


Expression of Toll-Like Receptor 4 and Matrix Metalloproteinase 8 in Gingival Crevicular Fluid in Patients with Periodontitis

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Abstract

Objective: To determine the expression of TLR4 and MMP8 in gingival crevicular fluid [GCF] in patients with periodontitis. **Material and Methods:** Clinical samples were collected from 23 gingival crevicular fluid of periodontal disease subjects (n = 14) and healthy periodontal subjects (n=9). Measurement of Clinical parameters of probing pocket depth (PPD), bleeding on probing (BOP), and clinical attachment loss (CAL) were included as diagnostic criteria. Pocket Depth (PD) and CAL were defined as present if the PPD was ≥ 4 mm and the CAL ≥ 1 mm. Expression of TLR4 and MMP8 in the gingival crevicular fluid of deep pockets (PD ≥ 6 mm), shallow pockets (PD 4–5 mm) and healthy periodontal sulcus (0–3 mm) were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Statistical analysis to compare the pocket was using Independent t-test and Mann-Whitney test. Correlation between mRNA expression and clinical parameters was analyzed using Spearman's correlation test. **Results:** Expression of TLR4 was higher in shallow pockets compared to the control group, but the difference was not statistically significant ($p > 0.05$). The expression of MMP8 was higher in shallow pockets compared to the control group, but the difference was not statistically significant ($p > 0.05$) either. There is no significant correlation between TLR4 and MMP8 with clinical periodontal parameters. **Conclusion:** TLR4 and MMP8 mRNA expression levels should not be used as a clinical biomarker in periodontitis diagnostic tools.

Keywords: Toll-Like Receptor 4; Matrix Metalloproteinase 8; Periodontitis; RNA, Messenger.

Introduction

Periodontitis is an inflammatory disease caused by multifactorial factors, such as microbial composition, host traits, and multiple modifiable risk factors that influence the severity and progress of periodontal disease [1]. Chronic periodontitis is the most common periodontal disease each year in Indonesia [2].

Proper identification, treatment, and preventive therapy are required to slow the disease progress in the future. The old classification has placed periodontitis into two categories: chronic and aggressive periodontitis [3]. However, in clinical practice, the ability to correctly differentiate between these categories has become a concern as diagnosis determines the appropriate therapy and maintenance care that should be applied [4]. Thus, many studies have been conducted to identify approaches to differentiate these categories, such as the characterization of biomarkers for host response [4].

The new classification of periodontitis had been employed by World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions using a case definition system, facilitating necessary patient treatment. It differentiates the four stages of periodontitis (Stages I-IV) and describes the severity (Interdental CAL at site of greatest loss, Radiographic Bone loss, Tooth Loss) and complexity (Local complexity such as Maximum probing depth, bone loss, furcation involvement, ridge defect, etc) of each stage's management according to clinical parameters [4].

Various etiologies, including host, environmental, and genetic factors, cause differential periodontal disease expression. Each component modifies the severity and progress of periodontal disease [5]. Periodontal pathogenesis initiated by lipopolysaccharides (LPSs) from periodontal pathogenic bacteria proliferating in host cells is recognized by Toll-like receptor 4 (TLR4), which stimulates the immune response to produce proinflammatory, prostaglandin, proteinase, and matrix metalloproteinases (MMPs); MMP-8, also known as neutrophil collagenase, destroys the collagen tissue of gingival epithelium [5,6]. Bacterial attachment and colonization occur within 3 hours, during which the bacteria undergo an adherence phase, and within the following 12 hours, undergo an internalization phase into the cell [7].

TLR4 is a human protein within the pattern recognition receptors family, whose activation triggers the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the production of proinflammatory cytokines responsible for activating innate immune cells. TLR4 recognizes LPS from gram-negative and some gram-positive cells [7]. TLR4 is expressed by monocytes, macrophages and leukocytes. TLR4 is expressed by gingiva epithelial cells as well [8]. Research on the association between TLR4 and periodontitis has produced different results; in Asia, an association has been shown between TLR4 and chronic periodontitis, whereas no such association has been reported in studies in other countries [6].

Matrix metalloproteinase 8 (MMP8) is positively correlated with the severity of chronic periodontitis [9]. MMP8 is primarily released by neutrophils in its inactive latent form, which is

subsequently activated by inflammatory mediators from independent hosts or via joint action triggered during periodontal inflammation. Moreover, MMP8 is expressed by macrophages as well as gingiva fibroblast, endothelial, epithelial, plasma, and bone cells [10]. A previous study showed high MMP8 levels in patients with chronic and aggressive periodontitis [10].

Although TLR4 or MMP8 have an important biological network in periodontitis, there is still a lack of knowledge of this cytokine in periodontal disease. The aimed of the study was to determine the expression of TLR4 and MMP8 in gingival crevicular fluid (GCF) in patients with periodontitis.

