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Protease inhibitor levels in periodontal health and disease

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Background and Objective: Our previous study showed that protease inhibitors were attenuated by the periodontal pathogen *Porphyromonas gingivalis* in cultured gingival epithelial cells. We hypothesize that fewer protease inhibitors would be present in more advanced periodontal disease sites, where the level of *P. gingivalis* may be high. The goal of this study was to investigate the relationship between the protease inhibitor [secretory leukocyte protease inhibitor (SLPI), elastase-specific inhibitor (ELAFIN) and squamous cell carcinoma antigen (SCCA)] levels in gingival crevicular fluid and the number of *P. gingivalis* micro-organisms in subgingival plaque.

Material and Methods: Plaque samples from subjects without ($n = 18$) and with moderate to advanced periodontitis ($n = 41$) were used to quantify *P. gingivalis* using real-time PCR. Protease inhibitor levels in the gingival crevicular fluid of all the subjects were determined by ELISA.

Results: P. gingivalis was detected in 68.3% of patients with periodontitis, while 16.7% of subjects without periodontitis had a detectable level of *P. gingivalis*. Patients with periodontitis and *P. gingivalis* in their plaque exhibited lower SLPI and ELAFIN levels ($p < 0.001$) compared with control subjects without periodontitis. Secretory leukocyte protease inhibitor was also reduced ($p < 0.05$) in gingival crevicular fluid of periodontitis patients without a detectable level of *P. gingivalis*. Periodontitis patients with high vs. low levels of *P. gingivalis* exhibited reciprocal mean levels of SLPI and ELAFIN concentrations.

Conclusion: The reduced concentrations of SLPI and ELAFIN may contribute to the loss of host protective capacity and increase susceptibility to breakdown from chronic infection. The work of this investigation may aid in finding diagnostic and prognostic markers in periodontal health and disease and may also help in finding pharmacological targets directed against periodontal inflammation.

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Periodontitis is a chronic inflammatory disease characterized by apical migration of epithelial attachment accompanied by loss of connective tissue and alveolar bone. It is classified based on different clinical measures, including the presence and severity of periodontal pockets and clinical attachment loss and the extent of alveolar bone loss. Bacteria associated with the initiation of periodontal disease are predominantly gram-negative anaerobic bacteria and spirochetes, notably *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (1), which belong to the red complex within subgingival biofilms (2). In particular, *P. gingivalis* plays a significant role in the progression of chronic periodontitis (3). It has a wide repertoire of virulence factors, including fimbriae (4), hemagglutinins (3) and proteases (gingipains; 5). The list of host proteins which are degraded by gingipains *in vitro* includes extracellular matrix components, such as laminin, fibronectin and collagen types III, IV, and

V (6). These proteases have been reported to account for at least 85% of the general proteolytic activity displayed by *P. gingivalis* (7). According to Imamura *et al.* (8) the protease gingipain R is able to promote plasma clotting by activating coagulation factors IX and X and prothrombin. Once the proteolytic cascade pathway is activated, thrombin not only plays a role in hemostasis by converting fibrinogen to fibrin, but also enhances vascular permeability (9) and induces leukocyte chemotaxis (10). At this point, the content in gingival crevicular fluid increases significantly, and polymorphonuclear neutrophils accumulate in actively resorbing periodontitis sites. Neutrophils have been implicated as playing a destructive role in the periodontal tissue breakdown process, due to high levels of lysosomal enzymes, generation of superoxides and reactive oxygen derivatives (11). One of these enzymes, namely neutrophil elastase, degrades important molecules of the periodontium during an inflammatory process. Its presence in gingival crevicular fluid is associated with periodontal inflammation and may be an indicator for the severity of inflammation at individual sites (12).

