Shiga toxins and apoptosis

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Abstract

The enteric pathogens Shigella dysenteriae serotype 1 and Shiga toxin-producing Escherichia coli (STEC) cause bloody diarrheal diseases that may progress to life-threatening extraintestinal complications. Although the S. dysenteriae and STEC differ in the expression of a number of virulence determinants, they share the capacity to produce one or more potent cytotoxins, called Shiga toxins (Stxs). Following the ingestion of the organisms, the expression of Stxs is critical for the development of vascular lesions in the colon, kidneys and central nervous system. It has been known for some time that following the intracellular routing of Stxs to the endoplasmic reticulum and nuclear membrane, the toxins translocate into the cytoplasm and target ribosomes for damage. However, numerous recent studies have shown that Stxs trigger programmed cell death signaling cascades in intoxicated cells. The mechanisms of apoptosis induction by these toxins are newly emerging, and the data published to date suggest that the toxins may signal apoptosis in different cells types via different mechanisms. Here we review the Stxs and the known mechanistic aspects of Stx-induced apoptosis, and present a model of apoptosis induction.

Keywords: Shiga toxin; Apoptosis; Programmed cell death; Microbial pathogenesis

1. Introduction

Secreted bacterial proteins have been defined as exotoxins based on the ability to cause cell death, mediate cell shape change, or disrupt normal cellular function. It has become clear, however, that many bacterial toxins are multifunctional proteins. This is particularly apparent when the actions of toxins are examined using different cell types. Bacterial toxins may activate cell signaling pathways leading to increased gene expression and/or arrest cell survival pathways. Thus, depending on the cell type utilized, toxins may cause necrotic or apoptotic cell death, may alter cell signaling pathways without causing cell death, or induce the expression of pro- or anti-inflammatory cytokines which in turn exacerbate local inflammation or the development of systemic disease (reviewed in [1,2]). This review will focus on recent advances in our understanding of cell death caused by Shiga toxins (Stxs), a family of structurally and functionally related exotoxins produced by the enteric pathogens Shigella dysenteriae serotype 1 and Stx-producing Escherichia coli (STEC). Although the enzymatic activity of the Stxs has been well described, the toxins may cause cell death by multiple pathways. We will review recent studies characterizing the interaction of Stxs with multiple cell types and offer future directions for research in the field.

2. The organisms

S. dysenteriae serotype 1 and STEC are etiologic agents of the bloody diarrheal diseases bacillary dysentery and hemorrhagic colitis, respectively. Shigellae are invasive bacteria capable of replication within the cytoplasm of colonic epithelial cells. STEC, in contrast, are non-invasive. Some, but not all, STEC attach to the intestinal epithelium in such a manner as to disrupt normal brush border. Filamentous actin and other cytoskeletal elements accumulate adjacent to adherent bacteria so that the organisms appear to be raised on pedestals [3]. These unique adherence structures are called attaching and effacing (A/E) lesions and STEC capable of causing A/E lesions are referred to as enterohemorrhagic E. coli (EHEC). The bacterial proteins necessary for A/E lesion formation,
as well as proteins for the type III secretion apparatus to secrete the adherence proteins, are encoded on a ~35 kb DNA insert called the locus of enterocyte effacement (LEE) pathogenicity island (reviewed in [4]). Thus, EHEC have acquired a large DNA fragment encoding the proteins mediating intimate adherence and cytoskeletal rearrangements.

Patients with bacillary dysentery or hemorrhagic colitis are at increased risk for developing life-threatening systemic complications, primarily acute renal failure and central nervous system (CNS) abnormalities. The acute renal failure that may follow infection with Stx-producing bacteria is called the hemolytic uremic syndrome (HUS) and is characterized by non-immune anemia, thrombocytopenia, and glomerular thrombotic microangiopathy with fibrin-enriched thrombi deposited within glomerular capillaries (reviewed in [5]). CNS abnormalities include lethargy, disorientation, seizures, paralysis and coma [6]. Children and the elderly are more likely to develop renal and neurological complications following infection with STEC.

