, and is involved in
the induction of the inflammatory immune response
(Ford et al.; Andrukhov et al.; Nussbaum & Shapira;
John et al.).

The purpose of this study is to analyze and com-
pare IL-6 and IFN-α levels in the gingival crevicular
fluid of HIV-1-positive and HIV-1-negative patients
with chronic periodontitis and different grades of tissue
destruction and inflammation.

MATERIAL AND METHOD

Study population. This study was approved by the
Ethics Committee for Research on Human Subjects of
the School of Medicine and Dentistry São Leopoldo
Mandic, Campinas/SP/Brazil (2012/0082).

Sample size was calculated according to test
power. The level of significance was 5 ‰, with an effect
size of 0.80. Considering a statistical power of 95 ‰,
sample size was set at 35 subjects per group. The
inclusion criterion was that patients had to be HIV-1,
with contamination diagnostic of more than 24 months,
asymptomatic, making regular use of TARV for at least
12 months, using the same medicine at the same
intervals. To be included in the groups, patients should
not present any systemic change. All subjects had to
have at least 20 teeth and not have been treated for
periodontal disease for about one year. All patients had
untreated advanced chronic periodontitis. Sex, race
and age were not used as inclusion and/or exclusion
criteria. Forty subjects, 34-60, were selected. Pregnant
women, infants, diabetics, smokers, patients using lo-
cal (mouthwashes) or systemic antimicrobial therapy,
hormone therapy, and any type of analgesic or anti-
inflammatory within the 30 days prior to sample

collection were excluded.

After the subjects signed the informed consent
form, they went through clinical examination, and samples
were collected from them. Thirty-five HIV-1 patients were
in the study group, and 35 patients were selected for the
control group. A tooth was probed by quadrants. Selected
periodontal sites for FCG collection had a probing depth
≥ 5 mm, and their X-rays showed great bone destruction.
The teeth of these sites were natural, healthy, without
prosthetics, and had no impairment in occlusion.

Blood samples were collected from the control
group patients for HIV testing, pursuant to Brazilian
regulation nº 59/GM/MS, January 28, 2003. Enzyme-
Linked Immunosorbent Assays (ELISA) (DiaSorin, anti-
HIV tetra Elisa, Biotest, Germany) were performed,
including recombinant antigens, one from the envelope
and two from the viral capsule. If seropositive, the
samples went through the serological triage once again, to detect anti-HIV antibodies through a Microparticle Immunoenzymatic Assay (AxSYM-System-Abbott, Germany), followed by confirmation by Indirect Immunofluorescence (Bio-Manguinhos Fiocruz, Brazil).

Crevicular fluid collection. The samples were collected randomly from two sites from different quadrants. An absorbent paper point (Mailefer, Destsply, SP, Brazil) was inserted into the gingival crevicular fluid at each site until slight resistance was felt. The paper point was then kept in place for 30 s to allow the fluid to be absorbed from the sulcus. Paper points that were visibly contaminated by blood were discarded. Each tip was placed in a sterile polystyrene tube (Eppendorf, Sigma, CA, USA) containing 250 µl of RIPA buffer supplemented with protease inhibitor to 1 % in the mixture (Sigma-Aldrich, Saint Louis, Missouri, USA). The tube was then sealed and identified with patient details and sample collection site. The samples were then frozen at -70 °C for the subsequent experiments.

Enzyme-Linked Immunosorbent Assay (ELISA). The samples were centrifuged for 15 min at 4 °C, at 10,000 g. The concentration of the inflammatory mediators (IL-6 and IFN-α) present in the gingival crevicular fluid was evaluated via ELISA, as per the manufacturers’ recommendations (eBioscience, 10240 Science Center, San Diego, CA, USA). Soon after that, 100 µl of detection antibody were added to all wells, with the exception of the blank well, mixed gently and incubated overnight (16–24 h) at 4 °C. The plates were washed 3 times; standards and supernatant were then added to their respective wells, in duplicate. After incubation, the plates were washed again and incubated with 200 µl of conjugate for 60 min at room temperature. After plates were washed three more times and 200 µl of substrate were added to them, they were incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 50 µl of stop solution, and the color, measured in an automated microplate spectrophotometer (Epoch, Biotek, Winooski, VT, USA). The total concentration of cytokines present was determined in picograms per milliliter (pg/ml). The results were calculated by using the standard curves created in each assay. All ELISA analyses were performed in a blind fashion in triplicate.

Statistical analysis. Data related to interleukin levels and clinical parameters of chronic periodontitis were submitted to descriptive and inferential analysis. The Chi-square test was used to assess proportion of patients from different genders in the two groups, positive or negative for HIV-1. The Student t test was performed to identify differences in age between the participants in both groups.

