Responses of Human Intestinal Microvascular Endothelial Cells to Shiga Toxins 1 and 2 and Pathogenesis of Hemorrhagic Colitis

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Enteric infection with Shiga toxin (Stx)-producing Escherichia coli (STEC) is associated with bloody diarrhea, often presenting as a characteristic clinical syndrome, hemorrhagic colitis (HC). STEC infections can lead to the development of hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (10, 16). The pathogenesis of HC, HUS, and TTP is characterized by a thrombotic microangiopathy related to endothelial damage. This damage is believed to be due to circulating bacterial exotoxins (Stxs), endotoxins, and cytokines related to endothelial damage. This damage is believed to be due to circulating bacterial exotoxins (Stxs), endotoxins, and cytokines related to endothelial damage. The increase in surface expression of the Stx receptor that leads to enhanced sensitivity to the toxins. Although the binding capacities of HIMEC for Stx1 and Stx2 were comparable, the binding affinity of Stx1 to HIMEC was 50-fold greater than that of Stx2. Nonetheless, Stx2 was more toxic to HIMEC than an equivalent amount of Stx1. The decreased binding affinity and increased toxicity for HIMEC of Stx2 compared to those of Stx1 may be relevant to the preponderance of Stx2-producing STEC involved in the pathogenesis of hemorrhagic colitis and its systemic complications. The differences between primary and transformed HIMEC in these responses were negligible. We conclude that transformed HIMEC lines could represent a simple physiologically relevant model to study the role of Stx in the pathogenesis of hemorrhagic colitis.

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MATERIALS AND METHODS

Toxin purification, iodination, and assay. Stx1 was purified from cell lysates of E. coli HB101-119B, an STEC expressing Stx1 only. Stx2 was obtained from the cecal carcinoma of the E. coli C600/S10 mouse with bacteriophage 9332. Both toxins were purified by affinity chromatography on a P1 blood group glycoprotein-Sepharose 4B column, as previously described (5).

Toxins were iodinated by incubating 10 μg of toxin in 0.2 M potassium phosphbate (pH 7.5) containing 1.0 μCi of 125I-labeled Bolton-Hunter reagent (ICN, Costa Mesa, Calif.) at 0°C with rocking, and the incubation was stopped after 1 h with 20 μl of 1 M glycine. Iodinated toxins, purified by Sephadex G-25 chromatography, retained full biological activity (as shown by cytotoxicity assay) and had radioactivity specific activities between 10,000 and 20,000 cpm/ng of protein.

Cytotoxicity was measured as the inhibition of protein synthesis in toxin-treated cells according to our previously published methods (13). Target cells were grown in 96-well plates at 37°C and incubated for 24 h with serial 10-fold dilutions of Stx1 or Stx2 or medium alone. [3H]leucine (1 μCi/100 μl) was added for 30 min, and the percent inhibition of incorporation of label into trichloroacetic acid-precipitable protein was measured. Cytotoxicity was expressed as the amount of toxin needed to inhibit leucine incorporation by 50% (TLC50). In some experiments, primary human intestinal microvascular EC (HIMEC) were incubated for 72 h with 40 μM of t-tre-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-SCN (PDMP), an inhibitor of neutral glycolipid synthesis which reduces Gb3 content of cells (12).

Binding of 125I-labeled Stx was measured following exposure of cells to labeled toxin for 1 h at 4°C, as previously described (15). Data were subjected to Scatchard analysis to determine the binding affinity and the number of binding sites per cell for each toxin and cell line. To assess movement of bound toxin from the cell surface, antibody rescue experiments were performed. HIMEC monolayers in 96-well plates were treated with Stx1 or Stx2 (100 pg/ml) at 4°C for 1 h and washed, fresh medium at 37°C was added, and the cells were transferred to a 37°C incubator. At times varying from 0 to 120 min the culture medium was changed (temperature shift), an excess of specific polyclonal Stx1 or Stx2 neutralizing antibody was added. [125I]leucine incorporation by the monolayers was measured after an overnight incubation at 37°C, as described above.

Isolation and maintenance of HIMEC. HIMEC were isolated from mucosal strips of intestine from surgically resected intestinal specimens from one patient by collagenase treatment and mechanical compression, as previously described (4). A transformed cell line was established from intestinal tissues from a second patient, treated with the Linker CMV retroviral construct, which encodes the simian virus 40 large T antigen and neomycin phosphotransferase enzyme (3). The cells were maintained in fibronectin-coated 75-cm2 flasks in MDCB 131 medium (Sigma) supplemented with 20% heat-inactivated fetal bovine serum, 2.5% (vol/vol) penicillin-streptomycin, heparin (50 μg/ml) (all from Gibco-BRL), and EC growth factor (50 μg/ml; Boehringer Mannheim). For all assays, cells were grown in fibronectin-coated 96-well microtiter plates and used when confluent.

