Expression of selected inflammatory proteins and metalloproteinases in periodontitis

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Abstract. – OBJECTIVE: Periodontitis is a chronic inflammatory disease caused by microbial dental plaque which leads to the destruction and loss of supporting tissues of the tooth. Microbial plaque alone, however, is not enough to cause the disease. The body’s response plays an important role, in which an imbalance between the pro-inflammatory and anti-inflammatory effects of cytokines leads to an inflammatory reaction.

PATIENTS AND METHODS: We detected changes in mRNA expression and protein levels of MIP-1α, and metalloproteinases (MMP-2, MMP-9) contributing to cascades in the initiation and progression of inflammatory bone resorption and destruction of periodontal soft tissues in patients with aggressive (AP) or chronic (CP) forms of periodontitis in comparison with healthy individuals (control).

RESULTS: MIP-1α mRNA levels were highest in AP (280 ± 23% higher than the control) also in comparison with CP. The difference in protein level was less pronounced. MMP-2 mRNA expression values were similar (300 ± 12% higher in comparison with control), but protein levels were lower, also when compared to CP. Only in CP MMP-9 mRNA levels were significantly higher than the control (30 ± 8%), while protein levels were again higher in AP. Both AP and CP showed a positive correlation between the level of MIP-1α and MMP-2 (0.879, and 0.954 respectively). However, a strong positive correlation was only found between the levels of MMP-2 and MMP-9 in CP (0.812).

CONCLUSIONS: MIP-1α, MMP-2 and MMP-9 mRNA expression, along with the concentration of proteins in saliva in patients with periodontal disease, is higher than in healthy individuals and correlates with the severity of the disease.

Key Words: Cytokine, Chemokine, Inflammatory protein, Metalloproteinase, Periodontitis.

Introduction

Periodontitis is defined as an inflammatory disease of supporting apparatus caused by specific microorganisms. The result of this inflammatory process is the destruction of the periodontal ligament and alveolar bone, followed by the formation of periodontal pockets, recesses or combinations thereof. Periodontitis is distinguished from gingivitis by the destruction of the connective epithelium. The loss of attachment associated with periodontitis can be continuous or in times of disease exacerbation. A microbial dental plaque is needed to form periodontitis, but the microbial plaque itself is not sufficient to induce the disease. Periodontitis is not a classic infectious disease. It is the result of an imbalance between risk factors and the consequent response of the organism. Recently, antimicrobial photodynamic therapy has been shown to be effective in the treatment of periodontitis.

Cytokines are a large group of small proteins with an important role in cell signalling, affecting other cells and occasionally the cell producing them. A comprehensive network of cytokines balances the pro-inflammatory and anti-inflammatory effects, and their imbalance can lead to an inflammatory reaction. MIP-1α, a chemotactant cytokine, is associated with leukocyte activation in response to damaged or inflamed tissue. Due to different chemokine-binding receptors, gingival tissue fibroblasts express higher levels of chemokines (MIP-1α/CCR3) and cytokines (IL-6) when exposed to bacterial lipopolysaccharide, viral protein or pro-inflammatory cytokine (IL-1β), compared to periodontal ligament fibroblasts. MIP-1α expression attracts circulating monocytes that express CCR5 and CCR2 receptors. It tends to attract cytotoxic T and B cells to inflamed tissue.
thereby increasing the expression of MIP-1α by gingival epithelial cells to promote an acute inflammatory response, and recruitment of B cells in the later stages of periodontal disease. In addition to chemotaxis, biological functions of MIP-1α include wound healing, stem cell inhibition, and maintenance of the effector immune response. The role of MIP-1α in osteoclast activation in aggressive periodontitis is well known. It is typified by the resorption of alveolar bone via the CCR1 receptor, which is predominantly expressed by osteoclasts. The result is higher levels of MIP-1α as one of the diagnostic criteria for aggressive periodontitis.

