Verocytotoxin Inhibits Mitogenesis and Protein Synthesis in Purified Human Glomerular Mesangial Cells Without Affecting Cell Viability: Evidence for Two Distinct Mechanisms

PETRA A. VAN SETTEN,*† VICTOR W. M. VAN HINSBERGH,† LAMBERT P.W. J. VAN DEN HEUVEL,* THEA J. A. N. VAN DER VELDEN,* NICOLE C. A. J. VAN DE KAR,* RAYMOND J. M. KREBBERS,‡ MOHAMMED A. KARMALI,§ and LEO A. H. MONNENS*

*Department of Pediatrics, University Hospital, Nijmegen, The Netherlands; †Gaubius Laboratory Toegepast Natuurwetenschappelijk Onderzoek-Preventie en Gezondheid, Leiden, The Netherlands; ‡Laboratory of Endocrinology and Reproduction, University Hospital, Nijmegen, The Netherlands; and §Department of Microbiology, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada.

Abstract. Acute renal failure is one of the hallmarks of the hemolytic uremic syndrome (HUS). Infection with a verocytotoxin (VT)- or Shiga-like toxin (SLT)-producing Escherichia coli has been strongly implicated in the etiology of the epidemic form of HUS. The functional receptor for these closely related toxins appears to be a glycosphingolipid, globotriaosylceramide (Gb3). Endothelial damage in the glomeruli and arterioles of the kidney induced by VT is believed to play a crucial role in the pathogenesis of HUS. However, little information is available regarding the effects of VT on mesangial cells, which also play an important role in glomerular function. In this study, the effects of VT on human mesangial cells in vitro were investigated. Mesangial cells were enriched by collecting hillock-shaped outgrowths derived from adult human glomeruli and subsequently purified by elimination of contaminating epithelial cells by immunoseparation with ulex europaeus lectin-I (UEA-I)-coated dynabeads. The obtained and subcultured mesangial cell populations were >98% pure. Their mesangial nature was established by the presence of α-smooth muscle cell actin in highly confluent cultures and the absence of cytokeratin or platelet/endothelial cell adhesion molecule-1. Mesangial cells bound VT to bands of Gb3 and a closely related glycolipid, which is similar to a glycolipid involved in the VT-dependent cytokine production in monocytes. VT did not induce the release of cytokines or chemokines in mesangial cells. In VT-susceptible cells, binding of VT to Gb3 causes cell death by the inhibition of protein synthesis. Although protein synthesis was inhibited in mesangial cells, all cells remained viable, both under basal and tumor necrosis factor-α-stimulated conditions. However, the marked reduction in protein synthesis may impair a proper response of the cells in conditions of increased demand of newly synthesized proteins. Furthermore, VT markedly inhibited DNA synthesis and proliferation of mesangial cells. The inhibition of mitogenesis was also found with the B-subunit of VT-1 alone, albeit to a lesser extent, without a significant effect on protein synthesis. Because the inhibition of protein synthesis involves the A-subunit, this suggests that two distinct mechanisms contribute to the effects of VT on protein synthesis and mitogenesis. Intracellular routing of VT (A- and B-subunits) may vary between cell types and result in differential effects on human mesangial cells when compared with other cell types. (J Am Soc Nephrol 8: 1877–1888, 1997)

Received December 26, 1996. Accepted May 6, 1997.
Correspondence to Dr. Leo A.H. Monnens, University Hospital Nijmegen, Department of Pediatric Nephrology, Geert Grooteplein 20, P.O. Box 9100, 6500 HB Nijmegen, The Netherlands.
1046-6673/0005-1877$03.00/0
Journal of the American Society of Nephrology
Copyright © 1997 by the American Society of Nephrology

Hemolytic uremic syndrome (HUS) is a clinical syndrome characterized by the triad hemolytic anemia, thrombocytopenia, and acute renal failure (1). Infection with a verocytotoxin (VT)-producing Escherichia coli is strongly implicated in the etiology of the epidemic form of HUS (2). Endothelial cell damage of glomeruli and kidney arterioles by interaction of VT with its receptor, resulting in a consecutive cascade of mutually influencing factors, plays an important role in the pathogenesis of HUS (3). Renal histopathological studies of HUS patients reveal swollen and detached endothelial cells and deposits of fibrin, all within the glomerulus (4). Pathological findings in the mesangial area of HUS patients are scarcely documented, reporting signs of mesangial disintegration associated with reparative changes varying both in severity and distribution. Mesangial hypercellularity is not a common feature of this syndrome (4–8). The intervening basement membrane is lacking at the point where the mesangium comes in closest contact with glomerular circulation (9) and, thus, only a single layer of
we hypothesized that VT and other plasma constituents may damage, fenestrated endothelium is separating glomerular cells in a paracrine manner and exert autocrine effects on mesangial cells themselves. In addition, mesangial cells not only produce a variety of factors involved in the regulation of cell proliferation, but also are target sites for the regulation of cell proliferation. In this study, the effects of VT on pure populations of human mesangial cells in culture, which were free of contaminating endothelial and epithelial cells, were investigated. Although human mesangial cells do possess a glycolipid receptor for VT, the toxin did not appear to affect the number of viable adherent human mesangial cells significantly. In contrast to human monocytes, in which VT induced cytokine release and also did not affect cell viability, human mesangial cells were not induced by VT to release cytokines. On the contrary, VT markedly inhibited DNA and, to a lesser extent, overall protein synthesis. Two distinct mechanisms appeared to be involved in these inhibitory effects. One was associated with the A-subunit, known to inhibit eukaryotic protein synthesis in other cell types (17,18), and the second mechanism was linked to the B-subunit of VT, which is normally required for the binding of the holotoxin to its functional receptor (19).

