Study on induction of apoptosis on HeLa and Vero cells by recombinant shiga toxin and its subunits

Saeid Bouzari · Mana Oloomi ·
Kayhan Azadmanesh

Received: 13 April 2009 / Accepted: 22 July 2009 / Published online: 9 August 2009
© Springer Science+Business Media B.V. 2009

Abstract Verotoxin (VT) or shiga toxin (Stx) produced by enterohemorrhagic *Escherichia coli* (EHEC) and *Shigella dysenteriae* is AB5 holotoxin with potent protein synthesis inhibitor. VT can induce both apoptosis and necrosis depending on the cell type, it has been shown that VT-induced apoptosis and cytotoxicity are distinct processes, and the A subunit can be necessary for apoptosis. In other words, the precise role of each subunit in apoptosis signaling has yet to be established. In this study, induction of apoptosis has been examined by using both recombinant A and B subunits, and recombinant Stx (rStx) with different doses in HeLa and Vero cells. For this purpose, the polymyxin B extract of constructs expressing A, B and AB5 recombinant proteins was used. Therefore, amounts greater than normally reported were used to induce desired effects on cell lines. The apoptotic effect of A and B subunits appear at higher doses than that of rStx. The highest apoptotic effect was observed for rStx at low concentration, compared to A and B subunits. A or B subunits separately cannot induce the signaling pathway stimulated by holotoxin though A subunit, does induce laddering pattern similar to holotoxin. We concluded that both subunits are important in complete death signaling pathway. Since different

concentration of A and B subunits and rStx was required in different assay, therefore, it could be emphasized that cell death or even apoptosis caused by either of the subunits or holotoxin depends on sensitivity or specificity of the assay and cell types used.

Keywords Apoptosis · Recombinant shiga toxin · Subunits · Cell lines

Abbreviations

AO/EB	Acridine orange/ethidium bromide staining
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
Gb3/CD77	Globotriaosylceramide
HUS	Hemolytic uremic syndrome
rStx	Recombinant Stx
Stx	Shiga toxin
StxB	B subunit of shiga toxin
VT	Verotoxin

Introduction

Shigatoxin (Stx), produced by enterohemorrhagic *Escherichia coli* (EHEC) is associated with hemolytic uremic syndrome (HUS) (Karmali et al. 1985). Stx consists of a single A subunit and a pentamer of B subunits (Jacewicz et al. 1986; Lingwood et al. 1998;

S. Bouzari · M. Oloomi (✉) · K. Azadmanesh
Molecular Biology Unit, Pasteur Institute of Iran, Pasteur Ave., 13164 Tehran, Iran
e-mail: manaoloomi@yahoo.com

Arab and Lingwood 1998; Endo et al. 1988). The B subunit of Stx (StxB) is responsible for the attachment of the holotoxin to the cell surface by binding to the functional receptor globotriaosylceramide (Gb3/CD77) (Jacewicz et al. 1986). The A subunit of Stx has N-glycosidase activity and cleaves a single adenine residue located in a prominent loop structure of the 28S ribosomal RNA of eukaryotic ribosome, which creates inhibition of protein synthesis and death of the cells resulting in cell cytotoxicity (Endo et al. 1988). In addition to having a direct effect on protein synthesis, protein toxins can also induce DNA cleavage and cause apoptosis-like changes in cells (Jones et al. 2000; Suzuki et al. 2000). Accumulated evidence suggests that Stx also induces apoptosis in several cell lines including Vero (Inward et al. 1995), Burkitt's lymphoma (Mangeney et al. 1993), ACHN; human renal tubular epithelium-derived cell (Taguchi et al. 1998) and astrocytoma (Arab et al. 1998), although the precise mechanisms of Stx-induced apoptotic signal pathways remain unclear (Nicholson 1999). Moreover, cell type specificity for the apoptotic signaling pathway has been demonstrated (Jones et al. 2000).

The rate of Stx uptake is dependent on the toxin concentration in several cell lines, and the increased rate observed at a higher concentration is strictly dependent on the presence of the A subunit of cell surface bound toxin (Torgersen et al. 2005). Thus, the presence of A subunit of the shiga holotoxin seems to be crucial for the uptake. Furthermore, both toxin subunits (A and/or B) might induce signaling that could mediate toxin internalization, and this toxin-induced signaling might differ depending on whether the intact toxin or the B subunit binds to Gb3. However, these signaling pathways are largely unknown (Torgersen et al. 2005).

