Expression Level of Osteonectin mRNA as Periodontal Healing Response after Scaling and Root Planing on Periodontitis Patients

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Abstract

Periodontitis is a chronic multifactorial inflammatory disease associated with plaque biofilms and host-mediated inflammation. The use of osteonectin (ON) for monitoring the healing response after treatment is unclear.

The present study aimed to evaluate the expression of osteonection mRNA as potential indicator of periodontal healing response after scaling and root planing (SRP) in periodontitis patients.

Gingival crevicular fluid (GCF) samples were collected from five periodontally healthy subjects and ten periodontitis patients with probing pocket depth (PPDs) of 4-6 mm at baseline and 1, 2, and 4 weeks (T0, T1, T2, and T3) respectively after SRP. The osteonectin mRNA expression levels were measured using quantitative real-time polymerase chain reaction (qPCR). The differences in the osteonection mRNA expression levels between the experiment groups were analyzed using the Friedman test. The correlations between changes in the osteonection expression levels and clinical parameters value were analyzed using the Pearson test.

Compared to healthy subjects, periodontitis patient expressed low at T0 levels of osteonectin mRNA, which increased at T1 but decreased at T2 and T3. The differences were statistically significant ($p \leq 0.05$). The osteonection mRNA expression levels were not significantly correlated with PPD and BOP value after SRP. Our findings suggest that the GCF levels of osteonectin mRNA were highest at 1 week after mechanical periodontal therapy and not correlated with PPD or BOP. Further studies with large sample size are required to clarify the involvement of osteonectin mRNA in periodontitis healing process.

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Introduction

Periodontal disease is one of the two most common oral diseases worldwide, with a high prevalence rate, and represents a major health problem.[1] In Asia, particularly in developing countries, the prevalence of periodontal disease has been reported to be over 90%.[2] According to Tadjoedin et al, from medical records documented form 2004-2014 that 75,2% of patients at Department of Periodontology, Dental Teaching Hospital, Faculty of dentistry,

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Universitas Indonesia experienced chronic periodontitis, in which 29% cases were localized and 46.2% were generalized.[3]

Periodontitis is a chronic multifactorial inflammatory disease associated with plaque biofilms and host-mediated inflammation, which is characterized by the progressive destruction of the tooth-supporting apparatus, resulting in the loss of periodontal tissue support.[4] Its primary features, such as the loss of periodontal tissue support, involve the destruction of the periodontal ligament and alveolar bone and the migration of the junctional epithelium.[5] It manifests through periodontal pocket, gingival bleeding, clinical attachment loss, and alveolar bone loss on radiography.[6]

Non-surgical periodontal therapies, such as scaling and root planing (SRP), have been shown to be able to stop the progression of periodontitis in shallow to moderately deep

pockets.[7] The approach of SRP aims to create an environment with reduced supragingival and subgingival plaques and calculus, in which periodontal pathogen recolonization of pockets can be more effectively prevented and plaque control can be facilitated.[8] In clinical practice, the prediction of disease progression and evaluation of the response to periodontal therapy are important.

Gingival crevicular fluid (GCF) is an exudate from the gingival sulcus and periodontal pockets, which contains a wide variety of cellular mediators, proteins, enzymes, and biochemical mediators that reflect the periodontal tissue metabolic status.[9] The extracellular matrix (ECM) is one of the GCF products that may reflect the periodontal tissue metabolic status. Among ECM glycoproteins, some proteins, such as osteonectin (ON), are thought to fulfill their biological functions by binding to matrix proteins, as well as to cell surface receptors and molecules, such as proteases and cytokines.[10] ON is also referred to as secreted protein acidic and rich in cysteine (SPARC) or basement membrane protein (BM-40).[11] ON plays a role in wound healing/repair by promoting granulation tissue formation through the modulation of fibroblast migration. ON has been predicted to modulate interactions between the cell and ECM.[12] The ON expression is the highest during development, angiogenesis, and re-expression associated with ECM remodeling in wound healing that involves changes in cell shape, adhesion, and motility.[12,13] ON is thought to promote inflammation by contributing to microvasculature permeability, which allows leukocytes to enter the ECM, and recruiting macrophages.[13,14]

