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The investigation of glutathione peroxidase, lactoferrin, myeloperoxidase and interleukin-1β in gingival crevicular fluid: implications for oxidative stress in human periodontal diseases

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Background: Human periodontal diseases are inflammatory disorders that are the result of complex interactions between periodontopathogens and the host's immune response. Two important and interrelated factors are involved in the pathophysiological progression of periodontal diseases, i.e. the activation of immune system and the production of oxygen radicals and their related metabolites. Increased production of oxygen radicals may contribute to oxidative stress, which is reported to be involved in many diseases, including periodontal diseases.

Objectives: The objective of this study was to investigate glutathione peroxidase, lactoferrin and myeloperoxidase, which play an essential role in free radical production and defenses, and the proinflammatory cytokine interleukin-1β (IL-1β), which is important in the regulation of immunological and inflammatory reactions in human periodontal diseases.

Methods: Gingival crevicular fluid (GCF) samples were collected from 27 subjects, 19 periodontitis patients and eight healthy controls, ranging in ages from 24 to 62 years. Clinical parameters were recorded. GCF glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β were analyzed by enzyme-linked immunosorbent assays (ELISA).

Results: The periodontitis sites exhibited significantly greater total amount of glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β than healthy sites. Total amount of glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β was positively correlated with plaque index, gingival index, probing depth and probing attachment level ($p < 0.05$).

Conclusion: The imbalance between the levels of myeloperoxidase/IL-1β and glutathione peroxidase/lactoferrin could result in tissue damage of reactive oxygen species (ROS) in periodontitis which is initiated and perpetuated by the chronic insults of periodontopathogens.

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An initiating event in the progression of human periodontal diseases is the host immune reaction to bacterial colonization in the subgingival area. The same host systems that provide protection and defense are responsible for destruction. Reactive oxygen species (ROS) and cytokines produced by activated phagocytes are potentially deleterious to the tissues (1, 2). ROS include oxygen free radicals and non-radical oxygen derivatives such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (2, 3). The imbalance between ROS and antioxidants in the human body, which leads to oxidative stress, makes a significant contribution to a variety of human diseases, such as arthritis, adult respiratory distress syndrome, heart disease, stroke, acquired immunodeficiency syndrome (AIDS), Alzheimer's disease, Parkinson's disease, alcoholism (4) and many others, including periodontal diseases (2). But the defense mechanisms against oxidative stress in the pathogenesis of periodontal diseases have received little attention. ROS can cause DNA and protein damage, initiate lipid peroxidation, oxidize important enzymes such as antiproteases, and stimulate proinflammatory cytokine release through depleting intracellular thiol compounds and activating nuclear factor κB (NF-κB) (2).

The antioxidant mechanisms are the evolutionary designs that avidly react with and annihilate ROS before they inflict oxidative damage to tissues and cells. Plasma glutathione peroxidase, an important extracellular antioxidant, is produced mainly in the kidney and has been detected in numerous human fluids (5, 6). Glutathione peroxidase is a selenium-containing enzyme that detoxifies hydrogen peroxide and various hydroperoxides using glutathione as a reducing agent (6, 7). Mates *et al.* reported that gene expression of glutathione peroxidase is up-regulated by H_2O_2 and other ROS (8). However, it has been demonstrated that some periodontopathogens have the capacity to utilize glutathione or cysteine as a source of hydrogen sulfide formation (2, 9–11). This may further potentiate the activation of NF-κB and thereby increase the release of proinflammatory cytokines, which may cause tissue damage (2).

A reaction of hydrogen peroxide with superoxide that forms highly reactive hydroxyl radicals (OH^-) is catalysed by iron or copper ions (2, 12). Therefore, the sequestration of metal ions plays an important role in the antioxidant defense (12). Lactoferrin is an iron-binding protein stored within the specific granules of polymorphonuclear leukocytes (PMNs) (13, 14). Myeloperoxidase is the content of azurophilic granules of PMNs (15). Hypochlorous acid (HOCl), the product of myeloperoxidase-catalysed reaction (3), is a powerful antibacterial agent (16), but is also capable of inactivating α1-protease inhibitor (15). The imbalance between proteases and antiproteases can lead to connective tissue destruction (15). Both lactoferrin and myeloperoxidase are considered to reflect the strength of oxidative stress.

