Differential cytotoxic actions of Shiga toxin 1 and Shiga toxin 2 on microvascular and macrovascular endothelial cells

Andreas Bauwens; Martina Bielaszewska; Björn Kemper; Patrik Langehanenberg; Gert von Bally; Rudolf Reichelt; Dennis Mulac; Hans-Ulrich Humpf; Alexander W. Friedrich; Kwang S. Kim; Helge Karch; Johannes Müthing

Summary
Shiga toxin (Stx)-mediated injury to vascular endothelial cells in the kidneys, brain and other organs underlies the pathogenesis of haemolytic uremic syndrome (HUS) caused by enterohaemorrhagic Escherichia coli (EHEC). We present a direct and comprehensive comparison of cellular injury induced by the two major Stx types, Stx1 and Stx2, in human brain microvascular endothelial cells (HBMECs) and EA.hy 926 macrovascular endothelial cells. Scanning electron microscopy of microcarrier-based cell cultures, digital holographic microscopy of living single cells, and quantitative apoptosis/necrosis assays demonstrate that Stx1 causes both necrosis and apoptosis, whereas Stx2 induces almost exclusively apoptosis in both cell lines. Moreover, microvascular and macrovascular endothelial cells have different susceptibilities to the toxins: EA.hy 926 cells are slightly, but significantly (∼10 times) more susceptible to Stx1, whereas HBMECs are strikingly (≥1,000 times) more susceptible to Stx2. These findings have implications in the pathogenesis of HUS, and suggest the existence of yet to be delineated Stx type-specific mechanisms of endothelial cell injury beyond inhibition of protein biosynthesis.

Keywords
Shiga toxin, endothelial cells, enterohaemorrhagic E. coli, single cell analysis, apoptosis, necrosis

Introduction
Endothelial cells line the inner surface of the vessels providing a barrier at the blood-tissue interface. Microbes have evolved numerous mechanisms to enter the host tissues and the vascular endothelium is a central target for pathogens and their virulence factors (1, 2). Pathogens attack the endothelium by a wide variety of strategies resulting in endothelial barrier dysfunction and cardiovascular disease (3). Vascular damage caused by enterohaemorrhagic Escherichia coli (EHEC), the major causes of haemorrhagic colitis and the life-threatening haemolytic uraemic syndrome (HUS) (4–7), is largely mediated by Shiga toxins (Stxs), which particularly injure microvascular endothelial cells in the kidneys, the brain and other organs (4, 5, 8, 9).

Stxs are AB5 toxins (10) composed of one A subunit of 32 kDa, which is the enzymatically active component of the toxin, and five identical B subunits (7.7 kDa each), which bind the cellular ligand glycosphingolipid globotriaosylceramide (Gb3Cer/CD77) (11). Stxs of EHEC consist of two major types, Stx1 and Stx2, which display 57% and 60% nucleotide sequence identity in their A and B subunits, respectively (12). The B subunits of Stx1 and Stx2 share identical binding specificity (13–15), and the A subunits identical N-glycosidase activity (16, 17).

After Stx release by the infecting EHEC in the intestine, the toxin is translocated across the gut epithelium into circulation (18), and transported to capillary endothelial cells. The mechanism of toxin delivery is still a matter of debate, although the role of polymorphonuclear leukocytes as a Stx carrier has been indicated (19, 20). Stx binds to the cell surface of endothelial cells and the AB5-Gb3Cer complex is internalised by various endocytic mechanisms (10, 21–23) and routed from the early endosomes through the trans-Golgi-network and the Golgi stacks to the endoplasmic reticulum and even to the nuclear envelope (24–27).
mechanism (28), the A subunit must be cleaved into the ∼27.5 kDa A1 and ∼4.5 kDa A2 fragments (29). In the cytosol, the catalytically active A1 fragment exerts its toxic function due to specific depurination of adenosine at a highly conserved loop position of the 28S rRNA of the 60S ribosomal subunit, which leads to the inhibition of protein synthesis and to the cell death (30–32). Additionally, binding of Stx1 to Gb3Cer was shown to induce activation of the Src family kinase Yes (33) and intracellular signals that mediate cytoskeleton remodelling (34). Recently, Fujii et al. found, that Stx2-induced apoptosis is mediated by C/EBP homologous protein (CHOP) (35). A growing body of evidence suggests that Stxs (like other ribosome-inactivating proteins) remove adenine moieties not only from rRNA, but also efficiently depurinate DNA. This effect leads to DNA damage in cultured cells and is likely to result from direct DNA-damaging activities and/or indirect DNA repair inhibition (31, 36–38) indicating the presence of more than one retrograde pathway (39).

Despite the subunit functional identity, Stx1 and Stx2 exhibit differential effects on various types of microvascular and macrovascular endothelial cells (40–44). However, none of the previous studies compared directly the effects of Stx1 and Stx2 using endothelial cells of different origin. Therefore, we performed a direct, comprehensive, and systematic comparison of cellular injuries induced by Stx1 and Stx2 on human brain microvascular endothelial cells (HBMECs) and macrovascular EA.hy 926 cells.

