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Minireview

MINIREVIEW

Pathogenic effects of glucosyltransferase from *Clostridium difficile* toxins

Yongrong Zhang and Hanping Feng*

Department of Microbial Pathogenesis, University of Maryland Baltimore, 650 W. Baltimore Street, Baltimore, MD 21201, USA

*Corresponding author: Department of Microbial Pathogenesis, University of Maryland Baltimore, 650 W Baltimore Street, Room 7211, Baltimore, MD 21201, USA.
Tel: +410-706-6328; Fax: +410-706-6511; E-mail: hfeng@umaryland.edu

One sentence summary: In this review, we summarize the pathogenic effects of glucosyltransferase domain of *Clostridium difficile* toxins in the past years.

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ABSTRACT

The glucosyltransferase domain of *Clostridium difficile* toxins modifies guanine nucleotide-binding proteins of Rho family. It is the major virulent domain of the holotoxins. Various pathogenic effects of *C. difficile* toxins in response to Rho glucosylation have been investigated including cytoskeleton damage, cell death and inflammation. The most recent studies have revealed some significant characteristics of the holotoxins that are independent of glucosylating activity. These findings arouse discussion about the role of glucosyltransferase activity in toxin pathogenesis and open up new insights for toxin mechanism study. In this review, we summarize the pathogenic effects of glucosyltransferase domain of the toxins in the past years.

Keywords: *Clostridium difficile*; glucosyltransferase; toxins; pathogenesis

INTRODUCTION

Clostridium difficile produces two glucosylating exotoxins, toxin A (TcdA) and toxin B (TcdB), that cause severe disease increasingly associated with global high rates of morbidity and mortality. In the United States, since the emergence of antibiotic-resistant, hypervirulent strains, the mortality of *Clostridium difficile* infection (CDI) has increased 400% between 2000 and 2007 and the infection led to 29 000 deaths in 2011 (Team 2013; Lessa *et al.* 2015). The clinical spectrum of CDI varied from asymptomatic carrier to fulminate colitis with multiple organ failure (Kelly and LaMont 2008; Bagdasarian, Rao and Malani 2015; Postma, Kiers and Pickkers 2015). The importance of the two toxins in disease is becoming increasingly clear, and evidence suggests that the toxins are the essential virulence factors (Lyras *et al.* 2009; Kuehne *et al.* 2010).

Both TcdA and TcdB belong to the large clostridial glucosylating toxin family. This toxin family includes TcdA and TcdB from *C. difficile*, lethal toxin from *C. sordellii*, alpha-toxin from *C. novyi* and toxin TpeL from *C. perfringens* (Aktories 2011). Among them, *C. difficile* toxins are the prototypes used to study structure and structure-related functions. The N-terminal glucosyltransferase (GT) was identified (Hofmann *et al.* 1997) as the main effector domain (TcdA: amino acid 1–544; TcdB: amino acid 1–543) (Just *et al.* 1994, 1995a,b,c; Chaves-Olarte *et al.* 1997), whereas the rest of the holotoxin was considered as a delicate delivery system to inject the effector into the host cells. The delivery system used by *C. difficile* toxins contains at least three other major domains including a cysteine protease domain, a translocation domain and combined repetitive oligopeptides also referred to as a receptor-binding domain. Therefore, the holotoxins were defined as ABCD-type toxins by Jank and Aktories (2008). The

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glucosylation reaction occurs intracellularly, following receptor-mediated cellular uptake, pH-dependent membrane insertion and intramolecular proteolytic autocleavage activated by inositol hexakisphosphate (von Eichel-Streiber, Sauerborn and Kuramitsu 1992; Qa'Dan, Spyres and Ballard 2000; Pfeifer *et al.* 2003; Ho *et al.* 2005; Rupnik *et al.* 2005; Egerer *et al.* 2007; Reineke *et al.* 2007; Genisyuerek *et al.* 2011; Kreimeyer *et al.* 2011). The autocleavage of the two toxins is not essential for the glucosylation reaction since both non-cleavable mutant TcdA and TcdB also lead to the glucosylation of target cells although in a reduced speed (Kreimeyer *et al.* 2011; Li *et al.* 2013, 2015). Monoglucosyltransferases inactivate members of the Rho GTPase family through the covalent transfer of glucose (Just and Gerhard 2004). This irreversible modification inactivates these small regulatory proteins, causing disruption of vital signaling pathways in the cells, and leads to actin cytoskeleton damage and cell death (Jank, Giesemann and Aktories 2007). The interaction between the bacterial virulent effector and host components will eventually present as disease symptoms, such as inflammation and diarrhea. This review will focus on the progress of research on the pathogenic role of GT of *C. difficile* toxins in the past decades.

