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Involvement of staphylococcal protein A and cytoskeletal actin in *Staphylococcus aureus* invasion of cultured human oral epithelial cells

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Following the coincidental discovery that β -actin isolated from renal epithelial cells was precipitated by staphylococcal protein A (SPA), the possibility that SPA and cytoskeletal actin filaments may be involved in *Staphylococcus aureus* infection of epithelial cells was considered. Therefore, to clarify the potential role of SPA and actin filaments in *S. aureus* infection, the invasion efficiency of *S. aureus* was determined quantitatively by measuring the number of cfu of viable organisms recovered from cultured KB cells. *S. aureus* invasion was found to be time dependent (0–60 min) and increased linearly when increasing numbers of bacteria were added (10^4 – 10^6 cfu/ml). However, significant variation in the level of invasion was noted in protein A-deficient *S. aureus* Wood 46. Cytochalasin B inhibited the invasion efficiency of *S. aureus* in a dose-dependent manner. The present study suggests that interaction of staphylococcal protein A and cytoskeletal actin filaments is involved in the *S. aureus* invasion of cultured KB cells, and this process may contribute, in part, to the intracellular movement, cell-to-cell spread and dissemination of *S. aureus* within human oral epithelial cells *in vivo*.

Introduction

While *Staphylococcus aureus* has not traditionally been considered to be an intracellular pathogen, previous studies have revealed that *S. aureus* cells may be actively internalised by phagocytosis and are capable of intracellular survival in epithelial [1] and endothelial cells [2, 3]. It has also been suggested that *S. aureus* cells interact or bind with the cell surface of bovine mammary epithelial cells [1] and are able to invade and appear free in the cytoplasm of cultured chicken osteoblast cells [4]. However, little is known of the mechanisms involved in the internalisation of *S. aureus* by the host cells.

On the other hand, it has been suggested that the continuous process of actin filament elongation provides the driving force for bacterial propulsion in infected cells or cytoplasmic extracts [5–7]. Several

unrelated intracellular bacterial pathogens, including *Listeria monocytogenes*, *Shigella flexneri*, rickettsiae and vaccinia virus, are known to share the ability to use actin polymerisation as a driving force for intracellular movement, cell-to-cell spread and dissemination within infected tissues.

Staphylococcal protein A (SPA) is used in a variety of immunological studies by taking advantage of its affinity for the constant (Fc) region of immunoglobulin, because type I Fc receptors are represented in *S. aureus* [8]. Recently, we found that SPA binds to cytoskeletal β -actin obtained from renal epithelial cells (Fig. 1) (unpublished observation). In an immunoprecipitation study with SPA-immobilised Sepharose 4B (Pharmacia Biotech), a protein (*c.* 43 kDa in SDS-PAGE) was precipitated by SPA and subjected to Lys-digestion to identify its amino-acid sequence. The SwissProt protein database revealed that the sequences obtained matched those of human β -actin cytoskeletal protein. This incidental finding led to the conclusion that the binding of SPA to mammalian epithelial actin filaments may play a role in the internalisation or dissemination of *S. aureus*, or both. To clarify this hypothesis, the

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Human β -actin: LTERGYSFTTTAEREIVRDIK
Obtained: LTERGYSFTTTAEREIVRDIK

Fig. 1. Interaction of staphylococcal protein A with cytoskeletal actin derived from renal epithelial cells. Cell lysates were precipitated with protein A-immobilised Sepharose-4B, and precipitated proteins were electrophoresed in SDS-PAGE 10%, blotted on polyvinylidene difluoride membrane. The amino-acid sequencing of the protein (*c.* 43 kDa) was determined by the Lys-digestion method. Lane **A**, staphylococcal protein A plus rabbit antibody; **B**, staphylococcal protein A alone.

present study examined the potential interaction of SPA and cytoskeletal actin in *S. aureus* infection of cultured human oral epithelial cells.

