Abstract

Background and aims. It has been reported that Type I hypersensitivity plays an important role in periodontal diseases. The aim of this study was to investigate the possible correlation between interleukin-1β, IL-6, and tumor necrosis factor-α as immunologic mediators and gingival clinical parameters in chronic and aggressive periodontitis.

Materials and methods. Clinical parameters including clinical attachment level (CAL), probing depth (PD) and bleeding index of 11 patients with moderate-to-advanced periodontitis were recorded; gingival tissue specimens from 12 chronic and 14 aggressive active sites, harvested from interproximal areas during their routine periodontal surgeries, were cultured with Fetal Calf Serum + RPMI + Amphotericin + Gentamicin in 96-well plates for 72 hours. The cytokines present in the culture media were quantified using enzyme-linked immunosorbent assay (ELISA) in each case and the results were statistically analyzed by ANCOVA, Pearson's and Spearman's rho.

Results. Mean values of CAL, PD, IL-1β, IL-6 and TNF-α were 6.8±1.3 mm, 6.5±1.2 mm, 111.23±143.4, 10.1±16.9 and 5.2±0.2, respectively. There were no significant differences between the three cytokine concentrations in aggressive and chronic periodontitis. There were no correlations between cytokine concentrations and clinical parameters. There were direct statistical correlations between IL-6 and TNF-α in both periodontitis types (p<0.05) and direct statistical correlations between IL-1β and TNF-α only in chronic periodontitis (p<0.05).

Conclusion. Regarding irritation due to bacterial products in both types of periodontitis and synergy among them, especially the correlation between TNF-α and both IL-1β and IL-6, TNF-α seems to play a more important role; however, further studies are strongly recommended.

Key words: Aggressive periodontitis, chronic periodontitis, interleukin-6, interleukin-1β, tumor necrosis factor-α.
Introduction

Periodontitis is a chronic infectious disease with a multifactorial nature, which is characterized by destruction of collagen fibers and other matrix constituents of periodontal ligaments and alveolar bone around the teeth in conjunction with formation of periodontal pockets. It has been established that the severity of periodontal disease is dependent on a dynamic equilibrium of interactions between the microbial challenge and host immune inflammatory responses. Virulence factors of periodontal pathogens, such as lipopolysaccharides, can stimulate inflammatory cytokine expression. Cytokines are small polypeptides with a wide range of inflammatory, metabolic and immunomodulatory properties, which are produced by a variety of cells, including the macrophage/monocyte system, dendritic cells, lymphocytes, neutrophils, endothelial cells and fibroblasts.

Several studies have demonstrated that some cytokines, such as interleukin (IL-1β, IL-6), interferon-γ, and tumor necrosis factor (TNF-α) are involved in T-helper-1 (TH-1) immune responses and induce cell-mediated immunity. All the periodontopathogenic bacteria as well as extracted lipopolysaccharides (LPS) have primarily been shown to stimulate monocytes to produce cytokines such as IL-1 and TNF-α. TNF-α is released in a rapid burst after LPS stimulation and IL-1 release is typically observed to occur shortly thereafter. IL-1 affects nearly every cell type, and often in concert with other cytokines or mediator molecules. IL-1 is a highly pro-inflammatory cytokine and the range between clinical benefit and unacceptable toxicity in humans is exceedingly narrow. There is no doubt that the balance between local levels of cytokines, stimulated in response to periodontopathogenic bacteria and their products, is important in determining the outcome of an immune response to a given pathogen. It has also been reported that in clinically healthy gingival tissue, inflammatory cytokines such as IL-1β, IL-6 and TNF-α are present in low quantities. This means that cytokines are prominent factors of normal tissue homeostasis. Evidence also suggests that interleukin can induce and activate metalloproteinase and tissue inhibitors of metalloproteinases, which are believed to be critical in initiating the collagenolytic cascade and regulation of connective tissue degeneration under both physiologic and pathologic conditions. Measurement of IL-1β, a pivotal pro-inflammatory cytokine, in gingival crevicular fluid (GCF) or tissues adjacent to periodontitis-affected sites in patients, has been suggested as a sensitive and important aid in monitoring the clinical severity of the disease. Although a number of studies have demonstrated that the biological activity of a variety of cytokines may be directly related to periodontal destruction such as periodontal attachment loss, destruction of collagen and alveolar bone resorption, biological mechanisms for the progression of periodontitis are not fully understood and still remain controversial despite much attention focused on the subject. In some studies, correlation analysis has shown that IL-1β level in inflamed periodontal tissue highly correlates with clinical parameters including gingival and plaque indices (GI and PI) and PICI as a degree of inflammation. However, the results of a study have shown that cultures from diseased sites are composed of cells with higher levels of constitutive CD40 expression, which may contribute to the increased IL-6 and IL-8 secretory phenotype. A study demonstrated that serum samples from chronic periodontitis patients had high individual variability of cytokine profiles, and no association between cytokine concentration and clinical parameters of periodontitis was found but the level of cytokines strongly correlated with the severity of periodontitis.