Materials and Methods
Isolation of Glomeruli
Studies were performed with human kidney tissue obtained from kidneys that had been surgically excised because of the presence of a localized neoplasia (donor age range, 45 to 70 yr). Written consent was obtained from the Medical Ethical Review Board of the University Hospital (Nijmegen, The Netherlands). Macroscopically normal portions of cortex, located at some distance (1 to 2 cm) from the neoplastic process, were dissected under sterile conditions. Glomeruli were subsequently isolated by a gradual sieving procedure and collected on top of sieves with openings sizes of 180, 125, 108, or 90 μm (Endecotts, London, England). Resulting pure populations of glomeruli were mildly treated with 0.1% (wt/vol) collagenase type 2 CLS (Worthington Biochemical, Freehold, NJ) for 30 min at 37°C. After collagenase treatment, the glomeruli were washed, centrifuged, and resuspended in complete media, which consisted of M199 (BioWhittaker, Walkersville, MD) supplemented with 10% (vol/vol) newborn calf serum (NBCS; GibCO, Grand Island, NY), 10% (vol/vol) human serum (HS; procured from a local blood bank), 2 mmol/L glutamine (ICN Biomedical, Costa Mesa, CA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 5 U/ml heparin (Leo Pharmaceuticals, Weesp, The Netherlands), and 150 mg/L of a crude preparation of endothelial cell growth factor (prepared form bovine brains as described by Maciag [20]) and plated on gelatin (1% Fluka BioChemika, Buchs, Switzerland)-coated wells (Costar, Cambridge, MA).

Primary Culture and Purification
In general, the glomeruli attached within a few days, after which outgrowth of predominantly epithelial and limited numbers of endothelial cells was noted. Epithelial cells displayed their typical polygonal, cobblestone morphology, whereas glomerular microvascular endothelial cells exhibited a slightly elongated morphology, with a nucleus containing one or two nucleoli and a dark perinuclear cytoplasm area surrounded by a bright halo. These endothelial cells, unless selectively collected, were rapidly overgrown by epithelial cells during the first days of culture. Within 10 to 30 d after the glomeruli were seeded, outgrowth of mesangial cells was noticed (Figure 1A), initially showing the characteristic spindle/stellate-shaped morphology and, later, the well recognized elongated conformation. These elongated cells exhibited a typical growth pattern of overcrossing hillock-forming cells (21); we selectively collected them by scraping them off the tissue culture plate (22), using the tip of a plastic pipette, and explanted them onto gelatin-coated six-well plates (Figure 1B). Whereas confluent populations of these mesangial cells were free of contaminating endothelial cells, the presence of small numbers of epithelial cells was not negligible (5 to 15%), as demonstrated by immunofluorescence studies, using monoclonal antibodies against endothelial (anti-platelet/endothelial cell adhesion molecule-1 [PECAM-1]) and epithelial cells (anti-cytokeratin 8), respectively. To further purify the mesangial cell populations, epithelial cells were eliminated by an immunomagnetic separation technique, using ulex europaeus lectin-1 (UEA-1) (Sigma, St. Louis, MO)-coated Dynabeads (M-450 tosyl-activated Dynabeads; Dynal, Oslo, Norway) that showed affinity for epithelial cells but not for mesangial cells. Similar results were obtained when a fluorescence-activated cell sorter analysis was performed using tetramethylrhodamine isothiocyanate-labeled UEA-I (Sigma), which consistently showed a much higher binding to epithelial cells than to mesangial cells. The immunomagnetic separation technique was performed by incubating trypsinized and subsequently washed cells with UEA-I-coated Dynabeads for 30 min at room temperature with occasional agitation. Epithelial cells that bound to the beads were collected with a magnetic particle concentrator, and the mesangial cells present in the supernatant were pooled and exposed to a second immunomagnetic separation technique to remove the last contaminating epithelial cells. UEA-I-negative mesangial cells were plated onto gelatin-coated wells and grown in a humidified atmosphere of 95% air and 5% CO₂. Resulting cell populations were highly purified populations of human mesangial cells (Figure 1C). Subculturing of these cells with a split ratio of 1:3 to 1:6 was successfully performed to at least 10 passages without observable changes in morphology and function.

Characterization of Human Mesangial Cells
Primary and serially passaged cultures were characterized using the following criteria:

1. Morphological criteria: Primary outgrowing mesangial cells resembled subcultured mesangial cells as assessed by phase-contrast microscopy; they initially displayed their characteristic spindle/stellate-shaped morphology and later showed the well recognized elongated form. Confluent and highly confluent cultures exhibited a typical growth pattern of overcrossing cells (Figure 1, B and C). Neither contaminating polygonal epithelial cells nor contaminating...
Effects of Vero cytotoxin on Human Mesangial Cells

1. Phase-contrast microscopy of primary culture of human mesangial cells. Human glomeruli were isolated by a gradual sieving procedure and mildly treated with collagenase. The glomeruli and glomerular remnants were then plated on gelatin-coated plates. After attachment, outgrowth of primarily endothelial and epithelial cells was observed. Within 10 to 30 d after the glomeruli were seeded, outgrowth of mesangial cells was noticed, showing their characteristic spindle/stellate-shaped morphology and, later, the well recognized elongated conformation (A); the elongated cells exhibited a typical growth pattern of overcrossing hillock-forming cells. Mesangial cell populations were enriched by collecting these hillock-forming cells (B). Contaminating epithelial cells were eliminated by performing an immunomagnetic separation technique, using ulex europaeus lectin I-coated Dynabeads, resulting in pure populations of human mesangial cells (C). Magnification, ×75.

