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The Fibrinolytic System in the Hemolytic Uremic Syndrome: *In Vivo* and *In Vitro* Studies

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ABSTRACT

Fibrinolytic parameters and von Willebrand factor (vWF) antigen were measured in the plasma of 10 patients with hemolytic uremic syndrome (HUS). Samples were taken at presentation and again 2 wk later, before and after infusion of 1-desamino-8-arginine vasopressin. Compared with the plasma values of healthy control children, levels of tissue-plasminogen activator (t-PA) antigen, plasminogen activator inhibitor type I (PAI-1) activity, and vWF as well as fibrin(ogen) degradation products were significantly elevated in the plasma of HUS patients on admission. No response of the fibrinolytic parameters and vWF were seen when 1-desamino-8-arginine vasopressin infusion was given on admission. After 2 wk, t-PA antigen and vWF had partially returned to basal values, and t-PA antigen increased rapidly again after 1-desamino-8-arginine vasopressin infusion. To investigate whether verocytotoxin contributes to the alteration of the fibrinolytic system found in HUS patients, purified verocytotoxin-1 (VT-1) was added to the media of cultured human endothelial cells. Addition of VT-1 alone did not change the production of t-PA, plasminogen activator inhibitor type I, and vWF antigen in these cells. However, when the endothelial cells were preincubated with tumor necrosis factor-α to increase the number of VT-1 receptors, VT-1 induced a marked decrease of the synthesis of t-PA, plasminogen activator inhibitor type I, and vWF. This was caused by a decrease in overall protein synthesis in the tumor necrosis factor-α– and VT-1–treated endothelial cells. We conclude from this study that the systemic fibrinolytic parameters measured in the plasma of HUS patients are probably not a direct effect of VT-1 on the endothelium but are sequelae of the disease in which the intestine and the kidney are predominantly affected. (***Pediatr Res* 36: 257–264, 1994**)

Abbreviations

HUS, hemolytic uremic syndrome
DDAVP, 1-desamino-8-arginine vasopressin
VT-1, verocytotoxin-1
VTEC, verocytotoxin-producing *Escherichia coli*
vWF, von Willebrand factor
t-PA, tissue-plasminogen activator
PAI-1, plasminogen activator inhibitor type I
u-PA, urokinase
NBCS, newborn calf serum
TDP, total fibrin(ogen) degradation products
FbDP, fibrin degradation products
FgDP, fibrinogen degradation products
TNF-α, tumor necrosis factor-α

HUS is characterized by the triad microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. The most common form of HUS seen in children is the epidemic form, which is preceded by acute, often bloody, gastroenteritis. Although the exact pathogenesis is not known, it is evident that the endothelium plays a central role. Histopathologic studies of the kidney show swollen endothelial cells that are detached from the glomerular basement membrane and bulging into the lumen of the capillaries, thrombi, and fibrin depositions. These thrombotic obstructions contribute to the acute renal failure in HUS patients (1). Since the beginning of the 80s, several studies have demonstrated that VTEC are the main cause of the epidemic form of HUS (2). *In vitro* studies on human endothelial cells have shown that verocytotoxins are able to damage endothelial cells and may lead to inhibition of the protein synthesis in the cell (3, 4).

The endothelial cells play an important role in the maintenance of the hemostatic balance in the blood (5). Besides having anticoagulant properties, the endothelial cells also prevent the aggregation of platelets and stimulate fibrinolysis. Endothelial cells are the principal source of t-PA in the blood. In addition, they synthesize and secrete PAI-1 (6). PAI-1 can also be liberated from platelets, but the platelet PAI activity is rather low and only

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contributes to a minor degree to a prolonged elevation of PAI activity in the circulating blood (7). Endothelial cells not only provide circulating t-PA under resting conditions, but they can also rapidly release t-PA upon stimulation by various agents including DDAVP. This results in a marked increase in the fibrinolytic capacity of the blood (8) and is also accompanied by a marked, transient release of vWF antigen from endothelial storage sites (9). In addition to the increase in circulating t-PA and vWF, the u-PA concentrations also rapidly increase in plasma after DDAVP infusion (10). The mechanisms by which DDAVP causes increases in t-PA, u-PA, and vWF are not completely understood, but it is likely that extrarenal V2-like receptors are involved in the effects of DDAVP (11).

