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Proteasome inhibitors prevent cell death and prolong survival of mice challenged by Shiga toxin

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ABSTRACT

Shiga toxin (Stx) causes fatal systemic complications. Stx induces apoptosis, but the mechanism of which is unclear. We report that Stx induced rapid reduction of short-lived anti-apoptotic proteins followed by activation of caspase 9 and the progression of apoptosis. Proteasome inhibitors prevented the reduction of anti-apoptotic proteins, and inhibited caspase activation and apoptosis, suggesting that the reduction of anti-apoptotic proteins is a prerequisite for Stx-induced apoptosis. A clinically approved proteasome inhibitor, bortezomib, prolonged the survival of mice challenged by Stx. These results imply that proteasome inhibition may be a novel approach to prevent the fatal effects of Stx.

1. Introduction

Stx-producing *Escherichia coli* (STEC), including O157, O104 and O111, causes diarrhea and hemorrhagic colitis in the gut. When Stx traverses the epithelium and passes into the circulation system, it occasionally causes systemic complications such as encephalopathy and hemolytic-uremic syndrome sometimes resulting in the death of the infected patients [1–4]. Therefore, the development of an antidote to prevent the lethal effects of Stx is urgently required.

Stx can be classified into two groups, Stx type 1 (Stx1) and type 2 (Stx2) [5,6], with Stx2 linked to severer diseases epidemiologically. The Stx holotoxin is composed of one molecule of the A-subunit that has RNA *N*-glycosidase activity and five molecules of the B-subunit. The pentameric B-subunit facilitates the binding of Stx to the cell surface receptors, globotriaosylceramide (Gal α(1-4)-Galβ(1-4)-Glcβ-ceramide (Gb3)/CD77) [6,7]. After internalization, Stx is transported from the early endosomes to the ER via the Golgi apparatus. Stx then catalyzes the removal of adenine at position 4324 of 28S rRNA, thereby inhibiting protein biosynthesis [8,9].

Several studies indicated that Stx induces apoptosis in some cells, implying that induction of apoptosis is, at least in part, crucial for vascular lesions and tissue damage after translocation of Stx into the circulation system [10]. These studies include Stx inducing rapid apoptotic cell death in several CD77-positive cell lines such as myelogenous leukemia cell line THP1, epithelial cell lines and Burkitt's lymphoma cell lines [11–14] in a mitochondrial pathway-dependent manner. In other studies, however, it was reported that Stx induces apoptosis through ER stress responses, including the activation of IRE1, PERK and ATF6, and an increase in the expression level of CHOP in THP1 cells [15,16]. In these reports, Stx treatment increased the expression of a death ligand, TRAIL and its cell surface receptor, DR5 which mediates activation of caspase 8. Garibal et al. reported that caspase 8-mediated cleavage of Bid is required for Stx1-induced apoptosis in Burkitt's lymphoma cells [17]. Induction of apoptosis by Stx through the activation of caspase 8, 6 and 3, but not the caspase 9-dependent mitochondrial pathway was also reported in HeLa cells [18].

Abbreviations: Stx, Shiga toxin; STEC, Shiga toxin-producing *Escherichia coli*; ER, endoplasmic reticulum; FLIP, FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; c-IAP1, cellular inhibitor of apoptosis protein 1; Mcl-1, myeloid cell leukemia 1; PARP, Poly(ADP-ribose) polymerase; PI, propidium iodide; BRZ, bortezomib; CHX, cycloheximide

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Thus, the cell death mechanism induced by Stx is complicated, and it is unclear whether the inhibition of apoptosis could be beneficial for preventing the fatal effects of Stx.

In this study, we investigated the cell death mechanism in THP1 cells and CD77 synthase transfected U937 cells. We found that the Stx induced apoptosis in CD77-expressing cells in a caspase-dependent manner with caspase 9 as the primary initiator caspase. Upon Stx treatment, apoptosis inhibitory proteins were rapidly downregulated, and proteasome inhibitors prevented the reduction of them and the progression of apoptosis. We also demonstrate that clinically approved proteasome inhibitor, bortezomib [19] suppressed Stx-induced apoptosis and prolonged the survival of mice challenged by a lethal dose of Stx.