Materials and methods

Cell cultures

HBMECs (45) and human umbilical vein endothelial cell (HUVEC)-derived EA.hy 926 cells (46) were cultured at 37°C in a humidified atmosphere containing 5% CO2. HBMECs were maintained in RPMI 1640 medium (Lonza, Cologne, Germany) supplemented with 10% fetal calf serum (FCS) (PAAS, Pasching, Austria), 10% Nu-Serum (Becton Dickinson Biosciences, Bedford, MA, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 1.0 U/ml MEM non-essential amino acids, and 1.0 μg/ml vitamins (Lonza). EA.hy 926 cells were propagated in DMEM:F12 medium (Lonza) containing 10% FCS.

Purification of Stx1 and Stx2

Stx1 and Stx2 were purified from E. coli C600(H19) carrying the stx gene from E. coli O26:H11 strain H19 (47) and from E. coli C600(933W) carrying the stx gene from E. coli O157:H7 strain EDL933 (48), respectively. Briefly, bacteria grown overnight in Luria-Bertani broth were harvested by centrifugation, the pellets were homogenised by sonication (Bandelin Sonopuls, Berlin, Germany) and subjected to extraction with polymyxin B (3000 U/ml) (Sigma-Aldrich, Taukirchen, Germany). After centrifugation, the supernatant containing crude Stx was concentrated by ultrafiltration (Vivaflow 200, Sartorius StediM Biotech, Aubagne, France) and subjected to chromatography on hydroxyapatite column followed by chromatofocusing (49, 50) using an automated AktaPrime (all GE Healthcare, Munich, Germany). The column fractions containing Stx were pooled, dialysed against phosphate-buffered saline (PBS) and aliquots were stored at −70°C until use. Purity of the Stx1 and Stx2 preparations was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the structural integrity of each toxin was checked by peptide mapping employing mass spectrometry (15). The purified Stx1 and Stx2 had protein concentrations of 1.9 mg/ml and 7.6 mg/ml, respectively. The CD20 values of Stx1 (0.0085 ng/ml) and of Stx2 (0.072 ng/ml) were determined using the Vero cell cytotoxicity assay (51) performed as described below for endothelial cells (i.e. toxin incubation for 48 hours [h]).

The used doses of each Stx were free of lipopolysaccharides as assessed with the Limulus assay (Bactitmm, Nijmegen, the Netherlands; detection limit 0.006 ng/ml).

Cell proliferation assay

Metabolic activity used as an equivalent for cellular growth was assayed using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1 assay, Roche Diagnostics, Mannheim, Germany). Stx1– and Stx2-caused reduction of metabolism was determined using subconfluent HBMEC and EA.hy 926 cell monolayers. Indirect measurement of the cell growth was achieved by conversion of the soluble sulfonated tetrazolium salt into blue formazan by metabolically active cells. For the WST-1 assay, 5 x 104 HBMECs or 7.5 x 104 EA.hy 926 cells per well were seeded in 96-well tissue culture plates (Corning Inc., Corning, NY, USA) and incubated, after an 24 h attachment phase, with Stx1 or Stx2 at various concentrations in a final volume of 200 μl for 48 h at 37°C. Twenty μl WST-1 solution were subsequently added to each well for 2 h at 37°C. The absorbance, which directly correlates with the metabolic activity of the cells, was measured in a microplate reader at 450 nm against 600 nm reference wavelength (OptysMR absorbance reader, DYNE Technologies, Worthing, West Sussex, UK; software: Revelation Quicklink 4.24). Results represent the means (standard deviations) from four-fold determinations shown as percentage of untreated control cells. The half maximal inhibitory concentration (IC50) was defined as the Stx concentration needed to reduce metabolic activity to 50% of that of control cells. It was calculated by linear regression of the adjacent values.

Cytotoxicity assay

The cytotoxicity assay was performed in 96-well plates (Corning Inc.) seeded with 1 x 104/well of HBMECs or 1.5 x 104/well of
EA.hy 926 cells. After 24 h incubation, 100 μl of 10-fold dilutions of Stx1 or Stx2 preparation in cell culture medium (starting with concentration of 10 μg/ml) or 100 μl of the medium as a control were added to confluent cell monolayers and incubated for 48 h (37°C, 5% CO2). After removing the medium with detached cells, remnant adherent cells were fixed with 2% formalin in PBS (70 μl/well) for 5 minutes (min), stained with crystal violet (0.13% crystal violet, 5% ethanol, 2% formalin in PBS) for 1 h, washed and dried on air. The cell detachment was determined using light microscopy and subsequently quantified by eluting crystal violet with 50% ethanol (30 min) and measuring the optical density (OD) of the eluate at 570 nm (OpsysMR absorbance reader). Results represent the means (standard deviations) of eight-fold determinations depicted as a percentage of untreated control cells. The toxin concentration, which caused a cytotoxic effect in 50% of the cells, was defined as CD_{50} and was calculated by linear regression of the adjacent values.

