Verotoxin-1 Promotes Leukocyte Adhesion to Cultured Endothelial Cells Under Physiologic Flow Conditions

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Hemolytic uremic syndrome (HUS), which is the most common cause of acute renal failure in infants and small children, is caused by verotoxin (VT)-producing Escherichia coli infection. Endothelial injury determines microvascular thrombosis and evidence is available from recent studies that suggests that leukocyte activation participates in endothelial damage. We studied here the effect of VT-1 on leukocyte adhesion to vascular endothelium under physiologic flow conditions. Human umbilical vein endothelial cells (HUVECs) were incubated for 24 hours with VT-1 (0.1, 1, and 10 pmol/L) and then exposed to a total leukocyte suspension in a parallel plate flow chamber under laminar flow conditions (1.5 dynes/cm²). Adherent cells were counted by digital image processing. Results showed that VT-1 dose-dependently increased the number of adhering leukocytes to HUVECs as compared with unstimulated cells. The adhesive response elicited by VT-1 was comparable to that of interleukin-1β (IL-1β), one of the most potent inducers of endothelial cell adhesiveness. Exposure of HUVECs to VT-1 did not affect the number of rolling leukocytes, which was similar to that of control values. To examine the role of adhesion molecules in VT-1–induced leukocyte adhesion, HUVECs were incubated with mouse monoclonal antibodies against E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) before adhesion assay. Functional blocking of E-selectin, ICAM-1, and VCAM-1 on endothelial cells significantly inhibited VT-1–induced increase in leukocyte adhesion. In some experiments, before VT-1 incubation, HUVECs were pretreated for 24 hours with tumor necrosis factor α (TNFα; 100 U/mL), which is known to increase VT receptor expression on HUVECs. The number of adhering leukocytes on HUVECs exposed to TNFα and VT-1 significantly increased as compared with HUVECs incubated with VT-1 and TNFα alone. These results suggest that VT-1 modulates leukocyte-endothelium interaction, thus increasing leukocyte adhesion and upregulating adhesive proteins on endothelial surface membrane.

MATERIALS AND METHODS

Endothelial cell culture. Endothelial cells were obtained from human umbilical veins (HUVECs) by collagenase digestion according to the method of Jaffe et al. The cells were grown in Medium 199 (GIBCO, Grand Island, NY) supplemented with 10% newborn calf serum (GIBCO), 10% human serum, and antibiotics. Cultures were grown at 37°C in 5% CO₂-95% air. Confluent HU-

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VECs were routinely used for experiments between the first and fifth passage. Cultured cells were identified as endothelial by their cobblestone morphology and the presence of von Willebrand factor, using indirect immunofluorescence microscopy. HUVECs were plated on 60-×-20-mm plastic coverslips (Thermoax; Nunc, Naperville, IL) coated with bovine gelatin and used 2 days after reaching confluence. HUVECs were incubated for 24 hours with control medium (M199 plus 10% fetal calf serum) or VT-1 (0.1, 1, and 10 pmol/L). Purified VT-1 was prepared in the laboratory of Dr M.A. Karmali (1.2 mg protein/mL; CD50 vero-cells: titer, 10^5 to 10^7). Endotoxin content of the VT-1 preparation was determined to less than 0.05 EU/mL using Limulus amoebocyte lysate assay (E-Toxic; Sigma Chemicals, St Louis, MO) at detection levels of 0.05 to 0.10 EU/mL. HUVECs stimulated for 4 hours with recombinant human interleukin-1β (IL-1β; 100 U/mL; 5×10^5 U/mg protein; Boehringer Mannheim, Mannheim, Germany) were used as positive control. Endotoxin contamination of VT-1 preparation was ruled out by experiments showing that the number of rolling cells on the HUVEC surface at 3.0 dynes/cm^2 was determined on a series of 16 consecutive images digitized during a 10-second interval. Adherent leukocytes were identified and counted at the end of 13 minutes of perfusion. All images were then superimposed by digital processing so that moving cells could be distinguished from their wake. Statistical analysis. Results are expressed as the mean ± SE. Statistical analysis was performed using analysis of variance and the Tukey-Ciccetti test for multiple group comparisons, as appropriate. Statistical significance was defined as P < .05.