An increase in ON expression was reported 3 days after incisional skin injury, and peak expression was noted at 7 days during skin wound healing. Increased ON expression has been shown to be associated with reduced angiogenesis and scar tissue formation.[15] On the other hand, a lack of ON expression has been shown to diminish mature collagen accumulation in the ECM of many connective tissues. In a previous study, ON-null exhibited decreased total collagen I content in the skin, bones, and periodontal ligament.[14] In periodontal disease, however, ON has been reported to be a marker for inflammation and to be elevated in GCF samples from sites with severe periodontitis.[16]

The production of ON in the cementum, PDL, and alveolar bone affected by periodontal disease makes ON a strong candidate for improving the regeneration of periodontal attachment that is lost owing to disease.[12] The use of ON for monitoring the healing response to non-surgical periodontal therapy needs to be explored. Therefore, the aim of the present study was to evaluate the expression level of osteonection mRNA as potential indicator of periodontal healing response after scaling and root planing (SRP) in periodontitis patients and to determine whether osteonectin could be used as potential biomarker for periodontal healing process as response to periodontal therapy.

Materials and methods

This study received ethical approval from the ethical committee of the institution. Subjects were selected from patient referred to Department of Periodontology, Dental Hospital School, Faculty of Dentistry, Universitas Indonesia. The patient criteria were age range 30–60 years and periodontitis stage I and stage II grade A defined as the presence of at least 1 site with probing pocket depth (PPD) 4–6 mm. Periodontally healthy subjects were selected as a control group. The exclusion criteria were systemic diseases, active infections, smoking, alcohol consumption, administration of drugs, such as antibiotics and antisteroids, pregnancy, and lactation. Written informed consent was obtained from each patient prior to participation after explaining all procedures.

All selected patients underwent a periodontal clinical examination, including the PPD, and BOP. Then, GCF samples were obtained from the selected periodontitis sites before (T0), 1 (T1), 2 (T2), and 4 weeks (T3) after SRP therapy by two calibrated examiners according to the research protocol using the intra-crevicular absorption technique.[17] GCF samples were collected from the periodontally healthy subjects only at T0. Periodontal Clinical parameters (PPD and BOP) were measured in all teeth, excluding the third molars. Six sites in each tooth (distobuccal, buccal, mesiobuccal, mesiolingual, lingual, and distolingual) were examined using a periodontal probe (PCP-UNC15; Hu-Friedy, Chicago, IL, USA) and only the maximum PPD recorded in each tooth was used for analysis.

GCF samples were obtained after isolation at the site with a cotton roll to prevent saliva contamination, and supragingival plaque was removed using a Gracey curette, without touching the margin gingiva. The crevicular site was air-dried and a paper point (no. 30) was gently inserted into the periodontal pocket 1–2 mm subgingivally for 30 seconds. The collection of GCF samples was sequentially repeated thrice with an interval of 30 seconds. The paper points were immediately placed into the same Eppendorf tube containing 300 µl of TE buffer solution. Then, the Eppendorf tubes were transported to the laboratory and stored at −80°C for future analysis.[18]

RNA was extracted from GCF using the GENEzol[TM] reagent (GZR100, Geneaid Biotech, New Taipei, Taiwan). The extracted RNA was further transcribed to cDNA using the synthesis kit ReverTra® qPCR RT Kit (TOYOBO Co., Ltd., Osaka, Japan). All procedures were performed according to the recommendations provided by the manufacturer. For qPCR, the cDNA purity and concentration were evaluated by spectrophotometry using the Metertech SP-8001 UV/Visible Spectrophotometer (Metertech Inc., Taipei, Taiwan).

Four commercial qPCR master mixes (AceSensiFAST[TM] SYBR® Hi-ROX Kit, primer forward, primer reverse, and nuclease free water) were used. qRT-PCR reactions were performed according to the product recommendations.