Cytoskeleton damage

Diarrhea is one of the most significant symptoms of CDI. Intestinal barrier dysfunction may be one of the causes of diarrhea. The intestinal epithelium functions as a barrier to selectively allow translocation of compounds as well as to defend against the invasive pathogens (Turner 2009). Rho GTPase family members play critical roles as switches to control the epithelial permeability by regulating the actin cytoskeleton. The GT domains of *C. difficile* TcdA and TcdB are able to inactivate Rho, Rac, and Cdc42 subfamilies by transferring a glucose moiety from cellular UDP precursors onto the threonine residue 35/37 in the GTP-binding domain (Just and Gerhard 2004). Cell rounding appeared to be the primary sign of Rho glucosylation-related cytoskeleton damage. *In vivo*, many gut pathogens and their toxins disrupt the epithelial barrier for colonization and invasion by targeting the Rho GTPase family (Krause-Gruszczynska *et al.* 2007). No significant evidence has delineated that glucosylation was related to *C. difficile* colonization. Nevertheless, Rho glucosylation increased the permeability of epithelium (Teichert *et al.* 2006). Loss of the epithelial barrier may lead to systemic dissemination of gut luminal contents. Full-length toxins were detected in systemic circulation (Steele *et al.* 2012; Yu *et al.* 2015) which may be a potential reason for multiple organ damage at the onset of infection (Carter *et al.* 2015). Tight junctions serve a barrier function to seal and regulate the movement of ions and solutes across a healthy epithelium. It is not clear whether TcdA and TcdB directly modify tight junction proteins (TJPs), but the abundance of TJPs were affected after TcdA treatment based on a proteomics study (Zeiser *et al.* 2013). The treatment of Caco-2 cells with GT-deficient TcdA does not significantly decrease transepithelial electrical resistance in comparison to wild-type TcdA (Teichert *et al.* 2006). *In vivo*, the GT-deficient TcdB did not disrupt mouse intestinal epithelia as the wild-type toxin did (Yang *et al.* 2015). These studies strongly support the critical role of GT-mediated Rho glucosylation in *C. difficile* toxin-induced epithelial permeability.

Cellular death

As cytotoxins, *C. difficile* TcdA and TcdB are well known to provoke cell death that may contribute to toxin-related pathogenesis. Both toxins are capable of inducing cytopathic effect (cell rounding) in less than 1 h, but cytotoxic effect (apoptotic cell death) does not occur until 24 h or even longer following toxin exposure. Recently, TcdB-related non-apoptotic cell death, referred to as necrosis and pyknosis, has been investigated.

Apoptosis

In the past decade, clear evidence has emerged that both toxins cause apoptosis via caspase activation that includes caspase-3, 8, 9 (Hippenstiel *et al.* 2002; Qa'Dan *et al.* 2002; Matarrese *et al.* 2007; Gerhard *et al.* 2008). Multiple cell lines including human intestinal epithelial cells, monocytes and fibroblasts undergo *C. difficile* toxin-mediated apoptosis (Qa'Dan *et al.* 2002; Solomon *et al.* 2005; Matarrese *et al.* 2007; Gerhard *et al.* 2008; Lica *et al.* 2011; Li *et al.* 2013). However, controversy exists in whether GT activity is the trigger for apoptosis. To differentiate GT-dependent and GT-independent effects, GT-deficient holotoxins were generated and widely used (Teichert *et al.* 2006; Jochim *et al.* 2011; Zeiser *et al.* 2011, 2013; D'Auria *et al.* 2015). Utilizing mutant TcdA D285/287N, activation of caspase-3 and induction of apoptosis in human colonic HT29 cells were shown to be dependent on GT activity (Gerhard *et al.* 2008; Kreimeyer *et al.* 2011). Qa'Dan *et al.* found caspase-3 activation in TcdB-treated HeLa and MCF-7 cells. But they also discovered a previously uncharacterized ability of TcdB to induce caspase-independent apoptosis in TcdB GT fragment-treated HeLa cells (Qa'Dan *et al.* 2002). An intrabody against GT activity was shown to completely prevent cells from experiencing TcdB-mediated cell death which suggested that GT is essential for the cytotoxic effects of holotoxins (Li *et al.* 2015).

