Elastase activity in GCF and saliva relates to both clinical parameters and protease inhibitor levels in patients with severe periodontitis

Introduction

Neutrophil elastase could contribute significantly to pathogenic mechanisms in human periodontitis as it is a highly destructive protease capable of degrading many extracellular matrix components and host defence molecules (see Owen and Campbell1 for a review). The activity of elastase in gingival crevicular fluid (GCF) has been associated with the severity and progression of periodontitis2-5. Elastase has also been found in whole mouth saliva (WMS) at levels related to the periodontal condition6-8. Following such results, measurements of elastase in GCF and WMS have each been proposed for diagnostic monitoring.
of the disease. Activities in the two oral fluids have, however, not previously been examined together and so an initial aim of the present investigation was to relate clinical assessments of periodontitis to elastase levels in both GCF and WMS from the same group of patients.

Elastase activity is inhibited irreversibly when it forms complexes with α-1-proteinase inhibitor (α1PI), known also as α-1-antitrypsin. Previous work has indicated that such complexes exist within GCF and suggest that this may be a particularly important mechanism for regulating elastase activity in the periodontium. However, there are other serine protease inhibitors, including secretory leukocyte protease inhibitor (SLPI), which has been found concentrated in glandular and mucous secretions, where SLPI is believed to be of particular importance in the protection of mucous membranes. SLPI has been described in parotid saliva (PS), It is released by cultured gingival epithelial cells, which are a potential source for the inhibitor found in GCF. SLPI therefore has potential to influence elastase activity in both the sub- and supra-gingival environments.

A further biochemical component of oral fluids potentially influencing oral elastase activity is the cysteine protease cathepsin B, which is found in GCF at levels that increase with the severity of periodontal disease. Cathepsin B can degrade SLPI in airway lining fluids and similar cleavage of the inhibitor in WMS has also been demonstrated recently. Therefore the influence of cathepsin B on relationships between elastase, its inhibitors and the periodontal condition was also explored.

In the present study, saliva was collected and GCF was pooled from the 12 deepest pocket sites of patients with severe periodontitis to allow multiple biochemical analyses that could be related to each other and to the clinical condition of the patients. The purpose of this study was to gain better understanding of the interactions between the various biochemical components of the oral fluids and the periodontal condition to assist future evaluations of their potential for diagnostic monitoring.

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**Material and methods**

**Patient selection**

Nineteen patients aged 30–65 years with severe chronic periodontitis and at least 20 teeth were enrolled in the study. All had a minimum of 12 sites on 12 different teeth with probing depth (PD) of 5 mm or more and radiographic evidence of at least 50% bone loss. Patients and sites with severe disease were selected to ensure that sufficient GCF was collected for multiple analyses. None had received periodontal treatment in the previous 6 months, antibiotics within the previous 3 months or had any medical conditions that might affect the periodontal condition. There were no current smokers and all former smokers had quit at least 5 years earlier. Suitable patients were identified from the initial six-point pocket chart and intraoral or panoramic radiographs taken for diagnostic and treatment planning purposes. The study was approved by the Guy’s Hospital Research Ethics Committee and all patients gave written consent to take part in the study. Although the patients were invited to participate at their initial consultation appointment, consent and samples were taken at a subsequent appointment as probing would have modified the GCF.

**Sample collection**

**Unstimulated whole mouth saliva**

A minimum volume of 2 ml unstimulated WMS was collected by passive drooling into a sterile universal with 1 ml of phosphate buffered saline containing 0.15 mol/l NaCl, 67 mmol/l phosphate and 0.1% Tween 20 (pH 7.4) (PBST). The time required for sample collection was recorded.

