High von Willebrand Factor Concentration Compensates a Relative Adhesion Defect in Uremic Blood

By Jaap J. Zwaginga, Martin J.W. Ijsseldijk, Nel Beeser-Visser, Philip G. de Groot, Jaap Vos, and Jan J. Sixma

Uremia is associated with a bleeding diathesis. We investigated platelet adhesion as a cause for the impaired primary hemostasis and the role of von Willebrand factor (vWF) in this process in uremic patients. Perfusions with blood with standardized hematocrit, platelet count, and free Ca\(^{2+}\) ions were performed over inverted and denuded endothelialized artery segments from human umbilical cords in a modified Baumgartner perfusion chamber. Platelet adhesion in patient perfusates was comparable with control adhesion. However, the high vWF levels present in uremic whole blood did not increase adhesion above the adhesion in control blood with lower vWF levels. These results suggested that a relative adhesion defect was present in patient blood. Control blood in which vWF levels were raised to uremic levels showed the high adhesion that uremic whole blood failed to show. Additionally, in perfusions with uremic plasma in which the initially high vWF level was normalized by dilution with vWF-depleted uremic plasma, adhesion was clearly lower than in normal plasma. Washed patient platelets did not differ from normal platelets in their association with purified vWF, via their adhesion receptors glycoprotein Ib and Iib-IIia. Patient platelets present in patient plasma showed a similar adhesion defect as control platelets, which were resuspended in the uremic plasma. Therefore, primary defects of uremic platelets were of minor importance for the observed adhesion defect in uremic whole blood. The adhesion defect was not dependent on the presence of uremic vWF; plasma of uremic patients depleted of vWF also inhibited adhesion, and the defect remained present when purified control vWF was added to vWF-depleted uremic plasma. The binding of uremic vWF to the vessel wall and its support of subsequent adhesion were not impaired. These results indicate that the observed adhesion defect was not due to abnormal vWF. Our current results suggest an unknown component present in uremic plasma that directly inhibits platelet interaction with artery segments; however, it has no effect on vWF binding to the vessel wall. High vWF levels in uremic plasma are able to compensate for the defect.

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quantitative or qualitative vWF disturbances correlated better with the capacity of plasma vWF^{50-51} to support adhesion in the perfusion system than with the measurement of the vWF antigen level or its activity to agglutinate platelets in the presence of ristocetin (vWF-RiCof).^{52}

In this study, we investigate the role of vWF as a possible cause of the impaired primary hemostasis in uremic patients with the help of a modified Baumgartner perfusion chamber. Perfusions with standardized hematocrit, platelet count, and free Ca^{++} ion concentration were performed on inverted and denuded arterial segments from human umbilical cords.^{30,49} It was found that plasma of uremic patients inhibited platelet adhesion when compared with control plasma with similar amounts of vWF. The defect was also present when control platelets were perfused in uremic plasma. vWF binding from uremic plasma to the vessel wall, which is necessary to support subsequent adhesion,^{29,53} was not impaired. Therefore, the decreased platelet adhesion is not due to intrinsic abnormalities of uremic vWF, but the high vWF levels present in uremic plasma may compensate for the defect. An unknown component present in uremic plasma seems to directly inhibit the platelet adhesion to artery segments.

MATERIALS AND METHODS

Patients. Twelve patients with clinically stable chronic renal failure, 8 women and 4 men, with age varying between 26 and 79 years, who received maintenance hemodialysis treatment two or three weeks a week for more than 3 months, were enrolled in the study.

The patients were not hypertensive or had hypertension that was medically controlled. Patients refrained from taking any medication with possible effects on platelet function unless stated otherwise. No androgens or immunosuppressive drugs were used; the participating men were not pregnant. There was no hematologic evidence of aluminum intoxication (acquired microcytosis without iron deficiency). Patients had no hepatitis and did not receive blood transfusions in the 2 weeks before the experiments. Their mean predialysis serum urea level was 33.9 ± 7.7 mmol/L (range 28.4 to 48.0), creatinine level (±SD) was 1,044 ± 205 μmol/L (range 730 to 1,400). Uremic plasma pools were derived from 18 patients randomly divided into three groups of six. These patients were typical for our population on hemodialysis (age range: 29 to 76; hemodialysis period range 5 to 132 months); they met the same requirements as the patients in Table 1; urea and creatinine levels fell within the ranges mentioned. Patients gave informed consent for blood collection necessary for the described experiments.

Laboratory tests. A set of laboratory tests was performed on each patient. Blood was obtained just before start of one of the regular hemodialysis treatments. Five milliliters of whole blood, anticoagulated with 2 mmol/L EDTA, was collected for determination of hematocrit and platelet count (Coultier-counter, Model-S, Harpenden, UK). All bleeding times were determined by one single experienced technician with a Simplate-II device (General Diagnostics, Morris Plains, NJ) using a venous pressure of 40 mm Hg and a horizontal incision on the volar surface of the forearm.^{5}

vWF antigen (vWF:R:Ag) was determined by an electroimmuno- diffusion assay using rabbit anti-vWF serum raised against human vWF^{54}; the ristocetin cofactor activity (vWF:RiCof) was measured with formalin fixed platelets and 1 mg/mL ristocetin sulphate (H. Lundbeck & Co, Copenhagen, Denmark).^{55} Factor VIII coagulant activity (FVIII:C) was measured in a one-stage clotting assay.^{56,57} Factor VIII and vWF related properties were determined in blood collected into 1/10 vol 130 mmol/L trisodium citrate. Reference plasma was a freshly frozen plasma pool from 40 normal subjects, stored at -70°C.

vWF purification and radiolabeling; vWF depletion of plasma. vWF was purified from control cryoprecipitates by gel filtration on Sepharose CL-4B (Pharmacia AB, Uppsala, Sweden).^{58} The vWF in the void volume fractions was precipitated by dialysis against 1.9 mol/L ammonium sulfate, pH 7.0, at 4°C and stored as ammonium sulphate suspension at 4°C until use. Precipitated protein was collected by centrifugation (2 minutes, 10,000 × g, 4°C). The pellet was dissolved in 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4, and further dialyzed against the same buffer. The purified vWF was used for radiolabeling, in binding studies, or in reconstituted perfusates. vWF purified from control cryoprecipitates was assayed for vWF antigen and vWF-ristocetin cofactor activity at the day of the experiments.