Conversely, there are also studies utilizing functional GT fragments which demonstrated that TcdB causes apoptosis in epithelial cells independent of GT activity (Qa'Dan *et al.* 2002; Matarrese *et al.* 2007). In addition to caspase activation, cytochrome C released from mitochondria leads to cellular apoptosis. TcdA caused damage of mitochondria within 5 min of treatment, which was earlier than Rho-glucosylation (He *et al.* 2000). TcdB was described to directly induce isolated mitochondria swelling in a calcium-dependent fashion. Interestingly, the GT fragment of TcdB failed to affect mitochondria even in the presence of Ca^{2+}, suggesting TcdB-induced mitochondria dysfunction was GT independent (Matarrese *et al.* 2007). Sun *et al.* (2014) reported that TcdB-induced endoplasmic reticulum (ER) stress in colonic cells in a GT-independent fashion. But TcdB-related ER stress was considered non-apoptotic due to lack of upregulation of C/EBP homologous protein, a classic mediator of apoptotic ER stress (Sun *et al.* 2014). Regardless of whether Rho GTPase glucosylation plays a role in cell apoptosis or not, the stage of cell cycle progression was implied as another determinant of cell demise. Proliferating HT29 cells displayed inhibited cell division (binucleation) and underwent apoptotic cell death with TcdB treatment (Lica *et al.* 2011). In contrast, TcdB triggered non-apoptotic cell death in confluent or terminally differentiated colonocytes (Lica *et al.* 2011).

Necrosis

Chumbler *et al.* (2012) found that TcdB caused rapid necrotic cell death in HeLa cells as well as human colonic biopsy independent of GT activity. Unlike apoptosis, necrotic death occurred when a higher concentration of either wild type or GT-deficient holo-TcdB was applied (Chumbler *et al.* 2012). More recently, the same group reported the related mechanism of TcdB-mediated necrosis. Although in necrosis GT activity might not be involved, necrosis occurred by a Rac1-dependent pathway (Farrow *et al.* 2013). TcdB provoked Rac1 activation prior to Rac1 glucosylation

(Farrow *et al.* 2013). Active Rac1 may assemble NADPH oxidase complex to mediate rapid production of cellular reactive oxygen species (ROS) which is lethal to cells (Farrow *et al.* 2013). Less research on necrosis of TcdA-treated cells has been reported. Early studies found that necrosis occurred to monocytes exposed to high doses of TcdA (Warny and Kelly 1999; Solomon *et al.* 2005).

Pyknosis

Pyknosis is another type of TcdB-mediated programmed cell death. The typical signs of TcdB-intoxicated cells undergoing pyknosis were chromatin condensation, nuclear blister, cell shrinkage and rapid loss of viability. Similar to necrosis, this phenomenon only happened to cells treated with high concentrations of TcdB (Wohlan *et al.* 2014). Both wild-type TcdB and GT activity-deficient TcdB D286/288N induced pyknotic cell death. Interestingly, glucosylation-mediated cytopathic effects (cell rounding) seemed to protect cells from pyknosis (Wohlan *et al.* 2014). Although GT activity is not required, the holotoxin structure of VPI10473 TcdB appeared to be a prerequisite for pyknosis since neither TcdBF from 1470 strain nor the chimera of TcdB harboring the GT domain of TcdBF was able to induce pyknotic effects (Wohlan *et al.* 2014). Increased production of ROS was also detected in pyknotic cells (Wohlan *et al.* 2014).

Inflammatory responses

Inflammation characterized by neutrophil flux and numerous cytokines for CDI is one of the hallmarks of the disease. Several proinflammatory cytokines have been indicated as predictors of susceptibility to CDI or biomarkers of human CDI disease severity (Jiang *et al.* 2006; El Feghaly *et al.* 2013; Connelly *et al.* 2014; Yacyshyn *et al.* 2014). Although in *in vivo* study multiple factors may contribute to inflammation (El Feghaly, Bangar and Haslam 2015), *C. difficile* toxins were demonstrated to be potent inducers of inflammatory responses *in vitro*. Several proinflammatory cytokines and chemokines produced by various cell lines exposed to *C. difficile* toxins, including IL-8, IL-1β, TNF-α, IL-16 and IL-23, were well documented (He *et al.* 2002; Na *et al.* 2005; Tixier *et al.* 2005; Kim *et al.* 2006; Meyer *et al.* 2007; Sun *et al.* 2009; Zemljic *et al.* 2010; Gerhard *et al.* 2011; Bobo *et al.* 2013; Hansen *et al.* 2013; Koon *et al.* 2013; Koon *et al.* 2014; Cowardin *et al.* 2015); however, some of the cytokines were reported to be induced independent of GT activity. Several studies showed that inflammasome activation was correlated to *C. difficile* toxin-induced inflammation (Ng *et al.* 2010; Xu *et al.* 2014; Cowardin *et al.* 2015). Ng *et al.* found that both TcdA and TcdB induced IL-1β through inflammasome activation. By utilizing GT activity-deficient full-length TcdB, the authors also demonstrated that the full-length structure of TcdB, but not the enzymatic activity of GT, was required to induce IL-1β. Thereafter, opposite results were reported by Xu *et al.*, who found that pyrin-mediated caspase-1 inflammasome activation was in response to the Rho-glucosylation activity of TcdB by using GT activity-deficient TcdB, which induced insignificant amounts of IL-1β in comparison to wild-type TcdB (Xu *et al.* 2014). Similarly, production of TNF-α by TcdA-treated macrophages was fully abolished when GT activity was disabled (Sun *et al.* 2009). Only wild-type TcdA with full functional GT was able to upregulate the mRNA of IL-8 and downregulate the mRNA of IL-16 in mast cells (Gerhard *et al.* 2011). Mitochondrial damage-derived ROS were involved in nuclear translocation of NF-κB and activation of the phoshporylated signal of p38 MAPK (He *et al.* 2002; Kim *et al.* 2005). NF-κB plays an important host mucosal protective role in response to TcdA by inducing colonocytes to produce IL-8, which initially recruits neutrophils (He *et al.* 2002). As we discussed in the previous section, mitochondrial damage can be independent of GT activity. Therefore, as a downstream reaction, IL-8 production might also be induced without glucosylation.

