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Fatal hemorrhage induced by subtilase cytotoxin from Shiga-toxigenic *Escherichia coli*

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ABSTRACT

Subtilase cytotoxin (SubAB) is an AB_5 type toxin produced by a subset of Shiga-toxigenic *Escherichia coli*. The A subunit is a subtilase-like serine protease and cleaves an endoplasmic reticulum chaperone BiP. The B subunit binds to a receptor on the cell surface. Although SubAB is lethal for mice, the cause of death is not clear. In this study, we demonstrate in mice that SubAB induced small bowel hemorrhage and a coagulopathy characterized by thrombocytopenia, prolonged prothrombin time and activated partial thromboplastin time. SubAB also induced inflammatory changes in the small intestine as detected by ^{18}F-fluoro-2-deoxy-D-glucose positron emission tomography imaging and histochemical analysis. Using RT-PCR and ELISA, SubAB was shown to increase interleukin-6 in a time-dependent manner. Thus, our results indicate that death in SubAB-treated mice may be associated with severe inflammatory response and hemorrhage of the small intestine, accompanied by coagulopathy and IL6 production.

1. Introduction

Infections with Shiga-toxigenic *Escherichia coli* (STEC) are characterized by gastrointestinal disease including hemorrhagic colitis, and may progress to systemic complications including hemolytic uremic syndrome (HUS) and cerebromeningitis. Toxins produced by STEC include Shiga toxins 1 and 2, and subtilase cytotoxin (SubAB). The latter toxin was recently discovered in O113:H21 strain 98NK2, which was responsible for an outbreak of HUS [1]. SubAB is now known to be produced by a variety of serotypes of STEC [2,3]. However, O157:H7, the most common serogroup implicated in hemorrhagic colitis and HUS, does not produce SubAB [3]. SubAB contains one 35-kDa A subunit (SubA), and a B subunit (SubB) complex, which consists of a pentamer of 13-kDa monomers. The catalytic A subunit is a subtilase-like serine protease, and B subunits bind to receptors on target cells (2). Potential SubAB receptors include *N*-glycosylated membrane proteins, α2β1 integrin [4], and glycans terminating in the sialic acid *N*-glycolylneuraminic acid (Neu5Gc) [5,6].

In Vero cells, SubAB treatment induced transient inhibition of protein synthesis, cell cycle arrest, and cell death [7]. These events were attributed to cleavage of the endoplasmic reticulum chaperone BiP by SubAB [8], which leads to ER stress secondary to accumulation of unfolded proteins. SubAB triggered the three major branches of the unfolded protein response (UPR) including the IRE1-XBP1, PERK, and ATF6 pathways [9]. SubAB induced phosphorylation of Akt and consequent activation of NFκB through the ATF6 pathway [10]. Moreover, ER stress by SubAB down-regulated gap junction expression and inhibited functional gap junction intracellular communication [11]. Thus, SubAB-mediated ER stress by cleavage of major chaperone BiP triggers a number of abnormalities in cellular functions.

Wang et al. demonstrated that intraperitoneal administration of SubAB in mice induced microvascular thrombosis and other histopathological changes in the kidney, brain, spleen, and liver, and also induced significant neutrophil infiltration and apoptosis in liver, kidney, and spleen [12]. These findings indicated that not only Shiga toxin 1 or 2 but also SubAB may be involved in human STEC disease. However, tissue tropism of SubAB or mechanism by which SubAB induced organ damage *in vivo* has not been determined. To define the precise mechanism of disease pathogenesis, we identified main target organs of SubAB using histopathological, biochemical, and ^{18}F-fluoro-2-deoxy-D-glucose positron emission

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tomography (^{18}F-FDG-PET) imaging analysis. In this report, we show that intraperitoneal administration of SubAB in mice induced severe inflammatory hemorrhage of the small intestine associated with increased interleukin-6 (IL6) mRNA and IL6 expression. A SubAB-associated coagulopathy was characterized by thrombocytopenia, prolonged prothrombin time, and activated partial thromboplastin time. SubAB induced splenic atrophy, hyperplasia of the juxtaglomerular apparatus (JGA) and mesangial proliferation in kidney, but not apoptosis.

2. Results

2.1. Effect of SubAB on mice

To determine the toxicity of SubAB, mice were injected intraperitoneally with 10 μg of SubAB, mutant SubA(S272A,H89A)B (mSubAB), which was catalytically inactive, or as a control, PBS. Serine 272 and Histidine 89 are located in the amino acids of catalytic site, in a conserved sequence domain of subtilase family members [1]. The single amino acid mutant SubA(S272A)B has slight BiP-cleavage activity after prolonged incubation with Vero cells. Therefore, we used a double mutated toxin, SubA (S272A, H89A)B, which contains replacements of both catalytic serine and histidine residues. SubAB-treated mice showed decreased movements by 36 h after injection and died within 72 h. When a lower dose of SubAB (5 μg) was injected into mice, death was observed between 5 and 7 days (data not shown). In contrast, as expected, mSubAB-treated mice were similar to controls. These data are consistent with a prior report, which showed that mice died at around 72 h after intraperitoneal injection of 10 μg (or 7.5 μg) of SubAB [13]. This lethal amount of SubAB is required for more than 100-fold that which would be required for Shiga-toxin 2 [14]. We investigated the quantity of SubAB produced by *E. coli* O29 in culture and associated with the organism. We found that 5×10^9 CFU bacteria produced about 2–3 μg of SubAB in culture supernatant and 2–3 μg of SubAB were associated with the bacteria (Fig. 1A). Both had BiP-cleavage activity in a HeLa cell assay (data not shown). Blood of SubAB-treated mice showed significant increases in BUN, AST, ALT, LDH, and WBC levels, and decreases in PLT and BS similar to a prior report [12]. In addition, we found a significant increase of AMY (Fig. 2a), and significant decreases of TP and ALB (Fig. 2b and c), compatible with pancreatic and hepatic abnormalities, respectively. T-Cho and TG at 48 h were significantly decreased (Fig. 2d and e). Further, we observed that blood collected at 48 h from SubAB-treated mice did not coagulate, which was consistent with an increased PT and APTT (Fig. 2e and f) and decreased Fib (Fig. 2g).

