Mechanism of *Clostridium difficile* Toxin A–Induced Apoptosis in T84 Cells

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This study is an investigation into the mechanism of *Clostridium difficile* toxin A–induced apoptosis in human intestinal epithelial cells. Toxin A induced apoptosis of T84 cells in a dose- and time-dependent fashion. Toxin A–induced apoptosis was completely inhibited by blocking toxin enzymatic activity on Rho GTPases with uridine 5′-diphosphate-2,3-dialdehyde by a nonspecific caspase inhibitor and was partially inhibited by caspase-1, -3, -6, -8, and -9 inhibitors. Caspases 3, 6, 8, and 9 and Bid activation were detected. Toxin A also induced changes in mitochondrial membrane potential and cytochrome c release at 18–24 h, a time course similar to caspase-9 activation. In conclusion, toxin A induces apoptosis by a mechanism dependent on inactivation of Rho, activation of caspases 3, 6, 8, and 9 and Bid, and mitochondrial damage followed by cytochrome c release. Toxin A proapoptotic activity may contribute to the mucosal disruption seen in toxin A–induced enteritis.

*Clostridium difficile*, an anaerobic gram-positive bacterium, causes antibiotic-associated diarrhea and pseudomembranous colitis and is the most common cause of nosocomial diarrhea [1–6]. *C. difficile* produces at least 2 large exotoxins involved in the pathogenesis of the disease, toxin A and toxin B. Although toxin B has potent cytotoxic activity in vitro, the enterotoxic activity of *C. difficile* in animals has been mainly attributed to toxin A (308 kDa). Purified toxin A causes disruption of the intestinal epithelium, intestinal secretion, intense mucosal inflammation, and hemorrhagic colitis when introduced in vivo to the intestinal lumen [7–12]. Most toxin A effects are related to its ability to monoglucosylate small GTP-binding proteins of the Rho family on threonine 37/35 [13]. This modification inactivates proteins Rho, Rac, and Cdc42, which leads to disruption of actin cytoskeleton assembly, cell retraction, loss of adhesion, and rounding of cells in tissue culture [13, 14].

Apoptosis, a form of physiological cell death, is critical for development and tissue homeostasis. In the intestinal epithelium, apoptosis maintains the balance between cell proliferation located in the crypts and cell elimination occurring in both crypts and villi [15]. However, unregulated apoptosis has been implicated in a growing number of clinical disorders. Morphologically, apoptosis is characterized by DNA fragmentation, membrane blebbing, cell shrinkage, and disassembly into membrane-enclosed vesicles, which are eliminated rapidly by phagocytosis, which prevents a host inflammatory response to intracellular components [16]. At the molecular level, classical apoptosis is caused by the activation of a family of cysteine proteases known as “caspases” that cleave their target proteins at specific aspartic acids [17, 18]. Two major apoptotic pathways have been defined: (1) the extrinsic pathway, where activation of a death receptor (e.g., Fas/CD95 and TNFRI/CD120a) by a ligand, such as tumor necrosis factor (TNF)–α or Fas ligand, leads to activation of initiator caspase 8 [19–21]; and (2) the intrinsic pathway, which is a consequence of cellular stress and cytochrome c release from mitochondria, which leads to caspase-9 activation [22–24]. Both pathways culminate in the activation of downstream executioner caspase 3 and caspase 6 [25, 26]. The activation of the executioner caspases results in a series of events, such as cleavage of cytoskeleton proteins [27–29], activation of nucleases, and subsequent DNA fragmentation [30, 31]. In addition, cross talk exists between the mitochondrial-mediated and receptor-mediated pathways. Caspase 8 has been shown to cleave and activate Bid, a proapoptotic Bcl-2 family member, which permeabilizes the mitochondria membrane and induces release of cytochrome c [20, 32]. Thus, Bid links the caspase-8 and caspase-9 pathways.

