Shiga Toxin 2 Causes Apoptosis in Human Brain Microvascular Endothelial Cells via C/EBP Homologous Protein[▽]

Jun Fujii,^{1*} Katie Wood,² Fumiko Matsuda,¹ Benedito A. Carneiro-Filho,³ Keilo H. Schlegel,² Takashi Yutsudo,⁴ Beth Binnington-Boyd,⁵ Clifford A. Lingwood,⁵ Fumiko Obata,² Kwang S. Kim,⁶ Shin-ichi Yoshida,¹ and Tom Obrig²

Department of Bacteriology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan¹; Department of Internal Medicine/Nephrology² and Center for Global Health,³ Department of Medicine, University of Virginia, Charlottesville, Virginia 22908; Discovery Research Laboratory, Shionogi & Co., Ltd., 2-5-1, Mishima Settsu City, Osaka 566-0022, Japan⁴; Molecular Structure and Function Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada⁵; and Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287⁶

Received 30 November 2007/Returned for modification 8 January 2008/Accepted 28 May 2008

Shiga toxin 1 (Stx1) and Stx2 produced by *Escherichia coli* O157 are known to be cytotoxic to Vero and HeLa cells by inhibiting protein synthesis and by inducing apoptosis. In the present study, we have demonstrated that 10 ng/ml Stx2 induced DNA fragmentation in human brain microvascular endothelial cells (HBMEC), with cleavage activation of caspase-3, -6, -8, and -9. A microarray approach used to search for apoptotic potential signals in response to Stx2 revealed that Stx2 treatment induced a marked upregulation of C/EBP homologous protein (CHOP)/growth arrest and DNA damage-inducible protein 153 (GADD153). Increased CHOP expression was dependent on enzymatically active Stx1. Knockdown of CHOP mRNA reduced the activation of caspase-3 and prevented apoptotic cell death. These results suggest that Stx2-induced apoptosis is mediated by CHOP in HBMEC and involves activation of both the intrinsic and extrinsic pathways of apoptosis.

In 1996, outbreaks of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infection occurred in Japan. In Sakai City, Japan, approximately 8,000 people were diagnosed with STEC infection and many developed hemolytic-uremic syndrome and central nervous system (CNS) complications (12, 28). CNS dysfunction is an important predictive factor for hemolytic-uremic syndrome and mortality in children (37, 38, 43). In 1994, we established a mouse model of STEC-induced CNS disorder by oral infection of Stx2c-producing *E. coli*. The model showed that STEC was associated with damage to capillary endothelial cells and nerve fibers of the brain cortex and spinal cord (10). We also reported that Stx2-injected rabbits developed brain edema, which indicated deterioration of the blood-brain barrier and the dysfunction of the brain microvascular endothelial cells (9).

The Stx family of toxins, including Stx1 and Stx2, comprise 1A and 5B subunit proteins (32). The A subunit has an N-glycosidase activity that removes adenine 4324 of 28S RNA of the 60S ribosomal subunit (6), rendering ribosomes inactive for protein synthesis (33). Each B subunit binds (with high affinity) to the glycosphingolipid globotriaosyl ceramide Gb₃ (CD77), which is present in specific host/mammalian cells (21, 22). CD77 antigen is also a marker for B-cell Burkitt's lymphoma (31, 44). Stx-mediated apoptosis in Burkitt's lymphoma may utilize the extrinsic pathway of Fas- or tumor necrosis factor alpha (TNF- α)-mediated apoptosis. However, there is no di-

rect evidence of the association of Gb₃ with the death domain. Nonetheless, Stx-induced apoptosis has been reported in cell types such as Vero cells (14), human renal proximal tubular epithelial cells (17, 18), human renal cortical epithelial cells (16), lung epithelial cells (42), and astrocytoma cells (1). It has been well demonstrated that Gb₃ was upregulated with TNF- α and interleukin-1 β (IL-1 β) (24).

Caspase activation is important in the process of apoptosis. Caspases are present as inactive proenzymes, most of which are activated by proteolytic cleavage at specific aspartic acid sites. Representing the extrinsic pathway of apoptosis, caspase-8 is activated in response to extracellular apoptosis-inducing ligands in a complex of the receptors and cytoplasmic death domain proteins (2). In the intrinsic pathway of apoptosis, caspase-9 is activated in response to the release of cytochrome *c* from the mitochondria (23) and caspase-9 is activated when complexed with extramitochondrial cytochrome *c*, dATP, and an apoptotic protease-activating factor (Apaf-1) (20). It is known that apoptosis in P3HR1 cells induced by the anti-CD77 monoclonal antibody is caspase independent and involves only the intrinsic pathway (41). It was also reported that Stx1- and Stx2-mediated apoptosis in Hep-2 cells is associated with the intrinsic pathway (15). We reported that Stx1 induction of apoptosis in HeLa cells requires caspase-6, -8, and -9 within 4 h, followed by activation of caspase-3, which leads to fragmentation of nuclear DNA (11). However, the mechanism of initiation of the apoptotic pathway induced by Stxs is still unknown.

The endoplasmic reticulum (ER) stress response is a signaling mechanism for the impairment of unfolded or misfolded proteins in ER and induces the activation of caspase-12 and c-Jun NH₂-terminal kinase and also induces C/EBP homo-

* Corresponding author. Mailing address: Department of Bacteriology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan. Phone: 81-92-642-6130. Fax: 81-92-642-6129. E-mail: junfujii@bact.med.kyushu-u.ac.jp.

[▽] Published ahead of print on 9 June 2008.

gous protein (CHOP) (49). In mice, caspase-12 appears to be localized to the ER and is activated in response to stimuli, such as ER stress inducer tunicamycin, and releases of ER Ca^{2+} stores, which leads to apoptosis. Expression of CHOP is regulated at the transcriptional level after ER stress, essentially by the RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK)/eukaryotic initiation factor 2 α (eIF-2 α) signaling pathway (35). Ergonul et al. showed that the combination of TNF- α and Stx1 caused apoptosis with phosphatidylserine (PS) exposure and DNA fragmentation in human brain microvascular endothelial cells (HBMEC) (7).

In the present report, HBMEC isolated from 4- to 7-year-old children were constitutively sensitive to Stx2. Using this cell line, we report here that Stx2 causes apoptosis via a CHOP-mediated signaling pathway and that this process may be important in the pathophysiological response of humans to Stx2 bacterial toxin.

MATERIALS AND METHODS

Purification of Stx1 and Stx2. Stx1 was purified from Stx1-hyperproducing *E. coli* as described previously (47), while Stx2 was immunoaffinity purified from a clinical isolate of STEC (26). Both toxins were determined to be free of detectable lipopolysaccharide by the Toxicolor test (Seikagaku Kogyo Co., Tokyo, Japan), sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and silver staining. A nontoxic Stx1 mutant (Stx1_{R170L}) was purified as described previously (34). The 50% cytotoxic dose (CD_{50}) of Stx1_{R170L} protein was 9,000-fold higher than that of native Stx1, as assessed on the basis of Vero cell cytotoxicity.