2. Immunological criteria: Indirect immunofluorescence studies of outgrowing as well as subcultured mesangial cells revealed intense staining of intracellular filaments with anti-α smooth muscle actin (Sigma; clone 1A4; 1:300; Figure 2A). However, whereas immunoreactivity with α-smooth muscle actin in primary cultured mesangial cells did not seem to depend on the cell density, immunoreactivity with α-smooth muscle actin in subcultured mesangial cells was predominantly observed when the cells were highly confluent. No differences were noticed when comparing F-actin
glomerular endothelial cells were observed in purified populations of mesangial cells by phase-contrast microscopy.

Figure 1. Phase-contrast microscopy of primary culture of human mesangial cells. Human glomeruli were isolated by a gradual sieving procedure and mildly treated with collagenase. The glomeruli and glomerular remnants were then plated on gelatin-coated plates. After attachment, outgrowth of primarily endothelial and epithelial cells was observed. Within 10 to 30 d after the glomeruli were seeded, outgrowth of mesangial cells was noticed, showing their characteristic spindle/stellate-shaped morphology and, later, the well recognized elongated conformation (A); the elongated cells exhibited a typical growth pattern of overcrossing hillock-forming cells. Mesangial cell populations were enriched by collecting these hillock-forming cells (B). Contaminating epithelial cells were eliminated by performing an immunomagnetic separation technique, using ulex europaeus lectin I-coated Dynabeads, resulting in pure populations of human mesangial cells (C). Magnification, ×75.

Figure 2. Indirect immunofluorescence studies of human mesangial cells (A), glomerular microvascular endothelial cells (B), and human renal epithelial cells (C) with antibodies against (respectively) α-smooth muscle actin, platelet/endothelial cell adhesion molecule-1 antigen, and cytokeratin 8. Magnification, ×350.
filaments (phalloidin-tetramethylrhodamine isothiocyanate; Sigma) of primary and subcultured mesangial cells at any cell density. The cultured mesangial cells showed no reactivity to the anti-PECAM-1 antibody (23) or to the anti-cytokeratin 8 antibody (24), excluding (respectively) contaminating endothelial and epithelial cells (data not shown). Glomerular microvascular endothelial cells did react with anti-PECAM-1 antibody (Figure 2B), whereas renal epithelial cells did with anti-cytokeratin 8 antibody (Figure 2C).

3. Functional criteria: Brown et al. (25) reported the synthesis of urokinase by human epithelial cells but not by human mesangial cells. Therefore, we measured urokinase-type plasminogen activator (u-PA) antigen in conditioned medium by a sandwich enzyme-linked immunosorbent assay (ELISA) previously described by van Hinsbergh et al. (26).

Cytotoxicity Assay

Mesangial cells were cultured in complete medium on gelatin-coated 24-well plates and grown until confluence. Subsequently, the cells were preincubated with or without tumor necrosis factor-α (TNFa; 10 ng/ml; Boehringer Mannheim, Mannheim, Germany) for 24 h. The next day, the medium was aspirated, and the cells were washed once. New medium, consisting of 20% fetal calf serum instead of 10% NBCS and 10% HS because of the potential neutralizing activity of NBCS and HS against VT, was added with different concentrations of VT-1. After 4 to 72 h, the cells were washed with phosphate-buffered saline (PBS) and adherent cells were released with trypsin/ethylenediamineeta-acetate (EDTA; 0.5 g trypsin 1:250/0.2 g EDTA in 1 L of Modified Puck’s Saline A; Gibco). Trypan blue was added, and viable, trypan blue-excluding cells were counted in a hemocytometer. To study the influence of cell density, cells at different degrees of cell density were preincubated with and without TNFa and tested for VT-1 (27), VT-2 (28) (kindly provided by Dr. M. A. Karmali), and VT-1 B-subunit (29) (kindly provided by Dr. J. L. Brunton, The Hospital for Sick Children, Toronto, Ontario, Canada) cytotoxicity as described above.

iodination of VT-1 and the Binding of {sup}125I-VT-1 to Human Mesangial Cells

VT-1 was radiolabeled with Na-{sup}125I according to the iodogen procedure (30). The radioactive activity of {sup}125I-VT-1 ranged from 10 to 12 μCi/μg protein. Mesangial cells were grown in 24-well plates until they reached confluence. The cells were then preincubated with TNFa for 24 h at 37°C, after which the cells were washed with M199/0.1% human serum albumin (pyrogen-free HSA; Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and incubated for 3 h with 0.25 to 16 nmol/L {sup}125I-VT-1 in M199/0.1% HSA at 0°C. After 3 h, the incubation medium was aspirated, and the cells were washed five times in M199 medium/0.1% HSA. Cell-associated {sup}125I-VT-1 was determined by solubilizing the cells in 400 μl of 1 mol/L sodium hydroxide at room temperature and counting radioactivity in a gamma counter. Nonspecific binding was assessed in parallel incubation by determining {sup}125I-VT-1 binding in the presence of a 100-fold excess of unlabeled VT-1. Cellular specific binding was calculated by subtracting the nonspecific binding from the cellular binding of {sup}125I-VT-1, as determined in the absence of unlabeled VT-1. All determinations were done in duplicate. Data were analyzed using the method described by Scatchard (31).

Glycolipid Extraction and Thin-Layer Chromatography

Confluent monolayers of mesangial cells were cultured in six-well plates for 24 h with and without TNFa. Subsequently, the cells were washed with PBS and trypsinized, after which neutral glycolipids were extracted and separated by methods described earlier by Lingwood et al. (32). After thin-layer chromatography (TLC), the silica gel TLC plate was soaked three times for 1 min in 0.01% polyisobutylmethacrylate (Polysciences, Warrington, PA) in hexane and air-dried, followed by overnight incubation in PBS/1% bovine serum albumin (BSA; Sigma) and 0.05% Tween 20. The plate was subsequently incubated with 50 ml of 1.5 nmol/L {sup}125I-VT-1 in 1% BSA and 0.05% Tween 20 in PBS for 4 h at 4°C. The plate was washed extensively with PBS supplemented with 1% BSA and 0.05% Tween 20, air-dried, and then analyzed by a Fuji BAS 1000 PhosphorImager (Leiden, The Netherlands).

