Molecular mechanisms of epithelial host defense in the airways

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CHAPTER 5

HOST DEFENSE EFFECTOR MOLECULES IN MUCOSAL SECRETIONS

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ABSTRACT

Mucosal secretions contain a range of defense effector molecules including antimicrobial peptides and proteinase inhibitors. These molecules play a central role in host defense against infection, and in a variety of immune and inflammatory reactions. The aim of this study was to analyze the levels of neutrophil defensins, the cathelicidin hCAP-18/LL-37, and the proteinase inhibitors secretory leukocyte proteinase inhibitor (SLPI), SKALP/elafin and cystatin M/E in various mucosal secretions and urine. We show here that especially seminal plasma is characterized by high concentrations of hCAP-18/LL-37, SLPI, SKALP/elafin and cystatin M/E. The results of this study demonstrate that each mucosal secretion is characterized by a unique profile of effector molecules, which may supply individual mucosal secretions with specific properties related to the control of local infection and inflammation.

INTRODUCTION

Epithelia play an important role in innate immunity by constituting a physical barrier to invading microorganisms and by synthesizing an array of defense effector molecules. These epithelia are lined by mucosal secretions that contain a variety of defense effector molecules, including antimicrobial peptides and proteinase inhibitors. In addition to their protective actions against invading microorganisms and the destructive effects of microbial and host proteinases on the epithelium, recent studies show that these defense effector molecules may regulate innate and adaptive immunity [1;2]. In addition to broad spectrum antimicrobial activity, these molecules have been demonstrated to activate epithelial cells [3], to mediate wound repair [4-6], and to display chemotactic activity towards a variety of cells from both innate and adaptive immunity [7]. It was shown that many cell types synthesize antimicrobial peptides, including epithelial cells and granulocytes [8]. Epithelial cells constitutively express some antimicrobial peptides, whereas the expression of other peptides is regulated by an array of stimuli including inflammatory mediators and bacterial products [9-11]. Toll-like receptors have been shown to mediate activation of epithelial cells by microbial products, and may also regulate expression of antimicrobial peptides by epithelial cells [12]. Whereas epithelial cells actively produce antimicrobial peptides, granulocytes only synthesize antimicrobial peptides in an early stage of differentiation followed by storage of these molecules in granules, the content of which may be released during activation [13]. Therefore, antimicrobial peptides that are present in mucosal secretions may be derived from epithelial cells or from granulocytes.

Various families of antimicrobial peptides have been identified, including the defensin family, which consists of both α- and β-defensins, and the cathelicidin family. The α-defensins are produced by neutrophils (human neutrophil peptide [1-4] HNP[1-4]), Paneth cells in the crypts of the small intestine and by epithelial cells of the female genital tract (human defensins-5 and -6 [HD-5 and -6]), while the human β-defensins (hBD1-4) have been demonstrated in various epithelial cells [8]. Expression of hBD1 was demonstrated to be constitutive, while hBD2-4 expression was induced by pro-inflammatory cytokines and microbial products [14-16]. The cathelicidin family of antimicrobial peptides is characterized by a conserved N-terminal domain and a variable C-terminal domain, which can be cleaved off by proteinases resulting in the release of the active peptide. hCAP-18 is the only human member of the cathelicidin family identified to date, and after cleavage by proteinases LL-37 is released. hCAP-18/LL-37 was demonstrated mainly in neutrophils, but also in various squamous epithelia and keratinocytes in inflamed skin, and inflammatory mediators were suggested to regulate hCAP-18/LL-37 expression [2].