Cell culture. HBMEC were isolated and cultured as previously described (40). HBMEC were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 10% NuSerum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 U/ml minimal essential medium with nonessential amino acids, 1 U/ml minimal essential medium with vitamins, and 5 U/ml heparin and incubated at 37°C in a 5% CO_2 atmosphere. Primary human renal proximal tubular epithelial cells (RPTEC) were purchased from Clonetics (Walkersville, MD). RPTEC were maintained in renal epithelial cell growth medium supplemented with human epidermal growth factor, hydrocortisone, epinephrine, insulin, tri-iodothyronine, transferrin, GA-1000, and FBS. Undifferentiated human leukemia THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% FBS.

Reagents and antibodies. General caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (fmk), caspase-1 inhibitor Z-Tyr-Val-Ala-Asp-fmk, caspase-2 inhibitor Z-Val-Asp-Val-Ala-Asp-fmk, caspase-3 inhibitor Z-Asp-Glu-Val-Ala-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 (Livermore, CA). Etoposide was purchased from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). Rabbit anti-human cytochrome *c* antibody was purchased from Research Diagnostic, Inc. (Flanders, NJ). Polyclonal antibodies against active caspase-3, -6, -8, -9, and Bid were purchased from Cell Signaling Technology (Beverly, MA). Anti- β -actin antibody and tunicamycin were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-FLICE-like inhibitory protein (FLIP) antibody (NF6) was purchased from Alexis Biochemicals (San Diego, CA). The annexin V-enhanced green fluorescent protein (EGFP) kit was purchased from BD Biosciences Clontech (Palo Alto, CA). 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was obtained from Molecular Probes (Eugene, OR). Recombinant active caspase-6 (rCasp-6) was purchased from Biomol Research Laboratory Inc. (Plymouth Meeting, PA).

Cytotoxicity assay. For time response experiments, HBMEC were dispensed into 96-well culture plates at a density of 10,000 cells per well (70 to 80% confluent cells). Cells maintained in medium alone served as the 100% viability control. The plates were incubated for 4 h, Stx2 (10 ng/ml) was then added, and cells were incubated for another 0, 6, 12, 18, and 24 h. Surviving cells were measured by a neutral red assay (25). To obtain toxin dose-response survival curves, HBMEC were dispensed into 96-well culture plates at a density of 5,000 cells per well. The plates were incubated for 4 h (nonconfluent cells) or for 24 h (confluent cells), and medium was replaced with fresh medium. Stx2 was added

to the plates at the concentration of 0.1 to 1,000 ng/ml. Eighteen hours later, cytotoxicity was measured by a neutral red assay.

Detection of Gb₃ in HBMEC by TLC/Stx1 overlay assay. Thin-layer chromatography (TLC) with a Stx1 overlay assay was carried out as previously described (46). Duplicate TLC plates were prepared by loading 1, 0.5, or 0.25 nmol each of a glycolipid standard mixture consisting of glucosylceramide, lactosylceramide, Gb₃ (Matreya, Inc., PA), and also extracts from HBMEC, RPTEC, and THP-1 cells (10^6 cells). The plates were exposed to an ascending solvent system of chloroform-methanol-water (60:36:8) and allowed to air dry for 30 min in a fume hood. The plate was used for Gb₃ detection by a Stx1 overlay assay as follows. The plate was immersed in 0.6% gelatin in warm water, incubated with gentle shaking overnight at 37°C, washed with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), incubated with purified Stx1 (0.1 $\mu\text{g/ml}$ in TBS), washed twice with TBS, reacted with monoclonal anti-Stx1 PH1 (1 $\mu\text{g/ml}$ in TBS), and then reacted with goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (diluted 1/2,000 in TBS).

Flow cytometric analysis for determination of apoptotic HBMEC. At 0, 6, 12, 18, and 24 h after addition of Stx2 (10 ng/ml), the cells were harvested by centrifugation at $200 \times g$ for 10 min, washed twice in phosphate-buffered saline (PBS), and resuspended in binding buffer. For extracellularly exposed PS detection, annexin V-EGFP (4 mg/ml) was employed, and for detection of nuclear membrane disruption, propidium iodide (PI; 2.5 mg/ml) was utilized. The reagents were added to the cells, and cells were incubated at room temperature in the dark. After addition of 500 μl of binding buffer, the cells were analyzed by a FACScan cytometer (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon laser (FL1) and a 568-nm argon-krypton laser (FL2). For detection of another apoptotic marker, the reduction of mitochondrial membrane potential, JC-1 dye was used in flow cytometry. Cells (5×10^5) were resuspended in 1 ml medium containing 100 μg JC-1 and incubated at 37°C for 10 min. The cells were washed twice in cold PBS, and samples were analyzed by the FACScan. JC-1 fluorescence was analyzed on the FL1 and FL2 channels for detection of the dye monomer and J-aggregate forms, respectively.

Transmission electron microscopy. Nonconfluent HBMEC grown in six-well Costar culture plates were treated with Stx2 (10 ng/ml) for 18 h. The cells were fixed and processed for electron microscopy as previously described (29). Briefly, the cells were fixed with 2% glutaraldehyde and then with 1% OsO_4 , dehydrated with ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined by electron microscopy in a JEM 2000EX instrument (JEOL, Ltd., Tokyo, Japan).

DNA fragmentation analysis of Stx2-treated HBMEC in the presence of caspase inhibitors. HBMEC were pretreated with caspase inhibitors (20 μM) for 30 min at 37°C, including a general caspase inhibitor and inhibitors of caspase-1, caspase-2, caspase-3, caspase-6, caspase-8, and caspase-9, before Stx2 (10 ng/ml) treatment. The cells were harvested by centrifugation at $200 \times g$ for 10 min. DNA was extracted according to DNA extraction kit instructions (Sepa Gene, Sankou-jyunyaku Co. Ltd., Tokyo, Japan). Briefly, the cell pellets were lysed with 0.3 ml hypotonic lysing buffer (10 mM Tris, 10 mM EDTA) containing 0.5% Triton X-100, and lysates were centrifuged at $13,000 \times g$ for 10 min to separate fragmented from intact chromatin. The supernatant containing fragmented DNA was transferred to a new microcentrifuge tube, and both pellet (intact DNA) and supernatant (fragmented DNA) were treated with 1 N perchloric acid at 4°C for 30 min. The precipitates were sedimented at $13,000 \times g$ for 20 min. The DNA precipitates were hydrolyzed by heating at 70°C for 10 min in 0.15 ml 1 N perchloric acid. The samples were transferred to the 96-well enzyme-linked immunosorbent assay plate, the A_{570} of the dye was measured, and the amount of DNA was quantified using the modified Burton method (5).

