Effects of TNFα on verocytotoxin cytotoxicity in purified human glomerular microvascular endothelial cells


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Effects of TNFα and verocytotoxin cytotoxicity in human glomerular microvascular endothelial cells. In the pathogenesis of the hemolytic uremic syndrome (HUS), endothelial damage of glomeruli and arterioles of the kidney appears to play a central role. Previous studies have shown that verocytotoxin-1 (VT-1) cytotoxicity on human vein endothelial cells requires additional stimuli, in particular the inflammatory mediator tumor necrosis factor alpha (TNFα). In this study the effects of VT on human glomerular microvascular endothelial cells (GMVEC) were examined. A reproducible method was developed for the isolation and purification of large numbers of highly purified GMVEC. The obtained GMVEC were over 99% pure; their endothelial origin was demonstrated by the expression of the endothelial antigens von Willebrand factor, EN-4, PECAM-1 and V.E-cadherin. Upon stimulation with TNFα the cells expressed the endothelial-specific adhesion molecule E-selectin. A limited number of fenestra structures was observed by scanning electron microscopy (SEM), suggesting glomerular origin of the endothelial cells. Cytotoxicity of VT-1 to GMVEC was evaluated by determination of the number of viable adherent cells and by assay of overall protein synthesis after exposure to varying concentrations of VT-1. In non-stimulated GMVEC, cytotoxicity of VT-1 was inversely related to the degree and duration of confluence, subconfluent cells being the most sensitive. In highly confluent GMVEC, VT cytotoxicity required pre-exposure of the cells to the inflammatory mediator TNFα, which induced an increase in the number of VT receptors on GMVEC. Thin layer chromatography of extracted glycolipids from the GMVEC showed binding of VT-1 to globotriaosylceramide (Gb3), known to be the functional receptor for VT. There were no major differences in protein synthesis inhibition with equal concentrations VT-1 and VT-2. In conclusion, in this study we provide a reproducible method to isolate, purify and culture well characterized human GMVEC on a routine basis. In vitro studies with these GMVEC demonstrate that VT cytotoxicity depends on the degree of confluence and the additional preexposure to the inflammatory mediator TNFα. These observations provide further insight into the complex events that may occur in glomeruli in the pathogenesis of HUS.

The hemolytic uremic syndrome (HUS) is characterized by renal failure, thrombocytopenia and hemolytic anemia [1]. Endothelial damage of glomeruli and kidney arterioles appears to play a pivotal role in the pathogenesis of HUS. Histopathological studies of the kidney in HUS patients reveal swollen and detached epithelial cells and deposits of fibrin, all within the glomerulus [2]. In severe cases of HUS endothelial cell damage is not limited to the kidney, but extends to other organs, such as brain and pancreas. Infection with a verocytotoxin (VT) producing E. coli has been implicated in the etiology of the epidemic form of HUS [3]. In vitro studies with umbilical and adult vein endothelial cells indicate that endothelial cells become more susceptible to VT if prestimulated with inflammatory mediators [4–7]. Induction of the globotriaosylceramide (Gb3) receptor, known to be the functional receptor for VT, proved to be the mechanism by which inflammatory mediators induce increased susceptibility to VT [6]. A number of observations have indicated morphological, biochemical and functional heterogeneity between macro- and microvessel endothelial cells, and even between microvascular endothelial cells derived from different organs [8]. Therefore, to test if non-stimulated glomerular microvascular endothelial cells (GMVEC) are more susceptible to verocytotoxin-1 (VT-1) than their venous counterparts, it would be desirable to directly study GMVEC. The isolation and culture of human renal [9] and glomerular [10–12] MVEC has been reported, but currently no procedure is available to culture human GMVEC on a routine basis. Here, we report on a reproducible method to isolate, purify and culture large numbers of GMVEC that are free of contaminating cells, and on the characterization of these cells. Furthermore, we describe the effects of TNFα and the degree of confluence on VT cytotoxicity for highly confluent GMVEC. We found that VT-1 cytotoxicity for highly confluent GMVEC requires pre-exposure of the cells to the inflammatory mediator TNFα, which is similar to previous findings on umbilical and adult vein endothelial cells [4–7]. These data seem to contrast to observations by Obrig et al [13], who showed that VT-1 had an identical toxic effect on TNFα-stimulated and non-stimulated renal MVEC. To explain these apparent differences, we analyzed the effects of VT-1 on GMVEC during maintenance in a confluent culture, and demonstrate that the sensitivity of GMVEC for VT-1 is markedly reduced in the post-confluent state.

