Valvular Heart Disease

Ultralarge von Willebrand Factor Fibers Mediate Luminal *Staphylococcus aureus* Adhesion to an Intact Endothelial Cell Layer Under Shear Stress

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**Background**—During pathogenesis of infective endocarditis, *Staphylococcus aureus* adherence often occurs without identifiable preexisting heart disease. However, molecular mechanisms mediating initial bacterial adhesion to morphologically intact endocardium are largely unknown.

**Methods and Results**—Perfusion of activated human endothelial cells with fluorescent bacteria under high-shear-rate conditions revealed 95% attachment of the *S aureus* by ultralarge von Willebrand factor (ULVWF). Flow experiments with VWF deletion mutants and heparin indicate a contribution of the A-type domains of VWF to bacterial binding. In this context, analyses of different bacterial deletion mutants suggest the involvement of wall teichoic acid but not of staphylococcal protein A. The presence of inactivated platelets and serum increased significantly ULVWF-mediated bacterial adherence. ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motif(s) 13) caused a dose-dependent reduction of bacterial binding and a reduced length of ULVWF, but single cocci were still tethered by ULVWF at physiological levels of ADAMTS13. To further prove the role of VWF in vivo, we compared wild-type mice with VWF knockout mice. Binding of fluorescent bacteria was followed in tumor necrosis factor–α–stimulated tissue by intravital microscopy applying the dorsal skinfold chamber model. Compared with wild-type mice (n=6), we found less bacteria in postcapillary (60±6 versus 32±5 bacteria) and collecting venules (48±5 versus 18±4 bacteria; *P*<0.05) of VWF knockout mice (n=5).

**Conclusions**—Our data provide the first evidence that ULVWF contributes to the initial pathogenic step of *S aureus*–induced endocarditis in patients with an apparently intact endothelium. An intervention reducing the ULVWF formation with heparin or ADAMTS13 suggests novel therapeutic options to prevent infective endocarditis. (Circulation. 2013;128:50–59.)

**Key Words**: blood flow velocity ■ cardiovascular diseases ■ endocarditis, bacterial ■ endothelium ■ infection ■ von Willebrand factor

*S aureus* is a major pathogen responsible for various infections in humans and is the most frequent cause of infective endocarditis in industrialized countries. Prerequisite for the pathogenesis of infective endocarditis is infection of the endocardium. Therefore, one of the first and essential steps is bacterial adhesion to the endothelium. In contrast to other pathogens causing infective endocarditis, *S aureus* appears to be able to bind not only to damaged but also to intact endothelium. Fibrinogen, fibronectin, and platelets, in combination with *S aureus* clumping factor and fibronectin-binding proteins, are involved in the pathogenesis of infective endocarditis. In addition, wall teichoic acids of *S aureus* have been implicated in adhesion to the endothelial cell (EC) layer. However, the molecular mechanisms of the very first adhesion steps of *S aureus* to the undamaged endothelium are still largely unknown.

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Clinical Perspective on p 59

Recruitment of host cells to the endothelium is a crucial step during inflammation and coagulation. It has been shown previously that under shear von Willebrand factor is a potent binding partner for platelets and a key molecule for the extravasation of leukocytes during inflammation on intact endothelium. VWF is a large multimeric glycoprotein that is produced mainly by ECs and stored in Weibel-Palade bodies. Acute activation of VWF is a large multimeric glycoprotein that is produced mainly by ECs and stored in Weibel-Palade bodies. Acute activation of VWF

In Vitro Perfusion Assay of Human Umbilical Vein ECs

In vitro experiments with primary human umbilical vein ECs (HUVECs) were performed under flow conditions, applying an air pressure pump system. This device allows a unidirectional, continuous perfusion of an intact endothelium. The general pump technique of the IBIDI air pressure pump (IBIDI GmbH, Munich, Germany) has been described previously. In brief, HUVECs (1×10⁶ cells per 1 cm²) were seeded on gelatin-coated µ-Slide I² Luer (IBIDI GmbH) and cultured under slight flow (1 dyne/cm²) to confluence in EGM2 medium (Lonza, Basel, Switzerland). Fluorescence-labeled bacteria (3.6×10⁷ CFU/mL) were resuspended in HEPES-buffered Ringer solution (10 mmol/L HEPES, 5 mmol/L glucose, 1 mmol/L calcium chloride, 1 mmol/L magnesium chloride, 5 mmol/L potassium chloride, 140 mmol/L sodium chloride), which was supplemented with 25% washed red blood cells to increase buffer viscosity and to approximate cellular constituents of physiological blood conditions. When indicated, platelets and serum were additionally added. Before the experiments, platelets were inhibited with prostaglandin and apyrase.

Methods

Bacteria

The staphylococcal strains and derivatives used in this study are listed in the Table. Staphylococcal strains were cultivated as described in the online-only Data Supplement. After cultivation, bacteria were stained either with FITC (Sigma-Aldrich, Steinheim, Germany) or TRITC (Sigma-Aldrich, Steinheim, Germany) as previously described. Optical densities of bacterial suspensions were adjusted to an OD₅₄₀ of 1 corresponding to 3×10⁹ colony-forming units (CFU)/mL.