Western blot analysis of Stx2-treated HBMEC. HBMEC were treated with Stx2 (10 ng/ml) for 0, 2, 4, 7, or 10 h. Cells were lysed, and proteins of interest were analyzed by Western blotting. Several caspases, c-FLIP_L, and Bid as well as internal control β -actin were detected by immunoblotting as previously described by Bitko and Barik (4). Cytosolic cytochrome *c* was quantified as described by Heibein et al. (13). Immunodetection was carried out using the ECL detection system (Amersham Pharmacia Biotech, United Kingdom). Protein expression was quantified by a LAS1000 Plus luminescent image analyzer (Fujifilm, Tokyo, Japan).

Cell-free assay using rCasp-6. Cell extracts were freshly prepared from HBMEC as described previously (30) with some modifications. Briefly, non-treated cells were harvested by centrifugation at $1,600 \times g$ for 5 min at 4°C. The cell pellet was washed twice with ice-cold PBS (0.1 M phosphate buffer with 0.15 M NaCl, pH 7.4), followed by a single wash with ice-cold caspase buffer (20 mM PIPES, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 250 mM sucrose, pH 7.2). After centrifugation, the cells were resuspended with two volumes

FIG. 1. HBMEC are sensitive to Stxs. (A) Cytotoxic effects of Stx1 or Stx2 (10 ng/ml) on nonconfluent HBMEC were assessed by a neutral red assay at 6, 12, 18, and 24 h after incubation, with the viability displayed at 0 h taken as 100%. (B) Confluent or nonconfluent HBMEC were prepared as described in Materials and Methods and exposed to different concentrations of Stx2. After 18 h, cytotoxicity was measured by a neutral red assay. Cells maintained in medium alone served as the 100% viability control. The error bars show the deviations from three independent experiments.

of ice-cold complete caspase buffer supplemented with protease inhibitors (100 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin) and transferred to a 2-ml Dounce homogenizer. The cells were disrupted with 50 strokes of a B-type pestle (Fisher Scientific Ltd., Nepean, ON, Canada). Cell disruption (>95%) was confirmed by examination of aliquots of a trypan blue-stained suspension under a light microscope. The nuclei were removed by centrifugation at 20,000 $\times$ g for 10 min at 4°C. rCasp-6 was added to the cell extracts, and extracts were incubated for 0, 15, 30, 45, and 60 min. These fractions were stored at -80°C until use. The ability of rCasp-6 to cleave caspase-8 and caspase-3 in the cell extracts was analyzed by immunoblotting as described above.

DNA microarray analysis of Stx2-treated HBMEC. At 0, 6, 10, and 19 h after addition of Stx2 (10 ng/ml), HBMEC were harvested as described previously and the total RNA was extracted using the RNeasy total RNA isolation system (Qiagen, Crawley, UK). Microarray experiments using the whole human genome oligonucleotide microarray (44K oligonucleotide DNA microarray; Agilent Technologies, Tokyo, Japan) were performed according to the manufacturer's protocol. The total RNA samples were used for the preparation of Cy5- and Cy3-labeled cDNA probes. A nontreated sample (0 h) was used as the control. The hybridized and washed material on each glass slide was scanned with a DNA microarray scanner (model G2505A; Agilent Technologies). The Feature Extraction and Image Analysis software (Agilent Technologies) was used to locate and delineate every spot in the array and to integrate the intensities, which were then filtered and normalized by using the locally weighted scatterplot smoothing method. Gene clustering analysis was performed with Genespring 7, version 1.1 (Silicon Genetics). The reproducibility of microarray analysis was assessed by two repetitions of dye swap in each experiment. mRNA expression differences between control HBMEC and HBMEC harvested at 6, 10, or 19 h were compared.

qRT-PCR of CHOP in Stx2-treated HBMEC. HBMEC were treated with either Stx1, Stx2, Stx1_{R170L}, tunicamycin, or etoposide for 0, 4, 7, 10, 13, and 16 h. Treatment of cells with the antibiotic tunicamycin, which prevents the transfer of the oligosaccharide chain from the dolichol-phosphate donor to specific asparagine residues on the nascent polypeptide chain (36), leads to the accumulation of misfolded nonglycosylated proteins in the ER and induces ER stress. The cells were harvested, and total RNA was extracted. Five micrograms of total RNA of each sample was used to generate cDNA using the ABI high-capacity cDNA archiving kit (Applied Biosystems, Foster City, CA). The oligonucleotides of forward and reverse primers and a TaqMan probe for human CHOP were designed using Primer Express software (Applied Biosystems). The sequences of the oligonucleotides were as follows: human CHOP (GenBank accession number NM_004083) forward primer, 5'-CTCTGGCTTGCTGACTGA-3'; reverse primer, 5'-GCTCTGGGAGGTGCTTGT-3'; reporter probe, CAGAACCAGCAGAGGTC. The real-time PCRs were carried out by following the manufacturer's protocol. Expression of mRNA for CHOP was measured by quantitative reverse transcription-PCR (qRT-PCR) using TaqMan gene expression assays with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). On each plate, an endogenous control gene (β -actin gene) and template-negative controls were also run in duplicate. CHOP mRNA expression was normalized with β -actin mRNA.

CHOP mRNA knockdown experiment using siRNA. Two different sequences of small interfering RNA (siRNA) against the CHOP/DDIT3, validated Stealth RNA interference (RNAi) DuoPak (Duplex1, siRNA-CHOP.1; Duplex2, siRNA-CHOP.2), were provided from Invitrogen (Carlsbad, CA). Stealth RNAi-negative control duplexes (NC-siRNA; Invitrogen) with GC content similar to that of each duplex siRNA were used as negative controls. Duplex siRNAs were transfected into HBMEC using Lipofectamine RNAiMAX (20 pmol/ml) according to the manufacturer's instructions 24 h prior to adding Stx2 (10 ng/ml). Knockdown of endogenous CHOP mRNA level was confirmed by qRT-PCR analysis as described above. After the transfected cells were incubated with Stx2 (10 ng/ml) for 18 h, immunoblotting was carried out with anti-caspase-3. Moreover, the siRNA-transfected cells were dispensed into 96-well culture plates at a density of 10,000 cells per well and were incubated with different concentrations of Stx2 (0.1 to 1,000 ng/ml) for 18 h. The neutral red cytotoxicity assay was performed to address the influence of CHOP on survival following exposure of cells to Stx2.

Statistical analysis. Basically, the assays were performed in triplicate in three independent experiments. Statistical analysis involved analysis of variance, which was followed by an unpaired of Student's *t* test and Bonferroni test with SPSS 15.0J. Statistical differences were considered to be significant at a *P* of <0.05. Statistical analyses for microarray data were performed using the Feature Extraction and Image Analysis software (Agilent Technologies).

RESULTS

Cytotoxicity of Stxs on HBMEC. HBMEC were incubated with Stx1 or Stx2, and cell viability was assessed by the neutral red assay. Either toxin at 10 ng/ml induced cell death in a time-dependent manner (Fig. 1A). The cell viabilities follow-

FIG. 2. Detection of the Stx1 receptor glycolipid (Gb_3) in RPTEC, HBMEC, and THP-1 cells in a Stx1 overlay assay. RPTEC, HBMEC, and THP-1 cell neutral glycolipids were prepared from 10^6 cells and separated by TLC. The relative Gb_3 concentrations were estimated to be 1.6 nmol (RPTEC), 0.4 nmol (HBMEC), and 1.4 nmol (THP-1) based on a standard of Gb_3 .