In holotoxin, the B subunit pentamer was suggested to more likely induce apoptosis (Nakagawa et al. 1999). This has been shown in Burkitt's lymphoma cells by the B pentamer from Stxs. The Stx B subunit alone also does not induce apoptosis in human renal epithelial cells (Williams et al. 1997), or in monocytic THP-1 cells (Kojio et al. 2000). Conversely, it has been shown that apoptosis is involved in the Stx-mediated cytotoxicity of the renal tubular and lung epithelium cell lines (Uchida et al. 1999). In this case, apoptosis was maintained at Stx concentrations, wherein protein synthesis inhibition was prevented; therefore, Stx-induced apoptosis and

cytotoxicity are two distinct processes (Gordon et al. 2000; Williams et al. 1999).

In different cell types, toxins may signal apoptosis via different mechanisms (Cherla et al. 2003). Therefore, there are highly Stx-sensitive cells with a variant receptor fatty acid, which plays a key role in the kind of intracellular trafficking and sorting of the toxin to the cytosol (O'Loughlin and Robins-Browne 2001). The different pathways of internalization can stimulate different signaling pathways ultimately resulting in cytotoxicity or apoptosis, yet the real role of each subunit in apoptosis induction is not known. The purpose of the present study was to examine the apoptotic effect of the polymyxin B extract of the recombinant constructs expressing Stx (rStx) compared to cloned recombinant A and B subunits.

Materials and methods

Holotoxin and its subunits preparation

Stx as holotoxin and its subunits were obtained from already cloned genes in pBAD expression vector (Oloomi et al. 2006). The obtained crude toxins were applied to Endotrap (profos AG) (endotoxin removal systems to remove LPS content). The samples were sterilized by filtering through low protein binding filter (Millex®-GV 0.22 µm filter unit, Millipore), and the protein concentration was estimated using Bradford method by absorbance at 280 nm with bovine serum albumin as the control (Protein assay Kit, Bio-Rad).

Cell culture

Vero and HeLa cells (National Cell Bank, Pasteur Institute of Iran) were grown in RPMI 1640 medium (Biosera), pH 7.4, supplemented with 5% heat-inactivated fetal bovine serum, (FBS) 1% (w/v) penicillin, and 1% (w/v) streptomycin at 37 °C in a 5% CO₂ atmosphere.

Cytotoxicity assay

Vero and HeLa cells with a concentration of 15×10^4 cell/mL were plated on 96-well cluster

plates and incubated to grow in complete RPMI 1640 medium. After 24 h, different concentrations of Stx and its subunits were added to the prepared medium, and cells were incubated for an additional 16 h. Then, the viability of cells was assessed using neutral red assay.

Neutral red cytotoxicity assay

The neutral red cytotoxicity assay was adopted by plating cells in 96-well plates and grown to become a confluence in complete RPMI (Biosera) medium. The cells were then washed in PBS and exposed to rStx and its subunits for 16 h. Two hundred microliters of freshly diluted neutral red in PBS were added to a final concentration of 50 µg/mL, and cells were incubated for an additional 3 h at 37 °C in 5% CO₂ incubator. Cells were then washed with 200 µL 1% CaCl₂ and 1% formaldehyde and solubilized in 200 µL 1% acetic acid in 50% ethanol. Adsorption in each well was read in a spectrophotometer at 540 nm (Awareness Technology, INC.). Results were expressed as neutral red uptake percent, and 100% represents cells incubated under identical conditions but without toxin treatment.

Flow cytometry

HeLa and Vero cells (1×10^6 cells per well) were plated in six-well plates. Cells were stimulated with medium alone, rStx1 (0.5–1 µg/mL) and Stx A and B subunits (4 µg/mL) for 16 h. Then cells were stained with Annexin V and propidium iodide (PI) using the Phosphatidyl Serine Detection™ kit to measure apoptosis (IQ Products, Groningen, Netherlands) according to the manufacturer's protocol. Briefly, cells were incubated with different concentrations of either rStx or its subunits for 16 h. Prior to staining, adherent cells were treated with trypsin and EDTA (BioSera), and cells were collected by centrifugation at $750 \times g$ for 5 min and were then washed with calcium buffer. Annexin V were added and incubated for 20 min. After washing with calcium buffer, PI was added and fluorescence was detected using a fluorescence-activated cell sorter (FACS Partec GmbH). Fluorescence parameters were gated using unstained and single-stained untreated control cells, and 10,000 cells were counted for each treatment.

