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Secretory leukocyte protease inhibitor and its potential interactions with elastase and cathepsin B in gingival crevicular fluid and saliva from patients with chronic periodontitis

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Background and Objective: Elastase is carried into the oral cavity by gingival crevicular fluid (GCF) from periodontal lesions. Our study investigated the regulation of elastase activity by secretory leukocyte protease inhibitor (SLPI) and the possible action of another GCF protease on this protective salivary component.

Material and Methods: Whole-mouth saliva (WMS), parotid saliva (PS) and GCF were obtained from 19 patients with periodontitis. The concentrations of active elastase and cathepsin B were determined using peptide substrates. SLPI and α1-proteinase inhibitor (α1PI) concentrations were determined using enzyme-linked immunosorbent assays (ELISAs). The molecular forms of SLPI were examined by immunoblotting.

Results: The molar concentrations of elastase, cathepsin B and α1PI were higher in GCF than in WMS and especially PS ($p < 0.0002$). The GCF SLPI concentrations were also higher than the WMS SLPI concentrations ($p < 0.05$). All WMS components increased with GCF content, significantly for elastase and SLPI ($p < 0.002$). In GCF, the concentration of α1PI was higher than the concentration of SLPI ($p < 0.0002$), while there was no significant difference for WMS. SLPI and elastase levels in GCF and WMS were inversely related ($p < 0.005$). In SLPI immunoblots, PS contained only the intact 14-kDa molecule of SLPI, while WMS also contained an 8-kDa fragment. For WMS there was a positive correlation between SLPI degradation and cathepsin B ($p < 0.002$). Incubation of WMS alone or of PS with GCF in the presence of cysteine proteinase activators caused SLPI immunoreactivity to shift to 8 kDa.

Conclusion: For GCF, serum-derived α1PI is the major elastase inhibitor, but in WMS SLPI probably reduces activity. The inflamed gingivae can be an additional source of SLPI in the oral cavity, but here the molecule is apparently cleaved by GCF cysteine proteinases, such as cathepsin B.

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Neutrophil elastase is a highly destructive enzyme, which can degrade many extracellular matrix components following the release of azurophil granules in response to inflammatory stimuli and cell lysis (1). Neutrophil elastase is found in the gingival crevicular fluid (GCF) of periodontitis patients, and high activities are associated with disease severity and progression (2–5). GCF also contains serum-derived α1-proteinase inhibitor (α1PI) or α1-antitrypsin, the main physiological inhibitor of neutrophil elastase (6). The complex of elastase with α1PI (elastase:α1PI) is readily detectable in GCF, and a significant proportion of both enzyme and inhibitor can be present in this form (7–11).

GCF flows from the gingival crevice, carrying elastase into the oral cavity where it has the potential to damage the mucosal surfaces and other soft tissues. Elastase has been detected in mixed whole mouth saliva (WMS) from periodontitis patients, with activity again being related to disease status (12–14). Here, an important regulator of the enzyme is likely to be secretory leukocyte protease inhibitor (SLPI), a low-molecular-weight protein that is associated with a number of glandular fluids (15), including saliva (16). Accordingly, the main objective of the present study was to assess the capacity of salivary SLPI, as compared with serum-derived α1PI, to moderate elastase activity entering the oral cavity from the inflamed gingiva.

As well as its inhibitory potential, SLPI has a wide range of other properties, including anti-inflammatory and antimicrobial action (17–19), and promotion of scarless wound healing (20,21), which could help to maintain oral health. However, any additional protective roles might be impaired by complexing to elastase or attack by other GCF proteases. In the airways, where SLPI is an important inhibitor of elastase (22–24), it can be cleaved by cysteine proteinases (25). GCF from patients with periodontitis similarly contains cysteine proteinases, such as cathepsin B and, like elastase, their activities increase in the presence of disease (2,26–28). Therefore, we also investigated cathepsin B activity and SLPI degradation in GCF and saliva.

Material and methods

Patients

Ninteen patients with severe chronic periodontitis (nine men and 10 women; age range 29–54 yr) were selected from those referred to Guy's Hospital for specialist treatment. All had a minimum of 20 teeth, at least 12 sites with pocket depth ≥ 5 mm and radiographic evidence of ≥ 50% alveolar bone loss. They had received no periodontal treatment in the past 6 mo and no antibiotic treatment in the previous 3 mo. None had any medical condition which could affect the periodontal tissues, and none had smoked within the past 5 years. The patients had no other inflammatory lesions of the oral mucosa and no evidence of salivary gland disease. Approval for the study was obtained from Guy's Research Ethics Committee, and all patients gave informed consent to participate.