Cytokine Production

Human mesangial cells were grown to confluence in 24-well plates and subsequently exposed to either lipopolysaccharide (LPS; Escherichia coli, serotype O55:B5, Sigma), TNFa, VT-1, VT-2, or B-subunit during a 24-h incubation period. Control cells were exposed to medium alone. After the indicated period of time, the supernatants were procured and centrifugated and stored at −20°C until they were assayed for the presence of cytokines. TNFa and interleukin 1-β (IL-1β) were determined by RIA as described previously (33). The sensitivity of both assays was 80 ng/ml. IL-6 was measured by ELISA as previously described by Van Setten et al. (33). The sensitivity of this ELISA was 20 pg/ml. IL-8 and monocyte chemoattractant protein-1 (MCP-1) were also determined by ELISA. In brief, ELISA plates (Merlin Diagnostic Systems, Rotterdam, The Netherlands) were coated overnight with (respectively) 0.5 μg/ml IL-8 monoclonal antibody and 3 μg/ml MCP-1 monoclonal antibody (R&D systems, Abingdon, Oxon, UK) in coating buffer. The next day, plates were washed three times with 0.5% (vol/vol) Tween/PBS, after which the plates were blocked for 1.5 h with 5% BSA in PBS. After being blocked, the plates were washed three times, and 50 μl of sample and standard (25 to 2500 pg/ml and 0.25 to 25 ng/ml) were added and incubated for 1 h at 37°C. Subsequently, the plates were washed, and biotinylated secondary antibodies (respectively, 3 μg/ml and 4 μg/ml; R&D Systems) were added for 45 min at 37°C. Plates were washed five times and incubated with streptavidin peroxidase (0.5 μg/ml) for 30 min at 37°C, after which the plates were washed again five times and exposed to o-phenylenediamine dihydrochloride (Sigma) for 30 min. The reaction was stopped by the addition of 2 mol/L H2SO4 (50 μl/well). Absorbency was read at 492 nm on a Titertek Multiscan. Preliminary experiments showed that a 1:10 to 100 dilution of the culture supernatants was needed to ensure that the results fell on the linear portion of the standard curve. The recovery rate was >95%, and parallelism was demonstrated for different dilutions. The sensitivities of the IL-8 and MCP-1 ELISA were 50 pg/ml and 0.25 ng/ml, respectively. All samples and standards were assayed in duplicate.

Cell Proliferation and Protein Synthesis

Cell proliferation and protein synthesis were determined by assaying the incorporation of {sup}3H-thymidine and {sup}35S-methionine into newly synthesized DNA and proteins, respectively. Mesangial cells were cultured in 24-well plates and grown until confluence. Subsequently, the cells were preincubated with or without TNFa for 24 h. The next day, the cells were washed and incubated with VT-1, VT-2, and B-subunit in medium containing {sup}3H-thymidine (1 μCi/ml complete medium) and {sup}35S-methionine (0.25 μCi/ml complete medium) during a 4- to 48-h incubation period. After incubation, the supernatant was aspirated, and the cells were washed with PBS and precipitated with
and 5% TCA, respectively. After solubilizing in 0.3 ml of 0.3 mol/L NaOH, the contents of each well were neutralized with HCl (60 μl of 1.5 mol/L) and counted in a liquid scintillation counter.

Results

Isolation and Characterization of Pure Cultures of Human Mesangial Cells

Primary outgrowths from glomeruli that were mildly treated with collagenase consisted of mostly epithelial and mesangial cells, the latter forming “hillocks.” Mesangial cell populations were enriched by collecting “hillock”-forming cells and further purified by selective elimination of contaminating epithelial cells by an immunomagnetic separation technique, using UEA-I-coated dynabeads. The cells were identified as mesangial cells because of their characteristic morphology by phase-contrast microscopy and on the basis of immunofluorescence studies. By phase-contrast microscopy, the mesangial cells initially showed their typical spindle/stellate-shaped morphology, followed by their characteristic growth pattern of overcrossing cells when reaching confluence (Figure 1). Immunofluorescence studies revealed intense staining of intracellular filaments with α-smooth muscle actin (Figure 2A), whereas no immunoreactivity was noticed with PECAM-1- and cytokeratin antibodies. In addition, epithelial cell contamination was made unlikely by the finding that the u-PA antigen concentration in conditioned media of cultured mesangial cells was present in very low quantities (<0.4 ng/24 h per 10^5 cells; three different cultures), whereas the presence of u-PA antigen in conditioned media of cultured renal epithelial cells was abundant (>500 ng/24 h per 10^5 cells; three different cultures).

Effect of VT-1 on the Number of Viable Human Mesangial Cells

To evaluate whether confluent human mesangial cells are sensitive to the toxic effect of VT-1, cells were exposed to various concentrations of VT-1 (1 pmol/L to 10 nmol/L) for 4 to 72 h, after which the number of viable adherent cells was assessed. After 24 h of exposure to VT-1 (Table 1), only a slight decrease in the number of viable adherent mesangial cells was observed. No further decrease in the number of viable adherent cells was observed after VT-1 exposure of the mesangial cells for 48 to 72 h (data not shown).

