Unrecognized Pattern of von Willebrand Factor Abnormalities in Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura

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Abstract. Heterogeneous abnormalities in multimeric structure and fragmentation of endothelial-derived von Willebrand factor (vWF) have been reported in hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). This study was conducted to establish whether different patterns of vWF abnormalities were associated with different clinical syndromes. Plasmatic levels of vWF antigen (vWF:Ag), vWF release from endothelial cells (EC) exposed to patient sera, and vWF multimeric pattern were studied during episodes and again in remission in three groups of patients with severe forms of HUS and TTP. Enhanced vWF fragmentation as defined by disappearance of high molecular weight and increase in low molecular weight forms was a consistent finding of the acute phases, and always normalized in remission in all three groups. Unusually large vWF multimers were found exclusively in plasma of relapsing forms of HUS and TTP both during and between relapses. Enhanced levels of vWF:Ag and serum capability to induce vWF release in vitro are markers of disease activity and may reflect systemic endothelial injury and consequent activation. Their presence discriminates acute single-episode cases from relapsing forms and, when failing to normalize with plasma therapy, predicts plasma resistance. Enhanced low molecular weight multimers that closely paralleled disease activity suggest a permissive role of fragmented vWF in the formation of microvascular thrombi. Finally, finding of unusually large multimers exclusively in relapsing forms of HUS and TTP even between relapses, when no other clinical signs of disease activity could be detected, suggests that they cannot be the only factor in microvascular thrombosis.

The pathogenesis of hemolytic uremic syndrome (HUS) and the related condition thrombotic thrombocytopenic purpura (TTP) is still poorly defined. These syndromes consist of hemolytic anemia, thrombocytopenia, and organ dysfunction (mostly kidney in HUS and central nervous system in TTP). Clinical manifestations are secondary to the formation of platelet thrombi in the microcirculation with endothelial cell detachment from basement membrane and proliferation, but no perivascular inflammation (1). These dramatic diseases still lack a specific treatment and mortality remains high—at least in the adult forms—despite the remarkably improved long-term survival reported since the introduction of plasma manip-
recovered after a single episode, but were found, even in recovery phases, in patients who had a tendency for the disease to recur. These latter data, albeit controversial (12), were however interpreted as suggestive of a persistent state of endothelial perturbation. In contrast to the above findings, we have previously found that the most consistent abnormality in circulating vWF in the acute phase of HUS and TTP consists of an increase of vWF fragments that would reflect an enhanced proteolytic fragmentation of the molecule (13). Discrepancies in the literature in vWF conformational changes in HUS and TTP can easily be attributed to the heterogeneity of patient population studied, as well as the very limited number of patients analyzed moreover in different phases of the disease. Thus far, vWF multimeric pattern analysis has been performed with different techniques without any quantitative analysis, making the comparison of different studies very difficult. Approaching such controversial matter in a more systematic way will help unravel another major issue of whether it was infusing or removing plasma that improved so remarkably the prognosis of HUS and TTP in the past 20 yr. There are recent data that the effective component of plasma manipulation is plasma infusion rather than removal (3) and that there must be a protease inhibitor(s) in normal plasma that helps handling vWF in a more physiologic way.

The present study was designed to characterize vWF changes in three different categories of HUS and TTP that reflected the diverse patterns of clinical manifestation in adults. Patients were retrospectively segregated into three groups: (1) with a single episode cured by plasma manipulation; (2) with prolonged disease who were eventually plasma-resistant; and (3) with chronic relapsing forms. We wanted to establish whether these three relatively well separated patterns of disease manifestation had common underlying abnormalities in vWF elaboration and/or processing that could possibly offer new clues to understanding pathophysiology of the disease process and predict early and long-term response to plasma.

Materials and Methods

Patients

Twenty-four patients (22 adults and two children) admitted to the Nephrology Division of Ospedali Riuniti di Bergamo or referred to the Clinical Research Center for Rare Diseases of the Mario Negri Institute “Aldo and Cele Daccò” entered the study (Table 1). All patients had laboratory evidence of thrombotic microangiopathy (TMA; platelet count <150 × 10⁹/L; serum lactate dehydrogenase concentration >460 IU/L, and fragmented erythrocytes in the peripheral blood smear). Eight adults and one child presented with the clinical features of HUS, including mild-to-severe renal insufficiency, hypertension, and only minor neurologic signs, such as headache or somnolence (14). In the remaining 15 patients (14 adults and one