**Stimulated parotid saliva**

A Lashley cup was used to collect saliva from one parotid gland. PS was collected in a sterile universal, which contained 1 ml of PBST. Once the Lashley cup was in place, the patient sucked a sugar-free sweet (Simpkins & Co, Sheffield, UK) to stimulate saliva secretion. A minimum volume of 2 ml was collected and the time required for sample collection was recorded.
Gingival crevicular fluid

GCF was collected from the 12 deepest pockets recorded on the previous six-point periodontal chart. Supragingival plaque at each site was scored as present or absent and gently removed using a curette before sample collection. The area was isolated and dried gently with cotton pledgets to avoid saliva contamination. GCF was collected for 20 seconds by placing a sterile Oraflow paper strip (Plainview, NY, USA) at the entrance of the pocket. Samples with visible blood or saliva were rejected and a different sample site with PD $\geq 5$mm selected. The volume of GCF on the strips was determined using a Periotron® 8000 (Oraflow, Plainview, NY, USA). Standard volumes of serum were used to calculate a calibration curve for the Periotron® 8000 using the MLCONVRT software supplied by the manufacturer. All the strips were pooled into one Eppendorf tube and 1.5 ml PBST was added immediately.

Clinical measurements

Sampled sites

After collection of GCF, the PD at the 12 sites was measured in mm using a force controlled probe (Electronic Periodontal Probe, Model 250, Vine Valley Research, Middlesex, NY, USA). Bleeding on probing (BOP) was scored as absent, discrete (pin point bleeding) or profuse if it spread out across the crevice. Recession at the sampled sites was recorded in mm.

Oral hygiene and gingivitis

The general level of plaque control and marginal gingival health were assessed on the buccal and lingual surfaces of the partial recording teeth of Ramfjord (16, 21, 24, 36, 41, 44). Plaque and marginal gingival bleeding were recorded as present or absent after running a probe gently along the gingival margin.

Sample analysis

GCF samples were vortex mixed for 30 seconds, the Oraflow strips eluted for 1 hour at 4°C and the strips were removed. The volumes of saliva samples were measured and PBST was added to adjust the dilution of each sample to 1:1 (saliva:PBST). Saliva samples were stored for 1 hour at 4°C. GCF and saliva samples were centrifuged for 10 minutes at 1000 g. The samples were divided into aliquots and frozen at -80°C until biochemical analysis. Details of the biochemical analyses are given in a further paper and are described briefly below.

Protease activity assays

Enzymes may occur in both active and inactive forms, but it is the active enzyme that has potential to cause tissue degradation and has therefore been measured in most previous periodontal studies. The elastase activity in samples was measured by fluorimetric assay using a substrate designed to be cleaved by neutrophil elastase. The GCF activity against this substrate is highly consistent with neutrophil elastase. Briefly, the substrate MeOSuc-Ala-Ala-Pro-Val-AMC (Bachem, St Helens, UK) was added to samples to a final concentration of 10 μmol/l in a volume of 2.0 ml and incubated for 1 hr at 37°C. The concentration of liberated AMC was read using an LS30 luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK).

Cathepsin B activity was measured by a similar procedure using the substrate Z-Phe-Arg-AFC (Enzyme Systems Products, California, USA) and 10 μmol/l of the inhibitor Z-Phe-Ala-CHN$_2$ (Bachem). Fluorescence readings were converted to ng of active enzyme by comparison with substrate hydrolysis by purified human leukocyte elastase (Sigma, Poole, Dorset, UK) and liver cathepsin B (Calbiochem, Nottingham, UK) under the same conditions. Molar concentrations were calculated from known molecular masses (elastase 30 kDa; cathepsin B 27 kDa).

Protease inhibitor ELISAs

SLPI concentrations were measured with a commercial sandwich ELISA kit (HyCult Biotechnology, supplied by Cambridge Bioscience, Cambridge, UK) using the manufacturer's instructions. Bound SLPI was detected using a biotinylated tracer antibody fol-
ollowed by streptavidin-peroxidase complex and tetramethylbenzidine (TMB) substrate. Absorbances were read at 450 nm with a microplate reader (Bio-Rad Laboratories, Hemel Hempstead, UK) and sample concentrations were calculated from a standard curve obtained from serial dilutions of SLPI.