Concomitant with the increase in proinflammatory enzymes in gingival crevicular fluid, epithelial cells start secreting protease inhibitors to protect the tissue from excessive damage. These protease inhibitors include secretory leukocyte protease inhibitor (SLPI), elastase-specific inhibitor (ELAFIN) and squamous cell carcinoma antigen (SCCA). Secretory leukocyte protease inhibitor regulates the activity of neutrophil elastase and can therefore protect local tissues from destruction during an inflammatory response (13). It is found in a variety of mucous secretions, including gingival crevicular fluid from sites of periodontal disease (14). Elastase-specific inhibitor, also known as skin-derived antileukoproteinase (SKALP), is thought to play a regulatory role in inflammation and is expressed in human epithelia of the tongue, palate, lingual tonsils, pharynx and gingiva (15). Elastase-specific inhibitor is directed against polymorphonuclear neutrophil-derived elastase and proteinase 3, and thus is involved in the maintenance of epithelial integrity by protecting tissues against excessive proteolysis (16). Squamous cell carcinoma antigen belongs to a group of serine protease inhibitors (SERPINS) and may serve as a marker for certain inflammatory conditions. Coexpression of SCCA1 and SCCA2 in the squamous epithelium of mucous membranes and other areas lined with squamous epithelium plays an important role in the co-ordinated regulation of certain serine and cysteine proteases associated with both normal and transformed cells (17). Squamous cell carcinoma antigen 1 inhibits papain-like cysteine proteinases, whereas SCCA2 inhibits chymotrypsin-like serine proteinases (18,19).

Our previous *in vitro* study found that SLPI, ELAFIN, SCCA1 and SCCA2 were induced in gingival epithelial cells in the presence of the common oral bacterium *Fusobacterium nucleatum* (20). We have also found that the secretion of SLPI and ELAFIN was significantly reduced in response to *P. gingivalis*, and that *P. gingivalis* supernatants digested recombinant SLPI, ELAFIN, SCCA1 and SCCA2, suggesting that degradation of protease inhibitors by *P. gingivalis* may result in decreased host protective capacity and higher susceptibility to *P. gingivalis* infection (21). This is in agreement with a study by another group, who reported that *P. gingivalis* gingipains, in particular Arginine Gingipain B, digested ELAFIN *in vitro* (22). Thus, we hypothesize that *P. gingivalis* in subgingival plaque of patients with chronic periodontitis decreases the amount of protease inhibitors in gingival crevicular fluid. Our aim in this investigation is to follow up the recent *in vitro* findings with *in vivo* studies to compare the level of protease inhibitors with the presence of *P. gingivalis* in periodontitis patients.

Material and methods

Study population

Human gingival crevicular fluid and dental plaque samples were obtained from subjects without periodontitis and from patients with generalized chronic periodontitis in accordance with the approval by University of Washington Institutional Review Board for research using human subjects. Periodontally diseased patients had at least two teeth with probing depth ≥5 mm, bleeding upon probing and attachment loss of ≥4 mm. Subjects without periodontitis had probing depth < 4 mm, without bleeding upon probing. Subjects were excluded from the study if they suffered from diabetes mellitus, were younger than 30 years of age, had their most recent periodontal maintenance treatment within the last 3 mo, or if they were treated with antibiotics for any medical or dental conditions within the last few months. Samples were collected from two teeth per subject, and the samples from the periodontitis group were later subdivided into a *P. gingivalis*-positive and a *P. gingivalis*-negative group according to the presence or absence of a detectable level of *P. gingivalis* in the plaque sample. Documented clinical data included probing depth, attachment level (the amount of attachment loss), plaque index and gingival index (23,24), bleeding on probing and suppuration. Probing depth was defined as the distance from the coronal-most margin of the free gingival margin to the most apical penetration of the periodontal probe (PCP-UNC 15; Hu-Friedy, Chicago, IL, USA) and was noted to the nearest millimeter. Attachment loss was defined as the distance from the cemento-enamel junction to the most apical penetration of the Marquis periodontal probe, which is marked every 3 mm between 3 and 12 mm. The presence of supragingival plaque was classified according to the plaque index defined by Loe (23). The severity of inflammation was classified according to the gingival index of Silness and Loe (24). Bleeding on probing and suppuration within 20 s were recorded dichotomously.

Harvest of gingival crevicular fluid and dental plaque

For the harvest of gingival crevicular fluid, two paper strips per site were

used. First, supragingival plaque was removed with a curette. Samples from patients with periodontitis were selected from the deepest sites, while those from healthy subjects were mainly from premolars and canines. As not all patients were fully dentate, we were unable to select one particular tooth for everyone. However, both mesiobuccal and distobuccal sides were considered for sampling, depending on tooth position and access. Each paper strip was placed into the gingival sulcus for 30 s according to the intracrevicular, superficial method by Loe and Holm-Pederson (25). The paper strips were then stored in small tubes and frozen at −80°C. Dental plaque was harvested using one paper tip ISO 50 (SybronEndo, Glendora, CA, USA) per site. Each paper tip was held in the gingival sulcus/periodontal pocket for 10 s, removed and put into 500 μL sterile water. The samples were centrifuged at 134 g. The paper tip was removed and the liquid centrifuged at 26,300 g for 10 min. The plaque pellicle obtained from centrifugation was kept at −80°C until DNA was extracted using Tris–EDTA buffer (26).