Methods

Isolation of glomeruli

Studies were performed with human kidneys not suitable for transplantation because of anatomical anomalies, technical or
were shaken vigorously to loosen up the glomeruli and liberate the
immunological reasons. Written consent was obtained from the
Medical Ethical Review Board of the University Hospital Nijmegen,
Netherlands. In general, the procedure was started as
soon as possible after explantation of the kidney, usually within 24
hours at 37°C. Following digestion glomerular remnants
were collected on top of the screens with opening sizes of 180, 125, 108,
90 or 53 μm and digested with 0.1% (wt/vol) collagenase type 2
CLS (Worthington, NJ, USA) in M199 (BioWhittaker, MD, USA)
for two hours at 37°C. Following digestion glomerular remnants
were shaken vigorously to loosen up the glomeruli and liberate the
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were shaken vigorously to loosen up the glomeruli and liberate the
endothelial cells. Subsequently the glomerular remnants were
washed, centrifuged and resuspended in complete media that
consisted of M199 supplemented with 10% (vol/vol) newborn calf
serum (NBCS; Gibco, Grand Island, NY, USA), 10% (vol/vol) human serum (HS; local blood bank), 2 mmol/liter glutamine
(ICN Biomedicals, Zoetermeer, The Netherlands), 100 IU/ml penicillin/0.1 mg/ml streptomycin and 5 μg/ml heparin (Leo Pharmaceuticales, Weesp, The Netherlands) and 150 mg/liter crude
gelatin (1%; Fluka BioChemika, Buchs, Switzerland) coated wells
(Costar Corp., Cambridge, MA, USA).

Primary culture and purification
In general the glomerular remnants attached within one to two
days, after which outgrowth of predominantly cells of epithelial
and endothelial origin was noted. Daily monitoring of proliferating
cells determined the exact timing of separating the endothelial
and epithelial cells, usually within three to eight days after plating
of the glomerular remnants. The purification procedure consisted
basically of three steps. First, selective trypsinization was per-
formed, allowing endothelial cells to detach and leaving the
epithelial cells attached to the plate. Selectivity in cell detachment
was reached by incubating the cells with trypsin/EDTA (0.5 g
trypsin 1:250/0.2 g EDTA in 1 liter of Modified Puck’s Saline A;
Gibco) at room temperature for a short incubation period, the
duration of which was determined during visual inspection of the
cell layer by phase-contrast microscopy (about 60 seconds). Sec-
ond, to relieve glomerular remnants, which proved to be a source
of contaminating cells when subcultured, trypsined cells and glomerular remnants were filtrated through a 38 μm sieve. The
filtrate was collected, washed and pelleted by centrifugation (200 × g). Third, the pellet was resuspended in Hanks’ balanced
salts (ICN Biomedicals)/10% (vol/vol) fetal calf serum (FCS;
Gibco) and incubated with a monoclonal antibody against
PECAM-1 (CLB, Amsterdam, The Netherlands) for 30 minutes
on ice. Excess of unbound anti-PECAM-1 monoclonal was re-
moved by washing for three times. Subsequently, an immunomag-
etic separation technique with dynabeads coated goat anti-
mouse antibodies (Dynal A.S; Oslo, Norway) and a magnetic
particle collector (Dynal A.S) was performed to selectively collect
glomerular endothelial cells. In general the number of beads used
was calculated according to the following formula: (total number
of cells) × (% estimated endothelial cells) × 3. The use of too
many beads per cell reduced the attachment of the endothelial
cells. PECAM-1 positive cells were plated on gelatin-coated wells
and maintained at 37°C with 5% CO₂ atmosphere. The medium
was changed every two to three days and cells were passaged with
trypsin/EDTA onto gelatin-coated wells at a split ratio of 1:2 or
1:3. In general it was necessary to repeat the immunomagnetic
separation technique once or twice or to perform additional
manual weeding to obtain pure cultures of GMVEC.

