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Short communication

The role of urokinase in innate immunity against *Staphylococcus aureus*

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Abstract

Urokinase (uPA) is a serine protease that not only displays fibrinolytic function but also promotes host leukocytes to home to inflammatory sites. We have recently demonstrated that staphylokinase (SAK), which is a fibrinolytic protein secreted by *Staphylococcus aureus*, forms complexes with human neutrophil peptides (HNPs), which are members of the defensin family and have anti-microbial properties, thereby inhibiting the bactericidal effects of the HNPs. The aim of this study was to assess whether endogenous uPA, which has fibrinolytic properties similar to those of SAK, binds to HNPs and interferes with SAK/HNPs interaction. To this end, an ELISA was used to analyze the interactions between uPA and HNPs. HMW uPA had the ability to bind to both HNP types. The biological consequences of the formation of this complex were analyzed with respect to its bactericidal properties. HMW uPA killed *S. aureus*, albeit at relatively high doses (50–100 μg/ml). In contrast, the binding of HMW uPA to HNPs had no impact on the bactericidal functions of the HNPs. Importantly, the addition of HMW uPA to SAK eliminated the ability of SAK to neutralize HNPs. Our results demonstrate that endogenous HMW uPA inhibits *S. aureus* growth both directly, by cytolysis, and indirectly, by abrogation of the neutralizing effect of SAK on the bactericidal activities of HNPs. These findings indicate novel functions of HMW uPA in the host defense against staphylococcal infections.

Keywords: Urokinase; *Staphylococcus aureus*; Human neutrophil peptides; Staphylokinase

1. Introduction

Urokinase-type plasminogen activator (uPA) is a serine protease that is secreted by almost all cell types as a single-chain zymogen (single-chain urokinase, scuPA), which is essentially inactive [1,2]. Thereafter, scuPA is cleaved by plasmin or by tumor-associated proteases to the active form, the two-chain urokinase (tcuPA), which is also called HMW uPA [3,4]. tcuPA is composed of a catalytic serine protease domain and a non-catalytic amino terminal fragment (ATF), which directs the binding of uPA to a specific, high-affinity cell surface receptor, uPAR [5]. The serine protease domain of uPA mediates plasminogen activation on cell surfaces, which plays a pivotal role in cell invasion and tissue remodeling by degrading extracellular membrane proteins [6,7]. In addition to its plasmin-dependent effects, uPA has protease-independent functions, such as mitogenic, migratory, and adhesive properties, which depend upon uPA/uPAR binding [8–10].

uPA expression is highly up-regulated in inflammatory diseases, such as rheumatoid arthritis (RA) [11–14], probably as the result of the actions of pro-inflammatory cytokines [15–17]. The essential role of uPA/uPAR in normal host defense against infection has been studied intensively in the rodent pulmonary inflammatory model. The absence of uPA results in the inadequate recruitment of leukocytes to the inflammatory site, down-regulation of macrophage antimicrobial function, failure to generate type 1 immune responses, and uncontrolled infection [18–20].

Human neutrophil peptides (HNP1-3) belong to the defensin family, which has broad-spectrum activity against bacteria, fungi, and some enveloped viruses [21]. HNPs are mainly packed in azurophilic granules of polymorphonuclear leukocytes (PMN) [22]. During phagocytosis, azurophilic granules fuse with phagocytic vacuoles, in which high concentrations of defensins consequently appear [23,24]. Aside from influencing microbial killing and regulation of the host adaptive immunity against microbial invasion [25], HNPs interact with fibrinolytic substances, and thereby inhibit their fibrinolytic properties [26,27]. Recently, we reported that staphylokinase (SAK), which is a thrombolytic protein

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secreted by most *S. aureus* strains [28], interacts with HNPs to block the bactericidal effects of HNPs [29].

Since (a) uPA has similar thrombolytic functions to tPA and SAK, and (b) both uPA and HNPs are strongly up-regulated at inflammatory sites, we investigated whether these two molecules could interact with each other and participate in host innate immunity against bacterial invasion. Our results show that uPA forms complexes with HNPs. However, the uPA/HNPs complex retains the bactericidal function that is mediated by HNPs. In addition, we demonstrate that uPA on its own inhibits *S. aureus* growth. These data indicate two novel functions of uPA in host innate immunity.

2. Materials and methods

2.1. Bacterial strains

Five *S. aureus* strains (LS-1 [30], Newman [31], RN6390 [32], P1 [33], and 1061 [34]) were used in this study. *Escherichia coli K-type, Streptococcus bovis,* and *S. epidermidis* strains were obtained from the Laboratory of Microbiology, Sahlgrenska University Hospital, Göteborg.