Because toxin A binds to apical receptors facing the lumen and is too large to be transported intact into the lamina propria or the plasma, it is presumed that the necroinflammatory reaction induced by toxin A is caused by primary derangement of the epithelial barrier [33]. Toxin A has been reported to induce apoptosis in intestinal epithelial cells, colonic lamina propria cells, and mast cells, but the mechanisms involved in activation of the...
caspase system remain unclear [34–37]. Epithelial apoptosis induced by the toxin could explain much of the pathogenesis of the colitis; thus, the study of its signaling pathways may improve the understanding of infection-associated apoptosis, as well as provide new molecular targets for therapeutic intervention strategies. The aim of the present study was to investigate the mechanisms of C. difficile toxin A–induced apoptosis in a human colonic epithelial cell line and to determine whether this apoptosis depends on the well-known enzymatic activity of toxin A, which leads to inactivation of Rho.

Materials and Methods

Materials. General caspase inhibitor (Z-Val-Ala-Asp-fmk), caspase-1 inhibitor (Z-Tyr-Val-Ala-Asp-fmk), caspase-2 inhibitor (Z-Val-Ala-Asp-Val-Ala-Asp-fmk), caspase-3 inhibitor (Z-Asp-Glu-Val-Val-Asp-fmk), caspase-6 inhibitor (Z-Val-Glu-Ile-Asp-fmk), caspase-8 inhibitor (Z-Ile-Glu-Thr-Asp-fmk), and caspase-9 inhibitor (Z-Leu-Glu-His-Asp-fmk) were purchased from Enzyme System Products. Fluorogenic substrate for caspase 1 (7-amino-4-trifluoromethyl coumarin; AFC), caspase 3, caspase 6, caspase 8, and caspase 9 was purchased from BIOMOL Research Laboratory. Rabbit anti–human cytochrome c antibody and human cytochrome c were purchased from Research Diagnostic. Horseradish peroxidase (HRP)–conjugated goat anti–rabbit IgG antibody was obtained from Amersham. Rabbit anti–human TNF-α MAb (0.75 μg/mL) was added, which was followed by a further incubation at room temperature (20°C–25°C) for 1 h. An HRP-conjugated streptavidin (1.25 mg/mL) diluted 1:10000 was added subsequently, and the plates were incubated for 30 min at room temperature, which was followed by the addition of 3,3’,5,5’-tetramethylbenzidine substrate solution and incubation for another 30 min (20°C–25°C). Then, stop solution (0.18 M H₂SO₄) was added, and the plates were read at 450–550 nm. The results were expressed as picograms per milliliter of TNF-α from the supernatant by comparing the absorbance of standard curves prepared with recombinant human TNF-α.

Dose response and time course of toxin A–induced apoptosis by annexin V assay. T84 cells were seeded onto 6-well plates (10⁴ cells/well), and, 24 h after plating, the medium was changed, and the cells were incubated with or without toxin A (1–1000 ng/mL). After 6, 18, 24, and 48 h of incubation with toxin A, the medium was discarded, the attached cells were washed with PBS, trypsinized, collected by centrifugation, and double stained with fluorescein isothiocyanate (FITC)–conjugated annexin V and propidium iodide (ApoAlert Annexin V Apoptosis Kit; Clontech).

Quantitation of DNA fragmentation. T84 cells were seeded onto 6-well plates (10⁵ cells/well), and, 24 h after plating, the medium was changed, and the cells were incubated with or without toxin A (100 ng/mL) for 6, 18, 24 and 48 h. The cells were harvested by centrifugation at 200 g for 5 min. The pellets were lysed with 0.3 mL of hypotonic lysis buffer (10 mM Tris and 10 mM EDTA containing 0.5% Triton X-100, and lysates were centrifuged at 13,000 g for 10 min, to separate intact from fragmented chromatin. The supernatant, containing DNA fragments, was placed in a separate microfuge tube, and both pellet and supernatant were treated at 4°C for 30 min in 1 N perchloric acid (PCA). The DNA precipitates were sedimented at 13,000 g for 20 min, were hydrolyzed by heating (70°C) for 10 min in 0.15 mL of 1 N PCA, and were quantitated by use of a modification of the diphenylamine method of Burton, as described elsewhere [39].