FIG. 3. Flow cytometric analysis of apoptotic markers and electron microscopic analysis. (A) Apoptotic and necrotic cells analyzed using annexin V and PI as markers. Stx2 (10 ng/ml)-exposed HBMEC were harvested at the time points shown and stained by annexin V-EGFP (4 mg/ml) and PI (2.5 mg/ml). The results are depicted as dot plots showing FL1 (488 nm/EGFP) and FL2 (568 nm/PI) channels. (B) Percentages of cells undergoing early apoptosis, late apoptosis, and necrosis were quantitated and represented in the bar graph. The error bars show the deviations among three independent experiments. *, $P < 0.05$ versus 0-h control (unpaired t test). (C) Decrease of mitochondrial membrane potential ($\Delta\psi$) induced by Stx2 in HBMEC was detected using JC-1. The results were depicted as dot plots with FL1 (488 nm/monomer JC-1) and FL2 (568 nm/aggregate JC-1) channels. The intact membrane/high $\Delta\psi$ -retaining population and reduced- $\Delta\psi$ population are gated and designated gate 1 and gate 2, respectively. (D) Percentages of gate 1 and gate 2 populations. A shift from gate 1 to gate 2 is the result of a decrease in $\Delta\psi$. The error bars show the deviations among the three independent experiments. *, $P < 0.05$ versus 0-h value (Bonferroni t test). (E) Typical chromatin condensations (arrows) were observed in the apoptotic cell at 18 h after incubation with Stx2 (10 ng/ml). Bars, 1 μ m.

ing Stx1 or Stx2 treatment were not statistically different in the time course assessed. When HBMEC were incubated with different concentrations of Stx2 alone for 18 h and assessed for cytotoxicity, a Stx2 dose response was observed in both confluent and nonconfluent cells (Fig. 1B). The CD_{50} in nonconfluent cells treated with Stx2 was less than 1 ng/ml Stx2, whereas the CD_{50} in confluent cells was more than 1,000 ng/ml Stx2.

Detection of Gb₃ by Stx1 binding. Similar amounts of cell-extracted neutral lipids from RPTEC, HBMEC, and THP-1 cells were loaded onto TLC plates and visualized by a Stx1 overlay assay. Gb₃ expression levels in HBMEC, RPTEC, and THP-1 cells were compared to standards and determined to be 0.4, 1.6, and 1.4 nmol/million cells, respectively (Fig. 2).

Flow cytometric analysis of exposed PS and of mitochondrial membrane potential. Following exposure of HBMEC to Stx2 for 0, 6, 12, 18, and 24 h, cells were harvested and stained with EGFP-conjugated annexin V for PS detection and with PI for nuclear membrane disruption. Quantification of cell death was analyzed by flow cytometry, as shown in the scatterplot (Fig. 3A; x axis, FL1/annexin V; y axis, FL2/PI). This assay identifies apoptotic and necrotic cells. Viable cells are negative for annexin V and PI and are shown at the lower left quadrant of Fig. 3A. Cells that are stained positive for annexin V and negative for PI, which are shown at the lower right quadrant in Fig. 3A, are indicative of early apoptosis. Cells in late apoptosis are both annexin V and PI positive and are shown at upper right quadrant in Fig. 3A. Necrotic cells, annexin V negative

and PI positive, are shown in the upper left quadrant in Fig. 3A. The results in Fig. 3A were summarized and quantified as percentages of the total cell population in Fig. 3B. A significant increase in early apoptosis was observed at 12, 18, and 24 h compared to the control (0 h). Late apoptosis was slightly increased in a time-dependent manner. However, the percentage of necrotic cells did not change during the time course. The change in the mitochondrial membrane potential is another marker of apoptotic cells and is detectable by JC-1, a cationic dye that accumulates in the mitochondria. When the mitochondrial membrane is intact, the dye forms aggregates that present as orange fluorescence (FL2), whereas in the damaged membrane a lower dye concentration occurs and the dye remains as a monomer that appears as green fluorescence (FL1). Thus, a population with a high membrane potential is indicated by gate 1 whereas a population with a low membrane potential is indicated by gate 2. The membrane potential is shown to decrease with a shift from gate 1 to gate 2 (Fig. 3C). The percentages of scatter population are summarized in Fig. 3D. The JC-1 aggregates decrease significantly at 12, 18, and 24 h compared to 0 h, following exposure to Stx2 (Fig. 3D, gate 1). This indicates that Stx2 induced a loss of mitochondrial membrane potential in HBMEC. Moreover, there is a correlation in timing between loss of mitochondrial membrane potential (Fig. 3D) and leakage of cytochrome c from mitochondria into the cytoplasm (see Fig. 4D) after Stx2 exposure.

Electron microscopic analysis. As shown in Fig. 3E, the cells exposed to Stx2 undergo morphological changes including

FIG. 4. Detection of DNA fragmentation and elements of the caspase cascade induced by Stx2 in HBMEC. (A) Stx2-induced DNA ladder formation and the decrease of the ladder due to caspase inhibitors in HBMEC. Lane 1, control; lane 2, Stx2 (10 ng/ml) alone; lane 3, Stx2 with a general caspase inhibitor; lane 4, caspase-1 inhibitor; lane 5, caspase-2 inhibitor; lane 6, caspase-3 inhibitor; lane 7, caspase-6 inhibitor; lane 8, caspase-8 inhibitor; lane 9, caspase-9 inhibitor. (B) The DNA fragments were quantified by a modification of the diphenylamine method of Burton (5) described in Materials and Methods. The error bars show the deviations among three independent experiments. *, $P < 0.05$ versus Stx2 alone. (C) Induction of caspase cascade proteins by Stx2 (10 ng/ml) in HBMEC was analyzed by Western blotting. Detected bands of FLIP_L, cleaved caspase-8, cleaved caspase-6, and cleaved caspase-3 as well as loading control, β-actin, are shown. Procaspase-1 was not changed (data not shown). (D) Bands of full-length Bid, cytochrome *c*, caspase-9, and the loading control, β-actin, are shown. Note that full-length Bid was reduced after 6 h of Stx2 (10 ng/ml) incubation with HBMEC, indicating an increase in active/truncated Bid (tBid). Cytochrome *c* release from mitochondria and cleavage of caspase-9 occurred following Bid activation. The experiment was repeated twice with similar results.

chromatin condensation (Fig. 3E, Stx2) in the periphery of the nucleus. The cellular apoptosis was also accompanied by cytoplasmic blebs (Fig. 3E, Stx2).

