Interaction of Shiga Toxin with the A-domains and Multimers of von Willebrand Factor*§

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Significance: Shiga toxin binding to VWF multimers specifically mediates increased secretion or persistence of von Willebrand Factor (VWF) in DHUS (3, 12–15). Thrombocytopenia characteristic of DHUS and accumulation of platelets atop intact (i.e. non-desquamated) glomerular endothelial cells in the renal circulation have been shown in sophisticated and carefully timed morphological studies (13, 15). These thrombi may be the result of increased secretion or persistence of von Willebrand Factor (VWF), a multimeric glycoprotein secreted by endothelial cells that mediates platelet adherence and aggregation (3, 4, 16). We hypothesized that the hyper-adhesive ultra-large VWF (ULVWF) multimers secreted from the endothelial cells play a role in the pathophysiology of DHUS, a speculation supported by data obtained from in vitro microfluidic flow models and in vivo primate studies (4, 16–18). ADAMTS-13 (a disintegrin and metalloprotease with thrombospondin domains 13), the plasma metalloprotease responsible for the cleavage of VWF into smaller less reactive forms, remains within a broad normal concentration range and does not provide an explanation for the pro-thrombotic pathologies during DHUS episodes (16, 19–21). We have investigated the binding and functional interactions among Shiga-like toxin, VWF, and ADAMTS-13 to determine the molecular explanation for thrombus formation in DHUS.

EXPERIMENTAL PROCEDURES

Plasma Preparation—Human blood from unmedicated healthy donors was drawn into final concentrations of 0.38% sodium citrate or 5 mM EDTA. Blood was centrifuged at 150 × g for 15 min at room temperature to isolate citrated and EDTA normal plasma (NP). All work on human VWF and human endothelial cells, including experiments in this study, have been specifically approved by the Rice University Institutional Review Board. Human tissues and blood samples were collected under a protocol approved by the Rice University Institutional Review Board. Donors provided their written informed consent to participate in the study.

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2 The abbreviations used are: HUS, hemolytic-uremic syndrome; Stx, shiga toxin; VWF, von Willebrand factor; ULVWF, ultra-large VWF; DHUS, diarrhea-associated hemolytic-uremic syndrome; HUVEC, human umbilical vein endothelial cell; NP, normal plasma; Ctx, cholera toxin.
**Antibodies, Proteins, and Toxins**—Stx-1 was fluorescently imaged using affinity purified polyclonal goat antibodies generated against a 20-amino acid sequence within Stx-1 B-subunits (SLT-1B, Bethyl Laboratories, Montgomery, TX) plus Alexa Fluor 594 (red) donkey anti-goat IgG (Invitrogen), and with an affinity-purified mouse monoclonal anti-SLT-1 (List Biological Laboratories, Campbell, CA) plus Alexa Fluor 594 (red) goat anti-mouse IgG (Invitrogen). VWF was fluorescently detected with either rabbit polyclonal anti-human VWF (Ramco Laboratories, Stafford, TX) plus Alexa Fluor 488 (green) chicken anti-rabbit IgG (Invitrogen) or with goat polyclonal anti-human VWF (Bethyl Laboratories) plus Alexa Fluor 488 (green) donkey anti-goat IgG (Invitrogen). Cholera toxin was fluorescently detected using rabbit polyclonal anti-cholera toxin whole antiserum (Sigma-Aldrich) plus Alexa Fluor 594 (red) chicken anti-rabbit IgG (Invitrogen).

Recombinant VWF single A-domain proteins (A1 (28 kDa), A2 (23 kDa), and A3 (23 kDa)) were expressed in *E. coli* and purified and checked for the monomeric state as previously described (22–26). Purity was confirmed through gel electrophoresis (4–15% SDS-PAGE) by Coomassie staining and Western blot detection using goat anti-human VWF, rabbit anti-goat IgG-HRP (Bethyl Laboratories), and chemiluminescence.

Shiga toxin-1 (Stx-1) was purchased from List Biological Laboratories and used primarily at 0.1 nM except in FRET assays where a range of Stx-1 concentrations were tested. Cholera toxin (Ctx, Sigma-Aldrich) with an analogous A (22 and 5 kDa) B₅ (10.6 kDa) subunit structure to Stx-1 was used as a control toxin at 0.125 μM (7). Toxin concentrations were consistent throughout experimentation. The purities of Stx-1 and Ctx were confirmed by 4–15% SDS-PAGE, Coomassie staining and Western blot detection (goat anti-SLT-1B plus rabbit anti-goat IgG-HRP, rabbit anti-Ctx plus goat anti-rabbit IgG-HRP), and chemiluminescence.

