Novel Subtilase Cytotoxin Produced by Shiga-Toxigenic Escherichia coli Induces Apoptosis in Vero Cells via Mitochondrial Membrane Damage

Gen Matsuura,1,2 Naoko Morinaga,1* Kinnosuke Yahiro,1,3 Reiko Komine,1 Joel Moss,3 Hideo Yoshida,2 and Masatoshi Noda1

Departments of Molecular Infectiology1 and Pediatric Surgery,2 Graduate School of Medicine, Chiba University, Chiba, Japan, and Translational Medicine Branch, NHLBI, NIH, Bethesda, Maryland3

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Subtilase cytotoxin (SubAB) is an AB5 cytotoxin produced by some strains of Shiga-toxigenic Escherichia coli. The A subunit is a subtilase-like serine protease and cleaves an endoplasmic reticulum chaperone, BiP, leading to transient inhibition of protein synthesis and cell cycle arrest at G1 phase. Here we show that SubAB, but not the catalytically inactive mutant SubAB (S272A), induced apoptosis in Vero cells, as detected by DNA fragmentation and annexin V binding. SubAB induced activation of caspase-3, -7, and -8. Caspase-3 appeared earlier than caspase-8, and by use of specific caspase inhibitors, it was determined that caspase-3 may be upstream of caspase-8. A general caspase inhibitor blocked SubAB-induced apoptosis, detected by annexin V binding. SubAB also stimulated cytochrome c release from mitochondria, which was not suppressed by caspase inhibitors. In HeLa cells, Apaf-1 small interfering RNA inhibited caspase-3 activation, suggesting that cytochrome c might form an apoptosome, leading to activation of caspase-3. These data suggested that SubAB induced caspase-dependent apoptosis in Vero cells through mitochondrial membrane damage.

Shiga-toxigenic Escherichia coli (STEC) is an etiologic agent of hemorrhagic colitis. Gastrointestinal disease caused by STEC may progress to systemic complications, including hemolytic uremic syndrome (HUS), which is characterized by thrombocytopenia, microangiopathic hemolytic anemia, and renal failure (13, 23). Shiga toxin 1 (Stx1) and Stx2 are both produced by STEC. HUS (22, 26).

SubAB is cytotoxic to Vero cells. BiP cleavage by the A subunit is necessary for Vero cell death (17, 18, 21, 22). BiP is known as a master regulator of ER function and homeostasis (11). SubAB induces ER stress (17, 27), as shown by activation of double-stranded RNA-activated protein kinase-like ER kinase (PERK) and eukaryotic initiation factor 2α (eIF2α), leading to transient protein synthesis inhibition and stress-inducible C/EBP-homologous protein (CHOP) induction, with cell cycle arrest in G1 phase as a result of downregulation of cyclin D1 (17).

Apoptosis, or programmed cell death, is a physiological event important in a diverse array of biological processes ranging from embryonic development to bacterial infection (7, 31, 33). Morphologically, cells undergoing apoptosis demonstrate nuclear/cytoplasmic condensation and membrane protrusions. Biochemically, apoptotic cells are characterized by reduction in the mitochondrial membrane potential, intracellular acidification, production of reactive oxygen species, externalization of phosphatidylserine residues in membrane bilayers, selective proteolysis of a subset of cellular proteins, and internucleosomal degradation of DNA, resulting in a typical fragmentation pattern (28). There are multiple potential participants described for ER stress-induced apoptosis; however, the precise mechanisms of ER stress-induced apoptosis have not been fully elucidated (29). Recently, SubAB-induced apoptosis was partially described (27). We report here that SubAB triggers apoptosis in Vero cells initiated via mitochondrial membrane damage, followed by activation of a caspase-dependent cell death pathway.