RESULTS
Effect of VT-1 on leukocyte adhesion to HUVECs under physiologic flow conditions. We have studied the adhesion of peripheral total leukocytes to control, VT-1-treated (0.1, 1, and 10 pmol/L), and IL-1β-treated (100 U/mL) HUVECs under flow conditions. We used VT-1 at concentrations (chosen according to previous studies) that did not affect cell morphology (Fig 1) and cell count after 24 hours of incubation (VT-1 at 10 pmol/L: 69.4 ± 2.0 × 10^6 cells v control: 65.5 ± 2.5 × 10^6 cells). As shown in Fig 2, exposure of HUVECs for 24 hours to increasing concentrations of VT-1 dose-dependently increased the number of leukocytes adhering to HUVECs after 13 minutes of perfusion, as compared with unstimulated control cells. On average 62 ± 7 leukocytes/mm² adhered to unstimulated HUVECs. Exposure of HUVECs to 0.1 pmol/L VT-1 did not modify the number of adherent leukocytes, which averaged 62 ± 4 leukocytes/mm². VT-1 at 1 pmol/L concentration significantly (P < .01) increased the number of adhering leukocytes as compared with control cells; on average, 181 ± 30 leukocytes/mm² adhered on the HUVEC surface. A similar adhesive response was observed after incubation with 10 pmol/L VT-1, with the number of adherent cells averaging 186 ± 20 leukocytes/mm² (P < .01 v control). On IL-1β-stimulated HUVECs, the mean number of firmly attached cells reached 226 ± 29 leukocytes/mm².

The possibility that VT-1-induced leukocyte adhesion could be due to endotoxin contamination of VT-1 preparations was ruled out by experiments showing that the number of leukocytes adhering to HUVECs exposed for 24 hours to LPS was similar to that of control HUVECs (66 ± 9 v 58 ± 9 leukocytes/mm²). Figure 3 depicts digitized images from a representative experiment at the end of 13 minutes of leukocyte perfusion. A limited number of leukocytes firmly adhered to control HUVECs. A more than threefold increase in leukocyte adhesion was observed on HUVEC monolayers exposed for 24 hours to VT-1 (10 pmol/L). A similar response was elicited by IL-1β, which is one of the most potent inducers of endothelial cell adhesive properties.

The number of leukocytes rolling at 3 dynes/cm² were re-
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Effect of VT-1 on endothelial cell morphology. HUVECs were incubated for 24 hours with medium alone (control) or in the presence of VT-1 (10 pmol/L).

Fig 1. Effect of VT-1 on endothelial cell morphology. HUVECs were incubated for 24 hours with medium alone (control) or in the presence of VT-1 (10 pmol/L).

Ported in Fig 4. On unstimulated static HUVECs, few rolling cells were detected averaging 2.3 ± 0.5 leukocytes/mm². Treatment of HUVECs for 24 hours with 1 or 10 pmol/L VT-1 did not affect the number of rolling leukocytes, which averaged 5.3 ± 2.1 and 3.4 ± 0.7 leukocytes/mm², respectively. However, on IL-1β-stimulated cells, the number of leukocytes rolling on endothelial surface significantly (P < .01) increased compared with that rolling on control and VT-1-treated HUVECs (58 ± 6 leukocytes/mm²).

Cell surface proteins involved in leukocyte adhesion to VT-1-stimulated HUVECs. To explore the molecular basis of VT-1-induced leukocyte adhesion to endothelial cells under flow, HUVECs exposed for 24 hours to VT-1 (10 pmol/L) were incubated for 20 minutes with MoAb anti-E-selectin, anti-ICAM-1, and anti-VCAM-1 immediately before performing leukocyte adhesion experiments. As shown in Fig 5, at the end of the perfusion assay the number of adherent leukocytes on HUVECs treated with VT-1 was significantly higher as compared with leukocytes adhering on control untreated HUVECs (191 ± 28 v 56 ± 6 leukocytes/mm², P < .01). MoAb anti-E-selectin significantly inhibited VT-1 induced increase in leukocyte adhesion (73 ± 17 v 191 ± 28 leukocytes/mm², P < .01). MoAb anti-ICAM-1 and anti-VCAM-1 also significantly reduced leukocyte adhesion to VT-1-treated HUVECs (100 ± 26 and 71 ± 15 leukocytes/mm², respectively; P < .01 v VT-1).