**Fluorescent Microscopy**—Fluorescent images were acquired using IP Lab software (version 3.9.4r4; Scanalytics, Inc., Fairfax, VA) on a Nikon Diaphot TE300 microscope equipped with a CFI Plan Fluor 60× oil numerical aperture 1.4 objective plus 10× projection lens (Nikon, Garden City, NY), SensiCamQE CCD camera (Cooke Corp., Romulus, MI), motorized stage, and dual filter wheels (Prior, Rockland, MA) with single band excitation and emission filters for FITC/TRITC/CY5/DAPI (Chroma, Rockingham, VT). The captured image dimensions were as follows: 78 μm × 58 μm or 688 pixels × 512 pixels (1 pixel = 0.113 μm).

**Fluorescent Staining of Stimulated Endothelial Cells**—Human umbilical vein endothelial cells (HUVECs) seeded on glass coverslips were washed with PBS, pH 7.4, and stimulated for 2 min with 100 μM histamine in PBS. To prevent HUVEC-released ADAMTS-13 from cleaving secreted ULVWF strings, cells were stimulated in a relatively high incubation volume to cell surface ratio (i.e. 1 ml of buffer to 10 cm² of cells) for a short time period. Following stimulation, cells were incubated with 0.1 nM Stx-1 in 1% BSA/PBS with rabbit anti-human VWF and chicken anti-rabbit IgG-488 for 15 min. Cells were fixed for 10 min with 1% p-formaldehyde in PBS, washed 3× with PBS, and then stained with either goat anti-SLT-1B plus donkey anti-goat IgG-594 or mouse anti-SLT-1 plus goat anti-mouse IgG-594 for 15 min. HUVEC nuclei (blue) were detected with 1 nM DAPI. In cholera toxin experiments, the histamine-stimulated HUVECs were incubated with 0.125 μM Ctx diluted in 1% BSA/PBS plus goat anti-human VWF and donkey anti-goat IgG-488 for 15 min. Cells were fixed for 10 min with 1% p-formaldehyde in PBS, washed 3× with PBS, and then stained with rabbit anti-cholera toxin plus chicken anti-rabbit IgG-594 for 15 min.

**Quantification of Stx-1 Bound to HUVEC-secreted/anchored ULVWF Strings**—HUVEC-anchored ULVWF strings detected with anti-VWF primary antibody plus fluorescent secondary IgG-488 were electronically traced in 488-nm (green) captured images at 600× magnification, and the emitted fluorescent intensity was measured and integrated along the line. The x- and y-coordinates of the traced ULVWF line were transferred to the corresponding 594-nm (red) captured images obtained using the detecting antibodies for Stx-1 (goat anti-SLT-1B or mouse anti-SLT-1) or in control experiments for cholera toxin (rabbit anti-cholera toxin) plus fluorescent secondary IgG-594 antibodies. The fluorescent intensity at 594 nm from these images was measured and integrated along the transferred line coordinates. To determine background 594-nm intensity, the line coordinates were trans-located ~20 pixels (~2.3 μm) parallel to its original position within each 594-nm image, and the fluorescent intensity was measured and subtracted.

**Measurement of Stx-1 Binding to Recombinant VWF A-domains**—Recombinant VWF A-domain proteins (A1, A2, A3) in concentrations ranging from 0.0625 to 1 μM in 50 mM bicarbonate buffer, pH 9.6, were immobilized overnight at 4 °C in a 96-well plate. The control wells were coated with 1% BSA in bicarbonate buffer, with subsequent dilutions to match the A-domain dilutions. The wells were washed with Tris-buffered saline, 0.05% Tween 20 (TBS-T) and blocked with 1–3% BSA/PBS for one hour at 37 °C. The blocked well were incubated with 0.1 nM Stx-1 in 1% BSA/PBS for 1 h at 37 °C. Subsequently, wells were washed 3× with TBS-T and incubated with goat anti-SLT-1B plus rabbit anti-goat IgG-HRP for 1 h at 37 °C. Bound Stx-1 was detected with 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance at 450 nm was determined with a Tecan Infinite M200 plate reader (Mannedorf, Switzerland). The experiment was conducted in triplicate.

**HUVECs**—Primary HUVECs were isolated from umbilical veins as described previously (4). Cells were seeded in flasks or glass coverslips coated with attachment factor (Invitrogen). HUVECs were grown in MCDB 131 (Invitrogen) and supplemented with 3% penicillin-streptomycin, 0.2 mM L-glutamine, and low serum growth supplement (Invitrogen). From previous studies, we know that the secretion and anchorage of hyper-adhesive ULVWF multimeric strings from HUVECs and glomerular microvascular endothelial cells are similar (4).