Sample collections

A 2–6 ml sample of unstimulated WMS was collected from each patient by passive drooling into a sterile universal container. Similar volumes of stimulated parotid saliva (PS) were obtained by placing a Lashley cup over the Stensen's duct from one gland and having the patient suck a sugar-free lemon or lime sweet (Simpkins, Sheffield, UK). In both cases, the collection times and volumes were recorded. The saliva samples were mixed initially with 1 ml of 67 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% Tween 20 (PBS-T); dilutions were subsequently adjusted to 1 : 1 with additional buffer.

GCF was obtained from the 12 deepest pockets recorded on a six-point periodontal chart at an initial visit. Supragingival plaque was noted and when present removed with a curette. The area was then isolated and dried gently with cotton wool rolls to avoid saliva contamination. Twenty-second samples of GCF were collected by placing sterile Periopaper strips (Oraflow, Plainview, NY, USA) at the entrance of the pocket. The volume of GCF on the strips was determined using a Periotron® 8000 (Oraflow), previously calibrated with the accompanying software and known volumes of serum. Samples containing visible blood or saliva were rejected and an alternative site selected. After collection of GCF, the probing depth was remeasured using a force-controlled probe (Electronic Periodontal Probe Model 250; Vine Valley Research, Middlesex, NY, USA) and bleeding after probing was noted.

The 12 GCF samples from each patient were placed together in a tube with 1.5 ml of PBS-T. The tube was vortexed for 30 s and the collection strips were allowed to elute for 1 h at 4°C, after which they were removed. For comparability, the saliva samples were also kept at 4°C for 1 h. Both GCF and saliva were centrifuged for 10 min at 1000 *g* to remove debris, and the supernatants were aliquoted and frozen at −80°C prior to analysis.

Protease assays

Elastase activities were measured by fluorimetric assay (26). Portions of sample were added to 0.1 M Tris-HCl, pH 7.5, containing 1.0 M NaCl and 0.1% Triton X-100. The reaction was then started by addition of the substrate MeOSuc-Ala-Ala-Pro-Val-AMC (Bachem, St Helens, UK) to give a final concentration of 10 μM in a volume of 2.0 ml. After 1 h at 37°C, the reaction was stopped by the addition of 20 μl of 20 mg/ml soybean trypsin inhibitor (Sigma, Poole, UK) and the concentration of liberated AMC was read using an LS30 luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Cathepsin B activity was measured by a similar procedure using the substrate Z-Phe-Arg-AFC (Enzyme Systems Products, Livermore, CA, USA) and the buffer 0.1 M 2-(*N*-morpholino)-ethanesulphonic acid (MES), pH 5.5, containing 2 mM dithiothreitol (DTT), 2 mM EDTA and 0.1% Triton X-100 (26). Excitation and emission

wavelengths were 405 and 505 nm, respectively. As salivary kallikrein also hydrolyses this substrate (29), a cysteine proteinase inhibitor (30) was used to differentiate cathepsin B activity, which was consequently determined from the difference in results between assays carried out in the absence and presence of 10 μM Z-Phe-Ala-CHN_2 (Bachem).

Protease activities were calculated initially in terms of μunits (i.e. pmoles of substrate hydrolysed per minute). They were converted to ng of active enzyme by comparison with substrate hydrolysis by purified human leukocyte elastase (Sigma) and liver cathepsin B (Calbiochem, Notts., UK) under the same conditions. Molar concentrations were estimated from known molecular weights (elastase, 30 kDa; cathepsin B, 27 kDa).

The ability of PS to inhibit elastase was determined with 1 ng of purified human leukocyte elastase (Sigma) or GCF eluate with equivalent activity. Portions (1–100 μl) of diluted PS (1 : 1 with PBS-T) were incubated with purified elastase or GCF in assay buffer for 10 min at room temperature, after which substrate was added and the assay continued as described above. The results were calculated as the percentage inhibition by comparison with the activity of controls without PS.

Inhibitor enzyme-linked immunosorbent assays (ELISAs)

SLPI concentrations were measured using a commercial sandwich ELISA kit (HyCult Biotechnology, supplied by Cambridge Bioscience, Cambridge, UK) using the instructions provided. Samples were diluted in a kit buffer and incubated in antibody-coated microplate wells for 1 h at 37°C. Sample and subsequent reagent volumes were 100 μl per well and, after each step, the wells were washed four times with 200 μl of a second kit buffer. The wells were next treated with biotinylated tracer antibody followed by streptavidin–peroxidase complex, each for 1 h at room temperature. Tetramethylbenzidine (TMB) substrate solution was then added and, after incubation for 20–30 min in the dark at room temperature, colour development was stopped with citric acid solution. Absorbances were read at 450 nm using a microplate reader (Bio-Rad Laboratories, Hemel Hempstead, UK) and sample concentrations were calculated by comparison to a curve obtained with successive dilutions (5.0–0.02 ng/ml) of SLPI standard from the kit.

