Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 661–673

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2012.01481.x

Human salivary cystatin SA exhibits antimicrobial effect against *Aggregatibacter actinomycetemcomitans*

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Ganeshnarayan K, Velliyagounder K, Furgang D, Fine DH. Human salivary cystatin SA exhibits antimicrobial effect against Aggregatibacter actinomycetemcomitans. *J Periodont Res 2012; 47: 661–673. © 2012 John Wiley & Sons A/S*

Background and Objective: Healthy subjects who do not have *Aggregatibacter actinomycetemcomitans* in their oral cavity may possess factors in saliva that might demonstrate antibacterial activity against the bacterium. The aim of this study was to identify and purify proteins from saliva of healthy subjects that might demonstrate antibacterial activity against *A. actinomycetemcomitans* and test the same against the bacteria.

Material and Methods: Saliva from 10 healthy volunteers was tested individually for its anti-*A. actinomycetemcomitans* activity. Among the 10 subjects, eight demonstrated anti-*A. actinomycetemcomitans* activity. Saliva was collected from one healthy volunteer who demonstrated the highest antimicrobial activity against *A. actinomycetemcomitans*. After clarifying the saliva, it was subjected to an affinity chromatography column with *A. actinomycetemcomitans*. The proteins bound to *A. actinomycetemcomitans* were eluted from the column and identified using mass spectrometry (MALDI-TOF/TOF MS). Among other proteins that bound to *A. actinomycetemcomitans*, which included lactoferrin, immunoglobulin A and kallikrein, cystatin SA was observed in significantly higher concentrations, and this was purified from the eluate. The purified cystatin SA was tested at different concentrations for its ability to kill *A. actinomycetemcomitans* in a 2 h cell killing assay. The bacteria were also treated with a proteinase inhibitor, leupeptin, to clarify whether the antimicrobial effect of cystatin SA was related to its protease inhibitory function. Cystatin SA was also tested for its ability to prevent binding of *A. actinomycetemcomitans* to buccal epithelial cells (BECs) in an *A. actinomycetemcomitans*–BEC binding assay.

Results: Cystatin SA (0.1 mg/mL) demonstrated a statistically significant antimicrobial activity against *A. actinomycetemcomitans*. The effect of cystatin SA decreased with lower concentrations, with 0.01 mg/mL showing no effect. The addition of monoclonal cystatin SA antibodies to the purified sample completely negated the antimicrobial effect. Treatment of *A. actinomycetemcomitans* with leupeptin resulted in no antimicrobial effect, suggesting that the antimicrobial activity of cystatin SA is independent of its protease inhibitory function. *A. actinomycetemcomitans* pretreated with cystatin SA showed reduced binding to BECs, suggesting a potential role for cystatin SA in decreasing the colonization of *A. actinomycetemcomitans*.

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Key words: *Aggregatibacter actinomycetemcomitans*; antibacterial; cystatin SA; saliva

Accepted for publication March 1, 2012

Conclusion: The present study shows that cystatin SA demonstrates antimicrobial activity against the periodontopathogen *A. actinomycetemcomitans*, and future studies determining the mechanism of action are necessary. The study also shows the ability of cystatin SA to reduce significantly the binding of *A. actinomycetemcomitans* to BECs.

Human saliva possesses an enormous array of proteins and factors that have a gamut of functions. Some of these functions are digestion, lubrication and buffering, and these are essential in maintaining the tooth and mucosal integrity (1). Research in the past few decades has focused on the protective role of saliva in preventing oral diseases such as dental caries and periodontal diseases. Saliva contains a lot of antimicrobial factors, many of which remain unexplored. The oral cavity contains over 600 different bacterial species (2), yet it remains relatively disease free. Saliva, apart from washing away the majority of micro-organisms and preventing colonization of bacteria, also contains many antibacterial proteins that are highly effective in killing micro-organisms. Some of the well-documented proteins in saliva with significant antimicrobial properties are lactoferrin (3–6), histatin (7–9), lysozymes (10,11) and secreted immunoglobulins. Another group of proteins found abundantly in saliva are the salivary cystatins.

Among the various diseases involving the oral cavity, localized aggressive periodontitis is a particularly severe form of gum disease characterized by rapid destruction of the supporting tissues of the periodontium, resulting in the early loss of teeth, and is most commonly found in otherwise healthy young adults. It is associated with minimal local factors such as plaque and calculus, and thus the disease is not commensurate with the amount of local factors present. *Aggregatibacter actinomycetemcomitans*, a gram-negative coccobacillus, has been implicated as the primary etiological agent of this form of disease (12). *A. actinomycetemcomitans* forms extremely tenacious biofilms *in vitro* (13) and shows autoaggregation (14), which plays a crucial role in colonization of the tooth surfaces. Localized aggressive periodontitis accounts for over 40,000 new cases annually in the US alone. Although it was traditionally thought to affect African Americans, recent surveys have shown prevalence of disease in Hispanics, Caucasians, Moroccans and Asians (15–17).

Previously, we have reported that the saliva of *A. actinomycetemcomitans*-negative subjects kills *A. actinomycetemcomitans in vitro* (18). We decided to investigate proteins isolated from saliva of healthy individuals that killed *A. actinomycetemcomitans* using an affinity column chromatography. In order to identify proteins in saliva that could potentially demonstrate antimicrobial activity against *A. actinomycetemcomitans*, we decided to test our hypothesis that for any protein to kill *A. actinomycetemcomitans* it must first bind to it. The salivary proteins that bound to *A. actinomycetemcomitans* were run on a gel, and the protein bands were excised and sent for protein identification using mass spectrometry (MALDI-TOF/TOF MS) at the Center for Advanced Proteomics (CAPR, New Jersey Medical School, UMDNJ). Among other proteins that bound to *A. actinomycetemcomitans* in the affinity column, a 14 kDa band was observed which was denser than other bands, and this band was identified as Cystatin SA. The other proteins in the column that bound to *A. actinomycetemcomitans* were identified also identified in a similar manner. These proteins included lactoferrin, immunoglobulin, kallikrein and galectin-3 binding protein. While we have previously demonstrated that lactoferrin exhibits significant binding to *A. actinomycetemcomitans*, lactoferrin did not exhibit significant antimicrobial effect against the clinical rough strains. Clinical strains of *A. actinomycetemcomitans* isolated from patients demonstrate a rough looking phenotype with an internal star structure, while some strains after multiple passages in the lab develop a smooth looking phenotype which may not be as virulent.

Cysteine proteases comprise a group of proteolytic enzymes that cleave peptide bonds by use of a reactive cysteine residue at the catalytic site. Examples include endoproteinases such as papain and lysosomal cathepsins such as cathepsin B, H and L. Cystatins are a group of cysteine protease inhibitors with significant cysteine protease inhibitory function (19). The salivary cystatins, which are the family 2 cystatins, include cystatin S, cystatin SN and cystatin SA. Previously, cystatins and sequences derived from cystatin C have been shown to possess antimicrobial properties (20–22). Cystatin C-based peptides have shown antibacterial properties against *Porphyromonas gingivalis* (23), one of the key pathogens in chronic periodontitis.

