Consequences of enterohaemorrhagic *Escherichia coli* infection for the vascular endothelium

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**Summary**

Microvascular endothelial damage underlies the pathological changes in haemorrhagic colitis and the haemolytic uraemic syndrome (HUS) caused by enterohaemorrhagic *Escherichia coli* (EHEC). Shiga toxins (Stxs) are presently the best characterised EHEC virulence factors that can cause the microvascular endothelium injury. Stxs are released by EHEC in the intestine, absorbed across the gut epithelium into the circulation, and transported to small vessel endothelial cells. Then, they presumably injure the host cell by inhibiting protein synthesis, stimulating prothrombotic messages, or inducing apoptosis. The net result is a multi-organ thrombotic process. Moreover, Stxs stimulate a variety of non-endothelial cells to produce and secrete inflammatory mediators (cytokines, chemokines, adhesion molecules) which could potentiate the effects of Stxs on endothelial cells. The association of HUS with Stx-negative *E. coli* strains stimulated intensive research on putative non-Stx virulence factors, which might also contribute to the pathogenesis of HUS and haemorrhagic colitis. Based on current data, cytolethal distending toxin, EHEC haemolysin, and subtilase cytotoxin might be such candidates.

**Keywords**

Enterohaemorrhagic *Escherichia coli*, haemolytic uraemic syndrome, microvascular endothelial damage, Shiga toxin, cytolethal distending toxin

**Enterohaemorrhagic *Escherichia coli*-associated diseases: Clinical course and postulated mechanisms of pathogenesis**

During the past two decades, enterohaemorrhagic *Escherichia coli* (EHEC) have emerged as worldwide causes of diarrhoea, haemorrhagic colitis, and the haemolytic uraemic syndrome (HUS) (1, 2). The most common EHEC serotype implicated is *E. coli* O157: H7 (2–5), but infections caused by other serotypes (especially O26: H11, O91: H21, O103: H2, O111: H8, O113: H21, O145: H28/H25, and O157: NM) have been increasingly identified (2, 4, 5–12). After a typical incubation period of three to four days (13), patients develop watery diarrhoea, usually accompanied by cramping abdominal pain. During the next several days, watery diarrhoea changes to bloody diarrhoea in most patients, though the frequency of bloody diarrhoea appears highest for members of the O157 serogroup (10). One week after the onset of diarrhoea, approximately 15% of patients under 10 years of age infected with *E. coli* O157: H7 develop a systemic complication, HUS. The frequency with which non-O157: H7 infections progress to HUS is not known, because most such infections remain under-diagnosed. HUS is the net effect of a variety of interacting factors, including virulence characteristics of the infecting EHEC strain, host factors, and environmental factors. Presently, there is no specific therapy for HUS. Antibiotic therapy should be considered with caution in patients with definite or possible EHEC infections, because antibiotic use during *E. coli* O157: H7 infection has been associated with an increased risk of developing HUS in children (14) and adults (15). Strategies for management of patients with *E. coli* O157: H7 infections have been recently reviewed (3).

HUS, one of the most frequent causes of acute renal failure in children, consists of thrombocytopenia, haemolytic anaemia and renal insufficiency (14). The precise mechanisms that cause these hematologic and renal effects are unknown. However, thrombotic microangiopathy from microvascular endothelial damage is the primary event in the complex cascade
leading from gastrointestinal infection to renal impairment (2, 3, 16).

How do EHEC infections damage the endothelium? In contrast to other bacterial pathogens which adhere to or invade the endothelium, such as Staphylococcus aureus (17) or Bartonella henselae (18, 19), EHEC infections are almost never bacteremic (3), so bacteria do not come in contact with endothelial cells. Thus, it is believed that HUS, and possibly also the bloody diarrhea/colic syndrome, result from vascular injury by the direct action of Shiga toxins (Stxs) (1, 2, 20–22), which are the major virulence factors of EHEC (1-3, 23). Because Stxs are common to EHEC (1, 2, 7), research on the pathogenesis of HUS has mainly focused on these toxins. However, reports of HUS cases associated with Stx-negative E. coli strains (24) combined with the fact that only a subset of Stx-producing E. coli cause severe human diseases (2) suggest that non-Stx virulence factors of EHEC may contribute to the pathogenesis of HUS.