Materials and methods

Bacterial strains and growth conditions

S. aureus ATCC 25923 was purchased commercially. *S. aureus* Wood 46 was kindly supplied by Dr Hideo Igarashi (Tokyo Metropolitan Research Laboratory for Public Health). Before each experiment, a single colony from a blood agar plate (Difco Laboratories, Detroit, MI, USA) was inoculated into a Brain Heart Infusion (BHI, Difco Laboratories) broth and grown overnight at 37°C with vigorous shaking. From this culture, 100 μ l were transferred into 10 ml of BHI broth and incubated for 8 h at 37°C with vigorous shaking. The overnight culture was centrifuged and the pellet was washed once with sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in the tissue-culture medium (without antibiotics; see below) to give a cell density of 10^5 cfu/ml unless otherwise specified.

Cell culture

An established human oral epidermoid carcinoma cell line, designated KB 100, was used. The KB cells were grown routinely in monolayers in Eagle's Minimum Essential Medium (EMEM, Gibco BRL, Grand Island, NY, USA) supplemented with heat-inactivated fetal bovine serum 10%, penicillin G 100 U/ml and streptomycin sulphate 100 μ g/ml. Before use, cells were seeded at 5×10^4 cells/well in 24-well tissue

culture plates (Costar, Cambridge, MA, USA) for binding and invasion assays and grown into confluent monolayers for 2 days in air at 37°C with CO₂ 5%.

Adherence and invasion assay

For bacterial adherence and invasion in cultured monolayers of KB cells, the methods of Isberg and Falkow [9] and Bayles *et al.* [1] were used with some modifications. Briefly, *c.* 16 h before beginning the experiment, the KB cells were washed three times with invasion medium (growth medium without serum and antibiotics) and held in this medium. Just before beginning the experiment, the medium was removed and the KB cell monolayers were washed once with invasion medium followed by a further addition of 1 ml of fresh invasion medium. Appropriate wells of KB cells were inoculated with 1 ml of invasion medium containing 10^5 cfu/ml/well (or other doses) of bacteria for the specified times at 37°C in air with CO₂ 5%. Subsequently, the medium was removed from the infected monolayers, before washing three times with sterile Ca²⁺- and Mg²⁺-free PBS to remove non-adherent bacteria. The KB cell monolayers were treated with trypsin 0.25% in Hanks's Balanced Salts Solution (Gibco BRL) and further lysed with Triton X-100 (Sigma) 0.025% in sterile distilled water. Cell lysates were serially diluted 20-fold and plated in triplicate on blood agar plates; the plates were then incubated overnight at 37°C and the cfu were counted. At this time, colonies of *S. aureus* were identified by Gram's staining, catalase and coagulase tests. To quantify membrane-adherent and intracellular bacteria, gentamicin was used to kill any extracellular bacteria [10]. After the KB cell monolayers had been incubated with bacteria for the specified times at 37°C in air with CO₂ 5%, supernates were removed and replaced with 1 ml of medium containing gentamicin (Gibco BRL), 100 μ g/ml followed by incubation at 37°C with CO₂ 5%. After 2 h, the supernates were removed and discarded. The KB cell monolayers were washed three times with sterile PBS, and any intracellular bacteria were recovered and counted as described previously.

Treatment with cytochalasin

To examine whether inhibition of bacterial invasion by cytochalasin B (CB) could occur, an invasion assay was done as described above, in the presence or absence of CB (Sigma). KB cell monolayers were incubated with normal growth medium containing CB at a given concentration for 30 min at 37°C in air with CO₂ 5%, followed by incubation of the KB cell monolayers with *S. aureus* (1×10^5 cfu/ml) in the presence of the same concentration of CB.

Electron microscopy

Transmission electron microscopy (TEM) was used to visualise *S. aureus* invasion of KB cells. The invasion

assay remained similar except that gentamicin was not added to the co-culture. The procedure for tissue processing was slightly modified from the procedures of Bayles *et al.* [1]. The infected cells were placed in glutaraldehyde 3% in 0.1 M cacodylate buffer containing sucrose 6%. Cells were post-fixed with osmium tetroxide 2% for 1 h. Cells were then scraped off the culture flask, centrifuged briefly and embedded in agar 1.5%. Small cubes of the agar were dehydrated, embedded in Epon/Spurrs resin mixture; 100-nm sections were cut with glass knives. After counter-staining with uranyl acetate 0.5% and lead citrate 1.0%, sections were examined by transmission electron microscopy (TEM; Zeiss, EM910).