Altered Stx2-induced DNA fragmentation by caspase inhibitors. HBMEC were pretreated with 20 μM caspase inhibitors for 30 min at 37°C and exposed to Stx2 (10 ng/ml). Preparation of DNA extracts was performed at 24 h after Stx2 treatment. Fragmented DNA was visualized as a DNA ladder (Fig. 4A) and quantified by the Burton method (Fig. 4B). A general caspase inhibitor completely blocked formation of the DNA ladder (Fig. 4A, lane 3, and B, Stx2+z-VAD-fmk), while specific inhibitors of caspase-1, caspase-3, caspase-6, caspase-8, and caspase-9 partially blocked DNA ladder formation (Fig. 4A and B). In contrast, the caspase-2 inhibitor did not significantly decrease DNA fragmentation induced by Stx2 (Fig. 4A and B).

Caspase activities and apoptosis-related proteins in Stx2-treated HBMEC. In the presence of Stx2, HBMEC expression of antiapoptotic protein c-FLIP_L decreased and disappeared completely at 6 h, followed by cleavage activation of caspase-8, caspase-3, and caspase-6 (Fig. 4C). It is known that de novo synthesis of c-FLIP_L is inhibited by protein synthesis inhibitors

FIG. 5. Activation of caspase-8 and -3 by exogenous rCasp-6 in HBMEC lysate. Active rCasp-6 or the PBS control was added to normal HBMEC extracts, and extracts were incubated for 15 min, 30 min, 45 min, and 60 min. Whole fragments and cleaved fragments of caspase-8 (A) and -3 (C) were detected in the cell extracts containing rCasp-6 by immunoblotting. Addition of PBS to cell extracts did not cause cleavage of caspase-8 (B) or caspase-3 (D).

TABLE 1. Changes in HBMEC mRNA expression after Stx2 treatment^a

Family and accession no.	Producer of mRNA	Change in mRNA expression (fold) ^b at:		
		6 h	10 h	19 h
Caspase family				
NM_033292	Caspase-1	1.00	1.17	1.23*
NM_032982	Caspase-2	0.98	1.28	0.85
NM_004346	Caspase-3	1.21	0.95	0.61*
NM_033306	Caspase-4	1.29	0.81	0.94
NM_004347	Caspase-5	1.21	1.04	0.92
NM_001226	Caspase-6	0.85	12.72▲	1.01
NM_033338	Caspase-7	0.91	0.88	1.09
NM_033356	Caspase-8	1.07	0.89	0.85
NM_001229	Caspase-9	1.67	1.98	2.63▲*
AF111344	Caspase-10	1.23	0.75	1.74
NR_000035	Caspase-12	1.37	0.82	0.75
NM_012114	Caspase-14	0.93	1.12	0.93
Bcl-2 family				
NM_000657	Bcl-2	1.01	0.95	0.96
NM_004322	BAD	0.96	1.26	1.02
NM_138764	BAX	0.93	2.17▲	1.39
NM_001196	BID	0.90	0.82	0.87
Death domain related genes				
NM_003824	FADD	1.09	1.03	0.96
U97075	FLIP, short form	1.34	1.68	1.63
NM_003879	CFLAR	1.44	1.40	1.06
XIAP and Apaf-1				
U45880	XIAP	1.15	0.95	1.01
NM_017523	XIAP-associated factor 1	2.49▲	1.21	0.61
NM_181861	Apaf-1	0.86	1.03	0.96
TRAF				
NM_005658	TRAF1	1.69	1.65	1.70
NM_021138	TRAF2	0.98	1.39	0.90
NM_145725	TRAF3	1.05	0.70	0.94
NM_004295	TRAF4	1.08	0.71	1.08
NM_004619	TRAF5	0.77	1.22	1.28
NM_145803	TRAF6	1.43	1.14	1.08
GADD and ATM				
NM_001924	GADD45A	4.44▲*	1.33	2.83▲*
NM_015675	GADD45B	3.58▲*	1.21	1.57
NM_138293	ATM	1.00	1.24	0.76
NM_014330	GADD34	3.23▲*	1.36	3.75▲*
eIF				
NM_004681	eIF-1AY	1.45	0.59	0.72
NM_004836	eIF-2AK3(PERK)	2.26▲*	1.39	2.04▲*
NM_032025	eIF-2A	1.06	0.96	1.05
BC013590	eIF-2B	1.23	1.00	1.34
NM_001967	eIF-4A2	1.55	1.02	1.64
CREB				
NM_134442	CREB1	1.01	0.96	0.96
NM_181715	CREB2	0.94	1.24	1.12*
NM_022769	CREB3	0.96	0.61	0.95
NM_004904	CREB5	9.75▲*	2.50▲	9.46▲*
ER stress related genes				
NM_004083	CHOP	4.99▲*	1.51	4.40▲*
NM_001675	ATF4	1.98*	1.55	2.74▲*
NM_005080	XBP-1	1.20	1.00	0.80
AF188611	BiP	0.77	1.05	0.60
NM_016306	HEDJ/ERdj3	1.00	0.98	0.93
NM_021158	TRIB3	0.87	0.86	0.89

Continued on following page

TABLE 1—Continued

Family and accession no.	Producer of mRNA	Change in mRNA expression (fold) ^b at:		
		6 h	10 h	19 h
Myd88 and TRIF				
NM_002468	MYD88	0.89	1.21	0.87
NM_014261	TRIF	0.96	1.22	1.00
p38				
NM_000584	IL-8	16.49 ^{▲*}	2.58 [▲]	14.08 ^{▲*}
p53				
NM_000546	TP53	1.27	1.20	2.56 [▲]

^a Abbreviations: FADD, Fas-associated death domain; CFLAR, caspase-8- and FADD-like apoptosis regulator; XIAP, X-linked inhibitor of apoptosis protein; TRAF, TNF receptor-associated factor; GADD, growth arrest and DNA damage-inducible protein; ATM, ataxia telangiectasia mutated; CREB, cyclic AMP response element binding protein; CHOP, C/EBP homologous protein; XBP-1, x-box-binding protein 1; HEDJ, human ER-associated DNAJ; ERdj3, endoplasmic reticulum-localized DnaJ homologues 3; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing beta interferon.

^b [▲], >2.0-fold increase versus 0-h value; *, *P* < 0.05 versus 0-h value.

including Stxs and cycloheximide (8). Because c-FLIP_L inhibits caspase-8 activity, a decrease in FLIP_L enhances the activation of caspase-8. The results suggest that protein synthesis inhibition by Stx2 induced caspase-8 activity in HBMEC (Fig. 4C), although, in this case, identification of the primary initiator of apoptosis is yet to be established. Nonetheless, activated caspase-8 cleaves full-length Bid to a shorter truncated Bid, tBid, and tBid is known to cause mitochondrial membrane rupture (48). Thus, the activation of Bid, a proapoptotic Bcl-2 family protein, is a link from the extrinsic pathway to the intrinsic pathway in apoptosis. Indeed, after 6 h of incubation with Stx2, a decrease in the full-length Bid was detected in a time-dependent manner, and this decrease resulted in an increase in tBid (Fig. 4D). The data indicate that Stx2 induces cleavage of Bid via caspase-8 activation. As a result of Bid activation, release of cytochrome *c* from mitochondria to the cytoplasm was detected after 12 h, which was also followed by caspase-9 cleavage (Fig. 4D). The time course of cytochrome *c* leakage matched the decreased mitochondrial membrane potential detected by JC-1 (Fig. 3D).

