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Up-regulation of tissue factor activity on human proximal tubular epithelial cells in response to Shiga toxin

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Background. The pathophysiology of hemolytic uremic syndrome (HUS) is incompletely established. Based on clinical studies demonstrating the presence of prothrombotic plasma markers in patients with HUS, we hypothesized that Shiga toxin might cause activation of the coagulation pathway by augmenting tissue factor, the major initiator of coagulation.

Methods. Human proximal tubular epithelial cells (PTECs) [human kidney-2 (HK-2 cells)] were exposed to Shiga toxin-1, and expression of tissue factor, cell detachment, protein synthesis, caspase-3 activity, and Shiga toxin-1 binding were examined.

Results. HK-2 cells expressed constitutive surface tissue factor activity and increased their tissue factor expression upon exposure to Shiga toxin-1. Shiga toxin-1 bound to HK-2 cells and inhibited protein synthesis. The up-regulation of tissue factor was dose- and time-dependent and strongly correlated with cell detachment and increase in caspase-3 activity caused by Shiga toxin-1 exposure. A general caspase inhibitor simultaneously inhibited HK-2 cell detachment and tissue factor up-regulation while mutant Shiga toxin-1 neither caused cell detachment, protein synthesis inhibition, nor increase in tissue factor activity. Tissue factor activity elicited by Shiga toxin-1 was abrogated by a monoclonal antitissue factor antibody. Calphostin C, a protein kinase C (PKC) inhibitor, partially blocked tissue factor up-regulation, indicating possible involvement of PKC-dependent mechanism.

Conclusion. These data, taken together, suggest a strong link between Shiga toxin–induced up-regulation of tissue factor activity, cytotoxicity, and apoptosis in HK-2 cells. The proximal tubule is a target of Shiga toxin in HUS, and it seems plausible that injured proximal tubular cells trigger the activation of the coagulation system, the formation of intrarenal platelet-fibrin thrombi, and the development of acute renal failure in HUS.

Key words: hemolytic uremic syndrome, human proximal tubular epithelial cells, Shiga toxin, tissue factor, tissue factor pathway inhibitor, cytotoxicity, apoptosis.

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Hemolytic uremic syndrome (HUS), a thrombotic microangiopathy, is characterized by nonimmune hemolytic anemia, thrombocytopenia, and acute renal failure [1]. The most common form of HUS is diarrhea-associated HUS, an important complication of hemorrhagic colitis caused by Shiga toxin-producing *Escherichia coli* [2]. Shiga toxin is considered the major cause of the tissue damage that occurs during the course of HUS [1, 3]. The most widely recognized tissue damage occurs within the kidney [1]. Glomerular endothelial cell swelling and detachment from the basement membrane, proximal tubular epithelial cell (PTEC) injury, mesangial expansion, and mesangiolysis have been observed, along with apoptosis or necrosis of glomerular and tubular cells [1, 3]. In the most severe cases, extensive cortical necrosis ensues [3].

The presence of localized platelet-fibrin thrombi is the most prominent hallmark of the intrarenal pathology in HUS, suggesting activation of coagulation and/or a decrease in fibrinolytic potential. Several authors reported that the levels of thrombin-antithrombin complexes, prothrombin fragment 1 + 2, and D-dimer (a product of the fibrinolysis of cross-linked fibrin) are higher in children with HUS as compared to children with acute renal failure from other causes [4, 5]. Recent work revealed high levels of plasminogen activator inhibitor type 1 (PAI-1) in HUS, indicating substantial inhibition of fibrinolysis [6]. Chandler et al [7] showed that HUS was preceded by thrombin generation and inhibition of fibrinolysis. Despite existing clinical studies, the mechanism of induction of a procoagulant state by Shiga toxin is only partially understood. A recent study suggested that Shiga toxin–induced decrease of thrombomodulin expression in glomerular endothelial cells might contribute to the local procoagulant state observed in HUS [8]. Tissue factor, a 47 kD transmembrane glycoprotein, is widely accepted as the initiator of coagulation and might play a central role in the procoagulant state in HUS [9]. Kamitsuji et al [10] reported elevated levels of circulating tissue factor

in children with HUS, and our group has demonstrated that Shiga toxin induces up-regulation of tissue factor on human glomerular endothelial cells in the setting of prior cytokine activation [11].