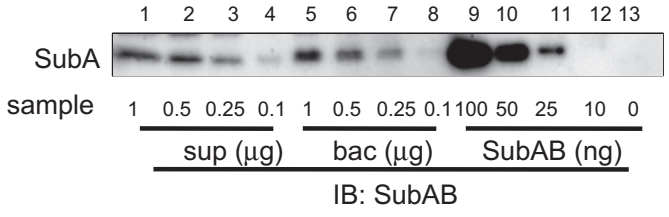

Fig. 1. SubAB levels in *E. coli* O29 culture supernatant or associated with bacteria. The indicated amounts of O29 culture supernatant (Sup), polymyxin B bacterial extract (bac) or purified recombinant SubAB as a standard were analyzed by SDS-PAGE in 15% gel, and transferred to PVDF membranes, which were incubated with anti-SubAB antibody followed by ECL detection. Lane 1 and 5, 1 μg; lane 2 and 6, 0.5 μg; lane 3 and 7, 0.25 μg; lane 4 and 8, 0.1 μg; lane 9, 100 ng; lane 10, 50 ng; lane 11, 25 ng; lane 12, 10 ng; lane 13, PBS as a control. Data are representative of two separate experiments.

2.2. ^{18}F-FDG PET imaging of SubAB-treated mice

In SubAB-treated mice, WBC levels were significantly increased compared to mSubAB and control mice, consistent with the conclusion that SubAB induced an inflammatory response. To determine the sites of inflammation following injection of SubAB, we conducted ^{18}F-FDG PET imaging with SubAB-treated, mSubAB-treated, and control (PBS)-mice. Under inflammatory conditions, the affinity and expression of glucose transporters are increased by various cytokines and growth factors, and uptake of FDG is increased [15–18]. Usually, the physiological uptake of ^{18}F-FDG in the brain, muscle including myocardium, and genitourinary tract exceed those of other organs. Hepatic and splenic uptake is generally low grade and diffuse. Uptake in stomach and bowel is variable [17,19,20]. In small animals, warming, fasting, and anesthesia influence uptake of ^{18}F-FDG [21]. Therefore, we performed ^{18}F-FDG PET in mice injected with SubAB, mSubAB, and PBS (control). At 24 h after injection, in SubAB-treated mice but not in mSubAB-treated or control mice, ^{18}F-FDG accumulated in the abdominal area corresponding to the small intestine (Fig. 3: circle). There were no obvious differences of ^{18}F-FDG uptake in kidney, liver, and spleen (Fig. 3). These data indicated that inflammation might be present in the small intestine of SubAB-treated mice.

2.3. SubAB induced severe hemorrhage in small intestine

We did not recognize any macroscopic changes in brain, heart, lung, kidney, pancreas, and colon for up to 48 h in SubAB-treated, mSubAB-treated, and control mice. However, we observed small bowel hemorrhage at 48 h in wild-type SubAB-treated mice (Fig. 4A and B). In addition, liver of SubAB-treated mice was pale compared with mSubAB-treated or control mice (Fig. 4C), compatible with ischemic changes in the SubAB-treated mice. Spleen of SubAB-treated mice showed a dramatic reduction in the size as reported previously [12].

2.4. Histopathological analysis of SubAB-treated mice

Organs (gut, kidney, brain, heart, lung, liver, spleen, and pancreas) were subjected to routine histological examination (HE staining) at 6 h, 24 h, and 48 h after injection of SubAB. We found significant bleeding into the small bowel of SubAB-treated mice. In the small intestine, inflammatory changes appeared as early as 6 h after wild-type SubAB injection and the damage was more severe with increasing time after injection (Fig. 5A). At 48 h after injection, neutrophil infiltration and associated hemorrhage were observed in the villus, muscularis mucosa, and muscle layer of the small intestine (Fig. 5A, d and i). These changes were observed in the upper part of the small intestine. No obvious differences between mSubAB-treated mice and control PBS-treated mice were seen at 48 h. In contrast to a prior report [12], we did not detect microvascular thrombosis and apoptosis in the kidney by TUNEL assay (data not shown). However, we found hyperplasia of the juxtaglomerular apparatus and proliferation of glomerular mesangial cells in the kidney at 48 h similar to prior report [12].

There was no obvious histopathological damage in organs except small intestine in SubAB-treated mice (data not shown). Initial inflammation and degradation of the small intestine was observed at 6 h in the villus of SubAB-treated mice. Using cy3-labeled SubAB, we investigated which regions in the small intestine bound SubAB. In addition, cy3-labeled SubAB was able to bind to Vero cells [22] and heat-treated SubAB (100 °C, 10 min) lost binding activity [4]. Each section of the small intestine of control mice was incubated with cy3-SubAB or with heat-inactivated cy3-SubAB as described in Methods. As shown in Fig. 5C, cy3-SubAB, but not heated cy3-SubAB,