**Protein synthesis inhibition assay**

The protein synthesis inhibition assay (Click-it AHA Alexa Fluor 488 Protein Synthesis HCS Assay, Invitrogen, San Diego, CA, USA) was performed in black 96-well plates with a transparent flat-bottom (Greiner bio-one, Frickenhausen, Germany) seeded with 1 x 10^4/well of HBMECs or 1.5 x 10^4/well of EA.hy 926 cells. After 24 h incubation, medium of confluent cell monolayers was exchanged for 100 μl of 10-fold dilutions of Stx1 or Stx2 in cell culture medium (starting with concentration of 1 μg/ml), or 100 μl of the medium alone (control) and the cells were incubated for 6 h (37°C, 5% CO2). After washing the cells with serum- and methionine-free RPMI 1640 medium (Gibco, Billings, MT, USA) [supplemented with 1 mM sodium pyruvate, 1.0 U/ml MEM non-essential amino acids, and 1.0 U/ml vitamins (Lonza)], the protein synthesis inhibition assay was performed according to the manufacturer’s instructions. Briefly, after culturing the cells in methionine-free

Figure 1: Different Stx1- and Stx2-caused growth inhibition of HBMECs (A) and EA.hy 926 cells (B). Cells were propagated in microtitre plates and subconfluent monolayers were exposed for 48 h to increasing concentrations of Stx1 or Stx2 as indicated. Cell viability was measured using the WST-1 assay. Results represent the means (standard deviations) from four-fold determinations shown as percentage of untreated control cells. IC_{50} values were calculated by linear regression of the adjacent values for HBMECs (Stx1: 5.95 ng/ml, Stx2: 0.73 ng/ml) and EA.hy 926 cells (Stx1: 6.76 ng/ml, Stx2: 3830 ng/ml). The horizontal black and white bars indicate a significant growth inhibition (p < 0.05) caused by the respective Stx. Significant differences (p < 0.05) between Stx1- and Stx2-mediated inhibition are marked with asterisks.
medium supplemented with the methionine-analogue L-azidohomoalanine (50 μM) for 30 min, the cells were fixed and permeabilised. Subsequently, the nascent azido-modified proteins were coupled with Alexa-Fluor 488-alkyne and detected utilising a microwell plate fluorescent reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) with appropriate filter sets. Resulting fluorescence values per well were averages from 10 independent measurements performed on a circle with 4 mm diameter. Results represent the means (standard deviations) from four independent determinations shown as percentage of untreated control cells. Cycloheximide (Sigma-Aldrich) in concentration of 1 μg/ml was used as a positive control.

Scanning electron microscopy (SEM)

HBMECs and EA.hy 926 cells were propagated on collagen-precoated Cytodex 3 microcarriers (GE Healthcare). Microcarriers covered with confluent HBMEC or EA.hy 926 cell monolayers were incubated in two independent experiments for 10 or 48 h with 500 ng/ml of Stx1 or Stx2, then washed gently with PBS (pH 7.2), fixed with 2.5% glutaraldehyde, washed with 0.1 M phosphate buffer according to Sørensen (pH 7.3) and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer. After dehydration in ethanol-gradient (30%, 50%, 70%, 90%, and twice absolute ethanol), the specimens were critical-point-dried, mounted on aluminium specimen stubs and sputter coated twice with 15 nm gold (52). SEM was performed with an S-450 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at 20 kV acceleration voltage using secondary electrons for imaging. The secondary electron micrographs were photographed from the photo-CRT of the SEM with a professional digital Nikon D200 camera (NIKON, Tokyo, Japan). The digital images were recorded with 3872 x 2592 pixels that are more than 10 Megapixels, which ensures that all generated information is recorded without any loss of resolution.

DNA fragmentation assay for determination of apoptosis and necrosis

A photometric enzyme immunoassay for the qualitative and quantitative determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) (Cell Death Detection ELISA PLUS, Roche Diagnostics) was employed to investigate Stx-induced apoptosis and necrosis in cell cultures. The DNA fragmentation assay was performed according to the manufacturer’s instructions with cell culture supernatants to detect DNA fragments released by cells, which underwent necrosis, and with lysed cells to determine the enrichment of mono- and oligonucleosomes in apoptotic cells. Specifically, after 48 h exposure of microtitre plate-grown confluent HBMEC and EA.hy 926 cell monolayers to increasing concentrations of Stx1 or Stx2, 20 μl aliquots of supernatants were withdrawn and examined for the occurrence of DNA fragments released from lysed cells to determine necrosis. Afterwards, 20 μl aliquots of cell lysate supernatants, obtained by 30 min incubation of the remaining adherent cells in lysis buffer, were used to quantify intracellular DNA fragments originating from apoptosis. After performing a sandwich ELISA against mono- and oligonucleosomes, the amount of peroxidase retained in the immunocomplex directly correlates with the content of DNA fragments in cell culture supernatants and lysates. This amount of peroxidase was quantified in a microplate reader at 405 nm against 600 nm as reference wavelength using 2,2’-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate. Quadruple wells were examined for each experimental condition. Background values (incubation buffer alone) were subtracted, and OD values of Stx-treated cells were normalised against those from untreated control cells to obtain an enrichment factor for apoptotic and necrotic cells in the culture.

