ADAM10 Mediates Vascular Injury Induced by *Staphylococcus aureus* α-Hemolysin

Michael E. Powers, Hwan Keun Kim, Yang Wang, and Juliane Bubeck Wardenburg

1Department of Microbiology, and 2Department of Pediatrics, The University of Chicago, Illinois

*Staphylococcus aureus* is a leading cause of bacteremia and sepsis. The interaction of *S. aureus* with the endothelium is central to bloodstream infection pathophysiology yet remains ill-understood. We show herein that staphylococcal α-hemolysin, a pore-forming cytotoxin, is required for full virulence in a murine sepsis model. The α-hemolysin binding to its receptor A-disintegrin and metalloprotease 10 (ADAM10) upregulates the receptor’s metalloprotease activity on endothelial cells, causing vascular endothelial–cadherin cleavage and concomitant loss of endothelial barrier function. These cellular injuries and sepsis severity can be mitigated by ADAM10 inhibition. This study therefore provides mechanistic insight into toxin-mediated endothelial injury and suggests new therapeutic approaches for staphylococcal sepsis.

Bacteremia and septic shock account for nearly 80% of invasive disease caused by methicillin-resistant *Staphylococcus aureus* [1]. The vascular endothelium functions as a potent barrier between the bloodstream and the tissues, preventing bacterial dissemination and contributing to homeostatic regulation of blood flow and coagulation. In the context of sepsis, both proinflammatory mediators and pathogen-induced endothelial injury impair vascular integrity, permitting extravasation of fluid, proteins, and cells into the tissues. Injured endothelial cells contribute to systemic inflammatory responses and serve as a nidus for microvascular thrombosis. Together, the triad of endothelial barrier disruption, profound inflammation, and impaired microvascular blood flow with coagulopathy culminate in impaired tissue oxygen delivery, a hallmark of sepsis [2]. As mortality from bacterial sepsis remains high, it is essential to better understand the molecular pathogenesis of this disease.

Staphylococcal α-hemolysin (Hla), a pore-forming cytotoxin secreted by almost all strains of *S. aureus*, directly contributes to endothelial injury [3, 4]. Recently, we defined A-disintegrin and metalloprotease 10 (ADAM10) as a eukaryotic receptor for Hla [5]. ADAM10 is a widely expressed zinc metalloprotease that principally regulates cellular adhesion and migration [6]. In the endothelium, ADAM10-mediated proteolysis of the extracellular domain of vascular endothelial (VE)–cadherin disrupts cadherin-dependent homotypic intercellular interactions [7]. As VE-cadherin provides the structural framework of the adherens junction, ADAM10 plays an important role in the regulation of endothelial permeability.

The physiologic importance of VE-cadherin in endothelial barrier integrity during infection is highlighted by London et al [8] wherein enhanced expression of VE-cadherin conferred protection against lethal polymicrobial or influenza A–induced sepsis. Previous studies have shown that Hla induces loss of VE-cadherin expression on the endothelial surface and compromises endothelial barrier function in vitro [4, 9, 10]. The molecular mechanism by which this occurs has yet to be determined. We hypothesized that Hla may contribute to lethal *S. aureus* sepsis through its ability to directly injure the endothelium, and that ADAM10 may provide the molecular link by which the toxin causes barrier disruption.

**METHODS**

Recombinant Hla and HlaΔ135L, a mutant that demonstrates normal cell binding but is incapable of heptamer pore formation [11], were prepared and purified as described elsewhere [5]. Human pulmonary artery endothelial cells (HPAECs; Lonza) were cultured in Endothelial Basal Medium-2 (EBM-2) BulletKit media (Lonza). *Staphylococcus aureus* strains were described elsewhere and propagated in tryptic soy broth (TSB) [12]. Anti-human VE-cadherin antibody (Santa Cruz Biotechnology) and Alexa Fluor–conjugated secondary antibodies (Invitrogen) were used according to the manufacturer’s protocols for LI-COR Imaging System detection. The ADAM10 inhibitor GI254023X was synthesized by OKeanos Tech according to a published synthetic path [13]. The inhibitor was...
resuspended in dimethyl sulfoxide (DMSO) and applied to cells at 20 μM in complete media 16–18 hours prior to experimentation or administered to animals via intraperitoneal route following dilution in 0.1 M carbonate buffer. Animals received 200 mg/kg/day in 2–4 divided doses beginning 3 days prior to delivery of purified Hla or infection, continuing throughout the course of observation.