<table>
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</tbody>
</table>

* TTP, thrombotic thrombocytopenic purpura; HUS, hemolytic uremic syndrome.
child), the disease was defined as TTP on the basis of severe neurologic signs at onset— including visual disturbances, paresis, confusion, or seizures — and only mild or no renal involvement (1). No patient reported a family history of the disease. A predisposing condition was identified only in one woman who was pregnant and who developed a clinical picture of severe HUS 2 d after a normal delivery. No patient had any evidence of systemic disease, including systemic lupus erythematosus, antiphospholipid syndrome, antineutrophil cytoplasmic antibody-positive vasculitis, scleroderma, malignant hypertension, or cancer. No recent exposition to drugs such as cyclosporin, mitomycin, or birth control pills was reported by any patient. Failure to detect free verotoxin or verotoxin-producing Escherichia coli (VTEC) in stools or an increased titer of antibodies against verotoxin VTEC lipopolysaccharides in the circulation reasonably excluded the possibility of a recent VTEC infection in the HUS patients. In particular, the VTEC serotypes O157, O111, and O26 (that account for the large majority of HUS cases in Italy) were titered for. The possibility of a recent VTEC infection was even more consistently excluded by a second evaluation 30 to 40 d after disease onset that, again, failed to document an increased titer of anti-verotoxin and anti-VTEC antibodies.

All adult patients, independent of clinical presentation, were given a course of daily plasma exchange as first-line therapy as soon as the diagnosis of thrombotic microangiopathy was established (15). The only exception were two patients (including one child) with chronic relapsing TTP who were already known to respond to plasma infusion alone and were therefore treated according to a standardized protocol of three daily infusions of fresh frozen plasma (3). The other child, who was diagnosed to have HUS, was treated by plasma exchange as well, because of the atypical (non-diarrhea-associated) presentation of the disease (16). As a rule, treatment was continued for at least 2 d after complete remission of the neurologic symptoms and normalization of the signs of hemolysis. On the basis of the clinical course and the response to therapy, three subgroups of patients were recognized. Group 1 consisted of seven adult patients (presenting in three cases with HUS and in four cases with TTP) who completely recovered within 10 d of plasma exchange and who never relapsed after recovery. These cases were classified as acute, single-episode TMA (14,15). Group 2 consisted of five patients (presenting with HUS in four cases and with TTP in one case) who did not recover with plasma therapy. In four cases, the microangiopathic process subsided only after a bilateral nephrectomy was eventually performed as rescue therapy. In four cases and with TTP in one case) who did not recover with plasma therapy. In four cases, the microangiopathic process subsided only after a bilateral nephrectomy was eventually performed as rescue therapy because of signs of life-threatening neurologic involvement (papilledema, hypertensive encephalopathy, and/or generalized convulsions) (17). The remaining patient recovered only after progression to anuric, terminal renal failure when no more perfusion could be documented by scintiscan evaluation in both kidneys. These cases were defined as plasma-resistant TMA (14,17). Group 3 included 12 patients (two presenting with HUS and 10 with TTP) who recovered within 10 d of plasma therapy, but experienced two or more episodes of disease recurrence defined by a further decrease in platelet count to <150 × 10⁹/L, an increase in serum lactate dehydrogenase concentration to >460 IU/L, or detection of fragmented erythrocytes in the peripheral blood smear with or without concomitant signs of renal or neurologic involvement 1 mo or more after complete recovery. Two of these patients had the chronic relapsing form of the disease with recurrent episodes every 30 to 40 d that always recovered by 3 d of plasma infusion. All cases of this group were defined as recurrent TMA (15,16).

All patients were studied twice: during the acute phase of the disease before any treatment, and after remission, at least 1 wk after the last day of plasma therapy. Remission of the microangiopathic process was defined by persistent increase in platelet count above 150 × 10⁹/L and normalization of the markers of hemolysis (serum lactate dehydrogenase <460 IU/L, no fragmented erythrocytes in the peripheral blood smear) for at least 1 wk after plasma therapy.