Acridine orange/ethidium bromide staining

Toxin-induced apoptosis was also assessed using acridine orange/ethidium bromide staining (AO/EB). Cells at the concentration of 1×10^5 cell/mL were plated on six-well cluster plates and incubated to grow in complete RPMI medium. After 24 h, Stx and its subunits with twice the concentration were added to prepared medium, and cells were incubated for an additional 16 h. For staining of treated cells, media were moved to centrifuge tubes. To detach the adherent layer, trypsin/EDTA solution was added to each well for several min at 37 °C. The detached cells were then transferred to the same tubes containing the initial media. Collected samples were washed, plated, and resuspended in the residual fluid. 25 µL of each sample were mixed with 2 µL of AO/EB solution (100 µg/mL), and cells were analyzed using a fluorescence microscope with 470 nm filter. For each sample, at least 1×10^5 cells were counted in different fields, and the percentage of apoptotic cells was calculated.

Hoechst staining

Cells were treated as described for acridine orange/ethidium bromide staining. The cells were then washed, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 for 20 min at 25 °C. The percentage of apoptotic cells was enumerated by fluorescence microscopic examination of the nuclear morphology of Hoechst 33342-stained cells (5 µg/mL of Hoechst dye).

DNA fragmentation assay

Fragmentation of the cellular DNA into low-molecular weight oligomers is characteristic of apoptosis. Cells grown to become a confluence were incubated with different concentrations of both rStx-1 and its subunits for 16 h. The cells were then washed with PBS and lysed with ice-chilled TE buffer (10 mM Tris, pH 7.4; 10 mM EDTA) containing NaCl 5 M and 0.6% NaCl. After 30 min of incubation in lysis buffer, the samples were centrifuged at $13,000 \times g$ for 20 min. Fragmented DNA remaining in the resulting supernatant solutions was treated with 2 µg of RNase (Gibco) per mL for 1 h at 37 °C and 0.2 mg of proteinase K (Gibco), and then adding 10 vol. of 95% ethanol and 1 vol. of 3 M sodium acetate. After overnight incubation, the

precipitated DNA was collected by centrifugation at $13,000\times g$ for 15 min. The DNA fragments were washed with 70% ethanol, resuspended in 20 μL of TE buffer and electrophoresed on a 1.2% agarose gel electrophoresis. DNA was visualized under ultraviolet light with ethidium bromide that was added to the gel.

Statistical analysis

All data are presented as the means $\pm$ the standard errors. Statistical comparison of the data among multiple groups was carried out by using one-way analysis of variance. Student's *t*-test was used if only two groups were compared. The level of statistical significance was defined as a *P* of <0.05 . (The two-tailed unpaired Student's *t*-test was used).

Results

Cell viability

Cell viability was measured after treatment of cell lines with rStx, A and B subunits after 16 h. Percentage of cell viability was measured for rStx, and it was

shown that with 1 $\mu\text{g/mL}$ and lower amounts of A and B subunits, cell viability does not vary, whereas using a higher concentration reduces cell viability. The percentage of cell viability with a different concentration of A and B subunits was compared with rStx (Fig. 1). Reduction of cell viability was statistically significant ($P < 0.05$) by rStx at a lower concentration (1 $\mu\text{g/mL}$). Although in similar concentration, the cell death for the A and B subunits was not significant. However, much higher concentration of A and B subunits (20 $\mu\text{g/mL}$) resulted in significant cell reduction ($P < 0.05$). On the other hand, comparing HeLa cell with Vero cells, the former was found to be more sensitive to cell death by rStx compared to vero cells.

Apoptosis activity

To determine the apoptotic effect of rStx and its subunits on Vero and HeLa cells, overnight (O/N) culture of cells was incubated with rStx and A and B subunits. The cells were detached by trypsin and washed with phosphate-buffered saline (PBS). Cells were treated with different concentrations of rStx and A and B subunits from 1 to 20 $\mu\text{g/mL}$. The collected cells were used for detection of apoptosis.

