Glycocalcin: A New Assay—The Normal Plasma Levels and Its Potential Usefulness in Selected Diseases

By J.H. Beer, L. Büchi, and B. Steiner

Platelet glycocalcin (GC) is the extramembranous portion of GPIbα that can be rapidly cleaved by enzymes such as calpain, plasmin, trypsin, elastase, etc. Quantitative cleavage will ultimately result in an acquired Bernard-Soulier–like bleeding disorder, and circulating GC may act as a potential inhibitor of platelet adhesion. We have developed fragmentations and the streptavidin-biotin system. First, the methodology was evaluated and standardized with special emphasis on the anticoagulant and the inhibitors (EDTA, prostaglandin E1 [PGE1], aprotinin, N-ethyl-maleimide), the mode of high-speed centrifugation (to avoid platelet microparticles), and the standards used (purified GPIb and GC). This assay was then used to analyze the GC levels of healthy subjects (2.04 ± 0.46 μg/mL) and of patients with selected diseases. The results of patients with aplastic anemia and thrombocytosis confirmed that GC levels are clearly dependent on the platelet count, which was the basis for the introduction of the GC index, the standardization of GC for a platelet count of 250 × 10^9/L. The GC index discriminates reliably patients with active immune thrombocytopenic purpura from those in remission. GC levels are elevated in patients on hemodialysis (3.62 ± 0.78 μg/mL, P < .001). The high GC index (6.93 ± 4.21, P < .001) in cirrhosis patients suggests an increased platelet turnover and/or abnormal proteolysis. In contrast to other groups, we have not found that recombinant tissue plasminogen activator (rtPA) treatment of patients with myocardial infarction increases GC levels. However, concentrations are elevated in leukemia and the highest levels found are approximately 40 μg/mL. These studies suggest that GC is a useful platelet marker in certain diseases, which directly reflects platelet damage and possibly platelet dysfunction.

Although several assays for GC, including ours, have been published in the past few years,4-11 a series of methodologic difficulties have resulted in variable values and have limited more widespread application. In fact, the normal concentrations proposed in the literature vary by a factor of 20, namely between 0.36 μg/mL plasma17 and 53.8 nmol/L (corresponding to ~7.3 μg/mL).10 In this study, we have first investigated the possible reasons for these differences, eg, the type of anticoagulant selected with or without enzyme inhibitors, the centrifugation procedure, the type of assay, the number and type of antibodies used, and the preparation and characterization of the standards. GC is a highly glycosylated molecule20,21 that is difficult to quantify in a regular protein assay, thus requiring amino acid analysis of the purified material for reliable standardization.

We have used the assay to determine the values of 116 normal adults and have analyzed selected patient groups to assess the impact of liver and kidney dysfunction, the influence of high and low platelet counts on the GC levels, and
the consequences of old age and severe arterial disease. Finally, we evaluated a series of patients with suspected increased levels of specific enzymes known to cleave GC from GPIba (plasmin, elastase), such as those with myocardial infarction treated with rtPA, and those with myeloid leukemia.

MATERIALS AND METHODS

Normal Donors and Patients

A total of 360 normal donors and patients were examined as summarized in Table 1. In addition to 116 normal adults (age: 18 to 90 years; mean: 38 ± 12), a group of nine children was examined to evaluate further the effect of age on GC levels. Detailed patient data are available from the author on request.

Materials and Procedures

Selection of MoAbs

All of the antibodies evaluated in the assay were murine IgG MoAbs. Antibody 6D1 was kindly provided by Dr B.S. Coller, SUNY, Stony Brook, New York. All MoAbs are from the IgG subclass, except for antibody Ib-4, which is an IgG2a. The MoAbs 6D1, Ib-6, Ib-23, and Ib-26 are all directed against the platelet GPIa/IIa was biotinylated as well and used as a control. All of the antibodies evaluated in the assay showed mutual competitive inhibition (data not shown). The combinations with the antibody 6FI and the combinations with the antibody 6D1, and Ib-26 with 6D1. Considerably less sensitivity was obtained with the antibody 6FI and the combinations with the same antibody as upper and lower antibody were all clearly negative.