**VWF Depletion of HUVEC Supernatant**—Cell supernatant was collected after 1–3 days from HUVECs maintained in serum-free MCDB 131 medium supplemented with insulin-transferrin-selenium (Invitrogen) plus glutamine and penicillin-streptomycin. Cell supernatant was depleted of VWF by overnight mixture at 4 °C with rabbit anti-human VWF IgG coupled to CNBr-activated Sepharose beads (Sigma-Aldrich). The anti-VWF coupled Sepharose beads were prepared following the manufacturer’s instructions.
Shiga Toxin and VWF

Measurement of Stx-1 Binding to Immobilized VWF Secreted from HUVECs—Samples of HUVEC supernatant (containing VWF) and VWF-depleted HUVEC supernatant were diluted in 50 mM bicarbonate and immobilized overnight at 4 °C on a 96-well plate. The wells were then blocked with 1–3% BSA/PBS overnight at 4 °C. The TBS-T washed wells were incubated with 0.1 nm Stx-1 for 1 h at 37 °C. The wells were washed, and the amount of bound Stx-1 was determined by immunoassay using goat anti-SLT-1B plus rabbit anti-goat IgG-HRP and 3,3′,5,5′-tetramethylbenzidine substrate detection at 450 nm. The experiment was conducted in triplicate.

Measurement of ADAMTS-13 Cleavage Rate by FRET Assay—Changes in the rate of ADAMTS-13 cleavage in the presence of Shiga toxin was measured by a FRET assay modified from a protocol by Kokame et al. (27). The FRETS-VWF73 fluorescent-quenching substrate (AnaSpec, Fremont, California) is a 73-amino acid peptide within the VWF A2 domain that contains the Tyr1605-Met1606 ADAMTS-13 cleavage site. The FRETS-VWF73 substrate was initially solubilized in 25% dimethyl sulfoxide to 100 μM and then further diluted to a working concentration of 2 μM in 5 mM Bis-Tris, 25 mM CaCl2, 0.005% Tween 20, pH 6.0 (Bis-Tris buffer). 2 μM FRETS-VWF73 substrate was incubated at 37 °C with and without 0.1 nM Stx for 15 min in white 96-well plates. Normal human citrated plasma (NP) samples were diluted 1:25 into Bis-Tris buffer. Samples of normal EDTA plasma (EDTA) diluted equally were used as a negative control. The reaction was started by the addition of diluted plasma at 37 °C to the FRETS-VWF73 substrate solution. Fluorescent intensities were collected every 2 min for 30 min with an excitation wavelength of 340 nm (bandwidth of 9 nm), an emission wavelength of 450 nm (bandwidth of 20 nm), and a gain of 100 using a Tecan Infinite M200 plate reader. Experiments were conducted in triplicate, and the reaction rates were determined by linear regression. In additional experiments, FRETS-VWF73 was incubated with a lower range of Stx concentrations from 1 × 10^{-11} M to 6.25 × 10^{-13} M.

RESULTS

Stx-1 Binds to Secreted and Anchored ULVWF Strings from Stimulated HUVECs—Stx-1 binding to endothelial cell-anchored ULVWF was demonstrated in HUVECs stimulated with 100 μM histamine and in the presence of either 0.1 nm Stx-1 or 0.125 μM Ctx under static conditions. Ctx, with an analogous AB5 subunit structure to Stx-1, was used as a negative control. Histamine was chosen as the stimulating agent to maintain consistency when comparing amounts of Stx-1 and Ctx binding upon HUVEC-secreted/anchored ULVWF strings. The cells were fixed and stained for either VWF and Stx-1 or for VWF and Ctx. Fluorescent microscopy demonstrated that Stx-1 bound along HUVEC secreted and anchored ULVWF strings, whereas Ctx did not bind (Fig. 1). Quantification of these images demonstrated that anchored ULVWF strings had 8-fold more Stx-1 attached than Ctx (Fig. 2A). These results were verified with both goat polyclonal and mouse monoclonal antibodies against Stx. The purity of toxins and specificity of antibodies are shown in Fig. 2B. In the absence of secreted ULVWF strings, Stx-1 (and Ctx) bound to the surfaces of unstimulated HUVECs (supplemental Fig. S1).

Stx-1 also Binds to Immobilized, VWF-rich HUVEC Supernatant—VWF-rich HUVEC supernatant samples with a range of VWF antigen from 100–750 ng/ml were immobilized and compared with samples completely and partially (50%) depleted of VWF antigen. Partial depletion of VWF reduced Stx-1 binding by 30%, and full depletion reduced binding by 90% (Fig. 3).

