Fatal outcome of bacteraemic patients caused by infection with staphylokinase-deficient Staphylococcus aureus strains

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Staphylokinase (SAK) is a plasminogen-activator protein produced by Staphylococcus aureus. SAK production was evaluated in vitro in S. aureus isolates from the bloodstream of patients with lethal (n = 56) and non-lethal (n = 57) bacteraemia and from anterior nares of healthy subjects (n = 48). Most isolates (93/161) produced SAK, and 68 % of SAK-producing isolates expressed both surface-bound and secreted types of SAK. SAK production was significantly less common among isolates from patients with lethal bacteraemia (39 %) than isolates from patients with non-lethal bacteraemia (68 %) or nasal carriage isolates (67 %) (P < 0·01). After adjusting for infection with methicillin-resistant S. aureus and APACHE II score, patients infected with SAK-deficient isolates were 4·3 times more likely to have lethal bacteraemia than patients whose infecting isolate produced high levels of SAK (>5 µg ml⁻¹), suggesting that in vitro SAK production was inversely associated with clinical outcome among patients with S. aureus bacteraemia. The high frequency of SAK production in nasal isolates and in cases with uncomplicated bacteraemia suggests that SAK may be one of the adaptive mechanisms of S. aureus symbiosis with the host.

INTRODUCTION

Staphylococcus aureus is a human pathogen that is a major cause of community- and hospital-acquired infections (Lowy, 1998). The pathogenicity of S. aureus is known to depend on a set of extracellular and cell wall-associated proteins that are not required for growth and cell division but are advantageous in particular environments (Foster & Hook, 1998; Joh, 1999; Winzer & Williams, 2001) and can influence the clinical severity of infection (Baba et al., 2002; Peacock et al., 2002; Gillet et al., 2002).

Several S. aureus proteins interact with host fibrinolysis. S. aureus interacts with host fibrinolysis by expressing plasminogen-binding moieties on the cell surface (Kuusela & Saksela, 1990) and by producing a plasminogen activator, staphylokinase (SAK) (Collen, 1998). SAK is carried by a converting phage that inserts into the β-haemolysin structural gene. Expression of SAK is positively regulated by accessory gene regulator (Agr) and negatively regulated by staphylococcal accessory gene regulator (Sar). The association between SAK expression and the outcome of S. aureus infections has not previously been evaluated.

In the present study, we assessed the pathogenic significance of SAK production for the invasive potential and outcome of staphylococcal infection using a collection of well-defined clinical isolates. Our results demonstrate a negative correlation between SAK production and the development of life-threatening complications and mortality during staphylococcal bacteraemia. In contrast, nasal isolates and strains of S. aureus giving rise to uncomplicated bacteraemia were more often SAK producers. These findings suggest that SAK is part of the adaptive mechanisms of S. aureus favourable for bacterial symbiosis with the host.

METHODS

Clinical characteristics of the patients. The study was approved by the Duke University Medical Center Institutional Review Board. Subjects permitting nasal swabbing provided written informed consent. Patient identification and selection methods have been described previously (Fowler et al., 2000). Briefly, daily reports were received from the microbiology laboratory about all patients at Duke University Medical Center with one or more blood cultures positive for S. aureus.
and clinical evidence of infection. The clinical charts of these patients were then reviewed within 36 h of detection of bacteremia to confirm the presence of a clinical infection. Clinical data were collected at the time of patient hospitalization by investigators and entered into an electronic database (Microsoft Access). The APACHE II score (Knaus et al., 1985) was evaluated for each patient on the day of the first blood culture yielding S. aureus. Exclusion criteria included S. aureus bacteremia in patients who were outpatients or less than 18 years of age, as well as those with polymicrobial infection, those with neutropenia (absolute neutrophil count less than 1 × 10^9 l⁻¹) or those who died before evaluation by the investigators. Only S. aureus isolates from patients meeting study definitions for either invasive bacteremia or uncomplicated bacteremia (see below) were included in the current investigation.

**Study definitions.** Specific clinical criteria were used to group bacterial isolates into three clinical categories: (i) lethal bacteremia, (ii) non-lethal bacteremia or (iii) nasal carriage isolates. Lethal bacteremia isolates were obtained from the bloodstream of patients with S. aureus bacteremia who died of their infection. Non-lethal isolates were obtained from the bloodstream of patients with clinically uncomplicated bacteremia, as defined by the presence of all of the following features: (i) clinically significant intraocular catheter-associated S. aureus bacteremia, (ii) no evidence of metastatic infection in internal organs, (iii) 14 days or less of intravenous antibiotic therapy and (iv) alive with no complications of infection 12 weeks after the initial positive blood culture. Nasal carriage isolates were obtained from the anterior nares of healthy non-medical university students by rotating a sterile Dacron fibre-tipped swab within both nostrils.