For each assay, the total reaction volume was 10 µl (7 µl of master mix and 3 µl of cDNA sample). For each sample, PCR was performed using primers for ON (forward 5′-ATC TAA ATC CAC TCC TTC CAC AG-3′ and reverse 5′-CAC CGT TAA TGT ATT CAC TTA AAT C-3′)[19] and human β-actin/ACTB (reference/control; forward 5′-TAA TGT CAC GCA CGA TTT CCC-3′ and reverse 5′-TCA CCG AGC GCG GCT-3′).[20] All analyses were performed in duplicate in MicroAmp[TM] Fast Optical 48-well plates using StepOne Plus (Applied Biosystems, Foster City, CA, USA). The following PCR amplification protocol was used for ON: initial reaction involving denaturation at 95°C for 5 minutes, followed by 45 cycles at 95°C for 20 seconds each and annealing at 60°C for 1 minute.[19] The following amplification protocol was used for β-actin: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles at 95°C for 15 seconds each, and 60°C for 1 minute.[20] In each sample, normalization was performed according to the β-actin content using the following formula: $2^{-(ON\Delta CT - ACTB \Delta CT)}$, where CT = (CT[ON] − CT[ACTB])$_{periodontitis\ patients}$ − (CT[ON] − CT[ACTB])$_{healthy\ subjects}$, ON represents osteonectin mRNA, and ACTB represents β-actin mRNA.[21]

A test for the validity of the normality assumption using standardized range statistics was performed. Differences in gene expression between the experimental groups (T0, T1, T2 and T3) were analyzed using the Friedman test. The periodontal clinical parameters were evaluated by comparing the differences in PPD and BOP at before (T0) to after (T3) SRP therapy using a paired t-test statistical analysis. The Spearman's correlation tests were used to assess correlations between the changes ON mRNA expression in GCF and periodontal clinical parameter values. All statistical analyses were performed using SPSS software. A *p*-value of <0.05 was considered to indicate a significant difference.

Results

Table 1 presents a distribution and clinical data of the periodontitis patients at the baseline (T0), comprising ten periodontitis patients with 22 tooth sites, aged between 30 and 60 years. While control group comprising five subjects aged between 30-31 years. The result of normality test (Shapiro-Wilk) of the ON mRNA expression level analysis was abnormal data distribution at T0 and T2. Accordingly, the Friedman test was used to comparative analysis ON mRNA expression level (Table 2). A comparison of the relative ON expression levels at baseline (T0) to 1 week (T1) after SRP, then 1 week (T1) to 2 weeks (T2) after SRP and 1 week (T1) to 4 weeks (T3) after SRP in the periodontitis group found significant differences (*p* ≤ 0.05). Accordingly, the relative ON expression levels at T0, T1, T2, and T3 shown in Figure 2. In periodontitis group, ON expression levels were highest at T1 and lowest at 2 weeks (T2) after SRP, and the levels were higher than those in the control group.

Sample No.	Sex	Age	Tooth Site	PPD (mm)	BOP
1	F	41	16, 17 disto buccal	5.5 ± 0.5	3 ± 1
2	M	57	47 disto lingual 27 mesio buccal	5 ± 0	2 ± 0
3	M	43	26 disto buccal 44 disto lingual 32 mesio buccal	5 ± 1	2.33 ± 0,33
4	M	43	16 disto lingual	5 ± 0	4 ± 0
5	M	35	26 disto lingual 34 mesio buccal 35 mesio buccal	4 ± 0	2.33 ± 0,66
6	F	32	26 disto lingual 27 mesio lingual	4 ± 0	4 ± 0
7	M	35	27 mesio buccal	4 ± 0	3 ± 0
8	F	33	15 disto lingual	4 ± 0	4 ± 0
9	M	31	16 mesio buccal 34 disto lingual 47 distolingual	5 ± 0	4.33 ± 0.33
10	M	36	17 disto buccal 36 mesio buccal 26 disto buccal 24 mesio lingual	4.5 ± 0.5	3.75 ± 0.25
Mean ± SD		38.1 ± 8.14		4.75 ± 0.59	3.27 ± 0.85

PPD, probing pocket depth; BOP, bleeding on probing

Table 1. Clinical Parameters of Sites.