Interleukin-1 (IL-1), a key mediator of the inflammatory process, affecting most cell types with potent proinflammatory and stimulatory properties, has many diverse biologic activities through its effects on the regulation of a variety of genes that are expressed during inflammation (17). IL-1 is capable of stimulating ROS production in some cell types (18, 19). Notably, IL-1β activates and is activated by NF-κB, contributing to the positive feedback loop for NF-κB activation (20). As discussed above, ROS also induce the activity of NF-κB. In this way, the severity of the diseases may be exacerbated by the persistent activation of NF-κB. Renard *et al.* evaluated the influence of glutathione peroxidase on IL-1-induced NF-κB activation and demonstrated that the inhibition of glutathione peroxidase was correlated with an overactivation of NF-κB (21). Therefore in this study we investigated their relationship in periodontal diseases.

The purpose of the present study was to improve our understanding of the pathogenesis of periodontal diseases by investigating glutathione peroxidase, an important antioxidant enzyme, lactoferrin and myeloperoxidase, which can reflect the strength of oxidative stress, and the proinflammatory cytokine IL-1β that has been implicated in human periodontal diseases. Furthermore, we correlated the four gingival crevicular fluid (GCF) substances with clinical parameters. The correlations between the three GCF markers, i.e. glutathione peroxidase, lactoferrin and myeloperoxidase, for oxidative stress and IL-1β will also be determined.

Materials and methods

Subject selection

Nineteen Chinese adult patients (11 males and eight females; aged from 31 to 62 years, mean age 45.1 ± 10.0 years) referred to the Department of Periodontics at the Kaohsiung Medical University Chung-Ho Memorial Hospital were recruited for this study. These patients were clinically diagnosed with chronic periodontitis and had at least 20 natural teeth. All patients were free of systemic disease and none had received periodontal therapy, such as scaling and root planing, prescribed antibiotics or anti-inflammatory medication within the preceding 6 months. Smokers were excluded from the study. Eight systemically and periodontally healthy subjects (three males and five females; aged from 23 to 29 years, mean age 26.6 ± 1.9 years) were selected as healthy controls. These healthy control subjects had a high standard of oral hygiene and history of regular prophylaxis. Informed consent was obtained from all participants and the protocol was approved by the institutional ethics review committee.

GCF sampling and periodontal examination

In order to eliminate the possibility of contamination with saliva, maxillary teeth were selected for sampling. Plaque index (22) was recorded. After removal of supragingival plaque, teeth to be sampled were isolated with cotton rolls to prevent contamination of saliva. A standard filter strip (Periopaper, IDE Interstate, Amityville, NY, USA) was inserted into the sulcus to the depth of 1–2 mm for 30 s. GCF

volume was immediately determined by a calibrated Periotron 8000 (Oraflow, Inc., NY, USA). Periopaper was removed and the wet portion was placed in a microcentrifuge tube containing 125 μl phosphate-buffered saline–0.1% Tween 20. The GCF samples were stored at −20°C until analysis. Samples visually contaminated with blood were discarded. Then gingival index (23) was recorded to assess the severity of gingival inflammation when probing depth and probing attachment level were measured. All clinical assessments were performed by the same investigator. The sampled sites were categorized according to the criteria described by Alpagot *et al.* (24), but we made a modification in order to have a more adequate division for gingivitis sites and periodontitis sites.

Group 1 (healthy sites, including sites with gingival recession): gingival index = 0, probing depth ≤ 3 mm and probing attachment level ≤ 1 mm.

Group 2 (gingivitis sites, including sites with gingival recession or pseudopocket formation): gingival index > 0, probing depth ≤ 3 mm and probing attachment level ≤ 3 mm.

Group 3 (periodontitis sites): gingival index > 0, probing depth > 3 mm and probing attachment level > 3 mm.

Sites were used as the experimental unit of observations. The gingivitis sites and the periodontitis sites were from periodontitis patients, whereas all the healthy sites were from the healthy control subjects.