2.2. Reagents

Todd-Hewitt broth (THB) and horse blood agar were obtained from Difco (Boule Nordic, Huddinge, Sweden). Human neutrophil peptides (HNP-1, 3442 g/mol; and HNP-2, 3371 g/mol) were purchased from Bachem (Feinchemikalien AG, Switzerland). Recombinant SAK (sakSTAR, 16000 g/mol) was a kind gift from Dr. Yves Laroche and Prof. Desiree Collen of the Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute of Biotechnology, Leuven, Belgium. Human high-molecular-weight (HMW) uPA was purchased from Medac (Hamburg, Germany). Human low-molecularweight (LMW)-uPA (residues: 136–411 containing the intact protease domain), amino terminal fragment (ATF)-uPA (residues 1–135 of the A-chain of uPA), and amino terminal growth factor-like domain (GFD, residues 1–43) were all purchased from American Diagnostica Inc. (Greenwich, CT). Bovine serum albumin (BSA) fraction V and *d*imethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.3. Analysis of uPA/HNP complex formation

The 96-well flat-bottom polyester ELISA plate (Nunc, Denmark) was coated overnight with HMW uPA (10 μg/ml) in carbonate buffer -(pH 9.6). The control wells were coated with 1% BSA. The plates were washed with PBS-0.05% Tween-20 and blocked with 0.5% PBS–BSA (pH 7.4) at 37 °C for 1 h. After washing, different amounts of HNP-1 (0.032–100 ng/ml) in 0.1% PBS–BSA (pH 7.4) were added to the uPA-coated wells. After a 1-h incubation at 37 °C and washing, uPA-captured HNP was detected by the addition of biotinylated anti-human HNP1-3 antibodies (Hycult Biotechnology, Enskede, Sweden) for 1 h at room temperature, followed by the addition of avidin-HRP and enzyme substrate. The difference in absorbance at 450 nm between the uPA-coated and BSA-coated wells was calculated to evaluate the formation of uPA/HNP complexes.

In order to determine the HNP-binding sites on HMW uPA, we coated equimolar amounts of different fragments of uPA (6.3 μg/ml LMW uPA, 3.3 μg/ml ATF, or 1 μg/ml GFD), and 10 μg/ml intact HMW uPA onto polyester ELISA plates, followed by the addition of increasing amounts of HNP-1 (6.4–4000 pg/ml). The protocol to detect HNP-1 binding to immobilized uPAs was similar to that described above.

To determine the specificity of HMW uPA/HNP binding, monoclonal uPA-specific antibodies (Hemochrom Diagnostica GmBH, Mölndal, Sweden) were added to block immobilized uPA. HNP-1 (0.2–1 μg/ml) was then added to interact with immobilized uPA followed by the addition of biotin-labeled HNP-specific antibodies and streptavidin-HRP, as described above.

To determine which fragment of soluble HMW uPA competes with immobilized uPA, HNP-1 alone (0.2–1 μg/ml) and HNP-1 mixed with either 63 μg/ml LMW uPA or 33 μg/ml ATF were added to the surface-bound HMW uPA. Detection of the solid phase HNP-1 was performed as described above.

2.4. Preparation of HMW uPA, HNP, and SAK mixtures

To allow reactions to occur between the three molecules, 5 μg/ml rSAK was incubated with 5 μg/ml HNP-1 and equimolar amount of HMW uPA in PBS buffer (pH 7.4) for 30 min at 37 °C. All these mixtures were used to evaluate the blocking effects of HMW uPA on SAK-mediated neutralization of the bactericidal properties of HNP-1.

2.5. Impact of HMW uPA on S. aureus growth

A standard number of bacteria (1×10^3 per ml in THB) were incubated with increasing concentrations (range 0–200 μg/ml) of HMW uPA. At specific time intervals, samples of the bacterial mixtures (0.1 ml) were spread on horse blood agar. After incubation for 24 h at 37 °C, the colonies were counted. The blocking effect of HMW uPA on the SAK-neutralizing bactericidal properties of HNP-1 was evaluated by comparing staphylococcal survival rates in the presence of mixtures of HMW uPA/SAK/HNP-1 and SAK/HNP-1 mixtures. The bacteria were treated with HNP-1/uPA mixtures, HNP-1, HMW uPA, SAK alone, and the equivalent volume of PBS as the control. The results were recorded as the percentage reductions in bacterial numbers for different treatment procedures as compared to the control (untreated) cultures.

