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Differential effects of periopathogens on host protease inhibitors SLPI, elafin, SCCA1, and SCCA2

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Objective: Secretory leukocyte peptidase inhibitors (SLPI), elafin, squamous cell carcinoma antigen 1 and 2 (SCCA1 and SCCA2) are specific endogenous serine protease inhibitors expressed by epithelial cells that prevent tissue damage from excessive proteolytic enzyme activity due to inflammation. To determine the effects of various periopathogens on these protease inhibitors, we utilized human gingival epithelial cells (GECs) challenged with cell-free bacteria supernatants of various periopathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum.*

Design: The gene expression and secretion of SLPI, elafin, SCCA1, and SCCA2 were determined using real-time PCR and ELISA, respectively. The direct effects of periopathogens and *P. gingivalis* gingipain mutants on these inhibitors were examined *in vitro* by Western Blot. The effect on the innate immune response of GECs was measured by expression of antimicrobial peptides: human beta-defenisin-2 (hBD2) and chemokine (C-C motif) ligand 20 (CCL20).

Results: We found that SLPI, SCCA2, elafin, hBD2, and CCL20 gene expression levels were significantly induced ($p < 0.001$) in response to *P. gingivalis*, whose virulence factors include cysteine proteases, but not in response to stimulation by other bacteria. *P. gingivalis* reduced the secretion of SLPI and elafin significantly in GECs, and degraded recombinant SLPI, elafin, SCCA1, and SCCA2. Differential degradation patterns of SLPI, elafin, SCCA1, and SCCA2 were observed with different bacteria as well as *P. gingivalis* mutants associated with the loss of specific gingipains secreted by *P. gingivalis.* In addition, pretreatment of GECs with SLPI, SCCA1, or SCCA2 partially blocked hBD2 and CCL20 mRNA expression in response to *P. gingivalis*, suggesting a protective effect.

Conclusion: Our results suggest that different periopathogens affect the host protease inhibitors in a different manner, suggesting host susceptibility may differ in the presence of these pathogens. The balance between cellular protease inhibitors and their degradation may be an important factor in susceptibility to periodontal infection.

Keywords: *gingival epithelial cells*; *host defense*; *periopathogen*; *protease inhibitor*

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Periodontal disease is an inflammatory disorder caused by Gram-negative anaerobic bacteria that affects tooth-supporting structures (1). The development and progression of this disease occurs from complex interactions between oral microflora and the host, affected by multiple factors, such as smoking, stress, illness, and genetic susceptibility (2). A number of emerging studies have reported that chronic periodontal disease could increase the incidence of various systemic diseases and conditions, such as preterm birth (3), cardiovascular disease (4), and respiratory infection (5). Thus, periodontal disease is not just an oral health problem, but a significant health risk. Although the strategies for prevention and therapies for periodontal disease have improved recently, effective prevention and treatment is still a challenge. Therefore, better understanding of mechanisms responsible for host defense responses against various periopathogens will contribute to developing effective future therapeutic strategies.

Numerous studies have demonstrated a close association between the pathology of periodontitis and development of a subgingival biofilm that includes *Porphyromonas*

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gingivalis, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, and *Fusobacterium nucleatum* (6, 7). However, the pathogenesis of periodontal disease is by no means fully elucidated. These putative pathogens produce a broad array of potential virulence factors: lipopolysaccharide (LPS), fimbriae, hemagglutinin, hemolysins, and gingipains from *P. gingivalis* (8–11); trypsin-like protease (12), sialidase (13), and hemagglutinin (14) from *T. forsythia*; and chymotrypsin-like enzyme and metalloproteases (15) from *T. denticola* and *A. actinomycetemcomitans*, respectively. These virulence factors contribute to the pathogenicity of these organisms and stimulate host cells to release inflammatory mediators that may actually promote the progression of disease (9). Specifically, *P. gingivalis*-derived gingipains (cysteine proteases) which include two arginine residue-specific enzymes, RgpA and RgpB, and a lysine residue-specific enzyme, termed Kgp (16–18), have been shown to exert pathophysiological effects through cleavage or degradation of host proteins, such as CD14 on human monocytes (19) and gingival fibroblasts (20), ICAM-1 on human oral epithelial cells (21), and secretory leukocyte protease inhibitor (SLPI) in gingival tissue of periodontitis associated with *P. gingivalis* infection (22).

Serine protease inhibitors play a critical role in host tissue homeostasis and the balance between proteases and their inhibitors contributes to maintenance of tissue integrity (23). As previously explained, that gingival epithelial cells (GECs) exposed to a cell wall preparation of *F. nucleatum* (FnCW) upregulated expression of multiple protease inhibitors as well as antimicrobial peptides and other potentially protective factors (24). The protease inhibitors elafin (or Skin-derived anti-leukoprotease), SLPI, SERPINB3 (or squamous cell carcinoma antigen SCCA1), and SERPINB4 (SCCA2) were among the most highly upregulated genes upon *F. nucleatum* exposure. These protease inhibitors potentially target two kinds of proteases, host cell-derived proteases such as neutrophil elastase, and pathogen-derived proteases such as the gingipains, and therefore may be important in controlling the extent of inflammatory tissue damage (22, 25). However, it is not clear how epithelial expression of these multiple protease inhibitors is modulated by various oral microorganisms, and how they might function in the complex environment of the periodontium. We hypothesized that various oral Gram-negative bacteria have differing effects on the expression and function of host protease inhibitors, and the effects could alter the balance between proteases and their inhibitors, thus might have an impact on pathogenesis of periodontal disease.