Radiolabeling of purified control vWF with 125I was performed with the Iodo-Gen method (Pierce Chemical Co, Rockford, IL).^{59} Free 125I was removed by dialysis at 4°C against 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4. The labeled vWF preparation had a specific activity of approximately 42 μCi/mg; and free 125I was less than 5%.

vWF-depleted plasma was obtained after immunoadsorption of vWF from plasma by CNBr-Sepharose (Pharmacia) coupled polyclonal antibodies raised against vWF. Plasma with levels of vWF

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age [yr]</th>
<th>HD (mol)</th>
<th>Diagnosis</th>
<th>Ht</th>
<th>Plt</th>
<th>Bt-Simpl</th>
<th>Bleeding Sympt</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>69</td>
<td>20</td>
<td>Analgetic nephropathy</td>
<td>29.9</td>
<td>197</td>
<td>&lt;30'</td>
<td>Bruising, epistaxis</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>36</td>
<td>3</td>
<td>Diabetic nephropathy</td>
<td>18.3</td>
<td>257</td>
<td>&gt;30'</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>55</td>
<td>3</td>
<td>Diabetic nephropathy</td>
<td>21.5</td>
<td>302</td>
<td>7.27&quot;</td>
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</tr>
<tr>
<td>4</td>
<td>M</td>
<td>63</td>
<td>26</td>
<td>Glomerulonephritis</td>
<td>26.5</td>
<td>222</td>
<td>&gt;30'</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
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<td>Analgetic nephropathy</td>
<td>26.6</td>
<td>502</td>
<td>&gt;30'</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>57</td>
<td>48</td>
<td>Cystic kidney disease</td>
<td>27.7</td>
<td>317</td>
<td>3.30'</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>60</td>
<td>4</td>
<td>Nephrosclerosis due to hypertension</td>
<td>26.0</td>
<td>320</td>
<td>24'50&quot;</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>26</td>
<td>94</td>
<td>Reflux nephropathy; neurogenic bladder</td>
<td>19.7</td>
<td>180</td>
<td>&gt;30'</td>
<td>Bruising</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>38</td>
<td>8</td>
<td>Nephrolithiasis</td>
<td>23.9</td>
<td>210</td>
<td>&lt;30'</td>
<td>Bruising, epistaxis</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>79</td>
<td>3</td>
<td>Renal failure of unknown etiology</td>
<td>16.0</td>
<td>14&quot;20&quot;</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>52</td>
<td>4</td>
<td>Renal failure of unknown etiology</td>
<td>24.7</td>
<td>286</td>
<td>&gt;30'</td>
<td>Bruising</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>63</td>
<td>*</td>
<td>Analgetic nephropathy</td>
<td>26.5</td>
<td>286</td>
<td>7'15&quot;</td>
<td>—</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; HD, hemodialysis period; Ht, hematocrit in percent (normal range F, 36% to 46%; M, 41% to 55%); Plt, platelet count (10^12/μL; normal range 150 to 300,000/μL); Bt Simp, Simplate bleeding time in minutes (') and seconds ("), normal range less than 8 minutes.

*Patient no. 11 was treated with peritoneal dialysis for 36 months before start of hemodialysis treatment.
lower than 0.1 U/mL antigen was accepted as vWF-depleted plasma.

**Platelet-vWF binding studies.** Blood of patients or control donors was collected into 1:10 vol of 110 mmol/L trisodium citrate, and platelet-rich plasma (PRP) was obtained by centrifugation (10 minutes, 150 x g, 20°C). Platelets were washed three times as previously described and resuspended to a concentration of 2 x 10^8 platelets/mL in Tyrode buffer (140 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH_2PO_4, 12 mmol/L NaHCO_3, pH 7.4) containing 1 mmol/L CaCl_2, 5 mmol/L glucose, and 3.5 mg/mL human albumin (HSA, Behringwerke AG, Marburg, Germany).

To study ristocetin-induced vWF binding to platelet glycoprotein Ib, radiolabeled vWF was used and diluted to various concentrations in Tyrode, containing CaCl_2 and HSA. A volume of 0.125 mL of this solution was first mixed with 2 μL of ristocetin solution (200 mg/mL), and then 0.125 mL of the platelet suspension was added. After 30 minutes' incubation at room temperature, the mixtures were centrifuged (1 minute, 10,000 x g) through 20% (wt/vol) sucrose in Tyrode buffer. The platelet pellet was counted in a gamma counter. The platelet-bound vWF was quantitated from the specific activity. As a control, the incubation was performed without ristocetin.

To study the vWF binding to platelet glycoprotein Ib/IIa, dilutions of purified unlabeled vWF were used. Human thrombin (Sigma Chemical Co, St Louis, MO) was added to a final concentration of 0.5 U/mL. Just after 200 μL of the vWF dilutions were incubated for 30 minutes with an equal volume of a suspension of patient or control platelets. The platelet pellet was centrifuged through sucrose, and the vWF remaining in the supernatant was measured with an enzyme-linked immunosorbent assay (ELISA).