In Vitro Perfusion Assay of Functionalized Surfaces

Functionalized surfaces coated either with human full-length recombinant wild-type VWF (rVWF) or VWF deletion mutants lacking the A1 domain (VWFdelA1), the A2 domain (VWFdelA2), or the A3 domain (VWFdelA3) were flushed with fluorescence-labeled S. aureus SA113 (3.6×10⁷ CFU/mL) for 4 minutes at 10 dynes/cm². To analyze the platelet-binding properties of these deletion mutants, we perfused washed, inactivated, and Celltrace Calcein Red-Orange, AM− (Invitrogen, Darmstadt, Germany) stained platelets over the functionalized surfaces. Bacterial and platelet adherence was analyzed by fluorescence microscopy using a microscope with ×40 objective (Zeiss, Jena, Germany). Adhesion was quantified at 12 fields of view for each microfluidic channel. More details of the VWF mutants and the coating are provided in the online-only Data Supplement.

Intravital Fluorescence Microscopy of the Mouse Dorsal Skinfold Chamber

After tumor necrosis factor-α stimulation of the tissue of the dorsal skinfold chamber, either VWF knockout mice or wt mice were challenged

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>Shear Rate Conditions, dynes/cm²</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus Cowan I</td>
<td>Wild type</td>
<td>2.5/10</td>
<td>ATCC 12598</td>
</tr>
<tr>
<td>S. aureus Cowan I SpA</td>
<td>SpA deficient</td>
<td>2.5/10</td>
<td>DU 5889²⁰</td>
</tr>
<tr>
<td>S. aureus SA113</td>
<td>Wild type</td>
<td>10</td>
<td>ATCC 35556</td>
</tr>
<tr>
<td>S. aureus SA113 tagO</td>
<td>WTA deficient</td>
<td>10</td>
<td>Reference 21</td>
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<tr>
<td>S. aureus SA113 srtA</td>
<td>Sortase A deficient</td>
<td>10</td>
<td>Reference 22</td>
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<tr>
<td>S. carnosus TM300</td>
<td>Wild type</td>
<td>10</td>
<td>Reference 23</td>
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SpA indicates staphylococcal protein A; and WTA, wall teichoic acid.

Table. Analyzed Staphylococcal Strains in Terms of the Deficiency of Adhesions, Applied Experimental Flow Conditions, and Origin
with fluorescence-labeled live *S. aureus*. Thirty minutes after injection, intravital fluorescence microscopy was performed as described previously.26 Quantitative offline analysis of bacterial adherence (given as clusters per 1 mm³ of endothelial surface) in postcapillary and collecting venules was performed as described previously.26 Bacterial adherence was analyzed in postcapillary and collecting venules. Detailed protocols are available in the online-only Data Supplement.

**Statistical Analysis**

In vitro perfusion assays with HUVECs were based on several independent batches of ECs; thus, VWF release and bacterial adhesion varied considerably between the experiments. Therefore, we normalized the result of the overall bacterial adhesion of each single experiment to 100%. Adhesion was classified into VWF-mediated and VWF-independent binding, and the percentage of binding of each class was calculated. To compare the results with another data set (eg, bacterial binding after ADAMTS13 addition), binding of the second group is represented relative to bacterial adhesion of the first group.

Values of the in vitro experiments performed with ECs are expressed as mean±SD. Values of the in vitro experiments performed on functionalized surfaces are expressed as mean±SEM. Statistical significance of the in vitro experiments was tested with the unpaired Student *t* test. All values of the in vivo experiments are expressed as mean±SEM. Differences between groups were tested with the unpaired Student *t* test and Mann-Whitney *U* test. Statistical tests were performed with SAS software (SAS Institute Inc, Cary, NC). Values of *P*<0.05 were considered statistically significant and are based on the unpaired Student *t* test.

**Results**

**Luminally Released VWF Mediates *S. aureus* Adhesion to Intact Endothelium In Vitro**

To analyze the impact of luminally released VWF on *S. aureus* adhesion to confluent endothelium, we flushed histamine-activated HUVECs with suspensions of 2 different *S. aureus* strains (Cowan I and SA133) at a shear stress of 10 dynes/cm². In agreement with previous publications, formation of ULVWF fibers on the intact endothelium was shear flow dependent (Figure 1A).12,27 Adherence of individual *S. aureus* cells or small bacterial clusters to an intact layer of ECs was almost exclusively mediated by ULVWF fibers (Figure 1A and 1B and Figure 1A and IB in the online-only Data Supplement). The highly dynamic process of the interaction between *S. aureus* with ULVWF fibers under high-shear conditions is illustrated in a live image (Figure II in the online-only Data Supplement) and by real-time movies showing the collapse and re-elongation of the ULVWF under stop-flow conditions and the waving of the fiber under continuous-flow (Movies 1 and 2 in the online-only Data Supplement). Entrapment of *S. aureus* by ULVWF fibers was also confirmed by high-resolution atomic force microscopy (Figure III in the online-only Data Supplement). Quantification of the fluorescence images proved that a total of 73±29 cocci per 1 mm³ of *S. aureus* SA113 (n=11) and 49±28 cocci per 1 mm³ of *S. aureus* Cowan I (n=7) adhered to the intact endothelium. In both cases, 95% of *S. aureus* colocalized with ULVWF, whereas VWF-independent adhesion was rare (96±2% of *S. aureus* SA113 and 95±6% of *S. aureus* Cowan I VWF mediated versus 4±2% of *S. aureus* SA113 and 5±6% of *S. aureus* Cowan I VWF independent, respectively [mean±SD]; Figure 1C).