MATERIALS AND METHODS

Cells and reagents. Vero (Vero-C1) cells were cultured at 37°C in a humidified 5% CO2 atmosphere in Eagle’s minimum essential medium (EMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. General caspase inhibitor (Z-VAD-FMK) was obtained subsequentially.
from BD Biosciences Pharmingen, caspase-8 inhibitor (Z-IETD-FMK) from R&D Systems, and caspase-3 inhibitor (Z-DQMD-FMK) from Calbiochem. Antibodies against cytochrome c were obtained from R&D Systems; antibodies against caspase-3, -7, and -8 and cleaved caspase-3 and -7 were from Cell Signaling; and antibodies against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were from Santa Cruz Biotechnology. Apaf-1 small interfering RNA (siRNA) and control siRNA were obtained from Santa Cruz Biotechnology. Apaf-1 antibody was purchased from Assay Designs.

Preparation of SubAB. Recombinant His-tagged SubAB and SubAB(S272A) were purified as previously reported (10).

Apoptosis assays. (i) Detection of apoptotic cells by DNA fragmentation. Cells (2 × 10⁶) were grown overnight and incubated with toxin for the indicated times. After incubation, cells were washed with phosphate-buffered saline (PBS) and lysed with lysis buffer (company supplied), and then DNA was isolated using the Apoptotic DNA Ladder kit (Roche Diagnostics). Isolated DNA was incubated with RNase (2 μg/ml) for 20 min at room temperature, and the amount was determined. DNA samples (2 μg) were loaded onto a 1% agarose gel, separated by electrophoresis, and then stained with ethidium bromide and visualized with UV light.

(ii) Detection of apoptotic cells by TUNEL assay. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using an in situ apoptosis detection kit (Wako Pure Chemical Industries, Osaka, Japan).

(iii) Detection of apoptotic cells by annexin V binding. An annexin V-Fluos staining kit (Roche Applied Science) was used. Briefly, 1 × 10⁶ cells were incubated with SubAB for indicated times, and cells were collected with trypsinization, washed once with PBS, and then incubated with annexin V-Fluos plus propidium iodide (PI) reagent for 15 min at room temperature. After treatment, cells were washed with PBS and cell fluorescence was measured using CellQuest software on a flow cytometer (Becton Dickinson). Fluorescence parameters were gated using unstained and single-stained (annexin V or PI) untreated and treated cells. Apoptosis was expressed as the percentages of annexin V-positive cells in 10,000 cells. Parameters were as follows: FSC voltage, E00; amp gain, 1.49; SSC voltage, 474; FL1 voltage, 474; FL2 voltage, 474; FL1, 59.9%; FL2, 45.4%; FL1.

Western blotting to detect caspases. Cells (5 × 10⁵ cells/well) were cultured overnight in 24-well plates with EMEM containing 10% FBS. Prior to treatment with SubAB, the medium was changed to EMEM containing 1% FBS. Cells were then treated with SubAB (100 ng/ml) for the indicated times. After treatment, cells were lysed with sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris [pH 6.8], 1% SDS, 10% glycerol, 2.5% mercaptoethanol, 0.001% bromophenol blue) and heated at 100°C for 5 min before separation by SDS-polyacrylamide gel electrophoresis. After electrophoresis at room temperature, separated proteins were transferred onto polyvinylidene difluoride membranes at 100 V for 1 h. Membranes were blocked with 5% nonfat milk in TTBS (20 mM Tris [pH 8.0], 137 mM NaCl, 0.1% Tween 20) for 30 min and then incubated with primary antibodies overnight at 4°C. After the membranes were washed three times for 5 min with TTBS, they were incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Bands were visualized using the Las 1000 (Fuji film). To investigate the effect of caspase inhibitors, cells were treated with inhibitors for 30 min prior to treatment with toxin and then incubated for 36 h with SubAB.

Detection of cytochrome c release from mitochondria. To evaluate the cytochrome c release from mitochondria into cytosol, cytosol was fractionated following the method described by Chen et al. (5) with slight modifications. Briefly, cells (1 × 10⁶) were treated with SubAB (100 ng/ml) in EMEM with 1% FBS for the indicated times, collected with a cell scraper, and homogenized for 5 min in buffer (75 mM KCl, 1 mM Na₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, 1 mM EDTA) containing 50 μM dithionitrobenzoic acid and protease inhibitor cocktail (Roche Diagnostics). Following centrifugation at 10,000 × g for 10 min, the supernatant was collected and then stored as cytosolic fractions. Mitochondrial contamination was detected using antibody against Tom20 (Santa Cruz), a mitochondrial marker. Cytochrome c was detected by Western blotting.