The individual course of the periodontal disease and variability of clinical parameters, which may lead to tooth loss in the absence of early and appropriate treatment, prompted us to undertake a complex study on the key cytokines responsible for the initiation and progression or suppression of the inflammatory response. This study was carried out to evaluate the gingival biopsy concentration of IL-1β, IL-6 and TNF-α and their correlation with clinical attachment loss in aggressive and chronic periodontitis patients. The results of this study can be one of the initial steps to determine correlation between clinical and immunological parameters in the aggressive and chronic types of periodontal diseases.

Materials and Methods

Patients

The study group consisted of 15 sites of 6 aggressive (3 females, 3 males; mean age of 31±2.58; age range of 28–34 years) and 14 sites of 4 chronic (1 female, 3 males; mean age of 43.8±4.99; age range of 39–49 years) periodontitis patients. The subjects were selected from the patients who had been referred to a private dental clinic in Qazvin, Iran.

On the basis of clinical and radiographic findings,
severe periodontitis was diagnosed in both groups by an experienced periodontist. In all the patients BOP (bleeding on probing) was positive and PD (pocket depth) and CAL (clinical attachment loss) were $\geq 5$ mm and $\geq 5$ mm, respectively.

All the patients were included in the study based on the following criteria:
1) no systemic disorders which could influence the course of periodontal disease
2) no use of antibiotics and narcotic substances since 2 months prior to the study
3) no treatment by any immunomodulatory drugs since 1 year prior to the study
4) no menstruation, pregnancy or nursing period during the time of surgery and sampling

Clinical Examination
The patients were clinically examined and clinical parameters of periodontium, including PD, CAL, and BOP were recorded using the Williams probe. The results were expressed as a mean value accompanied by standard deviation.

All the periodontal sites had been treated as a part of phase I periodontal treatment, including scaling, root planing and polishing 2–4 weeks prior to surgery. The study was approved by the Ethics Committee of Qazvin Medical Sciences University and necessity of the surgery was confirmed by a periodontist. All the patients were informed about the purpose of the study and signed written consent forms. All the tissue samples were taken from that part of tissue that had already been taken off.

Sampling of Gingival Tissue
Inflamed gingival tissues from the patients were biopsied during the flap debridement operations as their routine periodontal surgery with the use of a scalpel blade, and then the wound was secured with sutures. Periodontal pockets with PD $\geq 5$ mm and CAL $\geq 5$ mm and BOP were determined to have active disease and were chosen for the study. Each sample was transferred to a sterile microtube and stored for 24 hours at $-20^\circ$C prior to storage at $-70^\circ$C to start laboratory tissue preparation.

Tissue Preparation
500 $\mu$L of normal saline was added to each microtube and centrifuged at 3000 rpm for 5 minutes. The supernatants were discarded and the tissue samples were crushed with a sterile burnisher; then, 300 $\mu$L of sucrose buffer was added to each microtube and centrifuged at 3000 rpm for 10 minutes. Then the supernatant was collected with a sampler and was stored in the same coded microtube.

Cytokine Assays
Cytokine concentrations in tissue supernatants were measured by an enzyme-linked immunosorbent assay (ELISA) using commercially available Ucytech sets for human IL-1$\beta$, IL-6, and TNF-$\alpha$, (Utrecht, the Netherlands) according to manufacturer’s instructions.