**FIGURE 1.** Stx-1 binds to ULVWF secreted by, and anchored to, stimulated HUVECs. HUVECs seeded on glass coverslips were stimulated with 100 μM histamine for 2 min and incubated for 15 min with rabbit polyclonal anti-human VWF plus chicken anti-rabbit IgG-488 in the presence of 0.1 nm Stx-1. Endothelial cells (EC) were fixed, washed with PBS, and stained with goat polyclonal anti-SLT-1B plus donkey anti-goat IgG-594 (A–C) or with mouse monoclonal anti-SLT-1 plus goat anti-mouse IgG-594 (D–F). The white arrows in C and F point to areas on the ULVWF strings with high levels of Stx-1 binding. In G–I, the histamine-stimulated HUVECs were incubated with goat polyclonal anti-human VWF plus donkey anti-goat IgG-488 in the presence of 0.125 μM Ctx for 15 min. Following fixation and PBS washing, the cells were stained with rabbit polyclonal anti-cholera toxin plus chicken anti-rabbit IgG-594. Images were 600× with image dimensions of 78 × 58 μm. Cell nuclei were detected with DAPI (blue). White arrows in C and F point to areas on ULVWF strings with high level of Stx-1 binding. Images were selected from 6–12 experiments under each condition.

Stx-1 binds to ULVWF secreted by, andanchored to, stimulated HUVECs. HUVECs seeded on glass coverslips were stimulated with 100 μM histamine for 2 min and incubated for 15 min with rabbit polyclonal anti-human VWF plus chicken anti-rabbit IgG-488 in the presence of 0.1 nm Stx-1. Endothelial cells (EC) were fixed, washed with PBS, and stained with goat polyclonal anti-SLT-1B plus donkey anti-goat IgG-594 (A–C) or with mouse monoclonal anti-SLT-1 plus goat anti-mouse IgG-594 (D–F). The white arrows in C and F point to areas on the ULVWF strings with high levels of Stx-1 binding. In G–I, the histamine-stimulated HUVECs were incubated with goat polyclonal anti-human VWF plus donkey anti-goat IgG-488 in the presence of 0.125 μM Ctx for 15 min. Following fixation and PBS washing, the cells were stained with rabbit polyclonal anti-cholera toxin plus chicken anti-rabbit IgG-594. Images were 600× with image dimensions of 78 × 58 μm. Cell nuclei were detected with DAPI (blue). White arrows in C and F point to areas on ULVWF strings with high level of Stx-1 binding. Images were selected from 6–12 experiments under each condition.
Stx-1 Binds to Recombinant VWF A-domains A1 and A2—

Binding of Stx-1 to recombinant VWF A-domains (A1, A2, and A3) was assessed by ELISA. Stx-1 bound predominately to immobilized VWF A1 and A2 domains (Fig. 4A). Stx-1 binding to A1 was 15% greater than to A2, and 3-fold greater than to the A3 domain. The binding of Stx-1 to VWF A1 and A2 was concentration-dependent with saturation at 0.25 nM (H9262). Purity of A-domain proteins is shown in Fig. 4B.

Stx-1 Inhibits ADAMTS-13-mediated Cleavage of Tyr1605-Met1606 of the A2 Domain—The effect of Stx-1-VWF interaction upon ADAMTS-13-mediated cleavage of the Tyr1605-Met1606 peptide bond in the A2 domain was assessed using a synthetic FRETS-VWF73 substrate containing a 73-amino acid sequence of the A2 domain that includes the Tyr1605-Met1606 peptide bond cleaved by ADAMTS-13. The addition of 0.1 nM Stx-1 to NP reduced the ADAMTS-13-mediated cleavage rate of the FRETS-VWF73 substrate by 35% (Fig. 5). The cleavage of FRETS-VWF73 by plasma containing ADAMTS-13 inactivated by EDTA, which binds the Zn2+ and Ca2+ required for enzyme activity, is shown for comparison. The inset in Fig. 5B demonstrates that the inhibitory effect of Stx-1 is maintained even at lower concentrations.

DISCUSSION

We previously described a delay in ADAMTS-13-mediated cleavage of HUVEC-anchored ULVWF multimeric strings with adherent platelets under flowing conditions in the presence of nanomolar concentrations of Stx and suggested that this effect may contribute to the thrombotic microangiopathy in DHUS (4). The experiments described in this study demonstrate that Stx binds to the A1 and A2 domains of VWF and that binding results in a decreased rate of ADAMTS-13-mediated cleavage of the 1605–1606 peptide bond in the A2 domain of VWF. The results provide evidence in support of the importance of Stx-VWF interaction in the pathophysiology of DHUS.