Studies of HUS pathogenesis are hampered by the absence of a reliable animal model which recapitulates all the major lesions of HUS. A recently developed primate model (25) mimics best the histopathological and pathophysiological changes seen in humans. Moreover, the virulence of EHEC organisms precludes the performance of volunteer studies. Therefore, studies of the pathogenesis of HUS rely mostly on experimental data obtained in isolated systems, such as cell cultures (26), and on clinical-pathological observations in infected humans. Although microvascular endothelial cells are believed to be the major targets for Stxs (27–30), a variety of other cell types, such as monocytes and macrophages (31–33), circulating polymorphonuclear leukocytes (34–36), platelets (37), and renal proximal tubular cells (38) interact with Stxs and may thus play roles in the pathogenesis of HUS. This review will focus on the interaction of EHEC products with endothelial cells.

Histopathology and pathophysiology of HUS

Histopathology

HUS is characterised by widespread thrombotic microvascular lesions that are found in renal glomeruli, the gastrointestinal tract, and other organs, such as the brain and the pancreas (27–30, 39, 40). Glomeruli from patients with HUS show capillary wall thickening, endothelial cell swelling and detachment from the basement membrane, with deposition of fibrin thrombi in capillary lumens (27–30, 40). This finding suggests that injury to microvascular endothelial cells is the key event underlying the pathogenesis of HUS. Similarly, lesions of the small and large bowel during haemorrhagic colitis frequently consist of microangiopathy in the mucosa and submucosa, with haemorrhage, necrosis, and sloughing of cells into the lumen (41).

Prothrombotic coagulation abnormalities

The renal injury in HUS caused by EHEC O157: H7 is preceded by profound prothrombotic coagulation abnormalities (42), demonstrating that the vascular injury starts early in the course of the disease, before overt HUS develops. In this pre-HUS phase, considerable host injury is presumed to be initiated (43). Patient plasmas obtained after HUS have evolved demonstrable fibrinolytic inhibition manifested by elevated levels of plasminogen activator inhibitor (PAI)-1 activity (42, 44–47), increased intravascular generation of fibrin, as evidenced by elevated levels of circulating D-dimers (42, 44–46), and generation of thrombin, as suggested by increased levels of prothrombin fragment 1+2 (42, 44). Because patients with more severe prothrombotic abnormalities are at increased risk of HUS development (42), it is hypothesised that the development of HUS could be related to the degree of prothrombotic activation early in infection, and to the intensity of the coagulation response that subsequently develops (42). Also, elevations of interleukin-1 receptor antagonist (48), transforming growth factor beta-1 (49), platelet activating factor (50), P-selectin (51), vascular-cell adhesion molecules (51) and degraded von Willebrand factor multimers (40) during HUS demonstrate the presence of endothelial cell activation, or at least vascular injury, along with a prothrombotic state.

Activation of non-endothelial cells

In addition to endothelial cells, platelets (23, 37) and polymorphonuclear leukocytes (34, 35, 52–54) are also activated during HUS. A potential role for the polymorphonuclear leukocyte is possibly evidenced by leukocytosis (40, 55–57) which has been shown to be a risk factor for developing HUS (40, 55–57) and for the severity of renal failure during HUS (58, 59). Although the underlying mechanisms leading to leukocytosis remain unclear, increased circulating levels of granulocyte colony-stimulating factor (60) and interleukin (IL)-8 (54), which correlated with the white blood cell count, have been reported in children with HUS. Also, inhibition of spontaneous neutrophil apoptosis by Stx2, which could prolong their survival has been demonstrated (36). Neutrophils from patients with HUS have a higher capacity to adhere to cultured human endothelium and to induce endothelial injury by degradation of endothelial fibronectin by locally produced proteases (52).