Our data indicate that endothelium-derived ULVWF functions as a dominant binding partner for *S. aureus*. To further verify our results, we cut established ULVWF by the addition of recombinant ADAMTS13 (Figure 2A). Perfusion with ADAMTS13 caused a dramatic reduction of ULVWF fibers (red) released from an intact endothelial cell layer (bright field). Scale bars, 50 µm. B, Magnification of *S. aureus* SA113 (green) colocalized with ULVWF fibers (red). Scale bar, 20 µm. C, Quantification of *S. aureus* strains SA113 (n=11) and Cowan I (n=7) attached to an intact endothelium. Adhesion was classified into VWF mediated or VWF independent. Total bacterial adhesion was normalized to 100%. Data are expressed as mean±SEM (*P*<0.001).

**Fibrinogen Has No Effect on Initial VWF-Mediated *S. aureus* Binding to Endothelium**

Because recent studies showed that fibrinogen acts as a bridging molecule for *staphylococcal* adhesion to the endothelium,28,29 we analyzed the impact of fibrinogen on *S. aureus* adhesion under high shear flow. Although fibrinogen induced bacterial cluster formation as expected,28 neither VWF-mediated nor VWF-independent *S. aureus* adherence was affected (VWF-mediated, 95±6% without fibrinogen [n=7]; 95±6% with fibrinogen [n=3]); VWF-independent, 5±6% without fibrinogen versus 13±11% with fibrinogen [mean±SD]; Figure 3A). Figure 3B shows large adherent *S. aureus* clusters formed in the presence of fibrinogen that were entrapped by ULVWF.

**VWF-Mediated *S. aureus* Binding via Protein A Is Shear Flow Dependent**

To analyze the impact of SpA, a surface-associated molecule, on the bacterial interaction with VWF, we compared...
an isogenic SpA-deficient mutant with its parental wt strain (S aureus Cowan I). Within a paired experiment, both bacterial strains were perfused over activated HUVEC with a shear stress of 10 dynes/cm². Adhesion of the SpA mutant to VWF was significantly decreased at low shear flow (2.5 dynes/cm²) compared with the wt strain (54±3% S aureus SpA adhesion versus 93±3% S aureus Cowan I adhesion [mean±SD]; Figure 4A). However, at high-shear-flow conditions (10 dynes/cm²), S aureus SpA binding to VWF (116±9% [mean±SD]) was even significantly increased compared with the wt adhesion (95±6% [mean±SD]; Figure 4B). Figure 4C shows a representative immunofluorescence image of S aureus SpA binding to ULVWF fibers under high shear flow.

**Bacterial Binding to VWF Under High Shear Stress Is Multifactorial, Involving Diverse S aureus Virulence Factors**

Because protein A does not account for S aureus binding to luminally released VWF at high shear flow, we investigated the influence of other bacterial adhesion molecules. Therefore, suspensions with different bacterial mutants were perfused over activated HUVEC layers with a shear stress of 10 dynes/cm². The apathogenic S carnosus, which does not possess major S aureus surface proteins,29,30 showed a low VWF binding capacity (17±3% [mean±SD]; n=4) compared with S aureus SA113 (96±2% [mean±SD]; n=11; Figure 5). The S aureus mutants srtA and tagO are deficient in LPXTG-anchored surface proteins and wall teichoic acid, respectively.31,32 Binding of both mutants to VWF was significantly reduced compared with S aureus SA113 wt (S aureus srtA, 32±1% [n=4]; S aureus tagO, 44±2% [n=5; mean±SD]; Figure 5).

**The A1 and A3 Domains of VWF Mediate Bacterial Adherence**

VWF has binding sites for various molecules such as platelet glycoprotein Ib, collagens, or glycosaminoglycans like heparin. These functional binding sites are located mainly within the A-type domains consisting of A1, A2, and A3.28-31 The presence of unfractionated heparin reduced the binding of S aureus to VWF, suggesting the involvement of the VWF A1 domain (VWF mediated, 95±6% adhesion without [n=7] versus 16±2% adhesion with [n=3] heparin [mean±SD]; Figure 6A). However, heparin is not a specific blocker of the A1 domain but is able to influence other binding pathways of S aureus.32 Analysis of the VWF-independent bacterial adherence revealed a slightly reduced binding on heparin treatment (VWF independent, 5±6% adhesion without [n=7] versus 3±2% adhesion with [n=3] heparin [mean±SD]; Figure 6A), pointing to an interference of heparin with bacteria trapped by the endothelial glycosalyx.32 To further specify the contribution of the A-type domains for S aureus binding, we synthesized genetically modified recombinant VWF molecules lacking the A1 (VWFdelA1), A2 (VWFdelA2), or A3 (VWFdelA3) domain and perfused S aureus over surfaces functionalized with this recombinant VWF. S aureus SA113 (n=10) adhesion to VWFdelA1 and VWFdelA3 was...
significantly decreased compared with the adhesion to rwt-VWF (Figure 6B). Adhesion to VWFdelA2 was only slightly reduced (Figure 6B). Next, we perfused VWF-functionalized surfaces with S. aureus tagO (n=5) and S. aureus srtA (n=5). However, this gave no further insight into the molecular mechanisms responsible for the interaction between VWF and S. aureus because the adherence patterns were very similar in all mutants (Figure 6B).