A comparison of the clinical parameters of chronic periodontitis (clinical attachment level and probing depth) and IL-6 and IFN-α levels between HIV-1 positive and negative patients was carried out, by using the Student t and Mann-Whitney tests. The latter was used when the data did not conform to normality and homogeneity of variance.

The Spearman test was used to evaluate the correlation between interleukin levels and both clinical parameters of periodontitis. A significance level of 5 % was adopted. All statistical calculations were performed using the SPSS 20 program (SPSS Inc., Chicago, IL, USA).

RESULTS

According to the demographic data in this study, out of the 35 HIV-1 positive patients, twenty were male (58.3 %) and fifteen were female (41.6 %). The age range for the HIV-1 positive patients was 35-58 years, with a mean of 47.1 (±6.7) years. Out of the 35 HIV negative individuals, eighteen were male (52.9 %) and seventeen were female (47.1 %). The age range of the HIV negative group was 38-55 years, with a mean of 47.0 (±5.4) years. There was no significant difference between the HIV-1 positive and negative groups in terms of sex (p = 1.000). Likewise, there was no significant difference in age between the patient groups (p = 0.971). Clinical attachment level and probing depth were significantly higher (p < 0.05) in HIV-1 positive patients, as shown in Table I.

Table I. Mean values and standard deviation for the demographic data and clinical parameters for chronic periodontitis between HIV-1 positive and negative patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HIV-1 positive</th>
<th>HIV-1 negative</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47.1±6.7</td>
<td>47.0±5.4</td>
<td>0.971</td>
</tr>
<tr>
<td>Clinical attachment level (mm)</td>
<td>7.1±0.7</td>
<td>6.4±0.8</td>
<td>0.011†</td>
</tr>
<tr>
<td>Probing depth (mm)</td>
<td>6.6±0.7</td>
<td>5.9±0.7</td>
<td>0.015†</td>
</tr>
</tbody>
</table>

* Values based on the student t test, the data show a significant difference between groups. † Significance level of 0.05.
The IL-6 levels were significantly lower (p < 0.05) in HIV positive individuals, while IFN-α levels were significantly higher (p < 0.05), as shown in Table II.

Analysis of cytokine levels and clinical parameters (Table III) revealed an association between IFN-α and both clinical attachment level and probing depth in HIV-1 positive individuals (p < 0.05). On the other hand, there was no association between IL-6 levels and clinical parameters.

Table II. Mean values and standard deviation of cytokine profiles between HIV-1 positive and negative patients.

<table>
<thead>
<tr>
<th>Parameters (pg/mL)</th>
<th>HIV+</th>
<th>HIV-</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>8.81±4.72</td>
<td>35.56±18.92</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>INF-α</td>
<td>302.37±189.58</td>
<td>69.51±78.11</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* Values based on the Mann-Whitney test, data showing a significant difference between groups. Significance level of 0.05.

Table III. Correlation between cytokines (IL-6 e INF-a) and clinical parameters of chronic periodontitis, serology and viral load in HIV-1 positive and negative individuals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HIV+*</th>
<th>HIV-</th>
<th>INF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical attachment level</td>
<td>p = 0.292</td>
<td>p = 0.033† (r² = 0.615)</td>
<td>p = 0.219</td>
</tr>
<tr>
<td>Probing Depth</td>
<td>p = 0.134</td>
<td>p = 0.003† (r² = 0.46)†</td>
<td>p = 0.247</td>
</tr>
</tbody>
</table>

* p-values based on the Spearman correlation test, data shows a significant difference between groups. † Significance level of 0.05. ‡ Coefficient of determination.

**DISCUSSION**

The findings in this study revealed that HIV-1 infection may influence the severity of chronic periodontitis, not only due to immunological deficiency, but also in view of the higher levels of IFN-α found in the gingival crevicular fluid, which may be justified by its important role in viral infections. Lower levels of IL-6 were detected in HIV-1 positive individuals when compared to HIV-1 negative individuals with chronic periodontitis. This finding contradicts the findings by Ford et al. and Andrukhov et al., who reported an increase in IL-6 with disease progression.

Many studies have demonstrated that local cytokine production profile may be important in periodontal destruction (Ford et al.; Andrukhov et al.; Nussbaum & Shapira). However, an association between chronic periodontitis and HIV infection has not been wholly established yet, seeing as the results of many studies are contradictory as for the comparison between clinical attachment level and probing depth in HIV-1 positive and negative individuals (Mataftsi et al.; Segundo et al., 2011; Khammissa et al.).