Inflammatory response

HUS is associated with a marked inflammatory response as demonstrated by increased levels of various circulating inflammatory mediators, including interleukins, chemokines, soluble adhesion molecules, growth factors, and acute phase response proteins in patients with HUS (48, 54, 60–63). Moreover, inflammatory mediators including tumour necrosis factor (TNF)-α, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, basic fibroblast growth factor, and platelet activating factor are elevated in the urines of HUS patients (61, 63–65). The lack of correlation between serum and urine levels in the same patient suggests that these cytokines are produced locally within the kidney, rather than filtered from the bloodstream (63, 64). However, mechanisms underlying this process are presently not understood.

Virulence factors of EHEC which contribute to the endothelial cell injury

Shiga toxins

Stxs are believed to be the major precipitants of the endothelial cell injury during HUS (1, 2, 21, 22). This family of AB2 subunit toxins (66, 67) consists of two major types, Stx1 and Stx2 (68), and several Stx variants (69–75) (Table 1). Data from our labora-
Table 1: Shiga toxins (Stx) produced by human EHEC isolates and their association with HUS based on the data from our laboratory (1996–2004). *The stx subtypes in the EHEC isolates were determined as described previously (9, 69, 70) by using polymerase chain reactions (PCRs) with specific primers, restriction analyses of the PCR products with Haelll or HhaI, and sequence analyses of stx genes.

<table>
<thead>
<tr>
<th>Stx type</th>
<th>stx gene</th>
<th>Reference</th>
<th>Total no. of strains</th>
<th>No. (%) of isolates from HUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx 1</td>
<td>stxA</td>
<td>68</td>
<td>230</td>
<td>12 (5.2)</td>
</tr>
<tr>
<td>Stx2c</td>
<td>stxB</td>
<td>69, 70</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>Stx2d</td>
<td>stxBd</td>
<td>71</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Stx2</td>
<td>stxB</td>
<td>68</td>
<td>368</td>
<td>274 (74.5)</td>
</tr>
<tr>
<td>Stx2c</td>
<td>stxBc</td>
<td>72</td>
<td>45</td>
<td>14 (31.1)</td>
</tr>
<tr>
<td>Stx2d</td>
<td>stxBd</td>
<td>73</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>Stx2e</td>
<td>stxBdactivatable</td>
<td>74</td>
<td>38</td>
<td>3 (7.9)</td>
</tr>
<tr>
<td>Stx2e</td>
<td>stxBdactivatable</td>
<td>75</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

Renal cortex, colon, and brain are highly sensitive to cytotoxicity of Stxs (83, 94, 95, 98–100), in agreement with the involvement of these organs during HUS and haemorrhagic colitis. In contrast, large vessel endothelial cells, such as human umbilical vein endothelial cells (HUVEC) used as the first model to investigate the interaction of Stx with endothelium (96), and saphenous vein endothelial cells (101) are less sensitive to Stx. In one study, Stx2 was ca. 1000-fold more cytotoxic than Stx1 for microvascular cortical renal cells (98). Although this difference could explain the frequently observed association of HUS with infection by Stx2-producing EHEC (9, 76, 77), this observation was not confirmed for selectively purified glomerular endothelial cells (97).

In addition to their direct cytotoxicity for microvascular endothelial cells, Stxs exert a variety of other effects on endothelial and a spectrum of non-endothelial cells, which further contribute to the Stx-mediated endothelial cell injury (95) (Table 2). Specifically, human monocytes and macrophages (31, 32), glomerular mesangial cells (102), renal proximal tubular cells (38), large vessel endothelial cells (HUVEC) (103), microvascular endothelial cells (104) and intestinal epithelial cells (105, 106) produce after exposure to Stx various C-X-C cytokines which augment the action of Stx on endothelial cells by inducing expression of the Stx receptor Gb3 (23, 94, 95, 97, 100, 107, 108). A similar effect has been observed for bacterial lipopolysaccharide (109) and sphingomyelinase (110). Moreover, Stx1-mediated induction of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (111, 112), as well as Stx2-mediated induction of IL-8 and MCP-1 (113), trigger leukocyte adhesion to the endothelium (111, 113), enabling subsequent leukocyte-mediated endothelial injury (23, 52). Similarly, increased expression of P-selectin and platelet-endothelial cell adhesion molecule-1 (PECAM-1) in endothelial cells exposed to Stx1 (112) contributes to their prothrombotic response to toxaemia. Stx may also directly lead to endothelial cell activation with perturbed expression of endothelium-derived vasomediators, such as endothelin-1 (114). In addition, Stx stimulates expression of tissue factor, an important initiator of blood coagulation, in HUVEC (115). Also, a direct interaction between Stx and thrombocytes, which leads to their activation resulting in increased platelet aggregation, has been recently demonstrated (37). Stx also induces the binding of platelets to the cell membrane of HUVEC pretreated with TNF-α (37) contributing to the prothrombotic phenotype.