To prove the functionality of our VWF-mutants, we measured their capacity to bind platelets. As expected, we found a reduced platelet binding only toward VWFdelA1, whereas binding to VWFdelA2 and VWFdelA3 was not affected (n=5; Figure 6C).31

**Bacterial Binding Is Affected by Platelets and ADAMTS13**

Serum contains several proteins like immunoglobulins or plasmatic VWF that could interact with S. aureus, whereas platelets bind with high affinity via glycoprotein Ibα to the A1 domain exposed by stretched VWF under shear. An increased competition of the acting binding partners for specific VWF binding might be the consequence. Therefore, we further upgraded our in vitro flow device with inactivated platelets and serum lacking ADAMTS13 activity (Figure 7). However, we found an ≈2-fold increased bacterial adherence to the endothelium and an even more dominant role of ULVWF (n=5; Figure 7A). Immunofluorescence staining revealed that ULVWF fibers appear to be thicker and more robust in the presence of serum and platelets, implicating potentially more binding sites for S. aureus (Figure 7B), as hypothesized previously.33

On systemic inflammation, ADAMTS13 is consumed by overwhelming amounts of VWF released into the blood,13 suggesting also a reduced concentration of ADAMTS13 in the course of endocarditis. In addition, it has been proposed that interleukin 6, which was found to be elevated in the blood of endocarditis patients,34 could block ADAMTS13 activity. To understand the role of ADAMTS13 for the ULVWF-mediated adhesion of S. aureus, we analyzed bacterial attachment in the presence of platelets and inactivated serum supplemented with different amounts of recombinant ADAMTS13 (n=4) or serum with a physiological ADAMTS13 activity (n=4).

Figure 4. *Staphylococcus aureus* binding to von Willebrand factor (VWF) via protein A was shear flow dependent. A. Under low-shear-flow conditions, adhesion of S. aureus Cowan I SPA (staphylococcal protein A) was decreased compared with S. aureus Cowan I (n=3). B. Under high-shear-flow conditions, adhesion of S. aureus SpA was increased (n=5). S. aureus Cowan I adhesion was normalized to 100%, and adhesion of S. aureus SpA is represented relative to S. aureus Cowan I adhesion.

Data are expressed as mean±SD (P<0.05, **P<0.001). C. Representative fluorescence image of S. aureus Cowan I (green) and S. aureus SpA (red) bound to ultralarge VWF (ULVWF) fiber (white) under high-shear-flow conditions. Scale bar, 20 µm.

Figure 5. *Staphylococcus aureus* interaction with von Willebrand factor (VWF) comprises various adhesion factors. Percentage of adherent bacteria after perfusion over stimulated human umbilical vein endothelial cells. S. aureus SA113 adhesion was normalized to 100%, and bacterial adhesion of S. aureus tagO (n=5), S. aureus srtA (n=4), and S. carnosus (n=4) is represented relative to S. aureus SA113 (n=11) adhesion. Data are expressed as mean±SD (P<0.001).
S. aureus Adhesion to Undamaged Endothelium

Discussion

*S. aureus* is one of the main pathogens causing endocarditis. Initial adhesion to the endothelium is a crucial step for pathogenesis of this intravascular disease. Once bound to the vessel wall, *S. aureus* is able to overcome the EC barrier and to spread to the surrounding tissue, leading to the establishment of infection.

Most of the previously performed studies focused on bacterial adhesion at sites of vascular injury. However, *S. aureus* is also able to infect physically undamaged endothelium. The molecular mechanisms of initial *S. aureus* adhesion to intact endothelium are mostly unknown, although fibronectin and fibrinogen are required as bridging molecules. In contrast to those studies, we aimed to investigate bacterial adhesion to the intact endothelium during acute inflammatory conditions under distinct shear-flow conditions. Thus, we have established and adapted a microfluidic system to study activated ECs under arterial shear-flow conditions.

In the present study, we demonstrate that luminally released ULVWF acted as a predominant adhesive factor for *S. aureus*, mediating the binding of the bacterium to an intact EC layer under high-shear-flow conditions (Figures 1 and 2). Only <5% of the attached bacteria interact in a VWF-independent manner that might be attributable to EC surface associated fibronectin or surface-exposed glycosaminoglycans (Figures 1 and 6A). Previous studies demonstrated plasmatic fibrinogen as a potent binding partner of *S. aureus* that triggers the formation of bacterial clusters and bacterial adhesion to the endothelium. Therefore, we also investigated whether the presence of fibrinogen could affect the VWF-mediated interaction between the intact endothelium and *S. aureus*. Although fibrinogen led to the formation of bacterial clusters, we found that neither the VWF-mediated nor the VWF-independent amount of colony-forming units of endothelium-bound bacteria was increased in the presence of fibrinogen. Accordingly, our data suggest that binding of *S. aureus* to the endothelium is initiated by VWF and that VWF is able to not only sequester single bacteria but also entrap large bacterial clusters (Figure 3).