In addition to the defensin and cathelicidin families of antimicrobial peptides, proteinase inhibitors such as the secretory leukocyte proteinase inhibitor (SLPI), SKALP/elafin

and members of the cystatin family have been demonstrated in mucosal secretions [17-19]. SLPI was demonstrated to inhibit proteolytic activity of neutrophil elastase (NE), cathepsin G, trypsin and chymotrypsin, whereas SKALP/elafin inhibits activity of NE and proteinase 3. Both SLPI and SKALP/elafin were found to possess antimicrobial activity and are part of the cutaneous host defense system [20-23]. Cystatins are inhibitors of endogenous mammalian lysosomal cysteine proteases [24], and exogenous microbial cysteine proteases [25]. In addition to their proteinase inhibitory activity, some of the cystatins (A, C and S) were shown to have antimicrobial activity against bacteria and viruses [26;27]. Recently we reported that cystatin M/E, a new member of the human cystatin gene family, has an unusually tissue-specific expression pattern in which high expression levels are restricted to the skin [28]. The observed secretion of cystatin M/E in sweat, its expression at the interface of the internal and external milieu (cornifying layers of epidermis), and in bronchial epithelium (unpublished results) suggests that this cystatin is involved in host protection, but no direct antimicrobial activity against a small number of tested skin pathogens has been detected so far [28].

Previous studies have demonstrated defense effector molecules in mucosal secretions [17-19;29;30]. However, these studies are limited in either the number of mucosal secretions or effector molecules studied. Therefore, the aim of this study was to determine profiles of defense effector molecules in various mucosal secretions and urine. We show here that neutrophil defensins, cathelicidins and proteinase inhibitors are present in mucosal secretions lining various epithelia. Each secretion was shown to contain a specific profile of effector molecules, which may supply individual mucosal secretions with specific properties related to the local control of infection and inflammation.

MATERIALS AND METHODS

Mucosal secretions and urine

Mucosal secretions and urine were obtained from in total 20 healthy volunteers and include whole saliva collected after rinsing of the mouth with water, nasal secretions, tears collected after exposure to vapours of onions, sweat collected after exercise or sauna, semen, breast milk collected 1-3 month after birth and urine (morning urine excluded). The samples were analysed by ELISA for the presence of the following defense effector molecules: α-defensins (HNP1-3), β-defensin-2, LL-37, SLPI, SKALP/elafin, and cystatin M/E. The number of samples analysed varied for each secretion tested (saliva, n=5-9; nasal secretions, n=6; urine, n=5; tears, n=4-5; sweat, n=4-5; semen, n=4; and breast milk, n=4-6). The donors provided their material with informed consent, and the samples were anonymized afterwards.

HNP1-3 ELISA

Anti-HNP1-3 mAb were generated using conventional hybridoma technology[31]. Briefly, female Balb/c mice were immunized subcutaneously with a mixture of native and glutaraldehyde-cross-linked HNP1 in Freund's complete adjuvant and boosted with HNP-1 in Freund's incomplete adjuvant. Four days following injection of HNP-1 in the spleen, splenocytes were harvested and fused with SP2/0 myeloma cells. Hybridomas producing antibodies specific for HNP1-3 were subcloned by limiting dilution, tested for positivity by enzyme-linked immunosorbent assay (ELISA) using purified HNP1-3 as antigen and screened for specificity by Western blot analysis of neutrophil granule extracts. The generated antibodies recognise both HNP1, 2 and 3.

HNP1-3 concentrations in mucosal secretions and urine were determined using a sandwich-type ELISA. All steps were carried out at room temperature, and every incubation step was followed by washing with PBS containing 0.05% (v/v) Tween-20. Microtiter plates (Maxisorp, NUNC, Denmark) were coated overnight with monoclonal anti-HNP1-3 antibody (clone HNP-C1) in PBS. The next day, samples and standard HNP1-3 concentrations (isolated from purulent sputum as described previously [32], and starting at 100 ng/ml in three-fold dilutions) were diluted in PBS containing 0.05% Tween-20 and 0.01% (w/v) cetyltrimethylammonium bromide (CTAB) and added to the plate for 90 min. After incubation with a second biotin-labelled monoclonal anti-HNP1-3 antibody (clone HNP-A5) in PBS containing 0.05% Tween-20 and 0.5% (w/v) casein for 30 min, horse radish peroxidase (HRP)-labelled streptavidin in PBS containing 0.05% Tween-20 and 0.5% casein was added to the wells and incubated for 30 min. Finally, tetramethylbenzidine (TMB) was added as chromogenic substrate. The reaction was stopped by addition of 2.5 M H_2SO_4, and the data were read on an ELISA reader at OD 450 nm. The detection range of the ELISA was 0.4-100 ng/ml ***(Figure 1A)***.