Non-Stx virulence factors of EHEC that may contribute to endothelial cell damage

The finding that HUS can develop after infection with E. coli O157 strains which did not produce Stx (24) has stimulated research on non-Stx virulence factors that might play a role in the pathogenesis of HUS. Based on current data, cytotoxicus toxin (Cdt) (116), EHEC haemolysin (117), and subtilase cytotoxin (118) might be such candidates (Table 2). In EHEC, these molecules are usually not produced in combination by the same strain.
Table 2: Virulence factors of EHEC potentially involved in endothelial cell injury during haemolytic uraemic syndrome and haemorrhagic colitis.

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Target</th>
<th>Activity</th>
<th>Biological consequences for endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shiga toxin 1, Shiga toxin 2</td>
<td>Microvascular endothelial cells from kidneys, colon, brain</td>
<td>Direct cytotoxicity (necrosis or apoptosis)</td>
<td>Prothrombotic activation</td>
</tr>
<tr>
<td></td>
<td>Monocytes and macrophages, mesangial cells, renal proximal tubular cells, endothelial cells (microvascular and HUVEC)</td>
<td>Stimulation of cytokines and chemokines</td>
<td>Procoagulation state</td>
</tr>
<tr>
<td></td>
<td>Glomerular microvascular endothelial cells, HUVEC</td>
<td>Upregulation of chemokines, adhesion proteins and adhesion molecules</td>
<td>Adhesion of polymorphonuclear leukocytes</td>
</tr>
<tr>
<td></td>
<td>Platelets</td>
<td>Activation (increased aggregation)</td>
<td>Prothrombotic state</td>
</tr>
<tr>
<td>Cytotoxic distending toxin</td>
<td>Human brain microvascular endothelial cells, HUVEC</td>
<td>Nuclear DNA fragmentation</td>
<td>Inhibition of proliferation, cell death</td>
</tr>
<tr>
<td>EHEC haemolysin</td>
<td>Monocytes</td>
<td>Stimulation of cytokines</td>
<td>Upregulation of Gb3 on endothelial cells leading to the augmentation of Stx effects</td>
</tr>
<tr>
<td>Subtilase cytotoxin</td>
<td>Cerebellar cortex, renal cortex (glomeruli, tubuli), liver parenchyma (hepatocytes)</td>
<td>Not known</td>
<td>Microvascular thrombosis in the kidneys, brain, liver suggesting endothelial damage</td>
</tr>
</tbody>
</table>

**Cytotoxic distending toxin**

Recently, we identified (116, 119) in EHEC strains a novel toxin, which was characterised as a new member of the CDT family (120, 121), and designated CDT-V (119). CDTs are tripartite toxins (122) that demonstrate a unique mechanism of action on host cells by directly damaging their DNA (123). Through this mechanism, they cause cell cycle arrest (120, 121) and have been therefore classified as cyclomodulins (124). The DNA is damaged by the CdtB subunit, which has DNase I-like activity (123, 125), whereas CdtA and CdtC subunits mediate CDT binding to target cells and intracellular delivery of CdtB (122, 126).