In the inflammatory-altered periodontium, matrix metalloproteinases (MMPs) participate in various cascades and regulate the availability of many inflammatory signalling molecules. MMPs are a group of Zn-dependent proteases which play a role in the initiation and progression of inflammatory bone resorption and destruction of periodontal soft tissues in the process of periodontitis. Depending on the stimulus and local action, MMPs may increase or decrease the availability of signalling molecules by several mechanisms that may result in the loss of periodontal tissues. There is a positive correlation between the severity of periodontal disease and the concentrations of MMPs in sulcular fluid. Increased expression of MMP-9 has been shown to promote osteoclast formation and accelerate alveolar bone resorption by osteoclasts. MMP-2 (collagenase type IV) destroys type IV collagen, which subsequently leads to loss of attachment.

The aim was to detect the changes in protein and mRNA expression levels of selected MIP-1α, MMP-2, MMP-9 encoding inflammatory and matrix metalloproteinase proteins.

Patients and Methods

Patients and Sampling
The Ethics Committee of Louis Pasteur University Hospital approved the study under number 2018/EK/2010. After signing informed consent, 82 participants were included in this part of the study. Exclusion criteria were as follows: antibiotics use at the time of saliva collection, presence of other conditions such as immunodeficiency diseases, cancer, autoimmune diseases, immunosuppression after transplantation, malnutrition, or chronic kidney disease. Collection of saliva took place in the 1st Dental Clinic of the University Hospital and at the Dental and Maxillofacial Surgery Clinic - Academy Košice. Samples were collected in the morning between 7:00-9:00 and from January 2019 until June 2020. After periodontal examination, we asked the individuals to come for saliva sampling the next day after fasting, without smoking, drinking fluids and brushing their teeth. This time lag was important in preventing blood contamination of samples present during the periodontal examination.

For 10 minutes, the patients sat upright and spat freely-forming saliva into a container. The samples were kept on ice upon collection and immediately transferred to the laboratory located in the same building, where they were stored at -80°C prior to analysis.

Patients were divided into 3 groups according to clinical examination. The control group (Control) consisted of 43 healthy people. Their gingivae were without inflammatory changes, deposits of dental plaque, calculus, evidence of periodontal pockets or resorption of alveolar bone visible on X-ray. The second group consisted of 23 patients with chronic periodontitis (CP). In these patients, periodontal pockets were found in the oral cavity with a depth of up to 6 mm (values of 3 and 4 measured according to the CPITN) and alveolar bone resorption observed upon X-ray. Gingival bleeding upon stimulus, microbial plaque and calculus deposits were observed. The third group consisted of 16, predominantly young patients with aggressive periodontitis (AP), in which deep periodontal pockets were observed (over 6 mm). X-ray images showed horizontal resorption of the alveolar bone, and in some areas vertical or cup shaped. The gingivae were pale pink with possible point-like bleeding upon stimulus. Extensive damage to the periodontal tissues did not respond to relatively good dental hygiene, with a history of repeated inflammatory changes of the periodontium. Groups with periodontitis, either chronic or aggressive, represented a generalized form.

RNA Isolation and Analysis
RNA samples were isolated from patient saliva with RNeasy Micro Kit (Qiagen, Hilden, Germany), and changes in the expression of mRNA for MIP-1α, MMP2, MMP9 were detected. RNA samples were transcribed by reverse PCR into cDNA using First strand synthesis reverse H Minus M-MLV kit (Thermo Fisher Scientific, Waltham, MA, USA), followed by amplification using the SensiFast SYBR NO-ROX kit (Bioline, London, UK) 35 cycles (95°C/5 min, 95°C/15
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sec, 58–62°C/20 sec, and 72°C/25 sec) protocol using the corresponding specific primer sequences. Rotor-Gene Q-PCR Thermocycler (Qiagen, Hilden, Germany) was used for RT-PCR analysis. Due to the biological variability of the biological material samples, the analysed samples were measured in triplicate for each gene of interest. Changes in mRNA expression for the genes of interest were evaluated by comparative quantification, and Ct values using the Q Rotor Gene Software.

**Protein Determination**

Isolation of proteins from saliva samples was performed using the NORGEN RNA/Protein purification plus kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer’s manual. The commercial Human MIP1-α SimpleStep ELISA® kit (Abcam, Cambridge, MA, USA) a MMP-2 Human ELISA Kit (Abcam) was used for enzyme-linked immunosorbent assays. All reagents were incubated at room temperature for 1 hour before use. For MMP-2, the antibody was added only after incubation and washing, and for MIP1-α, the antibody was added after incubation (1 hour; 2.5 hours). Absorbance was detected at 450 nm on a standard microplate reader. To evaluate the results, a calibration curve was generated using the protein standard.