Results

Effect of cell numbers and time on S. aureus invasion of KB cells

To measure the efficiency of *S. aureus* infection of oral epithelial cells, the effects of different cell numbers and time on *S. aureus* invasion of KB cell monolayers were first examined by measuring the numbers of bacterial cells adhering to cells or becoming intracellular. When monolayers were incubated for 30 min with different numbers of bacteria (1×10^4 , 10^5 and 10^6 cfu/ml), an increase in the inoculum size resulted in an increase in the number of cfu recovered (Fig. 2a). Longer incubation times resulted in an increase in the number of cfu recovered (Fig. 2b). The cfu recovered increased in a time-dependent manner and approached 28.6% by 60 min, i.e., the percentage of the original inoculum which became bound and invaded the host cell membrane. TEM confirmed these results and clearly showed intracellular *S. aureus* in cultured oral epithelial cells (Fig. 3). Furthermore, treatment with lysostaphin (20 and 40 μ g/ml) dramatically reduced

Fig. 3. Transmission electron microscopy of KB cells infected with *S. aureus* ATCC 25923. Photograph represents a 60-min co-culture of cell monolayers with *S. aureus* as described in the invasion assay (magnification $\times 10\,000$).

the numbers of *S. aureus* recovered from cell monolayers (data not shown).

S. aureus invasion of KB cells

Further studies were conducted to differentiate between *S. aureus* adhesion and invasion of KB cells by using gentamicin treatment to kill adherent bacteria. Bacterial invasion of KB cells was estimated by quantifying the number of intracellular bacteria protected from gentamicin killing. Monolayers were incubated with *S. aureus* (1×10^5 cfu/ml) for 30 min, followed by co-culture in the presence of gentamicin 100 μ g/ml with or without CB (0.1–5 μ g/ml) for 30 min (Fig. 4). CB decreased the numbers of *S. aureus* protected from

Fig. 2. Kinetics of *S. aureus* ATCC 25923 infection of KB cells. Monolayers were incubated with different numbers of bacteria for 30 min (a) or 10^5 cfu/ml for the specified times (b), followed by measurement of colony forming units (cfu) recovered from cell monolayers. Each point represents means and SEM from 8 or 10 determinations done in triplicate.

Fig. 4. Dose-response of cytochalasin B on *S. aureus* ATCC 25923 invasion of KB cells. Cell monolayers were cultured with *S. aureus* (10^5 cfu/ml) for 30 min and washed three times with Ca^{2+} - and Mg^{2+} -free PBS to remove non-adherent bacteria, followed by additional co-culture in the presence of gentamicin with or without various concentrations of cytochalasin B for 30 min. Each point represents means and SEM from 8 or 10 determinations done in triplicate.

gentamicin killing in a dose-dependent manner; CB $1 \mu\text{g/ml}$ caused *c.* 50% decrease of the initially inoculated numbers of *S. aureus* (25.7×10^3 cfu/ml). However, cytochalasin D (CD) did not significantly change the numbers of *S. aureus* protected from gentamicin killing (data not shown).

In further experiments, cell monolayers were inoculated with *S. aureus* 1×10^5 cfu/well for the specified times and co-cultured for 2 h in the presence of gentamicin with and without CB (Fig. 5a). The recovered cfu of *S. aureus* in the absence of CB increased in a time-dependent manner and approached 17.4% by 60 min. In comparison, CB inhibited the numbers of *S. aureus* recovered from KB cells within 20 min ($p < 0.01$).

To confirm these results by means of different strategies, the KB cell monolayers were exposed to *S. aureus* 1×10^5 cfu for 30 min and co-cultured for several different time periods in the presence of gentamicin with and without CB. As shown in Fig. 5b, the number of cfu of *S. aureus* recovered after exposure to gentamicin was decreased by co-culture, and the decrease in cfu was sustained from 30 min to 2 h ($p < 0.01$).

