Glucosylation of Rho GTPases by *Clostridium difficile* toxin A triggers apoptosis in intestinal epithelial cells

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The intestinal epithelial cell line HT-29 was used to study the apoptotic effect of *Clostridium difficile* toxin A (TcdA). TcdA is a 300 kDa single-chain protein, which glucosylates and thereby inactivates small GTPases of the Rho family (Rho, Rac and Cdc42). The effect of TcdA-catalysed glucosylation of the Rho GTPases is well known: reorganization of the actin cytoskeleton with accompanying morphological changes in cells, leading to complete rounding of cells and destruction of the intestinal barrier function. Less is known about the mechanism by which apoptosis is induced in TcdA-treated cells. In this study, TcdA induced the activation of caspase-3, -8 and -9. Apoptosis, as estimated by the DNA content of cells, started as early as 24 h after the addition of TcdA. The impact of Rho glucosylation was obvious when mutant TcdA with reduced or deficient glucosyltransferase activity was applied. TcdA mutant W101A, with 50-fold reduced glucosyltransferase activity, induced apoptosis only at an equipotent concentration compared with wild-type TcdA at a 50 % effective concentration of 0.2 nM. The enzyme-deficient mutant TcdA D285/287N was not able to induce apoptosis. Apoptosis induced by TcdA strictly depended on the activation of caspasases, and was completely blocked by the pan-caspase inhibitor z-VAD-fmk. Destruction of the actin cytoskeleton by latrunculin B was not sufficient to induce apoptosis, indicating that apoptosis induced by TcdA must be due to another mechanism. In summary, TcdA-induced apoptosis (cytotoxic effect) depends on the glucosylation of Rho GTPases, but is not triggered by destruction of the actin cytoskeleton (cytopathic effect).

INTRODUCTION

*Clostridium difficile* is a pathogen that causes antibiotic-associated diarrhoea and severe pseudomembranous colitis. Toxin A (TcdA) and toxin B (TcdB) are the main pathogenic factors released by *C. difficile* (Just & Gerhard, 2004). The toxins are homologous glucosyltransferases that mono-glucosylate Rho GTPases (Rho, Rac and Cdc42) (Just et al., 1995). This glucosylation leads to functional inactivation. As Rho GTPases regulate the dynamics of the actin cytoskeleton (Hall, 1998), their inactivation leads to a reorganization of filamentous actin structures. Thus, the predominant effect of TcdA and TcdB is the breakdown of the actin cytoskeleton, accompanied by a change in cell morphology. Rho GTPase inhibition results in inhibition of migration, morphogenesis, cell division (Jaffe & Hall, 2005) and membrane trafficking (Ridley, 2001). Additionally, Rho GTPases are involved in transcriptional regulation and apoptosis (Aznar & Lacal, 2001; Symons, 1996). They play a critical role in apoptosis associated with morphological changes in cells (Coleman & Olson, 2002; Coniglio et al., 2001), or contribute in a pro- or anti-apoptotic manner as a response to genotoxic stress (Fritz & Kaina, 2006).

The apoptotic properties of TcdA and TcdB are well known. These toxins induce apoptosis in intestinal epithelial cells (Brito et al., 2002, 2005; Carneiro et al., 2006; Fiorentini et al., 1998; Liu et al., 2003; Mahida et al., 1998) and in all other cells tested such as neurons (Le et al., 2005), endothelial cells (Hippstenstiel et al., 2002), HeLa cells (Qa’Dan et al., 2002) and macrophages (Warny & Kelly, 1999). It is common sense that the executioner caspase-3 is involved in TcdA-induced apoptosis (Brito et al., 2002; Carneiro et al., 2006; Hippstenstiel et al., 2002; Le et al., 2005; Qa’Dan et al., 2002; Solomon et al., 2005). Additionally, activation of caspase-6, another executioner caspase, can also be observed (Brito et al., 2002; Carneiro et al., 2006). The initiator caspase-8 represents the extrinsic apoptotic pathway, which can be activated via transmembrane death receptors such as the Fas receptor. Caspase-9, representing the intrinsic pathway, is activated by an
apoptosome containing cytochrome c released from damaged mitochondria (Boatright & Salvesen, 2003). Both initiator caspases (8 and 9) are known to cleave procaspase-3 into the activated caspase-3. The activation of the intrinsic pathway by caspases has been described by different authors. Cytochrome c is released from mitochondria in response to treatment with TcdA or TcdB (Brito et al., 2002; Gerhard et al., 2005; Kim et al., 2005; Le et al., 2005; Liu et al., 2003). As a consequence, caspase-9 is consecutively activated (Brito et al., 2002; Carneiro et al., 2006; Hippenstiel et al., 2002; Le et al., 2005; Liu et al., 2003). However, caspase-8 also plays a role in TcdA-induced apoptosis (Brito et al., 2002; Carneiro et al., 2006). Intracellular effects of TcdA that trigger pro-apoptotic events are not known. The glycosylation of Rho GTPases as a prerequisite for apoptosis is controversial and is discussed below. Mutant forms of TcdA (Teichert et al., 2006) were used here to elucidate the impact of Rho glucosylation on the apoptotic processes.