VT-1 Binding to Mesangial Cells and the Effect of TNFα on the Number of VT-1 Receptors

Because no significant cytotoxic effects were observed, we wondered whether human mesangial cells, like other eukaryotic cells, do bind VT to their cell membrane. Therefore, binding experiments with ^125^I-VT-1 were performed. Binding of ^125^I-VT-1 to human mesangial cells was saturable (Figure 3A) and specific; ^125^I-VT-1 binding was entirely (95%) displaced by a 100-fold excess of unlabeled VT-1. Pretreatment of mesangial cells with TNFα resulted in an increase in ^125^I-VT-1 binding (Figure 3A). Scatchard plot analysis revealed that one type of binding site is involved in VT-1 binding to human mesangial cells (Figure 3B). In addition, Scatchard plot analysis showed that 24-h preincubation of confluent human mesangial cells with TNFα caused a twofold increase in the number of specific binding sites, whereas the apparent affinity of VT-1 did not change significantly.

Receptor Analysis by Thin-Layer Chromatography

To evaluate whether human mesangial cells bind VT-1 to the cell surface via a glycolipid (like other eukaryotic cells), neutral glycolipid extracts from both unstimulated human mesangial cells, as well as TNFα-stimulated human mesangial cells, were prepared. Neutral glycolipids were separated by TLC, after which the chromatograms were incubated with ^125^I-VT-1. After extensive washing, the bound ^125^I-VT-1 was detected by exposure to a phosphorimager. The radiolabeled VT-1 bound strongly to Gb3 and not to Gb4 when a standard preparation of neutral glycolipids was tested (Figure 4, lane B). In unstimulated and TNFα-stimulated human mesangial cells, binding of ^125^I-VT-1 occurred to three different bands (Figure 4, lane A and B). The upper two bands most likely represent Gb3 with different fatty acyl moieties (35,36). The third band, running slightly ahead of Gb4, has been observed previously in human monocytes, in which VT-1 induced cytokine release (33). The exact nature of this receptor has not yet been elucidated. The increase in ^125^I-VT-1 binding to the glycolipid extracts of TNFα-stimulated human mesangial cells indicates that the synthesis of VT-1 receptors is increased rather than only a reflection of a redistribution of VT-1 receptors from intracellular stores toward the plasma membrane. This pattern was consistently found with different preparations of human mesangial cells obtained from two different donors.

**Table 1. Effect of VT-1 on the number of viable adherent human mesangial cells**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Number of Viable Adherent Cells (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t = 4 h</td>
</tr>
<tr>
<td>VT-1 (1 pmol/L)</td>
<td>111 ± 7</td>
</tr>
<tr>
<td>VT-1 (10 pmol/L)</td>
<td>99 ± 11</td>
</tr>
<tr>
<td>VT-1 (100 pmol/L)</td>
<td>99 ± 8</td>
</tr>
<tr>
<td>VT-1 (1 nmol/L)</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>VT-1 (10 nmol/L)</td>
<td>106 ± 9</td>
</tr>
</tbody>
</table>

*Values are mean ± SD of three different experiments with three different populations of human mesangial cells. t, time.*
Effect of VT on the Production of Cytokines by Human Mesangial Cells

Because mesangial cells possess a VT-1 receptor similar to that of monocytes, which is believed to mediate VT-1–dependent cytokine induction (33), we studied whether VT also induces cytokine release in human mesangial cells. When conditioned media of two different populations of human mesangial cells were assayed for the presence of IL-6, IL-8, MCP-1, IL-1β, and TNFα, we observed that mesangial cells constitutively release only small amounts of IL-6, IL-8, and MCP-1, as was determined by sandwich ELISA, whereas TNFα and particularly IL-1β levels were not detectable by sensitive RIA. Upon stimulation with TNFα (10 ng/ml) for 24 h, the concentration of IL-6, IL-8, and MCP-1 increased to 15 ng/ml, 185 ng/ml, and 122.5 ng/ml, respectively (mean of two different donors). In addition, LPS induced the mesangial cells to release IL-6, IL-8, and MCP-1 (Table 2). Exposure of the two different donors’ human mesangial cells to VT-1 (0.1 pmol/L to 10 nmol/L), VT-2 (0.1 pmol/L to 10 nmol/L), or the B-subunit (1 µg/ml) alone did not enhance the production of the measured cytokines significantly (Table 2). Similar results were obtained with the human mesangial cells of a third donor, although the mean values of all five measured cytokines of this donor were lower, probably indicating individual differences in the capacity to secrete inflammatory mediators upon various stimuli.

Figure 3. Tumor necrosis factor α (TNFα) increases the specific binding of 125I-VT-1 to human mesangial cells. (A) Human mesangial cells of one representative donor were grown to confluence and pretreated with either control medium (open squares) or TNFα (10 ng/ml; filled squares) for 24 h. The cells were then incubated with increasing concentrations of 125I-VT-1 (0.25 to 16 nmol/L) for 3 h at 4°C, after which specific binding of 125I-VT-1 to mesangial cells was calculated as indicated in Materials and Methods. (B) Scatchard plot analysis of 125I-VT-1 binding to human mesangial cells preincubated with either control medium (open circles) or TNFα (10 ng/ml; filled circles) for 24 h.

Figure 4. 125I-VT-1 binding to glycolipids extracted from purified populations of human mesangial cells. Glycolipids were extracted from confluent human mesangial cells, separated by thin-layer chromatography, assayed for 125I-VT-1 binding, and visualized by a phosphorimager. Lanes A and B, Glycolipid extracts of 60 cm² human mesangial cells of one representative donor. Lane A, Control mesangial cells. Lane B, TNFα-treated (10 ng/ml) mesangial cells. Lane C, Glycolipid extracts of 10 × 10⁶ lipopolysaccharide-pretreated human monocytes. Lane D, Glycolipid extract of 75 cm² TNFα-pretreated human umbilical vein endothelial cells. Lane E, Standard neutral glycolipids: 2 µg of each glycolipid. Gb1, galactosylceramide; Gb2, lactosylceramide; Gb3, globotriaosylceramide; Gb4, globotetraosylceramide.
Effect of VT-1 and VT-2 on Cell Proliferation and Protein Synthesis

When mesangial cells were incubated with either control medium alone or control medium supplemented with VT-1, we observed that the number of cells increased under control conditions but not after exposure to VT. Therefore, we evaluated the effect of VT on cell proliferation and overall protein synthesis by measuring $^{3}$H-thymidine incorporation in DNA and $^{35}$S-methionine incorporation in newly synthesized proteins.