2.6. Statistical analysis

The differences in bactericidal effect between the treatment groups in the HMW uPA killing assay and HMW uPA

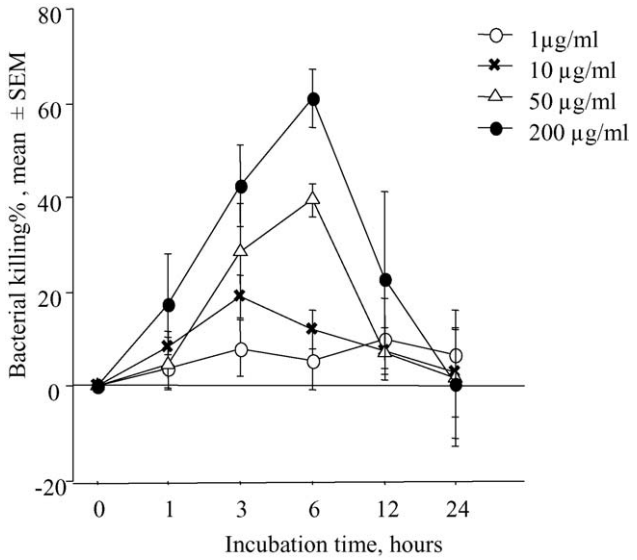

Fig. 1. Kinetics of *S. aureus* killing by HMW uPA. *S. aureus* strain LS-1 (10^3 CFU/ml) was incubated with increasing concentrations (0–200 µg/ml) of HMW uPA in vitro. Bacterial growth was monitored at the time intervals indicated on the *x*-axis.

blocking of SAK neutralization of HNP-1 experiment were analyzed by the Wilcoxon signed rank test. The data are presented as mean ± S.E.M. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. HMW uPA kills S. aureus in vitro

The influence of HMW uPA on bacterial growth was assessed using the *S. aureus* LS-1 strain. The bactericidal properties of uPA (0–200 µg/ml) with respect to the growth of LS-1 are shown in Fig. 1. At high concentrations (50–200 µg/ml), HMW uPA displayed dose-dependant bacterial killing. Indeed, the incubation of LS-1 with 50 µg/ml HMW uPA resulted in approximately 40% bacterial killing. Higher concentrations of HMW uPA led to 60% bacterial killing. With respect to the incubation time, the bactericidal effect of high-dose HMW uPA increased with incubation for up to 6 h (Fig. 1). In all subsequent experiments, 10^3 CFU/ml bacteria were incubated with potential inhibitors for 6 h.

To assess the killing effects of uPA on other Gram-positive and Gram-negative bacteria, one *E. coli* K-type, one *S. bovis,* and one *S. epidermidis* strain were exposed to HMW uPA (10–100 µg/ml) for 6 h in vitro. There was no visible effect of HMW uPA on the growth of these bacterial strains (data not shown), which indicates the specificity of killing by uPA.

3.2. HNP-1 forms complex with uPA through a binding site on the LMW fragment of the molecule

To assess possible interactions between HMW uPA and HNP-1, increasing amounts of the HNPs (32–4000 pg/ml) were incubated with HMW uPA (25 µg/ml) that was immobilized on polystyrene wells. The subsequent addition of HNP-specific antibodies led to a dose-dependent increase in the absorbance of the wells that were coated with HMW uPA (Fig. 2a), which indicates complex formation between HMW uPA and HNPs. The addition of higher levels of HNPs (10–100 ng/ml) did not give rise to higher absorbance values, which indicates saturation of binding. The monoclonal anti-uPA antibodies did not block HNP-1 binding to HMW uPA on the solid phase (data not shown), possibly due to limited epitope specificity.