Shigellae are distributed worldwide but the highest incidence of bacillary dysentery occurs in regions of overcrowding, malnutrition, and inadequate waste and drinking water supplies. Bacillary dysentery is primarily a disease of childhood with most patients being <5 years old. Shigellae cause endemic dysentery in Africa, Southeast Asia and the Indian subcontinent, where estimated incidences are 750–2000 cases per 1000 children per year [7]. Although both S. dysenteriae serotype 1 and STEC may be transmitted by ingestion of contaminated water or person-to-person spread, outbreaks of hemorrhagic colitis often occur after the ingestion of STEC-contaminated foods. Undercooked ground beef, unwashed vegetables, and unpasteurized fruit juices have been implicated in major outbreaks of bloody diarrhea. Thus, STEC infections are more prevalent in developed countries with widespread distribution systems for meats and vegetables. The most prevalent E. coli serotype associated with outbreaks of hemorrhagic colitis in the USA, Canada, UK and Japan is E. coli O157:H7. In the USA, it is estimated that there are ~73,000 cases/year of bloody diarrhea caused by E. coli O157:H7 and ~27,000 cases per year associated with non-O157:H7 serotypes [8].

3. Stxs

While S. dysenteriae serotype 1 and STEC differ in their epidemiology and expression of virulence determinants, they share the property of producing one or more Stxs (alternatively called verotoxins or verocytotoxins). The toxins belong to a group of structurally and functionally related cytotoxins. The prototype toxin of the Stx family is Stx produced by S. dysenteriae serotype 1. STEC may produce one or more related toxins designated Stx1c, Stx1d, Stx2, Stx2c, Stx2d, Stx2e or Stx2f [9,10]. Stx1 is most closely related to Stx, differing by only a single amino acid in the A-subunit of the toxin. Stx1 and Stx2 are approximately 56% homologous at the deduced amino acid sequence level, while the variants of Stx2 are 84–99% homologous to Stx2 [10]. The genes for Stx1, Stx2 and the Stx2 variants are encoded by lambdoid bacteriophages which lysogenize STEC (reviewed in [11]). The bacteria may express more than one Stx because they may harbor more than one Stx-encoding bacteriophage. The toxin-converting phages are important for horizontal transmission of stx genes and Stx-expressing Citrobacter freundii and Enterobacter cloacae strains have been described [12]. Although phage sequences can be detected adjacent to the genes encoding Stx in S. dysenteriae serotype 1, it appears that insertional sequences have rendered the prophage defective for excision [13].

All the Stxs are AB2 toxins consisting of a single ~32 kDa A-subunit in non-covalent association with a pentamer of identical B-subunits. The molecular mass of each B-subunit is ~7.7 kDa. X-ray crystallographic analysis demonstrated that the B-subunit pentamers form a doughnut-shaped structure with the carboxy-terminus of the A-subunit inserted into the central pore of B-subunits [14]. Toxin binding to cells is mediated by the B-subunits through interaction with membrane neutral glycolipids of the globo-series. For the Stxs causing disease in humans, the toxin receptor is globotriaosylceramide (Gb3) or CD77, while Stx2e, a toxin of veterinary importance, binds globotetraosylceramide (Gb4). Gb3 is expressed on epithelial and endothelial cells derived from a variety of sites in humans and animals (reviewed in [15]). Once Stxs bind their glycolipid receptors, the toxins are internalized via clathrin-coated pits and transported through the trans-Golgi network and Golgi apparatus to the endoplasmic reticulum (ER) and nuclear membrane. This pattern of intracellular trafficking is referred to as retrograde transport [16]. During retrograde transport, the A-subunit is cleaved by furin, a calcium-sensitive serine protease localized to the Golgi network. The resultant A-subunit fragments, A1 + A2, remain associated by a disulfide bond. An alternative mechanism of A-subunit processing involving the action of the protease calpain has also been described [17]. Once in the ER, the disulfide bond linking A1 + A2 is reduced, and the A1 fragment is translocated across the ER membrane into the cytoplasm via a mechanism which remains to be fully characterized. The A1 fragments of Stxs possess N-glycosidase activity and act to catalytically cleave a single adenine residue from the 28S rRNA component of the eukaryotic ribosomal 60S subunit. Following depurination, elongation factor 1-dependent aminoacyl-tRNA binding is inhibited and peptide elongation ceases (reviewed in [9]).