Quantitative real-time PCR for quantification of bacteria in dental plaque

The amount of overall bacterial load and of *P. gingivalis* in plaque samples was determined using quantitative real-time PCR (qRT-PCR). The oligonucleotide primer sequences for *P. gingivalis* and the TaqMan probe were previously described by Morillo *et al.* (26). The universal primer sequences for bacterial load and the probe were described by Nadkarni *et al.* (27). The qRT-PCR was conducted with the TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The volume used was 20 μL, comprising 13.2 μL of Master Mix and 6.8 μL extracted plaque DNA in sterile water. The amplification conditions were initial denaturation at 94°C for 10 min, followed by denaturation at 94°C for 60 s, annealing at 60°C for 60 s and elongation at 60°C for 45 s.

Determination of the levels of protease inhibitors in gingival crevicular fluid by ELISA

Each gingival crevicular fluid sample was gently thawed on ice prior to the elution process. For the analysis of SLPI and ELAFIN, we obtained a total of 400 μL eluate by two wash procedures, each with a 20 min rotation at 4°C in the elution buffer (200 μL of phosphate-buffered saline containing 0.1% bovine serum albumin). For the analysis of SCCA, we obtained a total of 250 μL eluate by two wash procedures, each with 125 μL of phosphate-buffered saline containing 0.1% bovine serum albumin. The difference in the total eluate volume was necessary because the optimal concentration range for each ELISA kit differed, based on our preliminary tests. All samples were centrifuged at 134 g for 5 min at 4°C between wash procedures. The following ELISA kits were used for analyses of protease inhibitors: Human SLPI Quantikine Immunoassay (R&D Systems, Minneapolis, MN, USA); Human Trappin-2/ELAFIN DuoSet ELISA Development kit (R&D Systems); and SCCA1 ELISA kit (Accurate Chemical & Scientific Corp., Westbury, NY, USA).

Classification of samples

Samples were classified as either without periodontitis or with periodontitis, according to their clinical parameters described in the inclusion criteria. After the quantity of *P. gingivalis* had been determined with qRT-PCR, the samples were further divided into diseased groups with or without the presence of *P. gingivalis*, based on the correlation between the standard curve for predicted bacterial number and threshold cycle number (C_t) generated from the qRT-PCR (26). The division into the three groups not only allowed the investigation of different protease inhibitor levels between healthy and *P. gingivalis*-infected sites, but also allowed the comparison of protease inhibitor levels between periodontally involved sites with and without *P. gingivalis*.

Data analysis

All experiments were performed in duplicate. Values are shown as the means ± SEM. Within groups defined by periodontal status and *P. gingivalis*, protease inhibitor levels were non-normally distributed and were therefore analysed using Mann–Whitney *U*-tests. These tests were preplanned to compare the healthy control subjects with each of the two groups with periodontitis defined by the presence vs. absence of *P. gingivalis*. Within the two groups defined in terms of low vs. high levels of *P. gingivalis*, protease inhibitor levels were normally distributed and were analysed using Student's unpaired *t*-tests. Analyses were performed using PASW (version 18; IBM, Somers, NY, USA). Statistical significance was established at $\alpha < 0.05$, two tailed.

Results

The amounts of total bacteria and of *P. gingivalis* in plaque samples of subjects with vs. without periodontitis

