Impaired Function of Platelet Membrane Glycoprotein IIb-IIIa in End-Stage Renal Disease

Meinrad P. Gawaz, Gustav Dobos, Michael Späth, Peter Schollmeyer, Hans J. Gurland, and Salim K. Mujais

ABSTRACT

Impaired platelet function and a bleeding tendency are well-recognized complications of chronic renal failure. Because the fibrinogen receptor GPllb-IIIa plays a central role in platelet aggregation and adhesion to the subendothelium, it was reasoned that a defect in this receptor may underlie the impaired platelet function in uremia. To test this hypothesis, the function of this receptor in the platelets of 11 uremic patients was studied. Aggregation studies were performed with flow cytometric techniques with anti-GPllb-IIIa conformation-specific monoclonal antibodies (mAb) (anti-LIBS1 and anti-PMI-1). Antifibrinogen and antithrombospordin mAb were used to characterize fibrinogen binding to GPllb-IIIa and the release of α-granules, respectively. Platelets from patients with chronic renal failure showed significantly decreased binding of conformation-dependent anti-LIBS1 mAb after ADP, phorbol myristate acetate, or RGD-peptide stimulation compared with normal controls, suggesting a defect related to the ability of the fibrinogen receptor to undergo a conformational change. Moreover, antifibrinogen and antithrombospordin binding to activated platelets were reduced in uremic patients, implying impairment of both ligand-binding and α-granule release. Hemodilatation partially restored GPllb-IIIa function, which may account for the observed effects of this therapy in restoring platelet aggregation. These findings indicate that platelets of patients with chronic renal failure reveal an aggregation defect at least partially due to an intrinsic GPllb-IIIa dysfunction and the presence of a putative uremic toxin that inhibits fibrinogen binding to GPllb-IIIa.

Key Words: Impaired platelet function, glycoprotein, ESRD

The bleeding tendency of uremia is considered to represent an acquired defect in primary hemostasis (1,2). Investigations to date have revealed a multiplicity of defects that may underlie this bleeding diathesis; however, a precise pathogenic framework continues to be elusive. Although defects in blood coagulation factors, alterations of the fibrinolytic system, and vascular abnormalities (1,2) have been considered to be contributory, platelet dysfunction has been the most consistently described hemostatic abnormality in patients with ESRD. Uremic platelets show a reduced adhesion to vascular subendothelium (3–5) and an impaired aggregation response to various stimuli (6,7). The altered interaction of adhesive macromolecules such as fibrinogen and von Willebrand factor with platelet membrane glycoproteins has been suggested to contribute to the aggregation and adhesion defects (8–10).

Platelet aggregation plays a central role in primary hemostasis (11–13). Under normal conditions, platelets circulate in a "resting" state, not interacting with each other or with other cells they encounter such as leukocytes or endothelial cells (13,14). Fibrinogen binding is a prerequisite for normal platelet aggregation (11,12). After platelet activation with various agonists such as ADP, epinephrine, or thrombin, the membrane glycoprotein complex GPIIb-IIIa undergoes a conformational change and is converted into a receptor that is capable of binding fibrinogen and other adhesive proteins that contain the amino-acid sequence RGD (Arg-Gly-Asp) (12). Plasmatic fibrinogen can then bind to its inducible receptor, enabling platelets to form microaggregates via fibrinogen “bridging.” This represents the first, but still reversible, step of platelet aggregation (14,15). Thereafter, platelets degranulate and release multiple compounds (ADP, thrombin, etc.) that in turn activate other platelets, thereby increasing the size of the platelet plug. Among these secreted compounds are

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adhesion molecules, such as thrombospondin (TSP), which after release from granules remain partially associated with the platelet membrane surface to further stabilize the platelet microaggregates (16,17). Because the fibrinogen receptor GPIIb-IIIa plays such a central role in platelet aggregation and adhesion to the subendothelium, we reasoned that a defect in this receptor may underlie the impaired platelet function in uremia. Conformation-dependent monoclonal antibodies (mAb) and newly developed flow cytometric techniques (18,19) have been found to be very useful tools in the study of platelet dysfunction in a variety of inherited and acquired diseases (18,19). We therefore used these techniques to examine GPIIb-IIIa function in the platelets of patients with ESRD.