2. Materials and methods

2.1. Reagents

RPMI1640, tunicamycin, PI, anti-α-tubulin antibody (B-5-1-2), anti-β-actin antibody (AC-74) and anti-FLAG antibody (M2) were purchased from Sigma. Z-VAD-fmk and MG132 were from the Peptide Institute. Etoposide was obtained from Bristol-Myers Squibb. Bortezomib was from LC Laboratories. The anti-Hsp90 antibody (68), anti-Mcl-1 antibody (22), anti-Bcl-x antibody and anti-Bcl-2 antibody (7) were from BD Biosciences. The anti-PARP antibody (46D11) and anti-β-catenin antibody were from Cell Signaling Technology. The anti-caspase 3 antibody (H-277), anti-CHOP antibody (R-20), anti-GAPDH antibody (FL-335) and anti-caspase 9 antibody (H-17) were from Santa Cruz. The anti-XIAP antibody, anti-caspase 8 antibody (5F7), anti-caspase 9 antibody (5B4) and anti-caspase 10 antibody (4C1) were from Medical & Biological Laboratories. The anti-c-IAP1 antibody was from R&D Systems. The anti-FLIP antibody and anti-Apaf1 antibody were from Enzo. The anti-Apollon antibody [20], recombinant Stx1 and Stx2 [21] and Stx1 A-subunit mutant (E167Q/R170L) [22] were prepared as described previously.

2.2. Cell culture

THP1 cells and U937 cells were purchased from the American Type Culture Collection and cultured in RPMI1640 containing 10% fetal bovine serum and antibiotics. Cells were grown in 5% CO_2 at 37 °C in a humidified atmosphere.

2.3. Measurement of the incorporation of PI

Cells were resuspended in staining buffer (SB, 3% calf serum in PBS (−)) containing 2 μg/ml PI. Fluorescence and phase contrast images were obtained by BIOREVO (Keyence). PI-positive cells were counted with FACSCalibur (BD Biosciences).

2.4. Mammalian expression vector for CD77 synthase and isolation of transformants

Human CD77 synthase cDNA [23,24] was amplified by polymerase chain reaction and inserted into the p3 × FLAG-CMV10 vector (Sigma). U937 cells were pulsed with a Gene Pulser II Electroporation System (BIO-RAD) at 250 V and 950 μF in the presence of 50 μg of the expression vector. After recovery for 48 h, the cells were selected with 1 mg/ml G418.

2.5. Measurement of cell surface CD77

CD77 synthase-transfected U937 cell clones were resuspended in SB containing fluorescein isothiocyanate (FITC)-conjugated anti-CD77 antibody (5B5, BD Biosciences) and incubated on ice for 20 min. The cells were then washed with SB twice and the expression of CD77 was measured with FACSCalibur.

2.6. Pull-down of initially activated caspases with biotinylated Z-VAD-fmk

Initially activated caspase was detected according to the previous report [25]. Briefly, 10^7 cells in 1 ml of culture media were pre-incubated with 50 μM biotinyl-VAD-fmk (MP Biomedicals) for 1 h, and then treated with the indicated apoptosis inducers. Following stimulation, cells were harvested and lysed in KCl lysis buffer (50 mM HEPES (pH 7.4), 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1% NP-40 and protease inhibitors). After centrifugation at >15,000*g* at 4 °C for 15 min, streptavidin-agarose was added to the supernatants and incubated at 4 °C overnight. Then, the agarose was washed four times with the KCl lysis buffer. Precipitated caspases were analyzed by Western blotting.

2.7. Immunoprecipitation (IP) and immunoblotting

After treatment, cell lysates were prepared with IP buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.5% Triton-X 100) supplemented with protease inhibitors and analyzed by immunoprecipitation and immunoblotting as described previously [26].

2.8. Animal experiments

All animal experiments were approved by the animal ethics committee of Doshisha University according to the guidelines for animal experimentation of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Pathogen-free female ICR mice were purchased from Japan SLC. Mice were housed under a 12 h light-dark cycle and fed a standard diet. Mice were injected intravenously with 0.1 ml of sterile saline solution supplemented with mannitol alone or with various doses of bortezomib prior to administration of a lethal dose of Stx2 (0.15 ng/g of body weight) as described in the legend to Fig. 6, and monitored at the indicated times.

2.9. Statistical analysis

For analysis of cell viability, PI positive cells were analyzed by unpaired two tailed Student's *t*-test.

3. Results

3.1. Stx induces apoptosis in a caspase-dependent manner

To gain insights into the mechanism of Stx-induced apoptosis, we first searched for cell lines that undergo rapid apoptosis upon Stx treatment. Among the 25 cell lines tested, THP1 showed the highest sensitivity to Stx-induced apoptosis (Table 1). Fig. 1A shows that Stx induced apoptosis-like morphological changes in THP1 cells as reported previously [27]. Cell death was confirmed by PI-staining and approximately 80% of the treated cells were PI-positive after 24 h. Both Stx1 and Stx2 induced caspase 3 activation and Poly(ADP-ribose) polymerase (PARP) cleavage 4 h after treatment (Fig. 1B). In our experiments, Stx did not induce the expression of CHOP, a marker of ER stress response [28] within 8 h following Stx treatment, whereas the glycosylation inhibitor tunicamycin induced CHOP expression 6 h after treatment, indicating that the ER stress response is not involved in the Stx-induced apoptosis under this condition. Stx1 (Fig. 1C) and Stx2 (Fig. 1D) induced caspase activation in a dose-dependent manner. The