Materials and methods

Reagents

Cysteine protease inhibitor tosyl-l-lysine chloromethyl ketone (TLCK) was from Sigma-Aldrich (St. Louis, MO). Recombinant human SLPI and elafin (trappin-2), and anti-human elafin were purchased from R&D Systems (Minneapolis, MN). Recombinant human SCCA1, SCCA2, and anti-SCCA1 and anti-SCCA2 were prepared as described previously (26, 27). Anti-human SLPI was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody anti-rabbit IgG conjugated with horseradish peroxidase (HRP) and anti-mouse IgG HRP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and Santa Cruz Biotechnology, respectively. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Human primary GECs and bacteria culture

Gingival biopsies were obtained from healthy patients who underwent third-molar extraction in the Department of Oral Surgery, School of Dentistry, University of Washington, in accordance with a University of Washington Institutional Review Board-approved study. The tissue was prepared for cell culture as described previously (28). Epithelial cells were cultured in keratinocyte growth medium (KGM) with 0.15 mM Ca^{2+} using the supplements from the KGM-bullet kit (Cambrex, Walkersville, MD) at 37°C in a humidified atmosphere containing 5% CO_2.

The bacterial strains used in this study are listed in Table 1. *P. gingivalis* wild-type and mutant strains were cultured under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) at 37°C in Trypticase soy broth (BBL, Sparks, MD) supplemented with 1 g of yeast extract, 5 mg of hemin, and 1 mg of menadione per liter. Appropriate antibiotics previously described were added to each culture of mutant of *P. gingivalis* (29, 30). *A. actinomycetemcomitans* (ATCC 700685) and *F. nucleatum* (ATCC 25586) were grown in Todd-Hewitt broth supplemented with 1 g of yeast extract per 100 ml at 37°C under anaerobic conditions. *T. forsythia* (ATCC 49610) was grown in 5% blood agar plates (Remel, Lenexa, KS) with a N-acetylmuramic acid (NAM) disk, and then bacteria were scraped from the agar surface using sterile cotton swabs and subcultured in heart infusion broth media (BBL, sparks, MD) supplemented with hemin, vitamin K, and l-cysteine under anaerobic conditions at 37°C (31). Bacterial purity was determined by microscopy and Gram staining, and numbers were estimated by absorbance measurement using the TECAN GENios Multidetection Reader, V.4.51 (Phoenix, Hayward, CA). The bacteria were cultured to late logarithmic growth phase. Cell-free Bacterial supernatants were collected

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Table 1. Bacterial strains used in this study.

Strains	Genotype	Source	References
T. forsythia 49610	Wild-type		
F. nucleatum 25586	Wild-type		
A. actinomycetemcomitans 700685	Wild-type		
P. gingivalis 33277	Wild-type		
P. gingivalis KDP112	rgpA$^-$/rgpB$^-$	Duncan, MJ	29
P. gingivalis W83	Wild-type	Macrina, FL	30
P. gingivalis V2577	kgp$^-$	Macrina, FL	30
P. gingivalis V2383	rgpA$^-$/kgp$^-$	Macrina, FL	30

by centrifugation at 800*g* for 5 min at 4°C and frozen at −80°C for all experiments. GECs were grown to 85% confluence and stimulated with various stimuli as indicated. Bacterial supernatants were used at an amount equivalent to the indicated multiplicity of infection (MOI). Each stimulation experiment was performed in duplicate and cells from two to four different donors were tested.

Quantitative real-time PCR (QRT-PCR)

Total RNA was isolated from GECs using RNeasy mini kit (Qiagen, CA) by standard procedures. cDNA was prepared from 0.5 μg of total RNA by standard techniques. Quantitative analysis of the cDNA was performed using iCycler (Bio-Rad, CA) and iQ5 according to the manufacturer's instructions. Quantitative real-time PCR (QRT-PCR) was conducted with SYBR Green Supermix kit from Bio-Rad (Hercules, CA). The amplification conditions were initial denaturation at 95°C for 12 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 57–65°C for 30 s, and elongation at 72°C for 60 s. Melt-curve analysis was performed to confirm that the signal was that of the expected amplification product and not possible primer–dimers. The sequences of oligonucleotide primers for human beta-defenisin-2 (hBD2), CCL20, SLPI, elafin, SCCA1 and SCCA2, and the calculation for relative fold changes were described previously (24).

Cytokine secretion assay

Cell culture supernatants after various stimulations were collected and the levels of the indicated proteins were measured with ELISA kits for SLPI and elafin (R&D Systems, MN) in accordance with the manufacturer's protocol. For DuoSet ELISA elafin, the detection limits of the assay for elafin in culture supernatants was 31.2 pg/ml. Intra-assay variation measured by repetitive testing in our laboratory was around 9.0 (CV%), while inter-assay variation was 10.0 (CV%). For Quantikine ELISA SLPI, the detection limits was 25 pg/ml, with intra-assay variation around 8.0 (CV%) and inter-assay variation around 8.0 (CV%). A standard curve was generated with each set of samples assayed. The linear region of the standard curve was obtained by a series of 2-fold dilutions in reagent diluents. The absorbance of the blank control (reagent diluents) was subtracted from the measured absorbance of the different standards and samples. Final concentrations in each sample were calculated as the mean of the results at the proper sample dilution yielding absorbance in the linear parts of the standard curves. Supernatants of cells from three different donors were tested, with duplicate samples from each donor.

Western Blotting

Recombinant SLPI, elafin, SCCA1, and SCCA2 were incubated at room temperature (RT) for 15 min with the indicated dilutions of *P. gingivalis* supernatants in a standard buffer (0.2 mM Tris, 0.1 mM NaCl, 5 mM $CaCl_2$, and pH 7.6) freshly supplemented with 10 mM cysteine (32). After incubation, samples were immediately denatured in SDS reducing buffer at 70°C for 10 min and separated by Nu-PAGE Bis–tris Gel (Invitrogen, Carlsbad, CA). Heat-denatured samples treated with or without protease inhibitors cocktail (Santa Cruz Biotechnology, Santa Cruz, CA) for detection of elafin and SLPI showed no difference using Western Blot. Primary antibodies used were anti-SLPI (Rabbit polyclonal antibody; 1:200 dilution), anti-elafin (Rabbit polyclonal antibody; 1:1,000 dilution), anti-SCCA1 and anti-SCCA2 (mouse monoclonal; SCCA1, 1:2,500 dilution; SCCA2, 1:1,500 dilution). Secondary antibody was anti-rabbit IgG HRP-conjugated antibody for SLPI; anti-mouse IgG HRP-conjugated antibody for SCCA1 and SCCA2. The signals were detected by chemiluminescence's (Thermo Fisher Scientific Inc., Rockford, IL) and exposure to Kodak X-ray film.