Noteworthy exposure of VT-1-treated HUVECs to MoAb anti-E-selectin and anti-VCAM-1 lowered the number of adhering leukocytes to control values (56 ± 6 leukocytes/mm²). Treatment with irrelevant MoAb did not affect leukocyte adhesion on VT-1-treated HUVECs (207 ± 63 v 187 ± 56 leukocytes/mm²).

The number of rolling leukocytes on VT-1-treated (10 pmol/L) HUVECs incubated with MoAb against E-selectin, ICAM-1, and VCAM-1 was similar to that observed on endothelial cells treated with VT-1 alone (3.4 ± 1.5; 4.8 ± 0.8; 3.1 ± 0.6 v 3.4 ± 0.7 leukocytes/mm²).

Effect of TNF on VT-1-induced leukocyte adhesion to HUVECs under physiologic flow conditions. In some experiments, before VT-1 (10 pmol/L) incubation, HUVECs were pre-exposed for 24 hours to TNFα at the concentration of 100 U/mL, which was previously shown to increase VT-1 receptor expression on HUVECs (Fig 6). VT-1 significantly enhanced leukocyte adhesion on HUVECs as compared with control cells (146 ± 15 v 45 ± 5 leukocytes/mm², P < .01). The number of leukocytes adhering to HUVECs pre-exposed to TNFα before challenge with VT-1 were significantly higher than that of HUVECs exposed to VT-1 alone (300 ± 13 v 146 ± 15 leukocytes/mm², P < .01). The number of leukocytes adhering on HUVECs exposed to TNFα (100 U/mL) alone averaged 201 ± 29 leukocytes/mm² (P < .01 v TNFα + VT-1).

With additional experiments we established that leukocyte adhesion on HUVECs reached a plateau at a TNFα concentration of 300 U/mL (control, 71.2 ± 6.8; 100 U/mL TNFα, 162.4 ± 13.5; 300 U/mL TNFα, 286.2 ± 13.6; 500 U/mL TNFα, 268.0 ± 10.7 leukocytes/mm²). When HUVECs pretreated with 300 U/mL TNFα were exposed to VT-1 (10 pmol/L) ± TNFα, leukocytes were detected averaging 201 ± 29 leukocytes/mm² (P < .01 v TNFα + VT-1).

Adhesion of total leukocytes on unstimulated (control) or VT-1-stimulated HUVECs under conditions of flow. HUVECs were incubated for 24 hours with VT-1 (0.1, 1, 10 pmol/L). HUVECs activated with IL-1β (100 U/mL) were used as a positive control. Leukocyte adhesion was measured at the end of 13 minutes of perfusion. Data are expressed as the mean ± SE. *P < .01 versus control.

Fig 2. Adhesion of total leukocytes on unstimulated (control) or VT-1-stimulated HUVECs under conditions of flow. HUVECs were incubated for 24 hours with VT-1 (0.1, 1, 10 pmol/L). HUVECs activated with IL-1β (100 U/mL) were used as a positive control. Leukocyte adhesion was measured at the end of 13 minutes of perfusion. Data are expressed as the mean ± SE. *P < .01 versus control.
An additive effect of VT-I on leukocyte adhesion was observed in only 4 of 8 experiments, with a percentage increase of 60.7% ± 8.3% over TNFα alone.