The absorbance of each well was read in a microplate spectrophotometer at 450 nanometers, and the tissue concentration of each cytokine was calculated from the standard curve included with each assessment kit.

Statistical Analysis
Data was analyzed by Kolmogrov-Smirnov test to determine normal distribution of data. According to the distribution, data was summarized by mean values and standard deviations. The groups were compared using $t$-test for all the parameters with normal distribution and Mann-Whiney test for the parameters that were not normal.

The correlation among clinical parameters was analyzed using Pearson’s correlation test. In addition, Spearman’s correlation test was used for the data that were not normally distributed.

Moreover, these tests were used to analyze correlation between clinical parameters and concentration of cytokines in the aggressive and chronic groups. Statistical significance was defined at $p<0.05$.

Results
Three samples were lost due to technical problems during laboratory procedures. The mean concentrations of IL-1$\beta$, IL-6, and TNF-$\alpha$ in the chronic group were 94.4$\pm$136.6, 13.1$\pm$22.8, and 5.2$\pm$0.1 pg/mL; in the aggressive group these values were 125.6$\pm$152.6, 7.5$\pm$9.6, and 5.3$\pm$0.3 pg/mL, respectively (Table 1). In all the collected samples, all the cytokines under study were present. The IL-1$\beta$ concentration was higher than the others.

According to data, the mean value of IL-1$\beta$, IL-6 and TNF-$\alpha$ were not significantly different between the aggressive and chronic groups.

The mean pocket depth and clinical attachment loss in aggressive and chronic periodontitis patients are presented in Table 1.

There was a significant correlation between IL-6 and TNF-$\alpha$ in all the samples. In addition, the correlation tests were run for both groups individually.
In the chronic group there was a significant correlation between IL-1β and TNF-α concentrations and also between IL-6 and TNF-α concentrations. In the aggressive group this correlation was significant between IL-6 and TNF-α concentrations (p<0.05).

Pearson’s and Spearman’s correlation tests indicated no significant correlation between gingival cytokine concentrations and clinical parameters (CAL and PD) in both aggressive and chronic periodontitis.

**Discussion**

In the present study, expression of key cytokines (IL-1β, IL-6, TNF-α) was analyzed; these cytokines seem to play an important role in the initiation and progression of periodontitis. In addition, the correlation between gingival concentration of IL-1β, IL-6, and TNF-α and CAL and PD in aggressive and chronic periodontitis patients was evaluated.

All the samples were collected from the sites with PD and CAL>5 mm. According to the clinical and radiographic findings, all the selected patients had generalized chronic advanced or aggressive periodontitis.

Limitation of the number of subjects, especially in aggressive cases and generalization of their disease, convinced us to collect more samples from different sites of each patient, which led to more general similarity among the samples. However, different results in previous studies can be partly attributed to the different samples in each periodontal patient from the severity point of view.

The concentration of IL-1β was more than the others in both of our groups, but Beklen et al showed in the macrophage/monocyte cultures of periodontitis patients that cell stimulation by IL-17 induces greater secretion of TNF-α compared to IL-1β. Donggari-Bagtzoglou & Ebersole showed that the level of IL-6 concentration was higher than IL-1β. In a study carried out by Lester et al, the order of cytokine concentration in gingival samples of adult periodontitis patients was TNF-α>IL-6>IL-1β.

There was no significant correlation between clinical parameters (PD, CAL) and cytokine concentrations in our study; however, Tobón-Arroyave et al found a positive correlation between these parameters and IL-1β levels in salivary samples of aggressive and chronic periodontitis patients. Furthermore, Lester et al found a correlation between CAL and concentration of these cytokines. They found a positive correlation between IL-6 and TNF-α and also between IL-1β and two other cytokines.

Stashenko et al showed a significant correlation between IL-1β and CAL in adult periodontitis patients. The differences in results can be attributed to different levels of bone loss in the present study and that study; the range of CAL in our study was 5–8 mm, whereas in that study it was >2.5 mm.

Our results are consistent with Hoe et al and Gorska et al; they reported that there was no significant correlation between IL-1β concentration and clinical parameters.

In the present study no significant difference was observed between chronic and aggressive groups as to cytokine concentrations. Considering the differences in CAL and PD between the two groups, even with coordination of these parameters using quasi-experimental method of analysis, no significant differences were observed.

