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New insights into Shiga toxin-mediated endothelial dysfunction in hemolytic uremic syndrome

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Abbreviations: HUS, hemolytic uremic syndrome; Stx, Shiga toxin; STEC, Shiga toxin-producing *E. coli*; SDF-1, stromal cell-derived factor-1; TCC, terminal complement complex; VTEC, verotoxigenic *E. coli*; EHEC, enterohemorrhagic *E. coli*

Shiga toxin-producing *E. coli* represents a significant global health concern, especially as hypervirulent pathogens surface amidst outbreaks of hemolytic uremic syndrome (HUS). Shiga toxin (Stx) is key in the microangiopathic events underlying the disease and its central role is underscored by the unprecedented HUS outbreak in Germany in 2011. The mechanisms of Stx-mediated endothelial dysfunction have been a major focus of research that has contributed to the current understanding of the pathogenic changes in endothelial phenotype leading to HUS. Among the newer concepts are Stx-mediated gene regulation in the absence of protein synthesis inhibition, a potential role for complement activation, and accumulating evidence for detectable serum markers before the onset of the classic clinical features of HUS. Further investigation of newer therapeutic targets and potential prognostic markers is essential to assess their utility in mitigating disease and/or predicting outcomes and will provide an improved overall understanding of HUS pathogenesis.

Introduction

Hemolytic uremic syndrome (HUS) is a thrombotic microangiopathy that is clinically characterized by thrombocytopenia, non-immune hemolytic anemia, and acute renal failure. Approximately 10% of cases are categorized as atypical HUS, a disorder associated with genetic or acquired deficiencies in complement regulation, among others.[1,2] Onset of atypical HUS in predisposed patients is often triggered by non-enteric infection, pregnancy, cancer, organ transplant, or drugs.[3] These patients unfortunately have a poor prognosis, with progression to end-stage renal disease in half of the cases and a mortality rate of 25%.[3] Typical HUS, also known as diarrhea-associated HUS (D+HUS), accounts for the majority of cases and is the most serious, life-threatening complication following gastrointestinal infection with Shiga toxin-producing *E. coli* (STEC).[1,3] Shiga toxin (Stx)-mediated endothelial dysfunction is thought to be a primary event underlying the microangiopathic changes that occur in HUS.[4-6] This review will cover the main events in STEC-mediated HUS pathogenesis, with a focus on Stx-induced endothelial dysfunction. The recent, unprecedented STEC outbreak in Germany will be discussed and emerging concepts in HUS pathogenesis and their implications for patient therapy will also be addressed.

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Overview of STEC-Mediated HUS

Karmali and colleagues' seminal reports 30 years ago made the association between STEC infection and sporadic cases of HUS.[7,8] Because Stx is also widely referred to as verotoxin, the terms STEC and verotoxigenic *E. coli* (VTEC) are used interchangeably. Enterohemorrhagic *E. coli* (EHEC) are a sub-group of STEC that have the ability to form attaching and effacing lesions (A/E lesions) that facilitate intimate adhesion to the intestinal mucosa.[9] Since Karmali's initial reports, Stx-producing *E. coli* O157:H7 has become recognized as the leading cause of HUS worldwide.[10] The principal route of infection is consumption of contaminated food and drinking water, although infection may also occur via direct contact with animals, person-to-person contact, and environmental exposure.[11,12] STEC-HUS is most prevalent in infants and young children and is, in fact, a leading cause of acute renal failure in the pediatric population.[13] STEC causes a spectrum of diseases, from uncomplicated infection to hemorrhagic colitis and HUS. For reasons not yet well understood, 5–15% of STEC cases progress to HUS.[10,14] The inability to predict which patients will recover spontaneously and which will develop HUS remains a significant dilemma for clinicians. The presence of STEC, especially Stx2-producing *E. coli*, in patient stools should immediately alert the physicians involved.