Figure 5, panels A and B, show the time- and concentration-dependent inhibition of $^{3}$H-thymidine and $^{35}$S-methionine incorporation in (respectively) newly synthesized DNA and proteins by VT-1 of one representative population of human mesangial cells of three. Four hours of exposure to VT-1 at concentrations ranging from 10 pmol/L to 10 nmol/L was sufficient to reduce the DNA- and protein-synthesis rate. This inhibitory effect increased after 12 and 24 h of exposure. At all times and concentrations tested, $^{3}$H-thymidine incorporation was inhibited to a higher extent than was $^{35}$S-methionine. The inhibitory effects of VT-1 on DNA and protein synthesis were dependent on the degree of cell density and on the preexposure to TNFa (10 ng/ml) as depicted in Figure 6. Four independent experiments with two different mesangial cell populations showed that subconfluent mesangial cells display the highest susceptibility. VT-1 susceptibility increased after preexposure to TNFa (Figure 6). Also, in this case, $^{3}$H-thymidine incorporation was inhibited to a higher extent than was $^{35}$S-methionine.

No major differences were observed when the effects of VT-1 and VT-2 were compared; both inhibited the overall synthesis of proteins, although VT-1 was slightly more potent than VT-2. After 24-h exposure to VT-1 and VT-2 (1 nmol/L), the mean overall protein synthesis in nonpretreated and TNFα-prestimulated highly confluent mesangial cells of three different populations of mesangial cells was, respectively, 72 ± 5 and 50 ± 2% of control for VT-1-exposed cells and 89 ± 5 and 72 ± 5% of control for VT-2-exposed cells (mean ± SD). Exposure of TNFα-treated human mesangial cells to the B-subunit of the VT alone resulted in a significant inhibition of $^{3}$H-thymidine incorporation, whereas $^{35}$S-methionine incorporation was not significantly affected. On a molecular base, the B-subunit alone inhibited DNA synthesis to a lesser extent than did the holotoxin (Figure 7). To evaluate the possibility that protein synthesis inhibition by VT underlies the inhibition of $^{3}$H-thymidine incorporation, we compared the effect of VT with that of the protein synthesis inhibitor cycloheximide (CHX). For proper comparison, we determined which CHX concentrations inhibited protein synthesis to a similar extent as did VT-2. On the basis that CHX (0.1 μg/ml) and VT-2 (0.1 nmol/L) inhibited protein synthesis 30 to 40%, only VT-2 inhibited $^{3}$H-thymidine incorporation (Table 3). Correspondingly, protein synthesis at a given proliferation rate was much more inhibited in cases of CHX treatment when compared with VT treatment (data not shown). This suggests that inhibition of DNA synthesis proceeds at least partly independently of protein synthesis inhibition.

### Discussion

The leading cause of acute renal failure in children is the epidemic form of HUS, the etiology of which is associated with a VT-producing Escherichia coli. Although the role of endothelial cell damage in the pathogenesis in HUS is well described in the literature, the involvement of mesangial cells is scarcely documented (6,37). The interaction of VT with purified human mesangial cells was the subject of this in vitro study. We have described a modified technique for the culture of highly purified human mesangial cells free of contaminating endothelial and epithelial cells. We demonstrated that human mesangial cells bind VT to three molecular forms of neutral glycolipids identical or closely related to Gb3. One band was comparable to the VT receptor present in monocytes, in which it has previously been shown that VT induces cytokine release (33). The two other bands are most probably Gb3 species with different fatty acyl moieties (35,36). Similar Gb3 species are present in TNFα-treated endothelial cells (3,34), in which it has been shown that VT induces cytotoxicity. In mesangial cells, VT did not induce cytokine release. VT markedly inhibited DNA and protein synthesis by two distinct mechanisms, but these effects were not associated with cell death.

The culture of human mesangial cells is, in several aspects, more difficult than that of rat mesangial cells. First, primary cultures of human mesangial cells are contaminated by epithelial and small numbers of endothelial cells. In particular, the epithelial cells continue to grow, even when mesangial cells appear after 10 to 30 d of culture. On the contrary, the culture

### Table 2. Effect of VT on the production of cytokines and chemokines by human mesangial cells

<table>
<thead>
<tr>
<th>VT</th>
<th>IL-1β (ng/ml)</th>
<th>TNFα (ng/ml)</th>
<th>IL-6 (ng/ml)</th>
<th>IL-8 (ng/ml)</th>
<th>MCP-1 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;0.08</td>
<td>0.19 ± 0.02</td>
<td>1.0 ± 0.1</td>
<td>2.4 ± 0.8</td>
<td>26.0 ± 0.0</td>
</tr>
<tr>
<td>LPS (1 μg/ml)</td>
<td>&lt;0.08</td>
<td>0.12 ± 0.01</td>
<td>36.9 ± 5.1</td>
<td>136.0 ± 38</td>
<td>91.5 ± 0.7</td>
</tr>
<tr>
<td>TNFα (10 ng/ml)</td>
<td>&lt;0.08</td>
<td>5.64 ± 2.34</td>
<td>15.0 ± 0.8</td>
<td>185.0 ± 15.6</td>
<td>122.5 ± 24.7</td>
</tr>
<tr>
<td>VT-1 (10 nmol/L)</td>
<td>&lt;0.08</td>
<td>0.18 ± 0.04</td>
<td>1.0 ± 0.02</td>
<td>1.4 ± 0.3</td>
<td>20.4 ± 0.8</td>
</tr>
<tr>
<td>VT-2 (10 nmol/L)</td>
<td>&lt;0.08</td>
<td>0.18 ± 0.01</td>
<td>1.0 ± 0.3</td>
<td>2.7 ± 0.1</td>
<td>24.1 ± 1.6</td>
</tr>
<tr>
<td>B-subunit (1 μg/ml)</td>
<td>&lt;0.08</td>
<td>0.16 ± 0.03</td>
<td>1.0 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>25.1 ± 2.8</td>
</tr>
</tbody>
</table>