Table 1. GC Levels in Normals and Patients: Donor Characteristics and Results

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>GC (µg/mL)</th>
<th>P</th>
<th>GC Index</th>
<th>P</th>
<th>Platelets (&lt;10¹¹/L)</th>
<th>WBC Count (&lt;10⁹/L)</th>
<th>Hemorheo (%)</th>
<th>Creatinine (µmol/L)</th>
<th>Age (yr)</th>
<th>Sex (%)</th>
<th>Sex (males)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Normal adults</td>
<td>116</td>
<td>2.04 ± 0.46</td>
<td>2.16 ± 0.66</td>
<td>246 ± 53</td>
<td>6.4 ± 5.3</td>
<td>419 ± 3.5</td>
<td>—</td>
<td>38 ± 12</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a Children</td>
<td>9</td>
<td>1.94 ± 0.48</td>
<td>0.53</td>
<td>1.76 ± 0.77</td>
<td>0.03</td>
<td>327 ± 139</td>
<td>7.8 ± 2.8</td>
<td>40.2 ± 10.8</td>
<td>9 ± 6</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b Twins</td>
<td>6</td>
<td>2.27 ± 0.61</td>
<td>0.22</td>
<td>2.0 ± 0.55</td>
<td>0.37</td>
<td>291 ± 86</td>
<td>5.9 ± 1.1</td>
<td>41.4 ± 2.7</td>
<td>—</td>
<td>33 ± 7</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>2 Diabetes patients</td>
<td>18</td>
<td>3.62 ± 0.75</td>
<td>&lt;0.01</td>
<td>3.65 ± 1.48</td>
<td>&lt;0.01</td>
<td>276 ± 70</td>
<td>7.2 ± 1.8</td>
<td>339 ± 5.6</td>
<td>674 ± 287</td>
<td>53 ± 13</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3a Atherosclerosis</td>
<td>13</td>
<td>3.98 ± 0.53</td>
<td>&lt;0.01</td>
<td>3.94 ± 1.66</td>
<td>&lt;0.01</td>
<td>242 ± 14</td>
<td>5.6 ± 1.9</td>
<td>28±3 ± 3.8</td>
<td>750 ± 304</td>
<td>67 ± 8</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>3b Cardiovascular</td>
<td>18</td>
<td>2.61 ± 1.48</td>
<td>0.13</td>
<td>6.9 ± 4.21</td>
<td>&lt;0.01</td>
<td>106 ± 48</td>
<td>6.6 ± 2.3</td>
<td>355 ± 5.6</td>
<td>90 ± 17</td>
<td>66 ± 11</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>4 Vascular disease</td>
<td>37</td>
<td>1.82 ± 0.42</td>
<td>0.01</td>
<td>1.95 ± 0.59</td>
<td>0.06</td>
<td>245 ± 50</td>
<td>7.6 ± 1.8</td>
<td>410 ± 4.7</td>
<td>90 ± 13</td>
<td>62 ± 12</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>4a Normal creatinine</td>
<td>68</td>
<td>2.23 ± 0.75</td>
<td>&lt;0.01</td>
<td>3.10 ± 0.36</td>
<td>&lt;0.01</td>
<td>267 ± 80</td>
<td>5.4 ± 1.9</td>
<td>371 ± 7.1</td>
<td>249 ± 196</td>
<td>70 ± 8</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>4b Elevated creatinine</td>
<td>17</td>
<td>2.32 ± 0.34</td>
<td>&lt;0.01</td>
<td>2.47 ± 0.66</td>
<td>0.15</td>
<td>237 ± 47</td>
<td>1.7 ± 3.2</td>
<td>418 ± 4.5</td>
<td>101 ± 18</td>
<td>57 ± 8</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>5 Myocardial infarction</td>
<td>14</td>
<td>2.32 ± 0.40</td>
<td>0.24</td>
<td>2.47 ± 0.66</td>
<td>0.15</td>
<td>237 ± 47</td>
<td>1.7 ± 3.2</td>
<td>418 ± 4.5</td>
<td>101 ± 18</td>
<td>57 ± 8</td>
<td>66</td>
<td></td>
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<tr>
<td>6 Thrombocytopenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6a Aplastic anemia</td>
<td>18</td>
<td>0.92 ± 0.74</td>
<td>&lt;0.01</td>
<td>2.50 ± 1.10</td>
<td>&lt;0.09</td>
<td>77 ± 67</td>
<td>6.1 ± 4.9</td>
<td>312 ± 10.2</td>
<td>84 ± 22</td>
<td>32 ± 15</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>6b Thrombocytopenic</td>
<td>61</td>
<td>2.4 ± 1.27</td>
<td>&lt;0.03</td>
<td>60.3 ± 93.0</td>
<td>&lt;0.01</td>
<td>45 ± 38</td>
<td>8.2 ± 4.1</td>
<td>38.0 ± 4.9</td>
<td>87 ± 19</td>
<td>60 ± 20</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>7 Infection</td>
<td>24</td>
<td>2.69 ± 0.54</td>
<td>&lt;0.01</td>
<td>2.64 ± 0.55</td>
<td>&lt;0.01</td>
<td>257 ± 63</td>
<td>7.2 ± 2.4</td>
<td>40.9 ± 3.1</td>
<td>86 ± 15</td>
<td>48 ± 16</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>8 Leukemia</td>
<td>12</td>
<td>11.2 ± 6.63</td>
<td>0.63</td>
<td>3.52 ± 1.88</td>
<td>&lt;0.01</td>
<td>242 ± 476</td>
<td>9.9 ± 4.1</td>
<td>41.2 ± 4.3</td>
<td>83 ± 28</td>
<td>53 ± 17</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia.
of GC. The purified GPIb/IX was functional, since it bound von Willebrand factor in a solid-phase assay using botrocetin as mediator (data not shown).

Purified GC was obtained from two sources. First, GC was purified from plasma using an MoAb Ib-23 immunoaffinity column. Alternatively, one third of a platelet concentrate was treated with three short bursts of ultrasonication (100 W, 20 seconds, 30 mL vol) to lyse the platelets. After centrifugation for 10 minutes at 30,000g, the supernatant was added immediately to the remaining two thirds of the platelet preparation for 1 hour at room temperature. It was assumed that during this time the liberated platelet calpain would cleave GC from intact platelet GPIb. The preparation was then centrifuged at 30,000g for 10 minutes, passed over a WGA column followed by a thrombin column as described above, and analyzed by SDS-PAGE, Western blotting, and ELISA.

To establish the normal GC plasma levels, plasma samples of 116 normal volunteers were analyzed individually in the assay and a pool was prepared from 100 donors, aliquoted, and frozen at −70°C. Blood was drawn from all donors by the same two persons under identical conditions. Blood was taken after informed consent was obtained and after the study was approved by the local ethical committee. The anticoagulant consisted of 1/10 vol of the blood (total, 5 mL) and contained 4 mmol/L EDTA and 0.5 µmol/L prostaglandin E3 (PGE3 Sigma), 5 mmol/L L-ethyl-maleimide (Sigma), and 50 U/mL aprotinin (Trasylol; Bayer, Leverkusen, Germany) (all final concentrations). The anticoagulant was freshly prepared and aliquoted at a 10× concentration in 0.5 mL and was kept frozen at −20°C in the styrofoam. It was thawed just before use.