Apaf-1 gene silencing in Vero cells. Vero cells (1 × 10⁵ cells) in a 12-well plate were cultured overnight (50 to 60% confluent) and were transfected with control siRNA (38 pmol) or siRNA for Apaf-1 (38 or 50 pmol) in Lipofectamine 2000 transfection reagent (Invitrogen) for 48 h following the company’s instructions. After incubation, cells were washed with PBS and dissolved with SDS sample buffer. Transfection efficiency was evaluated by Western blotting using Apaf-1 antibody.

RESULTS

SubAB induces Vero cell apoptosis. SubAB cleaves BIP and induces ER stress, as shown by activation of PERK and eIF2α, leading to transient protein synthesis inhibition and cell cycle arrest in G₁ phase as a result of downregulation of cyclin D1, resulting in cell death (17, 27). Treatment of Vero cells with SubAB, but not with a catalytically inactive mutant, SubAB(S272A), in which the critical A subunit serine was replaced by alamine, induced membrane blebbing followed by loss of adhesion and retraction from the matrix, leading to detachment (Fig. 1). The appearance was similar to that induced by tunicamycin (TM), an N-glycosylation inhibitor which causes ER stress, leading to apoptotic death in many cells. Stx1 also induces apoptosis (6, 10, 14), although bleb formation was not observed at this time point (Fig. 1). To investigate whether the cell death was due to apoptosis, DNA fragmentation was evaluated (Fig. 2). DNA fragmentation was detected in SubAB-, TM-, or Stx1-treated cells but not in untreated control cells or SubAB(S272A)-treated cells (Fig. 2). A typical DNA ladder was more clearly evident in cells detached from the matrix by treatment with SubAB than in cells still attached to the matrix (data not shown). Similarly, cells treated with SubAB, but not with SubAB(S272A), were TUNEL positive (data not shown). Further, flow cytometric analysis revealed that SubAB increased annexin V binding to Vero cells in a time-dependent manner, consistent with exposure of phosphatidylserine residues in the outer leaflet of the plasma membrane, which is an early event during apoptosis (Fig. 3A and B). In contrast, SubAB(S272A)-treated cells showed only a minor increase in apoptosis at 48 h.

SubAB activates caspase-3, -7, and -8. To determine whether SubAB-induced apoptosis was caspase dependent, we investigated caspase activation, as measured by cleavage of procaspase. Activated caspase-3, -7, and -8 were detected following incubation with SubAB at a concentration of 0.1 ng/ml or higher for 36 h (data not shown). Activated caspases following incubation with SubAB (100 ng/ml) occurred in a time-dependent manner (Fig. 4). Caspase-3 and -7 were detected at...
27 h, while caspase-8 was seen first at 33 h. The difference in the time-dependent appearance of caspase-8 and caspase-3 was significant.

A caspase-3 inhibitor inhibits cleavage of procaspase-8. To confirm the order of caspase activation, we blocked caspase activities with caspase inhibitors and examined the activation of caspase-3, -7, and -8 (Fig. 5). A general caspase inhibitor (Z-VAD-FMK) inhibited cleavage of procaspase-3, -7, and -8. A caspase-3 inhibitor (Z-DQMD-FMK) inhibited not only cleavage of procaspase-3 but also cleavage of procaspase-7 and -8. A caspase-8 inhibitor (Z-IETD-FMK) inhibited cleavage of procaspase-8 but did not have a significant effect on cleavage of procaspase-3. It partially inhibited cleavage of procaspase-7 at 100 μM. These results suggested that caspase-3 was upstream of caspase-8 and -7.

SubAB-induced apoptosis is suppressed by the caspase inhibitor Z-VAD-FMK. We next evaluated whether a caspase inhibitor suppressed SubAB-induced apoptosis in Vero cells. Apoptosis was evaluated by annexin V binding as shown in Fig. 3A. We investigated at 36 h of incubation. The percentage of annexin V-positive early apoptotic cells was suppressed significantly by incubation with Z-VAD-FMK (Fig. 6).