Pathogenic effects of GT in animal models

Although multiple pathogenic effects of *C. difficile* toxins have been identified *in vitro*, animal models are necessary to address which effects are more clinically relevant. The disease outcomes in terms of morbidity and mortality may vary due to host defensive responses (Giesemann, Guttenberg and Aktories 2008; Yu *et al.* 2015). The pathogenic effects of CDI have been widely studied in animal infection models. In the mouse infection model, disease manifestation ranged from local intestinal damage and inflammation to systemic toxemia and multiorgan damage. Lyras *et al.* (2009) and Kuehne *et al.* (2010)utilized genetically engineered *C. difficile* strains (A+B–, A–B+, A–B–) to establish infection models and demonstrated the critical roles of TcdA and TcdB in disease pathogenesis. However, no *C. difficile* strain modified to express GT-deficient toxin has been generated yet. Therefore, currently, investigation of the pathogenic effects of GT in animal infection models is not possible. D'Auria *et al.* (2013) developed a model similar to acute CDI by directly injecting toxins into cecum to study the downstream effects of the two toxins. Utilizing this particular model, we recently demonstrated that GT activity is required to induce severe fatal diseases (Yang *et al.* 2015). Unlike wild-type TcdB, TcdB-W102A/D288N (referred to as aTcdB) failed to induce clinic symptoms or death (Yang *et al.* 2015). Interestingly, mild inflammation occurred in high doses of aTcdB challenged mouse cecum although severe intestinal injury was not evident. Moreover, we developed a chronic disease model to mimic infection by using non-pathogenic *Bacillus megaterium* expressing toxins as surrogates. Repeated oral administration of *B. megaterium* expressing GT-deficient aTcdB failed to induce any clinical disease or death to animals, whereas surrogates expressing wild-type TcdB-induced persistent weight loss and 60% mortality (Yang *et al.* 2015). In addition to damage of the gastrointestinal tract, *C. difficile* toxins were able to leak into circulation and cause multiple organ damage (Steele *et al.* 2012; Carter *et al.* 2015). In another study, systemic injection of purified TcdB was shown to lead to rapid death (Wang *et al.* 2012; Li *et al.* 2015). Intraperitoneal injection of 100 times more aTcdB failed to cause either disease symptoms or death, indicating that systemic toxicity of TcdB in circulation was also abolished by disabling GT activity (Li *et al.* 2015). All these studies indicate a critical role of GT activity of *C. difficile* toxins in pathogenesis of the disease in animals.

CONCLUSIONS

The N-terminal GT domain of *C. difficile* toxins was primarily identified as the effector element of the holotoxins. They intoxicate mammalian cells by glucosylating a group of critical intracellular switches—Rho GTPases. Rho glucosylation will lead to cell morphology change that is ascribed to cytoskeleton reorganization. This particular effect in epithelial tissues is rendered as tight junction disruption and permeability increase. The permeable epithelium might be correlated with toxemia identified in animal infection models and patients with CDI. In the meanwhile, toxin-induced apoptosis may contribute to tissue damage as well as inflammatory responses *in vivo*. But both apoptosis and inflammatory responses were described bilaterally in terms

of the glucosylation dependence. It is hard to conclude that proinflammatory cytokines/chemokines were directly glucosylation driven because some upstream effects of apoptosis and inflammosome activation as well as mutual effects among cytokines may contribute to the complexity of cytokine responses. Recently identified GT-independent necrosis and pyknosis may also contribute to the tissue damage. Although plenty of studies using cell-based systems demonstrated both GT-dependent and independent effects, the GT-dependent effects seemed to be critical for pathogenesis in animal studies since GT activity-deficient mutant toxins do not trigger significant morbidity and mortality in animal models. Thus, the *in vivo* studies reinforce the essential role of GT in disease pathogenesis. Therefore, although *in vitro* study is necessary and helpful to understand the molecular mechanisms of the toxins, *in vivo* study is quite vital to verify *in vitro* findings and to develop therapeutic interventions.

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