Assays of glutathione peroxidase, lactoferrin, myeloperoxidase and interleukin-1β

After grinding the paper strips with microcentrifuge pestles, each sample was eluted in 500 μl phosphate-buffered saline containing 0.1% Tween 20 by centrifugation (1500 × *g*, 10 min) (25). Following elution, GCF samples were analyzed for plasma glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β using commercially available enzyme-linked immunosorbent assays (ELISA, R & D Systems Inc., MN, USA). Analyses were performed according to the manufacturer's recommended protocol. Concentration of each GCF substance was calculated with a standard curve obtained with each standard recombinant GCF substance. Total amount of each GCF substance was obtained by multiplying concentration and GCF volume.

Statistical analysis

The clinical parameters and levels of each GCF substance at healthy, gingivitis and periodontitis sites were expressed as mean ± standard deviation. The significance of differences within each group was assessed using the ANOVA test. Tukey–Kramer pairwise comparison was used for post-hoc analysis. A *p*-value less than 0.05 was considered statistically significant. The correlations between the levels of GCF substances and clinical parameters were calculated using Pearson's correlation. The JMP software package (Version 4.0.2, SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

Results

Characteristics of study groups

Table 1 shows the clinical parameters and GCF volume of each group. As expected, all the clinical parameters such as plaque index, gingival index, probing depth and probing attachment level reached statistically significant differences among the three groups ($p < 0.05$). A significant difference in GCF volume was also observed over all study groups ($p < 0.05$). Post-hoc analysis showed statistically significant differences in GCF volume between group 1 and group 2, group 1 and group 3 ($p < 0.05$), but not between group 2 and group 3.

GCF substance levels in periodontitis patients and healthy controls

Table 2 shows the levels of GCF substances in periodontitis, gingivitis and healthy sites. Total amounts of each GCF substance were significantly different among the three groups ($p < 0.05$). Total amounts of glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β were higher in periodontitis sites and gingivitis sites. Post-hoc analysis showed that the periodontitis sites exhibited significantly greater total amounts of glutathione peroxidase, lactoferrin, myeloperoxidase and IL1-β than healthy sites ($p < 0.05$). The gingivitis sites showed significantly greater total amounts of glutathione peroxidase, lactoferrin and IL-1β than healthy sites ($p < 0.05$). Moreover, total amounts of glutathione peroxidase, lactoferrin and IL-1β were significantly higher in periodontitis sites than in gingivitis sites ($p < 0.05$). As to concentrations, lactoferrin and IL-1β did not reach significant differences among the groups ($p > 0.05$). However, the concentration of glutathione peroxidase in periodontitis sites was higher than gingivitis sites and was the lowest in healthy sites, and the differences among the groups were significant ($p < 0.05$). Post-hoc analysis revealed significant differences between healthy sites and periodontitis sites ($p < 0.05$). The concentration of myeloperoxidase was higher in healthy sites than in periodontitis sites and was lowest in gingivitis sites, and the differences among the groups were significant ($p < 0.05$). Post-hoc analysis revealed significant differences between healthy sites and gingivitis sites ($p < 0.05$).

Correlations between GCF substances and clinical parameters

The severity of the disease was evaluated by clinical parameters such as plaque index, gingival index, probing depth and probing attachment level. We therefore correlated the levels of GCF substances with these parameters. The correlation between total amount and concentration of each GCF substance and clinical parameters is presented in Table 3. Total amounts of glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β were positively correlated with plaque index, gingival index, probing depth and probing attachment level ($p < 0.05$). In general, concentrations of these GCF substances did not show similar

Table 1. Clinical characteristics of study population

					Tukey–Kramer pairwise comparison		
	Group 1 ($n = 35$)	Group 2 ($n = 41$)	Group 3 ($n = 42$)	p-value from ANOVA	Group 1 vs. group 2	Group 1 vs. group 3	Group 2 vs. group 3
PLI	0.00 ± 0.00	1.66 ± 0.69	2.38 ± 0.76	< 0.0001	*	*	*
GI	0.00 ± 0.00	1.85 ± 0.48	2.48 ± 0.55	< 0.0001	*	*	*
PD (mm)	1.86 ± 0.55	2.68 ± 0.57	5.12 ± 1.04	< 0.0001	*	*	*
PAL (mm)	0.74 ± 0.44	2.85 ± 0.36	5.67 ± 1.37	< 0.0001	*	*	*
GCF volume (μl)	0.36 ± 0.33	0.62 ± 0.34	0.63 ± 0.37	0.0017	*	*	