* Values are the mean (ng/ml) ± SD of two different experiments with two different donors. IL, interleukin; TNFα, tumor necrosis factor-α; MCP, monocyte chemoattractant protein; LPS, lipopolysaccharide.
Figure 5. VT-1 inhibits cell proliferation and overall protein synthesis in human mesangial cells in a time- and dose-dependent manner. Human mesangial cells of one representative donor (of three) were grown to confluence and subsequently incubated with either control medium (circle), VT-1 (10 nmol/L; triangle), VT-1 (1 nmol/L; square), VT-1 (0.1 nmol/L; diamond), or VT-1 (10 pmol/L; plus sign) in the presence of 3H-thymidine and 35S-methionine. After 24 h, the incorporation of 3H-thymidine and 35S-methionine into (respectively) newly synthesized DNA (A) and 35S-proteins (B) was determined according to the descriptions in Materials and Methods.

Figure 6. The degree of cell density of cultured mesangial cells is related to inhibitory effects of VT-1 on cellular proliferation and overall protein synthesis. Human mesangial cells were grown to confluence, trypsinized, and split 1:5 onto gelatin-coated 24-well plates. Subconfluent (1), confluent (2), and highly confluent (3) mesangial cells were preexposed to either control medium (open bar) or TNFa (10 ng/ml; hatched bar), respectively, for 24 h. Subsequently, the cells were washed and exposed to either control medium or medium supplemented with VT-1 (1 nmol/L) in the presence of 3H-thymidine and 35S-methionine for 48 h, after which the incorporation of 3H-thymidine and 35S-methionine into (respectively) newly synthesized DNA (A) and 35S-proteins (B) was determined according to the descriptions in Materials and Methods. Data are expressed as percentages of control.

of rat mesangial cells is not hampered by contaminating epithelial cells, because these epithelial cells do not survive during the first weeks of culture. Secondly, no specific marker for human mesangial cells is available for immunoselection (38), whereas the anti-Thy-1 antigen is a specific marker for rat mesangial cells (38). Therefore, we have adapted the existing procedure to enrich the mesangial cell populations (22) by additional selective removal of the contaminating epithelial cells. An immunomagnetic separation technique that uses Dynabeads coated with UEA-I, which recognizes L-fucose residues, appeared to be a valuable ligand for removal of all epithelial cells from the mesangial cell culture. The avid binding of epithelial cells to UEA-I corresponds with immunochimical studies in vivo, which show that UEA-I binds to cell membranes of epithelial and endothelial cells in the kidney (39–43). Indeed, the resulting cell populations of human mes-
Figure 7. This figure shows the inhibitory effects of VT-1 and the B-subunit alone on cell proliferation and overall protein synthesis. Confluent layers of mesangial cells of one representative donor were trypsinized and seeded 1:5 onto gelatin-coated wells. After 3, 4, 8, and 11 d, the mesangial cells (representing cells with different cell densities) were pretreated with TNFα (10 ng/ml) for 24 h. A, Subconfluent mesangial cells; B, confluent mesangial cells; C, highly confluent mesangial cells. The cells were subsequently washed once and exposed to either control medium (circles), VT-1 (1 nmol/L; triangles; equivalent to 5 nmol/L B-subunit), or the B-subunit alone (1 μg/ml; squares; equivalent to 132 nmol/L B-subunit) in the presence of 3H-thymidine and 35S-methionine for 48 h, after which the incorporation of 3H-thymidine and 35S-methionine into (respectively) newly synthesized DNA (Panel A) and 35S-proteins (Panel B) was determined according to the descriptions in Materials and Methods.

Mesangial cells did not show immunoreactivity for PECAM-1 or cytokeratin 8, excluding endothelial and epithelial cell contamination, respectively. It seems unlikely that fibroblasts are present among mesangial cell cultures because mesangial cells were isolated from primary outgrowths of pure populations of glomeruli. This suggestion is strengthened by the observation that immunoreactivity for α-smooth muscle actin is present in the majority of highly confluent cultures of mesangial cells, whereas highly confluent cultures of fibroblasts do not express this antigen.

Previous studies have demonstrated that VT-1 affects endothelial cells (3,34) and monocytes (33,44) by different mechanisms. However, its effect on mesangial cells in vitro is poorly investigated. Only recently, Robinson et al. (37) reported on the binding of FITC-labeled VT-1 B-subunit to mesangial cells in vitro and the subsequent dose-dependent inhibitory effect of VT on mitogenesis of mesangial cells of one donor. Our report extends these observations and demonstrates that mesangial cells are not particularly susceptible to the cytotoxic effect of VT; however, they do bind VT to their cell membrane via different types of glycolipid receptors. One of these receptors is comparable to that on monocytes, in which it induces cytokine release. From a pathogenetic point of view, these inflammatory mediators play an important role in sensitizing glomerular microvascular endothelial cells to the cytotoxic effect of VT by induction of the Gb3 receptor on the cell surface (34). Besides, inflammatory mediators render the endothelium procoagulant and proinflammatory; these processes possibly contribute to the pathological findings observed in HUS patients. Chemokines may be involved in the pathogenesis of HUS by the recruitment of leukocytes into the kidney. In vivo data support the involvement of inflammatory mediators and chemokines in the pathogenesis of HUS, demonstrating elevated levels in urine and, to a lesser extent, in the plasma of HUS patients (45–48). In addition to monocytes, human mesangial cells have been reported to act as a source of inflammatory mediators and chemokines. The data presented here indicate that VT-1 does not induce the production of various cytokines or chemokines by human mesangial cells. As previously described, we have shown that LPS, which may play a role in the pathogenesis of HUS (33), induces human mesangial cells to produce and release cytokines and chemokines.