Gel electrophoretic analysis of DNA fragmentation. T84 cells were seeded onto 6-well plates (10⁵ cells/well), and, 24 h after plating, the medium was changed, and the cells were incubated with or without toxin A (100 ng/mL) for 24 h. After 6, 18, 24, and 48 h of incubation with toxin A, the medium was discarded, the attached cells were washed with PBS, trypsinized, collected by centrifugation, and double stained with fluorescein isothiocyanate (FITC)–conjugated annexin V and propidium iodide (ApoAlert Annexin V Apoptosis Kit; Clontech).

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Clostridium difficile toxin A induces apoptosis in a dose-dependent fashion. T84 cells were incubated for 24 h with toxin A (1–1000 ng/mL). Cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (annexin V–FITC) and propidium iodide (PI) and were analyzed by means of flow cytometry. Results are plotted as density plots with PI vs. annexin V–FITC. Viable cells have low annexin V–FITC (left lower quadrant), and apoptotic cells have high annexin V–FITC and low PI staining (right lower quadrant). Data are representative of 3 experiments. Bars on the graph (bottom right) represent mean ± SEM of the percentage of cells in apoptosis. *P < .05, vs. control group (analysis of variance–Bonferroni test).
Figure 2. Time course of Clostridium difficile toxin A–induced apoptosis. T84 cells were incubated for 6, 18, 24, or 48 h with toxin A (100 ng/mL). Cells were treated by adding fresh media containing toxin A (100 ng/mL). After incubating the cells with toxin for 6, 18, or 24 h, the cells were washed twice in PBS, detached, resuspended in medium (5 × 10⁶ cells/mL) containing JC-1 fluorescent dye (100 μg/mL), and incubated at 37°C for 10 min. Then, cell fluorescence was measured by use of the FACSscan cytometer (Becton Dickinson) that was equipped with a 488-nm argon laser (FL1) and a 568-nm argon-krypton laser (FL2). JC-1 fluorescence was analyzed on the FL1 and FL2 channels for detection of the dye monomer and J-aggregate forms.

Statistical analysis. Statistical analysis involved analysis of variance, which was followed by the Bonferroni test and unpaired Student’s t test. Statistical differences were considered to be significant at P < .05.

Results

Dose response and time course of toxin A–induced apoptosis by annexin V assay. The flow cytometric analysis shown in this study demonstrated that the incubation of T84 cells for 24 h with toxin A induces apoptosis in a dose-dependent fashion (figure 1). Apoptosis was analyzed by means of annexin V assay,
which detects the rearrangement of phosphatidylserine from the inner to the outer plasma membrane leaflet during early stages of apoptosis [42]. Annexin V is a cellular protein that avidly binds to phosphatidylserine. Apoptotic cells are positive for fluorescein isothiocyanate (FITC)-conjugated annexin V (annexin V–FITC) only (figure 1, lower right quadrants), and necrotic or late apoptotic cells are positive for both annexin V–FITC and propidium iodide (figure 1, upper right quadrants). The viable cells should stain negative for annexin V–FITC and propidium iodide (figure 1, lower left quadrants). The increase in the number of apoptotic cells is shown in figure 1 by an increase in the number of cells (density of plots) in the lower right quadrant. The treatment of T84 cells with 100 and 1000 ng/mL toxin A induced a significant increase in the percentage of apoptotic cells, compared with that of control (P < .05; 23% and 30% of cells were apoptotic, respectively). Because the treatment with 100 ng/mL toxin A induced apoptosis with smaller augmentation of necrosis than did treatment with 1000 ng/mL, we used this concentration (100 ng/mL) to examine the time course. The time course showed an increase in the number of apoptotic cells (density of plots in the lower right quadrants) after 6 h of treatment with toxin A and a greater increase 24–48 h after incubation with toxin A (figure 2).