Group 1, healthy sites; group 2, gingivitis sites; group 3, periodontitis sites.
PLI, plaque index; GI, gingival index; PD, probing depth; PAL, probing attachment level.
*p-value < 0.05 by Tukey–Kramer pairwise comparison.

Table 2. Summary statistics for levels of various substances in 30-s gingival crevicular fluid samples

	GPx [C] (ng/μl)	GPx TA (ng/site)	LTF [C] (ng/μl)	LTF TA (ng/site)	MPO [C] (ng/μl)	MPO TA (ng/site)	IL-1β [C] (ng/ml)	IL-1β TA (pg/site)
H	17.21 (12.67)	3.34 (1.20)	193.25 (168.12)	36.17 (15.35)	494.94 (558.17)	90.52 (60.20)	26.15 (31.69)	4.74 (3.01)
G	23.90 (28.35)	11.43 (8.14)	142.52 (105.02)	63.08 (33.38)	214.68 (173.99)	131.29 (160.84)	22.51 (17.81)	10.29 (7.56)
P	35.70 (35.89)	16.89 (9.76)	192.75 (156.54)	90.80 (53.00)	316.64 (254.58)	188.38 (187.36)	45.69 (75.76)	17.42 (13.89)
p-value from ANOVA	0.0171	< 0.0001	0.2009	< 0.0001	0.0041	0.0182	0.0851	< 0.0001
Tukey–Kramer								
H vs. P	*	*		*		*		*
H vs. G		*		*	*			*
G vs. P		*		*				*

H, healthy sites (group 1); G, gingivitis sites (group 2); P, periodontitis sites (group 3).
GPx, glutathione peroxidase; LTF, lactoferrin; MPO, myeloperoxidase; IL-1β, interleukin-1β; [C], concentration; TA, total amount.
The levels of GPx, LTF, MPO and IL-1β are expressed as mean (standard deviation).
*p-value < 0.05 by Tukey–Kramer pairwise comparison.

Table 3. Pearson's correlation coefficients between clinical parameters and various substances in 30-s samples of gingival crevicular fluid

	GPx [C]	GPx TA	LTF [C]	LTF TA	MPO [C]	MPO TA	IL-1β [C]	IL-1β TA
n	108	108	118	118	116	116	114	114
PLI	0.0788	0.5077*	−0.1272	0.4789*	− 0.2025*	0.3259*	0.0129	0.4741*
GI	0.1277	0.5473*	−0.0872	0.5449*	− 0.2082*	0.3493*	0.0813	0.5190*
PD	0.2749*	0.5620*	0.0139	0.4312*	− 0.1184	0.2254*	0.1596	0.4156*
PAL	0.2696*	0.5406*	0.0094	0.3968*	− 0.1178	0.1969*	0.1696	0.4165*

GPx, glutathione peroxidase; LTF, lactoferrin; MPO, myeloperoxidase; IL-1β, interleukin-1β; [C], concentration; TA, total amount.
PLI, plaque index; GI, gingival index; PD, probing depth; PAL, probing attachment level.
The data of diseased and healthy sites were pooled for analysis.
*Significant correlation ($p < 0.05$).

results with these parameters. The results suggested a positive and significant correlation between the severity of periodontal disease and total amounts of these GCF substances. In order to investigate whether oxidative stress is involved in the deleterious effects of the inflammatory process in periodontal disease, we correlated the three GCF markers for oxidative stress with the proinflammatory cytokine IL-1β. Positive correlations between total amounts and concentrations of glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β were noted ($p < 0.05$) (Tables 4A and B).