Platelet-bound vWF was calculated. Nonspecific binding was derived from incubation of vWF and platelets without thrombin. Both the binding of control vWF to patient or control platelets and the binding of control or patient vWF to control platelets were studied in this assay.

The ristocetin-induced binding of patient or control vWF present in their own plasma, to glycoprotein Ib of washed control platelets was studied as follows. Plasma of patients was assayed for vWF and diluted 1:3 in Tyrode/HSA. Control plasma assayed for vWF was used undiluted and diluted 1:1, 1:2, and 1:4 in Tyrode/HSA. First, 125 μL of the dilutions were mixed with 2 μL of ristocetin solution (200 mg/mL), and then incubated for 30 minutes with an equal volume of platelet suspension. After centrifugation of the platelets through sucrose, the vWF remaining in the supernatant was measured and platelet-bound vWF was calculated. As a control, the incubation was performed without ristocetin.

**Perfusion chamber and surfaces.** Perusions with steady flow were performed with a modification of the annular perfusion chamber. In the modified annular perfusion chamber (Fig 1), inverted artery segments isolated from the umbilical cord were mounted on two separate central rods. The two rods fitted in separate chamber segments that could be connected to each other. No systematic differences were observed between adhesion at the artery in the first or in the second chamber (not shown). Duplicate adhesion results were obtained in this way with one single perfusion. The human umbilical cords were obtained on the average within 6 hours after birth. Artery segments were isolated, stored, inverted, and mounted on the central rods in the perfusion chamber as described earlier.

**Perfusates: Blood collection and anticoagulation.** Citrated blood of patients was obtained by clean venepuncture, just before one of their regular hemodialysis treatments. Healthy human donors served as controls. Hematocrit and total blood volume were measured and plasma volume was calculated. The citrate concentration in plasma was subsequently adjusted to 16 mmol/L in patient plasma and to 19 mmol/L in control plasma. With these concentrations, we obtained comparable free Ca^2+ ion concentrations between 40 and 60 μmol/L in patient and in control plasma. Ca^2+ was measured with a Ca^2+ electrode in a specially constructed cuvette as developed by Akkerman et al. The lower detection limit was 5 μmol/L.

By addition of autologous platelet-poor plasma (PPP) to whole blood of controls and of autologous erythrocytes to whole blood of patients, perfusates were standardized on a fixed platelet number and hematocrit (>150,000 platelets/μL; Ht 30%). The PPP and erythrocytes were obtained by centrifugation (10 minutes, 3,000 x g, 22°C) of part of the collected whole blood of patients and controls. This standardization method allowed the study of platelet adhesion in whole blood perfusates independent of preexisting differences in platelet count and hematocrit. When the uremic hematocrit was raised to levels above 30%, then platelet count decreased concomitantly and adhesion was, therefore, hardly improved. Moreover, addition of uremic RBCs to obtain higher hematocrits required too much uremic blood.

Reconstituted perfusates were prepared from PPP, washed platelets, and washed RBCs. PPP was obtained by centrifugation of patient or control patient whole blood (10 minutes at 3,000 x g, 22°C). In some experiments, purified vWF was added (0.5 to 2.0 U vWF:RCoF/mL) to normal PPP or to vWF-depleted PPP. Washed platelets were prepared from control PRP as previously described. Washed platelets were added to the PPP in a concentration of 200,000/μL. Control RBCs of blood group O, rhesus positive, were prepared as follows. After whole blood centrifugation and collection of PRP and
Perfusions. Before the start of perfusions, the artery segments on the central rod in the annular perfusion chamber were rinsed with 25 mL prewarmed (37°C) 10 mmol/mL Hepes-buffered-saline, pH 7.4.

Perfusates prewarmed for 5 minutes at 37°C were then circulated for 5 or 10 minutes at 1,300 s⁻¹. After perfusion, the central rod with the inverted artery segments was removed from the annular chamber. Artery segments were subsequently fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) followed by 2.0% osmium tetroxide as postfixation, ethanol dehydration, and subsequent embedding in epon as described before. After each perfusion run the system was thoroughly rinsed with 30 mL of Hepes-saline.

To study the binding of plasma vWF to the vessel wall and its subsequent function in adhesion, we performed double perfusions. First, artery segments were perfused for 5 minutes at a shear of 1,300 s⁻¹ with PPP pooled from six uremic patients or controls. Also, vWF-depleted plasma pools with purified control vWF were used for preperfusions. The artery segments were then rinsed with 100 mL Hepes buffer, and a second perfusion was performed for 5 minutes at the same shear rate with reconstituted perfusates of control vWF-depleted plasma with control platelets and RBCs.

Evaluation. One-micrometer thick sections of the epon vessel wall segments were prepared and stained with methylene blue and basic fuchsin and evaluated by light microscopy (Dialux 20 EB, E. Leitz GmbH, Wetzlar, FRG) at a 1,000 × magnification. Evaluation was performed with a special constructed eyepiece micrometer in the ocular (Leitz). At least 1,000 intersection points at 10 µm intervals, evenly distributed over the total surface, were evaluated for each section.

Total adhesion to the vessel wall was defined as the percentage of the surface covered by platelets. Platelet adhesion was subdivided into contact platelets, spread platelets, and spread platelets with aggregate formation on top. The last category, aggregate formation, was further divided into aggregates with a minimum height of 2 µm and less than 5 µm in height, between 5 and 10 µm, and if present higher than 10 µm.