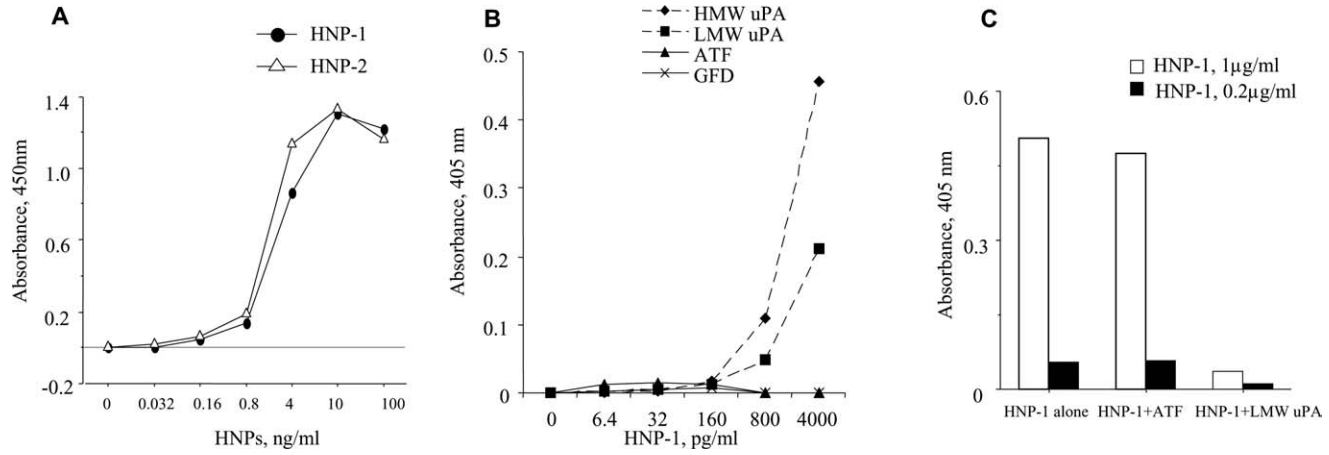

Fig. 2. Complex formation between HNPs and uPA.
a. HMW uPA (25 µg/ml) or 1% BSA was immobilized on solid phase. HNP-1 and HNP-2 were added at different concentrations, as indicated on the *x*-axis, followed by incubation with HNP-specific antibodies. Specific binding of HNP-1 and HNP-2 to immobilized molecules was expressed as the difference between the absorbance (450 nm) values of the HMW uPA-coated and BSA-coated wells. Similar results were observed in three separate experiments. *b.* LMW uPA is responsible for uPA binding to HNPs. Equimolar amounts of HMW uPA (10 µg/ml), LMW uPA (6.3 µg/ml), ATF (3.3 µg/ml), and GFD (1 µg/ml) were coated on solid phase. HNP-1 was added at different concentrations, as indicated on the *x*-axis, followed by incubation with HNP-specific antibodies. Specific binding of HNP-1 to immobilized molecules was registered as the absorbance at 405 nm. *c.* LMW uPA, but not ATF, competes with binding of HNP-1 to HMW uPA. Ten µg/ml HMW uPA was coated on the solid phase. Thereafter, HNP-1 (0.2–1 µg/ml) alone or HNP-1 mixed with equimolar amounts of soluble uPA fragments (63 µg/ml LMW uPA or 33 µg/ml ATF) in 1% BSA were allowed to react with immobilized HMW uPA, followed by detection with HNP-specific antibodies.

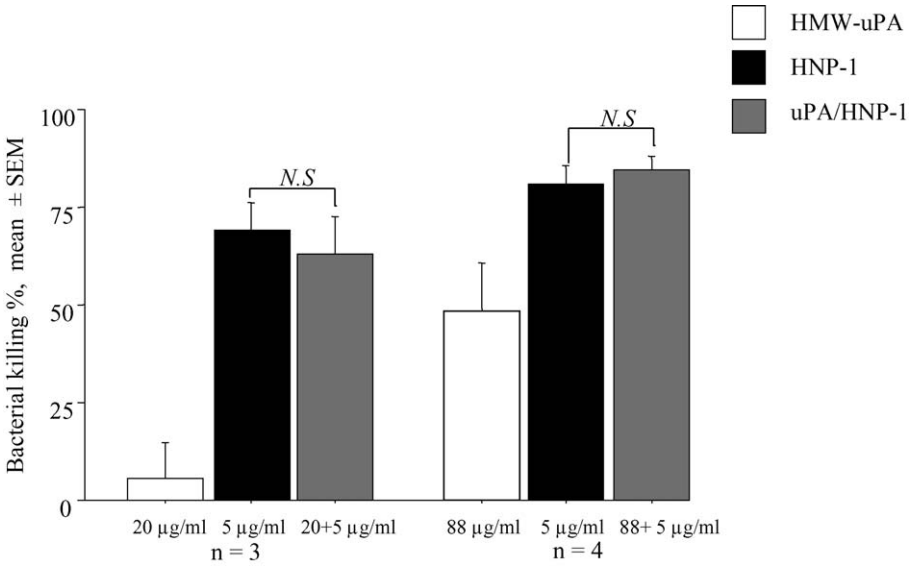

Fig. 3. HNPs complexed with HMW uPA retain their bactericidal properties. The growth of *S. aureus* was monitored following incubation of the bacteria with HMW uPA (20–88 µg/ml) alone, HNP-1 (5 µg/ml) alone, or mixtures of HMW uPA and HNP-1 (20 + 5 µg/ml or 88 + 5 µg/ml) for 6 h Bacterial killing was expressed as the percentage reductions in bacterial numbers for different treatment procedures as compared to the control (untreated) cultures as mean ± S.E.M. N.S. = not significant.