Effect of protein A on *S. aureus* invasion

To determine the role of SPA in *S. aureus* invasion of KB cells, cell monolayers were incubated with wild-type (ATCC 25923) and protein A-deficient *S. aureus*

Fig. 5. Inhibition of *S. aureus* ATCC 25923 invasion of KB cells by cytochalasin B. (a) Cell monolayers were incubated with *S. aureus* 10^5 cfu/ml for several different time intervals, followed by additional co-culture with (●) and without (○) cytochalasin B $1 \mu\text{g/ml}$ plus gentamicin for 2 h. (b) Cells were incubated with *S. aureus* 10^5 cfu/ml for 30 min, followed by additional co-culture with (●) and without (○) cytochalasin B ($1 \mu\text{g/ml}$) plus gentamicin for several time periods. Each point represents mean and SEM of cfu from eight separate experiments done in triplicate.

Wood 46 1×10^5 cfu/ml for the specified times, followed by co-culture with gentamicin for 2 h. As shown in Fig. 6, the numbers of strain ATCC 25923 recovered from cell monolayers increased in a time-dependent manner. However, the invasion efficiency – $(0.1\text{--}1.5) \times 10^3$ cfu/ml of *S. aureus* Wood 46 remained low throughout the incubation period. At 60 min, the numbers of strain Wood 46 recovered was only 7% of that of wild-type *S. aureus* ATCC 25923.

The invasion efficiency of *S. aureus* for cultured KB cells was estimated by determining bacterial numbers protected from gentamicin killing for 2 h [11]. As shown in Table 1, invasion efficiency increased during incubation; CB $1 \mu\text{g/ml}$ dramatically reduced its invasion efficiency. The invasion efficiency of protein A-deficient *S. aureus* Wood 46 was much lower than that of wild-type *S. aureus*.

Discussion

The present study provides evidence that SPA and cytoskeletal actin filaments feature in *S. aureus* invasion of human oral epithelial cells. KB cells were used as a model for gingival epithelial cells because they are known to be suitable for investigating

bacterial interactions *in vitro* [11, 12]. The results suggest that cytoskeletal actin in epithelial cells binds to SPA of *S. aureus*; *S. aureus* may adhere to and invade oral epithelial cells through interaction with SPA and actin filaments and *S. aureus* internalisation by oral epithelial cells may be mediated by polymerisation of actin filaments, which is a CB-sensitive process.

An immunoprecipitation assay with SPA-immobilised Sepharose 4B found that SPA precipitates a protein (*c.* 43 kDa) which has a high homology with human β -actin. This *in-vitro* finding suggests that SPA has a binding affinity with cytoskeletal actin filaments of mammalian epithelial cells *in vivo*, and this process may be involved in the pathogenesis and invasion or internalisation, or both, of *S. aureus*. Although there is no direct evidence suggesting that either mammalian actin filaments contain an SPA-binding domain, or that actin receptors are found in the surface of *S. aureus*, it is possible that the binding of SPA and actin filaments may regulate *S. aureus* infection of mammalian epithelial cells. This suggestion may be related, in part, to the previous report that intracellular pathogens develop a common mechanism to exploit the actin cytoskeleton as a means of facilitating their direct spread between cells [13].

Fig. 6. Efficiency of *S. aureus* ATCC 25923 (○) and Wood 46 (●) invasion of KB cells. Monolayers were infected with 10^5 cfu/ml/well of each strain for specified times, followed by additional co-culture for 2 h in the presence of gentamicin. Each point represents mean and SEM of cfu from eight separate experiments carried out in triplicate.