Fig. 1 Cytotoxicity assay: after 16 h of A and B subunit and rStx (AB) treatment, percentage of cell viability was measured in HeLa cells. Regarding AB holotoxin, cell viability was reduced significantly by increasing the concentration of holotoxin. For A and B subunits, cell viability reduction was

observed in higher concentration, i.e., 20 $\mu\text{g/mL}$ of B subunit is two times more cytotoxic than A subunit. Data shown are $\pm$ standard errors of the means from three independent experiments. 100% represent values of cells with medium (control)

The percentage of apoptotic cells were assessed by Hoechst, acridine orange/ethidium bromide staining, Flow cytometry and DNA fragmentation assay.

In flow cytometry, depending on the cell line used, percentage of apoptosis was different as it was shown in cell viability. In this experiment, HeLa cell was used and the percentage of apoptosis and necrosis was measured. Highest percentage of apoptosis was observed for B subunit (6.66%) with two times higher concentration (10 $\mu\text{g/mL}$), followed by 4.26% for rStx, and lowest percentage of apoptosis (2.78%) was observed for A subunit (Fig. 2).

On the other hand, the percentage of apoptotic cells was determined by counting 500 cells in a multiple randomly selected fields with light microscopy. First, cells were treated with 1 $\mu\text{g/mL}$ of rStx and 2 $\mu\text{g/mL}$ of A and B subunit, the concentrations at which most of the cells (80%) were alive. For rStx, A and B subunits, 12, 10 and 7%, respectively, of apoptotic cells were observed by Hoechst staining.

The concentration of A and B subunits (2 $\mu\text{g/mL}$) was twice higher than rStx (1 $\mu\text{g/mL}$) in this experiment (Fig. 3). Apoptotic cells by Hoechst staining in each group of A and B subunits and rStx are shown in Fig. 4.

With acridine orange/ethidium bromide staining, the nucleus of a live, normal cell stained bright green in a heterogeneous pattern, while apoptotic chromatin stained bright green in a homogeneous ‘beadlike’ pattern. Cells whose membranes were damaged (dead cells) were orange, but their pattern of nuclear staining was very similar to their live counterparts (Dwyer-Nield et al. 1998). In this experiment, with lower necrotic cell in the B subunit group, apoptotic percentage was higher though in another experiment with more live cells, percentage of apoptotic cells was similar in both A and B subunit groups (5, 7%, respectively) using HeLa cells. With Vero cells, a lower percentage of apoptosis (2%) was observed for both subunits (data not shown).

Fig. 2 Flowcytometry: cells were stained with Annexin V and propidium iodide (PI) using the Phosphatidyl Serine Detection™ kit to measure apoptosis. **a** Holotoxin AB, **b** B subunit, **c** A subunit, **d** normal cell line as controls were used in this experiment. Concentration of A and B subunits was two times higher than rStx. Percentage of apoptosis was 6.66% for B subunit, 4.26% for holotoxin and 2.78% for A subunit

Fig. 3 Percentage of apoptotic cells: after 16 h of *A* and *B* subunit and rStx (*AB*) treatment, percentage was calculated in HeLa cells. For the treatment of cells with rStx, *A* and *B* subunit, 12, 10 and 7% of apoptotic cells were observed. Twice the concentration of *A* and *B* subunits was used in this experiment. Negative controls are cells without any treatment and cells treated with preplasmic extraction of expression vector (pBAD) alone. The means $\pm$ standard errors of the means from four independent experiments are shown

Double concentrations of *A* and *B* subunits (12–15 $\mu\text{g/mL}$) than rStx (6 $\mu\text{g/mL}$) were also used in DNA fragmentation assay. DNA laddering observed for *A* subunit was similar to rStx, while no such laddering was shown for *B* subunit (Fig. 5a).

Fig. 4 Hoechst staining: induction of apoptosis in HeLa cells by rStx (*AB*), *A* and *B* subunits after 16 h incubation. Cells were incubated in RPMI 1640 medium with 5% FBS for 24 h. After washing, they were fixed with 4% paraformaldehyde and stained with Hoechst 33342 (5 $\mu\text{g/mL}$) for 20 min at 25 °C. The slides were then examined by fluorescence microscopy and photographed (green background is Hoechst staining and right, yellow background is the same panel with light microscopy). (Color figure online)

The DNA laddering formation in HeLa cells was more profound than that observed for Vero cells (Fig. 5b).