Histopathological manifestations of Stx-associated HUS include fibrin-rich microvascular thrombi primarily in the renal

glomeruli, although pre-glomerular arterioles and medium-sized vessels may also be affected.[6] Endothelial swelling and detachment from the underlying basement membrane and subendothelial deposits accompanied by vascular edema and narrowing of the vessel lumen are also observed.[10,15] The classical view argues that thrombocytopenia results from platelet consumption in thrombi and that red blood cells become fragmented as they flow through vessels with marked edema, fibrin deposits, and intravascular thrombosis, though newer evidence challenges these long-held beliefs.[16]

Although the kidneys are the prime targets, extra-renal complications may develop. Neurological involvement occurs in 10% to 25% of patients and is the most common cause of mortality during the acute phase of disease.[10,17] Although infrequent, cardiac complications are associated with a high risk of mortality in HUS patients (for a review, see ref. 18). Involvement of other organs such as the gastrointestinal tract, lungs, and pancreas is likely under-appreciated. The risk of mortality or end-stage renal disease associated with STEC-HUS is 12% and 25% of survivors experience long-term renal sequelae, including decreased glomerular filtration rate, proteinuria, hypertension, chronic kidney disease, and end-stage renal disease.[19-21]

Patients currently receive supportive care to manage symptoms, as no specific treatment exists for D+HUS. Antibiotic treatment is generally discouraged because of the potential to enhance the synthesis and release of Stx from EHEC and worsen disease severity.[22,23] Recent in vitro studies suggest that, at least for *E. coli* O104:H4, ciprofloxacin increases Stx2 production while other antibiotics either suppress or do not affect toxin expression.[24] The use of first-line plasma therapy in STEC-HUS is controversial[25,26] and a recent prospective study of 619 children with HUS identified an association between plasma therapy and poor long-term outcome.[21]

Volume resuscitation and re-expansion of extracellular fluid volume is also a key component of clinical management.[27,28] Ironically, the pathophysiology of the vascular leak and increased vascular permeability is poorly understood.

Unusual Stx-Producing Pathotype Emerges

E. coli O157:H7 is recognized as the leading cause of HUS globally.[10,29] From May to July 2011, Germany was at the center of an unprecedented outbreak of STEC-HUS that quickly garnered attention for its unusual epidemiologic characteristics, the organism's unexpected genetic composition and startlingly high virulence. Among 3816 people who fell ill in Germany, 845 developed HUS and 54 people died from HUS or gastroenteritis.[30] The higher than normal rate of progression to HUS (22%), together with the unusually high incidence in adult patients (88%) made this an extraordinary outbreak that required immediate action. Other atypical features of the disease included a longer incubation period and disproportionately greater frequency in women.[6,30] The clinical parameters are argued to reflect the implicated food source, salad sprouts, associated with infections during this epidemic.[31]

The pathogen was quickly identified as *E. coli* O104:H4, an enteroaggregative *E. coli* (EAEC) that colonizes the bowel in a characteristic "stacked brick" pattern. It normally causes watery diarrhea in children, travelers' diarrhea, and persistent diarrhea in HIV patients and had previously been associated with only a handful of sporadic cases of HUS.[32-34] Rapid genomic sequencing of the outbreak strain revealed it carried many common EAEC features but surprisingly, had acquired the *stx2* gene and the ability to produce extended-spectrum β-lactamase via horizontal gene transfer.[22,35-37] The dramatic pathology that accompanied the newly acquired ability of this organism to produce Stx2 underscores the fundamental role Stx plays in the pathogenesis of HUS.

Following the German outbreak, comparative genomic analyses were performed on O104:H4 isolates from historic cases, 2011 outbreak cases, and cases identified after the 2011 outbreak.[38] The authors concluded that, while the isolates were closely related and shared a common ancestor, the post-outbreak isolates were distinct from the outbreak strain. While all expressed Stx2, each isolate had a unique combination of virulence factors encoded on genomic islands, prophages, and plasmids, suggesting that O104:H4 isolates are widespread and have the potential to cause foodborne outbreaks in the future.[38]

Shiga Toxins

Shiga toxins, also commonly referred to as verotoxins, are implicated as key virulence factors in STEC-induced disease. The Stx family, comprising Stx1, Stx2, and their variants (Stx1c, Stx1d, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g),[39] belongs to a larger family of prokaryotic and plant A:B toxins that includes cholera, pertussis, diptheria, and ricin. Stx1 and Stx2 are encoded by distinct genes present in the late regions of lambdoid prophages and consist of a pentameric ring of receptor-binding B subunits linked to a single enzymatic A subunit.[40-42] *E. coli* strains expressing Stx2, including O157:H7, have historically been associated with more severe human disease.[30,40,43]