One potential binding partner of VWF expressed by *S. aureus* is protein A. In this context, Hartleib et al showed a specific interaction of SpA with VWF under low shear flow. Therefore, we compared the VWF binding capacities of an isogenic *S. aureus* wt/SpA strain pair within our experimental setting. In line with the previous results, we observed an SpA dependency at low-shear-flow conditions. However, at high-shear-flow conditions, we measured an SpA-independent *S. aureus* adhesion (Figure 4). Notably, these findings of a shear flow dependency are supported by results obtained by O’Seaghdha and coworkers showing that *Lactococcus lactis* derivatives expressing the SpA bind to recombinant human VWF under low shear rates but not under high shear rates. To reveal the potential bacterial binding partner for VWF under high-shear-flow conditions, we first determined the interaction between VWF and *S. aureus* srtA, which lacks all cell wall–anchored surface proteins. Adhesion of *S. aureus* srtA to ULVWF was higher than the adhesion of the nonpathogenic *S. carnosus*, which lacks all relevant surface-associated adhesins, suggesting the involvement of additional bacterial surface molecules for VWF adhesion (Figure 5). Therefore,
cleavage site for ADAMTS13. Our experiments indicate that the A2 domain has no adhesive properties but contains the ECs and released in complex with VWF. Interestingly, galectin 1 and 3 are both expressed by human agreement with our data, Li et al previously showed a heparin-dependent reduction of bacterial adhesion of Staphylococcus aureus with heparin revealed a 6-fold reduction of the VWF-mediated exposure of the A-type domains. Blocking of the A1 domain is immobilized mainly on stretched VWF fibers S aureus adhesion normalized to 100%, and adhesion in the presence of RBCs, platelets, and inactivated serum at 10 dynes/cm². Scale bars, 50 µm. C, Quantification of S aureus SA113 binding to endothelium in the presence of RBCs, platelets, and either ADAMTS13 active serum (n=4) or inactivated serum supplemented with 100 ng/mL (n=4) or 200 ng/mL (n=4) recombinant ADAMTS13 after perfusion of stimulated HUVECs at a shear stress of 10 dynes/cm². Bacterial adhesion in the presence of ADAMTS13 is represented relative to bacterial adhesion in the absence of ADAMTS13, which was normalized to 100%. Data are expressed as mean+SD. D, Quantification of the length of luminally released ULVWF fibers after perfusion with RBCs, platelets, and either ADAMTS13 active serum or inactivated serum supplemented with different concentrations of recombinant ADAMTS13. Total fiber amount was normalized to 100%. Data are expressed as mean+SD.

Bacterial binding is affected by platelets and ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motifs 13). A, Quantification of Staphylococcus aureus SA113 binding to endothelium in the presence of red blood cells (RBCs; n=11) and in the presence of RBCs, platelets, and inactivated serum (n=5) after perfusion of stimulated, confluent human umbilical vein endothelial cells (HUVECs) at a shear stress of 10 dynes/cm². Adhesion was classified into von Willebrand factor (VWF) mediated and VWF independent. S aureus adhesion was normalized to 100%, and adhesion in the presence of RBCs, platelets, and inactivated serum is represented relative to bacterial adhesion in the presence of RBCs. Data are expressed as mean+SD. B, Representative immunofluorescence images of ultralarge VWF (ULVWF) fibers on stimulated endothelium after perfusion with either RBCs or RBCs, platelets, and inactivated serum at 10 dynes/cm². Scale bars, 50 µm. C, Quantification of S aureus SA113 binding to endothelium in the presence of RBCs, platelets, and either ADAMTS13 active serum (n=4) or inactivated serum supplemented with 100 ng/mL (n=4) or 200 ng/mL (n=4) recombinant ADAMTS13 after perfusion of stimulated HUVECs at a shear stress of 10 dynes/cm². Bacterial adhesion in the presence of ADAMTS13 is represented relative to bacterial adhesion in the absence of ADAMTS13, which was normalized to 100%. Data are expressed as mean+SD. D, Quantification of the length of luminally released ULVWF fibers after perfusion with RBCs, platelets, and either ADAMTS13 active serum or inactivated serum supplemented with different concentrations of recombinant ADAMTS13. Total fiber amount was normalized to 100%. Data are expressed as mean+SD.

Figure 7. Bacterial binding is affected by platelets and ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motifs 13), A, Quantification of Staphylococcus aureus SA113 binding to endothelium in the presence of red blood cells (RBCs; n=11) and in the presence of RBCs, platelets, and inactivated serum (n=5) after perfusion of stimulated, confluent human umbilical vein endothelial cells (HUVECs) at a shear stress of 10 dynes/cm². Adhesion was classified into von Willebrand factor (VWF) mediated and VWF independent. S aureus adhesion was normalized to 100%, and adhesion in the presence of RBCs, platelets, and inactivated serum is represented relative to bacterial adhesion in the presence of RBCs. Data are expressed as mean+SD. B, Representative immunofluorescence images of ultralarge VWF (ULVWF) fibers on stimulated endothelium after perfusion with either RBCs or RBCs, platelets, and inactivated serum at 10 dynes/cm². Scale bars, 50 µm. C, Quantification of S aureus SA113 binding to endothelium in the presence of RBCs, platelets, and either ADAMTS13 active serum (n=4) or inactivated serum supplemented with 100 ng/mL (n=4) or 200 ng/mL (n=4) recombinant ADAMTS13 after perfusion of stimulated HUVECs at a shear stress of 10 dynes/cm². Bacterial adhesion in the presence of ADAMTS13 is represented relative to bacterial adhesion in the absence of ADAMTS13, which was normalized to 100%. Data are expressed as mean+SD. D, Quantification of the length of luminally released ULVWF fibers after perfusion with RBCs, platelets, and either ADAMTS13 active serum or inactivated serum supplemented with different concentrations of recombinant ADAMTS13. Total fiber amount was normalized to 100%. Data are expressed as mean+SD.