Gel electrophoresis, Western blotting and dot blot analysis for hCAP-18/LL-37

To determine the presence of intact and processed hCAP-18 in mucosal secretions and urine, gel electrophoresis and Western blotting were performed with a BioRad system (Hercules, CA) according to the manufacturers instructions. Samples were subjected to sodium dodecyl sulphate (SDS) poly acrylamide gel electrophoresis (PAGE) on a 16.5% Tris/Tricine gel and separated proteins were transferred to a polyvinylidene-difluoride (PVDF) membrane (BioRad). Non-specific binding sites were blocked overnight using buffer A, which consisted of PBS with 5% (v/v) heat-inactivated newborn calf serum (Gibco, Grand Island, NY) and 5% (v/v) skimmed milk (Campina melkunie, Eindhoven, The Netherlands). The next day, the membrane was incubated for 1 hour with monoclonal anti-LL-37 antibody 1.1C12 [3] in buffer A, followed by incubation for 1 hour with HRP-labelled goat anti-mouse antibody in buffer A. The enhanced chemoluminescent (ECL) Western blotting detection system (Amersham Pharmacia Biotech, Upsala, Sweden)

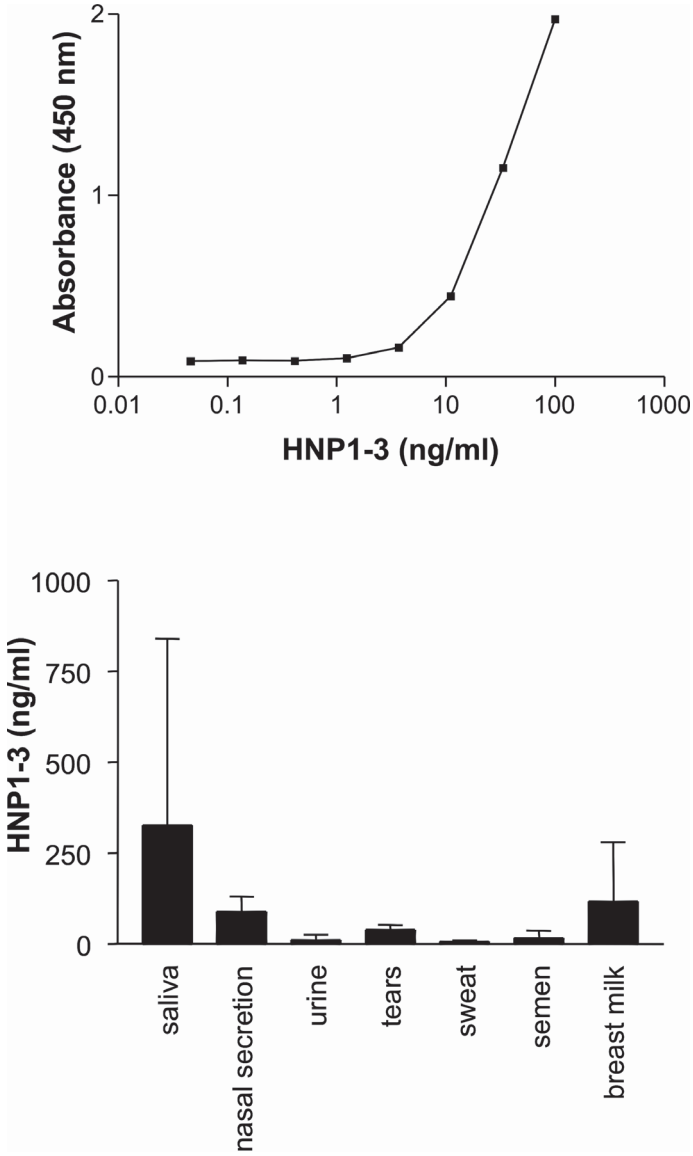

Figure 1: (A) Standard curve of the HNP1-3 ELISA. (B) HNP1-3 concentrations in mucosal secretions and urine as determined by ELISA. Data are expressed as mean ± SD.

was used to reveal immunoreactivity. To determine hCAP-18/LL-37 concentrations in the mucosal secretions, samples and standard concentrations of synthetic LL-37 [3] were spotted on a methanol-preincubated PVDF-membrane. After drying, the membrane was treated as described above for Western blot. Concentrations of hCAP-18/LL-37 in mucosal secretions were determined by densitometry. The detection range of the assay was 20-625 ng/ml.