The FRET VWF cleavage assay was conducted using plasma with ADAMTS-13 concentrations within a normal range, as found in DHUS patients (19–21, 28). The inhibitory effect of 0.1 nM Stx on ADAMTS-13-mediated cleavage of the Tyr1605-Met1606 peptide bond occurred within minutes of Stx addition and was likely the result of Stx binding to the VWF A2-derived 73-amino acid peptide due to the acute nature of this effect. This suggests that a delay in cleavage of VWF can be achieved without decreased ADAMTS-13 levels (26).

The importance of Stx-VWF interaction is further supported by results from our 2005 study (4) where secreted ULVWFs stimulated by Stx-1 and Stx-2 were visualized by adherent platelets. The presence of platelets upon Stx-occupied ULVWF indicate that Stx binding upon VWF does not obstruct platelet binding, meaning Stx-occupied ULVWF are still prothrombotic (4).

Stx-1 bound to surfaces of unstimulated HUVECs, presumably to Gb3 receptors in the absence of secreted ULVWF strings (1605–1606 peptide bond supplemental Fig. S1). Although Stx-1 and Stx-2 bind to Gb3 on both HUVECs and glomerular micro-
vascular endothelial cells and Stx-1 to the HUVEC-secreted/anchored ULVWF multimeric strings (as shown in this work), our data shows that Gb3 does not successfully compete with ULVWF for the binding to Stx-1 (4). Although the relative binding affinities of Stx for Gb3 and HUVEC-secreted/anchored ULVWF strings are not known, our study demonstrates that the binding affinity of Stx for the HUVEC-anchored ULVWF strings must be high. This is because after a 15-min incubation with 10⁻¹¹ M Stx-1, a sufficient quantity of the toxin attached to the HUVEC-anchored ULVWF strings to allow easy detection by immunofluorescence/microscopy, even when Gb3 binding sites on the HUVECs were also available.

The interaction between Stx and VWF (instead of Stx and ADAMTS-13) was clearly demonstrated by several means; Stx was capable of binding to HUVEC-secreted/anchored ULVWF multimeric strings, large soluble VWF multimers that were immobilized, and recombinant VWF A1 and A2 domains. The interaction between Stx and VWF A2 demonstrated in the ELISA likely explains the reduction in the rate of cleavage at Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ by ADAMTS-13 and may account for the persistence of hyper-adhesive HUVEC-secreted/anchored ULVWF strings for a time period sufficiently long to allow excessive platelet adherence and aggregation.

Stx-1 was further examined for binding to immobilized fibrinogen; however, the reactivity level was similar to that of its nonspecific interaction with immobilized BSA. This study focused on VWF because binding of Stx upon VWF creates an effect that is uniquely prothrombotic by preventing a natural cleavage, as opposed to binding upon other proteins, which may not produce an effect as heavily implicated in thrombosis.

The role of glycosylation in Stx-VWF binding (analogous to Stx binding on Gb3) was considered. Our experiments demonstrated that Stx binds to both non-glycosylated recombinant A-domain and glycosylated VWF multimers, indicating that the interaction can occur in the absence of carbohydrate chains. The interaction between Stx and VWF may also influence binding to platelet GPIbα. We have demonstrated previously that the structure of the A1 domain in isolated and full-length VWF is changed when bound to collagen (29). This conformational change is associated with an increase in binding capacity of VWF for GPIbα (30). We hypothesize that similar to collagen, the interaction between Stx and ULVWF alters the struc-
ture of the A1 domain and increases binding affinity for GPIbα. Furthermore, Stx, LPS, and cytokines, which are increased in DHUS, also increase secretion/anchorage of ULVWF multimeric strings from glomerular microvascular endothelial cells (4, 9, 16, 18, 31, 32). These mechanisms, together with the inhibitory action of Stx on ADAMTS-13 cleavage, presumably form part of the mechanism that contributes to the pathophysiology of thrombotic microangiopathy in DHUS.

In this study, we describe a novel and clinically significant molecular interaction between Stx and VWF. Stx binds to A1 and A2 or possibly to peptide regions near the A1-A2 junction. This binding obstructs the access of ADAMTS-13 to the Tyr<sup>1605</sup>-Met<sup>1606</sup> A2 cleavage site. The resulting delay in cleavage of endothelial cell-anchored ULVWF multimers, by increasing the time available for platelet adhesion, activation, and aggregation, provides a possible explanation for thrombotic microangiopathy in DHUS.

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