Osteonectin mRNA level expression	Median	(Min–Max)	P-value (Friedman test)	P-value (Wilcoxon test)
Before SRP	3.4876	(0.30-30.72)	0.042 *	
One week after SRP	20.1746	(0.73-51.44)		0.007 **
Two weeks after SRP	1.7052	(0.08-36.56)		0.037 **
Four weeks after SRP	9.5368	(0.17-26.37)		0.047**

Friedman test; *$P < 0.05$ indicates a significant difference
Post-hoc Wilcoxon test; **$P < 0.05$ indicates a significant difference
PPD, periodontal pocket depth; SRP, scaling and root planning

Table 2. Comparative Analysis of Osteonectin mRNA Expression in Periodontitis Patients Before and After Scaling and Root Planing.
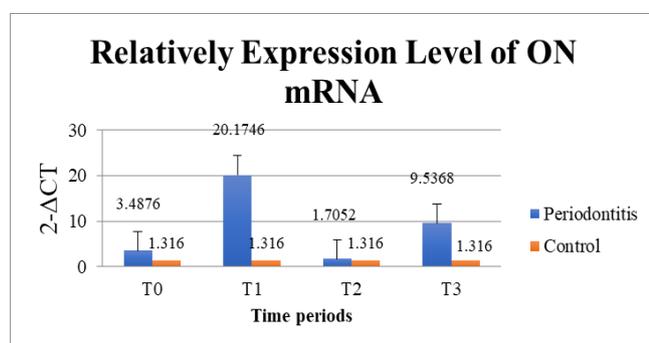
Figure 2. Expression Level of Osteonectin mRNA in Gingival Crevicular Fluids of Periodontitis Patients at Different Time Periods After Scaling and Root Planing Relative to Periodontally Health Subjects (Control Groups).

The clinical parameters include PPD and BOP was measured at T0 and T3. The result of normality test (Shapiro-Wilk) data exhibited a normal data distribution. Accordingly, the comparative analysis these variables were performed using paired *t*-test respectively. Table 3 presented the PPD and BOP was decreased over time from the baseline and the difference was significant ($p ≤ 0.05$).

Variable	Mean value (SD)	P-value
PPD		
Before SRP (T0)	4.75 ± 0.589	0.001*
Four weeks after SRP (T3)	3.1 ± 0.79	
BOP		
Before SRP (T0)	3.274 ± 0.848	0.001*
Four weeks after SRP (T3)	1.183 ± 0.873	

Paired *t*-test; *$P < 0.05$ indicates a significant difference
BOP, bleeding on probing; SRP, scaling and root planing; SD, standard deviation

Table 3. Comparative Analysis of Clinical Parameters Probing Pocket Depth and Bleeding on Probing Score in Periodontitis Patients Before and After Scaling and Root Planing.

Discussion

The primary objective of SRP is the effective reduction of supragingival and subgingival plaques and calculus, which will prevent the recolonization of pockets by periodontal pathogens, facilitate plaque control, and stop the progression of periodontal diseases in shallow to moderately deep pockets.[7,8] Osteonectin is a non-collagenous bone protein with a 40-kDa glycoprotein that is predominantly bound to hydroxyapatite.[23] Osteonectin expression level are high in immature bone and area associated with mineralization of collagen whereas a decrease expression level is observed in hemostasis.[14]

In the present study, we evaluated the expression levels of ON mRNA in GCF samples as potential indicator of periodontal healing response after scaling and root planing (SRP) with regard to clinical parameters on Indonesian Periodontitis patients. We found that ON mRNA expression levels in GCF samples from periodontitis patients increased at 1 week after SRP and gradually decreased 4 weeks after SRP. The findings of the present study might suggest periodontal wound healing in progress accordance with the findings by Trombetta et al. ON plays a role in wound healing/repair by

promoting granulation tissue formation through the modulation of fibroblast migration to facilitate he healing process. ON has been predicted to modulate interactions between the cell and ECM.[12] ON is thought to promote inflammation by contributing to microvasculature permeability, which allows leukocytes to enter the ECM, and recruiting macrophages.[13,14]

In these patients with periodontitis stage I-II, the observation of the highest ON expression levels at 1 week (T1) after SRP; followed by a decrease at 2 weeks (T2) and 4 weeks (T3) after SRP. The observation of the low ON expression level at baseline, suggests that a reduction in this factor due to chronic inflammation does indeed cause alveolar bone destruction. Although the ON expression level decreased again 4 weeks after SRP, it remained higher than at the baseline, may suggesting the continued expression of this gene during the tissue remodeling stage of the healing process.