Several studies have shown elevated levels of cystatin SA in patients with periodontal disease (24). A few other studies, however, showed either a decrease in concentrations or unaltered levels in periodontal disease (25,26). To date, there have been no previous reports of cystatin SA demonstrating antibacterial effects against *A. actinomycetemcomitans*. Based on these preliminary data, our aim was to investigate whether cystatin SA demonstrates antibacterial effect against the periodontopathogen, *A. actinomycetemcomitans*. We also determined to analyse the effect of cystatin SA on the binding of *A. actinomycetemcomitans* to buccal epithelial cells (BECs) to understand its role in the colonization of *A. actinomycetemcomitans*.

Material and methods

The study involved the collection and purification of cystatin SA from saliva of a healthy volunteer who did not

have *A. actinomycetemcomitans* in the oral cavity as evidenced by bacterial culture from plaque and buccal cell samples. One healthy volunteer was selected and asked to expectorate into a wide-mouthed 50 mL tube. The study also involved the collection of BECs by scraping the inner cheek surfaces with a sterile wooden tongue blade. The study was reviewed and approved by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey.

Bacterial strains and growth conditions

The bacterial strains used in the study are listed in Table 1.

Growth conditions for A. actinomycetemcomitans Seven different serotypes of *A. actinomycetemcomitans* have been identified (27–29). Five different serotypes of *A. actinomycetemcomitans*, including the clinical isolates DF2200, NJ1000, NJ9500, CU1000 and IDH781 belonging to serotypes a, b, e, f and d respectively were used in this study. While the majority of bacteria have a rough-looking phenotype, some have a smooth phenotype, which may have occurred as a result of several passages in the laboratory. IDH781 is a rough strain of *A. actinomycetemcomitans*, which belongs to serotype d and has been isolated clinically from a patient. IDH781 (serotype d) rough strains were cultured in 100 mm diameter polystyrene Petri dishes containing 20 mL of trypticase soy broth containing yeast extract (6 mg/mL), glucose (8 mg/mL), bacitracin and vancomycin. The culture plates were inoculated at 37°C for 48 h in air supplemented with 10% CO_2.

Growth conditions for Fusobacterium nucleatum American type culture collection (ATCC) 10953 was grown in crystal violet with erythromycin (CVE) agar agar containing trypticase peptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L), glucose (2 g/L), L-tryptophan (0.2 g/L), bacto agar (15 gm/L) and 1 mL of Crystal Violet (0.005 g/mL) supplemented with 4 mL of erythromycin (0.001 g/mL) and 50 mL of defibrinated sheep blood, and the pH was adjusted to 7.2.

Growth conditions for P. gingivalis A clinical isolate of *P. gingivalis* from a patient suffering from chronic periodontitis was isolated and grown in media containing trypticase peptone (15 g/L), yeast extract (5 g/L), NaCl (2.5 g/L), dextrose (2.5 g/L) and cysteine–HCl (0.65 g/L), supplemented with hemin (1 g/L) and menadione (100 mg/L). The cells were grown in an anaerobic chamber at 37°C for 3 d (MiniMACS Anaerobic workstation, Microbiology International, Frederick, MD, USA). *P. gingivalis* was confirmed prior to making a pure culture by PCR analysis using specific primers (16S rRNA).

Proteinase K treatment of anti-*A. actinomycetemcomitans* saliva

To understand whether the anti-*A. actinomycetemcomitans* factor in the saliva was a protein, we decided to investigate the effects of proteinase K treatment on the active saliva. To determine whether the anti-*A. actinomycetemcomitans* activity of saliva is retained after treatment with proteinase K, 20 μL of proteinase K was added to the clarified saliva of the healthy subject and incubated at 55°C for 2 h. After the 2 h incubation, the saliva was incubated with *A. actinomycetemcomitans* for 2 h, and the amount of *A. actinomycetemcomitans* recovered was estimated.

Table 1. Bacterial strains used in the study

Strain	Relevant characteristics	Source or reference
Aggregatibacter actinomycetemcomitans		
IDH781	Serotype d	(15)
DF2200	Serotype a	(13)
NJ1000	Serotype b	(13)
CU1000	Serotype f	(13)
NJ9500	Serotype e	(52)
JP2	Serotype b; smooth	(52)
Porphyromonas gingivalis	Clinical isolate	Present study
Fusobacterium nucleatum	ATCC 10953	(51)

Purification of cystatin SA

Based on the results of the identification of the anti-*A. actinomycetemcomitans* factor, the next set of experiments was focused on purification and characterization of cystatin SA.

Collection of whole unstimulated saliva— One volunteer was selected for purification of cystatin SA based on the results of the anti-*A. actinomycetemcomitans* activity. After obtaining the necessary consent, the subject was asked to expectorate into a 50 mL wide-mouthed tube, and this saliva was used for further purification. Fifty milliliters of whole unstimulated saliva was collected for the purification of cystatin SA.

Affinity column chromatography for purification of cystatin SA from clarified saliva— The saliva was centrifuged at 10,000*g* for 30 min in a wide-mouthed 50 mL collection tube to remove all organic debris, food and mucin. The clarified saliva was stored at −20°C until further use. Cystatin SA from the collected saliva was purified by using an affinity column chromatography of diethylaminoethyl-cellulose packed with *A. actinomycetemcomitans* as a substrate.

Preparation of A. actinomycetemcomitans and diethylaminoethyl column chromatography— Fifteen grams of diethylaminoethyl-cellulose was suspended in Tris buffered saline buffer with 5 mL of packed *A. actinomycetemcomitans* cells to yield a uniform suspension. *A. actinomycetemcomitans* (IDH781) was grown according to conditions mentioned under '*Bacterial strains and growth conditions*'. The bacteria were first fixed in 1% formalin and mixed with the diethylaminoethyl-cellulose in a stirrer for 10 min after ensuring that bacteria were dispersed in a single-cell suspension without any visible clumps. The *A. actinomycetemcomitans* mixed

with the diethylaminoethyl-cellulose was packed into a column with a 25 mL bed volume. After incubating with 2% bovine serum albumin to prevent nonspecific binding, the clarified saliva was loaded into the column and incubated for 2 h, to allow sufficient time for proteins in the saliva to bind to *A. actinomycetemcomitans* in the column. The column was then washed with Tris-buffered saline to wash away all the unbound proteins. Following this, the proteins in saliva bound to *A. actinomycetemcomitans* in the column were eluted using a 0.1 M Tris–glycine–HCl elution buffer at a pH of 2.3. The resulting fractions were collected in an automated fraction collector (Pharmacia Biotech, Piscataway, NJ, USA), and their absorbance was measured at an optical density of 280 nm in an ultraviolet spectrophotometer (UVMini-1240; Shimadzu Corporation, Kyoto, Japan). The protocol for the preparation of *A. actinomycetemcomitans* for the affinity column is summarized below:

Grow *Aa* at 37 C over night
↓
Cells washed and centrifuged at x5000 rpm for 5 mins
↓
Add formalin to a final conc. of 1%
↓
Incubate at R/T for 2 hours
↓
Wash cells with formalized saline 0.5% and centrifuged

The protocol for the affinity chromatography is summarized below:

DEAE cellulose Column Preparation

15g of DEAE cellulose suspended in STB with 5 ml of packed cells to yield a uniform suspension
↓
Column with a bed volume were poured and equilibrated with STB
Cells incubated with 2% BSA to prevent non specific binding
↓
Column Procedures
↓
Load clarified saliva onto a column and wash with STB buffer
↓
Elute with Glycine-HCL pH 2.3

Size exclusion chromatography followed by SDS–PAGE analysis and western blotting— After eluting the proteins with 0.1 M Glycine-HCl, the sample was subjected to a second size exclusion chromatography using 15 kDa MWCO filter (MilliporeInc, Billerica, MA, USA) to select for the 14 kDa cystatin SA. Proteins larger than 15 kDa will be retained in the column will the smaller molecular weight will flow through into the collection tube.