Time course of toxin A–induced apoptosis by quantitation of DNA fragmentation. The apoptotic effect of toxin A (100 ng/mL) on T84 cells was confirmed with analysis of DNA fragmentation in T84 cells treated with toxin A, showing 8%, 32.6%, 39.8%, and 33.5% of DNA fragmentation after treatment with toxin A for 6, 18, 24, and 48 h, respectively (figure 3). The fragmented DNA was quantified with the Burton method and was expressed as the percentage of DNA fragmentation per total DNA [39]. We also sought to quantify the DNA fragmentation among the attached and detached cells subsets after treatment with toxin A (100 ng/mL), to investigate whether T84 cell apoptosis was secondary to cellular detachment. The treatment of T84 cells with toxin A induced a substantial increase in the percentage of DNA fragmented, until 24 h after treatment with toxin A, in both subsets of cells, attached and detached (figure 3). At 24 h after treatment with toxin A, 17.3% of DNA fragmentation was observed among the remaining attached cells and 22.5% among the detached cells. At 48 h, most cells treated with toxin A had become detached from the culture dish, which explains why DNA fragmentation at 48 h among attached cells was 0%. These results suggest that the cells became apoptotic before detaching from the plate surface and also indicate that T84 cell apoptosis was not secondary to cellular detachment. This is in agreement with results obtained by using other types of transformed monolayer cells shown to lack adhesion-dependent regulation of apoptosis [43].

Effect of caspase inhibitors on toxin A–induced apoptosis by DNA fragmentation. Incubation of the cells with a nonspecific caspase inhibitor (20 μM) for 1 h before adding toxin A completely blocked toxin A–induced apoptosis, as shown by the absence of DNA laddering at 24 h after the addition of toxin A. The same preincubation time with caspase-1, -3, -6, -8, and -9 inhibitors, but not caspase-2 inhibitor, partially blocked toxin A–induced apoptosis, as shown by the reduction DNA laddering, compared with the effect of toxin A alone (figure 4).

Detection of caspase activity. The partial inhibition of toxin A–induced apoptosis by specific caspase inhibitors indicated
C. difficile toxin A activates caspase-3, -6, -8, and -9 activity. After treatment with toxin A (100 ng/mL) for 0, 6, 18, 24, or 72 h, T84 cells were lysed and were frozen and thawed repeatedly (54 times) using liquid nitrogen. Cell-lysate supernatants were used for the assay of caspase-proteolytic activity. Caspase proteolytic activity was monitored by use of the fluorogenic 7-amino-4-trifluoro-methyl coumarin (AFC)–peptide substrate specific for each caspase. Caspase cleaved AFC from the peptide and released free AFC that was detected in a kinetic microplate fluorescence reader at an excitation value of 380 nm and emission of 530 nm. Caspase activity was expressed as pM of AFC liberated/min/μg of protein. Data are representative of 3 experiments.

that toxin A was activating these proteases. Thus, we investigated the kinetics of cleavage activity induced by toxin A for each caspase. Caspase-3, -6, -8, and -9 cleavage activity was slightly increased 6 h after incubation with toxin A, became significantly increased, compared with that of control, at 24 h, and peaked at 48 h (figure 5). Caspase-3 activity (figure 5A) increased more quickly than the other caspases, reaching a higher level of activity. Caspase-6 activity (figure 5B) also reached higher levels, compared with caspase-8 activity (figure 5C) or caspase-9 activity (figure 5D). The higher levels of activity achieved by caspase 3 and caspase 6 might have resulted from the additive effect of both caspase-8 and caspase-9 pathways that activate these executioner caspases. Caspase-1 cleavage activity was not detected (data not shown).

Toxin A–induced cytochrome c release and change in mitochondrial membrane potential. Toxin A induced change in the mitochondrial membrane potential. JC-1 is a cationic dye that exhibits potential dependent accumulation in the mitochondria. The population of JC-1–positive cells, cells with decreased membrane potential, increased after treatment with toxin A (figure 6A). This was indicated by a time-dependent shift in the population of cells (density of plots) from the upper right quadrant to the lower left quadrant when cells were incubated with toxin A, compared with control cells incubated without toxin A; a 105% increase in the percentage of events in the lower left quadrant after 24 h of incubation with toxin A is seen, compared with control (incubation without toxin A; figure 6A).