METHODS

Platelet GPIIb-IIIa function was studied in 11 patients (five men, six women) with ESRD on chronic maintenance hemodialysis for 4 h thrice weekly. All patients gave informed consent, and the study protocol was approved by the Ethical Committee of our hospital. Causes of renal failure were chronic interstitial nephritis (N = 5), chronic glomerulonephritis (N = 4), and polycystic kidney disease (N = 2). Patients' hematocrit ranged from 25 to 36%, plasma fibrinogen ranged from 281 to 904 mg/dL, and platelet count ranged from 145 × 10^9 to 406 × 10^9/dL. None of the patients were known to have preexisting hemostatic disorders unrelated to their uremia, to suffer from infections, or to have received any medication known to affect platelet function (other than erythropoietin) 2 wk before the study. Six of the 11 patients were being treated with recombinant human erythropoietin (rHuEPO) for the correction of anemia at doses of 50 to 90 IE/kg body wt given iv three times a week for at least 3 months. Seven healthy individuals (four men, three women) with normal hematocrit, platelet count, and renal function served as a control group.

All ESRD patients enrolled in this study underwent regular hemodialysis treatment for at least 3 months (polysulphone membrane, four patients; PMMA membrane, one patient; cellulose acetate membrane, six patients) with synthetic dialyzers. Only new dialyzers were used. Because on subgroup analysis, we found no difference between the synthetic and cellular dialyzers for basal parameters or effects of dialysis, the data were pooled regardless of the type of membrane. Heparin was used as anticoagulant. In preliminary studies, we found no effect of heparin in the doses clinically used in hemodialysis on the flow cytometric assay. Predialysis blood samples were drawn from the access needle before a bolus injection of heparin and after the withdrawal of 3 mL of blood to clear the needle of any plasma or tissue components. Postdialysis blood samples were taken 240 min after the initiation of hemodialysis from the inlet blood line of the dialyzer. Platelet count, hematocrit, plasma fibrinogen, serum creatinine, and urea were determined in the samples by the hospital clinical chemistry laboratory by standard techniques. Blood samples for FACS analysis were drawn into plastic syringes containing citrate (ACD) (National Institutes of Health formula) as anticoagulant at a blood:ACD ratio of 7:1.

Platelet Flow Cytometry

For flow cytometric analysis, platelet-rich plasma was obtained from ACD-anticoagulated blood samples after the sedimentation of red and white blood cells at 180g for 20 min at room temperature. Thereafter, 5-μL aliquots of platelet-rich plasma were added to polypropylene tubes containing a saturating concentration (as determined in preliminary studies) of mAb dissolved in Tyrode's buffer (1% BSA, 2 mmol/L MgCl₂, 137.5 mmol/L NaCl, 12 mmol/L NaHCO₃, 2.6 mmol/L KCl; pH 7.4). ADP (final concentration, 10 M), phorbolmyristate (PMA; final concentration 2 nM), or GRGDSP (final concentration, 500 M) was added to achieve the indicated final concentrations in a total volume of 50 μL. Samples were incubated at room temperature in the dark for 30 min, diluted with 800 I of Tyrode's buffer, and then analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Platelets were identified in the forward versus side scatter plot (Figure 1) and 10,000 particles were analyzed for each sample. Light scatter and fluorescence data were obtained with gain settings in the logarithmic mode, and the data were analyzed with CONSORT 30 scientific software (Becton Dickinson). The mean intensity of fluorescence (MIF) (in arbitrary units) and the percentage of positive cells (%+) were used as quantitative measures of antibody binding (Figure 1). MIF was used to evaluate anti-CD41 binding because the antigen is expected to be universally present on platelets, and percent positive cells were used for the binding of the other mAb. As a control, a platelet-negative fluorescein isothiocyanate–conjugated immunoglobulin G1 monoclonal antibody (Dianova, Hamburg, Germany) was used.