SLPI ELISA

SLPI concentrations were determined by a sandwich-type ELISA as described earlier [33]. A monoclonal mouse-anti-SLPI antibody (clone 31) was used for coating, and a polyclonal rabbit-anti-SLPI antibody was used for detection. The detection range of the ELISA was 0.04-5 ng/ml.

SKALP/elafin ELISA

SKALP/elafin concentrations were determined by a sandwich-type ELISA using the monoclonal antibodies TRAB2F and TRAB2O. The detection range of the ELISA was 1-15 ng/ml [34].

Cystatin M/E ELISA

Cystatin M/E concentrations were determined by a sandwich-type ELISA using the purified polyclonal rabbit anti-cystatin M/E and guinea pig anti-cystatin M/E antibodies. The detection range of the ELISA was 0.156-5 ng/ml [35].

RESULTS

Defensins

Concentrations of neutrophil defensins (HNP1-3; ***Figure 1***) in various human mucosal secretions and urine were determined by ELISA. Highest HNP1-3 concentrations were detected in saliva compared to the HNP levels in other secretions; nevertheless nasal secretions and breast milk also contained readily detectable levels of HNP1-3 ***(Figure 1B)***. No hBD2 was detected in any of the secretions (data not shown).

hCAP-18/LL-37

The presence of the human cathelicidin hCAP-18 and its cleavage product LL-37 in various human mucosal secretions and urine was determined semi-quantitatively by Western blot analysis using a monoclonal antibody directed against LL-37. In every volunteer, hCAP-18 was demonstrated to be present in seminal plasma and saliva, mainly as the inactive precursor hCAP-18 ***(Figure 2A)***. Very low amounts of hCAP-18 were found in nasal secretions ***(Figure 2A)***, whereas no hCAP-18/ LL-37 was detected in tears, urine, sweat and breast milk (data not shown). In order to determine hCAP-18/LL-37 concentrations in mucosal secretions, a quantitative dot-blot analysis was used. Quantitation was performed using synthetic LL-37 as a standard ***(Figure 2B)***. Relatively high hCAP-18/LL-37 concentrations (~900 ng/ml) were detected in seminal plasma ***(Figure 2C)***, and also in saliva hCAP-18/LL-37 was demonstrated. Very low concentrations of hCAP-18/LL-37