### RESULTS

Laboratory tests. In Table I, sex, age, dialysis period in months, cause of renal failure, hematocrit, platelet number, platelet bleeding time, and clinical bleeding symptoms of the participating patients are listed. The platelet count was normal in all patients. The bleeding time was normal for 3 patients; 2 patients had bleeding times between 8 and 30 minutes; and 7 patients had bleeding times longer than 30 minutes, after which the measurement was stopped. Six of the seven patients with the longest bleeding times had symptoms of a bleeding tendency.

Perfusions over inverted umbilical arteries. Anticoagulation with 1/10 (vol/vol) 110 mmol/L trisodium citrate left higher free Ca²⁺ ion concentrations in patient blood with low hematocrit than in control blood with normal hematocrit. To obtain equal free Ca²⁺ concentrations in patient blood and control blood between 40 and 60 µmol/L, the citrate concentration was adjusted in patient blood to a plasma concentration of 16 mmol/L. Subsequently, the blood of patients and controls was normalized to a hematocrit of 30% and an equal platelet count of 150,000/µL. The perfusates were circulated for 5 minutes at a shear of 1,300 s⁻¹ over inverted umbilical arteries in a modified annular chamber (Fig 1). Table 2 shows the percentage of surface covered with platelets and with platelet aggregates, together with the levels of vWF in the perfusates. Comparing the patient with the control group, no altered adhesion or aggregate formation was observed.

The concentration of vWF:RA and vWF:RiCof was increased in 8 and 7 patients, respectively. Factor VIII, which circulates in complex with vWF, was increased in all patients.

vWF binding to glycoprotein Ib and IIIa of patient platelets. The amount of vWF that could be bound to glycoprotein Ib of patient and control platelets was measured. Washed platelets of 4 patients (no. 5 through 8) and 3 controls were incubated for 30 minutes with various concentrations (0.625 to 20.0 µg/mL) of purified 125I-radiolabeled vWF in the presence of ristocetin. The amount of platelet-bound label was measured in the platelet pellet and related to the initial vWF concentration (Fig 2A). A specific binding of vWF to the platelets was determined by leaving out ristocetin

### Table 2. vWF Parameters and Platelet Adhesion in Perfusions With Normalized Whole Blood

<table>
<thead>
<tr>
<th>No.</th>
<th>vWF:Ag</th>
<th>vWF:RiCof</th>
<th>Viii</th>
<th>Adhesion (sc %)</th>
<th>Aggr &lt;5 µm (sc %)</th>
<th>Aggr 5-10 µm (sc %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.6</td>
<td>2.1</td>
<td>3.2</td>
<td>44.3 ± 3.4</td>
<td>8.6 ± 1.1</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>1.9</td>
<td>3.1</td>
<td>23.6 ± 0.6</td>
<td>2.7 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>1.6</td>
<td>2.4</td>
<td>32.3 ± 2.1</td>
<td>4.8 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2.6</td>
<td>1.9</td>
<td>2.1</td>
<td>49.0 ± 2.0</td>
<td>9.0 ± 2.2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2.1</td>
<td>1.6</td>
<td>2.2</td>
<td>34.4 ± 3.4</td>
<td>3.1 ± 1.1</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2.2</td>
<td>2.7</td>
<td>2.3</td>
<td>38.1 ± 4.1</td>
<td>6.3 ± 1.4</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>1.2</td>
<td>1.8</td>
<td>36.4 ± 0.7</td>
<td>8.8 ± 0.8</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>8</td>
<td>2.3</td>
<td>2.0</td>
<td>1.8</td>
<td>43.9 ± 1.4</td>
<td>6.9 ± 0.8</td>
<td>0</td>
</tr>
</tbody>
</table>

Patients (mean ± SD, n = 8): 37.6 ± 8.1; 6.0 ± 2.3; 0.2 ± 0.3

Controls (mean ± SD, n = 5): 39.6 ± 2.2; 6.8 ± 1.0; 0.1 ± 0.0

Perfusions of 5 minutes at a shear of 1,300 s⁻¹ were performed with normalized blood of 8 patients (no. 1 through 8 in Table I) and 5 controls (Ht, 30% and platelet count ≥ 150,000/µL). Adhesion and aggregate formation are expressed in percentage surface coverage (% sc) ± SD (n = 4); vWF: Ag, vWF: RiCof, activity, and factor VIII coagulant activity are expressed in U/mL (normal range, respectively: 0.6 to 1.4, 0.4 to 1.5, 0.6 to 1.4 U/mL).
from the mixture. vWF bound to platelets (about 65%) increased linearly with initial vWF concentrations below 2.5 µg/mL. At vWF levels between 2.5 and 5 µg/mL, the slope of the binding curve decreased. No difference was seen between the amount vWF bound to patient or to control platelets.

The binding of purified vWF to glycoprotein Ib-IIIa on patient and control platelets was investigated. Purified vWF (1.2 to 18 µg/mL/10⁸ platelets) was incubated with washed platelets. The platelets were subsequently stimulated with thrombin (0.5 U/mL). After 30 minutes, the platelets with the bound vWF were spun down. The decrease in vWF concentration of the supernatant minus specific binding of vWF as determined by omitting thrombin gave the specific thrombin-induced vWF binding to platelet glycoprotein Ib-IIIa. vWF bound to GpIIb-IIIa increased linearly with initial vWF concentrations below 4.0 µg/mL. Above concentrations of 5.0 µg/mL, the slope of the binding curve decreased. No difference was seen between the amount vWF bound to patient or control platelets.