α1PI was measured by using a sandwich ELISA procedure based on that of Smith *et al.* (9), but with the following modifications. The capture antibody was rabbit anti-human α1PI (Sigma), diluted 1 : 2000, and the detecting antibody was sheep anti-human α1PI horseradish peroxidase conjugate (The Binding Site, Birmingham, UK) diluted 1 : 2000 and incubated for 1 h. The final reaction was carried out with 75 ng/ml TMB and 0.0075% H_2O_2 (both from Sigma), and colour formation was stopped, after 10 min, with 2 M H_2SO_4 (31). After measuring the absorbances at 450 nm, sample concentrations were estimated by comparison with a standard curve obtained with successive dilutions (125–0.97 ng/ml) of human plasma α1PI (Sigma). Molar concentrations of both inhibitors were calculated from known molecular weights (SLPI 12 kDa; α1PI 53 kDa).

Electrophoresis and SLPI immunoblotting

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) was conducted on precast 4–12% Bis-Tris gels (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Proteins were then transferred electrophoretically onto Protran nitrocellulose membranes (Schleicher and Schuell, London, UK). The membranes were treated initially with ProtoBlock (National Diagnostics, Hull, UK) for 60 min to prevent nonspecific binding. After this, and subsequent steps, the membranes were washed three times, for 5 min each wash, with Tris-buffered saline containing 0.1% Tween 20 (TTBS). Immunoprobing was performed for 120 min with affinity-purified goat anti-human SLPI (R & D Systems, Abingdon, UK), diluted 1 : 500, followed by 45 min with biotin-conjugated rabbit anti-goat immunoglobulins (Sigma) diluted 1 : 1000, and then 45 min with avidin and biotinylated horseradish peroxidase (AB-Complex/HRP; Dako, Ely, UK), diluted 1 : 4000; all dilutions were made in TTBS. Band visualization was by enhanced chemiluminescence (ECL) recorded photographically on Hyperfilm (Amersham Pharmacia Biotech, Bucks., UK). Band strengths were quantified by Kodak 1D image-analysis software (Eastman Kodak, Rochester, NY, USA) and for WMS the proportion of degraded SLPI was calculated as the percentage of the total absorbance of all observed molecular forms that was represented by inhibitor fragment(s).

To check SLPI processing in WMS, samples were incubated at 37°C, with and without 2 mM DTT and 2 mM EDTA as cysteine proteinase activators (25,26), prior to electrophoresis and immunoblotting. The action of GCF eluates on PS SLPI was also examined by incubating mixtures of the two in similar experiments.

Statistics

In the first instance, differences in the concentration of enzymes and inhibitors between the three oral fluids were examined using Kruskall–Wallis tests, and this was followed by Wilcoxon sign rank tests to establish where the differences lay. Wilcoxon tests were also used to compare the level of the two inhibitors in the fluids. Relationships between the different enzymes and inhibitors were explored with Spearman rank correlations. Statistical significance was accepted if the p-value was < 0.05.

Results

Patient and sample characteristics

For the 19 study patients, mean probing depths at the 12 GCF collection sites were all somewhat greater than the minimum of 5 mm specified as one of the criteria for severe periodontitis

(Table 1). Among patients, however, there were wide variations in the number of sampled sites exhibiting bleeding on probing and supragingival plaque. The total volumes of GCF collected ranged from 3.23 to 8.63 μl. Flow rates for WMS and PS, adjusted to the 20-s GCF sampling time, were both nearly two orders of magnitude greater (Wilcoxon $p < 0.0002$).

Protease and inhibitor levels

Protease and inhibitor levels were calculated as molar concentrations to simplify comparisons of their biochemical potential. All showed wide ranges in GCF and saliva samples from the 19 study patients (Table 2). The concentrations of active elastase and cathepsin B, and of immunoreactive α1PI, in GCF were all significantly greater than those in WMS, which in turn were higher than the very small amounts in PS (Wilcoxon $p < 0.0002$ in both cases). The levels of all these components in WMS increased in parallel with those in GCF, this relationship being significant in the case of elastase (Spearman rho = 0.749, $p < 0.002$) (Fig. 1A).

There was no significant difference in the concentration of SLPI between WMS and PS, and no significant correlation between the level of SLPI in these two salivas. However, the SLPI concentration in GCF was significantly higher than in WMS (Wilcoxon $p < 0.05$), and there was a highly significant correlation between the concentrations of SLPI in GCF and in WMS (Spearman rho = 0.805, $p < 0.001$) (Fig. 1B).

In GCF, the concentration of α1PI was significantly higher than that of SLPI; whereas in PS, SLPI levels were higher (Wilcoxon $p < 0.0002$ for both comparisons). In WMS, there was no significant difference between the values of the two inhibitors.