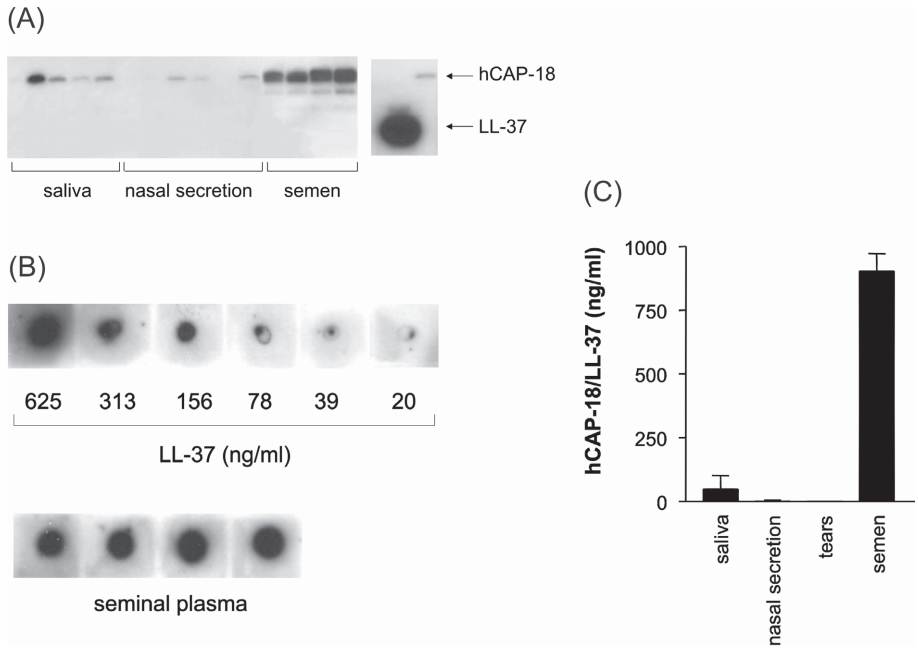

Figure 2: (A) Detection of hCAP-18 and LL-37 in saliva, nasal secretions and semen as determined by Western blot analysis. As controls, synthetic LL-37 and recombinant hCAP-18 were used (right panel). No hCAP-18/LL-37 was detected in urine, sweat and breast milk (data not shown). ***(B) hCAP-18/LL-37 dot blot analysis of both standard synthetic LL-37 and seminal plasma samples***, using the monoclonal anti-LL-37 antibody 1.1C12. ***(C) hCAP-18/LL-37 concentrations (based on a LL-37 standard curve) in mucosal secretions as determined by dot blot analysis.*** Data are expressed as mean ± SD.

were found in nasal secretions, while in tears, as expected based on the results of the Western blot analysis, no hCAP-18/LL-37 could be detected ***(Figure 2C)***.

Proteinase inhibitors

Concentrations of the serine proteinase inhibitors SLPI and SKALP/elafin ***(Figure 3)***, and the cysteine proteinase inhibitor cystatin M/E ***(Figure 4)*** were determined by ELISA. We found that the inhibitor profile is different for each body secretion, but consistent in every volunteer. All three proteinase inhibitors were present at relatively high concentrations in seminal plasma, could be detected in the other mucosal secretions, but were almost absent in sweat and urine in every volunteer.

DISCUSSION

Previous studies have demonstrated defense effector molecules in mucosal secretions covering epithelial surfaces. However, these studies are limited in either the number of mucosal secretions or effector molecules studied. We here report a comprehensive

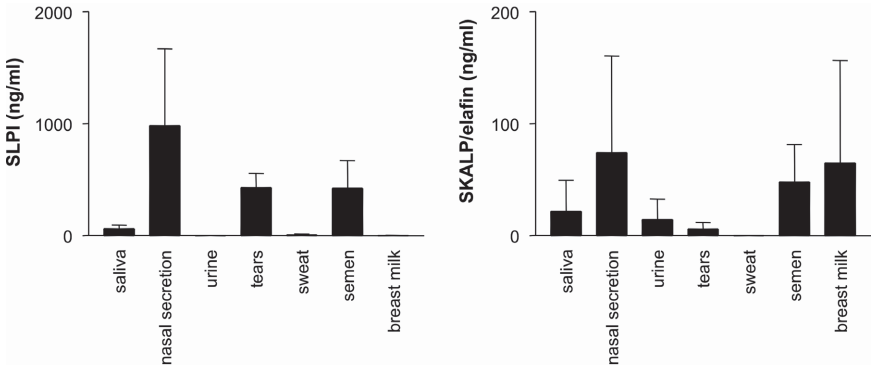

Figure 3: SLPI (A) and SKALP/elafin (B) concentrations in mucosal secretions and urine as determined by ELISA. Data are expressed as mean ± SD.