Data analysis

Each experiment was performed with GECs from different donors, and within an experiment, each test condition was performed in duplicate. Values are shown as the

means ± SD (standard deviation) (protein data from ELISA) or means ± SEM (standard error of the mean) (for quantitative gene expression data) from multiple experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) among the groups followed by the two-tailed *t*-test. All statistical analysis was performed using JMP for Windows Release 6.0 (SAS Institute, NC). Differences were considered to be statistically significant at the cut-off level of $p < 0.05$.

Results

SLPI, elafin, SCCA1, and SCCA2 gene expression in GECs is induced by P. gingivalis, but not by other bacteria

Gene expression of protease inhibitors SLPI, elafin, SCCA1, and SCCA2 was evaluated by using QRT-PCR after stimulation of GECs with the cell-free culture supernatants of *P. gingivalis*, *T. forsythia*, or *A. actinomycetemcomitans* equivalent to MOI 100 for 24 h. *P. gingivalis* KDP112 mutant (*rgpA* −/*rgpB* −) and *F. nucleatum* were used as control bacteria. TLCK, an inhibitor of the active sites of serine/cysteine proteases, pre-incubated with *P. gingivalis* supernatants served as an additional control. Wild-type *P. gingivalis* significantly induced mRNA expression of all four protease inhibitors ($p < 0.001$) compared to the unstimulated control and all other treatments (Fig. 1). *T. forsythia*, *F. nucleatum*, *A. actinomycetemcomitans*, *P. gingivalis* KDP112 and TLCK-treated *P. gingivalis* did not show any effects on the induction of SLPI, elafin, SCCA1, and SCCA2 gene expressions in GECs (inset Fig. 1). These results suggest that the importance of *P. gingivalis* in the induction of epithelial protease inhibitors.

GEC-secreted as well as recombinant SLPI and elafin are significantly degraded by P. gingivalis

We next examined the secreted levels of protease inhibitor proteins in GEC culture media in response to the cell-free supernatants of various periodontal pathogens using ELISA. We tested SLPI and elafin levels, since ELISA kits for SCCA were not available commercially. The levels of secreted SLPI and elafin were not significantly changed compared to the unstimulated control in response to *T. forsythia*, *A. actinomycetemcomitans*, *F. nucleatum*, and the *P. gingivalis* (*rgpA* −/*rgpB* −) mutant. TLCK pre-incubated with *P. gingivalis* supernatants also showed similar secreted levels of SLPI and elafin as the control. However, the levels of secreted SLPI and elafin were significantly decreased in response to wild-type *P. gingivalis* (Fig. 2A). The decrease in the secreted level of SLPI and elafin was verified by comparing effects of cell-free supernatants of *P. gingivalis* and whole bacterial cultures of *P. gingivalis* at the equivalent MOI (Fig. 2B). No significant difference was seen in GECs stimulated by the cell-free supernatants and whole bacterial culture. Secreted SLPI and elafin were only detectable with the lowest dose (MOI 10). Both SLPI and elafin were secreted by GECs in the absence of stimulants in a time-dependent manner from 2 to 48 h (Fig. 2C). The secreted levels of SLPI and elafin were very low in response to *P. gingivalis* (insets in Fig. 2C and D) and apparent loss of secreted

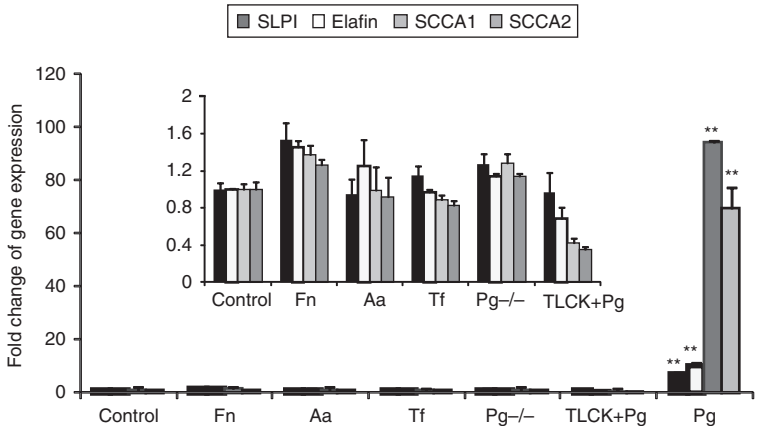

Fig. 1. P. gingivalis supernatant significantly induced mRNA gene expression of epithelial protease inhibitors (SLPI, elafin, SCCA1, and SCCA2). GECs were stimulated with cell-free supernatants of *P. gingivalis* (Pg), *P. gingivalis* mutant KDP112 (Pg −/−), *T. forsythia* (Tf) *A. actinomycetemcomitans* (Aa), and whole bacterial *F. nucleatum* (Fn) at MOIs of 100 or equivalent for 24 h. Change of mRNA expression was evaluated by QRT-PCR and results are expressed as fold change in gene expression compared with the unstimulated control after normalization with the housekeeping gene ribosomal phosphoprotein (RPO). The change of gene expression of SLPI, elafin, SCCA1, and SCCA2 was minimal in GEC stimulated by Fn, Aa, Tf, Pg −/− compared to the unstimulated control (inset of Fig. 1). TLCK pre-incubated with *P. gingivalis* supernatant blocked upregulation. The data are derived from three different cell donors tested in duplicate. Error bars indicate SEM (standard error of the mean).