Periodontal wound healing involves four overlapping stages: hemostasis, inflammation, proliferative and remodeling.[25] As its expression level peaks at 1 week, osteonectin is highly involved in inflammation and proliferation.[13,14] Furthermore, the observed high expression level of ON at 1 week after SRP suggest that SRP successfully eliminated inflammation and might promote to healing process. An increase in ON expression was reported 3 days after incisional skin injury, and peak expression was noted at 7 days during skin wound healing. Increased ON expression has been shown to be associated with reduced angiogenesis and scar tissue formation.[15]

Notably, Mccauley et al. ON has been reported to be a marker for inflammation and to be elevated in GCF samples from sites with severe periodontitis.[16] The result of present study was statistically significant, the present a profile osteonectin expression levels in humans that consistent with the findings of the previous animal studies.

An important clinical indicator for successful SRP is a reduction in the signs and symptoms of periodontal inflammation, such as PPD and the bleeding index. The high PPD and BOP score at baseline confirmed there is inflammation and resulting tissue destruction at baseline, respectively (Table 3). The improvements in clinical parameters such PPD and BOP score were support the findings of ON

expression levels in periodontitis patient. The findings of decreases in the bleeding index and PPD in our study were in accordance with the results in a previous study.[7] This previous study showed that PPD reduced by ±1 mm in moderate pockets (4–6 mm) 4 weeks after SRP when compared with the findings at baseline.[8,24]

GCF sampling was selected for the present study due to its simple, efficient, non-invasive nature, non-traumatic, and collected form both healthy and diseased sites.[17,26] Although this technique is limited by potential for contamination and difficult to determine volume. Collecting GCF using absorption method with paper point is more commonly used for subgingival plaque collection in microbilogical analysis. The time in which the paper point inserted in the periodontal pockets or gingival sulcus were 30 seconds to decrease the risk of blood or saliva contamination. [17]

It was thus important to correlate biomarkers expression level with clinical parameters. The correlation between ON expression levels in periodontitis patient and clinical parameters such PPD and BOP were no statistically significant correlation. For the ON expression and PPD, the correlation was positively, in other hand, negatively with BOP (Table 4). However, there are no similar studies for the comparison of our results. Notably, further studies with a similar methodology and larger sample size, which focus on ON mRNA expression levels, are needed to confirm our results and determine whether it could be used as a biomarker for clinical parameters in periodontitis patients. The limitation of this present study is amount of sample size.

Variable	Correlation coefficient (r)	P-value
ON expression level – PPD change	0.060	0.800
ON expression level – BOP change	-0.052	0.827

Spearman's test; *$P < .05$ indicates a significant correlation
ON, osteonectin mRNA; PPD, periodontal pocket depth; BOP, bleeding on probing; SRP, scaling and root planing

Table 4. Correlations of The Osteonectin (ON) mRNA Expression Level with Probing Pocket Depth and Bleeding on Probing in Periodontitis Patients Before and After Scaling and Root Planing.

Conclusions

In conclusion, SRP caused significant improvements in clinical parameters, and these improvements were accompanied by the up regulation followed down regulation of ON mRNA expression in GCF. The ON mRNA expression levels were significantly altered 1 week after SRP. The ON expression level can be detected in the GCF samples from both healthy and periodontitis sites. Our findings suggest the following: (1) ON mRNA expression levels in GCF is very low correlated with clinical inflammation parameters. Further studies with a large sample size are required to clarify the involvement of ON mRNA expression level as biomarker of periodontitis healing process.

Declaration of Interest

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