Flow cytometric determination of apoptosis and necrosis

The annexin-V-FLUOS staining kit (Roche Diagnostics) was used for quantification and differentiation of apoptosis and necrosis at a single cell level. HBMECs and EA.hy 926 cells were seeded in 12-well plates (Greiner bio-one) and grown (37°C, 5% CO2) until confluence. After 24 h of incubation the medium was exchanged for 1 ml of 10-fold dilutions of Stx1 or Stx2 in cell culture medium (starting with concentration of 1 μg/ml), or 1 ml of medium containing 1 μM staurosporin (Sigma-Aldrich) as apoptosis control, or 1 ml of medium alone (negative control) and incubated for 48 h (37°C, 5% CO2). After collecting the medium with detached cells, remnant adherent cells were dissociated using Accutase (PAA) and

Table 1: Synopsis of Stx1- and Stx2-caused effects on microvascular (HBMECs) and macrovascular (EA.hy 926) cells.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cell line</th>
<th>Shiga toxin 1 (ng/ml)</th>
<th>Shiga toxin 2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 a</td>
<td>HBMECs</td>
<td>5.95</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>EA.hy 926 cells</td>
<td>6.76</td>
<td>3830</td>
</tr>
<tr>
<td>CD50 b</td>
<td>HBMECs</td>
<td>8.38</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>EA.hy 926 cells</td>
<td>0.71</td>
<td>260</td>
</tr>
<tr>
<td>Apoptosis c</td>
<td>HBMECs</td>
<td>≥ 0.01</td>
<td>≥ 0.0001</td>
</tr>
<tr>
<td></td>
<td>EA.hy 926 cells</td>
<td>≥ 0.1</td>
<td>≥ 10</td>
</tr>
<tr>
<td>Necrosis d</td>
<td>HBMECs</td>
<td>≥ 1 *</td>
<td>≥ 10</td>
</tr>
<tr>
<td></td>
<td>EA.hy 926 cells</td>
<td>≥ 0.0001</td>
<td>≥ 1000</td>
</tr>
</tbody>
</table>

a 50% growth inhibitory concentration obtained by WST-1 proliferation assay (Fig. 1). b 50% cytotoxic dose determined by cytotoxicity assay (Fig. 2). c Stx concentration required for significant induction of apoptotic response (p < 0.05) measured by DNA-fragmentation (Fig. 5). d Stx concentration required for significant induction of necrotic response (p < 0.05) measured by DNA-fragmentation (Fig. 6). * only some lower concentrations induced significant necrosis.
washed with PBS, whereas untreated cells were washed with PBS containing 0.2% Triton X-100 as a control for the necrosis staining. As a modification to the supplier’s protocol, 1 x 10^6 cells were sequentially stained by annexin-V-fluorescein and 20 μl 7-aminoactinomycin D (7-AAD, Beckman Coulter) instead of propidium iodide to avoid electronic compensation resulting from the overlapping of the emission spectra. After forward scatter-side scatter gating for the exclusion of debris, the data from 1 x 10^4 cells were collected and analysed using a Beckman Coulter Cytomics FC500 (Becton Dickinson Biosciences). The percentages of all cells within the cell type-specific gates for apoptotic and necrotic cells were normalised against those from negative control to calculate an enrichment factor. Two independent experiments were performed.

Digital holographic microscopy (DHM)

An inverse microscope (iMIC, Till Photonics, Gräfelfing, Germany) with attached digital holographic microscopy module (engineered at the Center for Biomedical Optics and Photonics, Münster, Germany) (53) and an incubator (Solent Scientific Ltd., Segensworth, UK) for stabilised temperature was applied for digital holographic phase contrast imaging of Stx-treated single HBMECs and EA.hy 926 cells. The coherent light source for the recording of digital holograms was a frequency doubled neodymium-doped yttrium aluminium garnet (Nd:YAG) laser (Compass 315M-100, Coherent, Lübeck, Germany, λ = 532 nm). The experiments were performed in μ-dishes (ibidi GmbH, Martinsried, Germany) seeded with 3.2 x 10^4 cells, which then were exposed to 500 ng/ml of Stxs at 37°C, and digital holograms of single cells were recorded continuously every 3 min. The reconstruction of the di-