METHODS

Chemicals and reagents. The antibodies used were: polyclonal anti-caspase-3 antibody recognizing procaspase-3 and cleaved caspase-3, polyclonal antibody recognizing cleaved caspase-8 (Asp374) and polyclonal antibody recognizing cleaved caspase-9 (Asp315) (all from Acris Antibodies), monoclonal anti-Rac1 antibody (clone 102; BD PharMingen), monoclonal anti-Rac1 antibody (clone 23A8; Upstate), horseradish-conjugated anti-mouse IgG and anti-rabbit IgG (Rockland Immunoscience). The pan-caspase inhibitor z-VAD-fmk was from Merck Biosciences. The Bacillus megaterium expression system was from MoBiTec.

Cell culture. The colonic crypt cell line HT-29 was cultured under standard conditions in Dulbecco's modified Eagle's medium/F12 mix supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 μg/ml penicillin. For stimulation experiments, cells were seeded in Petri dishes (3.5 cm diameter) coated with fibronectin.

Preparation of toxins. All experiments were performed with expressed recombinant TcdA. The recombinant and genetically engineered mutants were expressed in a B. megaterium expression system as His-tagged fusion proteins. The constructs were sequenced to ensure that the sequence was identical compared with the reference gene of C. difficile TcdA, strain VPI 10463 (GenBank accession no. X51797), except for the desired mutations. Purification was performed by Ni²⁺ affinity chromatography as described previously (Burger et al., 2003). Toxins were stored until use in 10 mM Tris/HCl (pH 7.4), 50 mM NaCl at −80 °C. The recombinant toxins exhibited all features of the native TcdA, i.e. glucosyltransferase activity (except when specifically reduced by directed mutagenesis), cell binding, endocytotic uptake and autocatalytic cleavage of the catalytic domain (data not shown). Different conditions for expression and purification of native TcdA from C. difficile and of the recombinant TcdA did not induce alteration of any of these features (Gerhard et al., 2005).

Flow cytometry. The number of apoptotic or necrotic cells was counted by fluorescence-activated cell sorting (FACS). Detached and adherent cells, which were suspended by trypsinization, were pooled before FACS analysis. The DNA content was measured using the fluorescent nucleotide acid dye propidium iodide. Cells (0.5 × 10⁴) were fixed in 70% ice-cold ethanol for 30 min and washed once with 1% BSA in PBS. The DNA was stained with propidium iodide [150 μg ml⁻¹ in Tris/HCl (pH 7.4) containing 1% BSA and 1% Triton X-100]. RNA was removed by incubating the cells with 0.5% RNase for 30 min. The cells were then subjected to flow cytometry (FACScan flow cytometer; Becton Dickinson). A fluorescence area (FL2) of 400 was set to correlate with a 2σ set of chromosomes within the G1 phase. Cells found in the sub-G1 phase were considered to be apoptotic and necrotic due to the decrease in DNA content.

Western blotting. Activation of caspase-9, -8, and -3 was shown by Western blot analysis, using antibodies that recognize the cleaved and thus activated caspases. Whole-cell homogenates were resolved by SDS-PAGE and transferred to nitrocellulose. Immunodetection was performed in Tris-buffered saline containing 0.2% (v/v) Tween 20. The nitrocellulose was incubated overnight at 4 °C with primary antibodies, followed by the appropriate secondary antibody for 45 min at room temperature. Femto SuperSignal ECL Substrate (Pierce) was used for detection.

Statistical analysis. A two-sided Student's t-test was performed to determine significant effects. A value of P < 0.01 was considered significant and is indicated with asterisks in the figures. The t-test was only used for studies with n≥5 experiments. All values are given as means ± SD.

RESULTS AND DISCUSSION

All experiments were performed using expressed recombinant TcdA. The recombinant toxins exhibited all features of the native TcdA, i.e. glucosyltransferase activity (except when specifically reduced by directed mutagenesis), cell binding, endocytotic uptake and autocatalytic cleavage of the catalytic domain (data not shown). Different conditions for expression and purification of native TcdA from C. difficile and of the recombinant TcdA did not induce alteration of any of these features (Gerhard et al., 2005).