**Isolation and characterization of S. aureus strains.** Blood culture isolates were identified as S. aureus and stored for future use, as described elsewhere (Fowler et al., 2000). Blood and nasal cultures were identified as containing S. aureus by subculturing on sheep blood agar, Gram staining the isolate and then performing the Staph aurex test (Murex Diagnostics), a latex agglutination assay that detects S. aureus-specific proteins. Isolates were kept frozen (−70 °C) from the time of identification until they were analysed.

**Culturing of S. aureus strains for SAK production.** All assays were performed at the Department for Rheumatology and Inflammation Research, Göteborg University, Göteborg, Sweden, blinded to the source of the bacterial isolates. Prior to determination of SAK production, frozen S. aureus isolates were cultured on horse blood agar overnight and the bacteria within a single c.f.u. were used for further analyses.

One c.f.u. of each S. aureus strain was grown overnight in 2 ml Todd–Hewitt broth (THB; Difco) at 37 °C. Staphylococci were subsequently harvested by centrifugation at 4000 g for 10 min (4 °C). Supernatants were collected for further determination of secreted SAK activity. The bacterial pellet was assessed spectrophotometrically at 600 nm for bacterial density, washed with PBS (10 mM, pH 7.4) and used for evaluation of surface-bound SAK activity.

Staphylococci were resuspended in THB at 10⁹ c.f.u. ml⁻¹ and incubated for 4 h at 37 °C with 20 μg human Glu-plasminogen ml⁻¹ (Biopool). Excess plasminogen was removed by washing twice with PBS. The bacterial pellet was resuspended in Tris/HCl buffer (50 mM, pH 7.4) containing plasmid substrate (see below) and incubated for another 30 min to allow SAK-dependent activation of plasminogen attached to the bacterial surface.

**Determination of SAK activity.** SAK activity of bacterial supernatants was determined by incubation with 0.2 μM human Glu-plasminogen (Biopool) in Tris/HCl buffer (50 mM, pH 7.4) to allow SAK-dependent conversion of plasminogen to plasmin. Plasmin formation was evaluated by hydrolysis of 0.4 μM H-D-Val–Leu–Lys paranitroanilide (S-2251; Chromogenix), a specific plasmin substrate. The ΔA⁰₂₅ was compared with values obtained from standard dilutions of recombinant SAK (sakSTAR, a kind gift of Dr Desire Collen, Center for Thrombosis and Vascular Research, Leuven, Belgium). The absorbance reading for surface-bound SAK was measured after 30 min and for secreted SAK after 2 h of incubation with plasminogen. The incubation time was adjusted to give similar sensitivity in the assays, allowing the detection of ≥0.3 μg SAK ml⁻¹. Samples with absorbances below this level were considered negative. SAK levels ranging between 0.3 and 5 μg ml⁻¹ were arbitrarily assessed as low production and above 5 μg ml⁻¹ as high production. S. aureus strain Newman was used throughout as a positive control.

**Statistical methods.** The type of SAK production was assessed as none (i.e. below the detection level), surface-bound SAK alone or a combination of surface/secreted SAK. We looked at SAK production in a variety of ways. Our initial comparison of SAK production for both surface and secreted SAK was conducted by comparing the absorbances for the three clinical groups using Wilcoxon’s rank sum test. Following this, the frequency of SAK-producing S. aureus isolates within the groups was assessed by pairwise comparison. The comparisons were performed between the patients and controls, invasive cases and uncomplicated cases and uncomplicated cases and controls. For each of these comparisons, a χ² test was used.

Logistic regression analysis was also performed using case status (invasive or uncomplicated bacteremia) as the outcome and secreted SAK as an independent variable. Because the proportionality assumption was not met, secreted SAK was transformed into an indicator variable with three levels to fit the logistic model: <0.3 μg ml⁻¹ (none), 0.3–5 μg ml⁻¹ (low), >5 μg ml⁻¹ (high). To examine whether secreted SAK was a predictor of case status, a logistic model with case status as the outcome and secreted SAK was fitted. Furthermore, to determine whether the association between secreted SAK and outcome was independent of known risk factors for poor outcome, a logistic model with case status as the outcome and race, sex, APACHE II score, presence of methicillin-resistant S. aureus (MRSA) and secreted SAK was fitted. Backward selection was used to eliminate variables.