In case the epithelial cells seemed to overgrow the endothelial
cells in the primary culture from morphological point of view, the
protocol as described above was preceded by a negative immuno-
MVEC were seeded on gelatin-coated glass-coverslips and grown until confluence. Cells were fixed in 80% (vol/vol) acetone for 10 minutes at 4°C and rinsed with phosphate-buffered saline (PBS). The presence of von Willebrand factor was demonstrated by indirect immunofluorescence staining with rabbit anti-human von Willebrand antiserum [1:80 dilution in 10% (vol/vol) pig serum; DAKO; Glostrup, Denmark] and FITC-
swine anti-rabbit Ig (1:20 in PBS; DAKO). The presence of other
endothelial-cell specific antigens such as PECAM-1, EN-4 antigen
and VE-cadherin was investigated by incubating the fixed cells
with anti-PECAM-1 MoAb (10 μg/ml; CLB, Amsterdam, The
Netherlands), MoAb EN-4 [15] (10 μg/ml; Sanbio, Uden, The
Netherlands) and anti-VE-cadherin MoAb [16] (1:500; provided by
Dr. Dejana, Mario Negri Institute, Milan, Italy) in PBS/10%
bovine serum. After a 30-minute incubation period at room tem­
perature the cells were washed twice with PBS and incubated with
FITC-goat anti-mouse Ig (1:20; DAKO). After a 30-minute
incubation period at room temperature, cells were washed twice
with PBS and mounted with Vectashield (Burlingame, CA, USA)
under a coverslip and investigated using a fluorescence micro-
scope. Similar immunofluorescent assays were carried out using
MoAb to control for contamination with mesangial-cells (anti-a-
smooth muscle actin, clone 1A4; Sigma, Zwijndrecht, The Neth-
erlands) and epithelial cells (monoclonal anti-cytokeratin 8, M20;
provided by Dr. G. van Muijen, University Hospital Nijmegen,
The Netherlands [17]). The expression of the endothelial-cell
specific adhesion molecule E-selectin upon stimulation with TNFα was determined by cell-ELISA. GMVEC were grown until confluence in 96-well tissue culture plastic plates (Costar Corp.)
and subsequently exposed to TNFα (10 ng/ml) during a four-hour
incubation period. Cells were rinsed once with M199 and fixed in
0.025% glutaraldehyde. Following fixation wells were incubated with MoAb against E-selectin (ENA-2; gift of Dr. J. Leeuwen-
berg, University Hospital Maastricht, The Netherlands [18])
diluted in M199/5% FCS at a concentration of 10 μg/ml during 60
minutes at 37°C. The cells were washed twice with PBS/Tween

Characterization
Primary and serially passaged cultures were characterized using
three criteria.

(1.) Morphological criteria. Outgrowth from collagenase treated
glomerular remnants and subcultures were examined with a
phase-contrast microscope. Photomicrographs were made at
various passages using Technical Pan films.

(2.) Immunological criteria. The presence of endothelial cell-
specific antigens was determined by performing indirect immuno-
fluorescence studies with a panel of endothelial cell-specific
monoclonal antibodies (MoAb). GMVEC were seeded on gela-
tin-coated glass-cover slips and grown until confluence. Cells were
fixed in 80% (vol/vol) acetone for 10 minutes at 4°C and rinsed with
phosphate-buffered saline (PBS). The presence of von Willebrand
factor was demonstrated by indirect immunofluorescence staining
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diluted in M199/5% FCS at a concentration of 10 μg/ml during 60
minutes at 37°C. The cells were washed twice with PBS/Tween

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and incubated with peroxidase labeled, goat anti-mouse second antibody (1:1000; DAKO) for 30 minutes at 37°C. After washes with PBS/Tween o-phenylenediamine-peroxide was added. The reaction was stopped by the addition of 2.5 mol/liter H₂SO₄ (50 µl/well). Absorbency was read at 492 nm on a Titretek Multiscan. Absorbency with second antibody alone was subtracted from all absorbency values expressed. Expression of E-selectin was also analyzed by performing immunofluorescence studies and FACS analysis as described above with minor adjustments, in that the cells were incubated with the primary and secondary antibodies diluted in PBS plus (PBS plus Mg²⁺ and Ca²⁺) and cells were only fixed after incubation with primary and secondary antibodies.

(3.) Ultrastructure. Evaluation of human GMVEC by scanning electron microscopy was performed by the osmium-thiocarbohydrazide-osmium technique. GMVEC grown on gelatin coated glass-coverslips were rinsed with M199 and fixed for two hours at room temperature with 2.5% glutaraldehyde in 0.1 mol/liter cacodylate buffer. The cells were then rinsed carefully with 0.1 mol/liter cacodylate/6.8% sucrose pH 7.38 and postfixed with 1% OsO₄ in the same cacodylate buffer. Subsequently the cells were rinsed with aquabidest and immersed in 1% filtered thiocarbohydrazide-solution (TCH). Following immersion, alternate fixation in OsO₄ and immersion in TCH and fixation in OsO₄ were repeated after which the cells were selectively dehydrated in graded alcohol solutions and critical point dried. Cells were visualized with a SEM Jeol 1200 EX/2 at an accelerating voltage of 50 KV.