To study the binding site of HNP to uPA, increasing amounts of HNP-1 were interacted with three distinct fragments of uPA (LMW uPA, ATF, and GFD) immobilized on solid phase. Dose-dependent increases in HNP-specific absorbance were found in the wells that were coated with HMW uPA and LMW uPA (Fig. 2b). In contrast, no signal was detected in the GFD- and ATF-coated wells, which demonstrates that the serine protease domain of the uPA molecule is responsible for binding to HNPs.

In competition assays, HNP-1 alone or HNP-1 mixed with equimolar amounts of distinct uPA fragments (ATF or LMW uPA) were allowed to react with solid phase-bound HMW uPA. The addition of 63 µg/ml LMW uPA together with HNP-1 resulted in almost total abrogation of HNP-1-specific absorbance in comparison with the addition of HNP-1 alone. In contrast, the addition of equimolar amounts of soluble ATF did not affect the binding between HMW uPA and HNP-1 (Fig. 2c). These data strongly support the notion that LMW uPA contains the HNP-1 binding site.

3.3. uPA/HNP complex formation does not affect the bactericidal properties of HNP-1

Five *S. aureus* strains were used to investigate the impact of HMW uPA/HNP-1 complex formation on the bactericidal effects of HNP-1 (Fig. 3). The bactericidal effects on each staphylococcal strain were compared following 6 h incubation with HMW uPA/HNP-1 complexes or each component thereof. Incubation of these *S. aureus* strains with a low dose of HMW uPA (20 µg/ml) had no influence on bacterial viability. As expected, HNP-1 killed 69% of the staphylococci. However, despite complex formation between HNP-1/HMW uPA, bactericidal efficacy remained at 69% (*N.S*). In another experiment, we increased the concentration of HMW uPA to 88 µg/ml, which is equimolar to 5 µg/ml of HNP-1. Incubation of four *S. aureus* strains with 88 µg/ml HMW uPA reduced bacterial viability to 50%. While 81% of the bacteria were killed by HNP-1 alone, 85% of the bacteria were killed by the HNP-1/HMW uPA complex, which, suggests that complex formation between HNP-1 and HMW uPA does not affect the bactericidal effect of HNP–1.

3.4. Interaction between HMW uPA and SAK abrogates the effect of SAK on the bactericidal properties of HNP-1

Since HMW uPA binds to HNP-1 without influencing its bactericidal effect, we mixed HNP-1 (5 µg/ml) with an equimolar amount of HMW uPA (88 µg/ml) to study the consequences of HNP-1 to HMW uPA binding on SAK-neutralizing properties with respect to HNP-1 killing of staphylococci (Fig. 4). Incubation of *S. aureus* with HNP-1 resulted in 84.6% bacterial killing, and the mixture of HNP-1 and HMW uPA gave rise to a similar level of bacterial killing (84.5 ± 3.6%, *N.S*). In contrast, exposure to HNP-1 together with exogenous SAK decreased the killing rate to 36.5%, as previously demonstrated [29]. Importantly, the impaired HNP-1 bacterial killing caused by SAK was almost fully restored by adding equimolar amounts of HMW uPA (bacterial killing, 77 ± 8.4%, $P < 0.05$), which indicates that HMW uPA blocks SAK-mediated neutralization of HNP-1 bacterial killing. As a control, HMW uPA/SAK treatment gave rise to approximately 50% bacterial killing, and SAK alone treatment had no influence on staphylococcal growth.