Table 4A. Correlation matrix between concentration of each gingival crevicular fluid substance

Concentration	GPx	LTF	MPO	IL-1β
GPx	1.000*			
LTF	0.664*	1.000*		
MPO	0.295*	0.741*	1.000*	
IL-1β	0.763*	0.682*	0.430*	1.000*

Table 4B. Correlation matrix between total amount of each gingival crevicular fluid substance

Total amount	GPx	LTF	MPO	IL-1β
GPx	1.000*			
LTF	0.628*	1.000*		
MPO	0.479*	0.842*	1.000*	
IL-1β	0.530*	0.638*	0.579*	1.000*

GPx, glutathione peroxidase; LTF, lactoferrin; MPO, myeloperoxidase; IL-1β, interleukin-1β.
*Significant correlations at $p < 0.05$.

Discussion

In this study, we evaluated the three GCF markers for oxidative stress, i.e. glutathione peroxidase, lactoferrin and myeloperoxidase, and the proinflammatory cytokine, IL-1β, in periodontitis patients. The total amounts of glutathione peroxidase, lactoferrin, myeloperoxidase and IL-1β were higher in periodontitis sites and gingivitis sites than healthy sites, and were positively correlated with plaque index, gingival index, probing depth and probing attachment level ($p < 0.05$). Thus the three GCF markers for oxidative stress may reflect the severity of the disease. ROS have been implicated in the pathogenesis of periodontal diseases (2, 26). The susceptibility of the periodontium to oxidative injury will depend in part on the ability to upregulate protective ROS scavenging systems. If the increased levels of ROS overwhelm the antioxidant systems, the periodontium will be damaged.

Plasma glutathione peroxidase (extracellular glutathione peroxidase) is detected in a number of extracellular environments, such as milk, plasma, amniotic fluid and lung alveolar fluid (5, 27). To our knowledge, there have been no studies on GCF glutathione peroxidase in human periodontal diseases. A recent study by Comhair *et al.* demonstrated that human airway epithelial cells and alveolar macrophages of smoking individuals showed increased expression of extracellular glutathione peroxidase mRNA (28). They concluded that the extracellular glutathione peroxidase gene expression is induced by chronic exposure to cigarette smoke that contains numerous compounds including ROS. Because there are similar properties between the exposed location of the alveolar epithelium and the periodontal environment, ROS may exert similar impact on the adjacent tissues and cells. Although the extracellular glutathione peroxidase transcripts have been found in lungs (29) and epithelial cells with well-developed brush borders such as intestine and renal tubules (30), and the kidney proximal tubular cells are the main source for glutathione peroxidase activity in the plasma (31, 32), the source of extracellular glutathione peroxidase in GCF is unknown. The likelihood of local production by cells in the periodontal environment or recruiting inflammatory cells cannot be overlooked. Further investigation is necessary to resolve this enigma.

Several investigations and our previous study have suggested that levels of GCF lactoferrin increased in gingivitis and periodontitis sites, and were positively correlated with clinical parameters such as probing depth and GCF volume (13, 14, 33), which was consistent with the results of the present study. It was also reported that the GCF iron level increases in periodontally diseased sites (34, 35). Because iron may increase the oxidative stress through catalyzing the formation of ROS via the Fenton reaction (26) and enhance the growth of certain periodontopathogens (14, 34, 35), the increase of GCF iron may be harmful to the periodontium. Lactoferrin can deprive the bacteria of iron that is essential for their life (14). It also binds iron to prevent the progression of Fenton reaction (2, 36). Therefore the increased GCF lactoferrin in periodontitis sites may act as antimicrobial agent and preventive antioxidant (2).

It has been demonstrated that the level of myeloperoxidase is higher in periodontitis sites (37, 38). Yamalik *et al.* also found that the total myeloperoxidase activity and myeloperoxidase concentration in healthy sites were significantly lower than in periodontitis sites (15). Our results showed increased myeloperoxidase total amount in periodontitis sites. But, in contrast to Yamalik's study, the concentration was the highest in healthy sites. This may be due to the small GCF volume in the periodontally healthy sites as reported by Yamalik *et al.* (15). However in our study, data presented as total amounts seem to reflect the disease severity better than those presented as concentrations. Increased myeloperoxidase level may contribute to the efficacy of killing bacteria but, on the other hand, it may also lead to the increased formation of hypochlorous acid, which can be detrimental to periodontal health. Since hypochlorous acid can inhibit the activity of α1-antiprotease, the elastase is activated (15). This may further lead to connective tissue destruction. Notably, Yamalik *et al.* found a strong and positive correlation between myeloperoxidase and elastase-like activity (15).