Material and Methods

Study Design and Sample

This work is based on an observational study design, in which 23 gingival crevicular fluid (GCF) of individuals, aged 28-61 years (52% men and 48% women) where included between February 2018 and June 2018.

Data Collection

The clinical sample was collected from the patient in the Dental Teaching Hospital, Faculty of Dentistry, Universitas Indonesia (RSKGM FKG UI) and for the laboratorium work was conducted in the Laboratory of Oral Biology of the Faculty of Dentistry of the University of Indonesia.

Complete anamnesis was done by asking their chief complaint, medical history, information about the allergy, systemic condition, use of medication, smoking habits, tooth brushing habits, etc. The detailed information by checking their extraoral, intraoral, measuring their oral hygiene index, pocket depth, recession, clinical attachment loss, papilla bleeding index (PBI), radiographic bone loss was recorded.

The inclusion criteria were subjects with periodontitis stages II, III and IV [4]. The clinical parameters that include these criteria are pocket depth (PD) (≥ 4 mm), Papilla Bleeding Index (PBI), and Clinical Attachment Loss (CAL) (≥ 1 mm). Patients with a history of bruxism or smoking, intake of drugs during the last six months, pregnancy or breastfeeding, scaling ≤ 6 months or consumption of antibiotics ≤ 3 months were excluded.

Periodontal clinical examination for PBI and PPD were recorded using Periodontal Probe UNC 15 (Osung Mnd. Co. Ltd., Gyeonggi-do, Korea). PBI was recorded as local bleeding present thirty seconds after probing with a scale of 0-4 (from no bleeding to spread bleeding).

Radiographic measurements were used to take dental intraoral radiographs. Alveolar bone loss was measured from cemento-enamel junction to the alveolar crest and the pattern of the bone loss was observed whether it is horizontal or vertical bone loss. All of these measurements are important for the periodontal diagnostic.

After the examination, a sample of gingival crevicular fluid was collected from each individual after the removal of supragingival plaque. GCF was obtained in the study group from two sites of a random site that had deep pockets (PD ≥ 6mm) and shallow pockets (PD 4-5 mm) and in the control group, from the random site of the healthy periodontal sulcus (0-3 mm).

The tooth surface was dried and isolated by using a cotton roll, and subsequently, we inserted three paper points (no. 30) into the periodontal pockets and then held it for about 20-30 seconds. After that, the paper point was placed into a sterile Eppendorf tube containing 200 µl of a tris-EDTA buffer, refrigerated, and then immediately transferred to the oral biology laboratory for the analysis.

Total RNA extraction was performed using TRIzol™ Reagent (ThermoFisher Scientific, Waltham, MA, USA) according to the instructions provided by the company, followed by reverse transcription using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was amplified by qRT-PCR with specific primers. The qRT-PCR analysis was performed on the ABI StepOnePlus Real-Time PCR System with PCR master SYBR Green (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Each sample was amplified with PCR using TLR4 and MMP8 primers (Table 1). PCR conditions were set as follows: pre-denaturation at 95°C for 5 min; followed by 40 amplification cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The melt curve profile was set as at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. D-glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as a housekeeping gene, and a 2^{-ΔΔCt} folding transformation formula was used to analyze mRNA expression levels for each targeted gene in the oral samples.

Table 1. Sequences of TLR4, MMP8 and GAPDH primer.

Primer Name	Sequences	Reference
TLR-4	Forward: 5'- CAA GGG ATA AGA ACG CTG AGA- 3'	[7]
	Reverse: 5'- GCA ATG GTG TCT CTG GCA GGT GTA - 3'	
MMP-8	Forward: 5'- GCT GCT TAT GAA GAT TTT GAC AGA G- 3'	[11]
	Reverse: 5'- ACA GCC ACA TTT GAT TTT GCT TCA G - 3'	
GAPDH	Forward: 5'-GTT GTC TCC TGC GAC TTC A-3'	[12]
	Reverse: 5'-GCC CCT CCT GTT ATT ATG G-3'	

Statistical Analysis

The collected data of mRNA expression of TLR 4 and MMP8 between shallow and pocket depth were compared using the Mann-Whitney test and Independent T-test. Spearman rank correlation was used to analyze the association between mRNA expression and clinical parameters within each group. A p<0.05 was considered significant. Data analysis was performed by SPSS software program (SPSS Statistics/IBM Corp., Chicago, IL, USA).

Ethical Aspects

This study was approved by the Ethical Committee of Dental Research (KEPKG), Faculty of Dentistry, Universitas Indonesia (Protocol No. 090220218). All of the subjects signed their informed consent letter to participate in this study.

Results

Expression of mRNA of TLR4 and MMP8 in the shallow pocket and deep pocket was shown in Table 2 and Table 3.

Table 2. Comparison of TLR4 mRNA expression between the shallow pocket and deep pocket.