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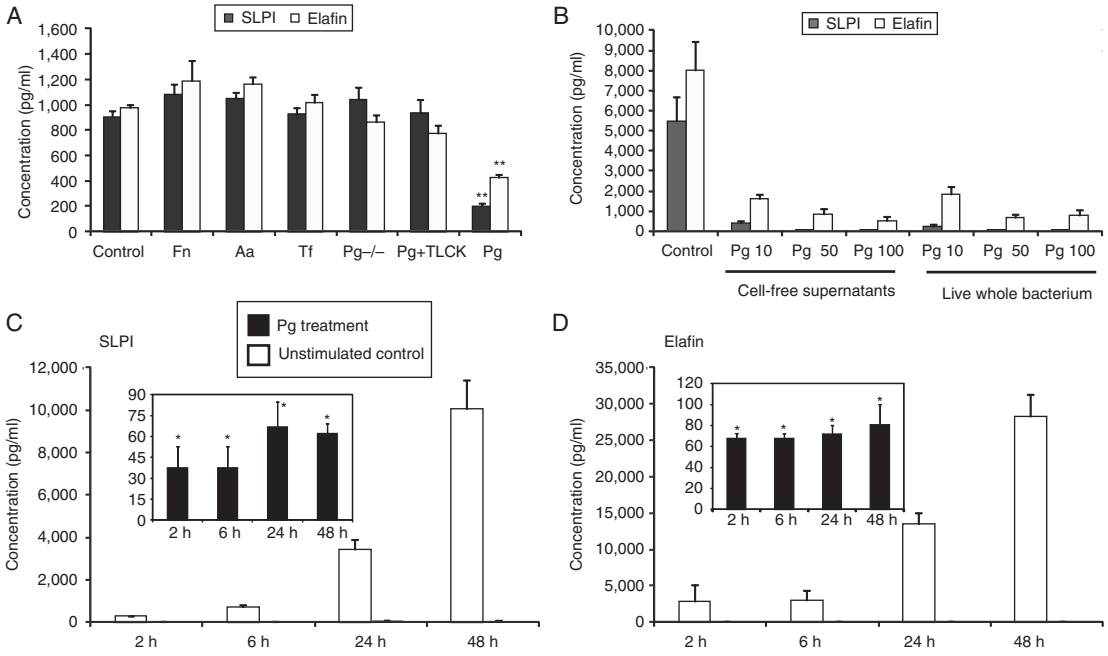

Fig. 2. Secreted SLPI and elafin are degraded by *P. gingivalis* supernatants. GECs were treated for 24 h (A and B) or for the time indicated (C) with various bacterial supernatants and the media analyzed by ELISA. (A) Secreted level of SLPI and elafin compared for various bacteria. GECs were stimulated with *P. gingivalis* (Pg), *P. gingivalis* mutant KDP112 (Pg −/−), *T. forsythia* (Tf) *A. actinomycetemcomitans* (Aa), and *F. nucleatum* (Fn) at MOIs of 100 or equivalent. (B) Secreted SLPI and elafin in media from GECs treated with graded doses of *P. gingivalis* supernatants and whole *P. gingivalis*. (C and D) Time course for the secreted levels of SLPI (C) and elafin (D) in GEC media in response to *P. gingivalis* supernatant stimulation (dark bar) and the unstimulated control (grey bar). The secreted level in response to *P. gingivalis* supernatant is shown with an expanded scale (insets). The results were expressed as means ± SD (standard deviation) of values from three independent experiments. (*) indicates significant difference ($p < 0.01$) compared with the control.

protein occurred as early as 2 h post-stimulation compared to the corresponding control. The secreted levels of SLPI and elafin differed between different batches of *P. gingivalis* used, but the trend remained the same.

We next tested if the proteases secreted by *P. gingivalis* directly affect the protease inhibitors *in vitro*. Recombinant SLPI, elafin, SCCA1, and SCCA2 were incubated with various dilutions of bacterial supernatants at RT for 15 min and analyzed by Western Blot using specific antibodies under reducing conditions. Each recombinant protease inhibitor was cleaved in response to dilutions of *P. gingivalis* supernatants in a dose-dependent manner (Fig. 3). With undiluted *P. gingivalis* supernatants, the recombinant proteins were completely degraded following 15 min incubation. The protease inhibitors remained intact or nearly so when *P. gingivalis* supernatant was diluted to 1,000-fold. Supernatants of *T. forsythia* had no effect on recombinant elafin and SLPI at all dilutions, but slightly degraded SCCA1 and SCCA2 at 1 and 30 dilutions. The degree of degradation was less than shown with *P. gingivalis*. Supernatants of *F. nucleatum* and *A. actinomycetemcomitans* had no effect on recombinant elafin, SLPI, SCCA1, and SCCA2 at all dilutions tested.

Differential degradation of SLPI, elafin, SCCA1, and SCCA2 by P. gingivalis gingipains

To determine whether the gingipains produced by *P. gingivalis* were responsible for the degradation of these protease inhibitors, we analyzed SLPI and elafin degradation in cell culture media by *P. gingivalis* supernatants alone and in the presence of TLCK, an inhibitor of the active sites of serine/cysteine proteases. Media from GECs cultured for 24 h and containing secreted SLPI and elafin were collected and incubated with *P. gingivalis* supernatants (1:10 dilution) in the presence of various concentrations of TLCK (0.125–12.5 mM) for 20 min at RT, and assayed for the remaining SLPI and elafin by ELISA. The SLPI and elafin concentrations decreased to 29% and 10% of the control, respectively, when incubated with *P. gingivalis* supernatants only (Fig. 4A). The degradation of both SLPI and elafin was blocked by TLCK in a dose-dependent manner. Thus, the degradation of these protease inhibitors is serine/cysteine protease-dependent.

SLPI and elafin degradation was prevented when *P. gingivalis* was heat-killed at 65°C for 60 min prior to incubating with GECs (Fig. 4B). The heat sensitivity of degradation supports the enzymatic degradation of these protease inhibitors.