Leupeptin and D-phenylalanyl-L-prolyl-L-arginine-chloromethylketone were not used because they appeared to be inhibitory in the assay, even if purified standard GPIb/IX was used as antigen instead of normal plasma. To compare this anticoagulant with EDTA alone and with citrate, blood from eight healthy donors was drawn into the three different anticoagulants and the GC levels were determined. The inhibitors used did not alter the results at the concentrations mentioned.

In an attempt to simulate the effect of enzymes potentially released after delays in sample preparation or under pathological conditions in vivo, which might affect the GC levels in vitro, we evaluated the effect of prolonged standing of whole blood on the GC levels from a series of normal donors over 24 hours at room temperature after the venipuncture using the anticoagulant as described above (n = 8) or EDTA alone (n = 6). To study the effect of prolonged standing under pathological conditions, we analyzed the samples of a patient with chronic myeloid leukemia (CML) with a high white blood cell count of 94 × 109/L and a platelet count of 463 × 109/L. His creatinine level was normal (86 µmol/L). The GC levels were measured immediately after venipuncture and after 12 hours of standing at room temperature in the presence of either EDTA alone or EDTA plus inhibitors.

In another series of experiments, the impact of a myeloid leukemia cell line (HL-60 cells, kindly provided by Dr A. Toher, Department of Hematology, University Hospital of Bern, Bern, Switzerland) on GC levels was analyzed in vitro using the different anticoagulants. The promyelocytic cell line was established from a patient with acute myeloid leukemia (AML) stage M2.26 HL-60 cells were grown in culture under standard conditions over 4 days. After washing twice in PBS, pH 7.4, 5 × 10^6 cells were added to 500 µL of control platelet-rich plasma (300 × 10^9 platelets/L) at room temperature. After 2 hours of incubation, the samples were centrifuged and the supernatant was analyzed for GC by ELISA. In another set of experiments, an equivalent number of HL-60 cells were lysed by sonication and then added to the platelets. Control experiments included the analysis of tissue culture supernatant and lysed HL-60 cells for GC by ELISA.

Evaluation of the centrifugation protocol. The presence of residual platelets and platelet vesicles in the plasma may give false high GC levels. We therefore performed a series of experiments designed to evaluate the effect of increasing centrifugation forces. Three to six aliquots of blood from 29 donors were drawn into EDTA plus inhibitors and were centrifuged using six different protocols as outlined in Fig 2. Method 5 was chosen (2,000g for 20 minutes + 6,000g for 20 minutes + 30,000g for 20 minutes) for the samples of all of the normal subjects and patients, since the results indicated a significant decrease of GC levels, but no further reduction was detected upon additional centrifugation at 100,000g for 60 minutes.

The assay was performed in flat-bottom, 96-well microtiter plates (M129B, cobalt 60-irradiated, Dynatech, Chantilly, VA). The plates were coated with MoAb Ib-6 (100 µL/well, 5 µg/mL in 50 mmol/L Na HCO3/Na2CO3, pH 9.4) for 12 hours at 4°C, washed three times for 2 seconds with 250 µL/well of PBS (20 mmol/L phosphate, 140 mmol/L NaCl, pH 7.4), containing 0.05% Tween-
gen-containing probes (plasma samples were diluted between 1:10 and 1:100,000). The latter antibody was used in combination with 6D1 as a negative control in all patient samples with GC levels greater than 4 μg/mL to exclude false-positives; (3) if the same antibody was used as lower and upper antibody: or (4) if unbiotinylated antibody 6D1 was used. Figure 3 shows that the standard curves of purified GPIb/IX and of dilutions of pooled normal plasma were parallel, thus making interference or cross-reactivity of other plasma proteins with the antibodies used unlikely. A standard curve of a GC preparation ran parallel to GPIb/IX (data not shown). The GC concentration was read from the standard curve obtained with GPIb/IX on each plate. A correction factor of 0.75 was introduced to account for the difference in molecular weights between GC (~135,000) and GPIb/IX (~180,000), and Heparin (1 U/mL) did not interfere with the test.

GC levels have been shown to correlate with the platelet counts in the steady-state and in the absence of increased platelet destruction. Since the results of our group of patients with aplastic anemia confirmed this finding, we have normalized the GC value in micrograms per milliliter for the individual platelet counts and have formed the glycocalicin index: GC = GC value (μg/mL) × (250 × 10^9 platelets/L)/(individual platelet count), similar to the method proposed by Steinberg et al., who calculated the glycoprotein index using glycoprotein A as percent of normal.

Analysis of GC in the urine. The urine of a healthy 36-year-old man with normal creatinine levels was collected into EDTA (4 mmol/L) and inhibitors (N-ethyl-maleimide 5 mmol/L, aprotonin 25 U/mL) over 24 hours (1.4 L), and an aliquot of 100 mL was dialyzed against PBS containing inhibitors, then against water, and finally lyophilized. It was redissolved in PBS (1 to 2 mL) and desalted over a PD-10 column (1 × 10 cm, Pharmacia, Uppsala, Sweden).