Variables	Mean (SD)	p-value*
Shallow Pocket/PD 4-5 mm (n = 8)	1.1700 (1.18)	0.682
Deep Pocket/PD ≥ 6 mm (n = 8)	0.945 (0.96)	

*Independent T-test.

According to Table 2, there is no significant difference statistically of TLR4 expression between shallow pocket and deep pocket (p=0.682). Figure 1 describes the expression compared to the control group.

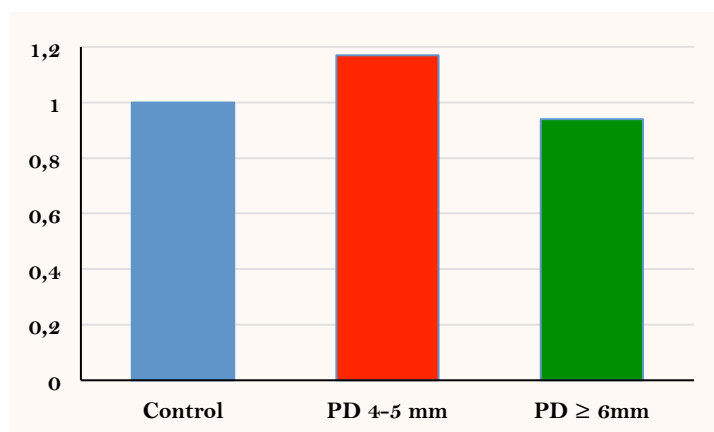


Figure 1. Expression of TLR4 of the shallow pocket and deep pocket compared to the control group.

The expression of TLR4 in the shallow pocket is increased by 1.17 fold compared to the control group. But, in a deep pocket, the expression of TLR4 is decreased 0.95 fold compared to the control group.

According to Table 3, there is no significant difference statistically of MMP8 expression between the shallow pocket and deep pocket (p=0.139). Figure 2 describes the expression compared to the control group.

Table 3. Comparison of MMP8 mRNA expression between the shallow pocket and deep pocket.

Variables	Median (Min.-Max.)	p-value*
Shallow Pocket/PD 4-5 mm (n = 8)	1 (0.01-3.67)	0.139
Deep Pocket/PD ≥ 6 mm (n = 8)	0.49 (0.07-2.2)	

*Mann-Whitney test.

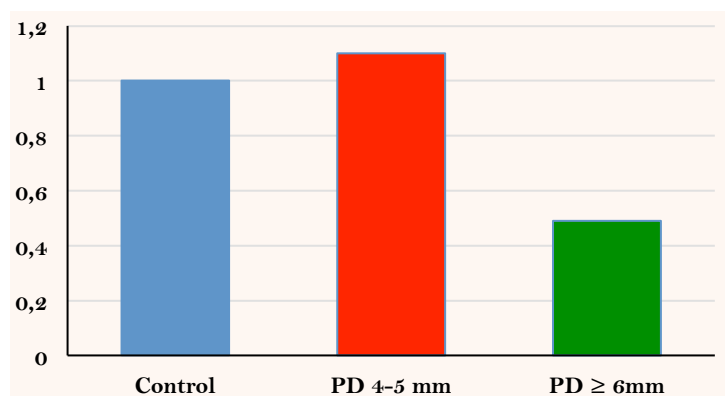


Figure 2. Expression of MMP8 of the shallow pocket and deep pocket compared to the control group.

The expression of MMP8 in the shallow pocket (4-5 mm) is increased by 1.1 fold compared to the control group. But, in the deep pocket (≥ 6 mm), the expression of MMP8 is decreased 0.49 fold compared to the control group.

The correlation between clinical periodontal parameters and gcf mRNA expression of TLR4 and MMP8 are shown in Table 4.

Table 4. Spearman's Correlation [r] between the gingival crevicular fluid expression of TLR4 and MMP8 and clinical periodontal parameters of the study group.

Parameters	r of TLR4	r of MMP8
PBI (p)	-0.406 (0.191)	-0.393 (0.206)
PD (p)	0.014 (0.966)	-0.235 (0.462)
CAL (p)	-0.182 (0.651)	-0.567 (0.05)

PBI: Papilla Bleeding Index; PD: Pocket Depth; CAL: Clinical Attachment Loss. Correlation was significant at $p < 0.05$.

There is a positive correlation between TLR4 and pocket depth, but the correlation is weak, and there is no significant correlation statistically. Correlation between TLR 4 and PBI negatively moderate but still no significant correlation statistically. A weak negative r correlation between TLR4 and CAL support by no significant correlation statistically. A weak negative correlation between MMP8 and PBI and PD statistically had no significant correlation. This happens between MMP8 and CAL even they have a strong negative correlation.