The study group consisted of 14 men, providing 21 sample sites, and 23 women, providing 38 sample sites. Among them, 18 sample sites were without periodontitis and 41 were periodontally diseased. The mean age of subjects was 57.41 ± 1.34 years. The clinical parameters of the group without periodontitis differed significantly ($p < 0.05$) from the periodontitis group (Table 1). The mean probing depth in the group without periodontitis was 2.94 ± 0.05 mm, the mean attachment level was 1.94 ± 0.06 mm, and the mean plaque index and gingival index were 0.17 ± 0.09 and 0.06 ± 0.06, respectively. This group did not have a detectable amount of *P. gingivalis* in the plaque. Among the periodontitis group, 25 samples had a detectable level of *P. gingivalis*, while 16 did not. The mean probing depth for the diseased group without detectable *P. gingivalis* in the plaque was 6.5 ± 0.27 mm, the mean attachment level was 7.44 ± 0.49 mm, and the mean plaque index

and gingival index were 1.44 ± 0.24 and 0.88 ± 0.15, respectively. For the periodontitis group with *P. gingivalis* in the plaque, the mean probing depth for these sites was 6.4 ± 0.23 mm, the mean attachment level was 6.68 ± 0.37 mm, and the mean plaque index and gingival index were 1.64 ± 0.19 and 1.56 ± 0.14, respectively. Among the diseased groups, periodontitis patients with *P. gingivalis* had higher numbers of total bacteria compared with those without *P. gingivalis* (Fig. 1). The difference in total bacterial count was statistically significant between the group without periodontitis vs. the *P. gingivalis*-positive group ($p < 0.001$) and between the two diseased groups ($p < 0.001$; Fig. 1).

Table 1. Clinical parameters of different samples sites

Group	Age (years)	Plaque index	Gingival index	Probing depth (mm)	Recession	Attachment level (mm)
Healthy ($n = 18$)						
Mean	59.33	0.17	0.06	2.94	0.00	1.94
SEM	1.85	0.09	0.06	0.05	0.00	0.06
Diseased without *P. gingivalis* ($n = 16$)						
Mean	54.63	1.44**	0.88**	6.5**	1.31*	7.44**
SEM	3.02	0.24	0.15	0.27	0.41	0.49
Diseased with *P. gingivalis* ($n = 25$)						
Mean	57.8	1.64**	1.56**	6.4**	0.8*	6.68**
SEM	2.14	0.19	0.14	0.23	0.2	0.37
Total ($n = 59$)						
Mean	57.41	1.14	0.92	5.37	0.69	5.44
SEM	1.34	0.14	0.11	0.24	0.15	0.37

*$p < 0.05$; **$p < 0.001$.

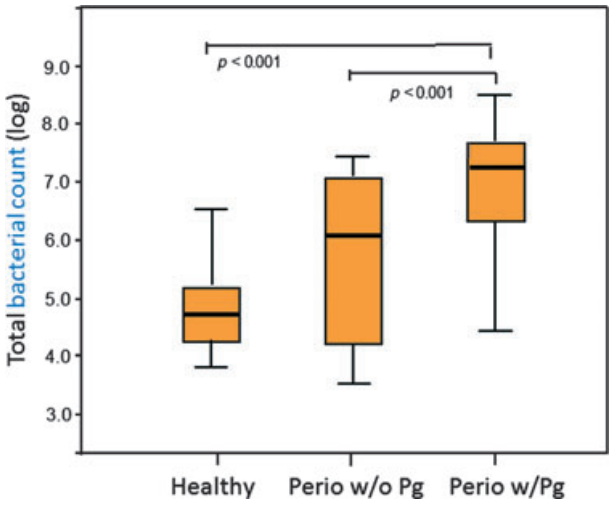

Fig. 1. The total bacterial count in plaque samples of the three groups. The top and bottom of each box indicate the 75th and 25th percentile, respectively. The band inside each box indicates the median bacterial count. The top and the bottom ends of the whiskers represent 98th and 2nd percentile, respectively. Planned comparisons of both diseased groups with the healthy group were conducted using nonparametric analysis. The amount of total bacteria in plaque is the lowest in the healthy group and increased in the diseased groups. Periodontal patients with *P. gingivalis* (Perio w/Pg) show a higher number of total bacteria compared with those without *P. gingivalis* (Perio w/o Pg).

Concentration of ELAFIN was reduced in the *P. gingivalis*-positive group

We examined the concentration of the protease inhibitor ELAFIN in gingival crevicular fluid samples of the three different groups using commercially available ELISA kits. The ELAFIN concentration ranged from 0 to 349.33 pg/mL, the mean concentration being 75.72 ± 92.05 pg/mL. Mann–Whitney *U*-tests indicated that the distribution of ELAFIN concentration of the diseased group with *P. gingivalis* (30.0 pg/mL) differed significantly from that of the healthy control subjects (86.21 pg/mL) ($p < 0.001$; Fig. 2). The ELAFIN concentration of the diseased group without *P. gingivalis* (44.0 pg/mL) was also decreased in comparison to the healthy control subjects, and the difference in this case approached significance ($p = 0.058$; Fig. 2). These results suggest that the level of the protease inhibitor ELAFIN is decreased in gingival crevicular fluid of periodontitis patients and, in particular, of *P. gingivalis*-infected patients.