The sample was then dialyzed against water using Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, USA 10,000 MWCO) to remove glycine from the eluate. After a 4-h dialysis, the sample was stored for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis.

For the SDS–PAGE analysis, the sample was mixed with an equal volume of SDS loading buffer and boiled for 10 min. After the samples were prepared, 25 μL (in triplicate) was loaded onto a 10-well 4–20% polyacrylamide gel (Pierce Inc., Appleton, WI, USA) and run with a constant voltage of 110 V until the dye front reached the bottom of the gel. After the gel was run, it was stained using Coomassie® Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA) and a second duplicate sample using Sypro Ruby stain. The third sample was transferred onto a polyvinylidene fluoride (PVDF) membrane for western blot analysis.

For the western blot analysis, the proteins on the gel were transferred to a PVDF membrane using a Bio-Rad Trans-blot® Semi Dry transfer machine with a constant voltage of 18 V for 45 min. This procedure allowed the proteins on the gel to be transferred to the PVDF membrane. After the gel was transferred, the PVDF membrane with the proteins was immersed in a blocking buffer of 2% bovine serum albumin for 2 h to prevent nonspecific binding. The bovine serum albumin was then washed away with phosphate-buffered saline (PBS; three times) and the membrane was then probed with an anti-cystatin SA monoclonal antibody (1:10,000 dilution; R&D Systems, Minneapolis, MN, USA) for 3 h. After the primary incubation for 3 h, the membrane was washed three times with PBS and incubated with a secondary anti-mouse IgG antibody (Bio-Rad) for 90 min. Following this, the membrane was washed again with PBS (three times) and probed with an alkaline phosphatase substrate conjugation kit to visualize for presence of cystatin SA.

To reconfirm the purity of the sample, the purified sample was sent for mass spectrometry analysis using the MALDO TOF/TOF MS (LTQ Orbitrap Velos; Center for Advance Proteomics Research, New Jersey Medical School, UMDNJ). This protein was then aliquoted to different concentrations of 0.1, 0.05 and 0.01 mg/mL and stored at −20°C until further use.

Cell killing assay

Cystatin SA was used at different concentrations (0.1, 0.05 and 0.01 mg/mL) to check for its antimicrobial effect against the afore-mentioned bacteria. The cell killing assay was performed in a 1.5 mL microcentrifuge tube containing 10^7–10^9 bacterial cells suspended in 1 mL of the three different concentrations of cystatin SA. The microcentrifuge tubes were gently agitated for 2 h and were washed with PBS three times. A 100 μL aliquot was removed, serially diluted and subsequently plated out for enumeration. The plates for *P. gingivalis*

and *F. nucleatum* were incubated in anerobic conditions for 3 d, while the plates for *A. actinomycetemcomitans* were incubated at 37°C in an atmosphere of air containing 10% CO_2 for 3 d, and the colonies calculated as colony forming units per milliliter (CFU/mL). All experiments were performed in duplicate on at least three different occasions.

Confocal microscopy of cystatin SA-treated A. actinomycetemcomitans *A. actinomycetemcomitans* IDH781 was grown on glass coverslips for 48 h according to conditions mentioned under '*Bacterial strains and growth conditions*'. After 48 h, the cells were washed in PBS and treated with either cystatin SA (0.1 mg/mL) or PBS for 2 h. After 2 h, the cells were washed and stained using the Live-Dead© bacterial assay (Invitrogen, Grand Island, NY, USA) and visualized using a confocal microscopy system (Inverted Axiovert 100 M SP; Zeiss LSM 50; Zeiss, Thornwood, NY, USA) using an inverted Zeiss microscope with a ×60 objective lens according to the established protocol (30). The kit utilized SYTO 9® (Invitrogen) and propidium iodide to differentiate between live and dead cells, which were visualized under a confocal microscope using a dual-emission filter at a constant magnification with laser settings of 488 nm for the argon laser and 543 nm for the He–Ne laser. If cystatin SA exhibits a bactericidal effect, it can be detected using the confocal microscope.

Antibody blocking assay Mouse monoclonal antibody against cystatin SA (R&D Systems) was added to cystatin SA in different concentrations, and this mixture was used in the cell killing assays to determine the effect of cystatin SA inactivation on the killing efficiency.

Leupeptin killing assay It can be argued that cystatin SA, by virtue of it being a cysteine protease inhibitor, prevents the protease function in these bacteria, thereby depriving them of nutrients required for growth, thus indicating that the antibacterial effect of cystatin SA is related to its protease inhibitory function. To clarify this possibility, the bacterial cells were treated with leupeptin hemisulfate, a synthetic derivative of the plant leupeptin. Leupeptin is a plant-derived cysteine protease inhibitor with no known antibacterial effect. *A. actinomycetemcomitans* IDH781 bacteria were treated with 0.1 mg/mL of leupeptin hemisulfate, similar to the cell killing assay described above, and examined for any antibacterial effect.

Collection of buccal epithelial cells The BECs were collected by scraping the inside of the cheeks of healthy volunteers with a sterile tongue depressor. The cells were suspended in 5 mL of PBS, washed once and resuspended in PBS. The cells were then counted using a hemocytometer to obtain dilutions of 10^3–10^4 cells/mL.

Buccal epithelial cell–A. actinomycetemcomitans binding assay Binding of *A. actinomycetemcomitans* to BECs was measured using the binding assay described previously (31). Briefly, 250 μL of bacterial cells were mixed with 250 μL of buccal cells in a 2 mL polypropylene microcentrifuge tube and incubated for 2 h. The ratio of the bacterial cells to the buccal cells is typically around 10^2–10^3 bacterial cells per buccal cell. After 2 h incubation, the bound cells were separated from the unbound cells using a 5% Ficoll gradient and centrifugation at the lowest possible speed for 5 min. The pelleted bacteria bound to the BECs were then serially diluted and plated out for enumeration.