Elastase inhibition

PS inhibited purified elastase and GCF with equivalent activity in a nonlinear, but dose-dependent, manner over the volume range 1–100 μl for 1 : 1 dilutions with PBS-T (Fig. 2A). Among the 19 patients there was a very strong correlation between the percentage inhibition of purified elastase activity obtained with a fixed volume of 2.5 μl of PS and SLPI concentrations determined by ELISA in the same samples (Spearman rho = 0.940, $p < 0.0001$) (Fig. 2B). In addition, there was a negative relationship between SLPI concentrations and active elastase in WMS and also GCF samples (Spearman rho = −0.670, $p < 0.005$ and rho = −0.644, $p < 0.01$, respectively) (Fig. 2C,D). By contrast, active elastase and α1PI concentrations increased together in WMS (Spearman rho = 0.677, $p < 0.005$), but not significantly

Table 1. Periodontal condition at the gingival crevicular fluid (GCF) collection sites and GCF, whole-mouth saliva (WMS) and parotid saliva (PS) flow rates for the 19 study patients

	Median of 19 patients	Interquartile range	Minimum–maximum
Mean probing depth/patient of 12 sampled sites	5.67 mm	5.33–6.42 mm	5.25–7.50 mm
No. of sampled sites bleeding after probing	6	3–7	1–12
No. of sampled sites with plaque	7	2–9	0–12
GCF flow rate from the 12 deepest pockets	4.76 μl/20 s	4.21–7.24 μl/20 s	3.23–8.63 μl/20 s
WMS (unstimulated) flow rate	348 μl/20 s	174–410 μl/20 s	102–898 μl/20 s
PS (stimulated, one gland) flow rate	374 μl/20 s	206–464 μl/20 s	132–826 μl/20 s

Table 2. Protease and inhibitor concentrations in gingival crevicular fluid (GCF), whole-mouth saliva (WMS) and parotid saliva (PS) from the 19 study patients

	GCF (12 deepest pockets)			WMS (unstimulated)			PS (stimulated, one gland)			Comparison of three fluids
	Median	Interquartile range	Minimum Maximum	Median	Interquartile range	Minimum Maximum	Median	Interquartile range	Minimum Maximum	Kruskal–Wallis *p*-value
Elastase (nM)	1430[a]	1216–1914	359 3208	64.2[b]	5.0–116.7	0.3 840.1	0.008	0.005–0.015	0 0.029	< 0.002
Cathepsin B (nM)	43.2[a]	31.8–72.6	5.8 123.8	1.02[b]	0.54–1.20	0.30 2.82	0.004	0–0.015	0 0.047	< 0.002
SLPI (nM)	100.9[a]	51.5–172.6	16.4 469.4	23.5	15.2–193.8	6.8 507.5	112.8[c]	69.8–171.8	49.8 303.7	< 0.07
α1PI (nM)	3545[a,d]	3049–4238	1604 9015	103.1[b]	64.6–163.9	24.5 316.5	1.74	1.21–2.37	0.73 5.99	< 0.002

[a] Significantly greater than WMS values: Wilcoxon $p < 0.0002$ (elastase, cathepsin B, α1-proteinase inhibitor), $p < 0.05$ (secretory leukocyte protease inhibitor).
[b] Significantly greater than PS values: Wilcoxon, $p < 0.0002$ (elastase, cathepsin B, α1-proteinase inhibitor).
[c] Significantly greater than PS α1-proteinase inhibitor values: Wilcoxon, $p < 0.0002$.
[d] Significantly greater than GCF secretory leukocyte protease inhibitor (SLPI) values: Wilcoxon, $p < 0.0002$.

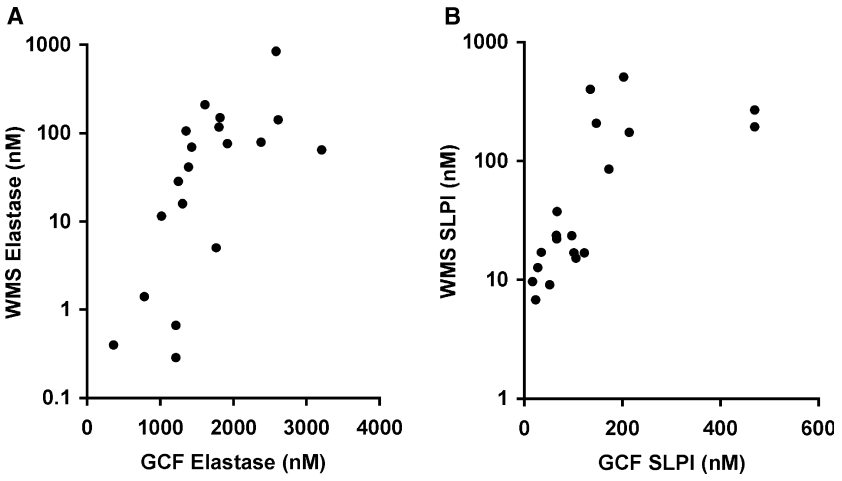

Fig. 1. Relationships between whole-mouth saliva (WMS) and gingival crevicular fluid (GCF) concentrations of (A) active elastase and (B) immunoreactive secretory leukocyte protease inhibitor (SLPI) in samples from the 19 study patients.

in GCF (Spearman rho = 0.235, $p = 0.32$).