The role of the fibrinolytic system in HUS has recently been highlighted by the report of Bergstein *et al.* (12), who suggest that a decrease in PAI-1 antigen during peritoneal dialysis in HUS patients contributes to the recovery of the patients. To get a better insight into the fibrinolytic system in HUS patients and to see whether the regulation of the production of the fibrinolytic proteins by the endothelial cell is disturbed, we have measured fibrinolytic parameters in plasma samples taken on admission and after 2 to 3 wk (convalescent phase) before and after stimulation with DDAVP. In addition, we have investigated whether verocytotoxin contributes to the change in the circulating fibrinolytic proteins and vWF. To that end, we have examined whether human endothelial cells in culture change their synthesis of fibrinolytic proteins when they are incubated with purified VT-1.

METHODS

Patients. Ten children (five females and five males; mean age, 27.2 ± 16 mo; range, 6.5–52.5 mo) were diagnosed as having HUS based on the presence of hemolytic anemia with burr cells in the peripheral blood smear, renal failure, and thrombocytopenia (13). HUS was preceded by diarrhea, usually blood stained. Seven patients needed peritoneal dialysis, which ranged from 1 to 10 d (mean 5.3 ± 3.3 d). In the blood of eight of these 10 patients, antibodies were detected against the most common VTEC serotype O157-O-antigen, indicating an infection with VTEC (14). In the feces of one of the other two patients, VTEC serotype O157:H7 was isolated. The main laboratory indexes of the patients are displayed in Table 1. In the first morning after admission to the hospital, before dialysis was undertaken, 0.3 μg/kg body weight DDAVP, diluted in 100 mL of saline, was administered i.v. over a period of 30 min. The same DDAVP infusion was given to nine of the 10 HUS patients in the convalescent phase, ranging from 10 to 21 d after admission (14 ± 4 d, mean ± SD). A 4.5-mL blood sample was collected immediately before and after the DDAVP infusion. Blood samples were transferred to plastic tubes containing 0.5 mL of 3.8% sodium citrate and immediately cooled on melting ice. Platelet-poor plasma, obtained by centrifugation at 4°C for 10 min at 3000 × *g*, was quickly frozen and stored at −70°C until assays were performed. In the same way, control blood samples were taken from 10 children with chronic renal failure (from six children immediately before the hemodialysis session and four children during peritoneal dialysis). Blood samples from 13 age-matched children in good clinical condition and with no underlying hematologic or renal disease were used as controls. This study was approved by the local Institutional Committee of the Department of Pediatrics and carried out according to the guidelines of the Institutional Review Board of the Sint Radboud Hospital in Nijmegen, the Netherlands.

Materials. DDAVP (Minrin) was obtained from Ferring Pharmaceuticals AB (Malmö, Sweden). Purified VT-1 was prepared in the laboratory of Dr. M. A. Karmali, Toronto, Canada (1.2 mg of protein/mL; CD_{50} vero cells: titer 10^{-8}-10^{-9} M) (15). Endotoxin content of the VT-1 preparation was < 0.05 endotoxin units/mL by Limulus amoebocyte lysate assay (E-Toxic, Sigma Chemical Co., St. Louis, MO) at a detection level of 0.05–0.1 endotoxin units/mL. MAb PH1 against VT-1 was a generous gift from Dr. C. Lingwood, Department of Microbiology, Hospital for Sick Children, Toronto, Canada. M199 medium supplemented with 20 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag *et al.* (16). Human serum was obtained from the local blood bank and was prepared from fresh blood of healthy donors, pooled, and stored at 4°C; it was not heat inactivated before use. NBCS and FCS were from GIBCO (Grand Island, NY) and Boehringer Mannheim (Mannheim, Germany), respectively; they were heat inactivated before use (at 56°C for 30 min). Heparin was purchased from Leo Pharma-

Table 1. *Laboratory indexes of patients known to have HUS**

	On admission (n = 10)	Convalescent phase (n = 9)
Hb (mmol/L)	4.5 ± 1.0 (3.4–6.5)	5.4 ± 0.7 (4.5–6.4)
Platelets (× 10^9/L)	63 ± 59 (23–203)	398 ± 159 (171–652)
Leukocytes (× 10^9/L)	17.2 ± 8.2 (7.6–33.8)	8.4 ± 2.5 (5.5–12.5)
Urea (mmol/L)	38 ± 17 (12–63.9)	6.3 ± 1.7 (3.9–9.6)
Creatinin (μmol/L)	386 ± 265 (139–928)	60 ± 14 (44–84)
Fibrinogen (g/L)	3.1 ± 1 (1.5–4.6)	Not determined
Presence of VTEC infection	9 patients	

* Values are mean ± SD with range in parentheses.

ceuticals (Weesp, the Netherlands). Penicillin/streptomycin was from Boehringer Mannheim. Human fibronectin was a gift of J. A. van Mourik, Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Human recombinant TNF-α was a gift from Jan Tavernier (Biogent, Ghent, Belgium). The TNF-α preparation contained 2.45×10^{-7} U/mg protein and less than 40 ng of lipopolysaccharide per mg of protein. ^{35}S-methionine was purchased from Amersham (Amersham, Buckingshamshire, UK).

