Tissue Factor–Dependent Procoagulant Activity of Subtilase Cytotoxin, a Potent AB₅ Toxin Produced by Shiga Toxigenic \textit{Escherichia coli}

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Subtilase cytotoxin (SubAB), produced by certain virulent Shiga toxigenic \textit{Escherichia coli} strains, causes hemolytic uremic syndrome–like pathology in mice, including extensive microvascular thrombosis. SubAB acts by specifically cleaving the essential endoplasmic reticulum chaperone binding immunoglobulin protein (BiP). BiP has been reported to inhibit the activation of tissue factor (TF), the major initiator of extrinsic coagulation. We hypothesized that the apparent prothrombotic effect of SubAB in vivo may involve the stimulation of TF-dependent procoagulant activity. TF-dependent procoagulant activity, TF messenger RNA (mRNA) levels, and BiP cleavage were therefore examined in human macrophage cells and primary human umbilical vein endothelial cells exposed to SubAB. In both types of cells, SubAB significantly increased TF-dependent procoagulant activity, induced TF mRNA expression, and mediated BiP cleavage. No effects were seen when cells were treated with a nonproteolytic mutant toxin, SubAA272B. Our results suggest that the procoagulant effect of SubAB may be dependent on both the up-regulation of TF expression and the activation of TF by means of BiP cleavage.

Shiga toxigenic \textit{Escherichia coli} (STEC) causes serious gastrointestinal disease in humans that can lead to potentially fatal hemolytic uremic syndrome (HUS). HUS is characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure, which are believed to be principally caused by Shiga toxin–mediated damage to the microvascular endothelium [1]. Of interest, some STEC strains produce an unrelated AB₅ toxin called subtilase cytotoxin (SubAB) [2]. These STEC strains lack the locus of enterocyte effacement, which is thought to contribute to the virulence of the better characterized O157:H7 strains. Although SubAB-positive STEC strains are less common than are strains that are positive for the locus of enterocyte effacement, SubAB-positive strains are nevertheless capable of causing severe disease in humans, including outbreaks of HUS [2, 3]. Remarkably, intraperitoneal injection of purified SubAB causes HUS-like pathology in mice, including extensive microvascular thrombosis and other histological damage in the brain, kidneys, and liver, as well as dramatic splenic atrophy [4]. These findings raise the possibility that SubAB directly contributes to pathology in humans infected with strains of STEC that produce both Shiga toxin and SubAB.

SubAB acts by binding to glycan receptors on the surface of target cells with its B pentamer, followed by internalization and retrograde transport to the endoplasmic reticulum [5]. Once in the endoplasmic reticulum compartment, the A subunit of SubAB (an extraordinarily specific subtilase-like serine protease) cleaves an essential endoplasmic reticulum Hsp70 fami-
Bip (also known as 78-kDa glucose regulated protein [GRP78]) is responsible for proper folding of newly synthesized proteins, and because Bip is the master regulator of the endoplasmic reticulum stress response, it is essential for the maintenance of endoplasmic reticulum homeostasis [7]. Prior studies have shown that overexpression of Bip in T24/83 cells, a prothrombotic human bladder carcinoma cell line, decreases thrombin generation by inhibiting cell surface tissue factor (TF) procoagulant activity, thereby suppressing the prothrombotic potential of the cells [8]. Bip has also been reported to negatively regulate TF procoagulant activity in both murine endothelial and macrophage cells by directly binding to and functionally inhibiting TF [9]. TF is a 47-kDa transmembrane glycoprotein and is the major physiological initiator of the extrinsic coagulation cascade. TF binds to the serine protease, factor VII/VIIa, leading to activation of factors IX and X and the subsequent generation of thrombin [10]. TF is implicated in many pathological conditions, including atherosclerosis [11], postoperative thrombosis [12], sepsis-linked disseminated intravascular coagulation [13, 14], chronic inflammation [15], and cancer [16]. Alternatively spliced human TF (asTF) is a soluble isoform of TF present in blood that may also contribute to procoagulant activity [17, 18]. Both TF and asTF have been detected in many cell types, including macrophages and endothelial cells [9, 18].