SLPI degradation

Western blotting for SLPI in WMS and PS showed immunopositive bands in the 6–14 kDa region (Fig. 3A). Higher-molecular-weight immunoreactivity probably occurred as a result of nonspecific labelling, because major salivary protein bands were present at these positions (data not shown). Parotid saliva samples from all 19 patients gave a single 14 kDa SLPI band corresponding to the apparent molecular weight of the native molecule in SDS–PAGE (32). By contrast, the 19 WMS samples, with only one exception, had a second, lower-molecular-weight band at 8 kDa, and in samples from three patients there was a trace of a third band at 6 kDa. For WMS there was a significant, positive correlation between the percentage of SLPI immunoreactivity represented by these degraded fragments and active cathepsin B in the same WMS samples (Spearman rho = 0.738, $p < 0.002$) (Fig. 4A). Furthermore, WMS SLPI concentrations (as measured by ELISA) tended to decrease with increasing cathepsin B concentrations, but this did not reach statistical significance (Spearman rho = −0.426, $p = 0.07$).

When WMS was incubated at 37°C for 3 h, there was sometimes a further shift in SLPI immunoreactivity from the native 14 kDa form to the 8 kDa fragment (Fig. 4B). This was more common and more pronounced when the cysteine proteinase activators DTT and EDTA (25,26) were included. Both the occurrence and extent of this effect generally increased with the amount of SLPI degradation in untreated samples. No change in SLPI band distribution was seen when untreated samples were kept at 4°C for 1 h, demonstrating that the initial observations were not an artefact of sample processing.

Incubation of PS with GCF in the presence of DTT and EDTA caused a progressive shift in the 14-kDa PS SLPI immunoreactivity to the 8 kDa fragment and this eventually disappeared altogether (Fig. 5A). No PS SLPI degradation was observed in the absence of the cysteine-proteinase activators (Fig. 5B). The GCF eluates themselves, which had been diluted more than 100-fold with PBS-T buffer (as compared with twice for the saliva samples), failed to show any SLPI bands in immunoblots (Fig. 3B).

Discussion

Some previous studies have investigated the balance between elastase and α1PI in GCF from gingivitis and periodontitis patients (7–11) and there have been a few separate reports of elastase (12–14) and α1PI (33) in WMS. SLPI has recently been measured in GCF before and after treatment (34), but has not been examined in the WMS of periodontitis patients. Hence, this is the first time that the levels of elastase and both inhibitors have been determined together in GCF and saliva samples from the same group of patients. The investigation clarified their origins and interactions and demonstrated SLPI degradation in WMS which appeared to involve GCF-derived cathepsin B.

The inflamed gingivae were probably the source of nearly all the elastase, cathepsin B and α1PI detected in the oral cavity. The concentrations in GCF were by far the greatest and these appeared to be largely responsible for the amounts found in WMS, with very much lower levels detected in PS. Neutrophils and monocytes migrating into the gingival crevice were probably the main source of elastase and cathepsin B, respectively (35), while α1PI would have derived from extravasated serum (6), possibly with some local production by macrophages (36).

Glandular saliva was expected to be a major source of SLPI in WMS. However, the concentrations of SLPI were actually higher in GCF, and the levels of SLPI in WMS were related to those in GCF rather than in PS. It would appear that in our group of patients with severe periodontitis, the comparatively large volumes of GCF entering the oral cavity (including contributions from disease sites additional to the 12 sampled) were sufficient to have a significant influence on the SLPI content of WMS. The composition of WMS would also have reflected output from salivary sources other than parotid glands, which provide ≈30–35% of WMS volume, depending on secretory activity (37).