Cell-free assay using rCasp-6. Based on the kinetic data of the immunoblotting, we investigated whether caspase-6 could directly cleave caspase-8 in a normal cell extract of HBMEC. The cell extracts without exposure to Stx2 were collected, and rCasp-6 was added to the cell extracts. As early as 15 min after adding rCasp-6, cleaved caspase-8 (43, 41 kDa) was detected, and after 30 min, cleavage of caspase-3 (17 kDa) was detected (Fig. 5). Thus, caspase-6 may function as an amplifier of the caspase-8–caspase-3-activated system.

DNA microarray and data analysis. To understand the upstream factors of caspase activation induced by Stx2, we analyzed the gene expression profiles of HBMEC treated with Stx2 for 6, 10, or 19 h and compared them to that for untreated control HBMEC. We examined the expression of ~44,000 genes using the whole human oligonucleotide microarray 44K. Among the genes that exhibited significantly altered gene expression between the Stx2-treated and untreated control cells, 51 were apoptosis-related genes (Table 1). The apoptosis-related genes could be classified into 12 groups, which are shown in Table 1. Among them, expression of ER stress-related genes such as the C/EBP homologous protein (CHOP/GADD153

[growth arrest and DNA damage-inducible protein 153]) gene increased significantly. The GADD45A, GADD45B, eIF-2AK3 (PERK), CREB5, and IL-8 genes were also significantly upregulated in response to Stx2 in HBMEC. It should be noted that GADD45A and GADD45B were previously associated with DNA arrest of HCT116 cells in S phase (3) and that IL-8 was associated with ribotoxic stress protein kinase c-Jun NH₂-terminal kinase/stress-activated protein kinase (39). Also, the eIF-2α/ATF4 (activating transcription factor 4) signaling pathway is known to play an essential role in the induction of CHOP in ER stress (35).

Verification of microarray results. The microarray results were further verified using qRT-PCR analyses with the TaqMan probe for CHOP. As shown in Fig. 6, the Stx1_{R170L} enzymatic site mutant did not cause an increase in CHOP mRNA expression, whereas native Stx1 or Stx2 caused a 10-fold increase in CHOP mRNA expression compared with Stx1_{R170L} (*P* < 0.05). Tunica-

FIG. 6. Validation of CHOP mRNA expression induced by Stx2 in HBMEC. HBMEC were incubated with Stx1 (10 ng/ml), Stx2 (10 ng/ml), Stx1_{R170L} (10 ng/ml), tunicamycin (1 μg/ml), or etoposide (100 μM) for the times indicated. The amount of CHOP mRNA expression was analyzed by qRT-PCR and depicted as the ratio of mRNA expression to Stx1 mRNA expression at 0 h. The error bars indicate the deviations among three independent experiments. *, *P* < 0.05 for Stx1 or Stx2 versus Stx1_{R170L} or etoposide (unpaired *t* test).

FIG. 7. CHOP knockdown in HBMEC reduces caspase-3 activation and increases viability induced by Stx2. (A) HBMEC were serially transfected with CHOP siRNA-CHOP.1 or -CHOP.2 or with NC-siRNA. Endogenous CHOP mRNA depletion was examined by qRT-PCR analysis. Note that with siRNA-CHOP.1 there was a 55% reduction in CHOP transcript levels compared to that for NC-siRNA (*, $P < 0.05$, unpaired t test). (B) Caspase-3 cleavage after 18 h of incubation with Stx2 (10 ng/ml) was decreased in the siRNA-CHOP.1-transfected HBMEC compared to that in NC-siRNA-transfected HBMEC. The cleaved caspase-3 was detected by immunoblotting with loading control β -actin. (C) The results additional experiments similar to those for panel B were quantified by densitometry and displayed as percentages of the cleavage in NC-siRNA-transfected cells (100%). Caspase-3 cleavage was statistically decreased in the siRNA-CHOP.1-transfected HBMEC compared to NC-siRNA-transfected HBMEC (*, $P < 0.05$, unpaired t test). (D) The viability of Stx2-treated HBMEC which were transfected with siRNA-CHOP.1, siRNA-CHOP.2, or NC-siRNA was measured by a neutral red assay. The siRNA-CHOP.1-transfected cells were significantly resistant to the toxin compared to the cell viability of NC-siRNA-transfected cells ($P < 0.05$, unpaired t test) at doses of Stx2 from 0.1 to 1,000 ng/ml. The error bars show the deviations among the 4 wells in 96-well culture plates. The experiment was repeated three times with similar results.

mycin, when used as a CHOP-inducing control, increased CHOP mRNA expression, which peaked at 10 h of incubation. The DNA-damaging topoisomerase II inhibitor etoposide did not induce CHOP mRNA, as reported previously (27). Etoposide treatment (100 μ M) for 18 h, known to induce apoptosis, resulted in 29% cell death, as measured by a neutral red assay (data not shown).

siRNA knockdown of CHOP. To test the involvement of CHOP-mediated apoptosis induced by Stx2 in HBMEC, CHOP was knocked down by RNAi. HBMEC were serially transfected with duplex siRNA oligonucleotides targeted against CHOP, and the extent of endogenous CHOP depletion was examined. qRT-PCR analysis of mRNA extracted from siRNA-transfected cells revealed a 55% (siRNA-CHOP.1) and 21% (siRNA-CHOP.2) reduction in CHOP transcript levels

compared to control cells identically transfected with a non-targeting siRNA oligonucleotide (NC-siRNA) (Fig. 7A). Knockdown construct siRNA-CHOP.1 produced a significant reduction of CHOP mRNA. These results accurately reflected a concordant reduction in Stx2-induced cleavage of caspase-3 in the CHOP knockdown cells, as measured by immunoblotting (Fig. 7B and C). Moreover, siRNA-CHOP.1-transfected cells were resistant to the toxin compared with NC-siRNA-transfected cells ($P < 0.05$, unpaired t test) (Fig. 7D).

DISCUSSION

As we reported previously, HBMEC are most likely a target for Stxs (9, 10); thus, we employed HBMEC to demonstrate the direct effect of Stxs on this cell type. Recently, Ergonul et

al. (7) reported that TNF- α - and Stx1-induced apoptosis in HBMEC was mediated by caspase-3. We also investigated the enhanced cytotoxicity of Stxs with TNF- α in the HBMEC. Neutral red assays were performed as described in Materials and Methods, and HBMEC were found to be sensitive to both Stx1 and Stx2 with TNF- α (data not shown). In the present study, to investigate whether cell death induced in HBMEC by Stx2 was by apoptosis or necrosis, we focused on five major biochemical features of apoptotic cells, which include (i) extracellular exposure of PS in the early stage of apoptosis, (ii) loss of mitochondrial membrane potential, (iii) DNA fragmentation in the final stage of apoptosis, (iv) activation of a caspase cascade, and (v) morphological changes. The results indicated that the death of HBMEC caused by Stx2 treatment is mostly due to apoptosis, and the caspase-1 inhibitor was shown to significantly reduce the formation of a DNA ladder.

