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Early *Staphylococcus aureus*-induced changes in endothelial barrier function are strain-specific and unrelated to bacterial translocation

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ABSTRACT

The vascular endothelium provides the critical barrier during hematogenous spreading of bacteria, a phenomenon that might contribute to severe diseases in humans including endocarditis and sepsis as known from infections by *Staphylococcus aureus*. Here we aimed to uncover early responses of the endothelium to *S. aureus* infection with respect to (a) inflammatory reactions such as paracellular endothelial barrier function and expression of cell adhesion molecule-1 (ICAM-1) and (b) translocation through the endothelium. After infection of the cultured endothelium with 22 different clinical isolates of *S. aureus* and two well-characterized lab strains a diverse and strain-specific change in para- and transcellular endothelial barrier function was observed. Bayesian data analysis revealed positive correlation of paracellular barrier function decrease followed by expression of ICAM-1 while these parameters negatively correlated with transcellular bacterial translocation. Translocating bacteria largely blocked TNFα-induced ICAM-1 expression indicating an active anti-inflammatory effect mediated by those strains probably due to intracellularly released virulence factors. Furthermore, the underlying background of barrier function decrease was investigated in more detail using two well-characterized lab strains, ls 8325-4 and ls 6850 and respective mutants. Barrier function decrease was found to be independent of early cell death and early release of virulence factors into the medium, but require internalization of live bacteria. The data show for the first time that endothelial cells respond diversely to infection with different strains of *S. aureus* and that translocating strains downregulate inflammatory response of the endothelium. Furthermore, data indicate that *S. aureus*-mediated activation of the endothelium reduces bacterial translocation.

Introduction

Systemic bacterial spread, an important pathophysiological parameter in severe infections, mostly occurs through the vascular system and requires bacterial translocation through the endothelial barrier. Endothelial barrier consists of individual endothelial cells that are connected by intercellular junctions, forming a functionally competent cell layer between the vascular system and the interstitial tissue. Bacterial spread through the endothelium might take place by direct or indirect opening of interendothelial cell junctions and/or transcellular through the endothelial cell body (Schnittler and Preissner, 2009; Chavakis et al., 2005; Bazzoni and Dejana, 2004). *S. aureus* is a Gram-positive bacterium causing a variety of infections ranging from minor skin infections to severe invasive diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, and sepsis (Lowy, 1998). *S. aureus*-caused bacteraemia might result in septic dissemination with formation of secondary infectious foci throughout the body (Cinel and Dellinger, 2007) and might lead to sepsis (Hetem et al., 2011). Spread of *S. aureus* through the endothelium (Edwards and Massey, 2011; Weidenmaier et al., 2004; Sheen et al., 2010) might be facilitated by opening of cell junctions in response to *S. aureus* α-toxin (Hocke et al., 2006), the formation of macroapertures, structures that develop in response to *S. aureus*-derived epidermal cell differentiation inhibitor (EDIN) via RhoA inhibition (Boyer et al., 2006), or induction of apoptosis as demonstrated for hemolytic invasive strains including the lab strain (ls) *S. aureus* 6850 (Haslinger-Loffler et al., 2005). A rapid interaction of *S. aureus* with the endothelium was demonstrated in vivo and in vitro. In particular, intravascularly injected *S. aureus* was found to adhere to the endothelium of postcapillary venules within 5 min (Kerdudou et al., 2006), appear in peripheral organ tissue within 3 h and be totally cleared from the blood within 6 h (Cheng et al.,

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2009). Furthermore, *S. aureus* internalization has been reported in cell culture models to occur within 10 min postinfection, and 80% of EC-bound bacteria are found intracellularly within 2 h (Schröder et al., 2006). However, the early endothelial responses to *S. aureus* infections and the underlying mechanisms have to be unraveled, in particular with respect to the entire endothelial barrier function.

Adhesion to and internalization of *S. aureus* in endothelial cells in vivo (Kerdudou et al., 2006) and in vitro (Tompkins et al., 1992; von Eiff et al., 1997; Que et al., 2005) can be mediated by fibronectin-binding proteins A and B (FnBPA and FnBPB) that bridge to $\alpha5\beta1$ integrin of the target cells, and seems to be critical for the virulence in a number of animal and cell culture models (Sinha et al., 1999; Sinha and Fraunholz, 2010). Apart from FnBPs that are expressed by the majority of *S. aureus* isolates (Minhas et al., 1995), invasion can also be promoted by other adhesins, such as Eap (Harraghy et al., 2003; Haggar et al., 2003) and AtlA (Hirschhausen et al., 2010). Purified FnBPs were shown to cause endothelial activation (Heying et al., 2007, 2009), a response characterized by expression of P- and E-selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and the release of pro-inflammatory mediators that together cause amplification of inflammation (Hunt and Jurd, 1998). In contrast, many *S. aureus* strains are able to prevent activation, e.g. through the immunomodulatory function of the Eap protein (Harraghy et al., 2003; Athanasopoulos et al., 2006), a mechanism that allows systemic spreading of the bacteria before the immune response takes place.

Here we addressed the unsolved question of whether diverse strains of *S. aureus* modulate the entire endothelial barrier function early in infection.