Concentration of SLPI was reduced in the periodontitis group

Next we examined the concentration of the protease inhibitor SLPI amongst different study groups. The overall SLPI concentration ranged from 202 to 5289 pg/mL, with a mean concentration of 1237.01 ± 1105.17 pg/mL. Both diseased groups showed a significantly reduced concentration of SLPI (755.52 pg/mL for the non-*P. gingivalis*-infected group and 623.13 pg/mL for the *P. gingivalis*-infected group) in gingival crevicular fluid compared with the group without periodontitis (1617.17 pg/mL; $p < 0.05$ and $p < 0.001$, respectively; Fig. 3). These results suggest that the concentration of SLPI is significantly reduced in sites with periodontitis, regardless of the presence or absence of *P. gingivalis*.

Concentration of SCCA1 showed little difference between study groups

Subsequently, we examined the concentration of the protease inhibitor SCCA1 using ELISA. The overall concentration ranged from 107.94 to 1575.17 pg/mL, with a mean concentration of 596.25 ± 451.96 pg/mL. The difference between the medians of 396.5 pg/mL for the healthy group, 479.78 pg/mL for the diseased group without *P. gingivalis* and 492.7 pg/mL for the diseased group with *P. gingivalis*

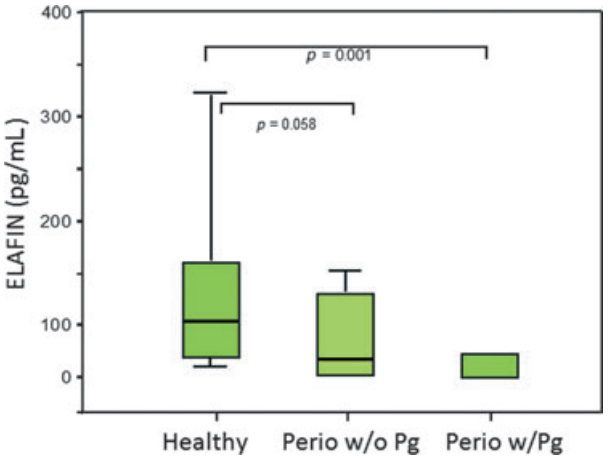

Fig. 2. Box plots of elastase-specific inhibitor (ELAFIN) concentration in gingival crevicular fluid of the three groups. The top and bottom of each box indicate the 75th and 25th percentile, respectively. The band inside each box indicates the median concentration. The top and the bottom ends of the whiskers represent 98th and 2nd percentile, respectively. Significance levels indicate results of preplanned Mann–Whitney *U*-tests of both diseased groups with the group without periodontitis.

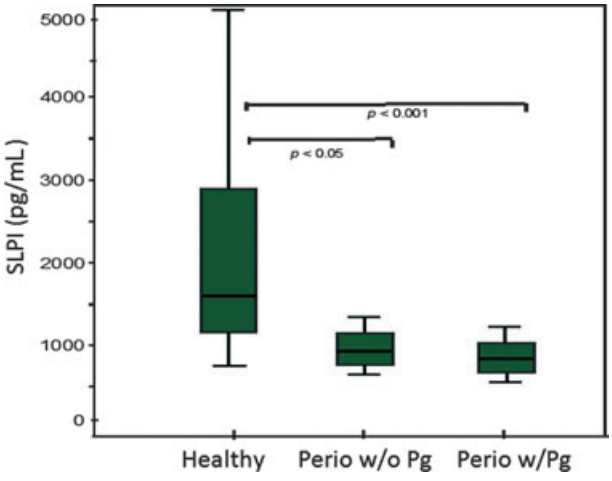

Fig. 3. Box plots of secretory leukocyte protease inhibitor (SLPI) concentration in gingival crevicular fluid of the three groups. Interpretation and significance levels are as described in the legend to Fig. 2.

was statistically not significant ($p = 0.108$; Fig. 4). The results of this experiment suggest that the concentration of the protease inhibitor SCCA1 is not affected by the disease status or by the presence or absence of *P. gingivalis*.