The invasiveness of Shigellae is critical for the development of bacillary dysentery. However, Fontaine et al. [18] orally administered either a toxigenic or atoxicogenic S. dy-
senteriae isogenic strain to rhesus monkeys and showed that the Stx-producing strain caused more damage to intestinal capillaries, suggesting that the toxin mediates vascular tissue damage caused by the invasive organisms. The characteristic intestinal damage caused by STEC includes hemorrhage and edema in the lamina propria with focal necrosis and neutrophil infiltration. Recently, it was shown that Stx1 and Stx2 are translocated across polarized intestinal epithelial monolayers via transcellular and paracellular mechanisms [19,20]. In this manner, Stxs may gain access to and damage colonic blood vessels. Stxs have also been shown to induce the expression of the neutrophil-specific chemokine interleukin-8 by human intestinal epithelial cells in vitro [21], suggesting that Stxs may participate in the process of gut inflammation. Incubation of fluoresceinated Stx1 with human whole blood resulted in almost exclusive binding of the toxin to neutrophils [22], and Stx2 was detected in the circulation of HUS patients associated with neutrophils [23]. Scatchard analysis using radiiodinated Stx1 showed that human neutrophils possess approximately $2.1 \times 10^5$ toxin binding sites per cell with a $K_d = 10^{-8}$ mol $^{-1}$. Interestingly, Stx1 could be transferred from neutrophils to the surface of tumor necrosis factor-$\alpha$ (TNF-$\alpha$)-treated human glomerular microvascular endothelial cells in vitro [22]. Collectively, these data suggest that the toxins (i) possess mechanisms to cross the intestinal epithelial barrier; (ii) induce a neutrophil-rich inflammatory infiltrate in the gut; and (iii) circulate in the bloodstream by ‘piggy-backing’ on neutrophils. The presence of Stxs in the circulation appears to be a critical determinant in the development of HUS and CNS complications, as much of the vascular histopathology can be reproduced in animals intravenously administered purified Stxs (reviewed in [24]). The toxins appear to target glomerular and CNS microvascular endothelial cells for damage.

4. Apoptosis

Apoptosis (programmed cell death) and necrosis are currently defined as the two major modes of cell death. In some instances, necrosis is a passive process characterized by the loss of plasma membrane integrity, cell swelling and inflammation due to the release of cellular contents into the periphery. In contrast, apoptosis is an active process generally characterized by morphological cell changes including cell shrinkage associated with cytoplasmic condensation and vacuolation, membrane blebbing, apoptotic body formation, chromatin condensation, nuclear fragmentation, and loss of cell adhesion [25]. However, as has been recently noted, when describing apoptotic versus necrotic changes, it is extremely important to discriminate between antemortem biochemical changes versus postmortem morphological changes in cells [26]. Apoptosis is initiated in response to stimuli which activate genetically programmed signaling cascades. Cytoplasmic constituents are not spilled into the extracellular milieu so that inflammation is not a characteristic of apoptotic cell death. Apoptotic bodies are generally internalized and processed by phagocytic cells.