Binding of patient vWF in the presence of ristocetin to normal platelets. The capacity of patient vWF to associate with glycoprotein Ib on normal platelets was investigated. Plasma dilutions of the 4 patients (1:3 in Tyrode) and 3 controls incubated for 30 minutes with washed control platelets (10⁸) in the presence of ristocetin. The amount of platelet-bound label was measured in the platelet pellet and related to the initial vWF concentration (control platelets, ○—○; patient platelets, ●—●). Nonspecific binding of vWF to platelets was determined by omitting ristocetin from the mixture. The curves for control platelets (∨—∨) and patient platelets (△—△) overlap. All values ± SD (n = 3). (B) Plasma dilutions of the same patients (1:3 in Tyrode) and three controls (1:1, 1:2, 1:4) were incubated for 30 minutes with washed control platelets (10⁸) in the presence of ristocetin. The incubation was stopped by spinning down the platelets. The total amount of platelet-bound vWF is calculated from the decrease in vWF concentration in the supernatant (control, ○—○; patient plasma ± SD, n = 4, *). Nonspecific binding of vWF is determined by omitting ristocetin in the binding assay with control plasma (○, n = 2).

Fig 2. (A) Washed platelets (10⁸) of 4 patients (no. 5 through 8 in Table 1) and 3 controls incubated for 30 minutes with various amounts purified ¹²⁵I-radiolabeled vWF factor in the presence of ristocetin. The amount of platelet-bound label was measured in the platelet pellet and related to the initial vWF concentration (control platelets, ○—○; patient platelets, ●—●). Nonspecific binding of vWF to platelets was determined by omitting ristocetin from the mixture. The curves for control platelets (∨—∨) and patient platelets (△—△) overlap. All values ± SD (n = 3). (B) Plasma dilutions of the same patients (1:3 in Tyrode) and three controls (1:1, 1:2, 1:4) were incubated for 30 minutes with washed control platelets (10⁸) in the presence of ristocetin. The incubation was stopped by spinning down the platelets. The total amount of platelet-bound vWF is calculated from the decrease in vWF concentration in the supernatant (control platelets, ○—○; patient platelets, ●—●). Nonspecific binding of vWF is determined by omitting ristocetin in the binding assay with control plasma (○, n = 2).

Fig 3. Washed platelets (10⁸) of 4 patients (no. 5 through 8 in Table 1) and 3 controls incubated for 30 minutes with various amounts purified vWF (1.2 to 18 µg/mL). The platelets were subsequently stimulated with thrombin (0.5 U/mL). After 30 minutes the platelets were spun down. (Top) The total amount of platelet-bound vWF is calculated from the decrease in vWF concentration in the supernatant (control platelets, ○—○; patient platelets, ●—●). Nonspecific binding of vWF is determined by omitting thrombin (control, ✰—✰; patient, †—†). When subtracted they give the specific thrombin-induced vWF binding to platelet glycoprotein Ib-IIIa at the bottom (control, □—□; patient, ♦—♦). All values ± SD (n = 3).
controls (1:1, 1:2, 1:3) were incubated for 30 minutes with washed control platelets in the presence of ristocetin. The incubation was stopped by spinning down the platelets with bound vWF. The remaining vWF in the supernatant was measured, and the amount of platelet-bound vWF was calculated (Fig 2B). A binding pattern comparable with the pattern seen in Fig 2A was obtained. Below 350 mU (±3.5 µg)/mL/10^9 platelets, vWF binding (about 80% of the added amount) increased linearly with concentration; binding leveled off above 500 mU (±5 µg). Binding of uremic vWF from the four patients closely followed the binding curve of normal vWF from control plasma to glycoprotein Ib of control platelets.

Perfusions at comparable vWF levels. Plasma was prepared from whole blood of 4 patients (no. 9 through 12, Table 1) and 4 controls. Extra vWF (0.5 and 1.0 U RCoF/mL) was added to the plasma of two controls. Perfusates were reconstituted with washed control platelets and RBCs. Table 3 shows adhesion on inverted umbilical arteries perfused for 3 minutes at a shear of 1,300 s⁻¹. Patients had high vWF levels ranging from 2 to 4.8 U RCoF/mL, while control plasma contained vWF in the normal range 0.4 to 1.5 U RCoF/mL. Addition of extra vWF to control perfusates increased adhesion. Regression curves were calculated from the control plasma and from the patient adhesion values at the different vWF levels. Compared with patient adhesion, it was found that control adhesion was significantly higher at vWF levels that were found in patient plasma.

Perfusates were also performed with fresh patient or control plasma pools with equal concentrations of their own native vWF. This was achieved as follows. Before perfusions, vWF levels of the pools were adjusted to 1 U/mL vWF: RCoF by mixing patient or control plasma pools with, respectively, vWF-depleted plasma of patients or controls. All vWF-adjusted pools were subsequently reconstituted with control platelets and RBCs. Table 4 shows that at a vWF concentration of 1 U/mL, uremic pooled plasma significantly inhibited adhesion of control platelets to artery segments.