Mitochondrial injury opens the mitochondrial transition pore, which allows the release of mitochondrial cytochrome c, a protein that can activate caspase 9 and cause apoptosis [44–46]. The present study detected a significant accumulation of cytosolic cytochrome c in T84 cells after 6, 18 and 24 h of exposure to toxin A, which indicates a leakage of cytochrome c from the damaged mitochondria (figure 6B).

Bid activation. Because Bid, a proapoptotic Bcl-2 family member protein, induces cytochrome c release and can be activated by caspase 8, we investigated whether toxin A could induce activation of Bid. Indeed, toxin A induced cleavage of Bid into a 15-kDa product after 6, 18, and 24 h (figure 7).

Effect of toxin A on TNF-α release by T84 cells. We found that toxin A induced TNF-α release by T84 cells. TNF-α release appeared to increase in cell supernatants at 24 h after incubation with toxin A and peaked and became significant at 48 h (figure 8).

Effect of anti TNF-α neutralizing antibody on caspase-8 activity. Treatment of T84 cells incubated with toxin A with neutralizing anti–human TNF-α antibody (20.8 μg; added every 12 h) did not influence caspase-8 activation induced by toxin A (data not shown).

Toxin A–induced apoptosis by DNA fragmentation and its
inhibition by blockage of its enzymatic activity on Rho. Toxin A is known to catalyze the monoglucosylation of Rho GTPases by transferring a glucose moiety from the cosubstrate UDP-glucose. The transfer of the glucose moiety is inhibited by an excess of UDP. We used a reactive UDP derivative, UDP 2',3'-dialdehyde, which reacts with free amino residues and thus inactivates glucosyltransferase activity of toxin A [38]. The treatment of T84 cells with toxin A (100 ng/mL) for 24 h induces apoptosis that can be detected by typical DNA laddering (figure 9). This appearance results from the cleavage of the DNA onto internucleosomal fragments each consisting of 180–200 bp by activation of endonucleases [15, 47]. The apoptosis induced by toxin A was blocked by the inactivation of toxin A with UDP 2',3'-dialdehyde in a dose-dependent manner, being completely blocked by the detoxification of toxin A with 10 mM UDP 2',3'-dialdehyde (figure 9, lane 2). Incubation of the toxin with the reducing buffer allowed toxin A to be fully active in inducing apoptosis (figure 9, lane 5), as seen by DNA laddering similar to the untreated toxin A (figure 9, lane 6).

Discussion

This study shows that *C. difficile* toxin A induces apoptosis in a human colonic epithelial cell line in a dose-dependent manner. Significant apoptosis was detected after incubation of T84 cells with toxin A (100 ng/mL) for 6–48 h. Toxigenic *C. difficile* is known to cause pseudomembranous colitis characterized by epithelial destruction and profound mucosal inflammation [7–12]. Because of the large size of toxin A (308 kDa), it has been postulated that the toxin must first act on and disrupt the colonic epithelial cells to induce intestinal inflammation. Because T84 cells have a phenotype characteristic of colonic crypt cells, which are important in the replacement of new cells after epithelial damage, the apoptotic effect of toxin A may have critical implications for the pathogenesis of the colitis induced by this pathogen. Toxin A–induced apoptosis has been reported in T84 cells and in organ cultures of human colonic biopsy specimens, but its mechanisms were unclear [35].

This study demonstrated for the first time the involvement of caspase-activating cascades in toxin A–induced apoptosis. Currently, there are 2 major caspase pathways known to regulate apoptosis. One results from a death signal transmitted by the binding of a death ligand, such as TNF-α or Fas ligand, to cell-surface death receptors (i.e., TNF-α or Fas receptors). In this pathway, the complex of death receptor and its ligand activates pro–caspase 8 to release activated caspase 8 [19]. Another caspase-activating cascade, the intrinsic pathway, occurs as a consequence of cellular stress that leads to depolarization of the mitochondrial membrane, release of cytochrome c, and subsequent activation of caspase 9 [22]. Both of these pathways result in activation of downstream executioner caspases 3, 6,
and 7, which cleave a number of vital proteins, resulting in apoptosis [48].