Isolation and culture of cells. Endothelial cells from human umbilical vein were isolated by collagenase treatment (17), cultured, and characterized as previously described (18); endothelial cells from human foreskin were isolated and characterized as described (19). The tissues for isolation of endothelial cells were obtained according the guidelines of the Institutional Ethical Committee of the Academic Hospital and St. Elisabeth Hospital in Leiden, the Netherlands. The endothelial cells were seeded in fibronectin-coated 10-cm^2 wells and cultured in M199 medium supplemented with 20 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4), 10% human serum, 10% NBCS, 2 mmol/L L-glutamine, 5 U/mL heparin, and 150 μg/mL crude preparation of endothelial cell growth factor under 5% CO_2 and 95% air at 37°C. When the cells reached confluence, they were detached by trypsin/EDTA and seeded in 2-cm^2 fibronectin-coated dishes with a split ratio of 1:3. The medium was renewed every 2 or 3 d. The cells in the experiments were used after two to four passages. Experiments of endothelial cells from umbilical cord and foreskin used in this study were isolated from three different donors.

In vitro experiments. For the experiments, cells were released with trypsin/EDTA, seeded in a split ratio of 1:3 in 2-cm^2 fibronectin-coated plates, and cultured in complete culture medium (400 μL/well). In the culture medium, 20% FCS was used instead of 10% human serum and 10% NBCS, because neutralizing activity of NBCS was found against VT-1 and because of potential neutralizing activity against VT-1 in human pooled serum. When confluence was reached, medium was renewed and the cells were preincubated for 24 h with or without TNF-α. After this preincubation period, media were renewed and media with or without VT-1 (17 pmol/L–17 nmol/L) were added. Twenty-four h later the media above the cells were collected and centrifuged, and the supernatant was stored at −20°C until fibrinolytic assays were performed. The cells were washed with M199 medium and released with trypsin/EDTA. The cells were diluted with 1:1 trypan blue dye solution and counted in a hemocytometer. Those cells that did not absorb trypan blue dye were recognized as viable cells. The experiments were done in duplicate wells.

Protein synthesis was determined by assay of the incorporation of ^{35}S-labeled methionine (0.25 μCi/mL added to complete culture medium) in ^{35}S-labeled proteins during a 24-h incubation period. After incubation, the cells were washed and the cellular ^{35}S-labeled proteins were precipitated by adding 10% trichloroacetic acid. Precipitated radioactivity was dissolved in 0.3 mL of 0.3 mol/L NaOH and counted in a liquid scintillation counter. Protein was determined according to the method of Lowry using BSA as a standard (20).

Fibrinolytic assays. Assay of t-PA antigen was performed with the ELISA Thrombonostika t-PA from Organon Teknika (Turnhout, Belgium) as described by Bos *et al.* (21). PAI-1 antigen in the patients' sera was determined using the commercially available TintElize PAI-1 kit from Biopool (Umeå, Sweden). For the *in vitro* assays, the Immulyse ELISA from Biopool was used. For the u-PA antigen determination, a sandwich ELISA was used as described by Binnema *et al.* (22). The assay measures the u-PA antigen present in plasma, irrespective of molecular form, *i.e.* single-chain u-PA, active u-PA, and u-PA in complex with inhibitors. The plasmin-activatable single-chain u-PA was measured with a biologic immunoassay (23). PAI activity was determined by a titration method with purified t-PA. Residual activity of t-PA was measured with a spectrophotometric assay, and the inhibitor activity was calculated from the amount of t-PA inhibited as described by Verheijen *et al.* (24). vWF antigen was measured with a modified ELISA adapted from Dr. J. Ingerslev, using pooled plasma from healthy donors as a reference (= 100%) (25). Fibrinogen was measured with the chronometric assay as described by Clauss (26). TDP, FbDP, and FgDP were measured with the sandwich-type enzyme immunoassays Fibrinostika TDP, Fibrinostika FbDP, and Fibrinostika FgDP, respectively, from Organon Teknika (Boxtel, the Netherlands). The data of TDP, FbDP, and FgDP are expressed as mg of fibrinogen equivalent unit/L (27). All measurements were done in duplicate.

Statistical analysis. Results are expressed as mean ± SD. The significance of differences between the different groups of patients was determined by using the Mann-Whitney test. The Wilcoxon test was performed for paired data when the fibrinolytic parameters before and after DDAVP infusion in HUS patients were compared. Pearson's coefficient correlation test was used to determine the correlations between fibrinolytic parameters, clinical severity, and laboratory indexes.