α1PI was also measured by a sandwich ELISA procedure based on that of Smith et al.12, but with the following modifications. The capture antibody was rabbit anti-human α1PI (Sigma, Poole, Dorset, UK) and the detecting antibody sheep anti-human α1PI horseradish peroxidase conjugate (The Binding Site, Birmingham, UK) both diluted 1 in 2000. The final reaction was carried out with 75 ng/ml TMB and 0.0075% H2O2 (both from Sigma) and colour formation was stopped with 2 mol/l H2SO4. Sample concentrations were estimated from a standard curve obtained with serial 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).

With the laboratory procedures used for the various analyses, the detection limits for GCF, WMS and PS respectively were for elastase 50, 0.25 and 0.005 nmol/l; for cathepsin B 4.0, 0.016 and 0.004 nmol/l; for SLPI 5.0, 0.02 and 0.02 nmol/l; and for α1PI 20, 0.08 and 0.08 nmol/l.

### Statistical analysis

Since the 12 GCF samples per patient were pooled to obtain sufficient GCF for multiple biochemical tests, clinical variables reflecting all 12 sites were developed. At the sampled sites a plaque score, a bleeding score and a profuse bleeding score were calculated as the number of sampled sites per subject with plaque present, bleeding after probing (discrete and profuse bleeding) or profuse bleeding after probing respectively. In addition, the mean PD and recession at the sampled sites were also calculated for each patient. To indicate the general level of oral hygiene and marginal gingival health, a plaque and gingival bleeding score per patient was calculated as the number of sites on the Ramfjord teeth with plaque or gingival bleeding.

The molar concentrations of all biochemical components were used in the analysis to enable standardisation between individuals and the three oral fluids. The distribution of elastase activity was examined and the molar concentration of elastase in GCF samples was Normally distributed and in WMS could be log transformed to a Normal distribution. The molar concentration of active elastase in GCF and log transformed molar elastase in WMS were used as the dependent variables in subsequent regression models.

Previous studies have related elastase activity to PD and clinical attachment loss in GCF2–5 and WMS7,8. In the present study, regression modelling was first used to examine the relationships between active elastase in GCF and WMS and the clinical condition, thereby allowing comparison of the data with previous findings. However, to better understand the relationships between elastase activity, the periodontal condition and the other biochemical components of the oral fluids, the biochemical variables were then added to the first significant regression model and a second model was developed. The purpose of this approach was to explore the possibility that the other biochemical variables may be modulating the relationship of elastase to the clinical condition.

In the first stage of the regression modelling, a stepwise approach was used to investigate whether age, gender, smoking history, the clinical variables or GCF volume could explain statistically the molar concentration of active elastase in GCF and WMS. Variables were removed from the model sequentially if they did not approach significance (p > 0.2). The other biochemical variables in the relevant oral fluids were then added to the first significant model to determine whether those biochemicals could account statistically for the significant relationship of elastase with clinical variables. In the case of GCF elastase, only biochemical variables in GCF were added to the clinical model. However, in the model representing WMS, the GCF and salivary biochemical variables were added as both could contribute to WMS enzyme and inhibitor values. The biochemical variables that did not approach statistical significance (p > 0.2) were removed sequentially to develop the second significant regression models. Statistical analysis was conducted using STATA™ data analysis software (STATA Corporation, Texas, USA) and statistical significance was considered present when p < 0.05.
Results

Nine men and ten women, mean age 45 years (SD 7.9) were enrolled in the study. The mean probing depth of sampled sites was 5.85 mm (SD 0.59) with 0.78 mm recession (SD 0.67) and nearly half of the 12 sites had plaque (mean 5.8, SD 3.8) and bled after probing (mean 5.7, SD 3.0). Oral hygiene and marginal gingival bleeding were assessed on the Ramfjord teeth and nearly half the tooth surfaces had plaque (mean 5.5, SD 0.96), but few had gingival bleeding (mean 1.84, SD 0.38).