Cytotoxicity assay

GMVEC were cultured in complete medium on gelatin coated 24-well plates and grown until confluence. Five days after reaching confluence (highly confluent) cells were preincubated with or without TNFα (10 ng/ml) for 24 hours. The next day the medium was aspirated and new medium with different concentrations of VT-1 and VT-2 (provided by Prof Karmali; Hospital for Sick Children, Toronto, Canada) was added. VT-1 and VT-2 were diluted in medium with 20% FCS instead of 10% NBCS and 10% HS, since previous studies have indicated that NBCS and HS may have neutralizing activity against VT-1 and VT-2. After 6 and 24 hours the cells were washed with PBS, released with trypsin/EDTA and subsequently viable, Trypan blue excluding cells were counted with a hemocytometer. In order to study the influence of confluence, cells at different degrees of confluence were preincubated with TNFα and tested for VT-1 cytotoxicity as described above.

Iodination of VT-1 and the binding of ¹²⁵I-VT-1 to human GMVEC

VT-1 was radiolabeled with Na⁻¹²⁵I according to the Iodogen procedure [19]. The radioactive activity of ¹²⁵I-VT-1 ranged from 10 to 12 µCi/µg of protein. GMVEC were grown in 24-well plates until they reached confluence. After five days the cells were preincubated with or without inflammatory mediators for 24 hours at 37°C. Subsequently the GMVEC were washed with M199 medium/0.1% human serum albumin (HSA) and incubated for three hours with 0.25 to 32 nmol/liter ¹²⁵I-VT-1 in M199 medium/0.1% HSA at 0°C. After three hours the incubation medium was aspirated and the cells were washed for five times in M199 medium/0.1% HSA. Cell-associated ¹²⁵I-VT-1 was determined by solubilizing the cells in 400 µl 1 mol/liter sodium hydroxide at room temperature and counting radioactivity in a gamma-counter. Non-specific binding was assessed in parallel incubation by determining ¹²⁵I-VT-1 binding in the presence of a 100-fold excess of unlabeled VT-1. Cellular specific binding was calculated by subtracting the non-specific binding from the cellular binding of 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 [20]. To investigate whether Gb3 is also the functional receptor for VT in GMVEC, TNFα-stimulated GMVEC were incubated with a MoAb against Gb3 [21] (MoAb anti-CD77; clone #38-13; Biodesign International, Kennebunk, ME, USA) one hour before and during assaying the binding of ¹²⁵I-VT-1.

Glycolipid extraction and thin layer chromatography (TLC)

Highly confluent GMVEC were cultured in six-well plates (20 cm²) for 24 hours with and without TNFα (10 ng/ml). Subsequently the cells were washed with PBS and trypsinized, after which glycolipids were extracted and separated by methods earlier described by Lingwood et al [22]. Following thin layer chromatography, the silica gel TLC plate was incubated with 50 ml, 1.5 nmol/liter ¹²⁵I-VT-1 in 1% BSA and 0.05% Tween-20 in PBS during four hours at 4°C. The plate was washed extensively with PBS supplemented with 1% BSA and 0.05% Tween-20, air dried and analyzed by a Fuji BAS 1000 phosphor-imager.

Protein synthesis

Protein synthesis was determined by assaying the incorporation of ³⁵S-labeled methionine (0.25 µCi/ml complete medium) in ³⁵S-proteins during an 8 to 24 hours incubation period with different concentrations of VT-1 and VT-2. After incubation, the cells were washed and ³⁵S-labeled cellular proteins were precipitated by adding 10% trichloroacetic acid. Precipitated proteins were dissolved in 0.3 ml 0.3 mol/liter NaOH, 60 µl 1.5 mol/liter HCl was added and precipitated radioactivity was counted in a liquid scintillation counter.

Results

Isolation and purification of human GMVEC

Incubation of pure populations of human glomeruli, which were isolated by a gradual sieving procedure with collagenase for two hours at 37°C followed by vigorously shaking, resulted in small clumps consisting of glomerular remnants. These remnants attached to gelatin-coated plates within one to two days. Following attachment, outgrowth of primarily endothelial and epithelial cells was noted (Fig. 1A), the latter easily overgrowing the GMVEC. As judged by phase-contrast microscopy the outgrowing endothelial cells demonstrated a slightly elongated morphology with a nucleus containing one or two nucleoli and a dark perinuclear cytoplasm area that was surrounded by a bright halo. Epithelial cells displayed the epithelial cell specific cobblestone pattern of densely packed polygonal cells. Besides the tendency of the contaminating epithelial cells to overgrow the endothelial cells, these cells seemed to inhibit the growth of endothelial cells, probably by producing growth inhibiting factor(s). Therefore, it was crucial to remove epithelial cells and purify the endothelial cell populations. The purification procedure consisted basically of three steps: selective detachment of most of the endothelial cells by trypsin-EDTA treatment; removal of glomerular remnants by...
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Identification and characterization of human GMVEC

Upon examination by phase contrast microscopy, confluent monolayers of human GMVEC exhibited a typical contact-inhibited morphology (Fig. 1C). They were not contaminated by spindle shaped and/or overcrossing mesangial cells [23], or by epithelial cells, which display a very regular, polygonal cobblestone morphology [24]. The endothelial and glomerular nature of the cells was confirmed by the expression of endothelial-specific antigens and by their ultrastructure.