RESULTS

Patients. Table 2 summarizes fibrinolysis parameters and vWF antigen values in the blood of children with HUS, children with chronic renal failure, and children with no underlying hematologic or renal disease (control children). Basal values of t-PA antigen were lower in control children (2.8 ± 1.5 μg/L) than in healthy adults (6.4 ± 2.3 μg/L) (21). These observations resemble the recently published fibrinolytic parameters measured in plasma of children by Andrew *et al.* (28). Basal values of u-PA antigen were higher in control children (4.9 ± 1.2 μg/L) than in adults (3.5 ± 1.2 μg/L) (23); basal values of vWF antigen and PAI-1 antigen were comparable to those in adults (29). In accordance with other studies

Table 2. *Fibrinolytic parameters and vWF measured in plasma of patients with HUS**

Parameter	HUS (n = 10) Admission	HUS (n = 9) Convalescent	CRF (n = 10)	Control (n = 13)
t-PA antigen (μg/L)	9.2 ± 3.9	4.5 ± 1.3†	2.5 ± 2.3‡	2.8 ± 1.5‡
PAI-1 antigen (μg/L)	68 ± 75	21 ± 6	27 ± 21	29 ± 13
PAI activity (IU × 10^3/L)	14.8 ± 11.4	5.2 ± 1.8†	5.9 ± 2.6‡	3.4 ± 0.8‡
u-PA antigen (μg/L)	5.2 ± 1.8	5.8 ± 1.2	5.6 ± 1.7	4.9 ± 1.2
scu-PA (μg/L)	2.5 ± 0.7	3.2 ± 0.5†	3.1 ± 1.3	3.2 ± 0.6
vWF (% of pooled plasma)	311 ± 92	166 ± 87†	180 ± 69§	105 ± 62‡
TDP (mg/L)	11 ± 8	2.7 ± 4.7†	0.9 ± 1.3§	0.26 ± 0.20‡
FbDP (mg/L)	6.7 ± 6.7	0.9 ± 1.3‖	0.9 ± 1.9§	0.07 ± 0.05‡
FgDP (mg/L)	3.9 ± 1.9	1.5 ± 2.9	0.5 ± 1.0‡	0.07 ± 0.09‡

* The data of HUS patients on admission (mean ± SD) are compared with data from the same patients 2 or 3 wk after admission (convalescent phase), with those of patients with chronic renal failure (CRF), and with those of the control group with no hematologic and nephrologic problems. To determine significant changes in the HUS patients, paired data obtained on admission and in the convalescence phase were compared with the Wilcoxon test. To compare the data of different groups of patients, the Mann-Whitney test was used. scu-PA, single-chain urokinase.

† $p < 0.05$ compared with data on admission.

‡ $p < 0.001$ compared with the HUS group on admission.

§ $p < 0.01$ compared with the HUS group on admission.

‖ $p < 0.01$ compared with data on admission.

(30), elevated vWF concentrations were found in children with acute and chronic renal failure.

The plasma of HUS patients on admission had significantly elevated levels of t-PA antigen, vWF antigen, and PAI activity compared with control children. PAI activity correlated very well with PAI-1 antigen (r = 0.71, Pearson's coefficient correlation). t-PA antigen levels measured from plasma taken on admission showed moderate correlation with PAI-1 antigen and vWF (r = 0.67 and r = 0.59, respectively; Pearson's coefficient correlation). No correlation was found for the fibrinolytic parameters and the clinical severity, white blood cell count, platelets, or leukocytes. Only vWf antigen correlated with the amount of white blood cells on admission.

After 2 to 3 wk of hospitalization, the increases in t-PA antigen and vWF antigen were reduced by about 75%, and the PAI-1 antigen levels had been normalized (Fig. 1). In contrast to children with HUS, children with chronic renal failure showed plasma t-PA antigen and PAI-1 antigen levels similar to those of control children; PAI activity and vWF antigen levels were higher in these patients than in the control group. Elevated levels of TDP, FbDP, and FgDP were detected in the plasma of HUS patients on admission. A decline in these degradation products was seen in the convalescent phase, but the values were not yet entirely normalized (Fig. 1).

The concentration of circulating t-PA, u-PA, and vWF can rapidly be increased by infusion of DDAVP. When DDAVP infusion was given upon admission to the HUS patients, we observed no significant increase in t-PA antigen or vWF antigen in the plasma (Table 3). Neither did the plasma concentration of u-PA antigen change in HUS patients during DDAVP infusion. When DDAVP was given to the same patients 2 to 3 wk later during the convalescent phase, an increase in t-PA antigen was found in all patients, whereas no increase was seen for vWF antigen or u-PA antigen (Table 3).