We showed that *C. difficile* toxin A–induced apoptosis was completely blocked by a nonspecific caspase inhibitor and was partially inhibited by caspase-1, -3, -6, -8, and -9 inhibitors. In addition, we found that incubation of T84 cells with toxin A activated caspase-3, -6, -8, and -9 activities, with enzymatic activity peaking at 48 h after incubation with toxin A. These findings are in agreement with the detection of the peak number of apoptotic cells by annexin V assay at 48 h. Because neither caspase-8 nor caspase-9 inhibitor was able to completely inhibit the apoptosis, it is possible that both pathways mediate toxin A–induced apoptosis. Indeed, both caspase-8 and -9 activities were significantly increased by toxin A. The involvement of the mitochondria/cytochrome c pathway also was demonstrated by the change of mitochondrial membrane potential and the release of cytochrome c into cytosol of toxin A–treated T84 cells. Cytosolic cytochrome c was detected at 18 and 24 h after incubation with toxin, which is consistent with the presence of caspase-9 cleavage activity at 24 h. In accordance with our finding, He et al. [49] have reported that toxin A induced mitochondrial damage, depletion of mitochondrial membrane potential, and cytochrome c release in CHO cells.

The mitochondria-mediated pathway of apoptosis is regulated by the Bcl-2 family of proteins. Some, such as Bcl-2 and Bcl-X, suppress apoptosis, whereas others, such as Bax, Bad, and Bid, promote it [50]. The Bcl-2 family members regulate apoptosis in several ways. For example, the cleavage of the apoptosis promoter Bid by caspase 8 releases a 15-kDa fragment that triggers the release of cytochrome c from mitochondria by inducing conformational change of Bax [20, 32, 51]. In this study, we also demonstrated that toxin A induced activation of Bid in T84 cells. Activated caspase 8 has been shown to induce release of cytochrome c through cleavage of Bid [20]. However, the activation of Bid was detected as early as 6 h after toxin A incubation, in accordance with early detection of apoptosis, and caspase-8 activity was considerable only after 24 h. Thus, it is likely that Bid participates in early signaling events that trigger apoptosis, but its activation might be independent of caspase 8.

Because toxin A has been shown to induce TNF-α release in macrophages and lamina propria cells [36, 52], we postulated that T84 cells were releasing toxin A–induced TNF-α and that this cytokine could be activating caspase 8 by an autocrine mechanism. Indeed, our findings are the first, to our knowledge, to show that toxin A elicits production of TNF-α in T84 cells. The kinetics of TNF-α production displayed a time course similar to that of caspase-8 activity, which suggests that this cytokine could contribute to caspase-8 activation in these cells. Previous reports also have shown that T84 cells produce TNF-α in response to bacterial invasion and hypoxia [53, 54]. However, treatment with neutralizing anti–human TNF-α antibody did not prevent caspase-8 activation (data not shown), which suggests that either the antibody did not neutralize TNF-α efficiently or caspase-8 activation is not mediated by this mechanism. The same antibody was not able to prevent apoptosis of lamina propria cells, even with detection of TNF-α produc-

![Figure 8](image_url), Detection of tumor necrosis factor (TNF-α) in supernatants from T84 cells stimulated with *Clostridium difficile* toxin A (100 ng/mL) or PBS (control). The concentration of TNF-α was determined by ELISA. Data are mean ± SEM of 4 samples measured in duplicate and are representative of 2 experiments. *P < .05, vs. control group (unpaired t test).