STEC release Stx in the gastrointestinal tract, after which the toxin translocates across the intestinal epithelium into the systemic circulation. The major Stx carrier in the blood has yet to be unequivocally identified. The toxin gains entry into susceptible cells via binding to cell-surface globotriaosylceramide (Gb_3).[44] The presence of Gb_3 targets Stx to the glomerular endothelium, where it causes major vascular injury. Endothelial cells from different vascular beds display varying degrees of sensitivity to Stx due, in large part, to the level of Gb_3 expression.[45] Once inside the cell, the Stx A subunit specifically removes an adenine residue within the highly conserved α-sarcin/ricin loop on eukaryotic 28S rRNA[46,47] and, in doing so, inhibits protein translation (**Fig. 1**).[46,48,49] In vitro and in vivo studies have shown that Stx-mediated cell damage may be accompanied by apoptosis.[50-53]

Shiga Toxin-Mediated Vascular Injury

While toxin-mediated ribosomal damage inhibits protein synthesis and can lead to cell death, Stx has profound effects on endothelial gene expression, effects that occur with little to no translation inhibition (**Fig. 1**).[5,16,43] Under normal conditions, the

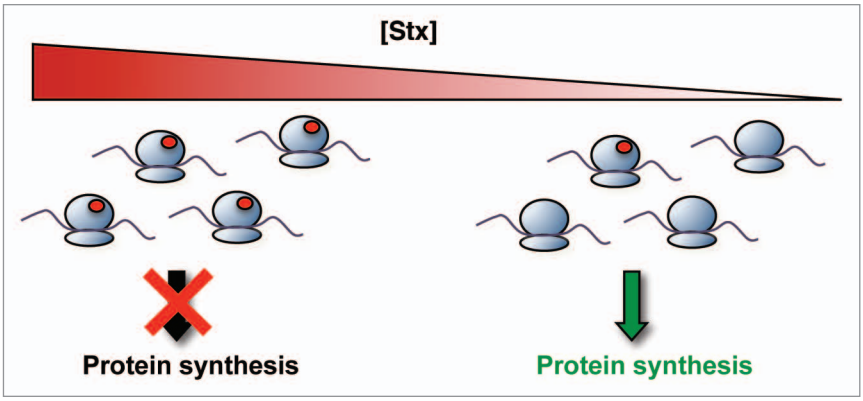

Figure 1. Molecular mechanisms of Stx pathobiology. Stx inactivates host ribosomes by removing a specific adenine residue from the 28S rRNA, a lesion depicted by the red circles in the figure. Overall protein synthesis is therefore inhibited. However, at lower concentrations that have only minor effects on global protein synthesis, Stx induces changes in gene expression that alter the endothelial phenotype. Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: *Current Opinion in Nephrology and Hypertension*,[6] copyright 2012.

endothelium has a vasodilatory, thromboresistant, anti-adhesive, and anti-inflammatory phenotype.[54,55] Recent findings have indicated that Stx elicits a very unique change in endothelial phenotype, in part through changes in patterns of expression of specific endothelial cell mRNAs.[16,56]

In an effort to understand the endothelial dysfunction mediated by Stx, several groups have studied the effects of Stx on vascular endothelial cells. Louise and Obrig have shown that Stx induces an increase in the ratio of plasminogen activator inhibitor-1 (PAI-1) to tissue plasminogen activator (tPA), suggesting that toxin treatment induced an endothelial phenotype favoring stabilization of fibrin clots.[57] Endothelial-derived tissue factor increases in the presence of Stx, likely as a result of increased expression of tissue factor, itself, and decreased activity of tissue factor pathway inhibitor.[16,58]