20 (PBS-T) in a microplate washer (easywasher 812 SW 1, SLT-Labinstruments, Grödig, Austria), blocked with 100 μL/well of 2% bovine serum albumin (BSA; Sigma) in PBS-T for 30 minutes, and washed three times, followed by the addition of 100 μL of the antigen-containing probes (plasma samples were diluted between 1:8 and 1:1,024 with PBS-T), or of the standards (plasma pool, diluted 1:8, 1:16, and 1:32; and purified GPIb/IX, from which a standard curve was obtained with eight values between 1.25 μg/mL and 0.01 μg/mL on each plate). After 1 hour, the wells were washed three times in PBS-T, then 100 μL of the second biotinylated antibody (6D1, ~1 μg/mL in PBS-T) was added for 1 hour, followed by washing and the addition of peroxidase-labeled streptavidin (1 μg/mL, Pierce, Rockford, IL) in PBS-T, containing 0.1% BSA, for 1 hour. After washing five times, the substrate, 2,2'-azino-di-[3-ethylbenzthiazoline-sulfonate (6)] diammonium-salt (ABTS; Boehringer, Mannheim, Germany) was added at a concentration of 1 mg/mL, freshly dissolved in 0.05 mmol/L phosphate, 0.1 mol/L acetate, pH 4.2, to which 2.5 mmol/L H₂O₂ was added just before use, and the optical density was read at 405 nm either during the first 10 minutes on a kinetic microplate reader (Vmax; Molecular Devices Corp, Menlo Park, CA) or, as an end point measurement after 30 minutes (Easyreader 340AT; SLT-Labinstruments). Kinetic experiments showed a linear increase in OD over a wide range of concentrations (20 to 500 ng/mL) and for at least 30 minutes. Control experiments gave background values (1) when buffer or GC-depleted plasma was added (full depletion was obtained by immunoaffinity chromatography of plasma over an MoAb Ib-23 column [1 mg MoAb/mL gel]); (2) when MoAbs were included that are directed against other platelet glycoproteins (antibody 6F1 and 10E5, which recognize epitopes on GPIb/IIa and GPIb/IIIa, respectively) or do not react with platelets (MOPC-21, an IgG, Sigma) (the latter antibody was used in combination with 6D1 as a negative control in all patient samples with GC levels greater than 4 μg/mL to exclude false-positives); (3) if the same antibody was used as lower and upper antibody: or (4) if unbiotinylated antibody 6D1 was used. Figure 3 shows that the standard curves of purified GPIb/IX and of dilutions of pooled normal plasma were parallel, thus making interference or cross-reactivity of other plasma proteins with the antibodies used unlikely. A standard curve of a GC preparation ran parallel to GPIb/IX (data not shown). The GC concentration was read from the standard curve obtained with GPIb/IX on each plate. A correction factor of 0.75 was introduced to account for the difference in molecular weights between GC (~135,000) and GPIb/IX (~180,000), and Heparin (1 U/mL) did not interfere with the test.

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The fractions (0.5 mL) were analyzed by Western blotting (using a polyclonal rabbit anti-GPIb/IX antibody and a secondary, gold-labeled goat antirabbit antibody) (Aurion, Wagening, Holland) and by ELISA. The experiment was repeated with another donor.

**Statistical analysis.** We used the Wilcoxon rank-sum number test to analyze the effect of different centrifugation procedures and the impact of prolonged standing in the presence of different anticoagulants. Student’s t test was used to analyze the significance of the differences between two groups (group of normals and specific patient groups), because the standard deviation and the size of the patient groups varied considerably, and the t test is less sensitive to differences in standard deviation. Differences were considered significant at $P < .05$. Values are given as means $\pm$ SD. Correlations were examined by least-squares regression analysis.

**RESULTS AND DISCUSSION**

**Evaluation of Methods**

The aim of the study was to minimize the in vitro platelet activation and the in vitro production of microparticles, the proteolytic degradation of GPIb and GC in vitro, and the elimination of the GPIb bound to platelets and microparticles.

In a series of experiments designed to compare different anticoagulants directly, we have found significantly lower levels of GC in citrated plasma (1.75 $\pm$ 0.65 $\mu$g/mL, n = 8) than in EDTA alone (2.20 $\pm$ 0.62) or in EDTA plus inhibitors (2.06 $\pm$ 0.74, $P < .05$, Wilcoxon rank-sum number test). Although some investigators have used citrate alone as anticoagulant, we decided to use EDTA and inhibitors, because of our analysis of the effect of prolonged standing on normal and patient samples, the incubation of PRP with myeloid leukemic cells, and theoretical considerations on enzymatic cleavage as outlined below. Furthermore, a recent report has demonstrated a direct interference of citrate in this particular assay.

During prolonged storage of whole blood from normal donors, the GC values remained relatively stable over the first 2 to 4 hours (in EDTA, as well as in EDTA + inhibitors). In the presence of inhibitors, GC levels rose quite uniformly over 24 hours to approximately 120% of the initial values and were significantly higher than the time-zero value ($P = .018$, Wilcoxon rank-sum number test). However, with EDTA alone, a small initial decrease over the first 6 hours was followed by an apparent increase and some more variation over time, especially after 24 hours. Two processes appear to be superimposed: (1) an initial decrease, perhaps due to further degradation of circulating GC, which leads to fragments that are no longer recognized by our antibodies used in the assay; and (2) an increase, due to continuing cleavage of GC from platelets.

Under pathological conditions, the differences obtained in the presence of EDTA with or without inhibitors can be significant. The analysis of a patient with chronic myeloid leukemia demonstrated that his GC levels increased after 12 hours of standing as whole blood from 18 $\mu$g/mL to 34 $\mu$g/mL in EDTA alone, but only to 20 $\mu$g/mL in EDTA in the presence of inhibitors. When normal platelet-rich plasma was incubated for 2 hours with HL-60 leukemia cells, the GC levels increased from 2.18 to 2.71 $\mu$g/mL in EDTA and doubled from 1.95 to 3.84 $\mu$g/mL in citrated plasma, but remained at 2.25 $\mu$g/mL in the presence of inhibitors. The addition of sonicated HL-60 cells resulted in even higher values, namely 2.90 $\mu$g/mL in EDTA and 4.35 $\mu$g/mL in citrate, but 2.35 $\mu$g/mL in EDTA plus inhibitors. The lysed cells and the cell culture supernatant itself were negative for GC in the ELISA.