Effect of cystatin SA on A. actinomycetemcomitans–BEC binding Different concentrations of cystatin SA (0.1, 0.05 and 0.01 mg/mL) were tested for their effect in interfering with the ability of *A. actinomycetemcomitans* to bind to BECs. *A. actinomycetemcomitans* bacteria were pretreated with different concentrations of cystatin SA for 2 h and the cells washed. The bacteria were then tested for their ability to bind to BECs using the *A. actinomycetemcomitans*–BEC assay (as above). An aliquot of the same was also plated out by serial dilution to determine the bacterial cell viability after exposure to the different concentrations of cystatin SA.

Statistical analyses

All experiments involved in the present study were performed in duplicate on at least three different occasions. All statistical analyses comparing the antibacterial effect of cystatin SA on *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* and the studies comparing the *A. actinomycetemcomitans*–BEC binding were analysed using Student's paired *t*-test for statistical significance with a confidence interval of 5% ($p < 0.05$).

Results

Proteinase K treatment of saliva results in loss of anti-*A. actinomycetemcomitans* activity

The study involved the identification of the anti-*A. actinomycetemcomitans* factor in the saliva of subjects who demonstrated anti-*A. actinomycetemcomitans* activity. The active saliva was treated with proteinase K, a broad-spectrum serine protease. Figure 1 shows the effect of proteinase K on the saliva of a healthy subject. The clarified active saliva was treated with proteinase K for 2 h at 55°C. The saliva was subsequently cooled to room temperature, incubated with *A. actinomycetemcomitans* for 2 h and compared with untreated saliva. The results show that while PBS did not show any anti-*A. actinomycetemcomitans* effect when compared with time 0, incubation with untreated saliva resulted in a 1 log reduction of *A. actinomycetemcomitans*. However, when the saliva was pretreated with proteinase K for 2 h at 55°C, the anti-*A. actinomycetemcomitans* activity was greatly reduced. These results indicate that the major anti-*A. actinomycetemcomitans* factor in the saliva is most likely to be a protein and is heat labile.

Purification of cystatin SA

Cystatin SA was purified from whole saliva of the healthy volunteer that

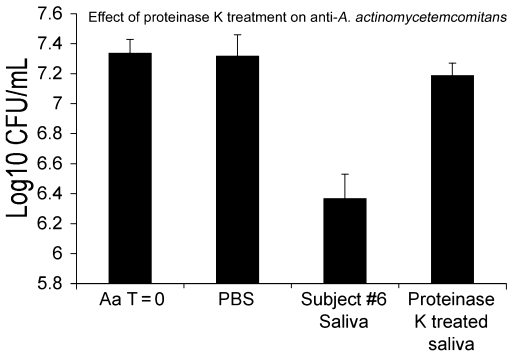

Fig. 1. The clarified saliva that demonstrated activity against *A. actinomycetemcomitans* was treated with proteinase K and compared with untreated saliva. While untreated saliva resulted in a statistically significant reduction in the amount of *A. actinomycetemcomitans* ($p < 0.05$), the proteinase - treated saliva did not show much antimicrobial effect. Values indicate the $\log_{10}$ of the mean number of colony-forming units (CFU) per milliliter for duplicate tubes. Error bars indicate range.

killed *A. actinomycetemcomitans*. The normal physiological concentration of cystatin SA in whole saliva is about 0.1–.2 mg/mL (24,25). The concentration of purified cystatin SA was estimated using the spectrophotometer using bovine serum albumin for the standard curve. The protein was diluted to 0.1, 0.5 and 0.01 mg/mL. The eluate was subjected to further purification procedures as described previously, and the purified protein was analysed by SDS–PAGE analysis and stained with Sypro Ruby stain, which is very sensitive and ideal for testing protein purity in a sample. Additionally, the purity of the sample was also confirmed by mass spectrometry analysis (LTQ Orbitrap Velos), which indicated that the sample was pure cystatin SA. The sample was also analysed by western blot using mouse monoclonal antibodies against cystatin SA (Fig. 2).

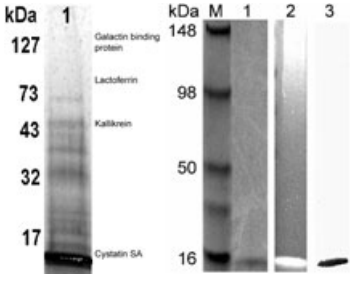

Fig. 2. The fraction from the *A. actinomycetemcomitans* column was analysed by SDS–PAGE. The SDS–PAGE analysis indicates several bands of different molecular weights. Cystatin SA (approximately 14 kDa) was observed at significantly higher concentrations when compared with other proteins in the sample. Subsequently, the protein was subjected to further purification procedures and subjected to analysis (right). Lanes are as follows: M, marker; 1, purified cystatin SA run on a gel and stained with Coomassie®; 2, gel stained with Sypro® Ruby; and 3, western blot analysis.

Cystatin SA demonstrates antimicrobial effect against *A. actinomycetemcomitans*

As this protein was purified from the saliva of a healthy subject that killed *A. actinomycetemcomitans*, we wanted to test the efficacy of this purified protein against *A. actinomycetemcomitans*.

Seven different serotypes of *A. actinomycetemcomitans* have been identified (27–29). Five different clinical strains of *A. actinomycetemcomitans* were tested, and cystatin SA demonstrated significant antimicrobial effect against all five different strains. The effect was dose dependent, with 0.1 mg/mL showing maximal effect (Fig. 3A). As there were no differences between the different clinical strains in terms of effects of cystatin SA, IDH781 (serotype d) was chosen as the reference strain for all further experiments. Treatment of rough strain IDH781 *A. actinomycetemcomitans* with 0.1 mg/mL cystatin SA resulted in > 1 log reduction, indicating a more than 90% kill of *A. actinomycetemcomitans* when compared with PBS treatment alone, which was found to be statistically significant ($p < 0.05$; Fig. 3B). This is a significant finding, because the rough strain is typically very difficult to kill and is the predominant form found in the oral cavity of patients with aggressive periodontitis. This killing was dose dependent, with the killing efficiency reduced when cystatin SA was diluted out. Treatment of *A. actinomycetemcomitans* with 0.01 mg/mL of cystatin SA did not show any antimicrobial effect against *A. actinomycetemcomitans*. Time point analysis of *A. actinomycetemcomitans* killing was done by treatment with 0.1 mg/mL cystatin SA for 30 min, 2 and 4 h. The results show that while there was some evidence of reduction at 30 min, the maximal effect was observed at 2 h and incubation for 4 h did not increase antibacterial activity (Fig. 4). To determine whether the antimicrobial effect was due to cystatin SA and to exclude the possibility of other proteins in the purified sample, we added cystatin SA monoclonal antibodies to cystatin SA in increasing concentrations and determined the effect of cystatin SA on cell killing. Figure 5 demonstrates the effect of addition of antibodies to treatment. Column 1 indicates PBS treatment; column 2 indicates treatment of cells with the fraction of saliva that actively kills *A. actinomycetemcomitans*, showing a 1.5 log reduction in viable bacteria; columns 3 and 4 represent addition of cystatin SA antibodies in different concentrations, showing that the addition of cystatin SA antibodies reduces the efficacy of killing; column 5 shows the effect of purified cystatin SA on *A. actinomycetemcomitans* killing, causing a 1 log reduction in cells, whereas the addition of different concentrations of cystatin SA antibodies (columns 7, 8 and 9) negate the killing efficiency of the protein. The addition of a control antibody (lactoferrin) did not significantly alter the efficacy of the purified cystatin SA.