SLPI can be synthesised by gingival epithelial cells (34,38) and these may have been the source of the GCF inhibitor. Production by the pocket epithelium, in particular, could potentially be stimulated by lipopolysaccharide from subgingival bacteria (39), elastase from migrating neutrophils (40) and cytokines from the inflamed tissues (41). GCF SLPI may also have

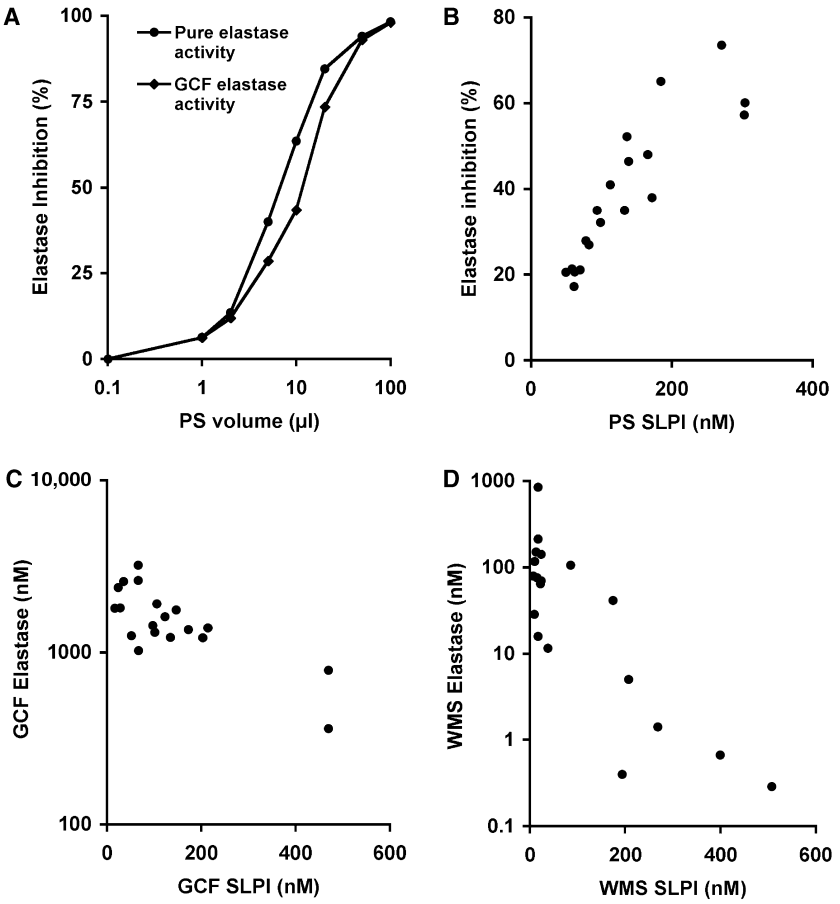

Fig. 2. (A) Inhibition of purified human leukocyte elastase (1 ng) and gingival crevicular fluid (GCF) eluate, of equivalent activity, by increasing volumes of parotid saliva (PS) [diluted 1 : 1 with phosphate-buffered saline containing 0.1% Tween 20 (PBS-T)] from the same patient. The graph is representative of results obtained with samples from three different patients. (B) Relationship between the percentage inhibition of purified human leukocyte elastase (1 ng) by 2.5 µl of diluted PS and secretory leukocyte protease inhibitor (SLPI) concentrations for the 19 study patients. (C) Relationship between active elastase and immunoreactive SLPI concentrations in GCF samples from the 19 study patients. (D) Relationship between active elastase and immunoreactive SLPI concentrations in whole-mouth saliva (WMS) samples from the 19 study patients.

derived from crevicular neutrophils themselves, as it is the major inhibitor of elastase in the cytosol of this cell type (32).

Despite relatively high levels of SLPI in GCF, the concentrations of α1PI were significantly greater. α1PI has a higher affinity for the enzyme than SLPI (42) and in GCF α1PI was almost certainly the main elastase inhibitor. The lack of any significant relationship between GCF α1PI and elastase in Spearman correlation may well have resulted from enzyme binding to another serum-derived inhibitor, α2-macroglobulin (α2M) (8,11). Unlike the elastase:α1PI complex, that with α2M is still active against small peptide substrates, such as that used here (8), whilst at the same time being resistant to inhibition by α1PI (11). The inhibitory capacity of GCF α1PI, furthermore, is not sufficient to inactivate all of the elastase available for release from neutrophils (7,9), which would probably have been lysed by the detergent (Tween 20) in our elution buffer.

The positive relationship between α1PI and elastase activity in WMS appears surprising but, in the presence of the additional salivary inhibitor, SLPI, this may have simply reflected the likelihood that the output of both α1PI and enzyme from the gingival crevice increased in patients with greater inflammation. An inverse relationship between extracellular elastase activity and α1PI has been demonstrated in GCF (11). However, that study used a mixed sandwich ELISA specific for the elastase:α1PI complex, whereas our procedure, with polyclonal capture and detection antibodies, could have detected total inhibitor, including complexes with other proteases. The SLPI ELISA, likewise, would have measured total amounts of inhibitor with enzyme complexes dissociating at the very low concentrations in the assay (information provided by the manufacturer).