In vitro experiments. It is generally assumed that endothelial damage by verocytotoxins plays a key role in the pathogenesis of the epidemiologic form of HUS. Inasmuch as the prolonged elevations of the plasma t-PA, PAI-1, and vWF levels suggest that the synthesis of these proteins in endothelial cells is enhanced, we examined whether VT-1 changes the production of t-PA and PAI-1 by human endothelial cells *in vitro*. Figure 2*A* shows that VT-1 did not significantly affect the production of t-PA and PAI-1 in nonstimulated confluent umbilical vein endothelial cells. To enhance the sensitivity of human umbilical vein endothelial cells for VT-1, we also studied these parameters in endothelial cells that had been preincubated for 24 h with TNF-α. This preincubation causes a marked increase in the expression of VT-1 receptors (4). Under these conditions, VT-1 markedly reduced the synthesis of t-PA and PAI-1 (Fig. 2*B*). In agreement with the known inhibitory effect of VT-1 on the cellular protein synthesis, a marked decrease in the synthesis of cellular proteins and in the number of cells was observed after incubation with VT-1. Similarly, the production of vWF antigen decreased after exposure of the cells to TNF-α and VT-1 (not shown). These effects were not found when VT-1 was inactivated by heat treatment before being added to the cells or when VT-1 was preincubated with MAb to VT-1. Similarly, in TNF-α–treated human microvascular endothelial cells, VT-1 induced a concentration-dependent decrease in cell number, PAI-1 production, and accumulation of t-PA (Fig. 3). These experiments indicate that VT-1 does not induce t-PA, PAI-1, or vWF production but rather decreases their synthesis in endothelial cells by its inhibitory effect on protein synthesis.

DISCUSSION

In this study, we report on the fibrinolytic parameters and vWF antigen in plasma of HUS patients, patients with chronic renal failure, and healthy children.

Elevated levels of t-PA antigen and PAI activity were found in the plasma of HUS patients on admission. These findings correspond with the recent observations in

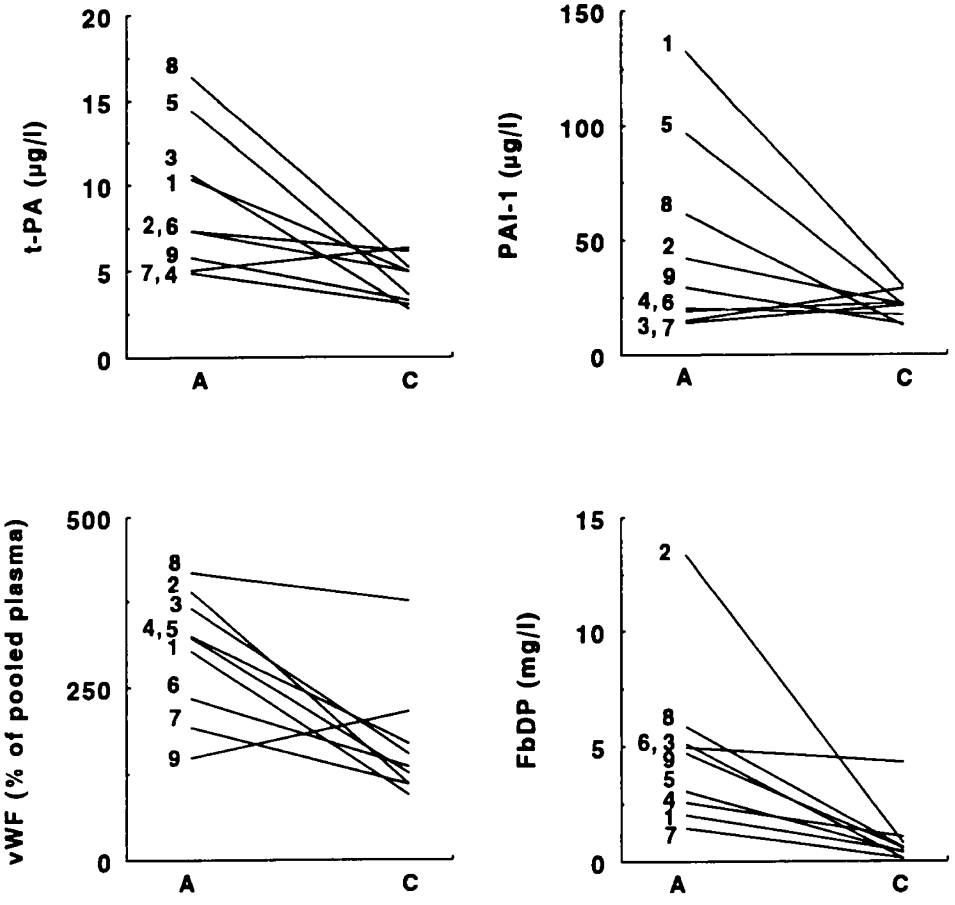

Figure 1. t-PA antigen, PAI-1 antigen, vWF antigen, and FbDP measured in the plasma of nine HUS patients, individually indicated by numbers. Measurements were done in plasma taken on admission (*A*) and in the convalescent phase of HUS (*C*).