For the confocal imaging analysis of cystatin SA-treated *A. actinomycetemcomitans*, after exposure to cystatin SA the cells were stained with a live-dead

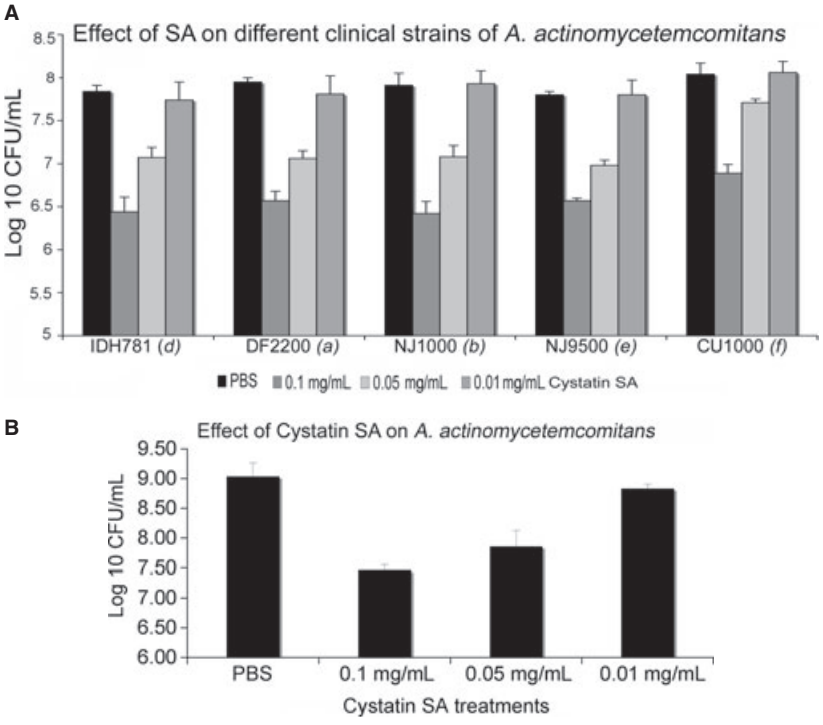

Fig. 3. (A) Effect of different concentrations of cystatin SA on different clinical strains of *A. actinomycetemcomitans.* (B) Treatment of *A. actinomycetemcomitans* IDH781 with different concentrations of cystatin SA in a 2 h cell killing assay. Cystatin SA at a concentration of 0.1 mg/mL showed a statistically significant (1.5 log; $p < 0.05$) reduction in the number of bacteria, while a concentration of 0.01 mg/mL did not have any effect.

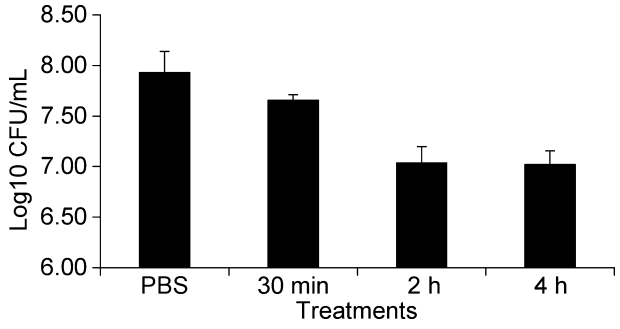

Fig. 4. Time point analysis of the antibacterial effect of 0.1 mg/mL of cystatin SA was carried out at 30 min, 2 and 4 h. The results show that while a marginal effect was observed at 30 min, incubation for 4 h did not show any increased effect when compared with the 2 h interval.

bacterial stain. The SYTO 9® stain (green) stains all cells, both live and dead, whereas the propidium iodide stain (red) stains all the cells with damaged cell membranes. The sample was excited at two different wavelengths using a laser at different wavelengths (488 nm for the argon laser and 543 nm for the He–Ne laser). A merge of both the images on the software revealed the relative proportion of live vs. dead cells in the sample. The confocal microscopy results show that the cells were killed when they were exposed to cystatin SA when compared with PBS-treated cells, as indicated by the merge figure (Fig. 6C). The analysis of the *z*-axis, however, showed that although the superficial layer of the biofilm was dead, the interior of the biofilm was still viable, suggesting that cystatin SA was not effective at penetrating the biofilm.

Cystatin SA demonstrates antimicrobial effect against *P. gingivalis*

A clinical isolate of *P. gingivalis* was treated with cystatin SA (0.1 mg/mL) and incubated for 2 h. The results of the cell killing assay show that treatment with cystatin SA results in a 3.5 log reduction in the number of cells, which was found to be statistically significant ($p < 0.001$). The antimicrobial effect of cystatin SA was observed even at the lower concentrations of 0.05 and 0.01 mg/mL (Fig. 7).

No effect of cystatin SA on *F. nucleatum*

The ATCC 10953 strain of *F. nucleatum* was grown as described previously and incubated with different concentrations of cystatin SA. The 2 h incubation with cystatin SA resulted in no antimicrobial effect against *F. nucleatum* when compared with PBS treatment even at the highest concentration (2 mg/mL; Fig. 8). The experiments were performed in duplicate on at least three different occasions.

Effect of leupeptin on *A. actinomycetemcomitans*

It can be argued that cystatin SA, by virtue of it being a cysteine protease inhibitor, prevents the protease function in these bacteria, thereby depriving them of nutrients required for growth, thus indicating that the antibacterial effect of cystatin SA is related to its protease inhibitory function. To clarify this possibility, the *A. actinomycetemcomitans* cells were treated with leupeptin hemisulfate (0.1 mg/mL), a synthetic derivative of the leupeptin. Leupeptin is another cysteine protease inhibitor with no known antibacterial effect. *A. actinomycetemcomitans* bacteria were treated with 0.1 mg/mL of leupeptin hemisulfate in a 2 h cell killing assay and examined for any antibacterial effect. The results of this experiment showed no difference when compared