Discussion

Shigatoxin is the main factor responsible for the pathogenesis of *Shigella dysenteriae* type 1 and enterohemorrhagic *Escherichia coli* (Stx-producing *E. coli*). This toxin belongs to a large family of bacterial toxins, which consists of two distinct subunits. The main known mechanism for the effect of Stx on different cells is inhibition of protein synthesis, which involves *A* subunit activity. Stx also contains five *B* subunits that carry the binding property of holotoxin to cell surface receptors, such as Gb3 and globotetraosyl ceramide (Gb4). It has been shown that Stx can cause apoptosis in Daudi cells (Burkitt lymphoma cell line) by a pathway independent of its effect on protein synthesis (Mangeney et al. 1993). It has been shown that the *B* subunit alone is enough to induce apoptosis, suggesting that the glycolipid receptor is the major mediator of this effect (O'Loughlin and Robins-Browne 2001). Furthermore, it was observed that

Fig. 5 DNA laddering: apoptotic activity was detected by agarose gel electrophoretic separation of DNA fragmentation after exposure to rStx, A and B subunits. Concentration of A and B subunits was two times higher than rStx. DNA was isolated and analyzed by ethidium bromide/agarose gel electrophoresis. DNA fragmentation in a dose-dependent manner was shown after 16 h treatment with rStx (AB), A and B subunits. **a** DNA laddering in HeLa cell. **b** DNA laddering in Vero cell

StxB generated intracellularly caused apoptosis (Fujii et al. 2003). It has also been shown that apoptosis or necrosis was induced in HeLa cells by internal expression of the StxB or A subunit, respectively, following transfection of the cells with the individual genes (Nakagawa et al. 1999). In our experiment, apoptosis was demonstrated by rStx morphologically. Through DNA fragmentation analysis and flowcytometry, apoptosis was also detected, which can proceed to necrosis. At a higher concentration, A subunit can induce apoptosis as shown in the DNA fragmentation experiment. Higher concentration of B subunit proceeds cells to more necrosis, while it has

been known that necrosis and apoptosis represent morphologic expressions of a shared biochemical network. The final form of a cell's death (apoptosis or necrosis) is highly dependent on its physiologic context (kind of signal) at the time when the death signal is received. Therefore, exact discrimination between apoptosis and necrosis is not very clear. In this regard, it has been known that the signaling cascade culminating in typical apoptosis is affected by a host of homeostatic mechanisms that in mice lacking individual apoptotic proteins results in a spectrum of necrotic morphologies (Zeiss 2003). It has also been demonstrated that purified StxB subunit triggered HEp-2 cell apoptosis, although higher doses were necessary compared to the holotoxins. In this experiment, it has also been shown that StxB subunit did not induce HeLa cell apoptosis, and retrograde transport of the holotoxin to the ER appeared to be essential for apoptosis induction. It should be noted that if apoptosis induction requires retrograde transport, the precise points in the trafficking process initiating apoptotic signals are currently unknown. In our study, B subunit causing DNA laddering was lower than A subunit. Similarly in this experiment, studying HeLa cell has suggested that toxin enzymatic activity is necessary to induce apoptosis through a mechanism completely independent of caspase activation (Cherla et al. 2003).

B subunit of Stx is supposed to be a convenient non-cytotoxic model (Arab and Lingwood 1998) of holotoxin internalization, and Stx A subunit lacks known trafficking motifs (Jackson et al. 1999); the A subunit might also modify holotoxin transport via unknown motifs. Nevertheless, it has been known that holotoxin initial internalization will be A subunit-independent, and two pathways have been identified as applicable to holotoxin endocytosis (Khine et al. 2004). Moreover, two distinct Gb3/CD77 signaling pathways leading to apoptosis have been triggered by Anti-Gb3/CD77 mAb and Stx (Tétaud et al. 2003). Therefore, different intracellular routing may play a pivotal role in cell susceptibility and triggering a different pathway for apoptosis. Stx-induced apoptosis and cytotoxicity are distinct processes (Gordon et al. 2000; Williams et al. 1999), although the A subunit can be necessary for apoptosis (Khine et al. 2004). In our study, the same result was also shown, as if A subunit can separately induce apoptosis morphologically in higher concentration by Hoechst staining and DNA laddering method. Just as

Torgersen et al. 2005 has shown, both toxin subunits (A and/or B) might induce signaling that could mediate toxin internalization, and this toxin-induced signaling might differ depending on whether the intact toxin or the B subunit binds to Gb3. Although B subunit induced apoptosis, the necrosis event was also evident; however, in the case of A subunit, more of apoptotic cells were observed than necrotic cells.