Indirect immunofluorescence microscopy revealed the presence of von Willebrand factor in a distinct granular pattern (Fig. 2A). Furthermore, the endothelial specific antigens EN-4, PECAM-1 and VE-cadherin antigen were present at the regions of intercellular contacts (Figs. 2 B, C, E). When the GMVEC were subcultured for more than 10 passages, the cells remained positive for all these markers. The cultured human GMVEC showed no immunoreactivity to the anti-cytokeratin 8 [17] antibody or to the anti-α smooth-muscle actin antibody, excluding the contaminating epithelial and mesangial cells, respectively (data not shown).

Upon stimulation with TNFα (10 ng/ml) for four hours, expression of the endothelial cell specific protein E-selectin was induced on human GMVEC, whereas unstimulated controls did not express E-selectin (OD, 3.24 ± 0.55 and OD, 0.15 ± 0.02, respectively, as determined by cell-ELISA; mean ± sd; N = 3). Similar results were obtained when E-selectin expression was determined by indirect immunofluorescence or FACS analysis (data not shown).

At the ultrastructural level, the cultured human GMVEC displayed small pores with a diameter of about 100 nm, as revealed by scanning electron microscopy (Fig. 3). These structures were not observed in human umbilical and iliac vein endothelial cells (data not shown), and probably represent fenestrae. The persistent presence of these fenestral structures in GMVEC in vitro, although to a lesser extent when quantified per square micrometer in vivo, points not only to the glomerular endothelial origin, but also to a certain degree of differentiation of the cultured GMVEC.

Effect of VT-1 on viability of human GMVEC

To evaluate whether GMVEC are sensitive to the toxic effect of VT-1, cell viability was estimated by counting viable adherent cells with a hemocytometer after an 8 to 24 hour exposure to various concentrations of VT-1. VT-1 at concentrations ranging from 1 fmol/liter to 10 nmol/liter did not significantly affect cell viability of three different populations of highly confluent human GMVEC when cultured under basal conditions over a 24-hour time period. At the highest dose tested, a 24 hour incubation with VT-1 (10 nmol/liter) caused 0%, 1% and 6% cytotoxicity, respectively, in these three different populations of highly confluent GMVEC. Similar to previous observations from our group, studying the effects of VT-1 on human umbilical vein and human femoral vein endothelial cells (HUVEC) [6], human GMVEC cultured and treated under identical conditions became susceptible to the cytotoxic effect of VT-1 after preincubation with the inflammatory conditions.
AT-1 binding to GVECE (1:600 of AT-1 and 1:7 of GVECE) was detected under basal conditions by immunofluorescence microscopy. No significant difference in AT-1 receptor expression was observed between GVECE-stimulated and untreated cells (data not shown). In order to determine the effect of GVECE on AT-1 receptor expression, we performed experiments on human GVECE. GVECE treatment increased the number of AT-1 receptors on human GVECE. The increase in AT-1 receptor expression was accompanied by an increase in GVECE binding. These results indicate that GVECE treatment upregulated AT-1 receptor expression on human GVECE. The increase in GVECE binding was more pronounced in GVECE-stimulated cells than in untreated cells.

Figure 4: Shows the increase in AT-1 receptor expression on human GVECE. A, B, and C show increased AT-1 receptor expression in GVECE-stimulated cells compared to untreated cells. The increase in AT-1 receptor expression was not observed in GVECE-stimulated cells. The figure also shows the increase in GVECE binding, which was more pronounced in GVECE-stimulated cells than in untreated cells. The increase in GVECE binding was accompanied by an increase in AT-1 receptor expression on human GVECE. The increase in GVECE binding was more pronounced in GVECE-stimulated cells than in untreated cells.
increase, respectively); interleukin-6 (0.9 ± 0.07) and interleukin-4 (0.9 ± 0.02) did not affect VT-1 binding to GMVEC.

Characterization of the ligand to which VT-1 binds on human GMVEC

To investigate whether globotriaosylceramide, which has been demonstrated to be the receptor for VT-1 on endothelial cells [25], lymphocytes [26] and human kidney [27], is also the receptor for VT-1 on human GMVEC, TNFα-stimulated GMVEC were incubated with a MoAb against Gb3 one hour before and during assaying the binding of 125I-VT-1 to human GMVEC. The anti-Gb3 MoAb inhibited the binding of 125I-VT-1 to TNFα-stimulated GMVEC in a concentration dependent way, but, probably because of the moderate affinity of the MoAb, no complete inhibition was reached at the concentrations used (18.3 ± 0.3 and 27.7% ± 0.6 inhibition at 10 and 20% (vol/vol) antibody solution, respectively).