thrombotic thrombocytopenic purpura and HUS patients by Monteagudo *et al.* (31). Simultaneously the plasma vWF concentration was enhanced. Elevated FbDP or FgDP found in the plasma on admission indicate that there is a systemic fibrinogenolysis and fibrinolysis secondary to an activated state of coagulation.

Nowadays it has become clear that VTEC infection is the major cause of the epidemic form of HUS. In nine HUS patients, we found evidence for a VTEC infection (14). Verocytotoxin can cause endothelial cell damage, which is the predominant feature of the glomeruli in the kidneys of patients with HUS. We demonstrated that addition of VT-1 to human endothelial cells that contained sufficient VT-1 receptors [as induced here by TNF-α pretreatment (4)] causes a decrease in the production of t-PA and PAI-1 antigens probably caused by an inhibitory effect of VT-1 on overall protein synthesis. Such inhibition of protein synthesis has also recently been observed in renal endothelial cells (32).

The t-PA, PAI-1, and vWF levels were elevated in the plasma of HUS patients on admission. This suggests that, in addition to hemorrhagic colitis and kidney failure, HUS patients display a general activation of the vascular endothelium. A similar pattern of elevated levels of t-PA, PAI-1, and vWF has been observed in the plasma of patients with sepsis, during which endotoxins and endog-

Table 3. *Effect of DDAVP on fibrinolytic parameters and vWF measured in plasma of patients with HUS**

Parameter	HUS (n = 10) on admission		HUS (n = 9) convalescent phase	
	Before DDAVP	After DDAVP	Before DDAVP	After DDAVP
t-PA antigen (µg/L)	9.2 ± 3.9	10.9 ± 5.0	4.5 ± 1.3	8.0 ± 3.3†
PAI-1 antigen (µg/L)	68 ± 75	44 ± 51	21 ± 6	17 ± 7
PAI activity (IU × 10^3/L)	15 ± 11	20 ± 27	5.2 ± 1.8	4.0 ± 1.4
u-PA antigen (µg/L)	5.2 ± 1.8	5.6 ± 1.4	5.8 ± 1.2	5.0 ± 0.9
scu-PA antigen (µg/L)	2.5 ± 0.7	2.5 ± 0.6	3.2 ± 0.5	2.9 ± 1.1
vWF (% of pooled plasma)	311 ± 92	373 ± 172	166 ± 87	211 ± 100

* The effect of DDAVP on fibrinolytic parameters and vWF was measured before and after treatment with DDAVP in the acute phase and in the convalescent phase. The data are expressed as mean ± SD. Statistical analysis of the paired data obtained on admission and in the convalescent phase was performed with the Wilcoxon test. scu-PA, single-chain urokinase.

† $p < 0.05$.