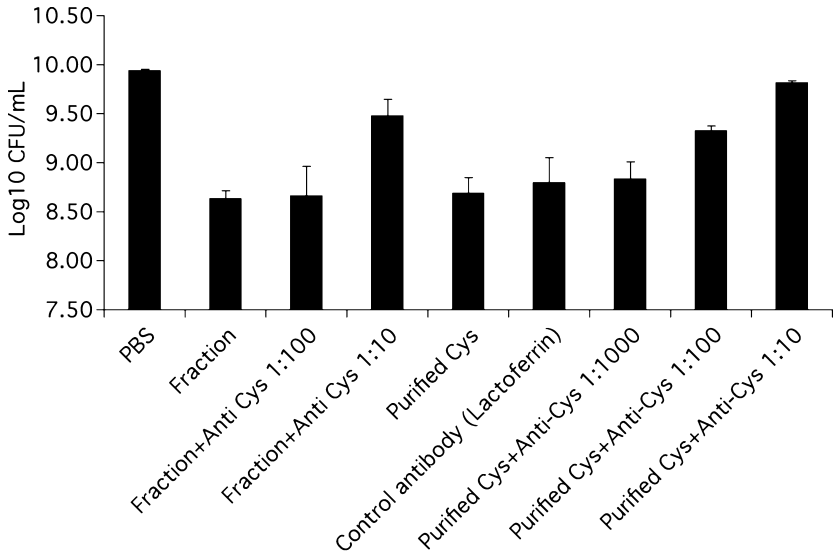

Fig. 5. The addition of cystatin SA antibodies to the either the fraction or purified cystatin SA reduces the killing efficiency. While the fraction contains other proteins, such as lactoferrin, kallikrein and galectin-3 binding protein, the purified cystatin SA did not contain any other protein. Values indicate the $\log_{10}$ of the mean number of CFU per milliliter for duplicate reactions. Error bars indicate range.

with PBS treatment alone, indicating that leupeptin, by itself, does not demonstrate antimicrobial effect (Fig. 9). It also suggests that the antimicrobial effect of cystatin SA is not related to its protease inhibitory function.

Pretreatment of *A. actinomycetemcomitans* with cystatin SA reduces the attachment of *A. actinomycetemcomitans* to BECs

To discover the functional significance of this interaction between cystatin SA and *A. actinomycetemcomitans*, we decided to test the effect of cystatin SA on the attachment of *A. actinomycetemcomitans* to BECs. The BECs were collected from healthy adult volunteers and were counted and diluted to 10^3–10^4 cells/mL. IDH781 *A. actinomycetemcomitans* cells were treated with different concentrations of cystatin SA and then incubated with the BECs for 2 h. After separation with a 5% Ficoll gradient, which allows only the bacterial bound cells to be pelleted, the bacteria bound to the BECs were plated out for enumeration. The assay was standardized using only bacteria without buccal cells to look for pelleted bacteria. Use of 10 mL of a 5% Ficoll gradient, which ensured that the bacteria did not pellet themselves by centrifugation alone, achieved this. Figure 10 shows the effect of different concentrations of cystatin SA treatments on the binding of *A. actinomycetemcomitans* to BECs. Treatment with 0.1 and 0.05 mg/mL virtually eliminated the binding of *A. actinomycetemcomitans* to BECs. However, as these concentrations showed lethal effect against *A. actinomycetemcomitans*, it is possible that the incubation with cystatin SA killed the cells, but 0.01 mg/mL of cystatin SA (a sublethal concentration) decreased the CFUs/BEC from 150 (PBS treatment) to 83.8 CFUs/BEC, resulting in a 45.5% reduction in *A. actinomycetemcomitans*–BEC binding when compared with PBS treatment (Fig. 10).

Discussion

Cysteine proteases are a group of proteases that cleave proteins with the help of a cysteine residue at their active site. They are usually involved in physiological functions such as cell turnover, clearance of debris from apoptotic cells, processing of secreted proteins and hormones and bone resorption (32). Some of the common cysteine proteases are the cathepsins, caspases and papain. These proteases are inhibited by a group of protease inhibitors called cystatins. Cystatins are comprised of three major families: the stefins, family 2 (the salivary cystatins) and family 3 (the kininogens; 1). Salivary cystatins have been the focus of research for the past few decades. Salivary cystatins are potent inhibitors of cysteine proteases, and their mechanism of action has been well characterized (32). The salivary cystatins are cystatin S, cystatin SA and cystatin SN. Though the proteins have a high sequence similarity, they are encoded by different genes (32).

In an effort to identify proteins from saliva that might demonstrate antibacterial effect against *A. actinomycetemcomitans*, we investigated the efficacy of saliva of healthy subjects showing anti-*A. actinomycetemcomitans* activity. Among 10 healthy subjects, eight demonstrated anti-*A. actinomycetemcomitans* activity to different extents. Two subjects demonstrated the highest anti-*A. actinomycetemcomitans* activity, and one of them was selected for identification of the anti-*A. actinomycetemcomitans* factor. A saliva sample collected from this individual was passed through an *A. actinomycetemcomitans* affinity column, and the fractions were tested for proteins that bound to *A. actinomycetemcomitans*. The anti-*A. actinomycetemcomitans* factor was further tested with proteinase K and assessed for its anti-*A. actinomycetemcomitans* effect. The loss of activity indicated that the active component in the fraction was indeed a protein. The major proteins that were identified in the sample were lactoferrin, galectin-3 binding protein, kallikrein and cystatin SA. Galectin-3 is a known proinflammatory mediator and helps to prime neutrophils for an oxidative burst (33), but there are no reports of a direct antimicrobial function. Kallikrein, though known to modulate immune responses, has no known antimicrobial function. Lactoferrin has previously been demonstrated to have significant antibacterial (6), antiviral (34) and antifungal properties (35,36). More

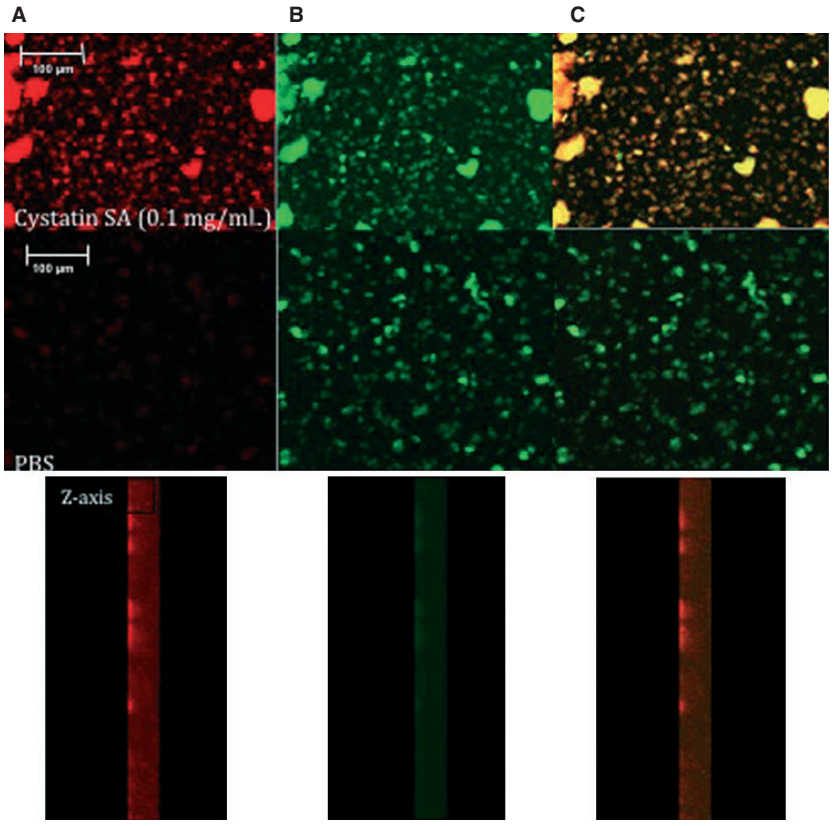

Fig. 6. Confocal microscopy of cystatin SA-treated compared with phosphate-buffered saline (PBS)-treated *A. actinomycetemcomitans* IDH781. (A) The He–Ne laser was focused at a wavelength of 543 nm, which is specific for propidium iodide. (B) The He–Ne laser was focused at a wavelength of 488 nm, which is specific for SYTO 9. (C) Merged image of (A) and (B), which is an indicator of the total live vs. dead cells. The PBS-treated cells appear greener than the cystatin SA-treated cells, which appear yellow (combination of red and green), indicating that there are a lot more dead cells in the sample when compared with the control. The *z*-axis panel shows that cystatin SA did not demonstrate complete penetration through the biofilm.