To further characterize the nature of the receptor for VT-1 present on human GMVEC neutral glycolipid extracts were prepared from both unstimulated and TNFα-stimulated human GMVEC and thin layer chromatography of these extracts was performed. After incubation of these thin layer chromatograms with 125I-VT-1 and extensive washing, the bound 125I-VT-1 was detected by exposure to a phosphor-imager. The radiolabeled VT-1 bound strongly to Gb3 and not to Gb4 when a standard preparation of neutral glycolipids was tested (Fig. 7, lane C). In unstimulated and TNFα-stimulated human GMVEC binding of 125I-VT-1 occurred predominantly to Gb3 (Fig. 7, lanes A, B). This pattern was consistently found with different preparations of human GMVEC obtained from two different donors. The increase in 125I-VT-1 binding to the glycolipid extracts of TNFα-stimulated human GMVEC indicates that the synthesis of VT-1 receptors is increased rather than that it only reflects a redistribution of VT-1 receptors from intracellular stores towards the plasma membrane.

Effect of VT-1 and VT-2 on protein synthesis

VTs may exert their cytotoxic effect by inhibiting the elongation factor-1 dependent binding of aminoacyl-tRNA to the ribosomes [28], a crucial step in the process of eukaryotic protein synthesis. To investigate whether VT cytotoxicity in human GMVEC was
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Fig. 5. VT-1 cytotoxicity is related to the degree of confluence of human GMVEC. Human GMVEC of one representative donor out of three were grown to confluence, trypsinized and seeded 1:6 onto gelatin coated 24 wells plates. Forty-eight hours before the indicated time points after trypsinization (as indicated on the x-axis) GMVEC were preincubated with either control medium (same culture medium without addition; open symbols) or TNFα (10 ng/ml; closed symbols) for 24 hours. Subsequently the cells were washed and exposed to either control medium or VT-1 (1 nmol/liter) for 24 hours, after which the number of viable adherent cells was counted with a hemocytometer. GMVEC indicated by A were categorized subconfluent (<50% confluent), by B “just reaching confluence” and by C “highly confluent” for respectively two, four and seven days. A. Effect of VT-1 on the viability of non-stimulated-(open symbols)- and TNFα-treated GMVEC (closed symbols). Symbols are: (♂, □) control cells; (♀, ◦) VT-1 treated cells. B. Effect of VT-1 on the number of viable adherent cells expressed as percentage of control (calculated by dividing the number of viable cells after VT-1 treatment by the number of viable adherent cells exposed to control medium × 100%). Symbols are: (♂) unstimulated GMVEC; (♀) TNFα-pretreated GMVEC. Similar results were obtained in two other experiments with two different donors.

Based on inhibition of protein synthesis, newly synthesized proteins were determined by assaying the incorporation of 35S-methionine into total cellular proteins. In agreement with cell- viability data, VT-1 alone did not or only slightly affect protein synthesis of highly confluent GMVEC over a 24-hour incubation period, whereas it inhibited protein synthesis in subconfluent and confluent cells (Fig. 8). When the highly confluent human GMVEC were preincubated with TNFα for 24 hours, the protein synthesis rate was inhibited similarly to subconfluent cells. Table 1 summarizes the results of protein synthesis inhibition by VT-1 in GMVEC differing in degree of confluence (N = 3). TNFα alone did not decrease overall protein synthesis.

When the effect of VT-1 and VT-2 on protein synthesis inhibition on TNFα-stimulated GMVEC of two different donors were compared, VT-1 was slightly more potent in inhibiting
endothelial cells or need the local exposure to inflammatory mediators to up-regulate Gb3 receptors and become susceptible to the cytotoxic effect of VT, as seen in venous endothelial cells.