Concentrations of SLPI and ELAFIN in gingival crevicular fluid are negatively associated with *P. gingivalis* counts in dental plaque

As ELAFIN and SLPI levels were decreased in gingival crevicular fluid of periodontitis patients with *P. gingivalis*, we performed analyses to determine whether the concentration of these protease inhibitors differed between periodontitis patients with high vs. low levels of *P. gingivalis*. Subdivision of the *P. gingivalis*-infected sites into two groups with high and low levels of *P. gingivalis* (cut-off point was C_t of 39) revealed lower mean values of protease inhibitors in the high-*P. gingivalis* group vs. the low *P. gingivalis* group. For SLPI, the mean concentrations were 861.76 ± 139.42 and 1219.81 ± 234.63 pg/mL, respectively, for high- and low-*P. gingivalis* groups ($p < 0.05$; Fig. 5A). For ELAFIN, the mean concentration was 16.95 ± 18.37 pg/mL in the high-*P. gingivalis* level group vs. 37.56 ± 16.34 pg/mL in the low-*P. gingivalis* group ($p < 0.05$; Fig. 5B). These data show that the amounts of *P. gingivalis* in dental plaque and the levels of protease inhibitors in gingival crevicular fluid are negatively associated.

Discussion

Our findings showed that host-derived protease inhibitors, which are released as a response to environmental and microbial stimuli, are decreased in concentration in periodontal pockets with *P. gingivalis*. Additionally, our study also showed that SLPI and ELAFIN were reduced in periodontitis patients who were not infected with *P. gingivalis*, suggesting that factors other than *P. gingivalis* may also play a role in the regulation of protease inhibitors, reflecting the complex features of the periodontal biofilm. Within periodontal biofilms, Socransky *et al.* described five different complexes, some of which were closely related to periodontal inflammation and periodontal destruction (2). Socransky and Haffajee suggested that the orange complex precedes the red complex in the subgingival plaque formation and that both complexes are closely associated. Contrary to the majority of other microbial species, all species of the red complex and most of the species of the orange complex show a significant increase in count with increasing pocket depth (28). In our previous *in vitro* study, exposure to *F. nucleatum*, a member of the orange complex, induced the expression of protease inhibitors, while *P. gingivalis*, a member of the red complex, degraded these protease inhibitors (21). The dominance of *P. gingivalis* in the degradation of protease inhibitors is important to note, because during the formation of dental plaque, protease inhibitors may be induced as a host-protective mechanism by the presence of bridging organisms, such as *F. nucleatum*, but may be rendered ineffective once protease-secreting pathogens are established.

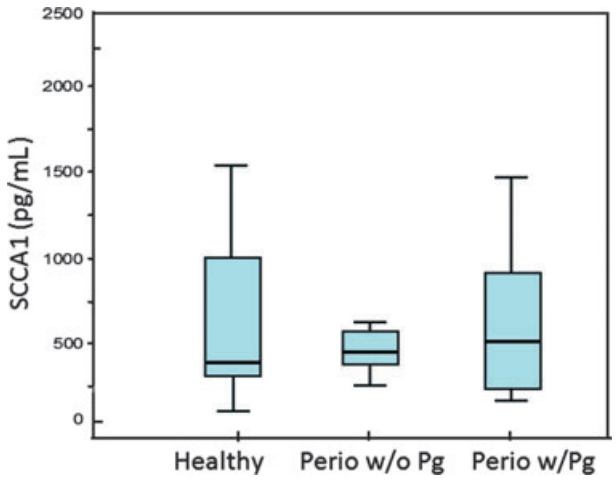

Fig. 4. Boxplots of squamous cell carcinoma antigen 1 (SCCA1) concentration in gingival crevicular fluid of the three groups. Interpretation is as described the legend to Fig. 2. No significant differences were found between the three groups.