**Statistical Analysis**

Data were analysed using SPSS Statistics version 26 (IBM, Armonk, NY, USA). Differences between groups were then assessed by a nonparametric Kruskal Wallis H-test and a Mann-Whitney U tests to determine pairs that differed. Statistically significant results were considered at $p<0.05$. Spearman’s correlation analysis was used to find interdependencies.

**Results**

Levels of mRNA for MIP-1α showed maximal values in the group of patients with AP, where expression was $280 \pm 23\%$ higher than the control ($p<0.001$). In the group of patients with CP, the level was higher by $49.6 \pm 12\%$ compared to the control, although not statistically significant. However, confirmation of the MIP-1α parameter as a specific marker was demonstrated by its significantly different mRNA expression between the two groups of patients. In the group with AP, the detected level was $230 \pm 9\%$ higher in comparison with CP ($p<0.01$) (Figure 1). The observed increased values showed a similar tendency at the protein level. In the group of patients with AP, MIP-1α protein levels were $89 \pm 16\%$ ($p<0.001$) higher than in the control group. CP showed a slight increase in protein level by $8.1 \pm 2\%$ compared to the control group. The difference between the two groups of periodontitis was less pronounced at the protein level than with mRNA by only $81 \pm 6\%$ (Figure 2). The correlation between mRNA and MIP-1α protein levels revealed a moderate interdependence in the AP group ($r_s = 0.500$, $p \leq 0.05$). A negative weak dependence was demonstrated in the CP group ($r_s = -0.185$, $p \leq 0.05$).
MMP-2 gene mRNA levels were similar to MIP-1α with a maximum value in AP (Figure 1). The level was 300 ± 12% (p<0.001) higher in comparison with the control. Surprisingly, changes at the mRNA level did not show the same extent at the protein level. A maximum value was detected in CP with a level 80 ± 10% (p<0.01) higher than the control (Figure 1). A comparison between groups with periodontitis showed a significantly lower level (by 60 ± 8% (p<0.001) in AP compared with CP. Monitoring the correlation between mRNA and MMP-2 protein levels revealed a moderate negative interdependence in the group of patients with AP (rs = -0.447, p≤0.05). A non-significant negative weak dependence was demonstrated in the group of patients with CP (rs = -0.303, p = 0.08).

mRNA levels were significantly higher only in the CP group, with a 30 ± 8% (p<0.01) increase when compared to control (Figure 1). At the protein level for MMP-9, the difference between the AP and CP forms manifested up to a 50 ± 13% (p<0.01) increase in the AP group (Figure 2). The largest difference was recorded in AP in comparison with control with a value of 74.8 ± 11% (p<0.01). A high positive correlation was detected in both groups between the level of MIP-1α and MMP-2 (rs = 0.879, p≤0.01 and rs = 0.954, p≤0.01 in the AP). A strong positive correlation was revealed only between the levels of MMP-2 and MMP-9 (rs = 0.812, p≤0.01) in CP.

**Discussion**

The dento-gingival interface is a unique place in which the calcified structure comes into contact with the external environment and where it is exposed to bacterial colonization. The predominant anaerobic, gram-negative bacteria induce activation of the immune response in the host and aggregation of polymorphonuclear cells and macrophages. Bacterial lipopolysaccharides in gingival connective tissue induce the activation of various inflammatory cells and stimulate the expression of pro-inflammatory and chemotactic cytokines.