For in vitro resuspension experiments, washed platelets were prepared from healthy volunteers by standard techniques and resuspended in Tyrode's buffer. Five-microliter aliquots were added to 40 μL of uremic plasma obtained either at the beginning or the end of a dialysis session to yield a final platelet concentration of 100,000/μL. Plasma obtained at the end of dialysis was corrected for hemocoagulation with Tyrode's buffer. Thereafter, 5 μL of RGDS peptide stock solutions was added to obtain the final

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peptide concentrations indicated in the Results section. For control studies, 5 µL of phosphate-buffered saline was used. After the addition of 5 µL of anti-LIBS1, mAb samples were incubated in the dark at room temperature for 15 min and evaluated by FACS analysis as described above. In control experiments, platelets of healthy volunteers were suspended in plasma from healthy volunteers. The pH of all plasma samples was adjusted to 7.4 with N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer.

mAb

Anti-CD41 is an activation-independent anti-GPIIb-IIIa mAb that detects both activated and non-activated GPIIb-IIIa on platelets. This mAb was obtained commercially as a fluorescein conjugate (Gentra Inc., Plymouth Meeting, PA). Anti-LIBS1 (LIBS, ligand-induced binding site) and PMI-1 are conformation-dependent mAbs that preferentially recognize ligand-induced epitopes on IIIa or IIb, respectively, which are only exposed when a ligand-induced conformational change of the receptor has occurred. These mAbs were kindly provided by Dr. Mark Ginsberg (RISC, La Jolla, CA). Anti-RIBS (RIBS, receptor-induced binding site) (provided by Dr. Mark Ginsberg) is a mAb that recognizes an epitope only exposed on immobilized fibrinogen. TSP-B7 (Sigma, St. Louis, MO) is a mAb specific for human platelet thrombospondin. Because TSP is released from α-granules after platelet activation and is considered to irreversibly stabilize the platelet-GPIIb-IIIa-fibrinogen-complex (16,17), we also examined the TSP plasma membrane expression in the studied patients after platelet activation. Anti-GPIIia (90BB10) and anti-GPIIb (98DFG) mAb, used for immunoblotting, were generously provided by Prof. Virtanen (University of Helsinki, Helsinki, Finland). The fibrinogen-mimetic peptide GRGDSP was purchased from Calbiochem (La Jolla, CA). All mAb used for flow cytometric analysis were conjugated with fluorescein isothiocyanate (Sigma) by standard methods to achieve fluorescein/protein molar ratios of 2 to 4.

Immunoblotting and Glycoprotein Staining

For immunoblotting, washed platelets were solubilized in Tris buffer containing 3% sodium dodecyl sulfate (SDS), 1 M leupeptin, and 1 mM polymethylsulfonyl fluoride. Samples to be run under reducing conditions were treated with 2% mercaptoethanol for 3 min at 90°C. Proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% resolving slab gels with a 4% stacking gel (20). Each lane was loaded with an equal amount of total protein (50 g), as determined by a Bradford protein assay (Bio-Rad). Resolved proteins were transferred electrophoretically to nitrocellulose paper (Bio-Rad) at 12 V for 30 min (Biometra Fast Blot, Göttingen, Germany) in pH 8.3 buffer containing 25 mmol/L Tris and 192 mmol/L glycine. The blots were blocked with 2% Tween 20 in Tris-buffered saline at pH 7.5, incubated with a mixture of anti-GPIIb (98DFG) and anti-GPIIIa (90BB10) mAb overnight at 4°C, and then incubated with a horse radish peroxidase–conjugated secondary goat anti-mouse antibody (Bio-Rad). 4-Chloro-1-naphthol (Sigma) was used as the peroxidase substrate. Glycoprotein staining of SDS-PAGE was performed by standard methods with periodic acid–Schiff’s (PAS) reagent (21).

Statistical Analysis

A t test for unpaired data was used to test for differences between normal control subjects and values obtained for patients before dialysis. Analysis of variance for repeated measures was used to test whether or not a given parameter changed after dialysis relative to predialysis values. Because some of the data was not normally distributed, the nonparametric Mann-Whitney and Wilcoxon tests were used in parallel. In all cases, the results of the parametric and nonparametric tests were compatible. All statistical testing was performed with the SPSS/PC+ statistical package (SPSS Inc., Chicago, IL). Data are presented as means ± SE for N observations.