The enzymes that may be involved in cleavage but also in the further degradation of GC include calpain, plasmin, elastase, trypsin, chymotrypsin, and the sulfhydryl proteases. Calpain may also cause shedding of microparticles from the membrane by intracellular effects. Calpain activity, in fact, is increased after platelet activation and mechanical disruption, as has been reported for thrombotic thrombocytopenic purpura patients who may have very high GC plasma levels. Calpain activity can be inhibited by EDTA, NEM, or, indirectly, by PGE$_2$, the latter probably via the inhibition of platelet activation. In fact, inhibition of platelet activation will effectively retard loss of GPIb from stored platelets.

Plasmin or rPA and plasminogen will cleave GC from platelets and activate the platelets at higher concentrations. A shift of intracanalicular GPIb to the surface will help to retain platelet function initially, but lead to the failure of platelets to agglutinate in the presence of ristocetin and to an acquired Bernard-Soulier–like defect in the later phase, as has been shown for stored platelet concentrates. Inhibitors of plasmin (aprotinin) resulted in a remarkable reduction of blood loss during surgery requiring cardiopulmonary bypass, and platelets from these patients seemed to retain their GPIb on the surface.

We therefore expected increased GC levels in the plasma of patients with acute myocardial infarction after treatment with rPA, as has been reported earlier. However, this was not the case in our study (Table 1, Fig 4), and may be explained by the concentration of rPA achieved, the short half-life, the plasminatic inhibitors, and the abundance of other substrates present. Low concentrations of rPA may, therefore, not generate sufficient plasmin activity. We cannot offer an explanation for the discrepancy; however, the relatively small increment of 17% reported appears to be insufficient to explain the transient hemorrhagic diathesis observed in these patients, and the potentially cleaved GPIb molecules will be replaced rapidly from the intracanalicular stores. On the other hand, plasmin may be important in other diseases such as myeloid leukemias, where increased urokinase levels have been reported and, in fact, high GC levels were found (see below; Table 1, Fig 5).

PGE$_2$ was included as a potent inhibitor of platelet activation, since, in addition to EDTA, PGE$_2$ significantly retards the loss of GPIb in stored platelets at concentrations of 300 nmol/L, and NEM will bind to and inhibit the sulfhydryl proteases.

The application of centrifugation forces between 2,000g for 20 minutes (obtained GC levels were set as 100%) and up to 100,000g for 60 minutes (Fig 2) indicated that GC levels could be reduced by an average of 14%. The sequential centrifugation mode was superior to the single-step high-speed centrifugation (Fig 2), because it appears to minimize the number of platelets first and then the platelet micropar-
This population can be completely eliminated by further treatment with rtPA. The box plot analysis gives the means, the 25th to the 75th percentile (box), and the 10th to the 90th percentile (error bars). Clearly no increase of GC after rtPA administration could be observed. However, the GC levels in myocardial infarction patients on admission are significantly higher (2.32 ± 0.4; P = .04) than those of the normal population (2.04 ± 0.48), suggesting increased enzymatic activity in the plasma of these patients.

Fig 4. Serial determination of GC levels in 14 patients with acute myocardial infarction before and 6, 24, and 48 hours after treatment with rtPA. The box plot analysis gives the means, the 25th to the 75th percentile (box), and the 10th to the 90th percentile (error bars). Clearly no increase of GC after rtPA administration could be observed. However, the GC levels in myocardial infarction patients on admission are significantly higher (2.32 ± 0.4; P = .04) than those of the normal population (2.04 ± 0.48), suggesting increased enzymatic activity in the plasma of these patients.

Particles, of which approximately 50% have been shown to carry GPIb on their surface and result in false-positivity for GC. In fact, the single-step high-speed procedure appeared even to increase the number of microparticles, possibly due to the increased shear rate. This is also supported by the recent observation that shear stress induces binding of von Willebrand factor to GPIb, initiates the calcium influx, and induces platelet aggregation, and by our results (Fig 2), which show lower values in samples centrifuged at 2,000g for 20 minutes followed by three times at 12,000g for 5 minutes as opposed to immediate centrifugation at 12,000g three times for 5 minutes each. Our findings are also in accord with earlier studies, which have shown that plasma samples from healthy donors after centrifugation at 12,000g for 10 minutes will still contain a small amount of microparticles (the equivalent of 4 × 10⁶ platelets/L), and this population can be completely eliminated by further centrifugation at 67,000g for 60 minutes. This number of microparticles will increase substantially in blood products of blood banks or in certain diseases. The aging process of blood banks or in certain diseases. The aging process of blood banks or in certain diseases. The aging process of blood banks or in certain diseases. The aging process of blood banks or in certain diseases. The aging process of blood banks or in certain diseases.

Fresh frozen plasma contains an enormous amount of microparticles, namely the equivalent of up to 40,000 platelets/μL. Whole blood clotting and platelet activation in particular, namely by the combination of collagen and thrombin or by the complement component C5b-9, will increase the microparticle formation 10- to 40-fold. This process appears to be calcium-dependent and supports the use of EDTA as a component of the anticoagulant. EDTA, on the other hand, increases the apparent platelet size due to the induction of a spheroid state, which in fact may favor vesiculation. In addition, the low calcium concentrations in plasma containing EDTA will inhibit the adherence of microparticles to platelets, blood cells, and artificial surfaces. Both mechanisms will increase the number of microparticles in plasma and this is supported by studies of Bessos et al., who found an increase in apparent GC levels by a surprising 100% after 1 day, if the platelets were stored in EDTA. However, this increase could be fully reversed by high-speed centrifugation (40,000g for 60 minutes), which indicates that the GC antigen measured was in fact GPIb on microparticles.