SubAB induces cytochrome c release from mitochondria. To investigate whether SubAB-induced apoptosis was induced via a mitochondrion-dependent pathway, cytochrome c release from mitochondria into the cytosol was investigated. The prepared cytosolic fractions were not contaminated by mitochondria, which was verified using mitochondrial marker, Tom20 (data not shown). Stx1, which is known to induce cytochrome c release (10, 14), was used as a positive control. SubAB induced cytochrome c release in a time-dependent manner (Fig. 7A). Caspase inhibitors did not affect cytochrome c release by SubAB (Fig. 7B), suggesting that changes in mitochondrial membrane permeability might be upstream of caspase activation by SubAB. In the intrinsic pathway, cytochrome c released from mitochondria triggers the formation of an apoptosome composed of Apaf-1, procaspase-9, and cytochrome c, leading to activation of caspase-9 and subsequent activation of caspase-3 (15, 32). We were unable to detect either procaspase-9 or
caspase-9 in Vero cells using human caspase-9 antibody (data not shown). Human anti-caspase-9 antibodies may not react with monkey caspase-9. HeLa cells, derived from human cervical cancer, exhibit almost the same sensitivity as Vero cells to SubAB, and SubAB induced cell cycle arrest at G1 in HeLa cells as well (17). In contrast to the case for Vero cells, cleavage of procaspase-9 into 39- and 37-kDa caspase-9 was observed in SubAB-treated HeLa cells. To confirm that apoptosome formation is necessary for caspase-3 activation, we transfected HeLa cells with siRNA against Apaf-1 (Fig. 8). The cleaved form of procaspase-9, especially the appearance of a 37-kDa band, was suppressed in cells transfected with Apaf-1 compared to cells treated with control siRNA, and similarly, procaspase-3 cleavage was suppressed in cells transfected with Apaf-1 siRNA. In contrast, cytochrome c release was not suppressed. These data suggest that in HeLa cells, SubAB induced caspase-3 activation via cytochrome c release, followed by apoptosome formation and caspase-9 activation.

**DISCUSSION**

There are two well-described caspase-dependent pathways that induce apoptotic cell death. One is the extrinsic pathway, in which binding of death receptors by death ligands is followed by recruitment of adaptor molecules and activation of caspase-8. The other is the intrinsic pathway, in which cytochrome c release from mitochondria triggers the formation of the apoptosome composed of Apaf-1, procaspase-9, and cytochrome c, which results in the activation of caspase-3. SubAB

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**FIG. 4.** Kinetics of SubAB-induced caspase activation. Cells were incubated with SubAB (100 ng/ml) for the indicated times. After incubation, cell were lysed and analyzed by Western blotting with specific anticaspase antibodies. The left panel shows a representative blot from three experiments. After quantification of caspase-3, -7, and -8 by densitometry of the blot, the percentage of caspase was calculated as caspase/(caspase + procaspase) × 100 (right panel). □, caspase-3; △, caspase-7; ○, caspase-8. Data are means ± standard deviations for three samples. *, P < 0.01; **, P < 0.02; and ***, P < 0.03 (versus caspase-8 at each time point).

**FIG. 5.** Effects of caspase inhibitors on cleavage of procaspase-3, -7, and -8. Cells were treated with a specific caspase-3 inhibitor (DQMD), a caspase-8 inhibitor (IETD), or general caspase inhibitor (VAD) (10 or 100 μM) for 30 min before incubation with SubAB (100 ng/ml) for 36 h. After incubation, cell were lysed and analyzed by Western blotting with specific anticaspase antibodies. Quantification of caspase-3, -7, and -8 was performed by densitometry, and percentages of control values were calculated as caspase in the presence of inhibitor/caspase of control cells without inhibitor × 100. Data are means ± standard deviations for three samples. *, P < 0.03 versus control without inhibitor.