Materials and methods

Cell culture, bacteria, and infection procedure

Endothelial cells were isolated from human umbilical veins (HUVEC) as described elsewhere (Schnittler et al., 1990) according to the principles outlined in the Declaration of Helsinki; the samples were treated anonymously; the procedure was approved by the ethics boards of the WW-University of Muenster (2009-537-f-S). The mothers of the donors were asked before delivery for agreement using the umbilical cords for research. Cells were cultured on cross-linked gelatin-coated cell culture supports as described elsewhere (Schnittler et al., 1990) in endothelial growth medium (EGM) (Promocell, Heidelberg, Germany) containing 100 μg/ml penicillin and 100 μg/ml streptomycin (PAA, Pasching, Austria), antibiotics that are highly efficient against extracellular bacteria, but has a much less profound effect on intracellularly located bacteria (Brunius et al., 1978). This medium is further referred to as EGM with antibiotics. Only cells of the first passage were used for experiments. Growth medium was replaced with antibiotic-free medium 12–18 h prior to infection.

Bacterial strains are listed in Table 1. Bacteria were cultivated as described elsewhere (Sinha et al., 2000). Briefly, bacteria were grown overnight in 15 ml tryptic soy broth (TSB) medium with aeration, centrifuged at 4 °C for 6 min at 4000 × *g*, washed twice with phosphate-buffered saline (PBS), and resuspended in 2 ml EGM without antibiotics. Bacteria at the indicated multiplicity of infection (MOI) were added to the HUVEC cultures and incubated at 37 °C, 5% CO_2.

To investigate the release of bacterial products, 10^7 bacteria were inoculated in 1 ml of EGM medium without antibiotics for 3 h or 5 h at 37 °C, 5% CO_2. Subsequently, bacteria were centrifuged at 4000 × *g* for 5 min and the supernatant was filter-sterilized before use. For preparation of the heat-inactivated bacteria, 10^7 bacteria were resuspended in 200 μl sterile PBS and incubated at 55 °C for 2 h. For formaldehyde fixation bacteria were resuspended in 1% formaldehyde/PBS after pelleting, incubated for 1 h, washed 3 times with PBS, and resuspended in 200 μl EGM without antibiotics. Heat- and formaldehyde treated preparations were further tested for total bacterial inactivation by plating.

Measurement of transendothelial electrical resistance (TER)

TER was determined by impedance spectroscopy equipped with TER analytical software (MOS-Technologies, Telgte, Germany) (Seebach et al., 2000). Briefly, HUVEC were cultured in measuring chambers to confluence and TER was automatically and continuously recorded. To compare the different changes in TER of different primary HUVEC cultures, the ΔTER according to the formula $\Delta TER = 1 - TER/TER0$ was calculated. TER/TER0 is the ratio between TER at a given time point and TER at the time point zero. Prior to infection, the bacterial suspension in EGM without antibiotics was warmed to 37 °C and added to the HUVEC at an MOI as indicated and incubated further at 37 °C, 5% CO_2.

Cell viability assay

Cells were grown in gelatin-coated 6-well culture plates (Greiner Bio-One, Kremsmünster, Austria) to confluence. Cells were infected at an MOI of 50 and incubated for 3 h. As a positive control, 1 mM H_2O_2 was applied to HUVEC cultures for 2 h; these were then washed with EGM and incubated for a further 6 h. For further control of apoptosis induction HUVEC were infected at a MOI of 50 with ls 6850 for 3 h followed by medium exchange and treatment with 20 μg/ml lysostaphin for 30 min. Cells were washed again with EGM with antibiotics and further cultured for additional 21 h. Subsequently, HUVEC were harvested by accutase treatment according to the manufacturer's instructions (PAA, Pasching, Austria) and centrifuged at 250 × *g* for 4 min. Pellets were washed with annexinV binding buffer, stained with annexinV-fluoresceine isothiocyanate (FITC) according to the manufacturer's instructions (Immunotools, Friesoythe, Germany), washed 3 times, resuspended in 100 μl of annexinV binding buffer, and subsequently incubated in 10 μg/ml propidium iodide for 5 min. Immediately after staining, cells were analyzed with a FACSCalibur instrument and CellQuestPro software (Beckton Dickinson, San Jose, USA). Quadrant statistics were used to determine the percentage of (a) double-positive (late apoptotic and necrotic), (b) only annexinV-positive (early apoptotic) and (c) double-negative cells (viable cells) (Vermes et al., 1995).

Labeling of bacteria and internalization assay

For FITC labeling bacteria suspensions were firstly fixed by 1% formaldehyde/PBS as described above and 10^9 bacteria were labeled with 200 μg/ml FITC (Sigma, Deisenhofen, Germany) in PBS for 30 min, washed twice with PBS, pelleted and resuspended in 500 μl EGM without antibiotics. OD_{540} was determined and subsequently adjusted to value 1 by addition of EGM. 100 μl of this suspension were added to HUVEC grown in gelatin-coated 12-well culture plates. After 3 h cells were harvested by accutase treatment (see below), washed with PBS and labeled treated with 10 μg/ml propidium iodide. Subsequently, fluorescence intensity of internalized bacteria was analyzed by flow cytometry using CyFlow Cube 8 instrument (Partec, Münster, Germany) and FCS software (De Novo Software, Los Angeles, USA). Internalization rate was calculated from the ratio between mean fluorescence of the infected cells to the mean fluorescence of untreated cells. Internalization ratio of *S. aureus* 8325-4 was set to 100%.