Discussion

Markers in Gingival crevicular fluid is a great diagnostic method for periodontal disease because its easy and simple for the operator. There are so many different results that use cytokine as the markers. The study of TLR4 and MMP8 as part of pro-inflammatory cytokine shows different results.

Periodontal pathogenesis started by periodontal pathogenic bacteria that proliferating in host cells and produce Lipopolysaccharide (LPS) is recognized by TLR-4 (Toll-Like Receptor-4) and stimulates the immune response to produce proinflammatory, prostaglandin, proteinase, and matrix metalloproteinases (MMPs), one of them is MMP-8 (Matrix Metalloproteinase-8) or also called neutrophil collagenase, which is responsible for collagenase by destroying the collagen tissue of gingival epithelium [10,13].

MMP8 and TLR4 expressions were consistent with the severity of Periodontitis, it consistent with periodontal pathogenesis which is initiated by LPS from pathogenic bacteria proliferating in host cells is recognized by TLR4, which stimulates the immune response to produce proinflammatory, prostaglandin, proteinase, and matrix MMPs, including MMP8 or neutrophil collagenase, which destroys the collagen tissue of gingival epithelium via collagenase activity.

In this study, we aimed to analyses TLR4 and MMP8 levels in GCF of periodontitis and their correlation with clinical parameters. In subjects with periodontal disease, TLR4 mRNA expression was overall higher in shallow pocket compare to the control group that has no periodontal pocket in it and there is a positive correlation between TLR4 and clinical periodontal parameter of pocket depth, even it's a weak correlation. It shows that TLR4 shows 1.17 fold higher than the control group. TLR4 polymorphism may be the etiology of chronic periodontitis in Asian people [6]. In a previous study, periodontitis tissue samples showed increased TLR4 levels in epithelial cell culture, which are present in higher densities under anaerobic conditions. Phenotypic changes in microorganisms, changes in oxygen levels, and interactions between microorganisms and their environment generate anaerobic conditions that support periodontal pathogenesis, which is supported by some authors, who demonstrated that TLR4 expression was increased in anaerobic conditions [14]. Even though there is no significant difference statistically, we can not throw away the results. The limitation of time and numbers of a subject that participates in this study may affect the results.

Surprisingly in deep pocket TLR4 are decreased 0.945 fold compare to the control group and there is a weak negative correlation with a clinical periodontal parameter of PBI and CAL and no significant difference statistically. We know that TLR4 was increased in anaerobic condition. Even though CAL is higher, but the long recession of the tooth affects the anaerobic condition that may result in higher CAL, but the condition is more aerobic than shorter CAL with no recession in the tooth surface. In this study, the CAL is higher due to pocket depth and long resesion that affects the microbial conditions.

MMP8 expression was increased in shallow pocket compare to the control group that had no periodontal pocket in it. The expression is 1.327 fold compare to the control group. MMP8 is stimulated by the immune response, and it plays a role in the healing process; however, MMP8 overexpression accelerates epithelial cell destruction. Immune responses are higher in the pockets due to anaerobic conditions, making pathogenic periodontal bacteria more resistant to the innate immune system. Bacterial LPS stimulates the immune system to respond and produce cytokines, as well as MMP8, which is responsible for destroying the collagen tissue of gingival epithelium. Even though there is no significant difference statistically as same as TLR4, we can not throw away the results. The limitation of time and numbers of subjects that participate in this study may affect the results.

MMP8 in the deep pocket was decreased 0.636 fold compare to the control group, but there is no significant difference statistically. The severity of periodontitis is affected by so many things;

one of them is the host response. Different cytokines with all other proteins collaborate and affect the severity. Other MMP like MMP9 affecting this condition and MMP8 may decrease in this condition. But it is likely that in a larger study population, this condition would not occur. MMP8 has a very weak negative correlation with clinical periodontal parameters. This result is supported by the recession of the tooth surface that may affect the microbial condition. This condition may indicate the expression depend on local inflammation [9].

Despite the limitation of this study, the data show us that the expression of TLR4 and MMP8 are higher in shallow pocket compare to the control group but with no significant difference statistically. It should also be noted that TLR4 and MMP8 cannot become a marker of the severity of periodontitis and do not seem to have diagnostic significance.

Conclusion

Expression of TLR-4 and MMP-8 in gingival crevicular fluid in a patient with periodontitis may indicate the activity of periodontal disease compared to the healthy periodontal subject. The lack of correlation between TLR4, MMP8 and clinical periodontal parameters shows that TLR4 and MMP8 cannot become a diagnostic marker for the severity of periodontal diseases. Further studies are needed to confirm the results of this study.

Authors' Contributions: SD designed the study, performed the data collection, experiment, data analysis and interpretation, and wrote the manuscript. YS and HS design the study, performed the interpretation, and reviewed the manuscript. BB performed the experiment, data analysis and reviewed the manuscript. All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest: The authors declare no conflicts of interest.

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