Isolation and maintenance of HSVEC. Human saphenous vein microvascular EC (HSVEC) were isolated by collagenase treatment of discarded saphenous vein segments after coronary bypass operations. Cells were maintained in gelatin-coated 75-cm2 flasks in medium 199 supplemented with 10% fetal bovine serum, 10% (vol/vol) penicillin-streptomycin, heparin (50 μg/ml) (all from Gibco-BRL), and retinoic acid-derived growth factor extracted from bovine retinas. For all assays, cells were grown in gelatin-coated 96-well microtiter plates and used when confluent.

Measurement of Gb3 content of cells. Total cellular Gb3 was measured by high-performance liquid chromatography of extracts of 75-cm2 dishes of cells, as described previously (14). Neutral glycolipids were isolated, benzoylated, and analyzed on a pellicular Zipax column (DuPont, Wilmington, Del.) by eluting with a gradient of 2 to 40% dioxane-hexane (46:54, vol/vol) in hexane at a flow rate of 2 ml/min. Eluted peaks were detected by absorption at 230 nm and analyzed with System Gold software (Beckman Instruments, San Ramon, Calif.).

Measurement of markers of cell activation. Cell adhesion molecules—intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin—were analyzed by 125I-labeled antibodies for HIMEC and HSVEC. Triplicate wells in 96-well microtiter plates were pretreated with medium containing different concentrations of LPS, TNF-α, IL-1β, Stx1 or Stx2, or medium alone for 24 h. Adhesion molecules expressed on the cell surface were measured by a modified enzyme-linked immunosorbent assay (ELISA). Cells were washed with phosphate-buffered saline (PBS) and fixed with 100 μl of 1 M HCl for 20 min, blocked with 200 μl of 1% gelatin in PBS (blocking solution) for 2 h, and treated with 50 μl of either anti-human VCAM-1 (1/2,000 dilution of 1 mg/ml; Endogen, Woburn, Mass.), anti-human ICAM-1 (1/500 dilution of 40 μg/ml; T Cell Diagnostics, Inc., Cambridge, Mass.), or anti-human E-selectin (1/2,000 dilution of 1 mg/ml; R&D Systems, Abingdon, United Kingdom) per well. As all three antibodies were murine immunoglobulin G (IgG) antibodies, a negative control of mouse IgG was included (DAKO Corp., Carpinteria, Calif.). Bound antibody was detected by the addition of 50 μl of peroxidase-conjugated anti-mouse IgG (1/6,000 dilution of 1 mg/ml; Promega, Madison, Wis.) for 1 h and developed with 100 μl of tetramethylbenzidine reagent (DAKO) per well for 10 to 30 min, but the color development time on a single plate was 30 min. All antibodies were preincubated in blocking solution, and the monolayers were washed five times with 200 μl of PBS-0.1% gelatin after each incubation. The reaction was stopped with 100 μl of 1 M HCl, and the A450 was measured on an ELISA plate reader, with wells treated with the negative control antibody considered blanks. Cells were considered to be activated when the A450 per cell in treated wells was significantly higher than in the corresponding medium-only wells. Cell number was determined by hemocytometer counting at a confidence level of 1% when subjected to Student’s t test.

Measurement of IL-8. IL-8 was measured in culture supernatants with a commercially available kit (Endogen), used according to the manufacturer’s instructions.

RESULTS

Expression of adhesion molecules and IL-8. ICAM-1 and VCAM-1 were expressed on the surface of resting cells of all three lines (Fig. 1), and their expression increased significantly (P < 0.001) 24 h following activation by TNF-α, IL-1β, or LPS and did not increase further at 48 h. E-selectin was not detected on resting cells but was expressed at high levels in all three lines following activation and was expressed maximally in the presence of either LPS or the combination of TNF-α and IL-1β. There was no evidence of activation when cells were incubated with Stx1 or Stx2; on the contrary, expression of ICAM-1 and VCAM-1 decreased in toxin-treated primary (Fig. 1A, treatments 6 and 7) and transformed (Fig. 1B, treatments 6 and 7) HIMEC (P < 0.001). Toxin treatment did not significantly affect expression of adhesion molecules on HSVEC (Fig. 1C, treatments 6 and 7). VCAM-1 and ICAM-1 decreased to lower levels on all cell lines pretreated with TNF-α and/or IL-1β and toxin (Fig. 1, treatments 7 and 8). In the presence of both TNF-α and IL-1β, inhibition of HIMEC adhesion molecule was greater for Stx1 (Fig. 1A and B, treatment 9) in HIMEC (P < 0.01).