A major biochemical feature of apoptosis is the internucleosomal fragmentation of genomic DNA, although it should be noted that DNA fragmentation is not a universal characteristic of apoptosis [27]. DNA fragmentation appears to be a multi-step process with DNA first cleaved into large fragments (50–200 kb) and subsequently degraded to nucleosomal units of multiples of 180–200 bp. The presence of fragmented DNA (‘DNA laddering’) is readily assessed using agarose gel electrophoresis. DNA fragmentation also forms the basis of the TUNEL staining assay in which the fragments are used as substrates for the enzyme terminal deoxynucleotidyl transferase. Although many stimuli may activate programmed cell death, most stimuli appear to signal through a common, tightly regulated pathway involving the sequential activation of proteases called caspases. Caspases are cysteine-dependent aspartate-specific proteases that exist as inactive precursors or pro-caspases. In general, caspase activation requires a single proteolytic cleavage of a latent single-chain zymogen to produce a heterodimer. The caspase heterodimers then self pair to form the active tetrameric molecule [28]. Caspases are unusual proteases in that aspartate specificity is rare, and many caspases possess the capacity to mediate auto-proteolysis. Programmed cell death takes place through two pathways categorized on whether apoptotic signals originate outside of or within cells. The extrinsic or extracellular pathway of programmed cell death generally requires the ligation of receptors of the tumor necrosis factor receptor (TNFR) superfamily containing cytoplasmic death domains. Caspase 8 is activated following its recruitment to the cytosolic face of the receptor-ligand complex. The intrinsic or mitochondrial pathway involves the disruption of mitochondrial membrane potential, the release of cytochrome $c$ and the activation of caspase 9. The intrinsic pathway of caspase activation is regulated by members of the Bcl-2 protein family, which include proapoptotic protein BH-3 interacting domain (Bid), Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer (Bak), and anti-apoptotic proteins such as Bcl-2 and myeloid cell leukemia-1 (Mcl-1). The intrinsic pathway is responsible for apoptosis in response to ionizing radiation and cytotoxic chemotherapeutic drugs. Activation of caspase 8 or caspase 9 by the extrinsic or intrinsic pathways leads to the activation of a common downstream caspase, caspase 3. Caspase 3 activates the DNase that executes the cleavage of DNA. Additional molecular details of caspase activation can be found in recent reviews [25,29].

Recently, a third mechanism of signaling for programmed cell death was described which requires the activation of caspase 12. In mice, caspase 12 appears to be
localized to the ER and is activated in response to stimuli which induce ER stress (such as brefeldin A or tunicamycin) and release Ca\(^{2+}\) stores. Mice lacking caspase 12 are resistant to apoptosis induced by ER stress but are sensitive to other death stimuli [30]. The human caspase 12 homolog has been cloned but contains loss of function mutations which render the protein inactive, suggesting that caspase 12 may not play a role in apoptosis induction in human cells [31].

5. Stxs and apoptosis

Numerous studies have examined the mechanism of cell death mediated by purified Stxs. Cultured cells or cell lines used in these studies include epithelial cells, endothelial cells, B-lymphoma cell lines, astrocytoma cells, monocyte cell lines, neutrophils and amniotic cell lines. These cell types respond differently to Stxs. For example, T84 cells, a human intestinal epithelial cell line, lack the toxin glycolipid receptors and are not killed by Stxs in vitro, yet are capable of toxin translocation from apical to basolateral surfaces [19]. Bovine crypt intestinal epithelial cells, in contrast, express Gb3 but escape killing by routing the toxins to lysosomes rather than to the ER [32]. Purified Stx2 has been shown to inhibit cell death of neutrophils, cells which normally undergo spontaneous apoptosis, suggesting that the toxins may increase the life span of cells capable of mediating extensive vascular damage [33]. While Stxs may not induce apoptosis in all cell types, there is ample evidence suggesting that apoptosis is critical in the development of vascular lesions and tissue damage following translocation of the toxins into the bloodstream. Based on the current available literature, a model for the progression of disease is shown in Fig. 1.

In contrast to the studies using T84 cells, Smith et al. [34] treated the human intestinal epithelial cell line, HCT8, with Stx1 and demonstrated the activation of the stress-activated kinase cascades JNK/SAPK and p38, and the activation of caspase 3 leading to apoptosis with DNA fragmentation. Toxin enzymatic activity was required for the activation of stress-activated kinases and cytotoxicity. Caspase 3 activation and cytotoxicity were blocked by the p38 kinase inhibitor SB202190. The signaling proteins necessary to link toxin enzymatic activity with activation of kinase and caspase cascades remain to be characterized.