In WMS, α1PI originating from GCF was evidently diluted, whereas SLPI in WMS was supplemented by contributions from glandular saliva. Consequently, the relative concentrations of the two inhibitors were much closer in WMS than GCF. Salivary SLPI clearly has the potential to regulate elastase, as PS inhibited both purified enzyme and GCF activity in a dose-dependent manner and this ability was very strongly related to measured SLPI content. In addition – and in contrast to α1PI – there was a significantly negative relationship between both WMS and GCF SLPI concentrations and active elastase. Thus, it seems likely that in the WMS of our periodontitis group, as for bronchoalveolar secretions of patients with respiratory tract diseases (22–24), SPLI played a role additional to α1PI in moderating elastase activity.

The effectiveness of WMS SPLI was, however, reduced by cleavage into one or more fragments equivalent to those seen in airway lining fluid from emphysema patients and produced *in vitro* by digestion with pure cysteine proteinases (25). Several lines of evidence suggested that GCF cathepsin B was responsible for the same process in the oral cavity. First, there was a positive correlation between enzyme activity and the percentage of WMS SLPI degradation in immunoblots. The potential for SLPI degradation in WMS was confirmed by its further

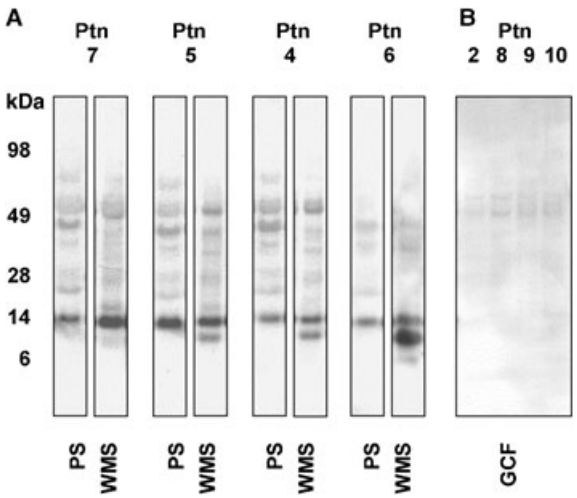

Fig. 3. Immunoblotting for secretory leukocyte protease inhibitor (SLPI) in parotid saliva (PS), whole-mouth saliva (WMS) and gingival crevicular fluid (GCF). (A) Four study patient (Ptn) samples show only the 14-kDa native molecule, while the corresponding WMS have increasing proportions of an 8-kDa fragment, with an additional trace of 6 kDa immunoreactivity for patient 6. (B) GCF samples from patients with the four highest SLPI concentrations, as measured in enzyme-linked immunosorbent assays (ELISAs). The eluates, which had been diluted more than 100-fold with phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) (as compared with a twofold dilution for the saliva samples), failed to show any SLPI bands in immunoblots. The positions of molecular weight markers are indicated on the left.

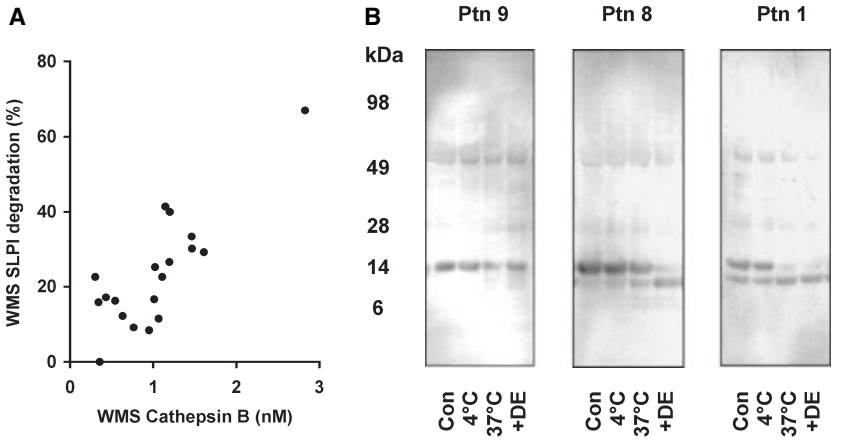

Fig. 4. (A) Relationship for untreated whole-mouth saliva (WMS) between active cathepsin B and the percentage of secretory leukocyte protease inhibitor (SLPI) degradation in immunoblots. (B) The effects of different treatments on WMS SLPI forms in immunoblots. Untreated portions (Con) are compared with those maintained at 4°C for 1 h (4°C) and incubated at 37°C for 3 h without additions (37°C) and in the presence of the cysteine proteinase activators dithiothreitol (DTT) and EDTA (+DE). The untreated sample from patient (Ptn) 9 contains only the native 14 kDa SLPI molecule and there is minimal degradation after incubation at 37°C. With patient 8, the untreated sample shows a small proportion of the 8 kDa fragment and only with inclusion of the cysteine proteinase activators during incubation at 37°C is there an appreciable increase. The untreated sample from patient 1 has approximately equal amounts of 14- and 8 kDa SLPI forms, and further fragmentation is seen at 37°C, even in the absence of the activators. None of the patient samples show any discernable change when kept at 4°C for 1 h. Positions of molecular weight markers are indicated on the left.

fragmentation during experimental incubations. This effect was enhanced by the addition of cysteine proteinase activators (25,26), and in their presence, PS SLPI was first cleaved and then digested entirely by GCF. The role of GCF-derived cathepsin B in WMS SLPI degradation was also indicated indirectly by a tendency for inhibitor concentrations to decrease in parallel with enzyme levels.