The cytotoxic effect of VT has originally been demonstrated to occur via inhibition of the elongation factor-1-dependent binding of aminoacyl-tRNA to the ribosomes by the A-subunit of VT (17,18), resulting in inhibition of eukaryotic protein synthesis, although an induction of apoptosis has also been observed (49–51). For the inhibitory effect on protein synthesis, the A-subunit has to be internalized by the cell, which requires the binding of the B-subunits of VT to the cell surface via its glycolipid receptor Gb3 (19). We showed that this receptor is also present on human mesangial cells and is upregulated after exposure to TNFα, similar to its expression on endothelial cells (3,34). Although VT-1 interaction caused complete inhibition of protein synthesis and cell death in vero cells and TNFα-treated endothelial cells (3,34), it inhibited protein synthesis in mesangial cells by approximately 75% without a significant effect on cell viability, either under basal conditions or after upregulation of the Gb3 moieties by TNFα. Apparently, the residual 25% of protein synthesis is sufficient for mesangial cells to survive. However, in conditions of increased demand of protein synthesis, the reduced capacity to synthesize new proteins may affect cell function.

In addition to reducing protein synthesis, VT-1 also inhib-
inated cell proliferation in human mesangial cells. One may anticipate that this is caused by the reduced protein synthesis rate. However, the inhibitory effect of VT-1 on proliferation was larger and faster than that on protein synthesis. Furthermore, the B-subunit, which did not significantly inhibit protein synthesis, also caused a dose- and time-dependent inhibition of cell proliferation. This points to an additional inhibitory effect of VT on cell proliferation, independent of the reduction of protein synthesis. This suggestion is strengthened by our observation that partial inhibition of protein synthesis by VT-1 was associated with a considerably higher inhibition of cell proliferation than a comparable inhibition of protein synthesis by cycloheximide, which is known to block the peptidyl transferase reaction on the ribosomes. The observations that the B-subunit of VT can induce apoptosis in Burkitt lymphoma cells (50) and affects cell proliferation of mesangial cells (this study) suggests that the B-subunit can, probably via interaction with Gb3, directly activate a signal transduction pathway.

The inhibitory effects on both overall protein synthesis and cell proliferation were dependent on the pretreatment with TNFα and the cell density. Several papers have reported that inflammatory mediators may sensitize cells (3,34) to the toxic effect of VT by an upregulation of the number of specific binding sites for VT. We extend this observation to mesangial cells, showing the constitutive expression of VT receptors with an increase in the number of VT receptors after TNFα pretreatment. The higher susceptibility of subconfluent cells when compared with confluent cells has also been described previously in the literature (3,52,53) and is in agreement with our findings. Most likely this higher susceptibility is related to the cell cycle-dependent exposure of Gb3 compounded with increased Gb3 turnover (53).

We conclude from this study with highly purified human mesangial cells that these cells bind VT-1 to specific glycolipid receptors but are not susceptible to the VT cytotoxicity. VT did not induce the release of cytokines but did inhibit DNA and—to a lesser extent—overall protein synthesis in mesangial cells. Two distinct mechanisms appeared to be involved in these inhibitory effects. One involves the effect of the A-subunit on overall protein synthesis. The second mechanism involves a B-subunit–linked inhibition of mitogenesis. The intracellular routing of VT (A- and B-subunits) toward the lysosomes, endosomes, Golgi and endoplasmic reticulum, or the nuclear membrane may vary between different cell types, possibly in relation to the fatty-acyl chain lengths of the glycolipid VT receptors (54–57).

The data presented here suggest that toxic and cytokine-inducing effects of VT on mesangial cells play a minor role in the development of VT on mesangial cells may play a minor role in the development of renal failure in HUS. However, the facilitated access of damaging plasma constituents, procoagulatory agents, infiltrating leukocytes, and their products may put a demand on mesangial cells to which they cannot adequately respond because of a reduced capacity to synthesize new proteins. In addition, the pathological findings in the mesangial area observed in some of the biopsies of HUS patients may be initiated by the effects of VT-1 on mesangial cells described in this report.

### Acknowledgments

We thank Prof. Dr. J. L. Brunton (Departments of Medicine and Microbiology, The University of Toronto, Toronto, Ontario, Canada) for providing us with purified B-subunit. This study was supported by grants from the Termeulen Fonds (Amsterdam, The Netherlands) and the Dutch Kidney Foundation (Grant C94.1344).

### References


### Table 3. Effect of the protein synthesis inhibitors VT and cycloheximide on cell proliferation and overall protein synthesis

<table>
<thead>
<tr>
<th>Donor</th>
<th>VT-2 (0.1 nmol/L)</th>
<th>CHX (0.1 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35S-Proteins</td>
<td>3H-Thymidine Incorporation</td>
</tr>
<tr>
<td>Donor 1</td>
<td>61.0% of C</td>
<td>44.3% of C</td>
</tr>
<tr>
<td>Donor 2</td>
<td>69.9% of C</td>
<td>49.8% of C</td>
</tr>
</tbody>
</table>

*CHX, cycloheximide; C, control.


55. Sandvig K, Ryd M, Garred O, Schweda E, Holm PK, van Deurs B: Retrograde transport from the Golgi complex to the ER of both Shiga toxin and the nontoxic Shiga B-fragment is regulated by butyric acid and cAMP. *J Cell Biol* 126: 53–64, 1994