The mean volume of GCF collected from the 12 deepest sites per patient was 5.42 μl (SD 0.39). The mean WMS volume collected was 3.9 ml (SD 0.20) and the mean volume of PS was 3.2 ml (SD 0.13). Further details describing the flow rates of the GCF and saliva samples can be found in Cox et al.\(^\text{20}\). The molar concentrations of active elastase in GCF and WMS are illustrated in Fig 1. GCF and WMS concentrations of cathepsin B, SLPI and $\alpha_1$PI are illustrated in Figs 2a, 2b and 2c respectively.

The levels of elastase and cathepsin B in PS were negligible (medians 0.008 nmol/l and 0.004 nmol/l respectively). $\alpha_1$PI levels in PS were also extremely low (median 1.74 nmol/l), but the levels of SLPI (median 112.8 nmol/l) were similar to those in GCF and WMS.

Comparisons of the concentrations of enzymes and inhibitors in the three oral fluids using univariate statistics have previously been reported by Cox et al.\(^\text{20}\). Regression analysis was used in this study to explore which clinical and biochemical variables could account statistically for the molar concentration of active elastase in GCF and WMS (Fig 1).

In the analysis of elastase activity, the patient’s age, gender, smoking history, levels of supragingival plaque and discrete bleeding after probing were removed from the developing regression model as they did not account significantly for the level of GCF elastase. The statistically significant regression model exploring the clinical interactions ($r^2 = 0.55$, $p < 0.007$) indicated a direct relationship between GCF elastase activity and the mean probing depth of the sampled sites, profuse bleeding at the sites and a variation dependent on the individual patient (Table 1a). Once GCF levels of cathepsin B, SLPI and $\alpha_1$PI had been added to the clinical model, the probing depth of the sites and individual variations were no longer signific-
significant explanatory variables and were removed from the final model (Table 1b). The apparent relationship with the profuse bleeding score was considerably reduced and did not reach statistical significance \((p = 0.056)\) in the new model \((r^2 = 0.79, p < 0.0002)\). The level of GCF cathepsin B was directly related to elastase and the GCF levels of the two protease inhibitors were inversely related to the level of elastase.

A similar approach was used to develop a regression model to explain the concentration of elastase in WMS (Fig 1). However, as the general levels of oral hygiene and marginal gingival bleeding could have contributed to the supragingival environment, these were also included in the clinical model development for WMS elastase. In the model relating clinical variables to WMS elastase \((r^2 = 0.57, p < 0.02)\), there was no effect of the individual patient, gender or past smoking habits. Pocket depth of the 12 deepest sites, the number of the deepest sites with bleeding after probing and gingival inflammation at the Ramfjord teeth also had no significant effect. However, age had a significant influence, with more elastase in younger patients. Profuse bleeding after probing and plaque at the deepest sites were significantly related to WMS elastase and gingival bleeding at the Ramfjord teeth almost reached significance (Table 1a).

## Table 1a

Regression analysis model with statistically significant clinical variables explaining the molar concentration of active elastase in gingival crevicular fluid (GCF) samples from 19 patients.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>SE</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual patient</td>
<td>51.2</td>
<td>22.9</td>
<td>100.0, 2.4</td>
</tr>
<tr>
<td>Mean probing depth</td>
<td>533.4</td>
<td>207.0</td>
<td>92.1, 974.6</td>
</tr>
<tr>
<td>No. of sample sites with profuse BOP</td>
<td>129.5</td>
<td>39.9</td>
<td>44.4, 214.5</td>
</tr>
</tbody>
</table>

Model \( r^2 = 0.55, \text{ adjusted } r^2 = 0.46 \)