RESULTS

Binding of Conformation-Specific Anti-GPIIb-IIIa mAb

Platelets of patients on regular hemodialysis treatment and of normal controls were incubated in the absence or presence of the agonists ADP, PMA, or...
the fibrinogen-mimetic RGD peptide (GRGDSP) (see Methods). These agonists are known to induce the activation state of the GPIIb-IIIa receptor, which is then assayed with the conformation-dependent mAb anti-LIBS1 and anti-PMI-1 (18,22). In addition, total GPIIb-IIIa expression was measured with a complex-specific mAb anti-CD41, which recognizes the activated and the nonactivated form of GPIIb-IIIa.

There was no difference in anti-CD41 binding to patient and control platelets (uremics, MIF = 188.9 ± 71.9; controls, MIF = 200.7 ± 85.6) (Figure 2). No significant difference was found in anti-LIBS1 and anti-PMI-1 binding to nonstimulated platelets of patients and controls (Table 1). All agonists induced a significant increase in percent positive cells for anti-LIBS1 in control platelets. This effect was most

Flow Cytometry Analysis

Figure 2. Binding of conformation-specific mAb (anti-LIBS1) to activated platelets. Binding of fluorescein isothiocyanate (FITC)-conjugated anti-LIBS1 to uremic and control platelets was measured in the presence of ADP (10 μM), PMA (2 nM), and GRGDSP peptide (500 M). In addition, total platelet GPIIb-IIIa was determined by complex-specific FITC-anti-CD41 binding. The histograms are of a representative patient and a control subject.
marked for RGD peptide (Table 1). There was a significant decrease in the binding of anti-LIBS1 to uremic platelets after stimulation with ADP, PMA, or GRGDSP compared with normal controls ($P < 0.05$) (Figure 2) (Table 1). The binding of anti-PMI-1 mAb to platelets was not significantly affected by the agonists used (Table 1), a finding suggesting a limited usefulness of this antibody for the evaluation of agonist-induced conformational changes in the GPIIb-IIIa complex, which we also observed in other studies (unpublished personal observations).

To examine whether the decreased antibody binding in uremics is due to a qualitative defect of the GPIIb-IIIa molecule, we further characterized the glycoprotein by immunoblotting studies after separating total platelet proteins by SDS-PAGE (Figure 3). As shown in a representative immunoblot (Figure 3), no difference in the reduced or nonreduced molecular mobility of GPIIb-IIIa was found in any of the patients studied when compared with normal controls (Figure 3). Furthermore, glycoprotein staining of total platelet proteins with PAS (21) revealed a similar GPIIb-IIIa staining pattern of uremics and controls (Figure 4).

**TABLE 1. Binding of conformation-dependent anti-GPIIb-IIIa mAb to nonstimulated and activated platelets of uremic patients ($N = 11$) and controls ($N = 7$) (percent positive cells)**

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>Agonist</th>
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<tbody>
<tr>
<td></td>
<td>ADP</td>
<td>PMA</td>
</tr>
<tr>
<td>Anti-LIBS1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>$4.3 \pm 1.9$</td>
<td>$14.1 \pm 9.3^*$</td>
</tr>
<tr>
<td>Controls</td>
<td>$6.4 \pm 5.5$</td>
<td>$50.3 \pm 33.5$</td>
</tr>
<tr>
<td>Anti-PMI-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>$2.8 \pm 2.9$</td>
<td>$8.4 \pm 2.5$</td>
</tr>
<tr>
<td>Controls</td>
<td>$4.5 \pm 4.5$</td>
<td>$10.1 \pm 4.9$</td>
</tr>
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</table>

* Significantly less than corresponding value in normal controls; $P < 0.05$.