Binding and functionality of vWF at artery segments. To investigate whether vWF binding to the vessel wall and its subsequent role in adhesion were impaired by uremic plasma, we performed double perfusions. In a first perfusion without platelets and RBCs, vWF was bound to the artery segment. The functionality of the vessel wall bound vWF was subsequently assayed in a second perfusion with vWF-depleted perfusates, in which normal platelets and RBCs were present. Adhesion in the second perfusion was proportional to the vWF level present in the first perfusion, and no differences were observed when artery segments were first perfused with fresh uremic or fresh control plasma pools (Fig 4B). In similar experiments (Fig 4A), the segments were first perfused with vWF-depleted plasma pooled from 6 patients or with vWF-depleted plasma of 6 controls. Equal adhesion was obtained in the second perfusion. Addition of purified vWF to these vWF-depleted plasma pools of patients and controls in the first perfusion increased the adhesion in the second perfusion similarly. Therefore, both the binding and the subsequent function of uremic vWF or purified vWF were not influenced by uremic plasma in the first perfusion. However, when vWF binding to the vessel wall is inhibited during the direct interaction of platelets with the vessel wall, this may cause the decreased adhesion and cannot be excluded with first perfusions with PPP. In vWF-depleted perfusates, vWF binding or its inhibition cannot play a role.

Table 3. Adhesion as a Function of vWF in Reconstituted Perfusates With Control or Patient Plasma

<table>
<thead>
<tr>
<th>Subject No</th>
<th>vWF-RCoF (U/mL)</th>
<th>Adhesion in %sc</th>
<th>Subject No</th>
<th>vWF-RCoF (U/mL)</th>
<th>Adhesion in %sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>2.0</td>
<td>46.6 ± 0.4</td>
<td>1</td>
<td>0.4</td>
<td>44.7 ± 3.3</td>
</tr>
<tr>
<td>11</td>
<td>2.6</td>
<td>51.2 ± 1.8</td>
<td>2</td>
<td>0.6</td>
<td>47.2 ± 2.2</td>
</tr>
<tr>
<td>12</td>
<td>3.9</td>
<td>57.8 ± 2.4</td>
<td>3</td>
<td>0.98</td>
<td>48.6 ± 2.1</td>
</tr>
<tr>
<td>10</td>
<td>4.8</td>
<td>53.0 ± 1.0</td>
<td>4</td>
<td>1.5</td>
<td>47.1 ± 0.9</td>
</tr>
</tbody>
</table>

Perfusions of 5 minutes were performed at a shear of 1,300 s⁻¹. Plasma of controls to which extra vWF was added was compared with plasma of patients in perfusates with washed control platelets and control RBCs (Ht, 40% and platelet count ≥150,000/µL). vWF-RCoF is expressed in units per milliliter. Adhesion is expressed in percentage surface coverage (%sc) ± SEM (n = 4). The influence of addition of vWF (+0.5 or +1.0 U/mL) to control plasma perfusates is given for controls no. 3 and 4. Regression lines for control and patient values were calculated; control adhesion was subsequently compared with patient adhesion at similar vWF levels by covariance analysis.

Table 4. Adhesion With Perfusates Reconstituted From Control or Patient Plasma Pools Adjusted to 1 U/mL vWF

<table>
<thead>
<tr>
<th>Pool No.</th>
<th>Patients</th>
<th>Adhesion (%sc)</th>
<th>Pool No.</th>
<th>Controls</th>
<th>Adhesion (%sc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.9 ± 0.4</td>
<td>58.4 ± 3.3</td>
<td>2</td>
<td>43.8 ± 2.4</td>
<td>54.4 ± 4.4</td>
</tr>
<tr>
<td>3</td>
<td>49.3 ± 1.9</td>
<td>55.3 ± 1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SEM (n = 4): 44.9 ± 2.2. Plasma was pooled from 6 controls or from 6 patients. Before perfusions, the vWF-RCoF activity was adjusted to 1 U/mL by mixing control and patient pools with vWF-depleted plasma of controls or of patients, respectively. Perfusates were subsequently reconstituted with washed control platelets and control RBCs (Ht, 40% and platelet count ≥150,000/µL). Perfusions of 5 minutes at a shear of 1,300 s⁻¹ were performed. Adhesion (%sc) for each pool is expressed in percentage surface coverage ± SEM (n = 4). Mean adhesion ± SEM of patient and control pools (n = 3) are compared: *P < .03 (student t-test).
Fig 4. (A) Artery segments were first perfused with vWF-depleted plasma pooled from 6 patients (■) or with plasma of 6 controls (○). Purified vWF was added to these plasma pools in different concentrations. Subsequently, second perfusions were performed with reconstituted perfusates with washed control platelets in vWF-depleted control plasma and RBCs (platelet count ≥150,000/μL; Ht 40%). Both perfusions were performed for 5 minutes at 1,300 s⁻¹. Platelet adhesion (in percent surface coverage [n = 4] ± SEM) obtained by addition of vWF to the preperfusate were compared with the adhesion at segments preperfused with vWF-depleted pools without extra vWF: (○), P < .05; (● ● ●), P < .01; (● ● ● ● ●), P < .001 (Student t-test). (B) First perfusions of artery segments were performed with fresh uremic (●) and control (○) plasma pools. The second perfusion was similar as described in (A). (C) First perfusions were now omitted. The vWF-depleted plasma pools of patients (A) or controls (B) with and without addition of purified vWF were now used directly in reconstituted perfusates with control platelets and RBCs. Adhesion values, which were obtained by addition of vWF to the perfusate, were compared with values at segments perfused with vWF-depleted pools without addition of purified vWF; (● ● ● ● ● ●), P < .001. We also compared the adhesion values obtained with control and patient perfusates at equal concentrations of added vWF; (+), P < .001.

Therefore, we prepared vWF-depleted plasma from both controls and patients and used this plasma in reconstituted perfusates. In Fig 4C, adhesion values are given that were obtained with vWF-depleted pooled plasma of patients and controls; these pools were used in perfusions with control platelets and RBCs. vWF-depleted plasma of uremic patients strongly inhibited the adhesion of control platelets when compared with adhesion obtained with pooled plasma of controls. Addition of purified control vWF resulted in improved adhesion; however, adhesion in uremic plasma remained inhibited as compared with control plasma with the same amount of purified vWF.