**FIG. 6.** Effect of caspase inhibitor on SubAB-induced apoptosis. Cells were treated with a general caspase inhibitor (VAD) (50 or 100 μM) for 30 min before incubation with SubAB (100 ng/ml) for 36 h, and apoptotic cells were analyzed by annexin V and PI staining as shown in Fig. 3A. Early apoptosis was expressed as the percentage of annexin V-positive and PI-negative cells. Data are means ± standard deviations for three samples. White bars, inhibitor alone; gray bars, inhibitor with SubAB. Data are means ± standard deviations for three samples. *, P < 0.02; **, P < 0.05 (versus SubAB without inhibitor).
is bound to cells, internalized by endocytosis, and transported from Golgi apparatus to ER in a retrograde manner. In the ER lumen, it cleaved BiP, leading to ER stress, which was demonstrated by activation of PERK and eIF2α, leading to transient protein synthesis inhibition and stress-inducible CHOP induction (17, 27). A catalytically inactive mutant, SubAB(S272A), did not cleave BiP and did not induce apoptosis. Therefore, SubAB-induced apoptosis is believed to be initiated not by cell receptor recognition but by ER stress resulting from BiP cleavage. The precise mechanisms of how ER stress results in the activation of caspases have not been fully elucidated (25, 29). Murine caspase-12 and human caspase-4, the counterpart of murine caspase-12, are candidates for involvement in the initial events of ER stress-induced apoptosis (12, 20); however, recent reports questioned their participation. Further ER stress-induced apoptosis required mitochondrion-dependent apoptosome formation by a caspase-12-independent mechanism (8, 24). SubAB induced changes in mitochondrial permeability and released cytochrome c in a caspase-independent manner, suggesting that caspase activation by SubAB might be downstream of the changes in mitochondrial membrane permeability. Similar to the case for the intrinsic pathway, in HeLa cells, a decrease in Apaf-1 expression clearly suppressed procaspase-9 and caspase-3 cleavage with no change in cytochrome c release, suggesting that apoptosis formation was necessary to induce caspase-3 activation. Treatment with SubAB might induce a similar pathway for caspase-3 activation in Vero cells. Caspase inhibitors did not suppress cytochrome c release by SubAB, also suggesting that there might be caspase-independent apoptosis, i.e., mitochondrial release of apoptosis-inducing factor (2, 4). Further studies are necessary to define the potential function of apoptosis-inducing factor and other factors.

We found that activation of procaspase-8, known as an initiator caspase, was downstream of caspase-3. We reached this conclusion because caspase-8 appeared later than caspase-3 and caspase-8-specific inhibitors did not suppress procaspase-3 cleavage, while a caspase-3-specific inhibitor suppressed procaspase-8 cleavage. However, this pharmacological approach may be compromised by possible off-target effects of the inhibitors. The use of a specific caspase siRNA may yield more conclusive results. Also, there is still a possibility that caspase-8 activation may partially occur independently of caspase-3. With regard to caspase-8, however, a recent report showed that unlike its proximal role in receptor signaling, in the mitochondrial pathway caspase-8 functions as an amplifying executioner caspase (9). Similar to the results in that report, cytochrome c release by SubAB was not suppressed by caspase inhibitors, and caspase-8 activation was a postmitochondrial event initiated by caspase-3. Therefore, caspase-8 might enhance the apoptotic signal initiated by mitochondria.

The mechanisms of how ER stress by SubAB induces mitochondrial damage have not been identified. Bcl-2 family proteins regulate apoptosis by controlling mitochondrial permeability. CHOP, a transcription factor, involved in ER stress-induced apoptosis that reduces expression of Bcl-2 (16), was activated in SubAB-treated cells. Further, ER stress also leads to increased cytosolic calcium levels, which activated m-calpain and resulted in cleavage of Bcl-XL (19). The study of
Bel-2 family proteins and their modulators may clarify signal transduction following ER stress (1).

Finally, we show a proposed activation pathway of SubAB in Fig. 9. SubAB first binds to cell membranes (step 1), is delivered to ER by endocytosis (step 2), and cleaves BIP (step 3), which is a necessary step in apoptosis. BIP cleavage triggers a mitochondrial membrane permeability change and induces cytochrome c release (step 4). Cytochrome c may form an apoptosome with Apaf-1 and procaspase-9 (step 5) and produce caspase-9 (step 6), caspase-8 (step 7), and caspase-8 (step 8) consecutively.

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