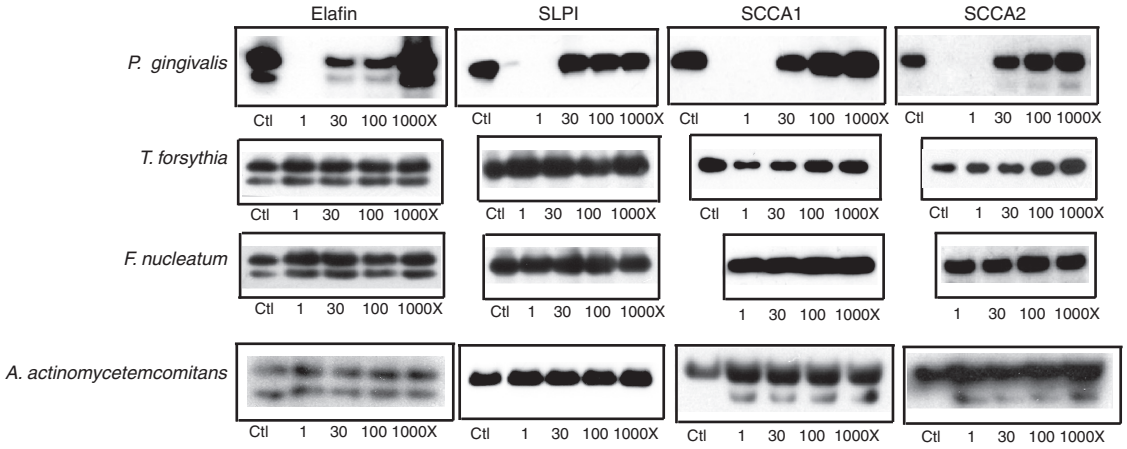

Fig. 3. Recombinant SLPI, elafin, SCCA1, and SCCA2 are degraded by *P. gingivalis* supernatants *in vitro* in a dose-dependent manner. Western Blot analysis for each protease inhibitor using a constant concentration of recombinant protease inhibitor incubated with cell-free supernatants of oral bacteria for 15 min at RT. The undiluted supernatant (1 ×) corresponds to MOI 100; increasing dilution factor is indicated below each protease inhibitor. Protein mixtures were denatured at 70°C for 10 min, separated by electrophoresis, and analyzed by Western Blot. Control: control, recombinant protein only. The controls shown with *P. gingivalis* also apply to the recombinant proteins treated with *T. forsythia* and *F. nucleatum*.

In order to identify the role of each gingipain in degradation of these protease inhibitors, we further utilized isogenic mutant strains of *P. gingivalis* lacking one or more combinations of proteases compared with the corresponding wild-type strain (Table 1). Cell-free supernatants of various mutants and the corresponding wild-type were incubated with recombinant elafin, SLPI, SCCA1 or SCCA2, and analyzed by Western Blots (Fig. 4C). Elafin, SLPI, SCCA1, and SCCA2 were significantly degraded by undiluted supernatants of wild-type W83 and 33277 *P. gingivalis*. Different wild-types (W83 and 33277) showed different potency against elafin. The mutant lacking lysine-specific gingipain (V2577, *kgp* −) and another mutant lacking both arginine- and lysine-specific gingipains (V2383, *rgpA* −/*kgp* −) showed minimal degradation of elafin by undiluted supernatants, compared to the corresponding wild-type W83 and control (Fig. 4C). Less difference was seen between the mutant lacking *rgpA* −/*rgpB* − (KDP112) and the respective wild-type 33277. These results indicate that the isoform Kgp is mainly responsible for the degradation of elafin although the combination of RgpB and RgpA may have a synergistic effect on elafin degradation. The mutant lacking the lysine-specific gingipain V2577 (*kgp* −) cleaved SLPI, while the one lacking both arginine-specific gingipains KDP112 (*rgpA* −/*rgpB* −) showed reduced ability to degrade SLPI, compared with the respective wild-type. The *rgpA* −/*kgp* − mutant (V2383) did not degrade recombinant SLPI. All these data suggest the degradation of SLPI is mainly RgpA-specific (Fig. 4C). The lysine-specific mutant V2577 (*kgp* −) and arginine-, lysine-specific double mutant V2383 (*rgpA* −/*kgp* −) cleaved both SCCA1 and SCCA2, similar to the corresponding wild-type. However, the double mutant (*rgpA* −/*rgpB* −) was unable to completely cleave either SCCA1 or SCCA2, suggesting the importance of RgpB in the degradation of SCCA1 and SCCA2. The data indicate different susceptibility patterns of these protease inhibitors to different gingipain isoforms, however, all of these protease inhibitors are degraded by one or more gingipains.

Functional effects of protease inhibitors on innate immune responses in GECs

In order to investigate changes in the expression of innate immune markers in response to changes in protease and/or protease inhibitor levels, we evaluated the gene expression of antimicrobial proteins hBD2 and CCL20 using QRT-PCR after stimulation of GECs with cell-free supernatants of *P. gingivalis* for 24 h (Fig. 5A and B) and whole bacterial cultures (data not shown). *P. gingivalis* supernatant-induced hBD2 and CCL20 at MOI equivalent to 100 and 200. We pretreated cells with recombinant SLPI, elafin, SCCA1, or SCCA2 at various concentrations for 1 h and then added *P. gingivalis* supernatants (MOI: 100) or supernatants plus TLCK for 24 h. The recombinant protease inhibitors alone did not alter the expression of hBD2 and CCL20 (data not shown). *P. gingivalis*-induced expression of hBD2 and CCL20 in GECs was reduced nearly to the unstimulated baseline level when TLCK was added (Fig. 5C–F). Pre-incubation of the supernatants with SLPI and SCCA1 partially blocked *P. gingivalis*-induced hBD2 expression compared with *P. gingivalis* alone ($p < 0.05$). Similarly, pre-incubation with SLPI, SCCA1, and SCCA2 blocked expression