Because SubAB cleaves Bip and because Bip is known to inhibit activation of TF on the surface of many cell types, we hypothesized that the apparent prothrombotic effect of SubAB observed in vivo may involve the stimulation of TF-dependent procoagulant activity. In this study, we examined TF procoagulant activity by measuring TF-dependent factor Xa generation and compared the effects of SubAB with those of Shiga toxin 2 (Stx2) in human macrophage (U937) and primary human umbilical vein endothelial cells (HUVECs). We also investigated Bip cleavage and TF and asTF gene expression in cells exposed to SubAB.

**METHODS**

**Purification of toxin.** SubAB and its nontoxic derivative (SubA<sub>272</sub>B) were purified as described elsewhere [2, 19]. Purification involved fusion of a His<sub>6</sub> tag to the C-terminus of the B subunit, which enabled purification by Ni-NTA chromatography under nondenaturing conditions. Stx2 was purified using an identical approach.

**Cell culture and toxin treatment.** All cells were grown at 37°C in 5% carbon dioxide in culture medium (Roswell Park Memorial Institute 1640 medium supplemented with 10 mmol HEPES, 0.2 mol L-glutamine, 10% heat-inactivated fetal calf serum, 50 IU/mL penicillin, and 50 μg/mL streptomycin). U937 cells were provided by Michael James (Rheumatology Unit, Royal Adelaide Hospital, Adelaide, Australia). HUVECs were harvested and incubated as described elsewhere [20]. HUVECs were used at <4 passages. For toxin treatment, cells were seeded into appropriately sized tissue culture plates; U937 6(1–2) /10<sup>6</sup> cells/mL or confluent monolayers of HUVECs were exposed to SubAB, SubA<sub>272</sub>B, or Stx2 at the indicated concentrations in culture medium containing 1% heat-inactivated fetal calf serum for the indicated times.

**Cell-based TF-dependent factor Xa generation assay.** In a 24-well culture plate, U937 cells (~1 × 10<sup>6</sup> cells per well) or HUVECs (confluent monolayer) were incubated with toxins at the indicated concentrations in 260 μL of culture medium containing 1% fetal calf serum with added phenol red at 37°C in 5% carbon dioxide for 1, 4, 6, 16, or 24 h. Factor VIIa (10 nmol/L; HFVIIa 2793AL; Enzyme Research Laboratories) and factor X (50 nmol/L; HFX 2942AL; Enzyme Research Laboratories) were then added, and trays were incubated at 37°C for an
Figure 2. Subtilase cytotoxin (SubAB)–mediated binding immunoglobulin protein (BiP) cleavage in human macrophage (U937) cells (A) and human umbilical vein endothelial cells (HUVECs) (B). The mobilities of immunoreactive native BiP (size, 72 kDa) and the cleaved C-terminal fragment (size, 28 kDa) are indicated. “M” denotes molecular size markers (Novex Sharp Protein Standards; Invitrogen). Stx2, Shiga toxin 2.
Figure 3. Induction of tissue factor (TF) and alternatively spliced TF (asTF) messenger RNA (mRNA) by subtilase cytotoxin (SubAB) and Shiga toxin 2 (Stx2). RNA was isolated from human macrophage (U937) cells or human umbilical vein endothelial cells (HUVECs) treated with 1 μg/mL SubAB or Stx2 for the indicated times. TF and asTF mRNA levels were measured using quantitative real-time reverse-transcription polymerase chain reaction. Results are expressed as the fold change in mRNA level relative to that in control (medium-only) cells, and data shown are the mean (± standard deviation) for triplicate assays.

trifuged (24,000 g for 30 min at 4°C), washed with acetone, and then redissolved in 2× LUG. Cell lysates or trichloroacetic acid–precipitated supernantant protein samples were boiled for 5 min and separated using SDS-PAGE [22].

Protein samples separated on SDS-PAGE gels (13%) were electroblotted onto polyvinylidene fluoride (Immobilon) or nitrocellulose membranes and were probed with goat anti-human BiP/GRP78 (c-20; Santa Cruz Biotechnology). Labeled bands were detected using antigoat immunoglobulin G conjugated to alkaline phosphatase or horseradish peroxidase with NBT/X-phosphate substrate (Roche) or chemiluminescent substrate (Sigma), respectively. A Novex Sharp prestained protein ladder (Invitrogen) was used as a molecular size marker.