For the examination of surface SAK, a logistic model with case status as the outcome and a binary indicator of surface SAK as the independent variable was fitted. As with secreted SAK, a multivariate logistic model with case status as the outcome and race, sex, APACHE II score, presence of MRSA and secreted SAK was fitted and backward selection was used to eliminate variables.

<table>
<thead>
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<th>RESULTS</th>
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<td><strong>Clinical and demographic characteristics of patient groups</strong></td>
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Patients with complicated bacteremia leading to death were more often over 65 years of age, more frequently females, more frequently infected with MRSA strains and had significantly higher APACHE II scores (Table 1).

Among the 56 patients with invasive S. aureus bacteremia, the following complications were identified: infective endocarditis (27 patients, 48 %), deep tissue abscesses (11, 20 %), meningitis (6, 11 %), septic arthritis (6, 11 %), empyema (5, 9 %), epidural abscess (5, 9 %), septic thrombophlebitis (4, 7 %), vertebral osteomyelitis (3, 5 %), pacemaker pocket infection (2, 4 %) and mycotic aneurysm (1, 2 %). According to study definitions, all patients with
invasive *S. aureus* bacteraemia died of their infection. All patients with uncomplicated bacteraemia had intravascular catheter-associated *S. aureus* bacteraemia, received 14 days or less of intravenous antibiotics and had no complications attributable to *S. aureus* at the time of the initial bacteraemia or at 12-week follow-up. Nasal strains were obtained from non-medical university students in their second decade of life.

**SAK production within the tested clinical isolates**

SAK production was detected in 93 of 161 (58 %) *S. aureus* strains tested (Table 2). This SAK production consisted of SAK being attached to the bacterial surface, referred to as surface-bound, and SAK being released into the fluid phase. Isolates with detectable surface-bound SAK also produced soluble SAK (Spearman’s correlation coefficient for surface-bound and soluble SAK, *r* = 0·71). Discordance between SAK production was observed in only 18 % (29/161) of *S. aureus* isolates, equally often in the patient and in the control groups. In all these discordant cases, isolated surface-bound SAK expression was present.

**Table 1.** Demographic characteristics of patients with uncomplicated *S. aureus* bacteraemia (*n* = 57) and with invasive *S. aureus* infection (*n* = 56)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Uncomplicated <em>S. aureus</em> bacteraemia</th>
<th>Invasive <em>S. aureus</em> bacteraemia</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt;65 years</td>
<td>17 (30 %)</td>
<td>33 (59 %)</td>
<td>0·0018</td>
</tr>
<tr>
<td>Male sex</td>
<td>37 (65 %)</td>
<td>26 (46 %)</td>
<td>0·0479</td>
</tr>
<tr>
<td>White race</td>
<td>41 (72 %)</td>
<td>35 (63 %)</td>
<td>0·3475</td>
</tr>
<tr>
<td>MRSA strains</td>
<td>14 (25 %)</td>
<td>34 (61 %)</td>
<td>0·0001</td>
</tr>
<tr>
<td>APACHE II score:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 10</td>
<td>25 (43 %)</td>
<td>3 (5 %)</td>
<td>0·0001</td>
</tr>
<tr>
<td>10–16</td>
<td>17 (30 %)</td>
<td>11 (19 %)</td>
<td>NS</td>
</tr>
<tr>
<td>16–21</td>
<td>11 (19 %)</td>
<td>15 (27 %)</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;21</td>
<td>4 (7 %)</td>
<td>27 (48 %)</td>
<td>0·0001</td>
</tr>
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</table>

NS, Not significant.

**Table 2.** SAK production in patients with uncomplicated *S. aureus* bacteraemia (*n* = 57) and invasive *S. aureus* infection (*n* = 56) and healthy nasal carriers (*n* = 48)