we analyzed the role of wall teichoic acids, which are highly abundant, surface-exposed glycopolymers incorporated into bacterial cell walls. Our data indeed suggest the attendance of wall teichoic acids because we found a significantly reduced adhesion of the S aureus tagO mutant compared with S aureus wt adhesion (Figure 5). Interaction of host lectins such as galectins and wall teichoic acid has already been debated and is the subject of ongoing investigations in our laboratories. Interestingly, galectin 1 and 3 are both expressed by human ECs and released in complex with VWF.

The A1 domain of luminally exposed, stretched VWF provides binding sites not only for the platelet receptor glycoprotein Ib but also for collagen and heparin. The A3 domain contains binding sites for collagen type I and III whereas the A2 domain has no adhesive properties but contains the ECs and released in complex with VWF.36 Interestingly, galectin 1 and 3 are both expressed by human ECs and released in complex with VWF.29 whereas its role in the onset of ULVWF-mediated binding of S aureus has not yet been investigated. Therefore, we performed experiments with different concentrations of ADAMTS13 to predict its role in the onset of ULVWF-mediated binding of S aureus. Although the amount and length of ULVWF fibers exposed on the endothelial surface were drastically diminished in the presence of physiological amounts of ADAMTS13 (744±168 ng/mL), we still found bacteria tethered by VWF. Assuming a consumption or inhibition of ADAMTS13 in affected patients, our experiments predict an even more significant contribution
of ULVWF to bacterial binding. In agreement with our in vitro data, we were also able to detect luminally released VWF fibers in the vessels of mice treated with tumor necrosis factor-α (TNF-α) exposure, suggesting the formation of ULVWF in vivo.

To further prove the pathological relevance of our results, we studied the interaction between human endothelium and *S. aureus* not only in the presence of red blood cells but together with platelets and serum. Although we expect a decoration of *S. aureus* with IgG or soluble VWF on the one hand and the occupation of the A1 domain by platelets on the other hand, we found an even increased contribution of ULVWF to bacterial binding. Interestingly, we also found that ULVWF strings decorated with platelets are thicker and more robust than ULVWF without platelets, suggesting an improved docking of bacteria. To investigate the pure effect of ULVWF for *S. aureus* adherence, we performed our flow experiments with inactivated platelets, preventing the exposure of active glycoprotein IIb/IIIa, a well-known binding partner for *S. aureus*.

The major drawback of current animal models for infective endocarditis is the artificial destruction of the endothelium prior to bacterial inoculation. We aimed to circumvent this strategy by applying our recently described in vivo model of the dorsal skinfold chamber. To measure the role of VWF for bacterial trapping, we compared wt mice with mice deficient in VWF. It is worth noting that the lack of VWF leads also to a lack of Weibel-Palade bodies, accompanied by a disturbed processing of various endothelium-derived factors such as P-selectin. Although P-selectin is a potent binding partner for leukocytes and potentially responsible for the anchorage of the ULVWF to the EC surface, it has recently been excluded as adhesion molecule for *S. aureus*. Applying our experimental in vivo setting together with intravital fluorescence microscopy, we showed a significant reduction of bacterial adhesion in VWF knockout mice (Figure 8). This further underlines the physiological relevance of VWF-mediated attachment of *S. aureus* to intact microvasculature.

**Conclusions**

We could show that binding of *S. aureus* to the intact EC layer is mediated predominantly by luminally secreted ULVWF fibers involving the A1 and A3 domains. Bacterial adhesion to VWF is resistant to even high-shear-flow conditions because shear flow–activated ULVWF fibers support *S. aureus* binding. Our data shed light on the initial early pathogenetic steps of *S. aureus* endocarditis in patients with an apparently intact endothelium. From our findings, one can speculate that therapeutic intervention with recombinant ADAMTS13, as is discussed for patients suffering from thrombotic thrombocytopenic purpura, or heparin could be a novel approach as adjunctive therapy besides the current antibiotic-based therapy for infective endocarditis.

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**Disclosures**

None.