**von Willebrand Factor Release from Endothelial Cells**

To test whether serum from patients with HUS/TTP was able to modulate vWF release from endothelium, human umbilical vein endothelial cells (HUVEC) were exposed “in vitro” for 24 h to serum diluted 1:2 with phosphate-buffered saline. The effect of serum obtained from patients during the acute phase of the disease was compared either with serum collected from the same patient during the remission or with serum from healthy subjects. At the end of incubation, supernatant was collected and vWF concentration was measured by sandwich enzyme-linked immunosorbent assay. Microplate wells coated with mouse antihuman vWF antibodies (kindly provided by Dr. Z. M. Ruggeri, The Scripps Research Institute, La Jolla, CA) were incubated with test samples. Rabbit anti-vWF polyclonal antibodies (Dakopatts, Glostrup, Denmark) were added to the wells followed by peroxidase-conjugated anti-rabbit IgG (Sigma Immuno Chemicals, St. Louis, MO). 1,2-Phenylenediamine in the presence of hydrogen peroxide was used as substrate. The data were extrapolated from a standard curve obtained with purified human vWF. The amount of vWF contained in the diluted serum was subtracted from that of cell supernatant. The results were normalized for the number of cells and expressed as micrograms of vWF released from 10⁶ cells.

**von Willebrand Factor Studies**

Five milliliters of blood was drawn as described previously (13) to evaluate plasmatic levels of vWF antigen (vWF:Ag) and to analyze the multimeric composition of the protein. Blood was collected in tubes containing one-tenth final volume of 110 mmol/L trisodium citrate as anticoagulant with 5 mmol/L ethylenediaminetetra-acetic acid, 6 mmol/L N-ethylmaleimide, 1 mmol/L leupeptin, and 200 kallikrein inhibitory units aprotinin per ml to inhibit the “in vitro” action of calcium-dependent cytokine proteases and serine proteases. Plasma vWF:Ag was measured by sandwich enzyme-linked immunosorbent assay. Data were extrapolated from a standard curve obtained with pooled normal plasma and expressed in arbitrary units, 1 U/ml being the concentration of pooled normal plasma prepared from at least 25 different donors (normal range, 0.50 to 1.50 U/ml). Multimeric pattern was analyzed by discontinuous sodium dodecyl sulfate agarose gel electrophoresis as described previously (18) with minor modifications (19). Resolving gels of 1.5% high-gelling temperature agarose HGT(P) (FMC Corp., Rockland, ME) for high molecular weight (HMW) multimers resolution, or 1.4% low-gelling temperature agarose LGT(P) type VII (Sigma) for low molecular weight (LMW) multimers resolution were used. After electrophoresis, the gel was fixed, washed, and dried. The dried gel was incubated with 125I-rabbit antihuman vWF antibodies (Dakopatts), washed, dried, and exposed for autoradiography.

Multimers were classified based on their electrophoretic mobility, and samples were applied at the top of the gel and electrophoresed toward the bottom. LMW multimers were defined as the fastest bands resolved that were positioned at the bottom of the gel, HMW were the multimers slower than the first 10 bands resolved, and UL multimers were defined as the ultra bands (close to the top of the gel) slower than HMW multimers found in normal plasma. Densitometric analysis was performed with computer-based digital image processing. Autoradiographs of the gels were acquired using a digitizing board. Multimers were resolved into a series of peaks and areas under the peaks.
calculated by specific functions of the software Image 1.55 (National Institutes of Health, Bethesda, MD). LMW multimers were defined as the area of two peaks at the bottom of the gel, and HMW multimers as the area after the first 10 peaks resolved. The UL multimer area was calculated together with the HMW area, because it is not possible to resolve extra peaks after the first 10. The corresponding area was computed and expressed as a percentage of the total area for each sample.

**Statistical Analysis**

Results are expressed as mean ± SD. The results were analyzed using the Wilcoxon test. The level of statistical significance was set at \( P < 0.05 \).

**Results**

**Patients’ Clinical Course**

**Group 1: Acute Forms of HUS and TTP Treated and Cured by Plasma**

These patients, who presented with the clinical features of acute HUS (\( n = 3 \)) or TTP (\( n = 4 \)), were given a course of daily plasma infusions (20 to 30 ml of fresh frozen plasma per kilogram of body weight) or exchanges (one to two plasma volumes exchanged per procedure) as soon as diagnosis was made, that was continued until complete remission of the microangiopathic process. Neurologic signs subsided within 3 d of treatment and renal function normalized within 1 wk. No patient recurred after remission.

**Group 2: Forms of HUS and TTP with Prolonged Course Who Were Eventually Plasma-Resistant**

One patient presented with the clinical features of acute TTP and four of acute HUS preceded by diarrhea in two cases. Despite daily plasma infusion or exchange—started as soon as diagnosis was established (see above)—thrombocytopenia and microangiopathic hemolysis failed to recover, renal function progressively deteriorated, and chronic dialysis was eventually needed to control uremia and fluid overload. Four patients developed severe hypertension that became progressively refractory to ultrafiltration and antihypertensive therapy (including \( \beta \)-blockers, angiotensin-converting enzyme inhibitors, calcium channel blockers, and arteriolar vasodilators) in three cases.