Small interfering RNA (siRNA) treatment was conducted as previously reported using 100 nmol of irrelevant or ADAM10-specific siRNA (Applied BioSciences) [14]. Cells were grown for 24 hours in complete media, then replated for experimentation. Flow cytometric analysis and lactate dehydrogenase assays were performed as described [5]. Metalloprotease assays were performed by plating 1.5 × 10⁴ HPAECs in 96-well dishes 24 hours prior to experimentation. Cells were incubated with recombinant Hla or phosphate-buffered saline (PBS) in unsupplemented EBM-2 media for the time periods indicated, washed with 25 mM Tris and pH 8.0 buffer, and incubated at 37°C with 10 μM fluorogenic peptide substrate (Mca-PLAQAV-Dpa-RSSSR-NH₂, R&D Systems). Fluorescence intensity was measured on a BioTek Synergy HT reader.

For detection of VE-cadherin in HPAECs, cells were plated at a density of 2.5 × 10⁵ per 6-well or 1 × 10⁵ per 24-well. Cell monolayers were rinsed in PBS and incubated with recombinant Hla in unsupplemented EBM-2 for the indicated times. DMSO or Ionomycin (5 μM) controls were added to monolayers 20 minutes prior to cell processing. For biochemical studies, cell lysates were immunoprecipitated with anti-VE-cadherin antibody and protein G sepharose (Pierce) at 4°C overnight, then analyzed by immunoblotting according to published protocols [5]. For immunofluorescence microscopy, rinsed monolayers were methanol-fixed for 10 minutes, stained, and visualized according to published protocols [5].

The effect of Hla on endothelial barrier function was measured with an electrical cell-substrate impedance sensing system (Applied BioPhysics). In total, 1 × 10⁵ HPAECs were seeded in 8W10E Cultureware and incubated at 37°C in a carbon dioxide incubator. Resistance of the monolayer was recorded until a stable resistance of approximately 600–1000 Ω was documented prior to the addition of purified toxin.

Lethal infection studies were performed in BALB/c mice (Charles River) as described, according to a protocol approved by the University of Chicago Institutional Animal Care and Use Committee [15]. To assess endothelial barrier disruption in vivo, 1 μg of endotoxin-free recombinant toxin was delivered by subcutaneous injection in 100 μL PBS, followed 3 hours later by the intravenous delivery of 100 μL 2% Evans blue dye. Mice were killed 30 minutes later and dye extravasation was quantified in excised skin by formamide extraction (65°C, 24 hours) and spectrophotometric detection (620 nm).

Statistical analysis of mortality studies was performed by log-rank test; all other statistical analyses were performed using the 2-tailed Student t test.

RESULTS AND DISCUSSION

To investigate the role of Hla in staphylococcal sepsis, we used a mouse model of intravenous infection that causes rapidly progressive, lethal disease [15]. Groups of 6-week-old mice were infected with 1 × 10⁸ colony forming units (CFUs) S. aureus Newman or 2.5 × 10⁷ CFUs USA300/LAC, representing a methicillin-sensitive clone and a highly virulent epidemic methicillin-resistant clone, respectively. Infection with wild-type strains of Newman and USA300/LAC led to acute lethality within 24–96 hours (Figure 1A). Previously characterized isogenic mutants of these strains [12] were less virulent, indicative of a role for Hla in the pathogenesis of sepsis (Figure 1A).

We have demonstrated that an association of Hla with ADAM10 is required for epithelial cytotoxicity, revealing that the extent of toxin-induced injury in epithelial cells varies directly with the level of ADAM10 cell surface expression [5]. As Hla has previously been demonstrated to cause injury to endothelial cells, we examined the requirement for ADAM10 in this process. HPAECs were treated with irrelevant or ADAM10-specific siRNA, followed by confirmation of knockdown by flow cytometry (Supplementary Figure 1A), then exposed to purified active Hla. ADAM10 knockdown cells were highly resistant to intoxication (Supplementary Figure 1B) compared with irrelevant siRNA-treated cells, extending our earlier observations on the requirement of ADAM10 expression for Hla-mediated injury to endothelial cells.

To examine whether an interaction of Hla with ADAM10 on endothelial cells affects the receptor’s metalloprotease activity, HPAECs transfected with irrelevant or ADAM10-specific siRNA were treated with 150 nM of purified, active Hla or HlaH35L[12]. Toxin-treated cells were subjected to analysis of metalloprotease activity via fluorescent peptide cleavage assay. Hla induced a rapid increase in enzymatic activity in irrelevant siRNA-treated cells (Figure 1B), whereas inducible metalloprotease activity was nearly absent on cells treated with HlaH35L or active toxin following ADAM10 knockdown (Figure 1B).