IL-8 production was measured in the supernatant medium of the same primary HIMEC monolayers used to measure adhesion molecules. IL-8 levels were greatly increased in TNF-α-treated cells compared to untreated cells (72.82 and 2.93 ng/105 cells, respectively) and were increased to an even greater extent in cells pretreated with TNF-α and IL-1β (180.3 ng/105 cells). Overnight exposure to toxin of cells exposed to TNF-α and IL-1β resulted in a sharp decrease in IL-8 production (40.33 and 59.61 ng/105 cells in the presence of Stx1 and Stx2, respectively).

Receptor glycolipid levels in EC lines. The total Gb3 content of resting confluent EC was determined once for each cell line. Gb3 content was similar in the primary and transformed lines (3.40 and 5.96 ng/105 cells) per well of peroxisome-conjugated antibodies and with the negative control antibody considered blanks. Cells were considered to be activated when the A450 per cell in treated wells was significantly higher than in the corresponding medium-only wells. Cell number was determined by hemocytometer counting at a confidence level of 1% when subjected to Student’s t test.

Binding of iodinated Stx1 and Stx2 to EC. Binding of iodinated Stx1 and Stx2 to EC is shown in Fig. 2. 125I-labeled Stx1 bound to all cells to a much greater extent than did 125I-labeled Stx2. Both toxins bound to unactivated HIMEC at much higher levels than to unactivated HSVEC, and the primary HIMEC bound more Stx1 and Stx2 than the transformed lines. Pretreatment of either HIMEC line with TNF-α or IL-1β failed to increase toxin binding capacity, whereas toxin binding to cyto-activated HSVEC increased almost to the levels found on HIMEC. Binding capacity and affinity parameters for both toxins and the three cell lines are shown in Table 1. The number of binding sites per cell was approximately the same for each toxin for a given cell line, although the binding capacity of HIMEC was 10-fold greater for both toxins compared to those of HSVEC. The binding affinity of Stx1 was 50-fold greater than the binding affinity of Stx2 for both HIMEC lines and for HSVEC.
Sensitivity of cells to Stx. Both primary and transformed HIMEC were highly sensitive to both Stx1 and Stx2 (Fig. 3). Both confluent and subconfluent (data not shown) HIMEC monolayers were equally toxin sensitive. A consistent finding was that both cell lines were more sensitive to Stx2 than to Stx1. Primary cells were more sensitive to both toxins than the transformed cells; thus, the TI50 for Stx1 was $10^{-2}$ and $10^{-3}$ ng/ml for Stx2 in primary cells compared to $10^{-3}$ ng/ml for Stx1 and $10^{-4}$ ng/ml for Stx2 in transformed HIMEC. Both cell lines appeared to be fully sensitized, and activation by incubating with TNF-$\alpha$ (2 ng/ml)–IL-1$\beta$ (2 ng/ml) overnight did not increase the cytotoxicity response of HIMEC to either toxin.

**TABLE 1.** Binding parameters of Stx to untreated EC

<table>
<thead>
<tr>
<th>Cell line (toxin)</th>
<th>Binding affinity (M$^{-1}$)</th>
<th>No. of binding sites/cell</th>
</tr>
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<tbody>
<tr>
<td>Primary HIMEC (Stx1)</td>
<td>$(2.4 \pm 0.8) \times 10^{6}$ A, D</td>
<td>$(7.7 \pm 2.0) \times 10^{5}$ F, H</td>
</tr>
<tr>
<td>Primary HIMEC (Stx2)</td>
<td>$(7.1 \pm 1.3) \times 10^{6}$ A, E</td>
<td>$(6.9 \pm 1.8) \times 10^{5}$ F, H</td>
</tr>
<tr>
<td>Transformed HIMEC (Stx1)</td>
<td>$(1.9 \pm 0.5) \times 10^{6}$ B, D</td>
<td>$(6.5 \pm 2.3) \times 10^{5}$ F, H</td>
</tr>
<tr>
<td>Transformed HIMEC (Stx2)</td>
<td>$(7.4 \pm 2.1) \times 10^{5}$ B, E</td>
<td>$(6.8 \pm 1.4) \times 10^{5}$ F, H</td>
</tr>
<tr>
<td>HSVEC (Stx1)</td>
<td>$(1.7 \pm 0.7) \times 10^{5}$ C, D</td>
<td>$(2.9 \pm 0.8) \times 10^{5}$ G, H</td>
</tr>
<tr>
<td>HSVEC (Stx2)</td>
<td>$(8.5 \pm 2.4) \times 10^{4}$ C, E</td>
<td>$(2.2 \pm 0.9) \times 10^{5}$ G, H</td>
</tr>
</tbody>
</table>