Suzuki et al. [35] reported that the Stx2A-subunit, but not Stx1 A-subunit, possesses a pentapeptide sequence (NWGRI) similar to Bcl-2 homology domain BH1. Thus, the Stx2 A-subunit may directly interact with mitochondrial Bcl-2 leading to activation of caspase 3 in Bcl-2 expressing human hepatoma (HepG2) cells. Many studies have documented that purified Stxs induce apoptosis in cultured primary epithelial cells, including human renal proximal tubule and renal cortical epithelial cells [36,37]. Apoptosis of renal cortical cells in vivo was demonstrated in biopsies taken from three children with HUS [37]. The biopsies showed extensive cortical necrosis and thrombotic microangiopathy, and TUNEL staining revealed apoptotic nuclei in tubules and glomeruli. A similar pattern of TUNEL staining was noted in the kidney tissues of mice fed Stx2-producing E. coli.

The mechanisms of Stx-induced apoptosis have been extensively studied using the human laryngeal epithelial cell line HEp-2 and the human cervical epithelial cell line HeLa. In HEp-2 cells, Stx1, Stx2 and Stx2c induce apoptosis by activating caspases 8, 9 and 3 [38]. Treatment of HEp-2 cells with the toxins resulted in increased poly(adenosine diphosphate (ADP)-ribose) polymerase (PARP) cleavage. PARP is a caspase 3 substrate, and the cleavage of PARP is thought to act in a proapoptotic manner by inhibiting DNA repair. Bid, a proapoptotic member of the Bcl-2 family, is cleaved to its active 15 kDa form by the action of caspase 8. Activated Bid (truncated Bid or tBid) is known to promote the oligomerization of the proapoptotic proteins Bak and Bax [39] that, in turn, trigger the release of cytochrome c from mitochondria and activate the downstream caspase 3. These data suggest that Bid acts as a link between caspase 8 and the intrinsic or mitochondrial pathway in Stx-mediated apoptosis. Purified Stx B-subunits triggered HEp-2 cell apoptosis, although higher doses were necessary compared to the holotoxins.
Stx1 or Stx2 treatment of HEp-2 cells resulted in the up-regulated expression of the proapoptotic Bax protein, and overexpression of the Bcl-2 protein protected cells from Stx-induced apoptosis [40]. Nakagawa et al. [41] transfected HeLa cells with plasmids expressing either Stx1 A- or B-subunits. Stx1 A-subunit-transfected cells died via necrosis as assessed by lactate dehydrogenase release and inactivation of reduced nicotinamide adenine dinucleotide (NADH)-generating dehydrogenases without DNA fragmentation. Stx1 B-subunit-transfected cells became apoptotic as evidenced by DNA fragmentation and caspase 1 and 3 activation. Fuji et al. [42] reported that Stx1 treatment of HeLa cells results in the activation of caspases 3, 6, 8, and 9, and pre-treatment of cells with caspase 3, 6, and 8 inhibitors, but not caspase 9 inhibitors, protected cells from apoptosis. Caspase 6 induces nuclear disassembly and chromatin condensation by activating lamin A. Bid activation and cytochrome c release were demonstrated in Stx1-treated HeLa cells. However, Stx1 treatment also upregulated the expression of the caspase 9 inhibitor X-linked inhibitor of apoptosis protein (XIAP) which suggests that the intrinsic or mitochondrial pathway and caspase 9 activation may not play a major role in apoptosis induction in this cell type. In contrast to the studies of Nakagawa et al., Stx1 B-subunits did not induce HeLa cell apoptosis, and retrograde transport of the holotoxin to the ER appeared to be essential for apoptosis induction [42].