4. Discussion

In a recent study, we showed that SAK produced by *S. aureus* interacts with and neutralizes α-defensins, and hypoth-

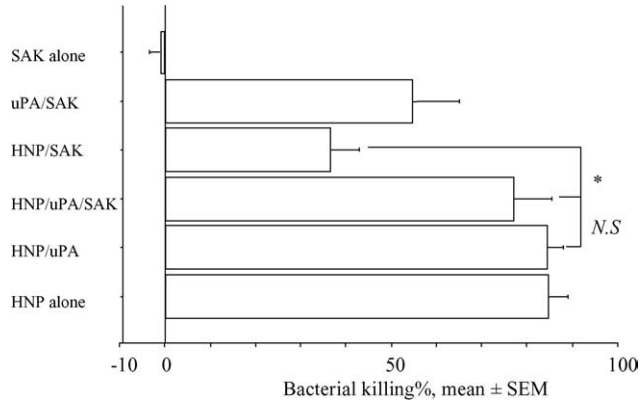

Fig. 4. HMW uPA counteracts the ability of SAK to neutralize the bactericidal effects of HNPs. Four *S. aureus* strains were cultured in vitro with SAK (5 μg/ml) alone, HNP-1 (5 μg/ml) alone, pre-mixed HNP-1/SAK (5 + 5 μg/ml), HNP-1/uPA (5 + 88 μg/ml), uPA/SAK (88 + 5 μg/ml), or HNP-1/uPA/SAK(5 + 88 + 5 μg/ml). After 6 h of incubation, bacterial growth was determined by viable counts. The percentage of *S. aureus* killing is presented as mean ± S.E.M.. * = $P < 0.05$; N.S. = not significant.

esized that this interaction was responsible for deficient bacterial killing during *S. aureus* infections [29]. In the present study, we propose that HMW uPA plays an important role in human innate immunity during the course of *S. aureus* infection by: (a) directly killing *S. aureus*, and (b) enhancing the sensitivity of SAK-producing *S. aureus* strains to α-defensins through the inhibition of SAK-mediated neutralization of the bactericidal properties of HNPs.

uPA participates in the network of inflammatory events required for the clearance of bacteria. Several lines of evidence suggest that uPA is involved in leukocyte recruitment, and thus mediates inflammation and bacterial clearance in pulmonary inflammatory responses [19,20,35]. However, during pulmonary infections, uPA-deficient mice have impaired anti-microbial activities even when the pulmonary macrophage number appears to be sufficient for host defense [18]. Importantly, our results suggest that uPA at high concentrations kills *S. aureus*, which suggests that uPA, in addition to affecting leukocytic recruitment, acts as an endogenous antibiotic. In local inflammatory sites, e.g. RA joints, the levels of uPA are strongly up-regulated [12–14,35], although extracellularly they do not quite reach bactericidal levels. However, in the *S. pneumoniae*-induced mouse model of pneumonia, the uPA concentrations in lung homogenates were increased to around 200 μg/ml [35], at which concentration uPA displays strong bactericidal effects in our in vitro system. Moreover, because of the uneven distribution of uPA, the local concentration of this molecule may be considerably higher. Could the bactericidal properties of uPA relate to plasmin activation? Data from our control experiment do not support this hypothesis, since neither plasminogen alone nor plasmin(ogen)/SAK complexes influenced *S. aureus* growth (data not shown).

The concentrations of defensins in inflamed or infected human tissues increase dramatically in both the acute and chronic inflammatory states [36,37]. Due to their abundance and broad-spectrum bactericidal activities, defensins have been considered as the first line in the innate host defense system [21]. However, defensin activity is neutralized by SAK produced by *S. aureus* [29]. Intriguingly, uPA, which is another molecule that is up-regulated at inflamed sites, also binds to α-defensins. Our data suggest that in this case, binding does not affect the bactericidal properties of HNPs. As a consequence of uPAR/uPA binding, the uPA/HNP complexes may be immobilized on the host cell surface, to defend the host against *S. aureus* invasion. We have shown that uPA inhibits *S. aureus* resistance to HNPs caused by SAK production. Is this due to HMW uPA competition with SAK for binding to HNPs? Both HMW uPA and SAK form complexes with HNPs, but only SAK blocks the bactericidal functions of HNPs, which indicates that functional regions of HNP react with SAK, and not with uPA. We could not abrogate completely the binding of HNP-1 to SAK on the solid phase, even by adding a 1000-fold excess of HMW uPA (data not shown).

In summary, we show that uPA acts as (a) an anti-microbial agent; (b) a complex-forming molecule with HNPs without influencing its functions; and (c) a potent inhibitor of *S. aureus* SAK, which is important for evading killing by anti-microbial peptides. Further understanding of these interactions may provide insight as to synergies between uPA already present at the site of staphylococcal infection and other anti-microbial therapies.

Acknowledgements

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