In this study, both clinical attachment level and probing depth were significantly higher in HIV-1 positive individuals with chronic periodontitis than in HIV-1 negative individuals with chronic periodontitis. Cotter et al. (2007) and Gibellini et al. (2008) evaluated cell function in the bone formation process in HIV-1 positive individuals, and found that osteoblasts are, in fact, affected, resulting in decreased osteogenesis. The mechanisms underlying this process are related to cellular apoptosis triggered by the virus. Such findings could explain, in part, the results of the present study in terms of the evaluation of clinical attachment level and probing depth, which revealed that HIV infection interfered with the natural progression of periodontal disease. However, this kind of association is not always described (Robinson et al., 2002; Guimarães et al., 2012). On the other hand, HIV infection leads to a disruption of the immuno-skeletal interface, affecting T-cell to B-cell communication and leading to elevated RANKL and diminished OPG production by B cells. The elevated RANKL/OPG ratio is biased in favor of increased osteoclast formation (Vikulin et al., 2010).

In addition to that, recent studies have shown how persistent inflammatory responses associated with HIV and ART may underlie disruption to the immuno-skeletal interface, possibly explaining the bone loss associated with both HIV infection and exacerbated bone loss as a result of using ART (Ofotokun et al.,
of the antibacterial defense response at these sites, may indicate decreased activity of the antibacterial defense response at these sites (Cotter et al., 2007; Gibellini et al., 2008; Segundo et al., 2011; Guimarães et al., 2012). Paradoxically, although it is important to highlight that the use of ART therapy has radically improved patient quality of life and survival, some of these benefits are offset by troublesome metabolic complications, including osteoporosis and elevated fracture prevalence (Ofotokun et al.).

Cytokine profile analysis in chronic periodontitis should not replace clinical examination and the tests performed in routine clinical practice. However, it could be used to complement and support clinical evaluation, to help in diagnosing and adopting a certain therapeutic intervention. Therefore, a combination of clinical data and cytokine levels in the gingival crevicular fluid may provide invaluable information on the local immunological response profile, hence aiding in understanding the pathogenesis of periodontitis, particularly in individuals with chronic infections, such as those that occur in HIV-1 patients.

IFN-α levels were found to be higher in HIV-1 positive individuals when compared to the negative population, which has also been reported in similar studies with experiments involving Rhesus and Cynomolgus monkeys. An increased level of this cytokine may indicate its importance in HIV infection, since, among other functions, it helps activate NK cells, leading to apoptosis of the infected cell (Ryder et al.).

When individually evaluating the cytokines within each group, with regard to clinical parameters (probing depth and clinical attachment level), IFN-α levels were significantly higher in HIV-1 positive patients, which may be explained by the fact that IFN-α is produced by dendritic plasma cells that are infected by the virus, which then act on other infected cells via the degradation of viral messenger RNA, hence interfering with viral replication (Teles et al., 2009; Puryear et al., 2013). Mathur et al. (1996), however, observed that IFN-α concentrations were significantly lower at the periodontitis sites, suggesting that a combination of lower IFN-α concentrations, alongside others, such as IL-8 at disease sites, may indicate decreased activity of the antibacterial defense response at these sites (Cotter et al., 2007; Gibellini et al., 2008; Segundo et al., 2011; Guimarães et al., 2012). Paradoxically, although it is important to highlight that the use of ART therapy has radically improved patient quality of life and survival, some of these benefits are offset by troublesome metabolic complications, including osteoporosis and elevated fracture prevalence (Ofotokun et al.).

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RESUMEN: La influencia de la citocina en la progresión de la periodontitis crónica en pacientes con el virus de la inmunodeficiencia humana (VIH) sigue siendo controvertida y poco investigada. Este estudio tuvo como objetivo analizar y comparar los niveles de interleucina-6 e interferón-α (IFN-α) en el líquido crevicular gingival de pacientes VIH-1-positivos y VIH-1-negativos con periodontitis crónica y diferentes grados de destrucción e inflamación tisular. Se obtuvieron muestras del surco crevicular gingival de 35 individuos VIH-1 positivos con periodontitis crónica y 35 pacientes seronegativos con periodontitis crónica. Se evaluaron la profundidad de sondado y el nivel de inserción clínica, así como los resultados del Ensayo Inmunoabsorbente Ligado a Enzimas para la confirmación del diagnóstico del paciente. Los análisis estadísticos se realizaron utilizando pruebas t de Student, Mann-Whitney y Spearman. Los niveles de IL-6 fueron significativamente más bajos, mientras que los niveles de IFN-a fueron significativamente más altos en los pacientes con VIH-1. El nivel de inserción clínica se asoció directamente con los niveles de IFN-α en los portadores del VIH-1, conectados a la profundidad del sondaje en estos pacientes. Los datos clínicos en asociación con los niveles de citocinas de los fluidos crevicales gingivales pueden revelar un patrón de respuesta inmunológica localizado, que puede contribuir a la comprensión de la patogénesis de la periodontitis en los portadores del VIH-1.

PALABRAS CLAVE: VIH-SIDA, enfermedad periodontal, citocina.

REFERENCES