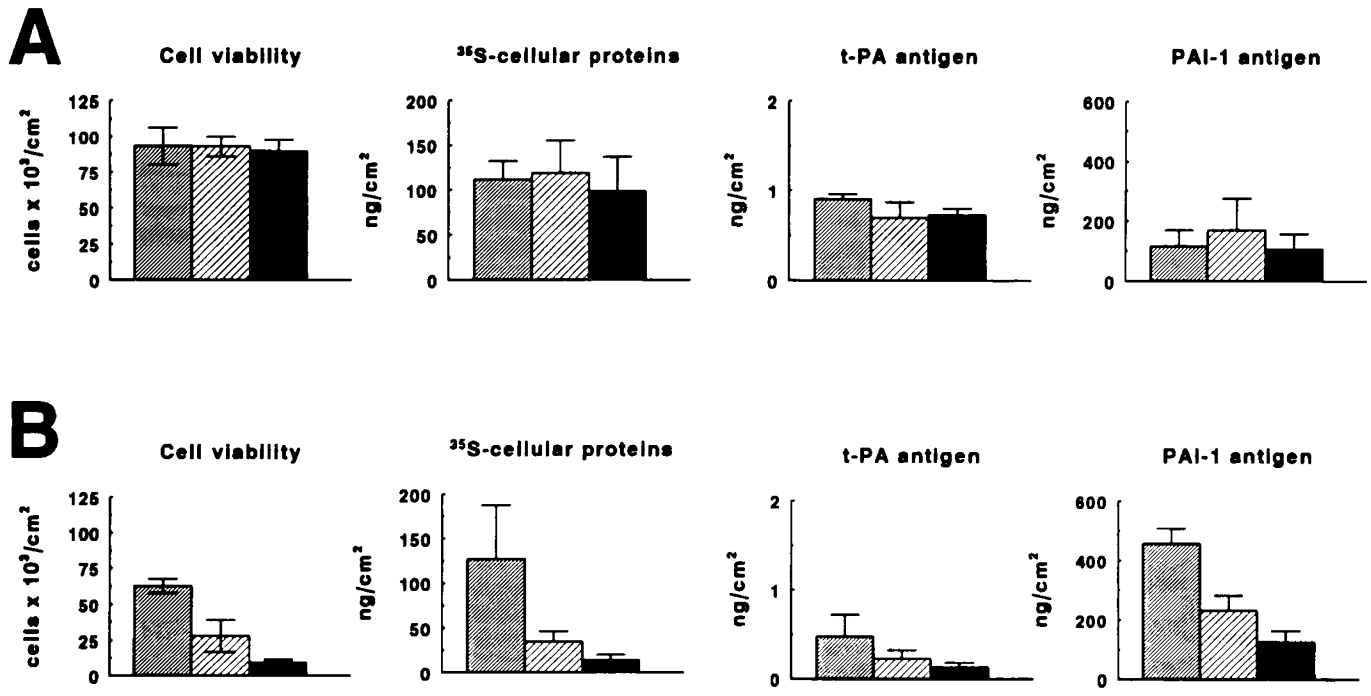

Figure 2. Confluent human umbilical vein endothelial cells in 2-cm^2 wells were preincubated for 24 h without (*A*) or with (*B*) 500 U/mL TNF-α and subsequently incubated in 0.4 mL of M199–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid medium supplemented with 20% FCS and the same amount of TNF-α, together with 17 pmol/L VT-1 (*hatched bars*) and 17 nmol/L VT-1 (*black bars*) and without VT-1 (*crosshatched bars*). The number of cells at the start of the preincubation was 9 × 10^4 cells/cm^2 for all wells. After preincubation, the number of cells was 9.3 × 10^4 cells/cm^2 for control cells (*A*) and 6.3 × 10^4 cells/cm^2 for TNF-α–treated cells (*B*). Because VT-1 caused cell death during the incubation with VT-1 and TNF-α and thus the number of viable cells changed during the incubation period, the production of t-PA and PAI-1 antigens are expressed per cm^2. Viability of the cells was determined by trypan blue exclusion test. Other experimental details are given in Methods. Data are expressed as mean ± SD of three experiments with endothelial cells from different donors.

enous mediators, in particular the inflammatory mediators TNF-α and IL-1, contribute to these alterations (33, 34). TNF-α, IL-1, and IL-6 were not elevated in the plasma of patients with a mild form of HUS (35, 36, unpublished observations), but we observed that they were elevated in patients with a severe form of HUS, *i.e.* in patients who also developed neurologic complications and pancreas involvement (N. van de Kar, unpublished observations). These data suggest that circulating cytokines may play a role in HUS but are unlikely to be significant in mild forms of HUS. On the other hand, locally produced inflammatory mediators in the kidney of HUS patients may escape detection in plasma because of their short biologic half-life (37). In favor of a local effect of inflammatory mediators, Siegler *et al.* (36) reported that TNF-α was elevated in the urine of HUS patients. Even if inflammatory mediators contribute locally to the increase of the t-PA, PAI-1, and vWF levels in plasma, it is likely that other factors that are generated in the injured intestines or kidneys are an important cause of the sustained elevation of the t-PA, PAI-1, and vWF concentrations in the blood of HUS patients (38–40).