Recently, Stx was shown to upregulate the chemokine stromal cell-derived factor-1 (SDF-1) in cultured endothelial cells and in children with STEC-HUS (**Fig. 2**).[16] SDF-1 was initially cloned from a bone marrow stromal cell line but is constitutively expressed in the kidney, spleen, liver, lung, heart, brain, and muscle.[59,60] CXCR4, the receptor for SDF-1, is expressed on a wide variety of cells, including cells within the hematopoietic lineage, endothelium, platelets, and neurons.[61-64] This chemokine pathway plays critical roles in the vasculature both during development and post-natal angiogenesis and repair.[65-68] SDF-1 has also been shown to enhance platelet activation induced by thrombin, resulting in increased platelet aggregation.[64,69] Antagonism of the SDF-1/CXCR4 pathway using AMD3100 (plerixafor), a small molecule antagonist of CXCR4, significantly improved kidney function and overall survival of mice injected with Stx, indicating that activation of this pathway contributed to Stx pathogenesis.[16] Furthermore, inhibition of CXCR4/SDF-1 normalized platelet levels in vivo and prevented formation of platelet strings on a Stx-treated endothelial monolayer in vitro (**Fig. 2**).[16]

Children with *E. coli* O157:H7-mediated HUS exhibit significant pro-coagulant changes, including evidence for increased thrombin generation and fibrin accumulation, prior to onset of HUS.[70] Stx was also shown to induce rapid release of ultra-large vWF multimers from endothelial cells in vitro and inhibited multimer cleavage by the metalloprotease ADAMTS13.[71] The sum of these data indicate that toxin-mediated endothelial dysfunction results in a pro-thrombogenic intravascular environment.

Stx also modulates the production of an important mediator of vascular tone, namely the vasoconstrictive peptide, endothelin-1 (ET-1).[43] In other studies, endothelial cells treated with Stx1 and cultured under flow demonstrated enhanced leukocyte adhesion as a result of upregulation of endothelial adhesion molecules E-selectin, ICAM-1, and VCAM-1.[72,73] Induction of chemokines, such as IL8 and MCP1, may also play a role in leukocyte adhesion.[74] In addition to the gene-specific studies described above, Matussek et al. reported the gene expression profiles of human umbilical vein endothelial cells exposed to Stx1 and Stx2.[56] These studies demonstrated that Stx stimulated mRNA and protein production of a variety of chemokines and cytokines, among other genes, which may serve to exacerbate Stx-induced endothelial damage. Importantly, Stx-mediated cytokine regulation alters cell sensitivity to the toxin as Gb_3 itself may be upregulated by cytokines.[45,75] The studies performed by Matussek et al. also demonstrated key differences in gene regulation by Stx1 compared with Stx2 that may, at least in part, explain the differences in virulence associated with each toxin.

Regulation of Endothelial Gene Expression by Shiga Toxin

Large-scale, unbiased gene expression profiling studies demonstrated that Stx profoundly affects endothelial gene expression and phenotype at concentrations that have only minor effects on protein synthesis.[16,56] Importantly however, the molecular mechanisms responsible for changes in endothelial phenotype in response to Stx remain largely unknown. There is growing evidence that Stx significantly alters RNA metabolism within the cell. Using endothelin-1 as a model, Bitzan et al. conducted nuclear run-on and actinomycin D experiments to study transcription and mRNA stability, respectively, and found that Stx increased ET-1 mRNA levels by stabilization of its mRNA transcript but not via transcription.[43] Recently, we demonstrated Stx-dependent upregulation of CXCR4 mRNA in endothelial cells.[16] Further investigation revealed a combination of enhanced transcription and mRNA stability contributed to the dramatic increase in CXCR4 mRNA levels.[16]

Because Stx is a potent ribosomal inhibitor, it is important to consider its effects on translation. Ribosome profiling