In contrast, the result of apoptosis induction by rStx is necrosis, which can be stimulated by A subunit or B subunit via different pathways. Induction of apoptosis in different cell types has been shown through different signaling mechanisms (Lee et al. 2005). On the other hand, different concentrations of toxin can induce different pathways of internalization depending on the concentration of surface Gb3 on each cell. However, in this study different applied techniques have been used that can detect variant process of apoptosis in different stages. The variability of detection can be the result of assay dependency. Collectively in our experiment, it was also shown that cell viability and induction of apoptosis in HeLa cells are much higher than Vero cells. Since the same trend was observed for Vero cells, the same data was not shown. In our study, different concentrations of A and B subunits or rStx was required to induce cell death or apoptosis, depending on assay employed or kind of cell type used. Therefore, it seems the discrepancy in the results is dependent on kind of assay or cell type employed for cell death or apoptosis purposes. Moreover, it has been shown that A subunit of rStx is one of the major parts in stimulation of apoptosis. In addition, A and B subunits of rStx are major parts of holotoxin though none of the two subunits alone can induce a complete form of death signaling pathway.

Acknowledgments This work was supported financially by a research grant No. 371 Pasteur Institute of Iran.

References

- Arab S, Lingwood CA (1998) Intracellular targeting of the endoplasmic reticulum/nuclear envelope by retrograde transport may determine cell hypersensitivity to verotoxin via globotriaosyl ceramide fatty acid isoform traffic. *J Cell Physiol* 177:646–660
- Arab S, Murakami M, Dirks P, Boyd B, Hubbard SL, Lingwood CA, Rutka JT (1998) Verotoxins inhibit the growth of and induce apoptosis in human astrocytoma cells. *J Neurooncol* 40:137–150
- Cherla RP, Lee SY, Tesh VL (2003) Shiga toxins and apoptosis. *FEMS Microbiol Lett* 228:159–166
- Dwyer-Nield LD, Thompson JA, Peljak G, Squier MK, Barker TD, Parkinson A, Cohen JJ, Dinsdale D, Malkinson AM (1998) Selective induction of apoptosis in mouse and human lung epithelial cell lines by the tert-butyl hydroxylated metabolite of butylated hydroxytoluene: a proposed role in tumor promotion. *Toxicology* 130:115–127
- Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K (1988) Site of action of a vero toxin (VT2) from *Escherichia coli* O157:H7 and of shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur J Biochem* 171:45–50
- Fujii J, Matsui T, Heatherly DP, Schlegel KH, Lobo PI, Yutsudo T, Ciralo GM, Morris RE, Obrigg T (2003) Rapid apoptosis induced by shiga toxin in HeLa cells. *Infect Immun* 71:2724–2735
- Gordon J, Challa A, Levens JM, Gregory CD, Williams JM, Armitage RJ, Cook JP, Roberts LM, Lord JM (2000) CD40 ligand, Bcl-2, and Bcl-xL spare group I Burkitt lymphoma cells from CD77-directed killing via verotoxin-1 B chain but fail to protect against the holotoxin. *Cell Death Differ* 7:785–794
- Inward CD, Williams J, Chant I, Crocker J, Milford DV, Rose PE, Taylor CM (1995) Verocytotoxin-1 induces apoptosis in vero cells. *J Infect* 30:213–218
- Jacewicz M, Clausen H, Nudelman E, Donohue-Rolfe A, Keusch GT (1986) Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J Exp Med* 163:1391–1404
- Jackson ME, Simpson JC, Girod A, Pepperkok R, Roberts LM, Lord JM (1999) The KDEL retrieval system is exploited by *Pseudomonas* exotoxin A, but not by shiga-like toxin-1, during retrograde transport from the Golgi complex to the endoplasmic reticulum. *J Cell Sci* 112:467–475
- Jones NL, Islur A, Haq R, Mascarenhas M, Karmali MA, Perdue MH, Zanke BW, Sherman PM (2000) *Escherichia coli* shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. *Am J Physiol Gastrointest Liver Physiol* 278:G811–G819
- Karmali MA, Petric M, Lim C, Fleming PC, Arbus GS, Lior H (1985) The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. *J Infect Dis* 151:775–782
- Khine AA, Tam P, Nutikka A, Lingwood CA (2004) Brefeldin A and filipin distinguish two globotriaosyl ceramide/verotoxin-1 intracellular trafficking pathways involved in vero cell cytotoxicity. *Glycobiology* 14:701–712
- Kojio S, Zhang H, Ohmura M, Gondaira F, Kobayashi N, Yamamoto T (2000) Caspase-3 activation and apoptosis induction coupled with the retrograde transport of shiga toxin: inhibition by brefeldin A. *FEMS Immunol Med Microbiol* 29:275–281
- Lee SY, Cherla RP, Caliskan I, Tesh VL (2005) Shiga toxin 1 induces apoptosis in the human myelogenous leukemia cell line THP-1 by a caspase-8-dependent, tumor necrosis factor receptor-independent mechanism. *Infect Immun* 73:5115–5126
- Lingwood CA, Khine AA, Arab S (1998) Globotriaosyl ceramide (Gb3) expression in human tumour cells:

- intracellular trafficking defines a new retrograde transport pathway from the cell surface to the nucleus, which correlates with sensitivity to verotoxin. *Acta Biochim Pol* 45:351–359
- Mangeney M, Lingwood CA, Taga S, Caillou B, Tursz T, Wiels J (1993) Apoptosis induced in Burkitt's lymphoma cells via Gb3/CD77, a glycolipid antigen. *Cancer Res* 53:5314–5319
- Nakagawa I, Nakata M, Kawabata S, Hamada S (1999) Regulated expression of the shiga toxin B gene induces apoptosis in mammalian fibroblastic cells. *Mol Microbiol* 33:1190–1199
- Nicholson DW (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 6:1028–1042
- O'Loughlin EV, Robins-Browne RM (2001) Effect of shiga toxin and shiga-like toxins on eukaryotic cells. *Microbes Infect* 3:493–507
- Oloomi M, Bouzari S, Arshadi M (2006) N-terminus leader sequence of shiga toxin (Stx) 1 is essential for production of active recombinant protein in *E. coli*. *Protein Pept Lett* 13:509–512
- Suzuki A, Doi H, Matsuzawa F, Aikawa S, Takiguchi K, Kawano H, Ohno S (2000) Bcl-2 antiapoptotic protein mediates verotoxin II-induced cell death: possible association between bcl-2 and tissue failure by *E. coli* O157:H7. *Genes Dev* 15:1734–1740
- Taguchi T, Uchida H, Kiyokawa N, Mori T, Sato N, Horie H, Takeda T, Fujimoto J (1998) Verotoxins induce apoptosis in human renal tubular epithelium derived cells. *Kidney Int* 53:1681–1688
- Tétaud C, Falguières T, Carlier K, Lécluse Y, Garibal J, Coulaud D, Busson P, Steffensen R, Clausen H, Johannes L, Wiels J (2003) Two distinct Gb3/CD77 signaling pathways leading to apoptosis are triggered by anti-Gb3/CD77 mAb and verotoxin-1. *J Biol Chem* 278:45200–45208
- Torgersen ML, Lauvrak SU, Sandvig K (2005) The A-subunit of surface-bound shiga toxin stimulates clathrin-dependent uptake of the toxin. *FEBS J* 272:4103–4113
- Uchida H, Kiyokawa N, Taguchi T, Horie H, Fujimoto J, Takeda T (1999) Shiga toxins induce apoptosis in pulmonary epithelium-derived cells. *J Infect Dis* 180:1902–1911
- Williams JM, Lea N, Lord JM, Roberts LM, Milford DV, Taylor CM (1997) Comparison of ribosome-inactivating proteins in the induction of apoptosis. *Toxicol Lett* 91:121–127
- Williams JM, Boyd B, Nutikka A, Lingwood CA, Barnett Foster DE, Milford DV, Taylor CM (1999) A comparison of the effects of verocytotoxin-1 on primary human renal cell cultures. *Toxicol Lett* 105:47–57
- Zeiss CJ (2003) The apoptosis-necrosis continuum: insights from genetically altered mice. *Vet Pathol* 40:481–495