There are a number of reports indicating that Stxs induce apoptosis in endothelial cells isolated from various anatomical sites, but the mechanistic aspects of Stx-induced endothelial cell apoptosis are just emerging. Pijpers et al. [43] showed that human foreskin microvascular endothelial cells (FMVEC) are sensitive to apoptosis induced by Stxs at concentrations as low as 10−100 nM. In nanomolar concentrations, purified Stx B-subunits also induced FMVEC apoptosis. A peptide aldehyde inhibitor of caspase 3 protected cells from apoptosis. In studies using the human dermal microvascular endothelial cell line HMEC-1, Stx1 and Stx2 were shown to rapidly (within 4 h) induce apoptosis and inhibit the expression of the anti-apoptotic Bcl-2 family member Mcl-1 [44]. Mcl-1 is normally constitutively expressed by endothelial cells. The decrease in detectable Mcl-1 levels was toxin dose and time dependent. A pan-caspase inhibitor did not prevent the decrease in the Mcl-1 protein levels, suggesting that caspases do not contribute to this phenomenon. The proteasome inhibitor lactacystin prevented the diminution of Mcl-1 levels and protected the cells from apoptosis. Using the same cells, another anti-apoptotic protein, FLICE-like inhibitory protein (FLIP), was also shown to be down-modulated by Stx1 [45]. FLIP is rapidly degraded by proteasomes and needs to be continuously synthesized for cell survival. These studies suggest a crucial role for proteasomes in the regulation of apoptosis. In the face of inhibition of de novo protein synthesis mediated by Stxs, labile anti-apoptotic factors are rapidly degraded, triggering the onset of apoptosis. FLIP is homologous to caspase 8, but is enzymatically inactive, thereby serving in a dominant negative fashion to prevent apoptosis via inhibition of caspase 8 activation [46,47]. Thus, the role of FLIP in protecting against Stx-induced apoptosis is consistent with previous findings implicating a caspase 8-dependent mechanism for Stx-mediated apoptosis.

6. Factors affecting Stx-induced apoptosis

Endothelial cells derived from the primary sites of Stx-mediated vascular damage, that is, human glomerular and brain microvascular endothelial cells appear to be relatively resistant to apoptosis induction by Stxs in vitro unless the cells are first treated with the proinflammatory cytokine TNF-α [43,48,49]. The mechanism of endothelial cell sensitization to apoptosis is not well understood, but may involve, in part, the capacity of proinflammatory cytokines to upregulate the membrane expression of GB1 on endothelial cells [50]. Another factor that may contribute to endothelial cell injury and/or dysfunction in HUS is the presence of endotoxin or lipopolysaccharides (LPS) in the circulation. LPS are known to be potent inducers of proinflammatory cytokine expression. Circulating anti-LPS antibodies and increased acute phase reactants in the sera of patients with HUS suggest that they may experience transient endotaxemia (reviewed in [5]). Human endothelial cells have been shown to be relatively resistant to apoptosis induced by LPS treatment in vitro. However, the pre-treatment of human dermal microvascular endothelial cells with Stx1, leading to decreased FLIP expression, increased the sensitivity of the cells to apoptosis mediated by LPS [45]. Collectively, these data suggest that multiple signals, Stxs, LPS, and cytokines, may be necessary to trigger apoptosis of the endothelial cells found at sites of major vascular damage.

7. Conclusions and future directions

It has become increasingly clear that Stxs induce apoptosis in some, but not all, cell types. The capacity of the toxins to trigger programmed cell death pathways may contribute to the development of bloody diarrhea and extraintestinal complications. An overall model of apoptosis induction mechanisms by Stxs derived from recently described studies is shown in Fig. 2. Apoptosis induction is complex, and may involve extracellular and intracellular signaling pathways. Although Stx-mediated activation of the upstream caspase, caspase 8, has been reported, it is not clear whether caspase 8 is activated by the extrinsic pathway acting through engagement of membrane associated receptors, or by intrinsic signaling proteins through the ribotoxic stress response. Receptor ligand complexes
directly interacting with caspase 8 following treatment of cells with toxins or purified B-subunits remain to be identified. If apoptosis induction requires retrograde transport, the precise points in the trafficking process initiating apoptotic signals are currently unknown. Studies using HeLa and HMEC-1 cells have suggested that toxin enzymatic activity is necessary to induce apoptosis through a mechanism completely independent of caspase activation. Such a mechanism may involve protein synthesis inhibition and proteasome-mediated degradation of anti-apoptotic proteins. Finally, it should be noted that Stxs may not trigger a uniform apoptotic signaling cascade in all cell types. Thus, the differential signaling events caused by the toxins in different cell types await further clarification. A better understanding of the mechanisms of apoptosis induction by Stxs may lead to the development of effective therapeutic strategies to interrupt the progression of disease from the bloody diarrheal phase to life-threatening complications.

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References