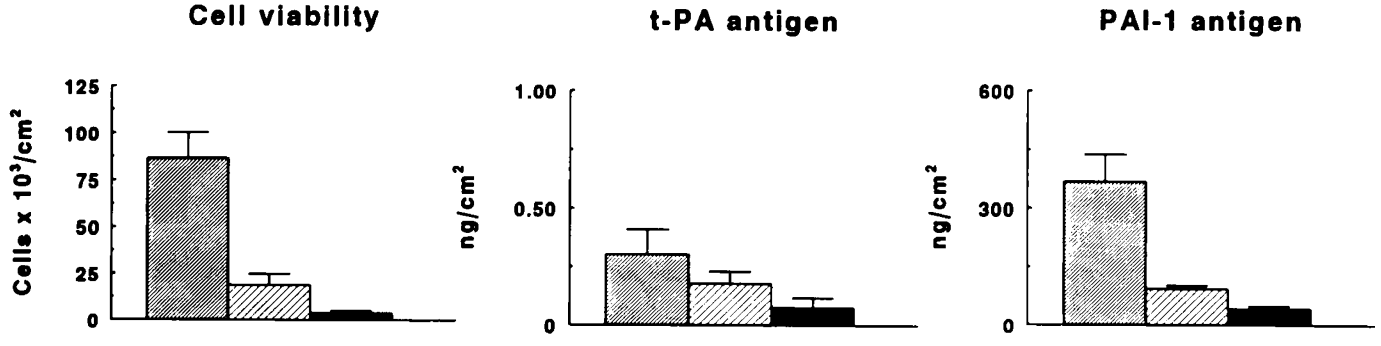

Figure 3. Confluent human foreskin microvascular endothelial cells in 2-cm^2 wells were preincubated for 24 h with 500 U/mL TNF-α and subsequently in 0.4 mL of M199–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid medium supplemented with 20% FCS and the same amount of TNF-α, together with 17 pmol/L VT-1 (*hatched bars*) and 17 nmol/L VT-1 (*black bars*) or without VT-1 (*crosshatched bars*). The number of cells after 24 h of preincubation with TNF-α was 8 × 10^4 cells/cm^2 for all variables. Because VT-1 caused cell death during the incubation with VT-1 and TNF-α and thus the number of viable cells changed, the production of t-PA and PAI-1 antigens is expressed per cm^2. Data given are expressed as mean ± SD of three independent experiments.

Activated leukocytes, which can be found in the peripheral blood in HUS (41), contribute to endothelial activation, and other factors such as activated complement and coagulation factors may be involved (42–45).

In our patients, no release of t-PA, u-PA, and vWF could be evoked by DDAVP infusion on admission. Unresponsiveness to DDAVP cannot be explained entirely by the renal problems seen in HUS, because patients with terminal renal insufficiency and anephric patients can still react to DDAVP infusion (46, 47). However, it is well known that patients with kidney diseases respond less pronouncedly to DDAVP than healthy volunteers, and some are nonresponsive (46, 47). Unresponsiveness of t-PA and vWF antigen release to DDAVP infusion has also been observed in patients with inflammatory bowel disease (Crohn's disease, ulcerative colitis) who have a normal level of t-PA and vWF before DDAVP infusion (48). It is therefore possible that the lack of response to DDAVP in HUS patients is caused by a simultaneous disturbance of the kidney and the intestine. On the other hand, it is unlikely that the lack of response of t-PA and vWF to DDAVP infusion is caused by the depletion of endothelial storage pools, because the biologic half-life of t-PA is very short and depletion of the t-PA should result in a drop in circulating t-PA. After 2 wk of hospitalization, not only were the plasma levels of t-PA, PAI-1, and vWF antigen normalizing, but also t-PA antigen increased upon DDAVP infusion. This indicates that the activation of the endothelium had decreased and that the response to DDAVP was recovering. It is of interest to note that a DDAVP-induced increase in t-PA antigen was also found in patients recovering from Crohn's disease or ulcerative colitis. Recently, Bergstein *et al.* (12) reported that elevated levels of PAI-1 during the clinical course were related to a poor outcome. In our study, all patients, including those with a high PAI-1 level on admission, completely recovered.

Although VT-1 causes a decrease of the production of fibrinolytic proteins by endothelial cells *in vitro*, the plasma levels of these proteins in HUS patients changed in the opposite direction. Although these data seem to be paradoxical, they do not really conflict. We hypothesize that the following events may occur in HUS. After a period of hemorrhagic colitis, the main organ affected in HUS is the kidney. The human kidney contains receptors for verocytotoxin (49), which is generally assumed to contribute to the damage of the endothelium in the glomeruli in the kidney. This damage can lead to a local procoagulant situation. In this condition, thrombin and inflammatory mediators are generated and polymorphonuclear leukocytes are activated, probably causing a general activation of the endothelium at sites distal to the affected areas. This is reflected in the elevated levels of t-PA, PAI-1, and vWF found in the plasma of the HUS patients in the acute phase of the disease. Additional studies will be necessary to prove this hypothesis.

We conclude from this study that the systemic fibrinolytic parameters measured in plasma of HUS patients are probably not a direct effect of VT-1 on the endothelium but are sequelae of the disease in which the kidney and the intestine are predominantly affected.

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