BOP, bleeding on probing
SE, standard error
95% CI, 95% confidence interval

## Table 1b

Regression analysis model with gingival crevicular fluid (GCF) constituents and clinical variables explaining the molar concentration of active elastase in GCF samples from 19 patients.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>SE</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin B</td>
<td>13.0</td>
<td>3.2</td>
<td>6.15, 19.88</td>
</tr>
<tr>
<td>SLPI</td>
<td>-2.2</td>
<td>0.7</td>
<td>-3.75, -0.72</td>
</tr>
<tr>
<td>( \alpha )1PI</td>
<td>-0.11</td>
<td>0.05</td>
<td>-0.22, -0.003</td>
</tr>
<tr>
<td>No. of sample sites with profuse BOP</td>
<td>59.1</td>
<td>28.3</td>
<td>-1.66, 119.84</td>
</tr>
</tbody>
</table>

Model \( r^2 = 0.79, \text{ adjusted } r^2 = 0.73 \) \(< 0.0002\)

\( \alpha \)1PI, \( \alpha \)-proteinase inhibitor
BOP, bleeding on probing
SE, standard error
SLPI, secretory leukocyte protease inhibitor
95% CI, 95% confidence interval
When the WMS cathepsin B and inhibitors were included in the model, PS levels of the same variables had no independent significant effect. WMS elastase was directly related to the amount of GCF elastase and to WMS α1PI, but was inversely related to WMS SLPI levels (Table 2b). However, the number of sites with profuse bleeding was not significantly related (p = 0.081).

**Discussion**

The present study shows similar relationships between the periodontal condition and elastase activity to those described previously in GCF2-5 and in WMS6-8. Cathepsin B2,21-23, α1PI11-14 and SLPI18 were also detected in GCF, as reported previously. However, no previous studies have attempted to examine elastase together with α1PI, cathepsin B and SLPI in GCF and WMS from the same patients. In addition, no other studies have attempted to examine how these enzymes and inhibitors might interact with the periodontal condition to explain elastase activity in the two fluids important for maintaining homeostasis of the sub- and supra-gingival oral environments, GCF and WMS respectively.

Elastase is unusual amongst the serine proteinases as it is stored in an active form within the azurophilic granules of neutrophils, which are the likely origin of much of the elastase in GCF. These granules may be discharged into the periodontal tissues or into the gingival crevice. Inflammation is like-
ly to be responsible for both bleeding after probing and the transit of neutrophils into the GCF and probably accounts for the association between bleeding and elastase activity. It is therefore no surprise that elastase levels in GCF rose with increasing levels of clinical inflammation in the present study and others. Profuse bleeding after probing was one of the clinical variables that accounted particularly significantly for the levels of elastase in GCF in the clinical model (Table 1a) and tended to have an effect even when cathepsin B and the two inhibitors had been included in the subsequent model (Table 1b). The relationship between GCF elastase and pocket depths described in some studies may in part be due to the larger volume of GCF collected from deeper pockets, and the correlation between pocket depth and elastase is stronger when the total elastase activity is examined than when elastase is expressed as a concentration. In the present study, even when the elastase activity had been standardised as molar concentrations, pocket depth still appeared to relate to the GCF elastase activity in the clinical model (Table 1a). However, once GCF cathepsin B, α1PI and SLPI concentrations had been added to the model, the apparent relationship of pocket depth and elastase activity was no longer statistically significant. A more highly significant and robust model (Table 1b) indicated that GCF elastase activity increased with increasing cathepsin B and decreased with increasing concentrations of both protease inhibitors. This suggests that the inflammatory process is most likely to determine the number of migrating neutrophils and thereby the amount of elastase activity which is subsequently modified by the other biochemical components.

The statistically significant inverse relationships between the two inhibitors and elastase activity suggest they may both reduce elastase activity in the subgingival environment and therefore have potential clinical significance, preventing further tissue damage by elastase. The increase of cathepsin B in parallel with elastase is not surprising, as both enzymes reflect the inflammatory process within the tissues. In addition, cathepsin B has the ability to degrade SLPI and evidently cleaves the inhibitor in WMS. Therefore it seems likely that degradation by cathepsin B reduced the ability of SLPI to regulate elastase activity in GCF. It is also probable that the low levels of cathepsin B in WMS were derived from the GCF since the levels of cathepsin B in PS were negligible.