DISCUSSION

Prolonged bleeding times associated with the uremic bleeding tendency strongly suggest a defect in platelet interaction with injured vessel wall. Plasma components such as vWF and fibronectin,29-32 and the presence of adhesive proteins in the vessel wall,33-34 are known to determine primary platelet adhesion. Plasma vWF has to bind to the vessel wall35,36 before it can associate with platelet glycoprotein Ib, and in this way contribute to platelet adhesion.34 Defective binding of vWF to glycoprotein Ib-IIIa on activated platelets will result primarily in decreased platelet aggregation, but adhesion at high shear rates might also be influenced.37

The mechanisms important for platelet adhesion are essential in hemostasis and are best investigated in perfusion studies. For this purpose, blood was collected from patients with chronic renal failure, just before one of the regular hemodialysis treatments, and used in perfusion studies. This patient group was selected because of their clinical stability, accessibility, the comparable state of uremia just before the hemodialysis treatment, and the absence of clinical complications with possible effects on hemostasis. In 9 of 12 patients participating in the study, bleeding symptoms and prolonged
bleeding times were present (Table 1). In accordance with other studies, high vWF levels in patient blood were observed. No clear discrepancy existed between the levels of antigen and ristocetin activity in our patients (Table 2).

To avoid the influence of decreased platelet transport caused by the low hematocrit in patients (Table 1), we standardized the hematocrit to 30% in both patient and control blood. With this hematocrit intermediate between normal control and uremic values, platelet adhesion was at a reasonable level. Also, the platelet count and the free Ca²⁺ ion concentration were standardized before perfusion. A plasma concentration of 50 ± 10 μmol/L free Ca²⁺ has been reported to ensure optimal platelet adhesion and spreading. By unclear mechanisms citrate proved to be a more efficient chelator in patient plasma than in control plasma. The normal concentration of 19 mmol/L gave in patient plasma Ca²⁺ levels between 25 to 40 μmol/L, which may impair platelet spreading. Therefore, we adjusted the citrate concentration to 16 mmol/L and 19 mmol/L in patient and control plasma, respectively. The normalized whole blood perfusions over inverted deendothelialized segments of umbilical arteries showed no differences in adhesion or aggregate formation between patient and control perfusates. However, the amount of vWF in the patient perfusates was much higher. It has been shown before that a normal vWF concentration in plasma is not sufficient for maximal adhesion; increasing the vWF concentration up to 4 U/mL still improves adhesion of platelets to artery segments. We saw no relation between the length of the dialysis period (range 3 to 94 months) and the platelet number, hematocrit, bleeding time, or, more important, the platelet adhesion in perfusions with whole blood of patients no. 1 through 8. Therefore, this factor was considered not to be important for the studied processes. The fact that high vWF levels in uremic whole blood are not associated with increased adhesion suggests the presence of a relative adhesion defect.

We further investigated whether the relative adhesion defect was also present in perfusates with only plasma of uremic origin. Reconstituted perfusates were prepared from uremic or control plasma to which control platelets and control RBCs were added. The availability of control RBCs allowed us to work at a hematocrit of 40% in the reconstituted perfusates; compared with perfusates standardized at a hematocrit of 30%, this resulted in higher adhesion values. By addition of 1.0 U/mL purified vWF to reconstituted perfusates with control plasma, adhesion increased significantly (Table 3). This control plasma with artificially raised vWF now showed higher platelet adhesion than patient plasma with similar vWF levels. In accordance, when uremic plasma pools were diluted with vWF-depleted patient plasma to 1 U/mL vWF:RiCof, platelet adhesion was significantly lower than control plasma perfusates with 1 U vWF/mL (Table 4). Though the patients in Table 3 have a relatively short hemodialysis period, a stable clinical status was present after 3 or more months of this treatment. Moreover, the results with this patient plasma were in agreement with those obtained in experiments with plasma pools (Table 4); in both cases, at equal vWF concentrations uremic plasma inhibited adhesion as compared with control plasma. The results with patient no. 8 through 12 were, therefore, not atypical in this sense. Normal platelets were used in the reconstituted perfusates. As in whole blood perfusates, the high vWF levels in uremic plasma were not able to increase adhesion above control values. Independent of the presence of uremic or normal platelets, at equal vWF concentrations, uremic plasma inhibited adhesion (Tables 3 and 4, Fig 4C). Therefore, intrinsic defects of uremic platelets were considered of minor importance for the relative adhesion defect in uremic blood. Moreover, the essential receptors for adhesions on the platelet membrane, glycoprotein Ib (Fig 2A), and IIb-IIIa (Fig 3) of washed uremic platelets bound purified vWF in a normal fashion. These observations were in agreement with others.

The failure of uremic plasma to give increased adhesion in the presence of high vWF levels might be due to an inhibitor of platelet vessel wall interaction. Abnormal vWF in uremic plasma should, however, be excluded as alternative explanation for the relative adhesion defect. Similar to vWF present in some FVIII-vWF concentrates, uremic vWF might be less effective per unit in supporting platelet adhesion. To test the functionality of uremic vWF, we first perfused artery segments with uremic plasma pools. The vWF that bound to the vessel wall was subsequently assayed for its adhesive qualities in a second perfusion with control platelets and RBCs in vWF-depleted plasma of controls. Adhesion in this second perfusion was similarly proportional to the vWF present in control or patient plasma pools that were used in the first perfusion (Fig 4B). Because of the equal support of adhesion, we conclude that vWF present in patient plasma bound equally well to the vessel wall as vWF from control plasma, and that vWF in uremic plasma is not altered in its potential support of platelet adhesion. In accordance, uremic vWF bound normally to glycoprotein Ib of control platelets (Fig 2B). Uremic vWF also had a normal multimeric structure as observed by sodium dodecyl sulphate agarose gel electrophoresis (not shown), which was seen before. These data suggest that uremic vWF is not only functionally but also structurally indistinguishable from control vWF.