**References**


In the past, there was a major shift in both the patient population and the microbial spectrum of infective endocarditis. Staphylococci, especially *Staphylococcus aureus*, are now the leading cause in industrialized countries. However, in >50% of patients, no previously known heart disease considered a classic risk factor is identified, suggesting alternative, unknown mechanisms for establishing disease. Von Willebrand Factor is luminally secreted on proinflammatory stimulation of endothelial cells and under high shear stress temporarily forms large fibers, entrapping platelets. We hypothesized that *S. aureus* also might be sequestered via this mechanism because it binds to von Willebrand Factor via protein A and to platelets via various surface proteins. In a microfluidic chamber, *S. aureus* adhered in a platelet- and protein A–independent manner to activated endothelial cells. Adherence was ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motifs 13) susceptible. Wall teichoic acid substantially mediated adherence under high shear stress. Platelets and serum substantially enhanced binding. Using recombinant von Willebrand Factor constructs, we were able to map the involved binding domains. Furthermore, we showed in an intravital microscopy model that circulating *S. aureus* also binds to von Willebrand Factor fibers on activated endothelial cells in vivo. To the best of our knowledge, this is the first study showing that *S. aureus* binds to morphologically intact but activated endothelial cells under high-shear-stress conditions that are thought to exist at the predilection sites for infective endocarditis. This finding sheds light on the initial steps during the pathogenesis of endocarditis and might provide the basis for novel strategies for prevention and treatment by targeting von Willebrand Factor fibrils.
Ultralarge von Willebrand Factor Fibers Mediate Luminal Staphylococcus aureus Adhesion to an Intact Endothelial Cell Layer Under Shear Stress
Karin I. Pappelbaum, Christian Gorzelanny, Sandra Grässle, Jan Suckau, Matthias W. Laschke, Markus Bischoff, Corinne Bauer, Marina Schorpp-Kistner, Christopher Weidenmaier, Reinhard Schneppenheim, Tobias Obser, Bhanu Sinha and Stefan W. Schneider

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SUPPLEMENTAL MATERIAL

for

Ultra-large von Willebrand factor fibers mediate luminal Staphylococcus aureus adhesion to an intact endothelial cell layer under shear stress

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Supplemental Methods

Cultivation of bacteria for *in vitro* assays

Staphylococcal strains were grown stationary in Mueller-Hinton broth (Roth, Karlsruhe, Germany) for 18h at 37°C. The culture media for the mutants contained appropriate antibiotics for marker selection (5 µg/ml tetracycline (Amdipharm, St. Helier, Jersey), 2.5 µg/ml erythromycin (Roth, Karlsruhe, Germany)).

Cultivation of bacteria for *in vivo* application

Bacteria cells of exponential growth phase cultures (A_{600nm} of OD1) of *S. aureus* strain Cowan I were washed twice with phosphate-buffered saline, fluorescent-labeled with 50 µM of CFSE (5-[6]-carboxyfluorescein diacetate succinimidyl ester (Life Technologies, Darmstadt, Germany) for 15 min at 37°C and 500 rpm, washed for an additional three times with PBS to remove unbound fluorescent dye and 100 µl of the fluorescent-labeled cell suspension (containing 6.4-8.9x10^7 cfu) were applied to the animals.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultivated to confluence in M199 medium (Invitrogen, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), antibiotics (penicillin and streptomycine; PAA Laboratories GmbH, Pasching, Austria), 5 U/ml heparin (Biochrom, Berlin, Germany) and 1% growth factor supplement derived from bovine retina as described previously.1
Immunofluorescence staining of VWF

HUVECs and bacteria were fixed with 4% paraformaldehyde in PBS for 10 min at 37°C under flow. For VWF staining endothelial cells were blocked for 1 h with 2% BSA. To avoid a co-staining of \textit{S. aureus} by antibody binding to protein A bacteria were blocked for 30 min with normal goat IgG (2.5 µg/ml) (R&D, Minneapolis, USA). Immunofluorescence staining was performed with anti-VWF rabbit antibody (15 µg/ml) (DAKO, Hamburg, Germany) and with anti-rabbit Alexa 350 (5 µg/ml) (Invitrogen, Darmstadt, Germany). Adherence of fluorescent-labeled bacteria to endothelial cells was analyzed by fluorescence microscopy using a microscope with 40 X objective and appropriate fluorescence filters (Zeiss, Jena, Germany). To quantify bacterial attachment to the intact endothelium 20 – 30 fields of view per experiment were analyzed. Analysis of images was performed with the software AxioVS40 V 4.8.0.0. (Zeiss, Jena, Germany).

\textit{In vitro} perfusion of functionalized surfaces

Human full length recombinant wild type VWF (rwtVWF) and VWF deletion mutants lacking the A1 (VWFdelA1: deletion of amino acid residues E1260-G1479), A2 (VWFdelA2: deletion of amino acid residues N1493-E1673) or the A3 domain (VWFdelA3: deletion of amino acid residues G1672-G1874) were expressed in 293-EBNA cells (Invitrogen, Darmstadt, Germany) as previously described.\textsuperscript{2} Perfusion experiments with functionalized surfaces were performed with the BioFlux 200 flow system (Fluxion, San Francisco, USA) as previously described.\textsuperscript{3} In brief, channels of 24 Well BioFlux plates were coated either with rwtVWF, VWFdelA1, VWFdelA2 or VWFdelA3 applying a solution of 100 µg/ml for 2h at 37°C. To remove excess VWF, channels were flushed with HEPES-buffered ringer solution.
(10 mM HEPES, 5 mM glucose, 1 mM calcium chloride, 1 mM magnesium chloride, 5 mM potassium chloride, 140 mM sodium chloride) which was supplemented with 25% washed red blood cells. To evaluate bacterial attachment, channels were flushed with HEPES-buffered ringer solution supplemented with 25% washed red blood cells and fluorescent-labeled *S. aureus* SA113.