Flow cytometry. To determine the time course of SubAB internalization in U937 cells, after SubAB treatment, U937 cells were washed and fixed in 1% paraformaldehyde at the indicated times. After permeabilization with 0.1% Triton X-100, intracellular SubAB was labeled using rabbit anti-SubA, followed by Alexa 488–conjugated antirabbit immunoglobulin G (Invitrogen). After 4 phosphate-buffered saline washes, cell suspensions were subjected to analysis by fluorescence-activated cell sorting (FACS); data from 10,000 cells for each sample were acquired using a FACSCanto and were analyzed using CellQuest Pro software (version 5.0.3; Becton Dickinson).

Fluorescence microscopic analysis. To determine the intracellular distribution of SubAB taken by HUVECs, cells were incubated for 1 h with SubAB (1 μg/mL), washed with ice-cold phosphate-buffered saline, and kept on ice. SubAB was labeled with anti-SubA and Alexa 488 conjugate, as described above. The cells were then washed and examined with an EVOS-fl high-resolution digital inverted fluorescence microscope system, and digital images were captured.

RESULTS

Internalization of SubAB by U937 cells and HUVECs. FACS analysis revealed that SubAB was rapidly internalized by U937 cells: the uptake peaked within 30 min of exposure to the toxin (Figure 1A). Fluorescence microscopic analysis also demonstrated that a significant amount of SubAB was internalized by HUVECs after 1 h of incubation. SubAB taken up by HUVECs was seen in a large juxtanuclear region of the cytoplasm and in puncta (Figure 1B). Both the kinetics of SubAB uptake and its distribution in these cell types are consistent with the findings reported previously for Vero cells [5].

SubAB-mediated BiP cleavage. Western blot analysis revealed substantial BiP cleavage in SubAB-treated U937 cells (Figure 2A) and in HUVECs (Figure 2B). This was evident
Figure 4. Stimulation of tissue factor–dependent factor Xa generation in human macrophage (U937) cells. U937 cells were incubated with subtilase cytotoxin (SubAB) or its nontoxic derivative (SubA_{A272B}) at the indicated concentrations for 1–24 h. Factor Xa generation was quantified using chromogenic substrate, and the absorbance at 405 nm (A_{405}) was read as described in the Methods. Data shown are the mean (± standard error of the mean) from triplicate assays. The differences between SubAB-treated cells and SubA_{A272B}-treated cells were analyzed using the Student t test. ***P < .001; **P < .01; *P < .05.

from decreased levels of the native 72-kDa protein and the appearance of a 28-kDa C-terminal fragment (the other BiP cleavage product, a 44-kDa N-terminal fragment [6], was not seen because the antibody used is specific for a peptide derived from the C-terminus). No BiP cleavage was detected in cells treated with a mutant toxin (SubA_{A272B}) [2] that lacks protease activity (data not shown) or in cells treated with Stx2 (Figure 2). Cleavage of BiP in SubAB-treated cells was apparent in the results of the earliest tests (30 min for U937 cells [Figure 2A] and 60 min and HUVECs [Figure 2B]). Furthermore, the cleaved 28-kDa C-terminal BiP fragment was detected in the cell lysate and in the culture medium (supernatant) from both U937 cells and HUVECs. However, the native BiP (size, 72 kDa) was not detected in the supernatant from HUVECs (Figure 2).

**TF mRNA induction.** RT-PCR revealed that TF and asTF are expressed in both U937 cells and HUVECs. Real-time RT-PCR analysis revealed that both SubAB and Stx2 increased TF and asTF mRNA levels in U937 cells. Maximal up-regulation of both isoforms (2–3-fold increase for TF and 3–4-fold increase for asTF) occurred at 1 h after toxin treatment in cells exposed to either SubAB or Stx2 (Figure 3). The response of HUVECs to SubAB was slower but much more profound than that to Stx2, with maximal up-regulation (∼18-fold increase for TF mRNA and ∼9-fold increase for asTF mRNA) occurring 4 h after toxin treatment. Of interest, however, the response level of HUVECs to Stx2 was much lower, not exceeding a 2.2-fold increase in up-regulation for either isoform at any time (Figure 3).

**Procoagulant activity.** SubAB induced significant increases
Figure 5. Comparison of the procoagulant effects of subtilase cytotoxin (SubAB) and Shiga toxin 2 (Stx2). Human macrophage (U937) cells were incubated with SubAB or Stx2 at the indicated concentrations for various times. 