Production of CDT-V by EHEC O157 (116) and particular non-O157 serotypes (O73: H18, O91: H21, O113: H21) (119), which have been associated with HUS (119, 127) prompted us to investigate biological effects of recombinant CDT-V from an EHEC O157 strain on human endothelial cells using HUVEC and two endothelial cell lines, EA.hy 926 (HUVEC-derived) and HBMEC (human brain microvascular endothelial cells) (128). In each of these cell cultures, CDT-V caused an irreversible G2/M arrest (Fig. 1), resulting in inhibition of cell proliferation, progressive cell distension and ultimately death. Moreover, CDT-V-treated cells displayed fragmented nuclei and increased levels of phosphorylated histone protein H2AX, indicating DNA damage followed by DNA repair response (128). Together, these data demonstrate that CDT-V directly injures human endothelial cells resulting in their death, and may thus contribute to the pathogenesis of EHEC-mediated diseases.

**EHEC haemolysin**

EHEC haemolysin, encoded on large plasmids of EHEC O157: H7 (117) and non-O157: H7 EHEC causing HUS (129–132), is a pore-forming cytotoxin (133) belonging to the RTX (repeats-in-toxin) family (117). Expression of the EHEC haemolysin during infection has been evidenced by the detection of a humoral immune response to this protein in sera of patients after HUS (117). A direct effect of EHEC haemolysin on endothelial cells has not been investigated. However, E. coli expressing cloned EHEC haemolysin operon induced IL-1β (134), one of the cytokines that upregulate Stx receptor Gb3 on HBMEC (100), from human monocytes. Thus, EHEC haemolysin might augment the effects of Stxs on endothelial cells. Although an attractive hypothesis, data to support a role of the EHEC haemolysin in the haemolytic anaemia of HUS patients are not available.

**Subtilase cytotoxin**

Subtilase cytotoxin (118) is a novel AB3 toxin encoded on a large plasmid of EHEC O113: H21. It is composed of an enzymatically active A subunit, which is a subtilase-like serine protease, and a binding B subunit, which is related to a putative exported protein from Yersinia pestis. Intraperitoneal injection of purified subtilase cytotoxin into mice causes extensive microvascular...
thromboses, and necrosis in the kidneys, brain, and liver (118), which resemble lesions in patients with HUS (27, 39). These data suggest that this toxin might contribute to the pathogenesis of human disease. Subtilase cytotoxin-encoding genes have been found in a proportion of EHEC O157 as well as in EHEC of non-O157 serogroups associated with HUS (118). Further studies of the interaction of this novel toxin with endothelial cells are warranted.

EHEC virulence factors with yet unknown effects on endothelial cells that might contribute to HUS and haemorrhagic colitis pathogenesis

Several putative virulence factors of EHEC probably contribute to the pathogenesis of EHEC-mediated diseases by mechanisms other than endothelial damage. However, effects of these molecules on endothelial cells have not yet been investigated.

Specifically, EspP (EHEC serine protease, plasmid encoded) (135), a serine protease from the family of autotransporter proteins encoded on a large plasmid of EHEC O157: H7, can cleave human coagulation factor V (135). This effect, which interferes with the blood coagulation cascade, was hypothesized to contribute to the mucosal haemorrhage observed in patients with haemorrhagic colitis (135). However, experimental data from animal models to support this hypothesis are not yet available.

EHEC O157: H7 (136) and O113: H21 (137) flagellins stimulate IL-8 secretion by intestinal epithelial cells. Because IL-8 is a potent neutrophil attractant, and polymorphonuclear leukocytes may be involved in Stx translocation through the intestinal barrier (79), this effect has potential implications for an effective delivery of Stx from the gut to the bloodstream, a step which is apparently critical for the HUS development. Moreover, the flagellin-mediated increased intestinal inflammation may cause significant local damage and contribute thus to the pathogenesis of haemorrhagic colitis.

Future perspectives

Because Stxs are the common denominator, and plausibly the major virulence factors of EHEC that are implicated in the pathogenesis of HUS, research resulting in various strategies to prevent human diseases (138–140) has focused on these toxins. In view of several putative non-Stx EHEC virulence factors that could potentially contribute to the microvascular endothelial injury, future research is necessary to delineate the role of these non-Stx molecules in the pathogenesis of HUS and to define precise mechanisms by which they contribute to the microvascular injury. This knowledge will form the basis for developing complex specific therapeutic and preventive measures for EHEC-mediated HUS.

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