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Shiga toxin 2a binds antithrombin and heparin, but does not directly activate platelets

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ABSTRACT

Escherichia coli-induced hemolytic uremic syndrome (eHUS) is a life-threatening complication of infection with Shiga toxin (Stx), in particular Stx2a-producing *Escherichia coli*. Enhanced coagulation activation with formation of microthrombi seems to be a key event in development of eHUS. Platelet activation has been postulated as a possible, but controversially debated mechanism.

The present study investigated the effect of Stx2a on plasmatic coagulation and platelets. Binding studies were initially performed with ELISA and co-immunoprecipitation and supported by quartz crystal microbalance with dissipation monitoring (QCM-D). Antithrombin (AT) activity was measured using the automated BCS XP® system. ROTEM® was used for functional coagulation testing. Platelet binding and activation was studied with FACS and light-transmission aggregometry.

We found binding of Stx2a to AT, an important inhibitor of blood coagulation, but only a mild albeit significant reduction of AT activity against FXa in the presence of Stx2a. QCM-D analysis also showed binding of Stx2a to heparin and an impaired binding of AT to Stx2a-bound heparin. ROTEM® using Stx2a-treated platelet-poor plasma revealed a significant, but only moderate shortening of clotting time. Neither binding nor activation of platelets by Stx2a could be demonstrated.

In summary, data of this study suggest that Stx2a binds to AT, but does not induce major effects on plasmatic coagulation. In addition, no interaction with platelets occurred. The well-known non-beneficial administration of heparin in eHUS patients could be explained by the interaction of Stx2a with heparin.

1. Introduction

Hemolytic uremic syndrome (HUS) caused by infection with Shiga toxin (Stx), in particular Stx2a-producing enterohemorrhagic *Escherichia coli* (EHEC) is the leading cause of acute kidney injury (AKI) in pediatric patients (Karch et al., 2005). In the full clinical picture of EHEC-induced HUS (recently termed eHUS) AKI is accompanied by microangiopathic hemolytic anemia and thrombocytopenia (Tarr, 1995).

It is well accepted that hemostatic abnormalities play an important role in the development and progression of eHUS, especially in the development of AKI (Lee et al., 2013; Nevard et al., 1997; Proesmans,

Abbreviations: eHUS, *Escherichia coli*-induced hemolytic uremic syndrome; Stx, Shiga toxin; EHEC, Enterohemorrhagic *Escherichia coli*; AT, antithrombin; AKI, acute kidney injury; t-PA antigen, tissue plasminogen activator antigen; PAI-1, t-PA-plasminogen activator inhibitor type 1; TF, tissue factor; vWF, von Willebrand factor; NOACs, new oral anticoagulants; PRP, platelet-rich plasma; APC, allophycocyanin; PE, phycoerythrin; LTA, light-transmission aggregometry; ROTEM®, rotational thromboelastometry; MFI, mean fluorescence intensity; CT, clotting time; SAP, serum amyloid P; QCM-D, quartz crystal microbalance with dissipation monitoring; PAV, polyamine; CHC, charged heparin conjugate

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2001). Patients with eHUS have an occlusion of the renal microvasculature due to endothelial damage and subsequent formation of microthrombi leading to ischemic kidney damage (George and Nester, 2014). They usually present with a strong rise of laboratory parameters indicating global coagulation activation such as increased levels of D-Dimer, tissue plasminogen activator antigen (t-PA antigen), t-PA-plasminogen activator inhibitor type 1 (PAI-1) complex and prothrombin fragments 1 and 2 (Ay et al., 2009; Chandler et al., 2002). Plasma levels of tissue factor (TF), the most important initiator of coagulation *in vivo* (Camerer et al., 1996), are markedly elevated in children with eHUS, through Stx induced release of TF (Bhowmik, 2001; Kamitsuji et al., 2000; Stahl et al., 2009). Additionally, there is also evidence for Stx-induced alterations in the protein C pathway (Mayer et al., 2015), an important inhibitor of coagulation.

On the other hand, there are data suggesting a major role of platelets and von Willebrand factor (vWF) in the development of eHUS; a widespread activation of platelets either through Stx itself or by the release of chemokines and other factors from endothelial cells has been repeatedly affirmed (Guessous et al., 2005; Karpman et al., 2001; Obrig and Karpman, 2012; Stahl et al., 2015; Sun et al., 2016).

The exact pathomechanism however, and the question whether this hypercoagulopathic status is mainly initiated via activation of platelets or the plasmatic hemostasis or a combination of both, is currently under debate. Thrombus formation is the result of a highly complex interaction between different physiological systems and extensive cross talk between coagulation and inflammation exists (Levi and van der Poll, 2005). Furthermore, it has been shown that complement, an important part of the innate immune system, might be involved in the pathogenesis of eHUS (Ehrlenbach et al., 2013; Orth et al., 2009; Poolpol et al., 2014). Additionally, it is notable that only 15% of patients infected with EHEC develop eHUS; it can be assumed that specific host related factors, such as polymorphisms or deficiencies of factors involved in hemostasis may lead to a clinically relevant change of coagulation after infection with EHEC.

Currently there is no consensus about how to treat coagulation abnormalities in affected patients or if they should be treated at all (Walsh and Johnson, 2018). In view of the clinically most relevant new oral anticoagulants (NOACs) with direct inhibition of either thrombin or factor Xa, new treatment strategies may emerge which could prevent or mitigate kidney failure in eHUS patients.

This study was designed to investigate which steps of the coagulation process are mainly disturbed by the presence of Stx; it was therefore assessed whether Stx2a activates platelets and in a second set of experiments we evaluated the effect of Stx2a on various factors of the plasmatic coagulation.