IL-1β has been comprehensively investigated in periodontal diseases. Our previous study and those of others have shown that the total amount of IL-1β in diseased sites is greater than in healthy sites (39–41) and is positively correlated with clinical parameters such as plaque index, gingival index, probing depth and probing attachment level (39, 40). IL-1β plays a role in the initiation and progression of connective tissue breakdown, and influences over 90 genes, such as genes of other cytokines, receptors for cytokines, acute phase reactants, growth factors, tissue remodeling enzymes, and genes for extracellular matrix components and adhesion molecules (17). It is notable that IL-1 is capable of stimulating ROS production in some cell types, including PMNs, macrophages, monocytes, pulmonary vascular smooth muscle cells, kidney mesangial cells and human fibroblasts (18, 19, 42). Studies have shown that both ROS and IL-1 regulate the expression of numerous genes through effects on several redox-sensitive transcription factors, such as NF-κB (20, 21). Various genes that are important in inflammatory responses are regulated by NF-κB, including those for cytokines, chemokines, adhesion molecules and oxidative stress-related enzymes (20, 43). Oxidative stress has been shown to induce NF-κB activation (20). As a result of the exaggerated activation of NF-κB, the degree of inflammatory response is perpetuated. It is therefore speculated that inhibition of NF-κB activity may be an effective strategy in the treatment of periodontitis. In our study, the increased level of the antioxidants glutathione peroxidase may exert at least part of their action through the inhibition of NF-κB, an attempt made to overcome the reactive oxygen species, thus alleviating the oxidative stress. In this study, we measured the total amount of glutathione peroxidase

in GCF, whereas in another study in our laboratory, we found decreased glutathione peroxidase activity in periodontitis sites. Hence the relationship between total amount and enzymatic activity of glutathione peroxidase needs further investigation.

Studies have investigated the relationship between antioxidants and cytokines in many cell types (44–46). Lortz *et al*. reported that overexpression of glutathione peroxidase may protect insulin-producing cells against cytokine-mediated autoimmune attack (44). Moutet *et al*. observed that glutathione peroxidase mimics were efficient inhibitors of IL-1 through the down-regulation of endothelial proinflammatory responses (45). Chen *et al*. demonstrated that in human peritoneal mesothelial cells, absence of induction of antioxidant enzymes such as glutathione peroxidase by inflammatory cytokines such as tumor necrosis factor-α and IL-1β may contribute to the susceptibility of these cells to oxidative damage (46). Renard *et al*. investigated NF-κB DNA-binding activity in cell lines stimulated with IL-1β and found that cells overexpressing glutathione peroxidase showed lower levels of NF-κB activation (21). In the present study, we found a positive and significant correlation between the total amount of glutathione peroxidase and IL-1β ($r = 0.530$, $p < 0.05$). However, further studies are warranted to explore the mechanism of their interaction in periodontal diseases. In general, with the increasing disease severity, the levels of myeloperoxidase and IL-1β that might be detrimental to the periodontal health were increased. On the other hand, the levels of glutathione peroxidase and lactoferrin that played protective roles were also increased. Because the results of ELISA only gave us information about the total amount and concentrations of GCF components, the enzymatic activity was unknown. However, the imbalance between the levels of myeloperoxidase/IL-1β and glutathione peroxidase/lactoferrin could result in tissue damage of ROS in periodontitis.

Based on our findings, the periodontitis sites exhibited greater total amount of antioxidants, glutathione peroxidase and lactoferrin, and a concomitant up-regulation of myeloperoxidase. The increased level of proinflammatory cytokine, IL-1β, may further exacerbate the disease. Further investigations are needed to determine the enzymatic activities of glutathione peroxidase and to elucidate the interrelationship between antioxidants and cytokines.

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