Figure 2: Differential cytotoxicities of Stx1 and Stx2 towards HBMECs (A) and EA.hy 926 cells (B). Microtitre plate-grown monolayers were treated for 48 h with Stx1 or Stx2 in indicated concentrations and cytotoxicity was quantified by measuring crystal violet absorption of remaining adherent cells. Results represent the means (standard deviations) of eight-fold determinations depicted as percentage of untreated control cells. CD_{50} values were calculated by linear regression of the adjacent values for HBMECs (Stx1: 8.38 ng/ml, Stx2: 0.1 ng/ml) and EA.hy 926 cells (Stx1: 0.71 ng/ml, Stx2: 260 ng/ml). The horizontal black and white bars indicate the Stx-caused significant cytotoxicity (p < 0.05) caused by the respective Stx. Significant differences (p < 0.05) between cytotoxicities of Stx1 and Stx2 are marked with asterisks.
Figure 3: Morphological changes of HBMECs caused by Stx1 and Stx2. Cells were grown on collagen-coated microcarriers and cellular injury was investigated by SEM. Confluent monolayers (Aa-Ad, controls) were exposed for 10 h and 48 h to 500 ng/ml of Stx1 (Ba-Bd and Ca-Cd, respectively) or 500 ng/ml of Stx2 (Da-Dd and Ea-Ed, respectively). The “a” panels show the microcarrier overview screens and the “b” panels examples of corresponding higher magnified partial views of the same microcarriers. The “c” and “d” panels of the A to E series show partial views of microcarriers from parallel cell cultures incubated under identical conditions. Bars represent 50 μm (SOU) or 5 μm (SU) as indicated in the micrographs. Original electron-optical magnifications of the microcarrier overview screens are x 870 (Aa), x 970 (Ba and Ca), x 950 (Da), and x 980 (Ea). Magnifications of partial detail views are x 4500 of panels A to E with exception of Cb (x 4400).

Cell cycle analysis

HBMECs and EA.hy 926 cells were seeded in T-75 tissue flasks (Greiner bio-one) and grown (37°C, 5% CO₂) until 50% confluence. After adding 500 ng/ml Stx1, Stx2 or cell culture medium (control) for 48 h, cells were detached using 5 mM EDTA in serum-free medium for 30 min. The cells were then washed with PBS and fixed with 70% ethanol in PBS (-20°C, 2 h). After washing with PBS the cells were resuspended in Nicoletti buffer (0.1% Triton X-100, 0.1% sodium citrate, 50 μg of propidium iodide/ml, 20 μg of RNase/ml) (57). After incubation on ice (30 min), the DNA content of the nuclei was determined by flow cytometry (Beckman Coulter Cytorics FC500, Becton Dickinson Biosciences). After forward scatter-side scatter gating for the exclusion of debris and doublets, the data from 1 x 10⁶ nuclei were collected and analysed.

Statistical analysis

The statistical analysis of results was performed using Student’s t-test; p = 0.05 was considered significant.

digitally captured holograms was performed by non-diffractive reconstruction (54, 55) with software developed at the Center for Biomedical Optics and Photonics. The resulting quantitative digital holographic phase contrast images were used to measure the cells’ shape and thickness (56). For DHM, the cells were cultured in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered medium. Three independent single cell analyses were performed for each cell type with each toxin, and representative measurements are shown in the results.
Results

To investigate whether or not Stx1 and Stx2 differentially injure microvascular (HBMECs) and macrovascular (EA.hy 926) endothelial cells, we performed growth, cytotoxicity, protein biosynthesis, apoptosis and necrosis assays, scanning electron microscopy (SEM), and digital holographic microscopy (DHM).

Differences in Stx1- and Stx2-mediated growth inhibition of endothelial cells

To monitor the growth of HBMECs and EA.hy 926 cells treated with Stx1 and Stx2, subconfluent monolayers were exposed to increasing doses (0.1 ng/ml to 10 μg/ml) of each toxin and assayed for metabolic activity using the WST-1 assay. Stx1 and Stx2 caused dose-dependent growth inhibition of each endothelial cell line (Fig. 1). The half maximal inhibitory concentration (IC$_{50}$) of Stx1 was similar for HBMECs (5.95 ng/ml) and for EA.hy 926 cells (6.76 ng/ml), but the IC$_{50}$ values of Stx2 for the respective cell lines were 0.73 ng/ml and 3.83 μg/ml, i.e. more than three orders of magnitude lower for HBMECs (Table 1). This demonstrates that Stx1 has a similar growth inhibitory potential against both cell lines, but Stx2 is > 1,000 times less potent against EA.hy 926 than against microvascular endothelial cells.

Differential cytotoxicities of Stx1 and Stx2 towards endothelial cells

Because Stxs are cytotoxins (30), we next investigated if the differences in the IC$_{50}$ values result from different cytotoxicities of Stx1 and Stx2. Both Stx1 and Stx2 were cytotoxic towards HBMECs and EA.hy 926 cells in a dose-dependent manner (Fig. 2). However, Stx1 was significantly more toxic than Stx2 to EA.hy 926 cells over the whole span of applied concentrations except for the uppermost
Inhibition of protein biosynthesis by Stx1 and Stx2

The protein biosynthesis inhibition caused by Stx1 and Stx2 (0.1 ng/ml to 1 μg/ml) on HBMECs and EA.hy 926 cells was quantified by fluorescence-labelling of nascent proteins after 6 h of toxin treatment. The maximal inhibition of protein biosynthesis was higher in HBMECs (reduction by ~50%) than in EA.hy 926 cells (reduction by ~25%), but no difference between Stx1 and Stx2 could be observed (see Suppl. Fig. 2 available online at www.thrombosis-online.com).