Table 1. Comparison of invasion efficiency of *S. aureus* strains ATCC 25923 and Wood 46 (10^3 cfu) for cultured KB cells

Culture conditions	Intracellular bacteria (10^3 cfu/ml) after incubation for (min)			
	10	20	30	60
<i>S. aureus</i> ATCC 25923 before gentamycin killing	2.1 (0.22)	5.2 (0.85)	9.4 (1.37)	28.6 (2.12)
after gentamycin killing				
without cytochalasin B	0.3 (0.06)	1.3 (0.34)	4.2 (1.19)	17.4 (2.07)
with cytochalasin B	0.04 (0.01)	0.08 (0.01)	0.2 (0.03)	3.4 (0.59)
<i>S. aureus</i> Wood 46 after gentamycin killing				
(without cytochalasin B)	0.01 (0.01)	0.04 (0.01)	0.3 (0.08)	1.5 (0.48)

KB cell monolayers were incubated with 10^5 cfu/ml of bacteria for each time period and washed three times with Ca^{2+} - and Mg^{2+} -free PBS, followed by additional co-culture with gentamicin $100 \mu\text{g/ml}$ for 2 h and the numbers of cfu protected from gentamicin killing were counted. Values represent the means (ZM) SE of 8–10 determinations made in triplicate

Bacterial adhesion is typically mediated through the interaction of bacterial ligands with the surface characteristics of host cells [14–16]. For example, extracellular matrix molecules, i.e., fibronectin, vitronectin, laminin and type IV collagen, can participate in the normal colonisation of sites by micro-organisms and in invasion during infections [17–19]. The role of cytoskeletal molecules, i.e., actin filaments, in invasion and internalisation of micro-organisms in mammalian cells has also been demonstrated [6, 7, 13, 19–21]. The invasion efficiency of protein A-expressing and -deficient *S. aureus* strains was significantly different. These differences suggest that unknown *de novo* processes may be involved in *S. aureus* infection in mammalian epithelial cells.

It has been shown that inhibition of actin elongation by CB [21, 22] concomitantly significantly inhibited the invasion of cultured KB cell monolayers by *S. aureus*. A similar result was also reported in renal epithelial cells [22]. It is possible that CB did not directly inhibit *S. aureus* invasion of cell monolayers, because the concentration of CB used in the present study had no apparent inhibitory effect on endocytosis of other micro-organisms [23]. Several pathogenic intracellular bacteria, including *L. monocytogenes*, *Shigella* and *Rickettsia* spp., use host cell actin cytoskeleton for propulsion [20, 23, 24]. These suggestions indicate that protein A may be necessary for binding of *S. aureus* to cytoskeletal actin filaments of KB cells.

It is generally considered that several factors, including the infective dose, regulate bacterial invasion of mammalian cells, i.e., high levels of infecting inocula are required to elicit invasion by micro-organisms that produce more chronic infections [10]. This seems to suggest that any bacterium may invade mammalian cell monolayers, if the infecting inoculum is sufficiently high. However, in the present study it was observed that the invasion efficiency of *S. aureus* Wood 46 was not increased, even when the bacterial inoculum level was increased (Fig. 6). A similar phenomenon has been observed in *Haemophilus arophilus* in cultured human oral epithelial cells [11]. In cultured bovine aortic endothelial cells, *S. aureus*-induced cytotoxicity depended on the size of inoculum and the length of incubation [3]. This may be due to intracellularisation of *S. aureus* and is consistent with the results obtained here (Fig. 2). No cytotoxicity induced by treatment of KB cells with *S. aureus* for 120 min was observed in the MTT assay (data not shown). The need for a high infective dose for invasion may indicate a different invasion tactic. The kinetics of *S. aureus* adhesion/invasion to KB cells revealed that a lag period occurred, suggesting that a factor or component involved in triggering invasion may have to attain a critical concentration for optimal invasion to occur. Furthermore, possible factors involved in *S. aureus* invasion may include bacterial and cellular metabolites or cell-surface receptors that have not been identified as yet.

In conclusion, this study has demonstrated that SPA has a binding affinity for epithelial actin filaments, and this is involved in the regulation of *S. aureus* invasion and internalisation by oral epithelial cells. Elongation of actin filaments by their polymerisation may mediate this process and thereby provide a novel regulatory role in *S. aureus* infection of oral epithelial cells. Similar types of in-vivo modulation caused by staphylococcal protein A and epithelial actin cytoskeleton may contribute to the pathogenesis of *S. aureus* infections.

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