Since Striker et al [10] and Green et al [12] described their methods to isolate and purify human glomerular endothelial cells, very few reports deal with glomerular endothelial cells in vitro, reflecting difficulties in the process of isolating and purifying large numbers of glomerular microvascular endothelial cells devoid of contaminating mesangial- and epithelial cells. In this report we have described an adapted procedure that is reproducible, relatively simple to perform and yields high numbers of GMVEC. It appeared to be crucial to avoid any contamination early in the process of purification because a minute contamination of epithelial or mesangial cells causes inhibition of GMVEC proliferation and rapid overgrowth of the contaminating cells. By several standard techniques the cultured cells have been characterized as pure populations of glomerular microvascular endothelial cells. The combined presence of the endothelial specific markers von Willebrand factor, V,E cadherin and EN-4 antigen and PECAM-1 (which is only encountered on endothelial cells, leukocytes and platelets), demonstrates the endothelial nature of our GMVEC. Upon inflammatory activation the endothelial cell specific leukocyte adhesion molecule E-selectin was also expressed. This agrees well with observations in vivo [29-33] showing an induction of E-selectin in glomeruli during inflammation. The presence of fenestral structures suggests that one of the specific properties of the glomerular endothelium is preserved in culture. Suboptimal expression of fenestrae in these human GMVEC may be due to the fact that they are cultured under static conditions. The need for dynamic flow to induce the expression of fenestrae in cultured glomerular endothelial cells was suggested by Ott, Olson and Ballermann [34] who, in contrast to our findings, did not observe fenestrae in bovine glomerular endothelial cells cultured under static conditions, but were able to induce the expression of fenestrae by shear forces.

Several papers have reported that HUVEC cultured under basal conditions are resistant to the cytotoxic effect of VT-1 [4-7]. When prestimulated with inflammatory mediators HUVEC become susceptible to the cytotoxic effect of VT-1 presumably by an up-regulation of the receptors for VT on the cell-surface [6]. Recently Obrig et al [15] have demonstrated that cell viability and protein synthesis of unstimulated renal endothelial cells was affected by VT with no alteration in sensitivity for VT when pre-exposed to TNFa or LPS. Furthermore, Obrig et al described high basal levels of VT receptor in renal endothelial cells reaching concentrations approximately 50 times higher than in HUVEC. These data suggest a higher relative sensitivity of renal endothelial cells versus HUVEC and a possible explanation for localized involvement of the kidney. In our study, cytotoxic and protein synthesis inhibitory effects of VT were found only on GMVEC that were subconfluent, just reaching confluence or pre-exposed to the inflammatory mediator TNFa, but not in the highly confluent GMVEC. Because Obrig et al have used cells one day after seeding in wells, it is likely that they studied the effect of VT on subconfluent or confluent renal endothelial cells.

Several papers have reported that subconfluent cells display a significant higher binding capacity of radiolabeled VT-1 and a higher susceptibility to the cytotoxic effect of VT than confluent cells [6, 35, 36]. Most probably this higher susceptibility of subconfluent and confluent cells is related to the cell-cycle

### Table 1. VT-1 inhibitory effect on overall protein synthesis is related to the degree of confluence of human GMVEC

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<th>Donor 1</th>
<th>Without TNFa</th>
<th>With TNFa</th>
<th>Donor 2</th>
<th>Without TNFa</th>
<th>With TNFa</th>
<th>Donor 3</th>
<th>Without TNFa</th>
<th>With TNFa</th>
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</thead>
<tbody>
<tr>
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<td>35</td>
<td>5</td>
<td>2</td>
<td>51</td>
<td>30</td>
<td></td>
<td></td>
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<tr>
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<td>10</td>
<td>8</td>
<td>69</td>
<td>32</td>
<td></td>
<td></td>
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<tr>
<td>Highly confluent (2 days)</td>
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<td>24</td>
<td>39</td>
<td>25</td>
<td>71</td>
<td>46</td>
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<tr>
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<td>33</td>
<td>71</td>
<td>33</td>
<td>82</td>
<td>48</td>
<td></td>
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<tr>
<td>Highly confluent (7 days)</td>
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<td>88</td>
<td>40</td>
<td>87</td>
<td>37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experimental design as described in the legend of Figure 8. The data of three independent experiments are given. The incorporation of $^{35}$S-methionine in proteins is expressed as % of control ($^{35}$S-methionine incorporation in proteins of VT-1 (1 nmol/liter) treated cells divided by $^{35}$S-methionine incorporation in proteins of control cells × 100%).

**Fig. 9. Effect of VT-1 and VT-2 on protein synthesis by "highly confluent" human GMVEC.** Human GMVEC were grown to confluency and maintained confluent for five days. Subsequently the cells were pre-treated without (open symbols) or with TNFa (11 ng/ml; closed symbols). After 24 hours of preincubation, the cells were washed and incubated with different concentrations (0.1 pmol/liter to 1 nmol/liter) of VT-1 (○, ●) and VT-2 (△, ▲) for 24 hours. Incorporation of $^{35}$S-methionine in proteins was determined as described in the Methods section. Data are expressed as the mean of two different experiments with two different donors (% of control).
dependent expression of Gb3 on the cell-surface compounded with increased Gb3 turnover [36]. Our observation that the degree of confluence of GMVEC affects susceptibility for VT corresponds with these findings. Since the half life of endothelial cells in the normal mature human body is approximately a hundred to thousand days [37] and autoradiographic analysis in glomeruli of rats demonstrated a constant rate of cell renewal of about 1% [38], we believe that from pathophysiological point of view, data on highly confluent cells are most appropriate in the study of the pathogenetic mechanisms of HUS.