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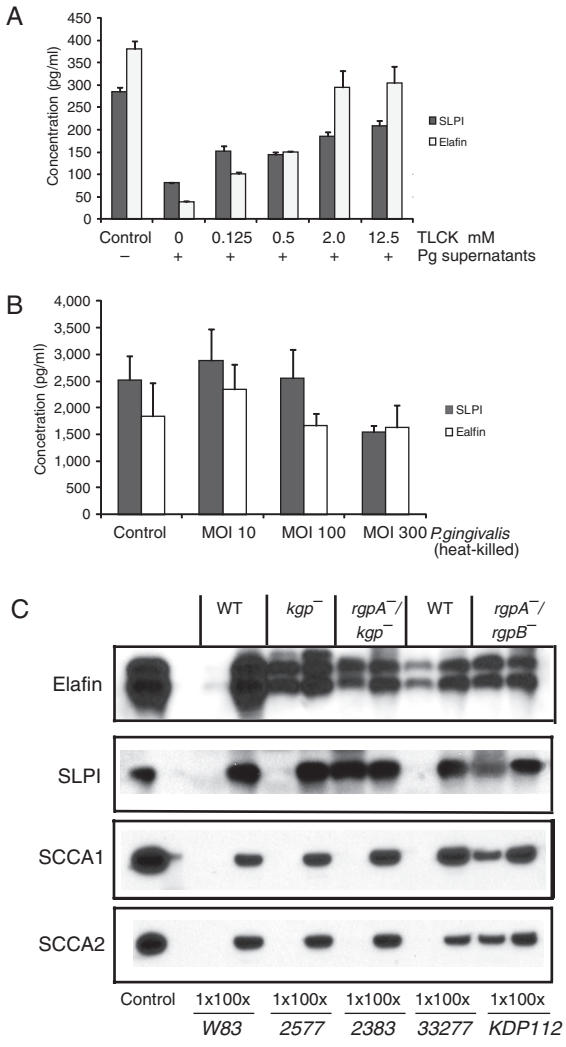

Fig. 4. The gingipains are responsible for the degradation of protease inhibitors. (A) The amount of SLPI and elafin is measured in the presence of various concentration of TLCK. Media from GECs cultured for 24 h and containing secreted SLPI and elafin were collected and incubated with *P. gingivalis* supernatants (1:10 dilution) in the presence of various concentrations of TLCK (0.125–12.5 mM) for 20 min at RT, and assayed for the remaining SLPI and elafin by ELISA. The data presented are means ± SD of three experiments. (B) The degradation of SLPI and elafin are heat sensitive. GECs were stimulated with heat-killed *P. gingivalis* equivalent to MOI 10, 100, and 300 for 24 h. The secreted level of SLPI and elafin was measured by ELISA. The results were expressed as means ± SD (standard deviation) of values from three independent experiments. (C) The degradation of recombinant protease inhibitors was compared using *P. gingivalis* mutants and corresponding wild-type strains *in vitro.* Incubation conditions are same as in Fig. 3. Supernatants used were undiluted (1×) and diluted 100-fold (100×). Recombinant SLPI, elafin, SCCA1, and SCCA2 in the absence of bacterial supernatant served as controls: Control the results are representative of two independent experiments.

of CCL20 compared with *P. gingivalis* alone ($p < 0.05$), while no significant alteration of hBD2 and CCL20 expression was found with elafin pre-incubation. The effect of these protease inhibitors to block innate immune responses in GECs suggests a protective role against *P. gingivalis.* In contrast, the protective effect of these inhibitors was lost when *P. gingivalis* supernatant was pretreated with any one of the four protease inhibitors then added to the cells, possibly due to degradation of the protease inhibitors by *P. gingivalis* supernatants (data not shown). The degree of hBD2 and CCL20 induction varied between cells from different donors, but the trend remained the same.

Discussion

In our attempt to compare differential effects of Gram-negative periodontal pathogens on host protease inhibitors, we observed that only *P. gingivalis* degraded multiple host protease inhibitors, such as SLPI, elafin, SCCA1, and SCCA2. Our data provide the strong evidence that *P. gingivalis* proteases, the gingipains, are implicated in the degradation of protease inhibitors and these degradation patterns are specific to gingipain isoforms.

Periodontitis is strongly associated with a complex of microbial species within the biofilm, suggesting that multiple microorganisms contribute to the pathogenesis of chronic periodontal disease. These putative periodontal pathogens express a variety of proteolytic enzymes which may be involved in the processes of periodontal disease (33, 34). The best characterized of these are gingipains from *P. gingivalis* (18, 35), but in addition, *T. forsythia* has a sialidase enzyme (13), *T. denticola* has a chymotrypsin-like enzyme, *A. actinomycetemcomitans* has metalloproteases (15), and *F. nucleatum* has hyaluronidase and chondroitinase activities (36). All these enzymes are released into gingival crevicular fluid (37). Therefore, it was of interest to us to explore the effect of various oral pathogens on epithelial protease inhibitors.

In this study, we observed that four epithelial protease inhibitors are significantly upregulated at the mRNA level only by *P. gingivalis* supernatants, however, the secreted protein level does not reflect mRNA level, suggesting rapid degradation in the culture media. This degradation was partially or largely blocked by TLCK, an inhibitor of gingipains. In addition, *P. gingivalis* supernatant was effective in degrading the four recombinant protease inhibitors. *P. gingivalis*-derived gingipains have been shown to cleave or degrade several types of host proteins and fit into the class of pathogenic bacteria in which proteases have multiple potent deleterious effects on innate immune responses as recently reviewed (38). Gingipains alter cell adhesion allowing bacterial penetration into the tissue (21), destroy protective innate immune