**Preparation of the mouse dorsal skinfold chamber and intravital fluorescence microscopy**

All experiments were conducted in accordance with the *German legislation on protection of animals* and the *NIH Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, Washington, USA) and were approved by the local governmental animal care committee. Fifteen to 20-week-old VWF knock-out mice (B.6.129S2-VWF<sup>tm1Wgr</sup>/J) and wild type (wt) mice (C57BL/6) (The Jackson Laboratory, Maine, USA) with a body weight of 22–25 g were used for the study. Dorsal skinfold chambers were prepared in mice as described previously in detail. After the surgery, the animals were allowed to recover from anesthesia and surgery for at least 48 h before the microcirculatory analyses. For intravital fluorescence microscopy, the animals were anesthetized (75mg/kg body weight ketamine intraperitoneal injection (i.p.) (Pharmacia GmbH, Erlangen, Germany) and 15mg/kg body weight xylazin i.p. (Bayer, Leverkusen, Germany)) and a fine polyethylene catheter (PE10, 0.28 mm internal diameter) was inserted into the A. carotis for application of bacteria and fluorescent dyes. For endothelial cell stimulation, the tissue of the dorsal skinfold chamber was exposed to TNF-α (topical application of 2000 U dissolved in 100 µL PBS) (Roche, Mannheim, Germany) for 30 min. Subsequently, the mice were attached to the microscopic stage. After analysis of the
intravascular bacterial adherence, 0.1 ml of 5% FITC-labeled dextran 150,000 was additionally injected for contrast enhancement by staining of blood plasma to determine microhemodynamic parameters, including vessel diameter (µm) and centerline red blood cell velocity (µm/s). Intravital fluorescence microscopy was performed by means of a Zeiss Axiotech microscope (Zeiss; Oberkochen, Germany) with a 100W mercury lamp attached to an epi-illumination filter block for blue (450-490nm excitation; >520nm emission wavelength), green (530-560nm; >580nm) and ultraviolet (330-390nm; >430nm) light and 10x and 20x long distance objectives. The microscopic images were recorded by a charge-coupled device video camera (FK6990; Pieper, Schwerte, Germany) and transferred to a DVD system for off-line evaluation.

**Atomic force microscopy (AFM)**

Plasmatic VWF (1 µg) (Merck, Darmstadt, Germany) was adsorbed to freshly cleaved mica (Science Service GmbH, Munich, Germany). Subsequently *S. aureus* Cowan I (1 µl of a suspension of an OD$_{540}$ of 1) was flushed. Intermittent contact mode AFM (the NanoWizard I, JPK Instruments, Berlin, Germany) was performed in air, applying a cantilever with a spring constant of 1 N/m and a resonant frequency of 100 kHz (NSC36, MicroMash, Tallinn, Estonia).
Supplemental Figures and Figure Legends

Supplemental Figure 1. VWF-dependent *S. aureus* adherence to intact endothelium. Stimulated, confluent HUVECs were flushed with bacteria at a shear stress of 10 dyne/cm². (A) Fluorescence image of FITC-labeled *S. aureus* Cowan I (green) bound to ULVWF fibers (red) released from an intact endothelial cell layer (bright-field). Scale bars correspond to 50 µm. (B) Magnification of *S. aureus* Cowan I (green) co-localized with ULVWF fibers (red). Regions of co-localization are represented in yellow. Scale bar corresponds to 20 µm.

Supplemental Figure 2. *S. aureus* adhesion to ULVWF fibers. Live image of fluorescent-labeled *S. aureus* SA133 (green) bound to ULVWF fibers (red) under high shear flow conditions (10 dyne/cm²). ULVWF fibers were stained with fluorescent-labeled antibody under flow conditions. Scale bars correspond to 50 µm.
**Supplemental Figure 3.** *S. aureus* is covered by ULVWF fibers. (A) High resolution surface topography of *S. aureus* Cowan I (arrowhead) that were entrapped by ULVWF fibers (arrow) obtained by atomic force microscopy. Height is false-color coded as indicated by the color bar on the right. Scale bar corresponds to 1 µm (B) Corresponding 3D image of *S. aureus* enveloped by ULVWF fibers. Scale bar corresponds to 1 µm.
Supplemental Figure 4. ULVWF fiber formation *in vivo* after stimulation. (A) Cryosections of TNFα-stimulated and unstimulated mouse tissue. ULVWF fibers (green) and endothelial CD31 (red) were stained with fluorescent-labeled antibody. Nuclei (blue) were stained with DAPI. Scale bars correspond to 20 µm. (B) Magnification of ULVWF fibers (arrows) stretched within the vessel lumen after TNFα stimulation (right). Without stimulation VWF are located within the vessel wall (left).

Supplemental Movie Legend

Supplemental Movie 1. *S. aureus* adhesion to ULVWF fibers after collapse and re-elongation under stop-flow. Perfusion of fluorescent-labeled *S. aureus* SA133 over histamine-stimulated HUVECs resulted in the formation of string-like structures that tethers bacteria. Interruption of flow (time 00:03:17.) lead to the contraction of ULVWF fibers until
flow starts again (time 00:09:85.). Timestamp in hh:mm:ss. as indicate. Arrow corresponds to 20 μm and arrowhead indicates the flow direction.

**Supplemental Movie 2. S. aureus adhesion to ULVWF fibers that are waving in the direction of flow.** Perfusion of fluorescent-labeled *S. aureus* SA133 over histamine-stimulated HUVECs resulted in the formation of string-like structures that tethers bacteria and waving under continuous flow. Timestamp in hh:mm:ss. as indicate. Arrow corresponds to 20 μm and arrowhead indicates the flow direction.

**Supplemental References**

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