**DISCUSSION**

The present results show that VT-I dose-dependently induced leukocyte adhesion to endothelial cells under flow conditions. The adhesive response elicited by VT-I was quite comparable to that of IL-1β, one of the most potent inducers of leukocyte-endothelial cell adhesion. Because leukocyte attachment consistently induces granule content release, the present study helps to clarify mechanism(s) by which VT-I damages the endothelium. These results are in harmony with data that in vitro HUS neutrophils adhere to endothelium more than normal neutrophils. Neutrophil-derived products (particularly elastase) that are released locally on neutrophil adhesion to endothelium degrade extracellular matrix, which would favor endothelial cell detachment from basement membrane, a typical feature of HUS. Formal evidence that this may occur in HUS derives from data that enhanced adhesion of neutrophils from HUS patients to endothelium in vitro is followed by degradation of endothelial cell fibronectin.

Events that regulate recruitment of leukocytes and subsequent migration are critically dependent on adhesive molecules constitutively expressed on the surface of the endothelium and leukocytes or induced by cytokines or flow condition. Early molecules involved in this process belong to the selectin family and are expressed on endothelial cells upon cytokine activation. Subsequent firm adhesion of leukocytes implies the interactions of leukocyte β2 and β1 integrin receptors with specific endothelial ligand, ie, ICAM-1 and VCAM-1, constitutively expressed on endothelial surface, whose level is further increased by cytokine activation.

With the present study we have established that functional
Fig 5. Effect of MoAb anti-E-selectin, anti-ICAM-1, or anti-VCAM-1 on VT-1-induced leukocyte adhesion on endothelial cells. Before the adhesion assay, VT-1-treated (10 pmol/L) HUVECs were incubated for 20 minutes with MoAb anti-E-selectin, anti-ICAM-1, or anti-VCAM-1. Data are expressed as the mean ± SE. *P < .01 versus control; **P < .01 versus VT-1.

Blocking of E-selectin, ICAM-1, and VCAM-1 with respective antibodies significantly reduced VT-1-mediated leukocyte adhesion to endothelial cells exposed to flow. An additional finding of the present study was that VT-1 inhibited the process of rolling that normally precedes adhesion of cells to the endothelium. We observed a similar phenomenon in tumor cell adhesion to the endothelium under flow. It was established that certain lines (ie, A375M and A2058 melanomas and the MG-63 osteosarcoma) firmly adhered on activated endothelial cells before any rolling could be seen, whereas other lines (HT-29M colon carcinoma and OVCAR-3 ovarian carcinoma) were capable of usual rolling before they adhered. It is tempting to speculate that VT-1 causes sudden expression of ICAM-1 and VCAM-1 at very high density on endothelial cell surface, which would promote immediate leukocyte adhesion not preceded by rolling. The last observation of the present study was that pre-exposure of HUVECs to TNFα before challenge with VT-1 significantly increased the number of adherent leukocytes under flow. This observation is consistent with several recent findings that TNFα does promote upregulation of endothelial VT-Gb receptor and supports VT-1 binding.

That TNFα plays a major role in VT-1-induced microvascular injury is documented by findings that mice genetically defective in TNFα production were less sensitive to the lethal effects of VT-1. These mice consistently had a longer mean time to death than did mice who were normally responsive. The molecular basis for the interaction between VT-1 and TNFα in determining extent and localization of microvascular lesions were recently addressed in an elegant experiment of mice bearing a chloroamphenicol acetyl-transferase (CAT) reporter gene that indicates TNFα synthesis. Upon VT-1 injection to the above-mentioned mice, CAT activity was selectively induced in the kidney but not in other organs, indicating a distinct potential of VT-1 of upregulating TNFα gene expression not equally distributed within all organs, which fits well with the unique sensitivity of the kidney to microvascular damage in HUS.

In conclusion, our results indicate that (1) VT-1 is a potent promoter of leukocyte adhesion to endothelial cells under flow conditions; (2) VT-1-induced leukocyte-endothelial interaction depends on upregulation of adhesion molecules that include E-selectin, ICAM-1, and VCAM-1, as documented by inhibition studies with respective specific antibodies; and (3) VT-1-induced leukocyte adhesion is enhanced by TNFα.

Despite the potential limitations of these in vitro studies as far as their theoretical implication in vivo, the present findings might be relevant to understand the nature of microvascular lesions in children with VTEC-associated HUS and open exciting new perspectives for treatment.

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REFERENCES


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