specifically, lactoferrin has been demonstrated to have significant anti-*Streptococcus mutans* activity (37). Lactoferrin binds avidly to *A. actinomycetemcomitans* and inhibits its binding to BECs when it exists in iron-saturated form but is not effective at killing rough clinical isolates of *A. actinomycetemcomitans* (38). There are many different antimicrobials that have been isolated from saliva. A few of these include histatins (9), lactoferrin (3) and immunoglobulins. Also of interest is the peroxidase-mediated killing by lactoperoxidase and myeloperoxidase systems in the saliva against *A. actinomycetemcomitans* (39) and many other organisms (40–42), including viruses (43).

Cystatins have been investigated for their antimicrobial properties since the late 1980s. Several reports have been published demonstrating antibacterial properties of cystatins. Naito *et al.* (44) reported that rat cystatin S specifically inhibited the growth of the anaerobic bacterium *P. gingivalis* due to a bactericidal effect. Bjorck *et al.* (20) synthesized a tripeptide derivative of the N-terminal sequence of chicken cystatin C and demonstrated significant antibacterial properties against different bacteria, including group A, B, C and G streptococci. Kasprzykowski *et al.* (21) synthesized peptidyl derivatives or cyclopeptides based on the amino terminal sequence of human cystatin C and demonstrated antibacterial effects against pathogenically important micro-organisms such as *Staphylococcus aureus* and *Streptococcus pyogenes* and concluded that the antibacterial effects of these compounds were not related to the protease inhibitory function. The avid binding of cystatin SA to *A. actinomycetemcomitans* and the evidence of antimicrobial function of cystatin SA led us to investigate its antimicrobial function against *A. actinomycetemcomitans* in order to understand its role in the disease process.

We tested the antibacterial effect of purified whole human cystatin SA against the gram-negative pathogen, *A. actinomycetemcomitans*, which is the primary etiological agent of localized aggressive periodontitis. There were no significant differences in the antibacterial effect of cystatin SA on different clinical strains of *A. actinomycetemcomitans*, which demonstrated significant bacterial killing (1.5 logs) against all clinical isolates that were tested. This indicates that the activity of cystatin SA is not serotype specific. Treatment of rough strain IDH781 with cystatin SA showed a potent antimicrobial effect. The effect was even greater with the smooth strain, JP2 (data not shown). However, the effect on rough strains of the bacteria is more clinically relevant and significant because this form of bacteria is routinely isolated from clinical cases of disease. The addition of cystatin SA antibodies to the sample resulted in a dose-dependent decrease in the antibacterial effect, confirming the potent antimicrobial function of this protein. This is the first report of the effect of purified cystatin SA against *A. actinomycetemcomitans*.

Pretreatment of cystatin SA with monoclonal antibodies against cystatin SA resulted in a dose-dependent decrease in the antibacterial effect, confirming the potent antimicrobial function of this protein. The addition of cystatin SA antibodies to the fractions in dilutions lower than 1:100 did not affect the antimicrobial activity of cystatin SA. It is possible that the use of a polyclonal antibody recognizing multiple epitopes could have had an effect at lower concentrations. Also, the addition of antibodies to cystatin SA to the fractions even at the highest concentration reduced the antibacterial activity but did not eliminate it completely. This

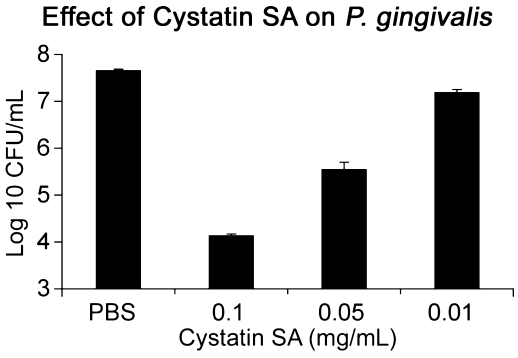

Fig. 7. Effect of cystatin SA on *Porphyromonas gingivalis*. Cystatin SA demonstrates significant antimicrobial activity against *P. gingivalis*, with a > 3.5 log reduction in the number of bacteria.

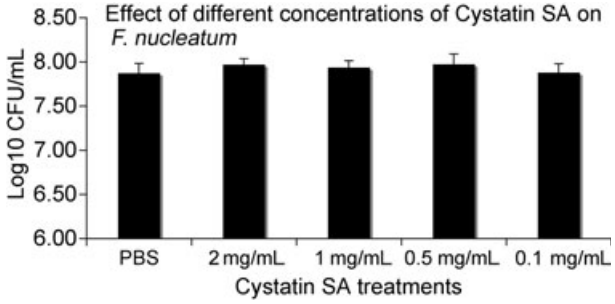

Fig. 8. Fusobacterium nucleatum was treated with higher concentrations of cystatin SA to look for any antimicrobial effect. However, even at 2 mg/mL there was no observed antimicrobial effect of cystatin SA.

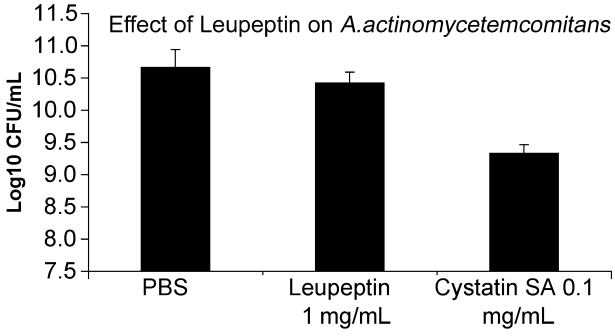

Fig. 9. Leupeptin killing assay. *A. actinomycetemcomitans* IDH781 cells treated with 0.1 mg/mL of leupeptin hemisulfate, a synthetic derivative of the plant-based leupeptin, showed an insignificant difference when compared with PBS treatment. However, treatment with cystatin SA resulted in a greater than a 1 log reduction in the number of cells, indicating that the antimicrobial effect of cystatin SA may not be related to its protease inhibitory function. Values on the vertical axis represent CFU per milliliter. Error bars indicate range.