2. Materials and methods

2.1. Purification of Stx2a, proteins, antibodies, platelet concentrates platelet-rich plasma (PRP) and plasma

Purification of Stx2a was done as described elsewhere (Zhang et al., 2008) and the purity level was evaluated by SDS-PAGE. Lipopolysaccharide contamination was determined to be 5.88 pg/μg using a method described elsewhere (Brigotti, 2012).

Stx2a was labeled with FITC using an Oyster-488 antibody labeling kit (Luminartis, Münster, Germany).

LPS from *E. coli* serotype O55B5 was obtained by Charles River Laboratories Inc. (Charleston, USA). The allophycocyanin (APC)-conjugated CD42b antibody (ab) and the phycoerythrin (PE)- conjugated CD62 P ab were purchased from BD Biosciences (San Jose, USA) and BioLegend (San Diego, USA), respectively; antithrombin (AT), factor Xa and protein C from Calbiochem (Darmstadt, Germany); thrombin and GPR peptide from Sigma Aldrich (Taufkirchen, Germany); ADP from Sigma Aldrich (Taufkirchen, Germany) and Chrono-Log (Haverton, USA); $CaCl_2$ from Siemens (Munich, Germany); collagen from Nycomed (Linz, Austria); heparin from G.L. Pharma (Lannach, Austria); rabbit anti-FXa ab from US Biological Life Sciences (Salem, USA); sheep anti-human AT ab from Acris (Herford, Germany); mouse anti-Stx2 ab from Hycult Biotechnology (Uden, The Netherlands); goat anti-mouse AP-conjugated IgG, goat anti-rabbit AP-conjugated IgG, donkey anti-sheep IgG and rabbit anti-human AT abs, p-nitrophenyl phosphate from Sigma Aldrich.

Platelet concentrates were obtained from the blood bank of the University Clinic Innsbruck. Platelets were checked to be not-activated by measuring CD62 P with FACS as detailed below. Blood samples for the preparation of plasma or platelet-rich plasma (PRP) were – after informed consent and with an existing ethical vote – collected from healthy volunteers. Blood was drawn in 10 mL tubes anticoagulated with buffered 3.2% citrate.

PRP for FACS analysis and light-transmission aggregometry (LTA) were obtained by centrifugation (135 g for 15 min) and used within 4 h following preparation. For rotational thromboelastometry (ROTEM®) analysis and co-immunoprecipitation citrated blood was centrifuged at 2500 g for 10 min. The obtained plasma was then pooled, swiftly portioned into identical aliquots of 1.5 mL per microcentrifuge tube and stored at −78 °C until analyzed.

2.2. Analysis of binding of Stx2a to either antithrombin (AT), thrombin or protein C and binding of Stx2a-bound AT to FXa by ELISA

To establish whether Stx2a binds to AT, microtiter plates were coated with Stx2a (1 μg per well) in 100 μL coating buffer (12,430 mg $NaHCO_3$, 5510 mg Na_2CO_3 in 1 l aqua dest.) overnight at 4 °C. After blocking with 1% (w/v) gelatine, each well was incubated with 1 μg of AT for 4 h at 37 °C. After additional washing steps, bound AT was detected with a primary rabbit anti-AT antibody (1:1000) followed by a goat anti-rabbit AP-conjugated IgG (1:1000), both diluted in PBS-T. The binding was detected with p-nitrophenyl phosphate as the substrate. AT alone was used as a positive control. As negative controls we coated BSA or serum amyloid P (SAP) (1 μg per well) in place of Stx2a followed by addition of AT (1 μg for 4 h) and detection with the above mentioned rabbit anti-AT antibody and goat anti-rabbit IgG.

Interaction of Stx2a with AT was further analyzed at different pH values (pH 4, pH 4.5, pH 5, pH 5.5, pH 6, pH 10). After blocking, AT (1 μg per well) was added to the wells in 100 μL PBS-T adjusted to respective pH values. The remaining steps were performed as described above.

To analyze the binding of Stx2a-bound AT to FXa, 1 μg of FXa was immobilized in 100 μL coating buffer. Simultaneously, 2.5 μg Stx2a and 2.5 μg AT were dissolved in 250 μL PBS-T and incubated in a micro centrifuge tube for 20 h at 37 °C. Afterwards, 100 μL of the Stx2a/AT-suspension was added to the immobilized FXa for 1 h at 37 °C. After washing, bound Stx2a/AT was detected with a primary rabbit anti-human AT antibody (1:1000) and a secondary anti-rabbit AP-conjugated IgG antibody (1:1000). Bound Stx2a/AT was detected by development with p-nitrophenyl phosphate as substrate. Controls were performed with BSA instead of Stx2a.

To evaluate whether Stx2a binds to protein C or thrombin, the microtiter plates were coated with the respective proteins (1 μg per well, each) in 100 μL coating buffer. After blocking, Stx2a (1 μg) was added for 4 h at 37 °C. Bound Stx2a was detected with a primary mouse anti-Stx2 antibody (1:100) followed by an anti-mouse AP-conjugated IgG (1:1000), both diluted in PBS-T. For negative control BSA was added to protein C or thrombin in place of Stx2a, for positive control Stx2a (1 μg per well) alone was coated. Photometric readouts were done at 415 and 490 nm.

2.3. Analysis of binding of Stx2a to AT by co-immunoprecipitation

To confirm the results of the ELISA test for Stx2a-AT-binding, co-immunoprecipitation using a kit from Thermo Fisher Scientific