<table>
<thead>
<tr>
<th>Morphological changes in</th>
<th>Cell line</th>
<th>Shiga toxin 1</th>
<th>Shiga toxin 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual cells b</td>
<td>HBMECs</td>
<td>Loss of microvilli</td>
<td>Membrane blebbing</td>
</tr>
<tr>
<td></td>
<td>EA.hy 926 cells</td>
<td>Loss of microvilli</td>
<td>Membrane blebbing</td>
</tr>
<tr>
<td>Monolayers c</td>
<td>HBMECs</td>
<td>Intercellular gaps Cell detachment</td>
<td>Irregular shape</td>
</tr>
<tr>
<td></td>
<td>EA.hy 926 cells</td>
<td>Intercellular gaps Cell detachment</td>
<td>None</td>
</tr>
</tbody>
</table>

*Altered morphology of individual cells and disintegration of cell layers were determined for HBMECs (Fig. 3) and EA.hy 926 cells (Fig. 4) by SEM. b Stx1-mediated necrotic loss of microvilli and membrane lesions were more prominent in EA.hy 926 cells than in HBMECs; the cell flattening was not detectable in HBMECs. Stx2-induced apoptotic membrane blebbing was more pronounced in HBMECs compared to EA.hy 926 cells. c Stx1-caused intercellular gaps and cell detachment were more distinct in EA.hy 926 compared to HBMEC monolayers.

Table 2: Stx1- and Stx2-caused morphological changes of HBMECs and EA.hy 926 cells.

Stx1 and Stx2 differ in apoptotic and necrotic potentials

To quantify the apoptosis- and necrosis-inducing capabilities of Stx1 and Stx2, we used a DNA fragmentation assay. For this purpose, endothelial cell monolayers grown on a microtiter plate were exposed for 48 h to increasing concentrations (0.1 pg/ml to 1 μg/ml) of Stx1 or Stx2. Nucleosomes released into the cell culture medium and intracellular nucleosomes (markers for necrosis and apoptosis, respectively) were quantified. The increase of the nucleosome concentration after Stx treatment was expressed as enrichment factor compared to untreated control to normalise the influence of the different natural apoptosis and necrosis rates of HBMECs and EA.hy 926 cells (the latter possess a higher natural apoptosis/necrosis rate).

As shown in Figure 5, both Stx1 and Stx2 induce apoptosis in each cell line, but to different extents. Stx1 induces a significant apoptosis at concentrations of ≥ 0.01 ng/ml and ≥ 0.1 ng/ml in HBMECs and EA.hy 926 cells, respectively. In contrast to this similar apoptotic potential of Stx1 against each cell line,
there are striking differences in apoptotic potential of Stx2. Specifically, whereas in HBMECs Stx2 causes apoptosis over the whole concentration spectrum (≥ 0.1 pg/ml), EA.hy 926 cells undergo apoptosis after exposure to 10^5-times higher concentrations of Stx2 (≥ 10 ng/ml) (Fig. 5B, Table 1).

The necrosis assay demonstrated that the major toxin inducing necrosis in both cell lines is Stx1 (Fig. 6A, B), which caused a significant release of oligonucleosomes from HBMECs in most and from EA.hy 926 cells in all concentrations used. In contrast, no significant necrosis could be detected in any of the cell lines after treatment with low concentrations of Stx2 (Fig. 6A, B).

Taken together, the apoptosis/necrosis assays demonstrate that Stx1 causes both apoptosis and necrosis through the whole range of concentrations used, whereas Stx2 preferentially causes apoptosis and only the highest toxin concentrations also induce necrosis. These data agree with morphological observations by SEM (Figs. 3 and 4).

Moreover, they confirmed results obtained with apoptosis and necrosis assays performed with flow cytometry of annexin-V-fluorescein- and 7-AAD-stained cells (see Suppl. Fig. 3 available online at www.thrombosis-online.com). In this assay we also detected apoptosis-inducing capabilities of Stx1 and Stx2 in both cell lines. Specifically, similar as in the DNA fragmentation assay (Fig. 5), both toxins induced apoptosis on HBMECs to the same extent, whereas EA.hy 926 cells showed also regarding apoptosis induction lower sensitivity to Stx2 (see Suppl. Fig. 3 A and B available online at www.thrombosis-online.com). Additionally, necrosis determined as presence of 7-AAD- and annexin-V-positive cells was almost exclusively induced by Stx1 in EA.hy 926 cells (see Suppl. Fig. 3D available online at www.thrombosis-online.com). However, there was a less pronounced difference between Stx1 and Stx2 effects on HBMECs in contrast to the DNA fragmentation assay. This effect could result from low amounts of intact cells gated (see Suppl. Fig. 3C available online at www.thrombosis-online.com).
sis-online.com) due to Accutase treatment (and thus potential damage of cells presensitised by Stxs) necessary to get the cells in suspension for flow cytometry. Both assays were able to detect apoptosis/necrosis induction triggered by Stx concentrations ≥ 0.1 pg/ml.