Although glomerular endothelial cell injury is considered central to the pathophysiology of HUS [1, 3], this injury depends on the concerted actions of Shiga toxin and inflammatory mediators, including bacterial products such as lipopolysaccharide (LPS) and cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), which are believed to induce globotriaosyl ceramide (Gb3) receptors on the surface of glomerular endothelial cells, to which Shiga toxin binds [12–15]. On the other hand, Gb3 receptors are prominent in renal tubules [16–18], and proximal tubular cells are exquisitely sensitive to Shiga toxin undergoing apoptosis when exposed to Shiga toxin in vitro [18–22]. Proximal tubular damage is evident in renal tissue during the early stages of HUS, raising the possibility that proximal tubules may be an important early target of Shiga toxin action [18–21]. Moreover, PTECs are a rich source of tissue factor and also produce tissue factor pathway inhibitor (TFPI) [23, 24], which, together with tissue factor, potentially regulates clotting in the microenvironment of renal tubules. Since tissue factor could be a major inducer of the thrombogenesis observed in HUS, and proximal tubular cells are a target of Shiga toxin in the kidney, we examined the role of proximal tubular cells in expressing tissue factor by studying their tissue factor functional activity with and without exposure to Shiga toxin.

METHODS

Reagents

Keratinocyte serum-free medium, human recombinant epidermal growth factor 1-53 and bovine pituitary extract were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Penicillin/streptomycin was purchased from BioWhittaker (Walkersville, MD, USA). Sterile tissue culture plasticware was purchased from Falcon Plastics (Cockeysville, MD, USA). Fibronectin was obtained from Becton Dickinson Biosciences (San Jose, CA, USA). Purified Shiga toxin-1, mutant Shiga toxin-1 (a toxin with a substitution mutation in the active site, Stx1E167D) and rabbit anti-Shiga toxin-1 antibody were kindly provided by Dr. Cheleste Thorpe (Tufts-New England Medical Center, Boston, MA, USA). A mouse monoclonal antibody against human tissue factor (antitissue factor) was provided as a courtesy by Dr. Yale Nemerson (Mt. Sinai Medical Center, New York, NY, USA). Human coagulation factors VII and X were obtained from Enzyme Research Laboratories (South Bend, IN, USA). Purified factor Xa was kindly provided by Dr. Arabinda Guha (Mt. Sinai Medical Center, New York, NY, USA). The human recombinant cytokine TNF-α and the general caspase inhibitor Z-VAD-FMK were purchased from R&D Systems (Minneapolis, MN, USA). Caspase-3 colorimetric protease assay kit was purchased from Medical and Biological Laboratories (Woburn, MA, USA). Spectrozyme Xa and rabbit antihuman TFPI (anti-TFPI) IgG were purchased from American Diagnostica (Greenwich, CT, USA). Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG was obtained from Accurate & Scientific (Westbury, NY, USA). ^{3}H-leucine was obtained from Perkin-Elmer (Boston, MA, USA). The scintillation cocktail was purchased from Beckman (Fullerton, CA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Renal PTECs. Human kidney-2 (HK-2) cells, an immortalized renal PTEC line (CRL-2190) derived from normal adult human kidney tissue, were a kind gift of Dr. Howard Trachtman (Schneider Children's Hospital of the North Shore-Long Island Jewish Health System, New Hyde Park, NY, USA). These cells were grown in complete medium consisting of keratinocyte serum-free medium, supplemented with recombinant epidermal growth factor (5 ng/mL), bovine pituitary extract (40 mg/mL), and penicillin/streptomycin (10 μg/mL). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air with medium changed every 2 days; cells were used between the 25th and 40th passage. All experiments were performed after cells had reached confluence (generally 5 to 7 days after passage) as judged by phase contrast microscopy.

Experimental conditions

HK-2 cells were plated on 12-well tissue culture plates that had been precoated with fibronectin (adsorbed from a 10 μg/mL solution for 1 hour at room temperature) and were grown to confluence in complete medium. At confluence, they were exposed to Shiga toxin-1 (complete medium containing Shiga toxin-1, 10^{-15} mol/L to 10^{-9} mol/L) for 2, 4, 6, 8, 10, 12, 22, 48, 72, and 96 hours to study their surface tissue factor activity as a function of Shiga toxin-1 concentration and duration of exposure. The concentrations of Shiga toxin-1 were chosen based both on the observations of Yagi et al [25] who detected Shiga toxin concentrations of this order of magnitude in plasma of patients with HUS, and on our observations that Shiga toxin-1 in concentration of 10^{-11} mol/L enhanced tissue factor activity on TNF-α stimulated human glomerular endothelial cells [11]. HK-2 cells were also studied following activation with TNF-α (20 ng/mL) for the same time periods. TNF-α was chosen because it has been implicated in the pathogenesis of HUS as an inflammatory