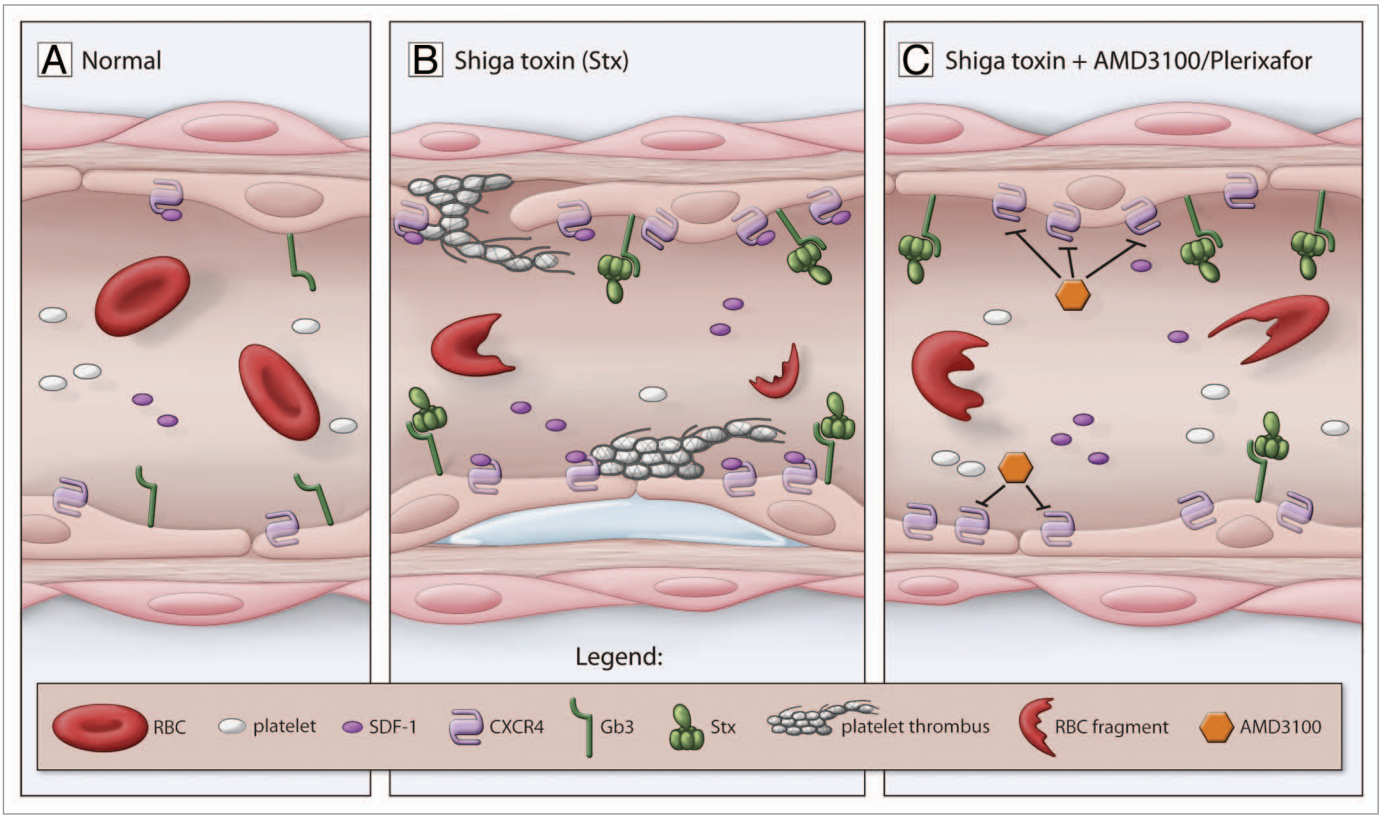

Figure 2. Contribution of the CXCR4/SDF-1 pathway to Stx pathophysiology. (**A**) Normal blood vessel. (**B**) Underlying HUS pathophysiology is detachment of the endothelium and exposure of the underlying basement membrane, subendothelial edema, increased platelet adhesion accompanied by thrombocytopenia, and red blood cell (RBC) fragmentation. Gb_3 on the surface of the endothelium binds Stx. Among the changes stimulated by Stx is upregulation of endothelial CXCR4 and increased blood SDF-1 levels. (**C**) Inhibition of CXCR4/SDF-1 interaction using AMD3100 (plerixafor) reduces Stx-mediated platelet adhesion to the endothelium in vitro and improves thrombocytopenia in vivo. Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: *Current Opinion in Nephrology and Hypertension*,[6] copyright 2012.

demonstrated higher association of CXCR4 transcripts with polyribosomes following Stx treatment, suggesting enhanced translation.[16] This effect was specific as the housekeeping gene cyclophilin A did not show the same pattern. These findings suggest that Stx-mediated gene expression is a complex process that affects many aspects of gene regulation. The various regulatory mechanisms are summarized in **Figure 3**.

