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

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Endothelial damage is characteristic of infection with Shiga toxin (Stx)-producing Escherichia coli (STEC). Because Stx-mediated endothelial cell damage at the site of infection may lead to the characteristic hemorrhagic colitis of STEC infection, we compared the effects of Stx1 and Stx2 on primary and transformed human intestinal microvascular endothelial cells (HIMEC) to those on macrovascular endothelial cells from human saphenous vein (HSVEC). Adhesion molecule, interleukin-8 (IL-8), and Stx receptor expression, the effects of cytokine activation and Stx toxins on these responses, and Stx1 and Stx2 binding kinetics and bioactivity were measured. Adhesion molecule and IL-8 expression increased in activated HIMEC, but these responses were blunted in the presence of toxin, especially in the presence of Stx1. In contrast to HSVEC, unstimulated HIMEC constitutively expressed Stx receptor at high levels, bound large amounts of toxin, were highly sensitive to toxin, and were not further sensitized by cytokines. 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.

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 thrombocytopenia and EC damage related to endothelial damage. This damage is believed to be due to circulating bacterial exotoxins (Stxs), endotoxins, and host-derived cytokines (tumor necrosis factor alpha [TNF-α] and interleukin-1β [IL-1β]), which may play a pivotal role by activating endothelial cells (EC) to respond to the toxins (18, 25).

Stx1 and Stx2 inhibit protein synthesis in a variety of EC of human origin (11, 15, 17, 20, 25, 26, 28). These data demonstrate that EC cultures derived from the endothelium of large blood vessels, such as umbilical and saphenous veins, do not constitutively produce large amounts of globotriosylceramide (Gb3), the Stx receptor glycolipid, and are not very sensitive to toxin unless activated by lipopolysaccharides (LPS) or certain cytokines (15, 17, 20, 26) that induce de novo synthesis of Gb3. It is the increase in surface expression of the Stx receptor that leads to enhanced sensitivity to the toxins. In contrast, human renal microvascular EC (HRMEC) constitutively express maximum amounts of Gb3, are highly sensitive to Stx1, and to fail to respond further after activation by LPS or cytokine (15, 20).

These data provide a potential explanation for targeting of glomeruli in HUS. Recently, another group using a different HRMEC line has reported just the opposite, that is, limited sensitivity of resting cells to Stx1 but marked activation following exposure to TNF-α (28). Preliminary reports suggest that responses of cerebral microvascular EC to Stx1 are also enhanced by TNF-α or IL-1β treatment (11, 23).

STEC colonize portions of the large intestine, and by using cultured human intestinal endothelial cell lines that develop tight junctions as an in vitro model, translocation of biologically active Stx has been demonstrated across this epithelial barrier (1). Microvascular EC in the intestine therefore will be the first endothelium to contact translocating toxin. Toxin-mediated EC damage may result in the characteristic bleeding of the HC syndrome associated with some STEC infections and gain access to the circulation to ultimately act on EC at distant sites such as the kidney and the brain.

EC isolated from different organs, and macrovascular and microvascular EC from the same organ, demonstrate considerable phenotypic heterogeneity (21, 22). Recently, microvascular EC isolated from human intestine have become available (4, 8, 9). The purpose of the present studies was to examine EC derived from the human intestine and determine their response to two E. coli-derived Stxs, Stx1 and Stx2. Our goal was to develop an in vitro model to study the pathogenesis of HC due to STEC infection.

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MATERIALS AND METHODS

Toxin purification, iodination, and assay. Stx1 was purified from cell lysates of E. coli HB101-H19B, an STEC expressing Stx1 only. Stx2 was obtained from the culture supernatant of E. coli C600(hglnr27, perA), a clinical isolate from a patient with bacteriotoxemia of 93SW. 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 phosphate buffer (pH 7.5), with 1 mCi of 125I (34). Labeled toxins were purified by gel filtration on a Sephacryl S-200 column. Both toxins showed an iodination efficiency of 95% and a binding activity of 98% as assessed by a cytotoxicity assay (23).

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. [H3]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% (TS50). In some experiments, primary human intestinal microvascular EC (HIMEC) were incubated for 72 h with 40 μM of either toxin. 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% (TS50).

Receptor glycolipids in EC lines. Total cellular Gb3 was measured by isolation 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 by treatment with the Linker CMVT 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.

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 upon increasing 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/ml/10⁶ cells, respectively) and were increased to an even greater extent in cells pretreated with TNF-α and/or IL-1β (180.3 ng/ml/10⁶ cells). Overnight exposure to toxins of cells exposed to TNF-α and IL-1β resulted in a sharp decrease in IL-8 production (40.33 and 59.61 ng/ml/10⁶ 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 (3,962 and 3,428 pmol/mg of cell protein, respectively) and was 10-fold greater than that of the HSVEC Gb3 content measured on a molar basis, whereas in HSVEC Gb3 accounted for less than 10% of the total neutral glycolipids.

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 cytokine-activated HIMEC 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-α–IL-1β (each at 2 ng/ml) overnight did not increase the cytotoxicity response of HIMEC to either toxin.

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

<table>
<thead>
<tr>
<th>Cell line (toxin)</th>
<th>Binding affinity (M⁻¹)</th>
<th>No. of binding sites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary HIMEC (Stx1)</td>
<td>$(2.4 \pm 0.8) \times 10^6$ A, D</td>
<td>$(7.7 \pm 2.0) \times 10^7$ F, H</td>
</tr>
<tr>
<td>Primary HIMEC (Stx2)</td>
<td>$(7.1 \pm 1.3) \times 10^4$ 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>$(6.3 \pm 1.4) \times 10^4$ F, H</td>
</tr>
<tr>
<td>HSVEC (Stx1)</td>
<td>$(1.7 \pm 0.7) \times 10^5$ C, D</td>
<td>$(2.9 \pm 0.8) \times 10^4$ G, H</td>
</tr>
<tr>
<td>HSVEC (Stx2)</td>
<td>$(8.5 \pm 2.4) \times 10^4$ C, E</td>
<td>$(2.2 \pm 0.9) \times 10^3$ 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.*
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^{-3} 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, Gn3, 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 Gn3 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 Gn3, 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 Gn3 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 Gn3 by blocking the biosynthetic pathway with PDMP we were also able to demonstrate upregulation of Gn3 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 Stx is a known property of EC lines (17, 20). More important, Gn3 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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REFERENCES


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