Investigation of Stx1- and Stx2-caused cell death on single cell level

To verify the different mechanisms of the Stx1 and Stx2 cytotoxicities and to gain a deeper insight into the dynamics of the cell death induced by each toxin, individual HBMECs and EA.hy 926 cells were analysed upon toxin exposure using quantitative DHM phase contrast, which permits non-destructive, marker-free, and quantitative long-term investigations of living cells.

For a HBMEC exposed to Stx1 (Fig. 7, left panels), the first reaction observed was the cell rounding after 20 h followed by an increase of the cell thickness by up to 50% after 35 h (Fig. 7C, left panel), with simultaneously decreased intracellular fluctuations. After 45 h the maximal cell thickness started to decrease as a sign of progressive cell disintegration. Finally, the cell death, accompanied by an extensive cell leakage, occurred after ∼51 h of Stx1 treatment (Fig. 7A, left panel). Neither signs of necrosis nor other morphological changes could be observed in any of three independent experiments performed with Stx2 even after a prolonged exposure time up to 65 h (Fig. 7B, right panels), indicating its lesser necrotic action compared to Stx1. However, no cell division occurred during the long-term cultivations with Stx2. The fact that Stx2 obviously prevented cells from entering mitosis might be explained by two different mechanisms: Stx2 could arrest the cell cycle, but the cell cycle analysis utilising FACS analysis excluded this possibility (data not shown). Therefore we interpret the absence of cell division with Stx2.

Figure 6: Necrotic responses of HBMECs (A) and EA.hy 926 cells (B) to Stx1 and Stx2. Microtitre plate-grown confluent monolayers were treated for 48 h with indicated concentrations of Stx1 or Stx2. The content of nucleosomes released into the cell culture supernatant served as a measure of necrosis. The supernatants were withdrawn from the same cell cultures whose lysates were used in the apoptosis assay (Fig. 5). Results are presented as the means (standard deviations) of four independent experiments and are expressed as "enrichment factors", which were calculated by dividing the necrotic value of the toxin-treated cells by the necrotic value of control cells. The horizontal black and white bars indicate significant necrosis (p < 0.05) induced by Stx1 and Stx2. Significant differences (p < 0.05) between necrosis induced by Stx1 and Stx2 are marked with asterisks.
division as apoptosis (demonstrated also by SEM and apoptosis assay), that cannot be directly visualised by DHM.

Initial Stx1-mediated cell rounding of an EA.hy 926 cell occurred already after 3 h and the cellular impairment progressed rapidly (Fig. 8, left panels). After a peak in the cell thickness at 8 h, the necrotic death with cell burst and cytoplasm release was observed after 20 h (Fig. 8A, left panel). Only slight morphological changes, such as a slowly progressive cell thickness, were visible after treatment of an EA.hy 926 cell with Stx2 at prolonged incubation time up to 65 h (Fig. 8, right panels). However, signs of necrosis were not apparent. Interestingly, while no cell division of the single cell under investigation occurred (a sign of apoptosis), an adjacent cell entered mitosis and divided into daughter cells as shown in Figure 8A (right panel, t = 64 h). These findings are in accordance with results of SEM and apoptosis assay, where EA.hy 926 cells showed also low susceptibility to Stx2. However, the M-phase in the dividing cell was abnormally prolonged (~6 h compared to ~0.5 h in control, data not shown).

Taken together, Stx1 induced cell swelling followed by necrotic cell death, which occurred earlier in macrovascular than in microvascular cells. In contrast, Stx2 induced no necrotic effects in any of the endothelial cell types, but prevented cells from cell division, which is caused by apoptosis.

Discussion

The new approach of visualising Stx-induced damage of endothelial cells using SEM of microcarrier-based cultures provides several advantages. The cells can be grown on a biocompatible collagen-covered surface in a pseudo-suspension culture under constant conditions regarding nutrition, pH, temperature and partial oxygen pressure. Moreover, the possibility to apply various extent of shear stress on the three-dimensional mini-monolayers enables to simulate the conditions during infection. To complement and expand the information gathered by analysis of the monolayers, we further applied quantitative DHM phase contrast to investigate effects of Stxs on living single cells. This marker-free and non-invasive imaging technique with the potential to visualise and quantify temporal cellular responses allowed us to monitor the whole sequence of morphological changes of single cells from the toxin exposure until the cell death, and to determine the mechanism of the death. Using these powerful visualisation techniques we verified and expanded the data obtained by the classical methods.

This study presents several new findings. First, we demonstrate that the protein synthesis is only partially inhibited at the highest toxin concentrations employed. This suggests that none of the measured parameters, i.e. metabolic inhibition, cell death and morphological changes may be directly related to generalised protein synthesis inhibition. Moreover, although no differences could be observed between Stx1 and Stx2 regarding the inhibition of protein biosynthesis, we found different mechanisms of Stx1 and Stx2 toxicities. Stx1 induces both, apoptosis and necrosis, while Stx2 induces almost exclusively apoptosis in both endothelial cell lines investigated. This conclusion is undoubtedly supported by results of SEM of mini-monolayers, DNA-fragmentation assay and DHM of single cells. Although the flow cytometric analysis of the toxin-treated cells appears to indicate that Stx2 causes a similar degree of necrosis as Stx1 in HBMECs, this effect results highly probably...
from the sample preparation procedure, which is designed for suspension cells. Specifically, enzymatic detachment of cells presensitized by Stxs led in case of HBMECs to cell injury and may result in false positive necrosis detection.