**Figure 3. Immunoblot of total protein from platelets of two normal controls (Lanes 1 to 4) and three representative uremics (Lanes 5 to 10). Samples were run under nonreducing (Lanes 1, 2, and 5 through 7) and reducing (Lanes 3, 4, and 8 through 10) conditions with 2% mercaptoethanol as the reducing agent. Proteins were separated on 7.5% SDS-PAGE. Each lane of the blotted gel contained equal amounts of protein (50 g). Immunodetection was performed in the presence of both anti-lib and anti-IIIa mAb. Standard molecular weight markers were myosin (200, 100, and 69 kd), ovalbumin (46 kd), bovine anhydrase (30 kd), and hen egg white lysozyme (143 kd).**

**Binding of Antifibrinogen and anti-TSP mAb**

To characterize fibrinogen binding to GPIIb-IIIa, nonstimulated and PMA-activated platelets of uremic patients and normal controls were further evaluated for anti-RIBS binding; anti-RIBS is an mAb that recognizes an epitope only exposed on immobilized fibrinogen (23). Anti-RIBS binding was similarly low in unstimulated platelets of uremic and control subjects (Table 2). PMA induced a significant increase in anti-RIBS binding in control platelets that was greater than that observed in PMA-stimulated uremic platelets (Figure 5) (Table 2).

Because TSP is released from $\alpha$-granules after platelet activation and is considered to irreversibly stabilize the platelet-GPIIb-IIIa-fibrinogen-complex (16,17) we also examined the TSP plasma membrane expression in the studied patients after platelet activation. Anti-TSP binding to nonstimulated platelets was similar in control and uremic platelets (Table 2). PMA led to a major fourfold increment in anti-TSP binding in control platelets, whereas a more modest increase was seen in the uremic platelets (Table 2).
Effect of Hemodialysis on Anti-GPIIb-IIIa, Antifibrinogen, and Anti-TSP mAb Binding

Because hemodialysis is known to improve platelet function and to correct bleeding time (1,24) we investigated the effect of hemodialysis treatment on GPIIb-IIIa function. There was no significant difference in anti-LIBS1 and anti-PMI-1 binding to nonactivated or ADP- or PMA-activated platelets before and after hemodialysis treatment (Table 3). However, anti-LIBS1 binding of RGD peptide-stimulated platelets after hemodialysis was significantly enhanced when compared with predialysis (P < 0.05; Table 3). The binding of anti-RIBS and anti-TSP mAb to activated and nonstimulated platelets did not change significantly after dialysis (data not shown).

Effect of rHuEPO on Binding of Conformation- and Complex-Specific Anti-GPIIb-IIIa mAb

The administration of rHuEPO has been reported to improve platelet function (25–27). Because the effect of rHuEPO may have been obscured by the overall presentation of the data from uremic subjects, we performed subgroup analysis comparing GPIIb-IIIa function in uremic patients with and without rHuEPO treatment. Platelets of rHuEPO patients had a significantly greater binding of complex-specific anti-CD41 mAb than did those of patients not receiving rHuEPO (with rHuEPO, MIF = 235.0 ± 38.8; without rHuEPO, MIF = 142.9 ± 67.7; P < 0.05), indicating a higher platelet membrane density of GPIIb-IIIa in the rHuEPO group. However, the binding of anti-LIBS1 and anti-PMI to the ADP-activated platelets of patients receiving rHuEPO treatment was not different from that of the group not receiving rHuEPO (Table 4).

Effect of Uremic Plasma Prehemodialysis and Posthemodialysis on RGDS-Induced LIBS1 Expression in Normal Platelets

To evaluate whether the effect of hemodialysis was related to the removal of uremic toxin(s), platelets from normal volunteers were suspended in uremic plasma obtained before and after hemodialysis, respectively. Normal platelets suspended in uremic predialysis plasma showed a defect in their response to RGDS compared with normal platelets suspended in normal plasma (Table 5). In contrast, normal platelets suspended in uremic postdialysis plasma showed a significantly improved response to RGDS that was not different from that observed in normal plasma.