Infections with VT producing *E. coli* most commonly involve isolates producing either VT2 alone or VT-2 with VT1. The structural genes of the two toxins share 58% overall nucleotide and 56% projected amino acid sequence homologies [39]. The toxins show similar physical properties such as toxin binding to Gb3 and inhibition of protein synthesis by the same mechanism, however, differences in biological activity have been observed. *In vitro* studies comparing the cytotoxic effects of VT-1 versus VT-2 on HUVVEC [40], Vero [41], HeLa [41] and Daudi cells [42] demonstrated reduced susceptibility to VT2. While performing studies with VT1 and VT2 hybrid toxins [43] it was found that the modulation in VT susceptibility is due to differences in the B subunit. Furthermore, Head, Karmali and Lingwood's study provided evidence for reduced binding affinity of VT2 for Gb3 when compared to VT1 [43]. This observation corresponds to our finding concerning the slightly reduced cytotoxicity of VT2 in GMVEC, but is in contrast with a recent study by Louise and Obrig [40] suggesting an increased susceptibility of renal endothelial cells for VT2. *In vivo* studies in mice [44] have demonstrated enhanced lethality of Shiga-like toxin-2 when compared to Shiga-like toxin-1 (toxins closely related to VT-2 and VT-1, respectively), suggesting that differences in properties other than direct target cell lysis are responsible for the observed heightened sensitivity of animals to VT-2.

Since *in vitro* data described in this report do not provide strong evidence for an intrinsic enhanced basal susceptibility of GMVEC to VT as a possible explanation for the preferential glomerular microvascular endothelial cell damage observed in HUS patients, the local availability of inflammatory mediators in glomeruli may contribute to local susceptibility for the cytotoxic effect of VT. A local role for inflammatory mediators was also suggested by Harel et al [45] reporting that Shiga-like toxin-1 injection into transgenic mice, bearing a chloramphenicol acetyltransferase (CAT) reporter gene coupled to a TNFα promoter, induced CAT activity (reflecting stimulation of the TNFα promoter) in the kidney but not in other tissues. Monocytes and macrophages, known to produce and release inflammatory mediators may be the source of locally produced glomerular inflammatory mediators. Of interest are recent *in vitro* studies of Testh, Ramegowda and Samuel [46] and Van Setten et al [47], which describe the interaction of VT with murine peritoneal macrophages and human monocytes, respectively. Both cells upon VT binding do not display protein synthesis inhibition but synthesise and release inflammatory mediators, suggesting a key role for monocytes in the process of endothelial cell damage in HUS. *In vivo* data supporting involvement of inflammatory mediators in the pathogenesis of HUS are provided by several papers demonstrating elevated concentrations of inflammatory mediators in plasma and urine among HUS patients [48–51]. Other possible local sources of cytokine production inside the glomerular capillaries such as mesangial [52, 53] and epithelial [54] cells need further investigation. It remains to be clarified for what reason inflammatory mediators are predominantly produced inside the glomerular capillaries.

Although our *in vitro* data strongly suggest that local availability of inflammatory mediators is essential for VT to result in a cytotoxic response, these data should be interpreted with some reserve. Since mainly children are affected by infection with a VT producing *E. coli*, it remains to be investigated whether GMVEC isolated from kidneys of juvenile origin display an increased sensitivity towards VT cytotoxicity when compared to GMVEC isolated from kidneys of adult origin. Of interest is the observation of Lingwood [55] that FITC labeled VT binds to human renal sections of infant < two years and not to the glomerulus of the adult. It should be noted, however, that Lingwood for the most part examined kidney sections of minimal lesion nephrotic syndrome patients, where locally released cytokines may induce the Gb3 receptor [56–58] resulting in the observed enhanced VT binding.

We conclude from this study with highly purified GMVEC, showing glomerular microvascular endothelial cell specific features, that VT cytotoxicity depends on the degree of confluence and the additional pre-exposure to inflammatory mediators. The latter are presumably locally produced by monocytes and not only up-regulate the receptor for VT, but also exert a variety of pro-inflammatory and coagulatory effects on the endothelium, resulting in a cascade of events of mutually influencing factors in the end leading to glomerular capillary damage, as observed in HUS patients.

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