Complement Activation in STEC-HUS

Mutations in complement regulatory genes are associated with atypical HUS and are found in 50% of patients.[76] Evidence for excessive complement activation in STEC-HUS is now also gaining attention. In vitro studies showed that relatively high concentrations of purified Stx incubated with normal serum induced formation of terminal complement complex (TCC) and that Stx bound factor H, resulting in delayed cell surface factor H activity.[77] Studies in 17 children with STEC-HUS showed that levels of the TCC were increased during the acute phase of disease and normalized within a month of patient discharge.[78] An independent analysis of 12 HUS patients in Sweden revealed elevated plasma levels of C3a and TCC during HUS compared with levels at recovery and pediatric controls.[79] Additional in vitro studies demonstrated C3 deposition on microvascular endothelial cells and subsequently an enhanced thrombogenic state as a result of Stx-mediated upregulation of P-selectin.[80] P-selectin inhibitory antibodies and P-selectin soluble ligand (PSGL-1) each reduced C3 deposition and thrombus formation.[80] These data support a role for complement dysregulation in STEC-HUS pathogenesis.

Eculizumab (Soliris®, Alexion Pharmaceuticals), a humanized monoclonal antibody that targets C5 to prevent formation of the membrane attack complex, was approved by the US FDA in September 2011 for treatment of atypical HUS. Publication of a study on the use of eculizumab in three 3-year-old patients suffering from severe STEC-HUS coincided with the 2011 HUS outbreak in Germany[81] and prompted off-label, compassionate use of the drug to treat severe cases of HUS.[1] While preliminary results appeared promising,[82] two groups have since reported no benefit of eculizumab treatment on short-term outcome.[83,84] These findings were based on retrospective analyses of cases where treatment strategy was dictated by disease severity and therefore, bias in patient outcome cannot be excluded. A prospective, controlled study with randomized treatment and control groups is essential in order to determine the efficacy of eculizumab therapy in STEC-HUS patients.