Most of the assays for GC reported so far have used standard centrifugation procedures. Some investigators have analyzed the effect of higher centrifugation forces on freshly drawn plasma samples from normal donors. One group described no difference between 1,100g for 15 minutes and 21,000g for 3 minutes (in citrated plasma); another found no difference between 2,000g for 10 minutes and 100,000g for 3 hours and passage through a 0.22-μm filter. On the other hand, Kunishima et al. found a reduction of approximately 6% between 2,000g for 10 minutes and 3,000g for 30 minutes using citrated plasma and inhibitors; some groups did not indicate the centrifugation force used. There are no data available on the effect of different centrifugation forces in patient samples, or of a serial centrifugation mode as proposed in our study.

We conclude that if microparticles can influence the GC levels to this extent in healthy volunteers, it appears even more justified to apply this centrifugation procedure to patient samples. The fact that the GC levels of the normal population obtained with our test were remarkably constant and had among the smallest standard deviations reported in the literature (2.04 ± 0.46 μg/mL) could indirectly support the proposed methodological procedure.

The intraassay variation was between 1.8% and 4.2% (mean of eight replicates on five different plates, 3.3% ± 0.9%) and the interassay variation was 7.1%. The intradonor fluctuations over time remained relatively small: the variation in seven healthy donors analyzed 10 times over 2 weeks was 8.5% ± 3.3%.

The analysis and quantification of GC in the urine by ELISA and Western blot studies indicated that GC is excreted into the urine in very small quantities (~0.5 μg/100 mL or 7.1 μg/24 h) in healthy controls (Fig 6).

Normal Donors

The mean GC value of 116 normal donors was 2.04 ± 0.46 μg/mL. Women had slightly but significant lower plasma levels (1.88 ± 0.38 μg/mL) than men (2.13 ± 0.47 μg/mL; P = .018, t test). The difference cannot be attributed
Fig 5. (A) GC and (B) GC index (GCI): in normal donors (n = 116) and patient groups. (A) Absolute GC values in µg/mL; (B) GCI is GC-normalized for the individual platelet count: [GCI = (GC / (250 X 10^9/mL)) / individual platelet count]. Horizontal lines give the 95% confidence interval of the population of normal controls. Anuric dialysis patients and patients with vascular disease and impaired kidney function had increased levels. The cirrhosis group was the most heterogenous one, with 30% to 40% of the patients having elevated levels. The highest GCI levels were found in the cirrhotics and a positive correlation with the severity of cirrhosis was detected (see text). The group of anurics on dialysis had partly increased levels with similar GC and GCI values. The patient groups with thrombocytopenia illustrate the influence of the platelet count and platelet turnover on GC levels. Only approximately 20% of patients within the group of active ITP had elevated GC levels, defined as higher concentrations than 95% of the normal population, as indicated by the upper horizontal line. However, if normalized for the platelet counts, the GCI provides an excellent discrimination between active disease and ITP in remission. The latter group showed slightly higher GC levels than normals, suggesting persistent platelet destruction in a significant percentage of these patients. Expectedly, the group with aplastic anemia had reduced and the group with thrombocytopenia increased GC levels, which, after normalization for the platelet counts, gave a GCI that was not significantly different from normals in the case of aplastic anemia. However, the GCI in thrombocytopenia was somewhat increased, suggesting that the GC levels in this group were not only increased because of the high platelet counts, but also because of increased platelet turnover in these patients. Patients with myeloid leukemia tended to have higher GC levels than patients with lymphoid leukemia, sometimes up to 40 µg/mL. Almost every leukemia patient had an increased GCI level. Values of up to several hundred could be reached and suggest increased proteolytic damage or platelet destruction. All but four patients were above 95% of the normal population. A large spread of the values may indicate that important subgroups within the leukemias exist.

to the dilutional effects of the anticoagulant, because higher hematocrits in men would result in the opposite. However, since soluble proteins generally circulate in plasma at approximately twice the concentrations compared with levels in whole blood, higher hematocrits in men probably account for the difference. A pool of 100 normals (25 determinations) gave 2.13 ± 0.15 µg/mL. There was no correlation of GC levels of normal donors with age (r = .092) or with the platelet count within the normal limits (r = .079). This is in accord with the larger standard deviation of the GC index (0.66) compared with the standard deviation of the absolute GC values (0.46 µg; if there was a correlation between the platelet count and GC in this group, a narrower standard deviation after normalization for the platelet count would be expected). We have performed amino acid analysis to determine the absolute concentration of our standard and found that it correlated with the standard GPIb/IX and GC measured by the protein assay described by Bradford et al. using γ-globulin as protein standard. However, this assay underestimates the GC concentration by approximately 40%. One reason might be the high glycosylation of GC. The GC values in children were not different from those in adults (P = .53), but a significantly lower index was found. The latter may have been due to difficult venipuncture and longer application of the tourniquet resulting in falsely increased platelet counts. Our normal values are in good agreement with those reported by Steinberg et al. However, other groups have reported substantially lower (0.36 ± 0.07 µg/mL) or higher concentrations (up to 9 µg/mL). Others have also found enormous variability within the normal group (0.4 to 4.9 µg/mL or 2.2 to 9.5 µg/mL). We believe that the differences of the mean values are due
to methodological aspects such as the production and characterization of the standard, whereas differences of standard deviations are more likely caused by differing amounts of microparticles or a variable degree of in vitro proteolysis.