A purpose of the present study was to determine the possible origin of Stx-induced apoptosis in human endothelial cells in vitro. We demonstrate here that the enzymatic activity of Stx1 and Stx2 is required for induction of CHOP mRNA expression in HBMEC. It should be noted that CHOP can be activated by the Ire1, PERK, and ATF6 pathways, which are three key components of the ER stress response (45). Recently Lee et al. reported that Stx1 treatment increased activation of the ER stress sensors IRE1, PERK, and ATF6 in the myelogenous leukemia cell line THP-1 (19). For HBMEC, further studies will be needed to determine the pathways that activate the CHOP promoter.

Our experiments do not address the possible role of an independent p38 mitogen-activated protein kinase pathway through the Stx2-induced ribotoxic stress response, though this pathway is known to be activated by Stx2 in eukaryotic cells (39). We have investigated the connection between CHOP and apoptosis, which is thought to be caused by proteins downstream of CHOP. Following siRNA knockdown of CHOP, a concomitant decrease in cleaved caspase-3 was observed. Further studies will be needed to determine how CHOP leads to activation of caspase-3. In the presence of Stx2, HBMEC expression of antiapoptotic protein c-FLIP_L decreased at 6 h, followed by cleavage of caspase-8 (Fig. 4C). c-FLIP_L inhibits caspase-8 activity, and a decrease in c-FLIP_L enhances the activation of caspase-8 in the death-inducing signaling complex. Initiation of caspase-8 activity could be directly induced by Stx inhibition of protein synthesis, as shown by the decrease in caspase-8 inhibitor c-FLIP_L. Activated caspase-8 cleaves full-length Bid, and the truncated Bid is known to cause mitochondrial membrane rupture (48). As a result of Bid activation, release of cytochrome *c* from mitochondria to the cytoplasm was detected after 12 h and was also followed by caspase-9 cleavage. While we observed activation of additional caspases in HBMEC in response to Stx2, the data do not allow one to assign a temporal order of activation of these apoptosis factors. However, exogenous purified caspase-6, when added to HBMEC lysate, was able to activate caspase-8 (Fig. 5), suggesting that this may also occur in whole cells.

In summary, we report that *E. coli* Stx induces CHOP, which interacts with the apoptotic cascade by mediating activation of caspase-3 in HBMEC.

ACKNOWLEDGMENTS

We thank Shinji Yamasaki (Osaka Prefecture University, Osaka, Japan) for providing the Stx1 mutant Stx1_{R170L}. We also thank Tomomi Gotoh (Kumamoto University School of Medicine, Japan) for a helpful discussion and Sharon Y. A. M. Villanueva (Kyushu University) for correcting the English. We thank Akemi Takade (Kyushu University) for technical support with electron microscopy.

This work was supported by grants-in-aid for scientific research as a part of U.S.-Japan Cooperative Medical Science Program (KH19A10010) from the Ministry of Health, Labor and Welfare of Japan and by U.S. Public Health Service grant AI24431 (to T.O.).

REFERENCES

- Arab, S., M. Murakami, P. Dirks, B. Boyd, S. L. Hubbard, C. A. Lingwood, and J. T. Rutka. 1998. Verotoxins inhibit the growth of and induce apoptosis in human astrocytoma cells. *J. Neurooncol.* **40**:137–150.
- Ashkenazi, A., and V. M. Dixit. 1998. Death receptors: signaling and modulation. *Science* **281**:1305–1308.
- Bhattacharjee, R. N., K. S. Park, S. Uematsu, K. Okada, K. Hoshino, K. Takeda, O. Takeuchi, S. Akira, T. Iida, and T. Honda. 2005. *Escherichia coli* verotoxin 1 mediates apoptosis in human HCT116 colon cancer cells by inducing overexpression of the GADD family of genes and S phase arrest. *FEBS Lett.* **579**:6604–6610.
- Bitko, V., and S. Barik. 1998. Persistent activation of RelA by respiratory syncytial virus involves protein kinase C, underphosphorylated I κ B β , and sequestration of protein phosphatase 2A by the viral phosphoprotein. *J. Virol.* **72**:5610–5618.
- Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315–323.
- Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi. 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.
- Ergonul, Z., A. K. Hughes, and D. E. Kohan. 2003. Induction of apoptosis of human brain microvascular endothelial cells by Shiga toxin 1. *J. Infect. Dis.* **187**:154–158.
- Erwert, R. D., R. K. Winn, J. M. Harlan, and D. D. Bannerman. 2002. Shiga-like toxin inhibition of FLICE-like inhibitory protein expression sensitizes endothelial cells to bacterial lipopolysaccharide-induced apoptosis. *J. Biol. Chem.* **277**:40567–40574.
- Fujii, J., Y. Kinoshita, T. Kita, A. Higure, T. Takeda, N. Tanaka, and S. Yoshida. 1996. Magnetic resonance imaging and histopathological study of brain lesions in rabbits given intravenous verotoxin 2. *Infect. Immun.* **64**:5053–5060.
- Fujii, J., T. Kita, S. Yoshida, T. Takeda, H. Kobayashi, N. Tanaka, K. Ohsato, and Y. Mizuguchi. 1994. Direct evidence of neuron impairment by oral infection with verotoxin-producing *Escherichia coli* O157:H⁺ in mitomycin-treated mice. *Infect. Immun.* **62**:3447–3453.
- Fujii, J., T. Matsui, D. P. Heatherly, K. H. Schlegel, P. I. Lobo, T. Yutsudo, G. M. Ciralo, R. E. Morris, and T. Obrig. 2003. Rapid apoptosis induced by Shiga toxin in HeLa cells. *Infect. Immun.* **71**:2724–2735.
- Fukushima, H., T. Hashizume, Y. Morita, J. Tanaka, K. Azuma, Y. Mizumoto, M. Kaneno, M. Matsuura, K. Konma, and T. Kitani. 1999. Clinical experiences in Sakai City Hospital during the massive outbreak of enterohemorrhagic *Escherichia coli* O157 infections in Sakai City, 1996. *Pediatr. Int.* **41**:213–217.
- Heibein, J. A., M. Barry, B. Motyka, and R. C. Bleackley. 1999. Granzyme B-induced loss of mitochondrial inner membrane potential (Delta Psi m) and cytochrome c release are caspase independent. *J. Immunol.* **163**:4683–4693.
- Inward, C. D., J. Williams, I. Chant, J. Crocker, D. V. Milford, P. E. Rose, and C. M. Taylor. 1995. Verocytotoxin-1 induces apoptosis in Vero cells. *J. Infect.* **30**:213–218.
- Jones, N. L., A. Islur, R. Haq, M. Mascarenhas, M. A. Karmali, M. H. Perdue, B. W. Zanke, and P. M. Sherman. 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.
- Karpman, D., A. Hakansson, M. T. Perez, C. Isaksson, E. Carlalmalm, A. Caprioli, and C. Svanborg. 1998. Apoptosis of renal cortical cells in the hemolytic-uremic syndrome: in vivo and in vitro studies. *Infect. Immun.* **66**:636–644.
- Kiyokawa, N., T. Taguchi, T. Mori, H. Uchida, N. Sato, T. Takeda, and J. Fujimoto. 1998. Induction of apoptosis in normal human renal tubular epithelial cells by *Escherichia coli* Shiga toxins 1 and 2. *J. Infect. Dis.* **178**:178–184.
- Kodama, T., K. Nagayama, K. Yamada, Y. Ohba, Y. Akeda, and T. Honda. 1999. Induction of apoptosis in human renal proximal tubular epithelial cells by *Escherichia coli* verocytotoxin 1 in vitro. *Med. Microbiol. Immunol.* **188**:73–78.