could be explained by the fact that additional proteins in the active fraction of saliva could be responsible for the antibacterial effect. However, the addition of antibodies to purified cystatin SA completely negated its anti-*A. actinomycetemcomitans* activity. This is the first report of the effect of purified cystatin SA against *A. actinomycetemcomitans*. To determine how quickly cystatin SA acts against *A. actinomycetemcomitans*, a time course analysis was performed. Cystatin SA was not effective at killing *A. actinomycetemcomitans* within 30 min, whereas 2 h incubation resulted in the maximal effect, and 4 h incubation did not significantly increase its activity. Another periodontopathogen, implicated in the etiology of chronic forms of periodontal disease, is *P. gingivalis*. Cystatin SA showed significant antibacterial activity against *P. gingivalis*. The effect on *P. gingivalis* was much greater (3.5 log reduction) than that observed for *A. actinomycetemcomitans* (1.5 logs), indicating that *P. gingivalis* is more sensitive to killing by cystatin SA than *A. actinomycetemcomitans*. Other studies have shown a similar effect of cystatin against *P. gingivalis* (23). In the clinical scenario, *P. gingivalis* is found predominantly in the subgingival environment, while cystatin SA is a salivary protein found in the supragingival environment; hence, the clinical relevance of this interaction is not clear at this point. It is possible that *P. gingivalis* does exist in a microenvironment in the supragingival plaque, from where it eventually migrates to the subgingival plaque. Additionally, *P. gingivalis* produces a lot of proteases, and cystatin SA might interact with the proteases on its cell surface and eventually kill it.

Interestingly, treatment of another periodontopathic bacterium, *F. nucleatum*, resulted in no antibacterial effect when exposed to physiologically relevant concentrations of cystatin SA. It is possible that cystatin SA shows specificity to certain bacteria or it is possible that the minimal inhibitory concentration for *F. nucleatum* is greater than for the other microorganisms. To clarify the latter possibility, *F. nucleatum* was treated with different concentrations of cystatin SA (2–0.01 mg/mL), but treatment with even the highest concentration had no antibacterial effect. It can therefore be concluded that *F. nucleatum* does not show sensitivity to killing by cystatin SA.

Studies of cystatin SA activity against *P. gingivalis* have shown that the antibacterial effect observed was not related to its protease inhibitory function (23). We decided to

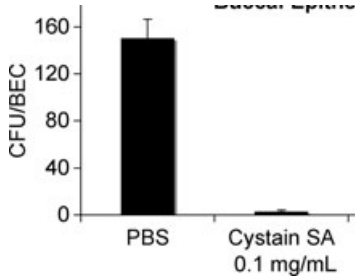

Fig. 10. Treatment of *A. actinomycetemcomitans* with cystatin SA reduced the ability of *A. actinomycetemcomitans* to bind to buccal epithelial cells (BECs). While PBS treatment resulted in a mean of 145 CFU per buccal cell, pretreatment of BECs with a low concentration of cystatin SA (0.1 mg/mL) resulted in a statistically significant reduction ($p < 0.05$; 45% decrease) in the binding of *A. actinomycetemcomitans* (85 CFU) to BECs.

investigate if this was true for *A. actinomycetemcomitans* as well. Leupeptin hemisulfate is a synthetic derivative of plant leupeptin, which is also a cysteine protease inhibitor. If the antibacterial effect of cystatin SA is to deprive the bacteria nutritionally, then treatment with leupeptin would have an effect similar to that observed with cystatin SA. The results showed that leupeptin did not demonstrate any antibacterial activity against *A. actinomycetemcomitans*, whereas cystatin SA did. This assay suggests that the effect of cystatin SA against *A. actinomycetemcomitans* is not related to its protease inhibitory function. The results from the confocal microscopy also confirmed that the cells were indeed dead due to disruption of their cell membranes, as evidenced by the live-dead bacterial staining. However, a 32-slice analysis of the *z*-axis in the confocal microscopy revealed that the cells in the biofilm continued to remain viable after the 2 h incubation. Although the surface of the biofilm was dead, cystatin SA was possibly unable to penetrate the biofilm. This is expected, because bacterial biofilms are typically hard to penetrate and show resistance to several antibacterial substances, including antibiotics (45). The antibacterial mechanism of action of cystatin SA remains unclear at this point. Cystatin SN, a closely related protein showing 60% sequence identity with cystatin SA was also tested against all the different bacteria, but was ineffective in killing any microorganism at the same concentration (data not shown). Cystatin SA is slightly more acidic than cystatin SN, and the presence of phosphorylation at four different serine residues (46) could be involved in its antibacterial effect.

Colonization of bacteria is the first step in disease production. Although aggressive periodontitis occurs in the subgingival environment, it is important for the bacteria to colonize prior to migration to the subgingival environment to cause disease. *A. actinomycetemcomitans* has been isolated from edentulous mouths and even predentate children, suggesting that the initial site for colonization of *A. actinomycetemcomitans* could be tissues besides the gingival environment (47,48). In the oral cavity, the buccal epithelial cells seem to be the reservoir for *A. actinomycetemcomitans*, which can bind and invade BECs and subsequently migrate to the subgingival environment, where it causes disease (49). To understand the role of cystatin SA, we decided to test the hypothesis that pretreatment of *A. actinomycetemcomitans* with cystatin SA might interfere in the ability of the bacterium to bind to BECs, thereby reducing colonization of the bacterium in the oral cavity. Our results suggest that treatment with cystatin SA at a low concentration (0.01 mg/mL) reduces the binding of the bacteria to the BECs by over 45%. The proteins on the surface of *A. actinomycetemcomitans* that are involved in specific adherence to BECs are an autotransporter adhesion (Aae) and an outer membrane protein 100 (ApiA; 31). It might be interesting to find out whether cystatin SA interacts with these proteins, thereby reducing the available Aae and/or ApiA to bind to the BECs.

Many studies have reported on the levels of cystatin SA in periodontal disease. Some studies have shown an increase, some have shown a decrease and some have shown no change, as reviewed by Dickinson (50). Most of the studies have focused on the quantity of cystatin SA in healthy subjects vs. those with periodontal disease. It is possible that there exists a difference in the function of cystatin SA among subjects, and this may or may not be related to the protease inhibitory function of cystatin SA. It is also possible that the cystatin SA present in the saliva may be complexed with other proteins due to its innate protease inhibitory function, and this may limit the amount of available cystatin SA to interact with the micro-organisms. Future studies need to be carried out to elucidate the mechanism of the antibacterial action of cystatin SA and to explore the therapeutic potential of cystatin SA in aggressive periodontal disease.

Acknowledgements

We wish to thank Dr Ramasubbu Narayanan for providing us with pure lyophilized samples of cystatin SN for the antimicrobial studies. We wish to acknowledge Dr Hong Li, Director of CAPR, UMDNJ for mass spectrometry analysis. We wish to thank Dr David Lagunoff, Director, Confocal Imaging facility for helping us with the confocal studies. Funding for this work was supported in part by grant number DE-017968 from the National Institutes of Health.

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