<table>
<thead>
<tr>
<th>Type of SAK production</th>
<th>Nasal <em>S. aureus</em></th>
<th>Uncomplicated <em>S. aureus</em></th>
<th>Invasive <em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted only</td>
<td>0</td>
<td>1 (1·7 %)</td>
<td>0</td>
</tr>
<tr>
<td>Surface only</td>
<td>11 (22·9 %)</td>
<td>12 (21·1 %)</td>
<td>6 (10·7 %)</td>
</tr>
<tr>
<td>Combined (surface/</td>
<td>21 (43·8 %)</td>
<td>26 (45·6 %)</td>
<td>16 (28·6 %)</td>
</tr>
<tr>
<td>secreted)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0·3–5 μg ml⁻¹)</td>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>High (&gt;5 μg ml⁻¹)</td>
<td>20</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>16 (33·3 %)</td>
<td>18 (31·6 %)</td>
<td>34 (60·7 %)</td>
</tr>
</tbody>
</table>
illness, comorbid conditions and infection with MRSA, individuals infected with non-SAK-producing *S. aureus* isolates were 4-3 times more likely to have lethal bacteraemia compared with individuals infected with *S. aureus* secreting high levels of SAK (>5 μg ml⁻¹). By contrast, the presence of combined surface-bound and secreted SAK production was eliminated from a multivariate model containing APACHE II score and infection with MRSA (OR = 2.65, P = 0.080).

**DISCUSSION**

The findings of the present study demonstrate an association between *in vitro* SAK production among *S. aureus* bloodstream isolates and the clinical outcome of patients from whom these isolates were obtained. After adjusting for confounding host and pathogen-specific characteristics, patients with *S. aureus* bacteraemia due to non-SAK-producing isolates were significantly more likely to die of their infection than patients with bacteraemia due to *S. aureus* isolates that produced SAK. This inverse correlation between SAK production and *S. aureus* mortality has interesting implications for our understanding of the host–pathogen interaction.

Lack of SAK production in staphylococci invading internal organs is unexpected, especially in view of a previously suggested proteolytic mechanism of harbouring of *S. aureus* in tissues (Lahteenmaki et al., 2001; Christner & Boyle, 1996). On the other hand, expression of SAK is followed by excessive plasmin formation. Plasmin-mediated degradation of extracellular matrix deprivies bacteria of the necessary adherent surface (Foster & Hook, 1998; Josefsson et al., 2001) and, thereby, decreases their invasive pathogenic potential. The high frequency of SAK production in nasal isolates suggests that production of SAK is an adaptive bacterial mechanism favourable for its symbiosis with the host. Expression of SAK may be an important property for *S. aureus* colonization of mucosal tissue. Nasal carriage of *S. aureus* is considered to be one of the major risk factors for a generalized staphylococcal infection (Harbarth et al., 2000; von Eiff et al., 2001). We observed that production of SAK is a feature that differentiates nasal isolates from staphylococci that invade internal organs. We have shown recently that SAK interacts with and neutralizes bactericidal peptides from human azurophilic granules (T. Jin, J. Mitchell, J. Higgins, T. Foster, A. Tarkowski and M. Bokarewa, submitted for publication), protecting *S. aureus* from the host innate immune system and facilitating bacterial survival.

There were several limitations to this investigation. It is possible that investigator awareness of the clinical source of infection may have biased the results. However, all isolations were performed at a separate site by investigators blinded to all clinical data. To guard further against analytical bias, all statistical investigations were also performed at a site separate from the source of the clinical isolates. Patient or bacterial characteristics other than SAK production may also have confounded these results. Despite adjustment of the patient groups for severity of illness and infection with MRSA, we could not exclude the possibility that these two factors made a major contribution to the patient outcome of septicemia.

The SAK gene-containing phage is inserted into the β-toxin gene, causing the loss of β-haemolysin expression. Thus, the increased mortality of bacteraemia with SAK-deficient isolates may be partly due to β-toxin production by these isolates. This suggestion seems less probable in view of recent studies that have shown a negligible impact of β-haemolysin on the virulence of staphylococcal strains (Nilsson et al., 1999; Dajcs et al., 2002). Additionally, we cannot exclude the possibility of a ‘hitchhiker effect’, in which the gene for SAK production is near an unknown gene that results in the observed differences.

Our study suggests an association between reduced SAK production *in vitro* among *S. aureus* bloodstream isolates and a worse overall clinical outcome in patients with *S. aureus* bacteraemia, indicating that important phenotypic differences exist in *S. aureus* isolates from different clinical settings. Keeping in mind that infection often occurs with one’s own strains, determination of SAK production is a valuable factor to predict the potential risk of invasive infection among nasal staphylococcal carriers. Future investigations are necessary in order to validate this observation and to determine whether such clinically relevant differences in pathogen phenotype are due to selective expression in response to differing conditions confronting the infecting isolate or to constitutive differences in the bacterial genotype.

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**REFERENCES**