The different Stx1- and Stx2-induced signalling pathways leading to apoptosis in cells from various origins have been partially clarified (reviewed in [58]). Most notably, Fujii et al. demonstrated that the apoptotic mechanism of Stx2 on HBMECs includes DNA fragmentation and cleavage of caspase 3, 6, 8 and 9, which is mediated by the CHOP upregulation (35). Stx1 also induced apoptosis in HBMECs, but the underlying mechanisms were not investigated (59). Similarly, although we demonstrate that Stx1 additionally induces necrosis in microvascular and macrovascular endothelial cells, the underlying mechanism(s) of the necrotic action remains unclear. Neither an inhibition of protein biosynthesis nor toxin-induced DNA damage seems to explain the sudden necrotic response of the cells to Stx1 ([Figures 7 and 8]). Moreover, neither the RNA cleavage resulting in inhibition of protein biosynthesis nor the induction of apoptosis via different pathways (58) can explain the heterogeneity of cellular responses caused by Stx1 and Stx2 and the distinct cell death occurring in both endothelial cell types. Thus, we postulate yet unknown mechanism(s) of the endothelial cell injury mediated by these toxins that require further investigation.

The second significant finding is the differential susceptibility of microvascular and macrovascular endothelial cells to Stx1 and Stx2. We demonstrate that both cell types react similarly in the growth inhibition and cytotoxicity assays to Stx1, but strikingly differ in their susceptibilities to Stx2 (Table 1). Specifically, comparison of the susceptibilities of the cell lines investigated to each Stx demonstrates that EA.hy 926 cells are ~10-times more affected by Stx1 than HBMECs, whereas HBMECs are >1,000-times more susceptible to Stx2 compared to EA.hy 926 cells (Table 1). Thus, the macrovascular endothelial cells are relatively resistant, whereas microvascular cells are extremely susceptible to Stx2. This finding parallels observations on glomerular microvascular endothelial cells (41). Although we have previously shown that EA.hy 926 cells express a higher level of Gb3Cer than HBMECs (60), this is probably not the only reason for the higher susceptibility of EA.hy 926 cells to Stx1. This is indicated by the fact that HBMECs, which have lower Gb3Cer content than EA.hy 926 cells (60), are substantially more susceptible to Stx2, which shares the receptor specificity with Stx1 (13–15). However, it should be noticed that EA.hy 926 cells are a fusion line (46) and generalisations for natural macrovascular cells are therefore limited.

The density of the accessible receptor on the cell surface does not directly correlate with the internalisation of the toxin (61). The newly discovered uptake mechanism of Stxs (23) indicates that for an efficient internalisation of the toxins the receptor conformation is important, which depends on the chemical characteristics of the Gb3Cer molecule (e.g. fatty acid composition and hydroxylation) (58, 62–65). Also, Stx2 and Stx1 differ in their stability within endosomes that transport the toxins to the Golgi complex (66). Altogether these studies suggest that the basis for the striking difference between the susceptibility of HBMECs to Stx1 and Stx2 might involve yet unknown differences in the internalisation and/or intracellular trafficking of these toxins (67).

The difference in the susceptibilities of the microvascular and macrovascular endothelial cells to Stx1 and Stx2 might be relevant...
to the pathogenesis of HUS. In particular, it allows us to hypothesise that the microvascular endothelial damage underlying HUS (68) is mainly triggered by Stx2. This is consistent with epidemiological studies which have shown that the risk of HUS development after an EHEC infection is significantly associated with the presence of Stx2 in the infecting strain (69, 70). Moreover, this is supported by studies using a baboon model of HUS (71), where intravenous administration of Stx2 triggered clinical and histopathological signs of HUS, whereas the same dose of Stx1 had no such effects. The greater susceptibility of microvascular endothelial cells to Stx2 combined with the fact that Stx2 mainly causes apoptosis on these cells, suggest that Stx2-induced apoptosis might be the major mechanism responsible for the damage of the microvascular endothelium during EHEC infection.

The third element of our data is that even within the family of endothelial cells there are big differences concerning the susceptibilities to Stxs. There is a growing body of evidence that Stxs’ bind- ings, internalisation, intracellular processing, and cytotoxic action are dependent on multiple cellular parameters (58), which differ for various cell types and tissues. This should be kept in mind while interpreting results of Stx studies performed with non-endothelial cells.

In summary, Stx1 and Stx2 differ in toxicities to microvascular and macrovascular endothelial cells, and these cells differ in susceptibilities to each toxin. Mechanisms underlying these differences need to be clarified.

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