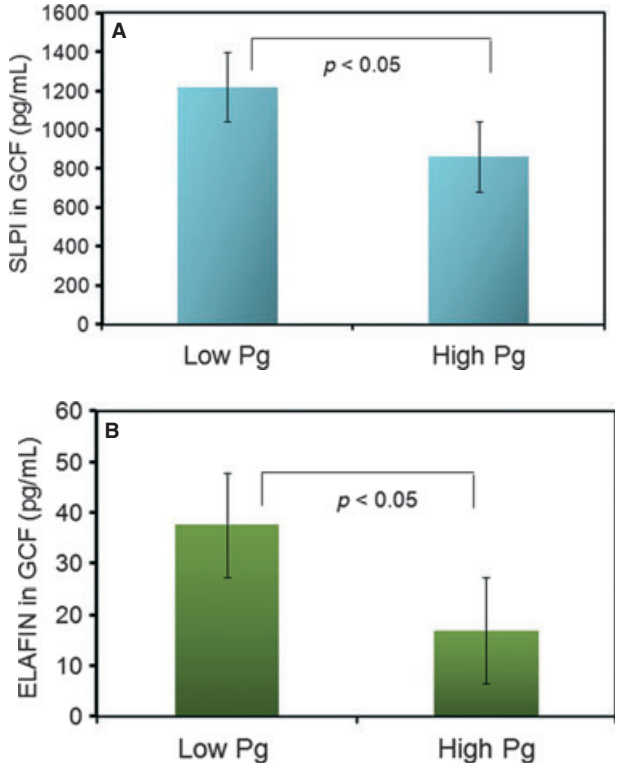

Fig. 5. Groups defined by high vs. low levels of *P. gingivalis* exhibited reciprocal mean levels of both SLPI (A) and ELAFIN concentrations (B). The cut-off point for high vs. low levels of *P. gingivalis* was set at C_t of 39. *p*-Values were obtained using Student's unpaired *t*-tests.

In an ecological community attempting to grow, such as dental plaque biofilm, virulence factors of different species take on a different significance (29). *P. gingivalis* proteases may have the most potent activity in tissue destruction (30) and, as shown in our *in vitro* studies, are able to degrade protease inhibitors most effectively (21). However, less potent trypsin-like proteases of certain *Capnocytophaga* species and *T. denticola* might play a secondary role in the environment of a microbiological network. A significant correlation between the increased total bacterial load and deeper pocket depth found in our study reinforces a notion that the biofilm influences the protease inhibitor concentration. Our data showed an inverse correlation between the number of *P. gingivalis* micro-organisms and the amount of SLPI and ELAFIN secreted. Our data also showed that the amount of these protease inhibitors was reduced in the periodontitis group without the presence of *P. gingivalis* but with clinical parameters of severe periodontitis, compared with the control group without periodontitis. Our previous report of *in vitro* studies using cultured gingival epithelial cells showed that *P. gingivalis* supernatant was able to degrade ELAFIN and SLPI, and that *P. gingivalis* mutants lacking proteases had a reduced capacity to degrade recombinant protease inhibitors (21). However, we acknowledge that there may be additional factors other than *P. gingivalis* proteases involved in the decreased level of protease inhibitors in gingival crevicular fluid. These additional factors might include host-derived proteases, such as cathepsins and neutrophil elastase. Secretory leukocyte protease inhibitor and elastase levels have been previously reported to be inversely correlated in periodontitis patients (31). The same study also reported that SLPI and cathepsin B are inversely correlated, although this correlation was statistically not significant (31). Taken together, our data suggest that other factors, in addition to *P. gingivalis* gingipains, are involved in the reduction of SLPI and ELAFIN, supporting the notion of the complexity, quantity and the bacterial interrelationship in the subgingival biofilm.

An earlier study compared SLPI concentrations in gingival crevicular fluid from active periodontitis patients and periodontitis patients in maintenance (5). Both study groups were *P. gingivalis*-positive, but its quantity differed. Secretory leukocyte protease inhibitor was significantly reduced in the group with a large amount of *P. gingivalis*. The authors attributed the finding to the proteolytic activity of *P. gingivalis* gingipain isoform Arginine Gingipain A (RgpA). The overall bacterial load in these samples was not

specified, but this is in agreement with our previous study using *P. gingivalis* mutant strains lacking various gingipains (21). Although RgpA may contribute to the degradation of SLPI, the bacterial biofilm may play a role as well. In particular, an increased neutrophil elastase level in the high *P. gingivalis* group in the study by Into *et al.* could be a reflection of a higher bacterial load of the respective plaque samples.