19. Lee, S. Y., M. S. Lee, R. P. Cherla, and V. L. Tesh. 2008. Shiga toxin 1 induces apoptosis through the endoplasmic reticulum stress response in human monocytic cells. *Cell. Microbiol.* **10**:770–780.
20. Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**:479–489.
21. Lingwood, C. A. 1996. Role of verotoxin receptors in pathogenesis. *Trends Microbiol.* **4**:147–153.
22. Lingwood, C. A., H. Law, S. Richardson, M. Petric, J. L. Brunton, G. S. De, and M. Karmali. 1987. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin in vitro. *J. Biol. Chem.* **262**:8834–8839.
23. Liu, X., C. N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**:147–157.
24. Louise, C. B., and T. G. Obrig. 1991. Shiga toxin-associated hemolytic-uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1 β , and tumor necrosis factor alpha on human vascular endothelial cells in vitro. *Infect. Immun.* **59**:4173–4179.
25. Louise, C. B., and T. G. Obrig. 1992. Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells in vitro. *Infect. Immun.* **60**:1536–1543.
26. Louise, C. B., and T. G. Obrig. 1995. Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. *J. Infect. Dis.* **172**:1397–1401.
27. Mandic, A., J. Hansson, S. Linder, and M. C. Shoshan. 2003. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. *J. Biol. Chem.* **278**:9100–9106.
28. Michino, H., K. Araki, S. Minami, S. Takaya, N. Sakai, M. Miyazaki, A. Ono, and H. Yanagawa. 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *Am. J. Epidemiol.* **150**:787–796.
29. Morris, R. E., G. M. Ciraolo, and C. B. Saelinger. 1992. Validation of the biotinyl ligand-avidin-gold technique. *J. Histochem. Cytochem.* **40**:711–721.
30. Nie, Z., B. N. Phenix, J. J. Lum, A. Alam, D. H. Lynch, B. Beckett, P. H. Krammer, R. P. Sekaly, and A. D. Badley. 2002. HIV-1 protease processes procaspase 8 to cause mitochondrial release of cytochrome c, caspase cleavage and nuclear fragmentation. *Cell Death Differ.* **9**:1172–1184.
31. Nudelman, E., R. Kannagi, S. Hakomori, M. Parsons, M. Lipinski, J. Wiels, M. Fellous, and T. Tursz. 1983. A glycolipid antigen associated with Burkitt lymphoma defined by a monoclonal antibody. *Science* **220**:509–511.
32. O'Brien, A. D., and R. K. Holmes. 1987. Shiga and Shiga-like toxins. *Microbiol. Rev.* **51**:206–220.
33. Obrig, T. G., T. P. Moran, and J. E. Brown. 1987. The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. *Biochem. J.* **244**:287–294.
34. Ohmura, M., S. Yamasaki, H. Kurazono, K. Kashiwagi, K. Igarashi, and Y. Takeda. 1993. Characterization of non-toxic mutant toxins of Vero toxin 1 that were constructed by replacing amino acids in the A subunit. *Microb. Pathog.* **15**:169–176.
35. Oyadomari, S., and M. Mori. 2004. Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* **11**:381–389.
36. Parodi, A. J. 2000. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. *Biochem. J.* **348**(Pt. 1):1–13.
37. Rooney, J. C., R. M. Anderson, and I. J. Hopkins. 1971. Clinical and pathological aspects of central nervous system involvement in the haemolytic uraemic syndrome. *Aust. Paediatr. J.* **7**:28–33.
38. Siegler, R. L. 1995. The hemolytic uremic syndrome. *Pediatr. Clin. N. Am.* **42**:1505–1529.
39. Smith, W. E., A. V. Kane, S. T. Campbell, D. W. Acheson, B. H. Cochran, and C. M. Thorpe. 2003. Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. *Infect. Immun.* **71**:1497–1504.
40. Stins, M. F., F. Gilles, and K. S. Kim. 1997. Selective expression of adhesion molecules on human brain microvascular endothelial cells. *J. Neuroimmunol.* **76**:81–90.
41. Tetaud, C., T. Falguieres, K. Carlier, Y. Lecluse, J. Garibal, D. Coulaud, P. Busson, R. Steffensen, H. Clausen, L. Johannes, and J. Wiels. 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.
42. Uchida, H., N. Kiyokawa, T. Taguchi, H. Horie, J. Fujimoto, and T. Takeda. 1999. Shiga toxins induce apoptosis in pulmonary epithelium-derived cells. *J. Infect. Dis.* **180**:1902–1911.
43. Upadhyaya, K., K. Barwick, M. Fishaut, M. Kashgarian, and N. J. Siegel. 1980. The importance of nonrenal involvement in hemolytic-uremic syndrome. *Pediatrics* **65**:115–120.
44. Wiels, J. 2000. CD77. *J. Biol. Regul. Homeost. Agents* **14**:288–289.
45. Xu, C., B. Bailly-Maitre, and J. C. Reed. 2005. Endoplasmic reticulum stress: cell life and death decisions. *J. Clin. Invest.* **115**:2656–2664.
46. Yiu, S. C., and C. A. Lingwood. 1992. Polyisobutylmethacrylate modifies glycolipid binding specificity of verotoxin 1 in thin-layer chromatogram overlay procedures. *Anal. Biochem.* **202**:188–192.
47. Yutsudo, T., N. Nakabayashi, T. Hirayama, and Y. Takeda. 1987. Purification and some properties of a Vero toxin from *Escherichia coli* O157:H7 that is immunologically unrelated to Shiga toxin. *Microb. Pathog.* **3**:21–30.
48. Zamzami, N., C. El Hamel, C. Maise, C. Brenner, C. Munoz-Pinedo, A. S. Belzacq, P. Costantini, H. Vieira, M. Loeffler, G. Molle, and G. Kroemer. 2000. Bid acts on the permeability transition pore complex to induce apoptosis. *Oncogene* **19**:6342–6350.
49. Zinszner, H., M. Kuroda, X. Wang, N. Batchvarova, R. T. Lightfoot, H. Remotti, J. L. Stevens, and D. Ron. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* **12**:982–995.