The effects of saliva on the periodontal condition would operate predominantly in the supragingival environment, where it can help limit gingivitis. WMS contains factors from parotid, submandibular, sublingual and minor salivary glands and from any inflammatory lesions of the oral mucosa. The patients in the present study had no mucosal inflammatory lesions other than periodontitis and no salivary gland pathology which might have modified the constituents of saliva.

GCF from deep pockets and from the marginal gingivae could contribute to WMS elastase, although it has been argued that the relatively low volume of GCF would be too diluted by saliva to raise levels greatly. However, in the clinical model, the inflammatory status of deep pockets was significantly related to elastase activity in WMS (Table 2a), although the low levels of marginal gingival inflammation in this study had no effect. There was also apparently a significant effect of plaque in the clinical model (Table 2a) and it is possible that elastase-like enzymes from bacteria within supragingival plaque could contribute to elastase activity in WMS. However, plaque was no longer a significant contributor to the regression model once the GCF, WMS and PS enzymes and inhibitors had been included in the subsequent model (Table 2b).

None of the PS enzymes or inhibitors or the GCF inhibitors had a significant influence on WMS elastase when the same variables in WMS were used in the model, although it should be remembered that the parotid gland and periodontal tissues were still the origin of some of the WMS enzymes or inhibitors. However, the regression model suggests that GCF elastase activity made a significant contribution to the WMS elastase level (Table 2b). As might be expected, when WMS SLPI concentrations increased, WMS elastase activity decreased. However, as WMS elastase increased, WMS α1PI also increased. This might be because our assay with the peptide substrate detects enzyme bound to α-2-macroglobulin, which is resistant to α1PI. Greater output of both elastase and α1PI from the gingival crevice as inflammation increased could then have led to the direct statistical relationship in WMS. In the patients
examined in the present study, parotid saliva inhibited the activity of purified elastase and elastase in GCF from the same individuals in vitro. The inhibitory activity of PS was probably derived from SLPI since PS levels of α1PI were minimal and there was a significant relationship between the concentration of SLPI in PS and the ability of the respective PS to inhibit elastase activity. It is interesting that the statistical model of WMS elastase (Table 2b) suggests none of the PS enzymes or inhibitors had an independent influence on WMS elastase activity once they had interacted with the materials from all sources that contribute to WMS and the WMS levels of the materials after interactions were included in the model.

Throughout the regression analyses, the biochemical variables were more strongly related to elastase activity than the measured clinical variables. This may be because the biochemical variables are more clearly defined and more readily quantified than the clinical assessments of the disease. Ideally, comprehensive sets of clinical variables and comprehensive sets of biochemical variables should both on their own account for the elastase levels. However, in practice it is not possible to measure every single potential influence, and so quantified biochemical factors might be able to replace currently unmeasurable aspects of the clinical condition, in particular episodes of active destruction. The patients selected for this study had severe periodontitis to allow collection of sufficient GCF to permit multiple biochemical analyses. This approach would limit any future longitudinal study evaluating the use of biochemical mediators as predictors of disease progression for patients with existing periodontitis. The multiple analysis approach would also be difficult to apply to a longitudinal study of the initiation of destructive disease.

In summary, statistical regression analyses support the concept that GCF SLPI and α1PI have significant inhibitory effects on GCF elastase activity, and this may be important to the regulation of elastase activity and tissue destruction in disease. WMS elastase activity appears to be derived mainly from GCF, and elastase activity in saliva may be inhibited by the concentration of SLPI in WMS. The development of clinical regression models indicated that our data were comparable with previously described relationships between elastase levels and the periodontal condition. However, the elastase levels were related more strongly to biochemical than clinical variables in our statistical models. The data serve to illustrate how important it is to include the widest possible range of interacting clinical and biochemical variables when trying to evaluate GCF or WMS components as potential diagnostic indicators in periodontitis.

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**References**