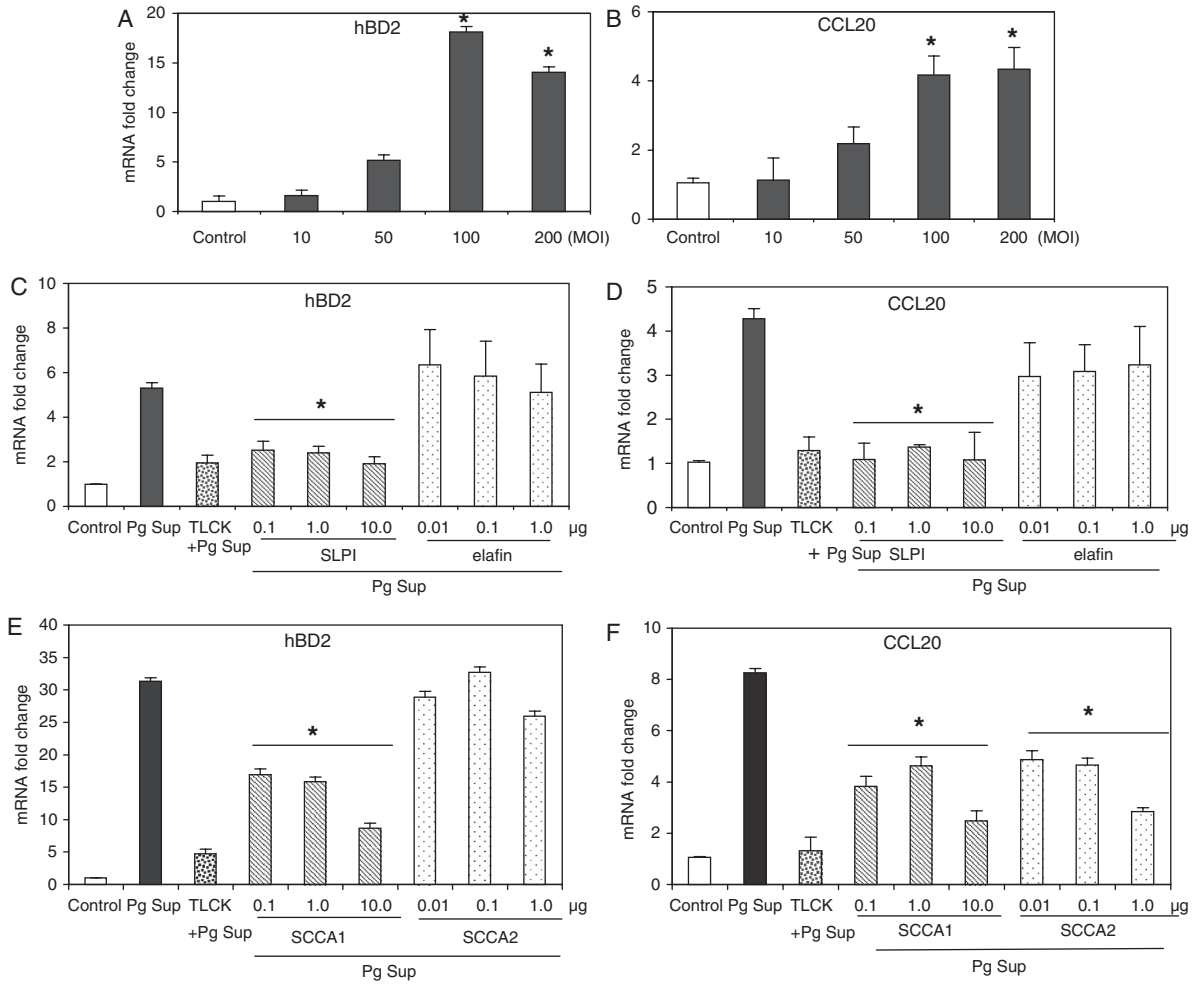

Fig. 5. Functional effects of protease inhibitors on innate immune responses of GECs. (A and B) The fold change for hBD2 (A) and CCL20 (B) mRNA expression was evaluated by QRT-PCR after GECs were stimulated for 24 h with *P. gingivalis* supernatants at increasing MOI equivalents. hBD2 and CCL20 mRNA levels were compared with unstimulated control (unfilled bar) after normalization with the housekeeping gene ribosomal phosphoprotein (RPO). Data show means ± SEM (standard error of the mean) for three donors evaluated in duplicate. (*) indicates the significant difference versus the respective unstimulated control ($p < 0.01$). (C–F) SLPI, elafin, SCCA1, and SCCA2 partially attenuated mRNA gene expression of hBD2 and CCL20 in GECs. hBD2 and CCL20 mRNA fold change evaluated by QRT-PCR is shown for GECs that were pre-incubated with the indicated concentration of recombinant SLPI, elafin, SCCA1 or SCCA2 at 37°C for 1 h, then exposed to *P. gingivalis* supernatants (MOI equivalent 100) for 24 h. Data are from duplicate samples using GECs from three donors. The gene expression of hBD2 and CCL20 varied between different batches of *P. gingivalis* or primary cells from different donors used, but the trend remained the same. Controls without protease inhibitor pre-incubation include unstimulated GECs (unfilled bars); GECs stimulated with *P. gingivalis* supernatants (Pg Sup) (black bars) and with *P. gingivalis* supernatants (Pg Sup) plus TLCK (dashed bars). Data are expressed as means ± SEM. (*) indicates a statistically significant difference compared with the stimulation of GECs by *P. gingivalis* supernatants ($p < 0.05$).

responses including the antimicrobial peptides α- and β-defensins (39), and suppress expression of IL-8 (40), thus hindering the role of neutrophils that are the first line of essential host defense against invading pathogens in the gingival sulcus (41). Our results show that yet another protective epithelial innate immune response, the expression and function of multiple protease inhibitors, is altered by *P. gingivalis*. In addition, our preliminary data (data not shown) utilizing siRNA showed that the induction of gene expression of all four protease inhibitors was associated with protease-activated receptor 2 (PAR2), a G-protein-coupled receptor activated by *P. gingivalis* proteases, further suggesting the role of gingipains in the regulation of protease inhibitors.