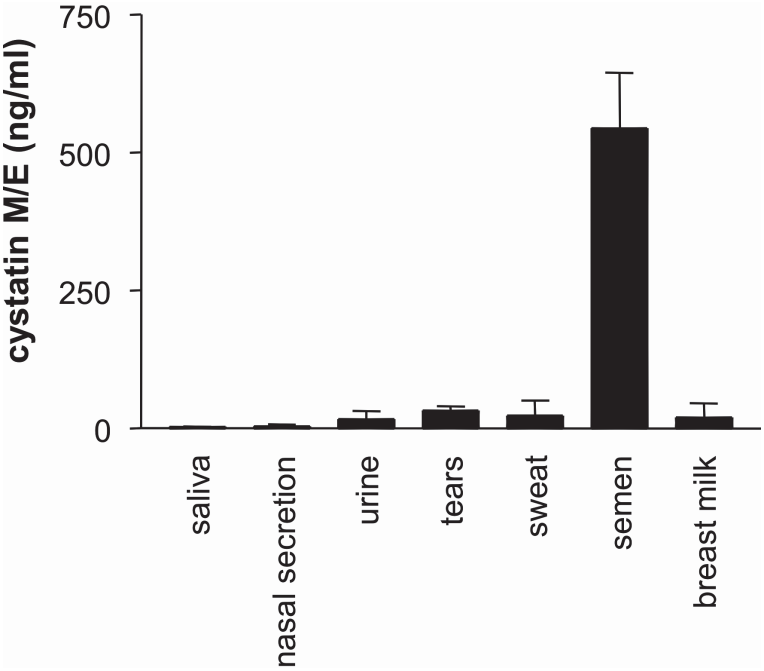

Figure 4. Cystatin M/E concentrations in mucosal secretions and urine as determined by ELISA. Data are expressed as mean ± SD.

study of several host defense proteins in nearly all accessible mucosal secretions and urine. We demonstrate that each of these fluids is characterized by a specific profile of antimicrobial peptides and proteinase inhibitors.

Expression of antimicrobial peptides has been shown to be regulated by a range of mediators including pro-inflammatory cytokines [11], microbial [9] and neutrophil products [36]. Increased levels of neutrophil defensins were demonstrated in airway secretions from patients with inflammatory lung diseases, including cystic fibrosis [37] and chronic bronchitis [38]. Furthermore, in skin of patients with inflammatory skin diseases, expression of

SLPI, cystatin M/E, SKALP/ elafin and LL-37 was increased [18;21;35;39]. In addition, tracheal aspirates of mechanically ventilated newborn infants with pulmonary or systemic infections were shown to contain increased hCAP-18/LL-37 levels as compared to non-infected newborns [40]. Finally, expression of SLPI, cystatin M/E and LL-37 was increased during the process of wound healing [5;6;35].

In addition to regulation at the expression level, also the processing and activity of host defense molecules may be subject to regulation. Proteolytic processing of precursors of antimicrobial peptides is crucial for release of these active peptides, and proteinase inhibitors may affect the cleavage of precursor antimicrobial proteins by proteinases. Furthermore, the proteinase inhibitor α_1-proteinase inhibitor (α_1-PI) was demonstrated to block neutrophil-defensin-induced IL-8 expression and release [41], and apolipoproteins, which are present in serum, were shown to bind and inhibit antimicrobial activity of LL-37 [42]. Finally, local salt concentrations, proteolytic degradation and synergistic interactions [43] between defense effector molecules may affect the activity of these molecules. The regulation of both expression and activity of defense effector molecules suggests that the microenvironment of mucosal secretions may play an important role in the functional properties of mucosal secretions.

Previous studies have demonstrated the presence of antimicrobial peptides in mucosal secretions. Seminal plasma was shown to be characterized by high concentrations of both hCAP-18/LL-37 [44] and SLPI [45]. We show here that in addition to hCAP-18/LL-37 and SLPI also other host defense effector molecules, including SKALP/elafin and cystatin M/E, are present at relative high concentrations in seminal plasma. Furthermore, while saliva and nasal secretions have been demonstrated to contain various host defense effector molecules including HNP1-3 [30;46;47], LL-37 [48;49], SKALP/elafin [50] and SLPI [47;51-53], we show that cystatin M/E is (almost) absent in saliva and nasal secretions. In urine, which has been demonstrated to contain HNP1-3 [54], SKALP/ elafin [18] and SLPI [55], and in sweat, we show relatively low concentrations of host defense effector molecules. In addition, we show that in addition to HNP1-3 [56] and SLPI [57], also SKALP/elafin and cystatin M/E could be detected at relatively low levels in human tears, while no hCAP-18/LL-37 was detected. Finally, we show HNP1-3, SKALP/elafin and cystatin M/E in breast milk, while we could not demonstrate any secreted SLPI. This is in contrast to others, which have found SLPI [51;58] and HNP1 [59] in breast milk. Concentrations of host defense effector molecules in mucosal secretions as reported in previous studies [17;44], show some marked differences as compared to our study. For example, SLPI was demonstrated in nasal lavage, saliva, tears, seminal plasma and breast milk [17] at higher concentrations, and also hCAP-18 levels in seminal plasma were shown at higher concentrations as compared to our study [44]. In addition, the presence of hCAP-18/LL-37 was reported in human sweat samples [60], whereas we did not detect hCAP-18/LL-37 in the sweat of each individual. These discrepancies in concentrations of antimicrobial peptides determined in different