Glucosyltransferase activity of TcdA is crucial for induction of apoptosis

TcdA (3 nM) induced morphological changes in HT-29 cells and the cells rounded up within 8 h (Fig. 1a). The kinetics of Rac glucosylation, which was assayed using a specific antibody (Genth et al., 2006), was in strong correlation with cell rounding (data not shown). The activation of caspase-3 followed the glucosylation of Rho GTPases. The results of Western blot analyses with specific antibody recognizing uncleaved procaspase-3 (35 kDa) as well as the cleaved and thus activated caspase-3 (12 kDa) are shown in Fig. 1(b). There was a strong correlation between caspase activation (Fig. 1b, upper panel) and Rac1 glucosylation (Fig. 1b, middle panel). Total Rac1 is shown in the lower panel. The maximum activation of caspase-3 was detectable only when Rac1 was quantitatively glucosylated. The 50% effective concentration required to activate caspase-3 after 36 h was approximately 50 ng TcdA ml⁻¹ (0.2 nM). Recombinant TcdA with mutations that specifically affect the glucosyltransferase activity (Fig. 2a) is an ideal tool to distinguish between glucosyltransferase-dependent and -independent effects. Different mutants

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were used to check for glucosyltransferase-independent effects. The purity of toxin preparation (Fig. 2b, left panel) and the absence of cleaved N-terminal glucosyltransferase (Fig. 2b, right panel) were checked by SDS-PAGE and Western blot analysis with specific anti-TcdA1–542 antibody, respectively. TcdA W101A had 50-fold reduced glucosyltransferase activity compared with wild-type TcdA, and was applied in an equimolar or equipotent ratio to distinguish between Rho glucosylating effects and those that are independent. The double mutant TcdA D285/287N does not glucosylate Rho GTPases when applied to cells, even at the highest achievable concentration of 0.3 μM (Teichert et al., 2006). The cytotoxic effect of TcdA resulting from caspase activation was measured by flow cytometry. As shown in Fig. 2(c), 1 nM TcdA induced apoptosis in 28 ± 6% of cells after 36 h incubation compared with untreated cells (5 ± 2%). The double mutant TcdA D285/287N, which had no Rho glucosylating properties in a cell culture assay, did not induce apoptosis (4 ± 3%). Mutant TcdA W101A led to a slight and non-significant increase (8 ± 4%) in the number of apoptotic cells at a concentration of 1 nM. However, at a 50-fold concentration, TcdA W101A had similar apoptotic features (21 ± 15%) to wild-type TcdA. Thus, the apoptotic effect of TcdA correlates with the glucosyltransferase activity that

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is displayed intracellularly. Destruction of the actin cytoskeleton alone by latrunculin B (5 μg ml⁻¹) did not induce apoptosis in HT-29 cells. Fig. 2(d) shows the activated, i.e. cleaved, caspase-3 after treatment of cells with either wild-type TcdA, TcdA W101A or TcdA D285/287N (1 nM each). This set of experiments showed that apoptosis was induced only when Rho GTPases were maximally glucosylated (compare with Fig. 1b). Breakdown of the actin cytoskeleton was a sequel of Rho glucosylation, but did not cause apoptosis.

Cells treated with TcdA (1 nM) arrested at the G₂/M transition as a sequel to the breakdown of the actin cytoskeleton (Fig. 3). After 12 h, about 17% of cells were found in this phase. At 24 h, the cells started to become apoptotic with a maximum of 42% of cells after 48 h. However, an arrest within the G₂/M transition did not necessarily induce apoptosis, as could be seen by the latrunculin B experiment. Latrunculin B induced arrest within the G₂/M phase in 14% of cells without increasing the number of apoptotic cells.

**TcdA-induced apoptosis depends on the activation of caspases**

HT-29 cells were subjected to flow cytometry to measure DNA content. Cells with a DNA content less than that of cells with a 2n set of chromosomes (G₁ phase) were considered apoptotic. Fig. 4(a) shows representative FACS data of untreated HT-29 cells and cells treated with 1 nM TcdA in the presence or absence of the pan-caspase inhibitor z-VAD-fmk (50 μM). The caspase inhibitor fully prevented the apoptotic outcome of TcdA treatment, as could be seen by a decrease in the number of cells within the sub-G₁ phase. The ability of TcdA to arrest cells within the G₂/M transition was observed when cells were not allowed to become apoptotic. Compared with untreated cells, TcdA induced a strong cell-cycle arrest in the G₂/M phase when apoptosis was blocked by z-VAD-fmk. Thus, there was no caspase-independent cell death of TcdA-treated cells.