Increased MIP-1α production in macrophages at periodontal and bone resorption sites has been detected in several studies. Elevated levels of this protein may reveal the hidden presence of subclinical inflammation on clinically healthy patients with periodontitis. During non-surgical periodontal treatment, salivary MIP-1α levels decrease. Detection of this marker, associated with periodontal bone remodelling, shows high sensitivity (95%) and specificity (97%) in saliva. At the mRNA level, a significant increase was detected in our study compared to control (p<0.05). This was also reflected in an increase at the protein level compared to control (p<0.001). When comparing the aggressive and chronic forms of periodontitis, a difference was detected in mRNA levels (p<0.05), which was also reflected at the protein level, where the difference was less pronounced. The results of our measurements are consistent with the results of several publications, such as Thunell et al., Fine et
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al found that the mean MIP-1α level was 18-fold higher in patients with periodontitis than in the control groups (p<0.0001). MIP-1α also showed a significant correlation with periodontitis (AUC = 0.94; p<0.0002). CART analysis revealed that, of the markers monitored, only MIP-1α was a significant discriminator of periodontitis. The threshold value for periodontitis was 1.12 pg/ml, which corresponded to a sensitivity of 94.9% and a specificity of 92.7%. MIP-1α/CCL3 concentrations in saliva also showed a strong positive correlation with clinical parameters of periodontal disease. In addition to correlating with disease severity, salivary MIP-1α/CCL3 levels have been shown to reflect response to treatment. Monitoring MIP-1α/CCL3 levels in biological fluids provides a great opportunity to diagnose various inflammatory diseases and conditions in their early stages. In addition, inhibitors of MIP-1α/CCL3 and its receptors may be potential therapeutic targets in the treatment of these diseases.

Matrix metalloproteinases (MMPs) play an important role in physiological and pathological processes, including degradation and regeneration of connective tissue due to the inflammatory response. The main source of MMP-2 found in saliva is polymorphonuclear leukocytes. MMP-2 acts by activating cell proliferation but also by inhibiting apoptosis. MMP-2 also affects fibroblast growth factor-1 (FGF), leading to the release of the active domain of its receptor, giving MMP-2 the potential to modulate the mitogenic and angiogenic activities of fibroblast growth factor. Elevated levels of MMP-2 have been detected in the gingival crevicular fluid (GCF) of patients with periodontitis. Our results at the mRNA level indicate a maximum level of MMP-2 in the aggressive form of periodontitis (p<0.001), which resulted in a slight decrease in the level of MMP-2 protein compared to the control (p<0.01). When comparing the aggressive and chronic forms of periodontitis at the protein level, we detected a significantly higher level in the chronic form (p<0.001). The results correlated with the results of Bostanci et al who demonstrated significantly higher levels of MMP-2 in the GCF of periodontal patients compared to healthy individuals. Further study has shown that IL-1β can promote the development of chronic periodontitis by increasing MMP-2 expression. Song et al described increased MMP-2 expression levels in patients with untreated periodontitis compared to patients with treatment.

MMP-9 is one of the most abundant MMPs in saliva, sulcular fluid or gingival tissue in periodontal diseases. Rai et al, in their comparison of sulcular fluid in healthy individuals and in patients with periodontitis, observed higher levels of MMP-9 in the group with periodontitis. In our study, we observed significant changes in MMP-9 expression in both chronic and acute periodontitis. Higher expression was also noted in patients with acute periodontitis compared to control (p<0.01). At the protein level for MMP-9, there was a significant increase in the AP group in comparison with CP and control (p<0.01). Maeso et al also observed higher levels of MMP-9 in the sulcular fluid of patients with periodontitis compared to the healthy group, but these were not statistically significant. Lin et al, however, describe upregulation of MMP-8 and MMP-9 expression in inflamed gingival tissue compared to healthy tissue.

Conclusions

The expression and concentration of the detected inflammatory proteins was higher in patients with periodontitis than in healthy individuals. The highest expression of MIP-1α and MMP-2 was in patients with aggressive periodontitis. At the protein level, differences between the chronic and aggressive forms were not significant for MIP-1α, but the MMP-2 values were lower in the chronic form. In contrast, MMP-9 expression was highest in patients with the chronic form, though protein levels were lower compared to the aggressive form. The levels of MMP-2 and MMP-9 showed strong positive correlation in chronic periodontitis.

The limitation of the study is the low number of patients sampled due to the epidemiological situation with COVID-19 at the time of sampling which still persists.

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Authors’ Contributions

MR, PU, JV contributed to the study conception and design. Material preparation, data collection and analysis were performed JK, ZŠ, ZK, KK. The first draft of the manuscript was written by JV, PU and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.
The authors declare that they have no conflicts of interest.

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