DISCUSSION

The major findings of this study are: (1) The GPIIb-IIIa function of platelets from chronic renal failure patients is impaired, most likely as the result of a conformational change and fibrinogen-ligand-binding defect of GPIIb-IIIa, suggesting a pathophysiologic role in uremic platelet dysfunction. (2) Hemodialysis improves the fibrinogen-ligand binding of GPIIb-IIIa, indicating the removal of a uremic inhibitor during dialysis treatment. (3) The defect is reproduced in normal platelets when they are incubated in uremic predialysis plasma but not in postdialysis plasma. (4) Chronic renal failure patients treated with rHuEPO for the correction of anemia show an increased GPIIb-IIIa expression, implying that rHuEPO treatment could ameliorate platelet function in uremic patients.

TABLE 2. Binding of conformation-dependent antifibrinogen mAb (anti-RIBS) and anti-TSP to platelets of patients (N = 11) and controls (N = 7) (percent positive cells)

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Anti-RIBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3.8 ± 2.9</td>
<td>3.7 ± 1.6</td>
</tr>
<tr>
<td>PMA</td>
<td>7.6 ± 7.4</td>
<td>26.2 ± 12.6</td>
</tr>
<tr>
<td>Anti-TSP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>10.9 ± 12.4</td>
<td>11.1 ± 10.8</td>
</tr>
<tr>
<td>PMA</td>
<td>27.4 ± 15.2</td>
<td>49.4 ± 22.3</td>
</tr>
</tbody>
</table>

* Significantly less than corresponding value in normal controls; P < 0.05.
Flow Cytometry Analysis

![Flow Cytometry Analysis Diagram](image)

Figure 5. Binding of anti-fibrinogen (anti-RIBS) and anti-TSP mAb to uremic and control platelets. Binding of fluorescein isothiocyanate-conjugated anti-RIBS and anti-TSP was determined on nonactivated platelets and in the presence of PMA (2 nM).

These findings indicate that platelets of patients with chronic renal failure reveal an aggregation defect at least partially due to an intrinsic GPIIb-IIIa dysfunction and the presence of a putative uremic toxin that inhibits fibrinogen binding to GPIIb-IIIa.

Defective hemostasis in patients with chronic renal disease has been attributed to a variety of causes but remains poorly understood (1,2,4,5,9,10,28). The uremic bleeding tendency has been associated with platelet dysfunction, which may be caused by abnormalities of platelet membrane glycoproteins (8,9,29). GPIIb-IIIa is the major glycoprotein found on the platelet plasma membrane and is an absolute requirement for normal platelet aggregation (1,12,30,31). The platelet aggregation process is dependent on the binding of fibrinogen to activated...
TABLE 3. Binding of conformation-dependent anti-GPIib-IIIa mAb to nonstimulated and activated platelets of uremic patients prehemodialysis and posthemodialysis (percent positive cells)

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<tr>
<td></td>
<td>None</td>
<td>ADP</td>
</tr>
<tr>
<td>Anti-LIBS1</td>
<td></td>
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</tr>
<tr>
<td>Predialysis</td>
<td>4.3 ± 1.9</td>
<td>14.1 ± 9.3</td>
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<tr>
<td>Postdialysis</td>
<td>6.3 ± 3.4</td>
<td>19.6 ± 7.8</td>
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<tr>
<td>Anti-PMI-1</td>
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<td></td>
</tr>
<tr>
<td>Predialysis</td>
<td>2.8 ± 2.9</td>
<td>8.4 ± 2.5</td>
</tr>
<tr>
<td>Postdialysis</td>
<td>3.4 ± 1.9</td>
<td>8.8 ± 2.7</td>
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</table>

* Significantly greater than corresponding value predialysis; *P* < 0.05.