SLPI is degraded more effectively by cathepsin L than cathepsin B (25). Previous studies have reported the presence of cathepsin L in GCF (26), and a substrate analogous to that used in the present study has been utilised for measurement of its activity (27). An attempt was therefore made to assess the contribution of cathepsin L to substrate cleavage by additional assays with a selective inhibitor of cathepsin B, CA-074 (43). However, in general, cysteine proteinase activity resistant to this inhibitor made up only about 2% of the GCF and 1% of the WMS total. Hence, the great majority of activity detected under the conditions of the present study was cathepsin B, and this enzyme appeared to be causing salivary SLPI degradation. On the other hand, the cleavage of SLPI in the oral cavity could also be brought about by bacterial cysteine proteinases, especially Arg-gingipain from *Porphyromonas gingivalis*, which has a similar peptide substrate specificity to cathepsin B (44) and which degrades other host protease inhibitors (45). More detailed biochemical investigations are therefore needed to confirm the identity of the enzyme(s) responsible.

To summarise, we found that in saliva, SLPI probably plays a role in controlling elastase activity originating from GCF. A proportion of salivary SLPI would be consumed in the resulting complex and some appeared to be broken down by other protease(s) from the periodontal pocket. However, GCF was also a source of additional inhibitor, which helped to maintain salivary levels. Therefore, it seems that, in the group of patients recruited for this study, the potentially beneficial effects of SLPI, in relation to infection, inflammation and wound healing in the oral cavity (17–21), were not, in

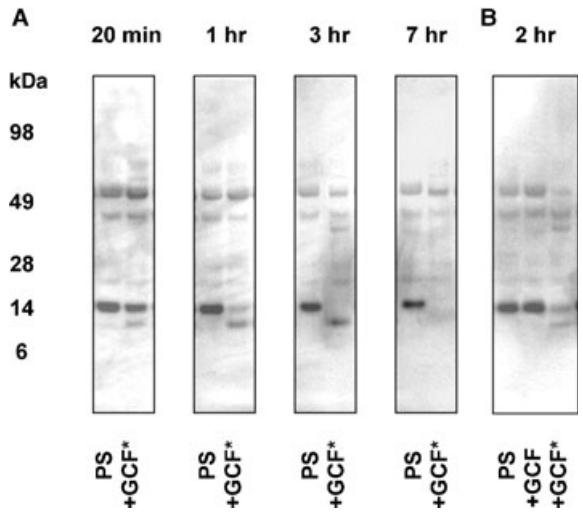

Fig. 5. Incubations at 37°C of parotid saliva (PS) alone or with gingival crevicular fluid (GCF) from the same patient in the absence (+GCF) or presence (+GCF*) of the cysteine proteinase activators dithiothreitol (DTT) and EDTA. (A) After the addition of activators, the blots show a progressive shift in secretory leukocyte protease inhibitor (SLPI) immunoreactivity, with time, from the native 14 kDa form to an 8-kDa fragment, which eventually disappears altogether. (B) After incubation for 2 h, no SLPI degradation is seen in the absence of the activators. The positions of molecular weight markers are indicated on the left. The immunoblots are representative of results obtained with samples from three different patients.

general, compromised by the presence of periodontitis. Nevertheless, there were individual variations in GCF protease levels and WMS SLPI degradation and so it is feasible that this could occur, particularly in patients with more severe disease.

The focus of our study was on the enzyme:inhibitor balance in the oral cavity and it is unlikely that salivary SLPI enters the subgingival compartment. On the other hand, GCF SLPI probably derives from gingival epithelial cells (34,38) or neutrophils (32) and we have identified the inhibitor in immunoblots of gingival homogenates (S. W. Cox & B. M. Eley, unpublished observations). Consequently, it is quite possible that interactions with elastase and cysteine proteinases, similar to those proposed here for WMS, could also take place in the periodontal pocket or tissues. The relationships between protease and inhibitor levels in GCF and saliva, and the periodontal condition of the present group of patients, will be the subject of a separate report (V. Booth *et al*., manuscript in preparation). In addition, further studies would be required to investigate the potential of SLPI to modulate the disease process.

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