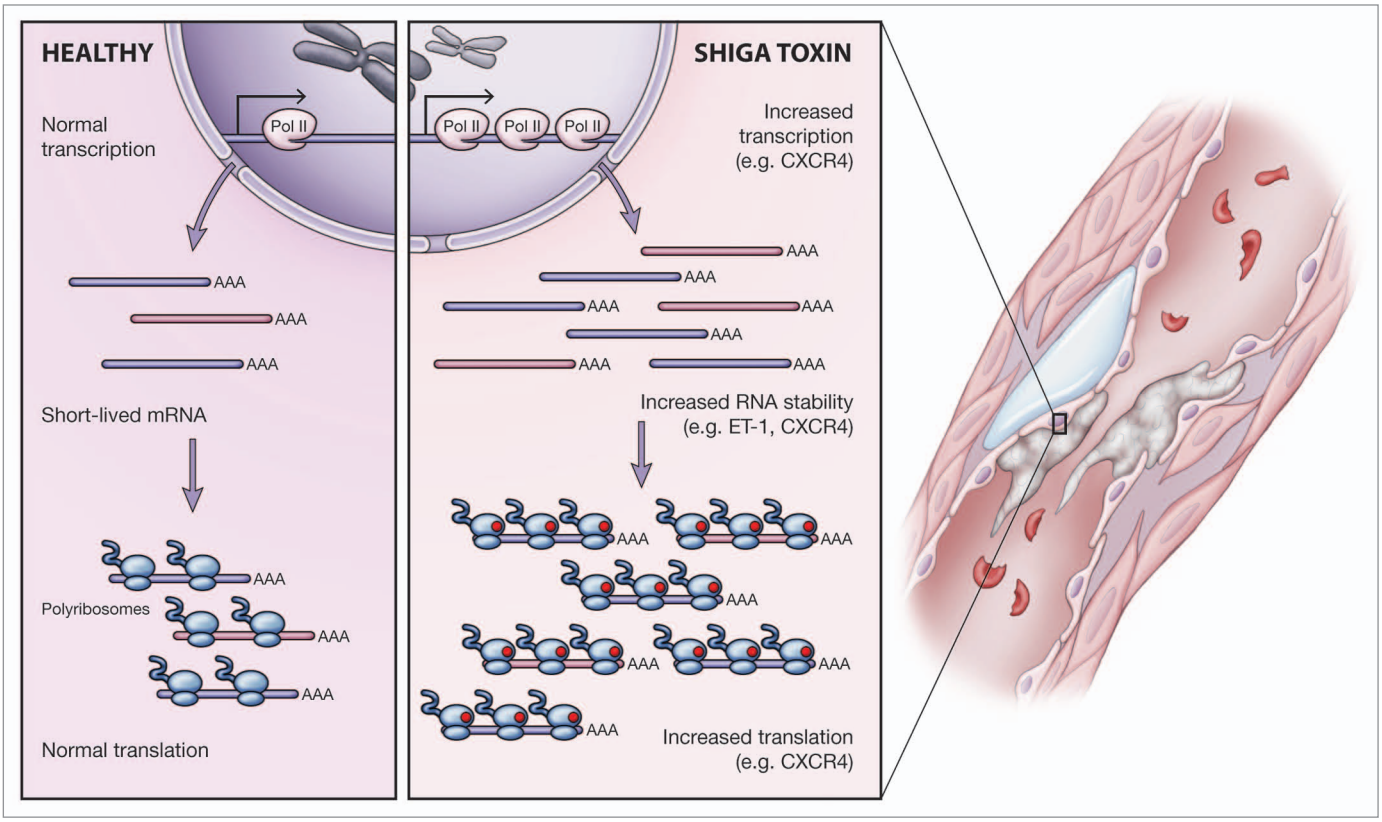

Figure 3. Mechanisms of endothelial gene regulation by Stx. Investigation into the mechanisms by which Stx affects gene expression in the endothelium revealed multi-level regulation. Stx may increase expression of select transcripts by upregulating transcription but also by enhancing the stability of short-lived mRNAs. Additionally, despite its ribosome inactivating properties, Stx has transcript-specific effects on translation, whereby it increases association of target mRNAs with polyribosomes.

Evidence of Early Vascular Dysfunction in Predicting Patient Outcome

One of the major obstacles facing clinicians is the inability to predict which patients will develop life-threatening complications from STEC infection. Recent advances in understanding the underlying pathophysiology of HUS have brought to light several significant concepts that may provide insight on the matter. In a prospective study of children with *E. coli* O157:H7 infection, Chandler et al. reported evidence of elevated thrombin generation and diminished fibrinolysis when patient hematocrit, platelet, and serum creatinine levels were still normal.[70] These studies are particularly important because they indicate that prothrombotic coagulation abnormalities precede onset of HUS and these changes are detectable in a patient's bloodstream before the classical features of the disease are clinically evident.

Other studies demonstrated that additional key mediators of vascular homeostasis and function exhibit important changes prior to the onset of the hallmark clinical features. Plasma levels of the chemokine stromal cell-derived factor-1 (SDF-1) exhibited a 4-fold increase in children who were later diagnosed with HUS compared with children with uncomplicated infection.[16] More recently, Page et al. described the disruption of angiopoietin-1 and angiopoietin-2 homeostasis in *E. coli* O157:H7-infected children.[85] Dysregulation was evident before the onset of HUS and became more pronounced with disease progression. The increased Ang2:Ang-1 ratio observed in HUS may represent an important mechanism of microvascular barrier disruption.[85] Taken together, the above findings not only identify potential targets for therapeutic development, but also may represent key, early warning signs that a patient is at higher risk of developing complications from STEC infection and should be monitored closely in order to mitigate severity of disease. Large-scale studies are required to determine the suitability of these molecules as biomarkers, but further investigation is warranted.

Conclusion

Recent events involving an alarmingly virulent pathogen underscore the important public health threat posed by STEC. Advances in our understanding of the pathogenic mechanisms involved are imperative for better patient treatment strategies. Studies focused on the molecular mechanisms of endothelial dysfunction continue to provide valuable insight into the thrombotic microangiopathy at the heart of Stx-mediated HUS. The accumulating evidence of uncontrolled complement activation is intriguing and supports the need for a multi-center, controlled trial of complement inhibition in STEC-HUS patients. A novel concept

emerging from recent literature indicates that detectable changes in vital mediators of endothelial function occur in patients prior to the onset of HUS. While it may be difficult to discriminate conclusively between uncomplicated infection and infection that will progress to HUS using a single biomarker, it would be worthwhile to explore the suitability of combinations of markers as a more definitive predictive test. Together, these recent advances in our understanding of Stx-mediated disease provide attractive potential prognostic and therapeutic avenues worth exploring.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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