**Caspase-3 plays a central role in TcdA-induced apoptosis**

Apoptosis induced by TcdA can be abolished by blocking caspase activity. The hierarchy of caspases involved in TcdA-induced apoptosis is not known so far. We applied the pan-caspase inhibitor z-VAD-fmk (50 μM) to check which caspase is the primary one that is cleaved by a non-
caspase protease, and which caspases are downstream of other caspases. Treatment of cells with TcdA led to cleavage of procaspase-3. Western blot analysis showed a reduced specific signal for procaspase-3 and an increase in the 12 kDa fragment of the active caspase-3 (Fig. 4b, upper panel). Surprisingly, procaspase-3, the precursor of the executioner caspase-3, was cleaved, even in the presence of z-VAD-fmk. Compared with controls, there was less procaspase-3 and, in addition, two intermediate fragments of 20 and 17 kDa were generated. The signals can be interpreted as follows: firstly, cleavage of procaspase-3 was performed by a non-caspase protease and further auto-cleavage resulting in the 12 kDa fragment was inhibited by z-VAD-fmk. Cleavage of procaspase-8 and procaspase-9 was completely inhibited by z-VAD-fmk, indicating that these caspases are activated by an upstream caspase (Fig. 4b, middle and lower panels, respectively).

TcdA induced activation of caspase-3, -8 and -9 in HT-29 cells and thereby triggered apoptotic cell death. Activation of the executioner caspase-3 strongly correlated with the glucosylation of Rac1. Significant cleavage of procaspase-3 was detected when TcdA was applied at concentrations that were sufficient to achieve quantitative glucosylation of the Rac1 pool. It is not known which GTPase is the crucial one for induction of activation of caspases when glucosylated. The kinetics of TcdA-catalysed Rac and Rho glucosylation are almost identical (Teichert et al., 2006), and thus Western blot analysis of non-glucosylated Rac1 is an ideal surrogate for the intracellular action of TcdA (Genth et al., 2006). The correlation of the extent of glucosylation of Rho GTPases and the increasing activation of caspase-3 strongly suggest that inhibition of Rho GTPases is a cause of apoptotic cell death. A single report has described the Rho-independent effect of TcdA (Kim et al., 2003), and it is likely that TcdA exhibits functions additional to its glucosyltransferase activity when applied to cells. To study the effect of glucosylation events in TcdA-induced cell death, we used a set of TcdA mutants that differed solely in their glucosyltransferase activity. These experiments clearly showed that apoptotic cell death strictly depended on the glucosyltransferase activity of these toxins in our model system. No effect on caspases or cell viability was observed when TcdA W101A (with reduced glucosyltransferase activity) or TcdA D285/287N (no glucosyltransferase activity) was applied at concentrations higher than that of wild-type TcdA sufficient to induce maximum effects. It is not known whether inhibition of Rho, Rac, Cdc42 or a combination of these GTPases is responsible for the TcdA-induced apoptosis. Rho GTPases are positively involved in transduction of receptor-mediated apoptotic signals (Brenner et al., 1997), and can also be negatively involved (Esteve et al., 1998; Rattan et al., 2006). Inhibition of Rho by exoenzyme C3-catalysed ADP ribosylation (Bobak et al., 1997; Ikeda et al., 2003; Li et al., 2002), as well as dominant-negative Rac1 and Cdc42 mutants, induces apoptosis (Lassus et al., 2000). Specific glucosylation of Rac/Cdc42 by lethal toxin from Clostridium sordellii also induces apoptosis (Linseman et al., 2001; Petit et al., 2003). The predominance of either these Rho GTPases may be the reason for the different sensitivities, and indicates the difficulties in estimating the relative contribution of these Rho GTPases to the apoptotic properties of Rho-glucosylating toxins.

Caspase-3 is the central player in TcdA-induced cell death. Inhibition of the executioner caspase-3 prevented an apoptotic outcome for cells treated with TcdA for at least 48 h. The most important finding was that the first cleavage of procaspase-3 into intermediate fragments was carried out by a protease that was not susceptible to a pan-caspase inhibitor. This indicates that a non-caspase protease triggers activation of the cascade of caspases, where caspase-3 is simultaneously the initiator and executioner caspase. Caspase-8 and -9 are also involved, but seem to participate only in an amplifying manner.

The results of this study are summarized in a schematic diagram (Fig. 5). Treatment of cells with TcdA causes inhibition of Rho GTPases. This causes a reorganization of the actin cytoskeleton, which in turn results in arrest at the G2/M transition. This effect is referred to as the cytopathic effect. Glucosylation of Rho GTPases also causes activation of caspases, which is independent of the effect on the actin cytoskeleton. Active caspase-3 induces apoptosis, i.e. the cytotoxic effect of TcdA. By application of different mutants of TcdA, we have shown that the glucosyltransferase activity of TcdA is responsible for both the cytopathic and the cytotoxic effect.

**Fig. 5.** Schematic diagram of the cytopathic and cytotoxic effects of TcdA. Glucosylation of Rho GTPases induces reorganization of the actin cytoskeleton with subsequent G2/M arrest (cytopathic effect). Independently of the cytoskeleton, Rho glucosylation results in activation of caspase-3 to execute apoptosis (cytotoxic effect). Caspase-8 and -9 participate in an amplifying manner.
REFERENCES