* Letters next to values correspond to statistical significance for comparisons as follows: A and B, $P < 0.007$; C, $P < 0.02$; D, $P > 0.04$; E, $P > 0.7$; F, $P > 0.8$; G, $P > 0.3$; H, $P < 0.001$. 

FIG. 1. The expression of adhesion molecules by resting and activated EC was measured by an ELISA. VCAM-1 (open bars), ICAM-1 (dark bars with light hatching), and E-selectin (light bars with dark hatching) are shown. Data represent $A_{450}$ after subtraction of background absorbance in the presence of the negative control IgG. (A) Primary HIMEC; (B) transformed HIMEC; (C) HSVEC. Cells were exposed to one of the following treatments: 1, medium alone (resting level); 2, TNF-$\alpha$, 2 ng/ml; 3, IL-1$\beta$, 2 ng/ml; 4, TNF-$\alpha$, 2 ng/ml, plus IL-1$\beta$, 2 ng/ml; 5, E. coli O55:B5 LPS, 1 $\mu$g/ml; 6, Stx1, 10 ng/ml; 7, Stx2, 10 ng/ml; 8, TNF-$\alpha$, 2 ng/ml, plus IL-1$\beta$, 2 ng/ml, and Stx1, 10 ng/ml; 9, TNF-$\alpha$, 2 ng/ml, plus IL-1$\beta$, 2 ng/ml, and Stx2, 10 ng/ml. Data shown are from one representative experiment of three separate studies and are expressed as the mean changes in $A_{450}$ of the triplicate measurements of each value (error bars, standard deviations).

FIG. 2. Binding of iodinated Stx1 (A) and Stx2 (B) to resting or cytokine-activated EC lines. Data are expressed as the means ± 1 standard deviations (error bars) of triplicate data points of one representative experiment of three separate studies. Symbols: open triangles, primary HIMEC; solid triangles, primary HIMEC activated with TNF-$\alpha$–IL-1$\beta$ (each at 2 ng/ml); open circles, transformed HIMEC; solid circles, transformed HIMEC activated with TNF-$\alpha$–IL-1$\beta$ (each at 2 ng/ml); open squares, HSVEC; solid squares, HSVEC activated with TNF-$\alpha$–IL-1$\beta$ (each at 2 ng/ml).
Pretreatment of primary HIMEC over 3 days with 40 μM PDMP, an inhibitor of Gb3 synthesis, neither was cytotoxic nor reduced basal leucine incorporation into protein (data not shown). However, this treatment rendered the cells resistant to Stx1 (Fig. 4). Inclusion of TNF-α (5 ng/ml) with PDMP for the final 2 days of the incubation period did not alter the response to the toxin. However, if TNF-α was added following removal of PDMP the cells recovered their sensitivity more rapidly than did cells in the presence of medium alone (Fig. 4). In contrast, HSVEC were relatively resistant to both toxins (TI50: approximately 10⁻¹ ng/ml for both), but their sensitivity increased significantly following cytokine pretreatment.

Antibody rescue of toxin bound to HIMEC at 4°C is shown in Fig. 5. The addition of an excess of antibody at time zero after washing and warming cells to 37°C protected the cells; however, there was still considerable cytotoxicity, indicating rapid uptake of toxin. There was no significant difference between Stx1 and Stx2 in the time course of antibody rescue. The ability of the added antibody to reduce toxicity decreased as a function of time following toxin removal, and after 120 min cytotoxicity was the same in cells with and without added antibody.

**DISCUSSION**

These studies were undertaken to determine if microvascular EC of intestinal origin were sensitive to Stxs and could serve as a model to study the pathogenesis of STEC-related bloody diarrhea. The intestinal capillary network is the first EC target to be encountered as small quantities of Stx translocate across the intestinal epithelial cell layer. The exquisite sensitivity of HIMEC in vitro to Stx1 and Stx2 is consistent with a role in the pathogenesis of HC. These EC are also the most likely gateway to the systemic circulation for Stx to reach the kidney and brain in the pathogenesis of HUS and TTP, and toxin-mediated local EC damage could facilitate dissemination to the more distal targets.