Aggressive periodontitis is associated with neutrophil dysfunction, such as a deficiency of adhesion and reduced chemotaxis and phagocytosis activity (32,33). In addition, hyperfunctional neutrophils in a primed state may lead to abnormal tissue injury due to abnormal signal transduction mechanisms (34). In that case, hyper-responsive neutrophils could defeat the protease inhibitors and render the host more susceptible to periodontal breakdown. Guentsch *et al.* (35) and Giannopoulou *et al.* (36) showed in their clinical studies that patients with aggressive and chronic periodontitis released significantly more elastase from granulocytes than healthy control patients. As we know that SLPI, ELAFIN and SCCA are susceptible to bacterial proteases, particularly to the different isoforms of *P. gingivalis* gingipains (21), there might also be a dose-dependent susceptibility to neutrophil elastase, their usual target. An *in vitro* study design would help to decipher the effect of neutrophil elastase on these protease inhibitors. A study has shown that ELAFIN is cleaved by its cognate enzyme, neutrophil elastase, at excessive concentrations in sputum from individuals with cystic fibrosis, and that ELAFIN was degraded more quickly in *Pseudomonas aeruginosa*-infected sputum than in *P. aeruginosa*-negative sputum (37). According to Joseph *et al.* (38), *P. aeruginosa* is able to induce the expression of chemoattractant interleukin-8 and therefore increase recruitment and activation of neutrophils at inflammatory sites. Assuming the two-fold interaction between serine protease inhibitors and neutrophil elastase, two contributing factors might be involved in the reduced amount of protease inhibitors in our two periodontally involved groups. On the one hand, bacterial infection with its biofilm leads to the release of proinflammatory cytokines, which results in vast recruitment of potent neutrophils. On the other hand, neutrophils in a primed state are hyperfunctional (34) and contribute to excessive neutrophil elastase in sites of periodontal disease. Thus, the two contributors, host-derived neutrophil elastase and *P. gingivalis*-derived gingipains, might be involved in the reduction of host protease inhibitors. This reflects the various degrees of reduction of host protease inhibitors between the diseased group with and without *P. gingivalis* infection. The lower concentrations of SLPI and ELAFIN found in the gingival crevicular fluid of periodontitis patients with *P. gingivalis* infection suggests that two contributors might be responsible for this reduction.

While SLPI and ELAFIN concentrations in gingival crevicular fluid presented a similar pattern in periodontal disease, SCCA1 concentration was unaffected by the presence of bacterial biofilm and therefore not significantly different between the three groups tested. Unlike ELAFIN and SLPI, whose activities tend to be inhibitory in inflammation, the activity of SCCA may be more pronounced in tissue repair and cell differentiation. Kato (39) described an interaction of SCCA with cathepsin L as a function to protect against infection in the skin indirectly, via inhibition of the final differentiation of the epidermis. Katagiri *et al.* (40) proposed a novel ultraviolet protection mechanism in human skin due to an SCCA1-mediated blockade of ultraviolet-induced keratinocyte apoptosis. Squamous cell carcinoma antigen was found to be upregulated in psoriatic skin (41), in atopic dermatitis (42) and in bronchial asthma (43). Interestingly, these noninfectious inflammatory diseases are associated with type I hypersensitivity immune reactions with involvement of IgE, T-helper 2 cells and mediating cytokines interleukin-4 and interleukin-10. In contrast, the inflammatory cell infiltrates in periodontal disease are composed of a mixed T-helper 1 and T-helper 2 cell profile, with respective predominance at different phases of the disease (44). Therefore, the role of SCCA in inhibition and regulation of infectious inflammation and in host defense might not be pronounced, but instead may be directed towards tissue differentiation and protection. Furthermore, SCCAs have been reported to be intracellularly localized as serine protease inhibitors, unlike other protease inhibitors (45). Therefore, the level of SCCA1 might be integrated with multiple factors *in vivo*. Further studies are needed to explore the mechanisms behind the regulation of SCCAs.

In conclusion, we showed that the concentrations of the serine protease inhibitors SLPI and ELAFIN are reduced in periodontitis, and that *P. gingivalis* plays a role in the reduction of these protease inhibitors, although it does not appear to be the sole cause. In contrast to SLPI and ELAFIN, the concentration of SCCA1 was unaffected by the periodontal pathogen and the associated host response, and therefore SCCA1 might not be as involved in the innate immunity process in periodontal disease. The work of this investigation may aid in finding diagnostic and prognostic markers in periodontal health and disease and may also help the search for pharmacological targets to support the host immune responses directed against periodontal inflammation.

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