The mean difference of GC levels within the pairs of six identical twins was significantly smaller (0.29 ± 0.31 µg/mL, n = 6) than if the difference was taken from the nonrelated twin population (0.78 ± 0.34 µg/mL, n = 6; P = .013). Together with the relatively small intradonor variation (8.5% ± 3.3%), this constellation suggests that the individual GC levels may be influenced by genetic factors and remain remarkably stable over time. This is also in agreement with studies that have found a similar stability of the expression of GPIb on the platelet surface of Bernard-Soulier patients within the same family.49

The half-life of GC is not known, but a simple calculation may help to analyze the results and to discuss possible kinetistics: the level of approximately 2 µg/mL of GC corresponds to approximately 9 × 1012 molecules/mL (assuming a molecular weight for GC of approximately 135,000). Interestingly, this high number is similar to the number of GPIb molecules that can be expected on the platelet surface in the same volume (~6 × 1012, assuming 25,000 molecules per platelet and 2.5 × 108 platelets/mL). This number must at least be doubled to account for the intraplatelet pool of GPIb14 (and our unpublished observations). If we assume that a platelet loses 50% to 100% of the total GPIb during its lifespan, or an average of 5% to 10% daily, the whole GC pool will have a turnover time of 5 to 10 days under steady-state conditions. This could indicate a half-life of 2.5 to 5 days. If the intraplatelet stores of GPIb are taken into consideration, an even longer half-life appears possible, thus suggesting a major difference compared with other platelet markers.

Patients

Surprisingly, the patients with vascular disease (Table 1, Fig 5) showed a decreased level, provided that their kidney function was in the normal range (1.82 ± 0.42 µg/mL, P = .01). This apparent paradox may be explained by GC binding to immobilized von Willebrand factor,50 which is abundantly present in the subendothelium; arteriosclerotic lesions may increase the GC clearance in vascular disease. However, if the kidney function was impaired, even at an intermediate creatinine level only (none of the patients were dependent on dialysis), GC levels increased to 3.23 ± 0.75 µg/mL (P < .001).

Patients on stable hemodialysis had clearly higher values (3.62 ± 0.75) than normals. In particular, the anurics were approximately 5 SD higher (3.96 ± 0.53) than the normal controls, despite an apparently minimal GC clearance. A reduced metabolism of GC in the kidney is a possible explanation.

The GC index in uremia was almost identical to the GC levels, which may indicate a prolonged half-life rather than an increased cleavage from platelets or platelet destruction. However, the latter possibility cannot be ruled out and, in fact, is supported by the study of Sloand et al,5 who found a decreased platelet-GPIb level in uremic patients on hemodialysis. Patients with liver cirrhosis represented a more heterogeneous population with respect to GC levels. Their absolute values varied considerably and the mean was only insignificantly increased (2.63 ± 1.47 µg/mL, P = .13); however, it was approximately sixfold higher than would be predicted from the (reduced) platelet counts (mean, 105 × 109/L ± 48), thus resulting in a high GC index (6.93 ± 4.21, P < .001) and suggesting increased platelet destruction and/or reduced GC clearance. Subgroup analysis gave no differ-
ences of GC or GC index levels between patients with and without splenomegaly (as determined by ultrasonography), with and without elevated liver enzymes, or with posthepatic or alcohol-induced cirrhosis. However, the GC index increased with the severity of cirrhosis as expressed by the Child-Pugh score\(^2\) (score A, GC = 3.98 ± 1.86; n = 5; score B, GC1 = 6.66 ± 4.5, n = 10; score C, GC1 = 10.4 ± 5.1, n = 3). Subgroup C was significantly different from A (\(P = .038\)).

Ordinas et al\(^6\) have found a reduced GPI level in platelets from cirrhosis patients. Together with our data, this may favor a mechanism of increased proteolytical enzyme activity in plasma resulting in higher GC cleavage from platelets, rather than a reduced GC clearance by the liver. Increased serum enzyme activity has been found in patients with posthepatic cirrhosis\(^2\) and, in particular, evidence of increased plasmin activity.\(^5\)

The close correlation between the platelet count and the GC levels in the group of patients with aplastic anemia (Fig 7) in this assay, and the fact that the linear regression went almost through zero, supports the concept of the platelets as major source of GC (\(r = .95\)). The correlation between the platelet count and the GC levels could be extended by the inclusion of the group of normals (\(r = .80\)) and the patients with essential thrombocytosis (\(r = .82\)) in which the GC levels could reach up to 26 \(\mu\)g/mL. The spread made it obvious that the latter group was more heterogenous. The GC index was significantly increased (3.52 ± 1.88, \(P < .001\)), which may indicate increased platelet destruction. The patients with aplastic anemia, on the other hand, did not have significantly different GC indexes from normals (2.5 ± 1.1, \(P = .069\), Fig 5B), with a tendency toward increased levels.