Both primary and transformed HIMEC lines expressed EC adhesion markers and responded to cytokines and LPS with increased surface expression. HIMEC constitutively produced the inflammatory chemokine IL-8 (2), and this too was stimulated nearly 27-fold when cells were treated with TNF-α. Stx1 increases inflammatory cytokine production by human macrophages (24), and a cytokine-mediated burst in IL-8 could be relevant to recruitment of neutrophils to the lamina propria of the intestine in HC, thereby explaining the elevated levels of IL-8 in serum of patients with diarrhea-associated HUS (6).
Inflammatory cytokines and LPS did not increase expression of the Stx receptor glycolipid, Gb3, or increase binding of toxin, as they do in macrovascular EC (15, 17, 20, 21, 27). The two HIMEC lines constitutively produced threefold greater levels of Gb3 than did Vero cells, the most toxin-sensitive cell line we have previously studied in our laboratory (14). Interestingly, Stx1 bound to all cell lines to a much greater extent than Stx2, although the number of receptors was the same for both toxins. This difference is presumably a consequence of the higher binding affinity of Stx1 for the receptor. However, despite this, HIMEC were more sensitive to inhibition of protein synthesis by Stx2 than Stx1. This differential effect of the two toxins was not noted in HSVEC. While the mechanism is uncertain, we have no evidence that this is due to a difference in the rate at which the toxins are internalized following binding to their cellular receptor, since we found no difference in the time course for specific antibody neutralization of toxin bound to the cell surface at 4°C. These findings may be clinically important, since epidemiological data suggest that HUS is more likely to follow infection by Stx2-producing organisms than following infection by STEC producing Stx1 only (7). If less Stx2 is needed to damage the intestinal endothelium and if the binding affinity of Stx2 is also lower in vivo than that of Stx1, more toxin may be able to access the bloodstream to reach the kidneys and the brain.

The characteristics of the intestinal mucosal microvascular EC reported here (high constitutive production of Gb3, exquisite sensitivity to toxin [more so to Stx2 than Stx1], and no upregulation by LPS or cytokines) are in agreement with those originally reported for HRMEC (18, 19, 21). Others, however, have reported that a homogeneous preparation of HRMEC does not produce high levels of Gb3 and that TNF-α increases the sensitivity to Stx1 (27, 28). The difference in the results may be due to conditions of study, such as cell density, since subconfluent cells may be more sensitive to Stx than confluent cells, or may be related to cell-cell interactions in nonhomogeneous cell lines. In the present study of HIMEC, the lines were highly homogeneous and were not contaminated by other cell types (4) and there were no density-dependent differences in response to either toxin. The HIMEC lines also responded to inflammatory cytokines and LPS by increasing expression of adhesion molecules, and when we first depleted cellular Gb3 by blocking the biosynthetic pathway with PDMP we were also able to demonstrate upregulation of Gb3 by TNF-α during the recovery phase. We remain cautious in generalizing from these results until more HIMEC derived from more individuals can be studied.

Finally, we found only minor differences between the primary and transformed HIMEC lines, for example, a greater induction of cell adhesion markers by IL-1β than TNF-α in primary cells, with the reverse in transformed cells, and a somewhat greater sensitivity of primary cells to the two toxins. However, the two lines were obtained from different donors, and donor variability in the response to Stxs is a known property of EC lines (17, 20). More important, Gb3 was fully expressed in the two lines, and while both were highly sensitive to both toxins, they were more sensitive to Stx2 than to Stx1 even though Stx1 bound to a much greater extent to both cell lines. Microvascular cells are in vivo targets for Stx and thus represent a more biologically relevant investigative target than macrovascular cells. A major problem is that primary HIMEC are difficult to grow. The overall similarity in response to Stx1 and Stx2, cytokines, and LPS between the primary and transformed HIMEC make the latter an attractive model for studying toxin-mediated pathophysiological changes in vitro. This will allow us to address many unanswered questions relative to the role of toxin in the pathogenesis of STEC-related thrombotic microangiopathy.

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FIG. 5. Antibody rescue of primary HIMEC exposed to Stx1 (open bars) or Stx2 (hatched bars) at 4°C. Cells were then warmed to 37°C, and at various times afterward, an excess of antibody was added. Percent cell survival was determined by comparing leucine incorporation by treated HIMEC to that by untreated cells (no toxin). Cells exposed to toxin and incubated overnight without antibody (DN—no Ab) are included to demonstrate maximum cytotoxicity. Data are expressed as the means of triplicate measurements of each data point from one experiment (error bars, standard deviations).

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