![Figure 9](image_url), Inactivation of *Clostridium difficile* toxin A by treatment with UDP-dialdehyde prevents toxin A–induced apoptosis. Toxin A was treated with UDP-2′,3′-dialdehyde (DH; 0.1, 1, and 10 mM; lanes 4, 3, and 2, respectively) dissolved in modification buffer (20 mM Tris-HCl [pH 7.2], 150 mM NaCl) at 37°C for 3 h, followed by reduction with 4 mM NaBH₄CN. This procedure is described to block the enzymatic activity of toxin A on Rho GTPases. For controls, the cells were untreated (lane 1) or were treated with regular toxin (lane 6) or with toxin A treated with reducing buffer (lane 5). DNA was extracted from cells cultured in 6-well culture plates by use of a DNA extraction kit (Sepa Gene, Sankoujyunyaku), and gel electrophoretic analysis of DNA fragmentation was performed. DNA size markers are shown in bp on both sides. +, Presence; −, absence.
tion [36]. Because the Fas receptor is constitutively expressed on the basolateral membrane of healthy colon epithelium [55], it is reasonable to assume that the Fas-dependent pathway might be involved in toxin A induction of caspase-8 activation and apoptosis. However, there is no report in the literature about induction of Fas ligand by toxin A. The mechanism of toxin A induction of caspase-8 activity, including Fas ligand and Fas receptor expression, is the focus of ongoing investigations in our laboratory.

Most toxin A effects are linked to its ability to catalyze the transfer of glucose from UDP-glucose to GTP-binding Rho proteins, rendering these signaling proteins nonfunctional [13]. Inactivation of Rho leads to disaggregation of actin-containing stress fibers, loss of adhesion, and cell rounding of tissue culture cells [33]. In our study, we treated toxin A with a reactive UDP derivative, UDP-2′,3′-dialdehyde, which has been described to inactivate the glucosyltransferase activity of C. difficile toxin A on Rho [38]. The transfer of the glucose moiety by the toxin to Rho is inhibited by a surplus of UDP. It has been proposed that UDP-dialdehyde reacts with free amino residues and inactivates toxin by binding to its catalytic domain, resulting in alkylation of toxin A [38]. Thus, to study the involvement of Rho inactivation in the toxin A–induced apoptosis cascade, we blocked the enzyme activity of toxin by treatment with UDP-2′,3′-dialdehyde and evaluated its ability to induce apoptosis. We found that the induction of apoptosis by toxin A was abolished by the detoxification of toxin with UDP-dialdehyde in a dose-dependent manner, as observed by the DNA fragmentation ladders. These data indicate that toxin A–induced apoptosis in T84 cells is mediated through its inactivation of Rho. In accordance with our data, it has been demonstrated that inactivation of Rho by exoenzyme C3 of C. botulinum led to induction of apoptosis in lymphocytes and also that overexpression of a dominant negative form of Rho or an inactive form of Rho induced apoptosis in smooth-muscle cells [56]. Additionally, it has been demonstrated that activation of Rac and Rho inhibited apoptosis induced by growth factor deprivation, DNA damage, and loss of cell adhesion [57–60]. In apparent contrast to these studies are reports that show overexpression of constitutively active forms of Rho induces apoptosis in fibroblasts and leukemia cells [61, 62]. Therefore, we believe that Rho might trigger distinct cell survival and/or apoptotic pathways, depending on the cell type and the stimuli. Our findings showing that inactivation of Rho is critical for toxin A–induced apoptosis, which support other evidence that placement of Rho within signaling pathways is required for cell survival [56, 60].

In summary, our studies show that C. difficile toxin A induces apoptosis in T84 cells in a time- and dose-dependent manner. We demonstrated that the 2 main caspase-activating pathways, caspase-8 and cytochrome c/caspase-9 activation, mediate toxin A induction of apoptosis, and we also showed a possible link between both pathways through Bid activation induced by toxin A. Our data also suggest that toxin A–induced apoptosis is dependent on monoglucosylation of Rho protein catalyzed by toxin A. These findings provide important information on basic biological responses by epithelial cells to injury and suggest that toxin A–induced apoptosis may play a role in the pathogenesis of C. difficile colitis. The clarification of mechanisms of toxin A–induced apoptosis opens potential new approaches to nonantibiotic pharmacological interventions for treating the growing problem of nosocomial C. difficile colitis.

Acknowledgments

We wish to thank William Ross and Kailo Heinz Schlegel for assistance with the flow cytometry and David Lyerly (Techlab, Blacksburg, VA) for the toxin A used in these experiments.

References