The influence of uremic plasma on binding of purified control vWF to the vessel wall and its subsequent function in adhesion was also investigated. Preperfusions were performed with vWF-depleted patient plasma with or without purified vWF. In the second perfusion with vWF-depleted control perfusates, adhesion was again proportional to the vWF levels in the first perfusion. The adhesion was not different from that obtained on segments first perfused with purified vWF in vWF-depleted plasma of controls (Fig 4A). Therefore, by the double perfusion experiments, it appeared that the vessel wall binding and subsequent function of uremic vWF (Fig 4B) from uremic plasma pools and of purified normal vWF (Fig 4A) in vWF-depleted uremic plasma was not inhibited. When used directly in reconstituted perfusates, the same patient pools diluted with vWF-depleted plasma were less able to support platelet adhesion than the control plasma pools with equal vWF concentrations (Table 4). The vWF-depleted patient plasma also inhibited adhesion of control platelets in reconstituted perfusates (Fig 4C). The addition of 1.5 U/mL of purified vWF to the
uremic vWF-depleted plasma resulted in increased adhesion. While with native uremic vWF concentrations of 1 U/mL, adhesion is about 20% lower than in control plasma (Table 4), the same concentration of purified vWF in uremic plasma results in an adhesion decrease of 44% (Fig 4C). The small effect of purified vWF in uremic plasma as compared with the capacity of the same vWF to increase adhesion in control plasma suggests that purified vWF is less effective in or better inhibited by uremic plasma.

When vWF binding to the vessel wall is inhibited during the direct interaction of platelets with the vessel wall, this may still cause decreased adhesion. We investigated this mechanism in reconstituted perfusates with vWF-depleted plasma of patients and controls. If disturbed vWF binding to the vessel wall causes the adhesion defect, then it should not be observed under these conditions. However, vWF-depleted patient plasma still inhibited platelet adhesion (Fig 4C). Moreover, the observed compensation of the adhesion defect by high levels of vWF in patient perfusates requires undisturbed binding of vWF from plasma to the vessel wall. Therefore, the unknown toxin in uremic plasma most likely inhibits the direct interaction of platelets with the adhesive proteins present in the vessel wall.

Two explanations for the uremic adhesion defect can be derived from our data. First, a uremic plasma factor inhibits the direct association of platelets with vWF bound to or initially present in the vessel wall. The vWF binding to platelet glycoprotein Ib is especially essential for adhesion. The defective ristocetin-induced platelet agglutination, which has been reported for PRP of patients with nonfixed platelets, may confirm this explanation.

The binding of vessel wall bound vWF to glycoprotein Ib is essential for adhesion. Because the ristocetin-induced binding of vWF to platelet glycoprotein Ib (Fig 2B) of fixed platelets is undisturbed by uremic plasma, one might conclude that the uremic adhesion defect might be due to inhibition of platelet adhesion, which is not dependent on vWF. However, in the ristocetin-induced binding studies, vWF was bound to glycoprotein Ib of fixed platelets under static conditions to prevent platelet activation and agglutination. Therefore, the results under these conditions cannot be compared entirely to the platelet association with vessel wall bound vWF in perfusion studies; the high shear forces in perfusion experiments require a much stronger and faster interaction between vWF and platelets. Hence, interference with this binding is more critical than binding under static conditions.

Bleeding symptoms and prolonged bleeding times in uremic patients can depend on a large group of factors. As our findings show, defects in primary platelet adhesion are probably of minor importance in uremic bleeding, while the usually high levels of vWF in uremic blood are able to compensate this defect. Though initially high vWF levels in patients need not be a guarantee against bleeding (Table 1), patients with low vWF activity may especially benefit by DDAVP or cryoprecipitate administration. These therapies, which increase plasma vWF, also reduce uremic bleeding. Whether the increase in vWF by these therapies also causes improved platelet adhesion should be investigated separately. However, our experiments seem to agree with this mechanism; high vWF levels resulted in extra binding to the vessel wall and compensated for the uremic inhibitor of primary platelet adhesion. The toxins in uremic plasma did not interfere with the vWF binding that is necessary for platelet adhesion. Moreover, the preperfusion experiments indicate that binding of inhibitors to the vessel wall is not likely to be responsible for the adhesion defect. However, it is not impossible that the vessel wall itself is altered by chronic uremia. The high vWF levels may reflect changes in endothelial metabolism. The understanding of uremic hemostasis should, therefore, also include investigations about the capacity of uremic subendothelium to support platelet adhesion.

Beside platelet adhesion, aggregate formation is also important for normal hemostasis. In our experiments, aggregate formation decreased at the same rate as total adhesion; the percentage of aggregate deposition remained unchanged at about 15% of the total adhesion (results not shown). Perfusions should be performed over more thrombogenic surfaces to show if uremic toxins also inhibit aggregate formation independent of platelet adhesion. The nature of the uremic plasma factor, which inhibits platelet adhesion to vessel wall, is under investigation presently.

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High von Willebrand factor concentration compensates a relative adhesion defect in uremic blood

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