Mengal et al found a correlation between IL-1β and IL-6 in blood samples of aggressive periodontitis patients. They reported that IL-1β was found only in the samples of patients with aggressive periodontitis but IL-6 was found in all the samples. The differences between our results and the results of that study might be attributed to different samples from blood versus gingival tissues.

In some previous studies, the important role of LPS in induction and secretion of cytokines have been emphasized, due to the high level of inflammatory mediators, especially LPS in gingival tissue samples, where the presence of these cyto-

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### Table 1. The mean pocket depth and clinical attachment loss in aggressive and chronic periodontitis patients

<table>
<thead>
<tr>
<th>Group</th>
<th>CAL</th>
<th>PD</th>
<th>TNF-α</th>
<th>IL-6</th>
<th>IL-1</th>
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<tbody>
<tr>
<td>Chronic Mean</td>
<td>6.00</td>
<td>6.00</td>
<td>5.17</td>
<td>13.08</td>
<td>94.44</td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.85</td>
<td>1.21</td>
<td>0.13</td>
<td>22.81</td>
<td>136.59</td>
</tr>
<tr>
<td>Minimum</td>
<td>5</td>
<td>5</td>
<td>5.01</td>
<td>3.45</td>
<td>2.56</td>
</tr>
<tr>
<td>Maximum</td>
<td>8</td>
<td>9</td>
<td>5.39</td>
<td>84.89</td>
<td>57.54</td>
</tr>
<tr>
<td>Median</td>
<td>6.00</td>
<td>6.00</td>
<td>5.16</td>
<td>6.86</td>
<td>49.41</td>
</tr>
<tr>
<td>Aggressive Mean</td>
<td>7.43</td>
<td>7.00</td>
<td>5.28</td>
<td>7.53</td>
<td>125.62</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.34</td>
<td>1.04</td>
<td>0.28</td>
<td>9.56</td>
<td>152.65</td>
</tr>
<tr>
<td>Minimum</td>
<td>5</td>
<td>5</td>
<td>4.97</td>
<td>3.20</td>
<td>19.77</td>
</tr>
<tr>
<td>Maximum</td>
<td>10</td>
<td>8</td>
<td>6.05</td>
<td>38.83</td>
<td>519.73</td>
</tr>
<tr>
<td>Median</td>
<td>7.50</td>
<td>7.00</td>
<td>5.22</td>
<td>4.14</td>
<td>60.88</td>
</tr>
<tr>
<td>Total Mean</td>
<td>6.77</td>
<td>6.54</td>
<td>5.23</td>
<td>10.09</td>
<td>111.23</td>
</tr>
<tr>
<td>N</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.34</td>
<td>1.21</td>
<td>0.23</td>
<td>16.86</td>
<td>143.45</td>
</tr>
<tr>
<td>Minimum</td>
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<td>5</td>
<td>4.97</td>
<td>3.20</td>
<td>2.56</td>
</tr>
<tr>
<td>Maximum</td>
<td>10</td>
<td>9</td>
<td>6.05</td>
<td>84.89</td>
<td>519.73</td>
</tr>
<tr>
<td>Median</td>
<td>7.00</td>
<td>7.00</td>
<td>5.20</td>
<td>4.28</td>
<td>52.38</td>
</tr>
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</table>
kines was reasonable. Roberts et al. reported that presence of mRNA among all the inflammatory cytokines relevant to TNF-α was considerable in the gingival tissue of adult periodontitis patients. The results of their study seem to verify ours.

It has been reported that bacterial stimulation, especially by LPS, may increase secretion of inflammatory cytokines by tissue inflammatory cells. It seems there is some synergism between IL-1β and TNF-α and also between IL-6 and TNF-α, inducing more effects of TNF-α in this field. Lack of correlation between clinical parameters and cytokine concentration can also be relevant to other destructive factors like IL-17 which may induce inflammatory cytokines. Moreover, some factors like IL-1Ra and TGF-β have a controlling effect on inflammation and inhibit cytokine effect to some extent. More studies are required to identify the specific role of each cytokine in the initiation and progression of periodontal diseases.

Acknowledgements

We would like to thank Qazvin University of Medical Sciences for its financial support of this study.

References

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