All patients progressively developed severe neurologic signs with bilateral papilledema, and generalized convulsions and coma in two cases. In one case, the microangiopathic process completely subsided and platelet count normalized shortly after (7 d) the onset of anuria. The other four patients, who retained some degree of renal function, continued to have clinical and laboratory signs consistent with a still active and progressive disease. Of these, three had severe, refractory hypertension, but one had a BP in the normal range without any antihypertensive therapy. On the basis of biopsy evidence of extensive platelet trapping in the kidney, it was reasoned that kidney microvasculature could sustain the process of platelet consumption, and kidney removal could have improved platelet count and limited disease progression. In particular, extensive structural damage of renal microvessels associated with narrowing of the lumina may determine major changes in fluid shear stress. In turn, rising of shear stress induces protease-dependent vWF fragmentation (20), increasing \( \text{in vivo} \) the circulating LMW vWF multimers fraction (17). vWF multimers fragmented by the protease calpain may bind avidly to receptors on activated platelets (21) and sustain microvascular thrombosis even in other vital organs, in particular the brain (17), a process that may be favored by, but not necessarily dependent on, increased BP. Bilateral nephrectomy was therefore considered as the last available rescue therapy to stop the disease in these desperately sick patients in imminent danger of death. Actually, bilateral nephrectomy was followed by normalization of the neurologic status within 48 h, and complete remission of the microangiopathic process, with normalization in platelet count and arterial BP within 7 d (17). Two patients received a kidney transplant and none recurred after discharge.

**Group 3: Forms of HUS and TTP with Frequent Relapses**

Patients of this group were admitted because of recurrences of frequently relapsing HUS (\( n = 2 \)) and TTP (\( n = 10 \)). As soon recurrence was diagnosed, patients were given a course of daily plasma infusions or exchanges (see above) that was continued until complete remission of the microangiopathic process. Neurologic signs subsided within 3 d of treatment and renal function normalized within 1 wk. All patients had at least one additional recurrence, within 3 mo after discharge.

**von Willebrand Factor Antigen**

In the acute phase of the disease, plasma vWF:Ag was significantly elevated over controls in patients of group 1 and 2 (2.61 ± 0.75 and 2.31 ± 1.05 U/ml, respectively) but normal in patients of group 3 (1.51 ± 0.51 U/ml) (Figure 1).

Normalization of vWF:Ag was achieved in remission (as defined by platelet count >150 × 10⁹/L and lactate dehydrogenase <460 IU/L) in group 1, whereas in group 2 hemato logic and clinical remission were still associated with vWF levels higher than normal (2.23 ± 0.85 U/ml). Patients of group 3 still had normal vWF levels at remission.

**vWF Release from Endothelial Cells**

The effect of sera from patients and control subjects of stimulating vWF release from HUVEC is given in Figure 2. Sera taken from patients of groups 1 and 2 during the acute phase of the diseases induced the release of vWF from HUVEC to a significantly higher extent than control sera. In remission, the property of serum to induce vWF release normalized in group 1, but remained above control values in group 2. Sera taken from patients of group 3 significantly (\( P < 0.05 \)) inhibited rather than stimulated vWF from HUVEC in the acute phase of the disease, but this activity normalized in remission.

**vWF Multimeric Pattern Studies**

Acute HUS and TTP, independently from the various clinical patterns, were all characterized by very similar vWF multimeric abnormalities, consisting of enhanced fragmentation of the molecule as revealed by disappearance of the HMW...
multimers (Figure 3) paralleled by an increase in the LMW forms (Table 2) but without any evidence of UL multimers in groups 1 and 2. In remission phases of the disease, multimeric structure of vWF was almost indistinguishable from normal plasma, and fragmentation was completely normalized in group 1 and very close to normal in group 2, as revealed by gel showing the HMW (Figure 3, lane 4) and by densitometric analysis (Table 2). Only patients of group 3 with frequent relapsing HUS and TTP had UL multimers in the circulation during the acute phase of the disease which, however, persisted in remission in all patients (Figure 4). The presence of UL forms was evidenced by densitometry with an increase of the percentage area of the HMW multimers (Table 2), rendering this group not comparable with healthy subjects in both phases of the disease. However, also in this group an increased fragmentation was present, as revealed by the relative increase of LMW area percentage (Table 2) during the acute phase with respect to remission.