The mean GC level in patients with active immune thrombocytopenic purpura (ITP) was increased and was even above the values observed in the normal population (2.44 ± 1.27, \(P = .003\)), but at a mean platelet count five times lower than normal; many patients had even less than 10 \(\times\) 10\(^9\) platelets/L. Accordingly, the GC index increased to very high levels of up to more than 400 (mean, 60.3 ± 93, \(P < .001\)). It was remarkable that only two of 61 GC indexes from patients with ITP overlapped with the levels of ITP patients in remission. This excellent discrimination appears to be useful in clinical practice, and the GC indexes could be an aid in the classification of disease activity in thrombocytopenic disorders and will indicate increased destruction, as has been reported earlier.\(^7\) One patient with active ITP with an autoantibody against GPIb (platelet count, 21 \(\times\) 10\(^9\)/L) had an unusually low GC level (0.04 \(\mu\)g/mL). The value was even lower than in the patients with aplastic anemia, who had the lowest platelet counts. It is therefore our suspicion that the autoantibody competed with one of the MAb's used in our assay. However, we cannot exclude a structural abnormality of this patient's GPIb at this point. The group of ITP patients in remission did not completely normalize their GC levels (2.69 ± 0.54 \(\mu\)g/mL, \(P < .001\)). The fact that their index was somewhat above the normal population as well (2.64 ± 0.55 \(\mu\)g/mL) at a similar platelet count (246 \(\uparrow\) 257 \(\times\) 10\(^9\)/L) may indicate that some patients with ITP thought to be in remission on the basis of their platelet counts had a compensated degree of immune-mediated platelet destruction or a subclinical relapse.

Serial determinations of GC indexes from patients with ITP in remission will show whether an increasing index predicts an early relapse (at a compensated degree of platelet destruction) in these patients. An alternative explanation could be the absence of inhibitors other than EDTA in the samples of these patients. We considered this possibility as less likely, because our control experiments did not show a difference compared with normals, the patient samples were immediately centrifuged, and calpain, which might have been liberated under conditions of platelet destruction, was effectively inhibited by EDTA.\(^7\)

We chose patients with leukemia as another group with potentially elevated GC levels for several reasons. Cell destruction will result in the liberation of enzymes such as leukocyte elastase from the myeloid cell lines, which has been shown to easily cleave GC from platelets.\(^4\) Activation of coagulation and fibrinolysis with clinical bleeding has been reported in myeloid (especially in promyelocytic) forms, as well as in lymphocytic leukemia.\(^3\) We hypothesized that elevated GC levels and reduced platelet GPIb levels could contribute to the bleeding problems in these patients.

Not unexpectedly, we found high, sometimes even extremely elevated, mean GC levels in all myeloid and lymphocytic forms compared with what would be expected from the platelet counts. This is reflected, in some cases dramatically (up to 700), by increased GC indexes. Although the patient groups were very heterogenous with respect to the type of leukemia and platelet and white blood cell counts (Table 1), it appeared that the myeloid leukemias tended to have higher values than lymphocytic leukemias. In fact, the highest levels, which were approximately 40 \(\mu\)g/mL, were found in patients with myeloid leukemia. There were two patients within the group of AMLs with megakaryocytic leukemia (M7) with extremely high GC values (9.1 and 40.0 \(\mu\)g/mL). No subgroup (within stages M1 to M6) could be found that was significantly different from the others. However, clearly, the highest GC index levels were found in stage M2 (57.8, 49.2, 47.9), except for one patient with megakaryocytic leukemia (M7; GC index, 714). A contribution of GC, respective of the lost GPIb molecules from platelets to the well-recognized coagulation disorder in these patients, appears possible.

CMLs often present with thrombocytosis, which may result, as in patients with essential thrombocytosis, in massively elevated GC levels (10.4 \(\mu\)g/mL ± 7.7). However, in contrast to the latter group, CML patients also had higher GC indexes (14.5 ± 12.9) than expected from their platelet counts (Table 1).

The functional consequences of very high levels of circulating GC on hemostasis are not well established. We have found two patients with GC levels of greater than 40 \(\mu\)g/mL in the group of myeloid leukemias and several others were in the range of 10 to 20 \(\mu\)g. It is interesting that both patients with a GC level of 40 \(\mu\)g/mL had mucocutaneous bleeding as the initial symptom. It is not known at which concentration GC can impair normal coagulation in vivo and what the respective contributions of GC in plasma and of the
GPIb loss from the platelet surface are. Earlier studies have demonstrated that GC will interfere with the interactions of thrombin with fibrinogen in a fibrinogen-clotting assay at concentrations of 0.1 to 0.5 pmol/L, corresponding to approximately 13 to 65 µg/mL. This is in accord with recent findings of Jandrot-Perrus et al., who showed blockade of fibrinogen-clotting activity of thrombin and thrombin-binding to fibrin at an inhibitory constant for half maximal inhibition for GC of 0.1 µmol/L (≈13 µg/mL). Binding of thrombin to washed platelets was reduced at 60 to 180 nmol/L (8 to 24 µg/mL). A recombinant fragment of GC (rGPIb-221-318), which contains the von Willebrand factor binding site of GPIb, fully inhibited the binding of von Willebrand factor to paraformaldehyde-fixed washed platelets at 1.5 µmol/L in the presence of ristocetin, which would correspond to approximately 200 µg/mL of intact GC on a molar basis. Ristocetin-induced agglutination of fixed and washed platelets could be partially inhibited at concentrations of 400 to 850 nmol/L (≈55 to 115 µg/mL). These reports show that an inhibitory effect can be observed between 10 and 200 µg/mL in a single system. We have shown that this range of concentrations can be found in vivo. Since GC is able to interfere with at least three important mechanisms of coagulation, namely (1) thrombin-fibrinogen interactions, (2) thrombin-platelet interactions, and (3) von Willebrand factor–platelet interactions, we conclude that even lower concentrations could be sufficient for functional inhibition and clinical bleeding. A lower than normal number of GPIb molecules on the platelet surface (due to GC cleavage) and a low platelet count may further reduce the threshold for a coagulation disorder. To give an idea of the latter aspect the GC index may be useful.

In summary, we have developed and standardized a reliable assay for GC which may be diagnostically useful for blood banks and in several diseases, particularly in ITP.

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Glycocalcin: a new assay--the normal plasma levels and its potential usefulness in selected diseases

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