To examine the role of ADAM10 in toxin-mediated endothelial barrier disruption, electrical cell-substrate impedance sensing (ECIS) was used to measure transendothelial electrical resistance. HPAEC monolayers treated with 75 nM of Hla demonstrated a rapid and progressive loss of resistance (Figure 1C), detectable upon treatment with concentrations to 30 nM. Neither PBS nor HlaH35L altered monolayer resistance. Toxin-induced loss of endothelial barrier function
was prevented by ADAM10 knockdown (Supplementary Figure 1C).

The above data, in light of existing observations, suggested that ADAM10 indeed provides the mechanistic link between Hla and endothelial barrier disruption. ADAM10-mediated VE-cadherin cleavage leads to the release of an N-terminal extracellular fragment, whereas an approximately 35-kD C-terminal intracellular fragment (CTF) remains tethered to the membrane [7]. To examine whether toxin treatment resulted in this specific cleavage event, HPAECs were treated with 225 nM of purified Hla or Hla_H35L and cell-associated metalloprotease activity was measured using a fluorogenic substrate assay. C, HPAECs were treated with the indicated concentrations of purified Hla, and barrier resistance was continuously measured using an electric cell-substrate impedance sensing (ECIS) system. D, Immunoprecipitation and immunoblot analysis of full length (FL) and C-terminal cleavage fragment (CTF) of vascular endothelial (VE)–cadherin in HPAEC treated with 225 nM Hla or the negative control dimethyl sulfoxide (DMSO) and positive control ionomycin. E, Immunofluorescence microscopy of VE-cadherin (green) in HPAEC treated with 150 nM Hla. Nuclei were visualized with DAPI (blue). F, Miles assay in BALB/c mice demonstrating Evans blue dye extravasation from the vasculature into the skin following subcutaneous injection of purified toxin (1 μg per mouse). Abbreviations: IgG, immunoglobulin G; PBS, phosphate-buffered saline.

Figure 1. Role of Staphylococcus aureus α-hemolysin in lethal infection and endothelial barrier disruption. A, BALB/c mice (n = 10) were inoculated with wild-type (WT) or toxin-deficient (Hla-) S. aureus Newman (1 × 10^8 colony-forming units [CFUs], upper panel) and USA300/LAC (2.5 × 10^7 CFUs, lower panel) via retroorbital intravenous route and observed for acute lethal disease. B, Human pulmonary artery endothelial cells (HPAECs) transfected with either irrelevant (irr) or ADAM10-specific siRNA (A10) were treated with 150 nM purified Hla or Hla_H35L, and cell-associated metalloprotease activity was measured using a fluorogenic substrate assay. C, HPAECs were transfected with the indicated concentrations of purified Hla, and barrier resistance was continuously measured using an electric cell-substrate impedance sensing (ECIS) system. D, Immunoprecipitation and immunoblot analysis of full length (FL) and C-terminal cleavage fragment (CTF) of vascular endothelial (VE)–cadherin in HPAEC treated with 225 nM Hla or the negative control dimethyl sulfoxide (DMSO) and positive control ionomycin. E, Immunofluorescence microscopy of VE-cadherin (green) in HPAEC treated with 150 nM Hla. Nuclei were visualized with DAPI (blue). F, Miles assay in BALB/c mice demonstrating Evans blue dye extravasation from the vasculature into the skin following subcutaneous injection of purified toxin (1 μg per mouse). Abbreviations: IgG, immunoglobulin G; PBS, phosphate-buffered saline.
of the active toxin (PBS, 0.025 ± 0.001; HlaH35L, 0.049 ± 0.017; Hla, 0.161 ± 0.027; P = .0004 PBS vs Hla and .00005 HlaH35L vs Hla).

Pathogen-induced microvascular injury contributes to widespread vascular permeability and uncontrolled inflammation, compromising blood flow to vital organs and impairing organ function [2]. We reasoned that prevention of S. aureus–mediated endothelial injury may limit the deleterious effects of endovascular infection and examined whether the use of a small molecule metalloprotease inhibitor of ADAM10 could prevent Hla-mediated endothelial barrier disruption. HPAECs were treated with 20 μM of the ADAM10 metalloprotease inhibitor GI254023X, followed by the addition of active Hla [16]. GI254023X prevented VE-cadherin cleavage as revealed by immunoblot analysis (Figure 2A) and immunofluorescence microscopy (Figure 2B) when compared with treatment with PBS or the DMSO vehicle and Hla. ECIS studies revealed complete protection of HPAECs from Hla-mediated barrier disruption in the presence of GI254023X (Supplementary Figure 1E).