**Discussion**

The first finding of the present study is that in groups 1 and 2, vWF:Ag levels were consistently elevated during the acute phase of the disease in patients with severe adult HUS and TTP but not in the relapsing form. vWF:Ag in plasma can therefore help to distinguish patients who will not recur after the first episode from those who will tend to relapse. High levels of circulating vWF:Ag, however, will not tell whether a given patient will respond to plasma. According to our present find-
of HUS and TTP, vWF:Ag levels were normal even in the acute phase of the disease and their serum was never capable of stimulating vWF release from cultured endothelium above values of normal serum. The differences in vWF:Ag levels between patients with single-episode and recurrent or chronic relapsing forms may be directly linked to the still completely unknown underlying pathologic mechanism that gives rise to these two manifestations of HUS and TTP.

As for the UL vWF forms, we, at variance with Moake et al. (12), were never able to find such multimers in patients with single episode. One may speculate that UL multimers cannot be detected in the circulation due to their property of avidly binding to platelet receptors (30). UL forms were only found in group 3 either during and between episodes. Finding of UL multimers even when patients were in complete clinical remission argues against their possible role in promoting platelet thrombi. This is also consistent with recent data that an elastase inhibitor α1-antitrypsin effectively eliminated UL multimers from the circulation but failed to improve platelet count and never induced a remission in a patient with frequent relapsing TTP (24). The presence of UL multimers in frequently relapsing patients may be explained by recent findings documenting a vWF cleaving protease deficiency—constitutional (31) or acquired due to the presence of antibodies against this protease (32)—in patients with relapsing TTP. The most innovative of the present findings was a quite consistent increase in the LMW forms during the acute phase of the disease—but never in remission—in all three groups of patients studied. This pattern conceivably reflected an enhanced fragmentation of vWF molecule dependent on an excess proteolytic activity already documented by previous studies (33,34). Data that fragmented vWF binds directly to vWF receptors on activated platelets with a high affinity (21) raises the issue of whether fragmented vWF rather than UL multimers causes microvascular thrombosis. In contrast to plasma-responsive patients, evidence of vWF fragmentation was more conclusive in the circulation of plasma-resistant patients who also had a more prolonged disease. Recent data that vWF susceptibility to fragmentation increases in response to increasing levels of shear stress (20) can be taken to suggest that in plasma-resistant forms more shear stress in the microvasculature sustains disease activity by further supporting vWF break-down. The above interpretation is consistent with data that in the latter group, removal of the kidneys, a major site of vascular bed occlusion and augmented shear stress, reversed to normal the process of vWF fragmentation and invariably induced remission of the disease (17). Information derived from the present study done in severe forms of HUS and TTP in all likelihood will not apply to classical postdiarrheal HUS of children.

This study has documented: (1) Enhanced levels of vWF antigen in the acute phases of most forms of HUS and TTP, with the exception of patients with frequent relapses. This can be used to distinguish single-episode cases from those with a tendency to recur. Failure of vWF:Ag to normalize after plasma manipulations may help to identify plasma-resistant cases, and can possibly be used as an indicator to stop the treatment. (2) Evidence of vWF fragmentation in the acute
Table 2. Densitometric analysis of vWF multimers

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<th>Remission</th>
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<td>HMW</td>
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<td>HMW</td>
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<td>Plasma-resistant</td>
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<td>14.89 ± 2.52(^d)</td>
<td>36.00 ± 4.54(^d)</td>
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<td>LMW: 18.91 ± 1.93</td>
<td>HMW: 25.43 ± 2.83</td>
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\(^{a}\) Data are mean ± SD and are expressed as percent of total area of the sample lane. In relapsing group, HMW area also contains UL multimer area (see Materials and Methods). vWF, von Willebrand factor; LMW, low molecular weight; HMW, high molecular weight.

\(^{b}\) \(p < 0.05\) versus remission.

\(^{c}\) \(p < 0.05\) versus control subjects.

\(^{d}\) \(p < 0.001\) versus control subjects.

Figure 4. Autoradiographic image of UL multimers from a patient with recurrent TTP (1.5% HGT(P) agarose gel for the resolution of HMW multimers). The origin of the gel is at the top. Arrow indicates the position of UL multimers. Lane 1, normal plasma; lane 2, patient plasma during the acute phase; lane 3, patient plasma at remission.

Acknowledgments

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