studies may be explained by donor and assay differences, by the cationic properties of antimicrobial peptides, which may affect detection of these peptides, and by masking of epitopes in secretions that may have obscured their detection. Furthermore, testing small groups of subjects in different studies, including our study, may affect the study results. In addition, it should be noted that concentrations of defense effector molecules in mucosal secretions might underestimate the concentration of these molecules present at the mucosal surface.

Why do mucosal secretions, which all cover epithelial cells and may constitute a first line of defense against invading pathogens, differ in their content of host defense effector molecules? This may be explained by the complex role these peptides play in infection and inflammation. It has been proposed that antimicrobial peptides may induce various activities in epithelial cells. Indeed, both HNP1-3 and LL-37 were shown to activate and chemoattract both inflammatory and immune cells [61], to enhance IL-8 release from airway epithelial cells [3], which may result in chemotaxis of inflammatory cells, and to induce cell death at relatively high concentrations [62]. In addition, antimicrobial peptides including SLPI, HNP1-3 and LL-37 were shown to be involved in wound repair [4-6]. In the process of wound healing, several functions of antimicrobial peptides have complementary and synergistic effects: in addition to promoting cell proliferation, differentiation and migration, antimicrobial peptides may protect the wound from infection. Also in seminal plasma, which was demonstrated to contain high concentrations of defense effector molecules, multiple activities may be displayed. While antimicrobial peptides may prevent infection following sexual intercourse, these peptides may also play an important role in reproduction as suggested by a study showing that rat β-defensins affect motility of sperm cells [63]. In addition to antimicrobial peptides, also proteinase inhibitors may be involved in multiple biological processes by displaying antimicrobial activity and by regulating proteinase activity. Neutrophil serine proteinases contribute to host defense through their effects on ingested microorganisms, but may also be involved in degradation of extracellular matrix proteins. Finally, proteinases play an important role in host defense by affecting mucociliary clearance [64], phagocytosis of pathogens [65] and T-cell function [66], and by mediating the processing of precursor antimicrobial proteins to the active peptides [67;68]. Different proteinases may regulate processing of antimicrobial proteins at different anatomical sites, as demonstrated for hCAP-18. Neutrophil-derived hCAP-18 was demonstrated to be cleaved by proteinase 3 [67], resulting in the release of the active peptide LL-37, while hCAP-18 present in seminal plasma was cleaved by the prostate-derived serine proteinase gastricsin resulting in release of ALL-38 [68], a peptide slightly larger that LL-37 with similar antimicrobial activity. Human sweat was demonstrated to contain serine proteinases that process LL-37 in several smaller antimicrobial peptides, displaying lower inflammatory activity as compared to LL-37 [69]. Specific profiles of host defense effector molecules in mucosal secretions may thus reflect tissue-specific

requirements, and there might not be a simple answer on the relevance of these specific profiles in the different secretions.

In conclusion, this study demonstrates profiles of defense effector molecules in various human mucosal secretions and urine. Although mucosal secretions covering different epithelia share their function of constituting a first line of defense against invading microorganisms, we show that mucosal secretions and urine display different profiles of antimicrobial peptides and proteinase inhibitors, which may be explained by the variety of functions these peptides may play in infection and inflammation. Regulation of expression and activity of antimicrobial peptides in mucosal secretions may determine the functional effect of these peptides in host defense and inflammatory processes.

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