To examine the ability of GI254023X to inhibit Hla-mediated endothelial barrier disruption in vivo, mice treated for a 3-day period with GI254023X via intraperitoneal injection were subjected to a Miles assay following subcutaneous injection of active Hla, demonstrating dye extravasation into the skin (C) and following intravenous injection of S. aureus strain Newman to evaluate lethal disease progression (D) (n = 18). Abbreviation: IgG, immunoglobulin G.

Despite extensive research, the precise pathophysiologic mechanisms that underlie bacterial sepsis remain enigmatic, and many strategies evaluated to combat this disease process have failed to achieve clinical efficacy. Widespread endothelial dysfunction and systemic coagulopathy appear to constitute a final common pathway in sepsis that compromises tissue oxygenation. We provide new mechanistic insight into how S. aureus targets the endothelium through the formation of a toxin-receptor complex. The interaction of Hla with ADAM10 enables the toxin to both cause lytic injury to endothelial cells via its pore-forming capacity and rapidly upregulate ADAM10 enzymatic activity. Although

---

**Figure 2.** ADAM10 inhibition prevents endothelial barrier disruption by *Staphylococcus aureus* α-hemolysin. A and B, HPAECs treated with 20 μM GI254023X, an ADAM10 inhibitor, for 16–18 h. Vascular endothelial (VE)–cadherin cleavage following Hla administration observed by Western blot analysis (A) and immunofluorescence microscopy (B) of VE-cadherin (green) and nuclei (blue). FL, full-length VE-cadherin; CTF, C-terminal fragment. C and D, BALB/c mice treated with 200 mg/kg GI254023X/day or dimethyl sulfoxide (DMSO) control were examined in a Miles assay following subcutaneous injection of active Hla, demonstrating dye extravasation into the skin (C) and following intravenous injection of *S. aureus* strain Newman to evaluate lethal disease progression (D) (n = 18). Abbreviation: IgG, immunoglobulin G.
the precise molecular mechanism by which toxin-receptor binding stimulates the metalloprotease is not yet known, these studies demonstrate that the toxin can utilize a native cellular mechanism that governs endothelial permeability for a pathologic purpose. In the sequence of events that culminate in overt sepsis, it is plausible to envision that S. aureus first utilizes specific virulence factors to counter bloodstream host defenses and associate with the endothelium; subsequent secretion of Hla by S. aureus may then act either locally or systemically to cause endovascular injury. The resultant loss of integrity of the endothelial barrier and concomitant inflammatory responses may facilitate pathogen dissemination and the life-threatening sequelae of sepsis.

The independent effects of genetic alteration of ADAM10 expression and ADAM10 inhibition on Hla-induced injury together highlight the potential of small molecule inhibitors as a novel host-targeted approach to modify disease. Although our inhibitor studies are limited by the fact that GI254023X also impairs the function of the matrix metalloproteases MMP 9 and 13 [16], they provide rationale for optimization of an ADAM10 inhibitor for anti-infective purposes. The partial virulence defect observed with an Hla-deficient mutant in sepsis and the modest effect of ADAM10 inhibition in vivo together suggest that the development of successful therapies for S. aureus sepsis will require an understanding of the unique contribution of multiple virulence factors to disease. Knowledge of how these factors function in a coordinated sequence of pathogenic events may permit the design of combinatorial strategies that directly interfere with the ability of S. aureus virulence factors to disturb endovascular homeostasis, providing benefit in the management of this refractory disease process.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank O. Schneewind for discussions and comments on the manuscript, C. Labno and the Integrated Microscopy Facility for microscopy support, and Eddie Chiang for assistance with siRNA-mediated knockdown in HPAECs.

Financial support. This work was supported by the Departments of Pediatrics and Microbiology at the University of Chicago. The authors acknowledge membership in and support from the Region V “Great Lakes” Regional Center of Excellence (National Institutes of Health award 2-U54-AI-057153). M. P. was partially supported by National Institutes of Health grant T32 GM007183.

Potential conflicts of interest. J. B. W. has the potential to receive royalties from Novartis Vaccines and Diagnostics in relation to patents owned by the University of Chicago. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References