TABLE 4. Binding of conformation-specific mAb to platelets of hemodialysis patients receiving and not receiving rHuEPO treatment (percentage of positive cells)

<table>
<thead>
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<tr>
<td>Anti-LIBS1</td>
<td></td>
<td></td>
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<tr>
<td>With rHuEPO (N = 6)</td>
<td>3.7 ± 0.9</td>
<td>12.4 ± 10.2</td>
</tr>
<tr>
<td>Without rHuEPO</td>
<td>5.1 ± 2.4</td>
<td>15.9 ± 8.0</td>
</tr>
<tr>
<td>Anti-PMI-1</td>
<td></td>
<td></td>
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<tr>
<td>With rHuEPO (N = 6)</td>
<td>4.3 ± 3.6</td>
<td>5.8 ± 3.0</td>
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<tr>
<td>Without rHuEPO</td>
<td>1.2 ± 0.3</td>
<td>3.2 ± 0.8</td>
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TABLE 5. Effect of uremic plasma predialysis and postdialysis on RGDS-induced LIBS1 expression on normal platelets

<table>
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<tr>
<th>Plasma</th>
<th>RGDS concentration (mM)</th>
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<tr>
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<tr>
<td>Control (N = 5)</td>
<td>6.6 ± 3.1</td>
</tr>
<tr>
<td>Predialysis (N = 5)</td>
<td>18.0 ± 6.6</td>
</tr>
<tr>
<td>Postdialysis (N = 5)</td>
<td>11.0 ± 5.1</td>
</tr>
</tbody>
</table>

* Significantly greater than corresponding value predialysis; *P* < 0.05.

GPIib-IIIa (11,12). The expression of the fibrinogen receptor on GPIib-IIIa requires previous activation with agonists such as ADP, thrombin, epinephrine, or PMA (11,12). This study was conducted to test the hypothesis that uremic platelet defect may be due to an impaired GPIib-IIIa receptor function.

When platelets of patients with chronic renal failure were stimulated with ADP, there was a significant decreased binding of anti-LIBS1, indicating that the GPIib-IIIa of uremic patients exhibits a conformational change defect (Table 1; Figure 3) (18,22). This defect was not specific to ADP stimulation, because uremic platelets activated with PMA also showed a decrease in anti-LIBS1 fluorescence (Figure 3; Table 1). This abnormality was not due to a reduced number of GPIib-IIIa receptors found on the
plasma membrane because there was no difference in complex-specific anti-CD41 binding to uremic and control platelets (Figure 3). Recent reports suggest that the binding of other activation-specific mAb, e.g., PAC1 (9) and 7E10 (29), to uremic platelets is also significantly reduced after activation. Because activation-dependent epitopes exposed on the GPIb-IIIa molecule after fibrinogen binding may mediate transmembrane interactions between cytoskeletal elements and polymerizing fibrin (postoccupancy events) (22), a defect in GPIb-IIIa conformation may not only lead to impaired platelet aggregation but may also affect normal thrombi formation and clot contraction (22). A defect of these postoccupancy events necessarily results in defective primary hemostasis and might play an important pathophysiologic role in uremic bleeding tendency.

It has been shown that the adhesive glycoproteins von Willebrand factor (vWF) (10) and TSP (30,32) are structurally altered in uremia. Whether an impaired GPIb-IIIa function is due to an altered structure or glycosylation of the fibrinogen receptor had not been examined previously. We did not find any difference in the molecular mobility of IIb or IIIa in the nonreduced or reduced form in our immunoblotting studies (Figure 3). Furthermore, there was no difference in the glycoprotein PAS staining of IIb and IIIa of uremic total platelet proteins (Figure 4). Thus, it seems unlikely that the impaired GPIb-IIIa function is a consequence of transcriptional defect or posttranscriptional modification involving glycosylation or proteolytic clipping, as was suggested for TSP in uremia (30,32).

Because anti-LIBS1 recognizes an epitope on IIIa only when fibrinogen or fibrinogen-mimetic peptides are bound to GPIb-IIIa (18,22), the impaired up-regulation of the anti-LIBS1 epitope found in uremic platelets could be due to a deficit in ligand recognition. Therefore, platelets were incubated with a fibrinogen-mimetic peptide (GRGDSP) known to induce a conformational change of GPIb-IIIa (18,22). Ligand binding was assayed with anti-LIBS1 (18,22). Platelets from uremic patients showed a significant twofold decrease of anti-LIBS1 binding in the presence of this peptide compared with those from normal controls (Table 1; Figure 2), indicating that the interaction of RGD peptide with the ligand pocket of GPIb-IIIa is impaired. In addition, anti-RIBS binding to activated uremic platelets was significantly reduced compared with normal control (Table 2), suggesting that the binding of the macromolecule fibrinogen to GPIb-IIIa is also reduced in uremia—a finding consistent with recent reports (9).