Serine protease inhibitors target multiple proteases and are involved in multiple immunomodulatory functions, for example, to trigger keratinocyte proliferation and differentiation as well as leukocyte attraction and activation (23, 42). Despite the crucial importance of protease inhibitors in immune modulation, little is known about

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their role in periodontal health. SLPI is constitutively produced at many mucosal surfaces and by neutrophils and macrophages (43), while elafin is mainly induced in skin (44, 45) or lung epithelium (46) under inflammatory conditions. In addition to their activity as protease inhibitors, SLPI and elafin have antimicrobial activity against Gram-positive and -negative pathogens (47, 48), anti-HIV-1 activity (42, 49, 50), and anti-inflammatory functions (47, 51–54). SCCA1 and SCCA2, members of the ovalbumin-serpin family of serine protease inhibitors, have been reported to be co-expressed widely in normal epithelial tissues, such as tonsil, airway, vagina, uterine cervix, and keratinocytes (55, 56). Both SCCA1 and SCCA2 are detected normally in the cytoplasm of squamous epithelial cells (55) and inhibit serine proteases of endogenous origin, such as cathepsin G and chymase, as well as extrinsic origin such as cysteine proteases derived from house dust mites (57, 58). SCCAs, as primarily intracellular serine protease inhibitors, might act on proteases from *P. gingivalis* after it invades epithelial cells.

It is emerging that a number of diseases are associated with an imbalance between proteases and their specific inhibitors. These imbalances may be due to excess protease secreted by bacterial infection or by inflammatory cells. For example, the increased matrix metalloproteinase (MMP) or tissue inhibitor of metalloproteinase (TIMP) ratio was reported to be associated with periodontitis (59), cardiovascular disease (60), and lung disease (61). When the ratio of protease to protease inhibitor favors the proteases (excess protease); the proteases may alter the immunomodulatory properties of the inhibitors or cleave the inhibitor as shown for elafin when complexed with neutrophil elastase (62). The results of the present study demonstrated degradation of SLPI, elafin, SCCA1, and SCCA2 by the oral pathogen *P. gingivalis* in addition to SLPI, as previously reported (22); this would be expected to change the ratio of proteases and their inhibitors as *P. gingivalis* infection is established and progresses. Reduction of serine protease inhibitor secretion has been previously related to bacterial infection. For example, SLPI was reduced in vaginal secretions and sputum (63) in the presence of bacterial infection. Elafin has been found in lower levels in *P. aeruginosa*-positive as opposed to *P. aeruginosa*-negative sputum in cystic fibrosis patients. Our study is the first to show differential degradation of host SLPI, elafin, SCCA1, and SCCA2 by various major periodontal pathogens. We suggest that this reduction in host protective capacity contributes to increased susceptibility to inflammatory periodontal disease once *P. gingivalis* becomes a component of the subgingival biofilm.

Our previous study showed that the multiple protease inhibitors were induced by cell wall preparation of *F. nucleatum*, but this phenomenon was not observed by live *F. nucleatum* in this study, possibly due to a dose effect. Live bacterial culture of *F. nucleatum* exhibits plasmin protease activity (64, 65), which may also influence the expression of those protease inhibitors. *T. forsythia*, whose main virulence factor is a trypsin-like protease, slightly degraded SCCA1 and SCCA2 in this study. Thus, a main player in the pathogenesis of *T. forsythia* may not be a protease, but the organism's ability to penetrate host cells or induce apoptosis (66).

By utilizing protease mutants of *P. gingivalis* we gained information about the type of gingipain that degrades each of the host protease inhibitors tested. Our results suggest that Kgp is mainly responsible for elafin degradation, with some contribution from RgpB. Our data are comparable to a recent finding by Kantyka et al. that compared the anti-human neutrophil elastase activity of elafin in the presence *P. gingivalis* gingipains. In Kantyka's study, all three gingipains (RgpA, RgpB, and Kgp) were found to degrade elafin, with RgpB being the most efficient gingipain (67). Furthermore, in our study, RgpA is most effective in degrading SLPI, which is consistent with the findings of Into et al. (22). RgpB is most effective in degrading SCCA1 and SCCA2, however, SLPI, SCCA1, and SCCA2 are all slightly susceptible to cleavage by Kgp as indicated by their reduction in stability in the double mutants KDP112 (*rgpA* −/*rgpB* −). The different susceptibility patterns are undoubtedly due to specific cleavage sites within the protease inhibitors. We also sought to determine if the protease inhibitors have an effect on the response of GECs to *P. gingivalis*, in other words, do these protease inhibitors function in a protective manner for epithelial cells? Our preliminary data found that SLPI inhibits the proteolytic activity of *P. gingivalis* supernatants in a dose- and time-dependent manner (data not shown). Furthermore, we also found that pretreatment of GECs with SLPI, SCCA1, and SCCA2 resulted in reduced expression of 'alarm' signals hBD2 and CCL20 gene expression when these cells were exposed to *P. gingivalis*. This is possibly due to the protease inhibitors influencing inflammatory signaling pathways. For example, SLPI has been found to suppress NF-κB activation, elafin has been shown to inhibit LPS-induced MCP-1 in monocytes by inhibiting AP-1 and NF-κB activation, and SCCAs specifically suppress the kinase activity of JNK1 (51, 68, 69). The pretreatment with those inhibitors might attenuate inflammatory responses.

The progress of periodontal disease is associated with an increased proteolytic activity in gingival sites (33, 34). These proteases originate from both host-derived inflammation and periopathogens, and contribute to the pathogenesis of periodontal disease. Within the complex dental biofilm, it is important to define which putative periopathogens are dominant in the destruction of periodontal tissue during the chronic inflammation. In this study,

we have found that *P. gingivalis* was most effective at degradation of multiple host proteases inhibitors and this may be due to the fact that *P. gingivalis* has most protease activity and a greater number of proteases than any of the other bacteria, as reviewed by Eley and Cox (37). The cleavage of these protease inhibitors is not essential for the survival of periopathogens, but their loss could make the host more susceptible to progressive infection. An extensive review of effects of bacterial proteases on innate immunity categorizes deleterious effects on cytokines, chemokines, antimicrobial peptides, complement, and inflammatory signaling pathways (38). The present study demonstrates that the balance of proteases and their inhibitors may also be an important factor in the innate immune response and provide initial evidence for the development of preventive therapies for chronic periodontal disease using protease inhibitors.

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