A, Dose-dependent response of the effect of Stx2 on tissue factor (TF)–dependent factor Xa generation at 4 h. B, Time courses of TF-dependent factor Xa generation by U937 cells treated with low doses of SubAB and Stx2 alone and in combination. Factor Xa generation was quantified using chromogenic substrate, and the absorbance at 405 nm ($A_{405}$) was read as described in the Methods. Data shown are the mean (± standard error of the mean) from triplicate assays. The differences between Stx2-treated cells and control cells were analyzed using the Student $t$ test. *** $P<.001$; ** $P<.01$; * $P<.05$.

in TF-dependent factor Xa generation in U937 cells (Figure 4). The procoagulant effect of SubAB was both dose-dependent and time-dependent, with maximal stimulation occurring at 4–6 h (Figure 4). Stx2 also stimulated TF-dependent factor Xa generation in U937 cells to a similar extent and with similar kinetics (Figures 5A and 5B). Of interest, there was no evidence of procoagulant synergy between SubAB and Stx2. Responses were not significantly greater in U937 cells treated with a combination of SubAB and Stx2 (both used at doses of either 0.1 or 0.01 μg/mL) than in those treated with either toxin alone (Figure 5B). The basal level of TF-dependent factor Xa generation elicited by untreated U937 cells and that elicited by cells treated with either SubAB or Stx2 was significantly higher when cells were assayed rather than the respective culture supernatants (Figure 6). This finding indicates that the toxin-induced procoagulant activity is largely attributable to activation of cell membrane–bound TF, rather than to the soluble aTF.

TF-dependent factor Xa generation was also stimulated by treatment of HUVECs with either SubAB or Stx2. However, the time course was markedly delayed in comparison with that for U937 cells. Statistically significant stimulation was not observed before 6 h after toxin treatment, and maximal stimulation of procoagulant activity occurred at 16–24 h (Figure 7). Procoagulant activity response levels were significantly greater for both SubAB and Stx2 than for control cells at 6–48 h. At 6, 16, and 24 h, SubAB elicited significantly stronger procoagulant responses than did Stx2. However, the response elicited by Stx2 was still maintained at its maximal level at 48 h, at which time it was significantly greater than that elicited by SubAB. The nonproteolytic SubA272B did not elicit significant TF-dependent procoagulant activity levels, relative to those of untreated HUVECs, at any time (Figure 7).

**DISCUSSION**

This study showed that SubAB and Stx2 significantly increase TF-dependent procoagulant activity in both human macrophages and endothelial cells. The procoagulant effect of SubAB appeared and levels peaked earlier in macrophages than in HUVECs, although the maximal levels achieved were similar. Real-time RT-PCR results showed that TF and aTF are expressed in both cell types; this is consistent with previous findings in
Figure 7. Stimulation of tissue factor–dependent factor Xa generation in human umbilical vein endothelial cells (HUVECs). HUVECs were incubated with 1 μg/mL subtilase cytotoxin (SubAB), its nontoxic derivative (SubA<sub>A272</sub>B), Shiga toxin 2 (Stx2), or medium only (control) for 1–48 h. Factor Xa generation was quantified using chromogenic substrate as described in the Methods. Data shown are the mean (± standard error of the mean) from triplicate assays. The differences between treatments were analyzed using the Student t test. ***: P < .001; **: P < .01; *: P < .05. A<sub>405</sub>, absorbance at 405 nm.

murine and human cells [9, 18]. Furthermore, TF and asTF mRNA was up-regulated by SubAB and Stx2 in both cell lines. The induction of TF and asTF mRNA in U937 cells was detected earlier than that observed in HUVECs, which is consistent with the earlier onset of toxin-induced TF-dependent procoagulant activity in U937 cells. However, the fold increases in mRNA levels for both TF and asTF in SubAB-treated or Stx2-treated HUVECs were much greater and were sustained for a much longer time than those in similarly treated U937 cells. This apparent discrepancy between the extent of gene up-regulation and actual TF-dependent procoagulant activity responses in the 2 cell types could be explained by lower baseline TF and asTF mRNA levels in HUVECs compared with those in U937 cells, such that absolute postinduction mRNA levels are similar. The findings are also consistent with a significant proportion of the procoagulant activity response being attributable to release and/or activation of preexisting TF.