α-Granule release seems to be impaired in uremia (33), and TSP plays an important role in platelet aggregation by stabilizing the fibrinogen-GPIb-IIIa-platelet complex (16,17). We observed significantly reduced TSP expression on uremic platelets after PMA stimulation in comparison to normal controls, indicating a reduced release of TSP (Figure 5, Table 2). This decrease in TSP expression is most probably due to an acquired storage pool deficiency because the TSP content of uremic platelets is reduced in uremia (28,32). Thus, we conclude that platelet aggregation in uremia is impaired both because of GPIb-IIIa dysfunction, which causes an impaired first phase of aggregation, and because of a reduced α-granule release of TSP required for the irreversible stabilization of platelet macroaggregates (second phase of aggregation).

Hemodialysis has been shown to affect platelet function (24) and to correct bleeding time (1). We found no difference in anti-LIBS1 binding to nonactivated or ADP- or PMA-activated platelets before and after dialysis (Table 3). However, there was a significant increase in anti-LIBS1 binding to platelets in the presence of fibrinogen-mimetic peptide after hemodialysis. Thus, the increase of LIBS1 expression after RGD peptide stimulation of postdialysis platelets could be due to the removal of a dialyzable substance present in uremic plasma. This possibility was further tested by suspending platelets from normal volunteers in uremic plasma obtained before and after hemodialysis, respectively. Normal platelets suspended in uremic predialysis plasma showed a defect in their response to RGDS compared with normal platelets suspended in normal plasma (Table 5). In contrast, normal platelets suspended in uremic postdialysis plasma showed a significantly improved response to RGDS that was not different from that observed in normal plasma. Therefore, it seems likely that hitherto unidentified substances present in uremia inhibit fibrinogen binding to GPIb-IIIa and thus affect platelet aggregation. Interestingly, uremic toxins have been shown to inhibit platelet aggregation (34). Moreover, uremia and hemodialysis are associated with an increased fibrinolytic activity and an increase of fibrinogen degradation products (1). Thus, it is tempting to speculate that small peptides, most likely derived from degraded fibrinogen, that have been isolated from the dialysate (35) might interfere with fibrinogen binding to GPIb-IIIa in uremia. The removal of this/these inhibitor(s) during hemodialysis could be responsible for the improved platelet function found after dialysis treatment (1,24).

The treatment of chronic renal failure patients maintained on regular hemodialysis with rHuEPO has been reported to transiently increase platelet counts, shorten skin bleeding time, and improve platelet aggregation (25). Moreover, platelets from patients receiving rHuEPO seem to be more activated during hemodialysis, which could pose problems in terms of dialyzer blood clotting and arteriovenous
fistula thrombosis (28). We found that platelets of rHuEPO-treated patients had a significantly greater binding (about 1.5-fold) of activation-independent anti-CD41 than did those of patients not receiving rHuEPO, implying that rHuEPO treatment increases the number of GP IIb-IIIa molecules found on platelet plasma membrane. rHuEPO increases megakaryopoiesis in vitro (26). The administration of rHuEPO to uremic patients might, therefore, have an effect on thrombopoiesis in addition to erythropoiesis in uremic subjects. Thus, it is reasonable to suggest that the improved platelet function found in patients with rHuEPO is not solely due to rheologic changes (25).

Uremic thrombopathy and the bleeding tendency of chronic renal failure are of multifactorial origin. However, we and others have found that platelet membrane proteins play a crucial role in the defective platelet function found in chronic renal failure patients. These data suggest that platelet aggregation and thus primary aggregation are impaired in uremic patients, most probably secondary to impaired GP IIb-IIIa function and fibrinogen binding (reversible first phase) and a reduced TSP release (irreversible second phase). This platelet dysfunction can be partially corrected by hemodialysis treatment (removal of anti-GP IIb-IIIa antagonist). Characterization of the putative uremic antiplatelet substance requires further study.

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