Previous studies showed that TF expression and its procoagulant activity are increased by exposure to lipopolysaccharide [23–25]. However, in the present study, we used toxins purified from a lpxM-negative recombinant E. coli host strain, the lipopolysaccharide of which has negligible endotoxic activity. Moreover, TF-dependent procoagulant activity was not induced by treatment with the nonproteolytic SubA<sub>A272</sub>B, which was purified under conditions identical with those for SubAB and Stx2. Thus, the effects seen in this study are not the result of contaminating endotoxin.

The absence of TF-dependent procoagulant activity in cells treated with SubA<sub>A272</sub>B also indicates that proteolytic cleavage of the endoplasmic reticulum chaperone BiP (the only known
substrate of SubAB [6]) is essential for the phenomenon. BiP has been shown to negatively regulate TF procoagulant activity in both cell-based and non–cell-based coagulation assays [8, 9]. The inhibitory effect was not mediated by a decrease in cell surface TF protein levels, which suggests that retention of TF in the endoplasmic reticulum or attenuation of TF synthesis are not the causes [8]. Pozza and Austin [26] proposed that BiP inhibits TF activation either directly by formation of a BiP-TF complex, which locks TF into a latent or encrypted conformation, or indirectly by sequestering intracellular calcium and attenuating apoptosis. In the present study, the former explanation seems to be more likely, because significant SubAB-dependent TF procoagulant activity responses were evident by 4 and 6 h for U937 cells and HUVECs, respectively; this timing is too rapid for an apoptosis-dependent mechanism. In Vero cells, which are highly susceptible to SubAB, apoptosis is not evident until ~30 h [27], and for U937 cells, significant apoptosis is not seen until 48 h (H. Wang et al, unpublished data, 2010). In contrast, cleavage of BiP occurs rapidly in SubAB-treated U937 cells and HUVECs, and this may directly trigger dissociation of BiP-TF complexes. SubAB-mediated BiP cleavage may also contribute to TF-dependent procoagulant activity indirectly through downstream effects on cellular signaling pathways. The toxin rapidly induces an unfolded protein response in Vero cells by activation of the PKR-like endoplasmic reticulum kinase, inositol-requiring enzyme 1, and activating transcription factor 6 endoplasmic reticulum stress signaling pathways [27]. The toxin is also known to induce proinflammatory cytokine responses by activation of NF-κB by means of phosphorylation of Akt in rat renal epithelial cells [28]. Proinflammatory cytokines were previously shown to augment cell surface TF activity after exposure of human glomerular endothelial cells to Shiga toxin [29]. Thus, SubAB may share certain mechanisms with Shiga toxin in the up-regulation of TF-dependent procoagulant activity. However, the capacity of SubAB to cleave BiP and thereby directly mediate the release and/or activation of cell surface TF is a prothrombotic mechanism unique to this novel cytotoxin.

In summary, SubAB and Stx2 significantly increase TF-dependent procoagulant activity in human macrophages and endothelial cells in vitro. Both toxins are similarly effective but did not appear to exert a synergistic effect at the doses tested. The procoagulant effect of SubAB may be dependent not only on the up-regulation of TF expression but also on BiP cleavage-dependent activation of preexisting TF. Disturbance of the coagulation cascade may be critically important in triggering HUS in humans. TF-dependent factor Xa generation activates thrombin, which in turn increases fibrin deposition and platelet aggregation, leading to thrombotic microangiopathy and thrombocytopenia. Moreover, deposition of fibrin clots in glomerular capillaries leads to renal insufficiency and to physical damage to erythrocytes. This triad of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure is the defining hallmark of HUS. We previously reported that intraperitoneal injection of purified SubAB induces HUS-like pathology in mice; there was extensive microvascular thrombosis, hemolytic anemia, thrombocytopenia, and renal insufficiency, as well as extensive ischemic damage to multiple organs, including the kidneys, liver, and brain [4]. Thus, SubAB-mediated stimulation of TF-dependent procoagulant activity may directly contribute to thrombotic microangiopathy in patients with HUS caused by gastrointestinal infection with SubAB-producing STEC strains.

References