New Enzyme-Linked Immunosorbent Assay for Glycocalcin in Plasma
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A new sandwich-type enzyme-linked immunosorbent assay for quantifying glyocalcin, a proteolytic fragment of platelet membrane glycoprotein Ib, is described. The assay is based on the use of two monoclonal antibodies raised against glycoprotein Ib and involves the avidin–biotin technique. The detection limit is 7 μg/L and the range of glyocalcin determined in plasma is 0.01 to 1 mg/L. Assay time is 2 h. The intra-assay CV ranged from 3.6% to 5.2%, the interassay CV from 5.4% to 8.0%. Analytical recovery of purified glyocalcin added to a plasma pool averaged 96%. In 36 healthy subjects, the mean glyocalcin concentration in plasma was 0.36 (SD 0.07) mg/L (2.7 nmol/L). We conclude that this assay is suitable for measuring glyocalcin in plasma and is also more sensitive and precise than the previously published immunoassays based on competitive binding assay.

Additional Keyphrases: platelet membrane glycoprotein Ib • biotin–avidin interaction • reference value • idiopathic thrombocytopenic purpura

Platelet membrane glycoprotein Ib plays an important role in primary hemostasis by serving as the receptor for von Willebrand’s factor bound to the subendothelium of damaged blood vessels (1–3). It also binds thrombin (4) but the physiological function is not clear. Glycoprotein Ib is the third most prominent glycoprotein in platelet membranes (21 000–26 000 copies per platelet) (2, 5). This molecule has an apparent molecular mass of 170 000 Da on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (under nonreducing conditions). It consists of two disulfide-linked subunits, α-subunit (Mr 143 000) and β-subunit (Mr 23 000) (6). Glyocalcin is a carbohydrate-rich, hydrophilic fragment (Mr 135 000) that is readily cleaved from the external portion of the α-subunit of glycoprotein Ib by a calcium- and thiol-dependent protease (6, 7). Glyocalcin circulates in normal plasma and increases in concentration during conditions associated with increased platelet activation and destruction in vivo (8, 9).

An immunoradiometric assay (competitive binding assay) for glyocalcin in plasma was described by Coller et al. (8, 9). The inherent disadvantages of assays involving radioisotopes, however, include the following: a relatively short half-life of label, semiquantitative nature of the assay, requirement for expensive special instruments, and difficulty in automating the procedure. These problems may be overcome by using enzyme immunoassays. An enzyme-linked immunosorbent assay (ELISA) described previously for glyocalcin (10) was based on immunoinhibition; however, it was used only for experimental studies and has not been validated clinically.3

We describe here a noncompetitive, sandwich-type ELISA for quantifying glyocalcin in plasma by using two different monoclonal antibodies and the avidin–biotin technique (11, 12).

Materials and Methods
Reagents
Diatupe-H anticoagulant was obtained from Diagnostica Stago Co., Asnieres, France. Protein A–Sepharose CL-4B was from Pharmacia Inc., Uppsala, Sweden. Vectastain Elite ABC Kit (containing avidin DH and biotinylated horseradish peroxidase H) was from Vector Labs. Inc., Burlingame, CA. N-Hydroxysuccinimidobiotin was from Pierce Chemical Co., Rockford, IL. Bio-Rad Protein Assay Kit, with bovine serum albumin (BSA) as a standard (Protein Standard II), was from Bio-Rad Labs., Richmond, CA. BSA (Cohn Fraction V powder, 96–99% pure) was from Sigma Chemical Co., St. Louis, MO. Other reagents were of analytical grade.

Plasma Samples
Blood was drawn from the antecubital vein through a 19-gauge needle into a plastic syringe. We took samples from 36 healthy volunteers (30 men, six women, ages 22–42 years) and from five patients, two with aplastic anemia (a 31-year-old man and a 37-year-old woman), and three with idiopathic thrombocytopenic purpura (two males, one female, ages 6–40 years). The diagnosis of aplastic anemia was based on internationally recognized criteria (13) and that of idiopathic thrombocytopenic purpura on conventional criteria (14). Specimens were anticoagulated with one volume of Diatupe-H (per liter, 0.11 mol of citric acid, 15 mmol of theophylline, 3.7 mmol of adenosine, and 0.198 mmol of dipyriramole, pH 5.0) to nine volumes of whole blood (15). Samples were immediately placed on ice and centrifuged at 3000 × g for 30 min at 4 °C within 1 h. In some experiments blood was centrifuged at greater centrifugal force. Plasma samples were either tested immediately or stored at −70 °C.

Procedures
Monoclonal antibodies. The two murine monoclonal antibodies used in the assay, HPL-7 (IgG1) and HPL-16

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3 Received August 6, 1990; accepted November 15, 1990.

3 Nonstandard abbreviations: ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
(IgG_{2a}), which are specifically reactive with human platelet membrane glycoprotein Ib, have been previously described in detail (16–18). HPL-7 does not compete with HPL-16 for binding with glycoprotein Ib, as shown by an antibody-binding inhibition assay. The IgG fraction of HPL-7 and HPL-16 was purified from ascitic fluid by Protein A-Sepharose CL-4B affinity chromatography (19).

**Glycocalcin.** Purified glycocalcin was generously provided by Dr. Makoto Handa, Keio University, Tokyo, Japan. The glycocalcin was purified by a two-step procedure involving immunofinity chromatography with the anti-glycoprotein Ib murine monoclonal antibody coupled to agarose and then with wheat germ agglutinin coupled to agarose (20). Sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis of purified glycocalcin showed a single band with an apparent molecular mass of 140 000 Da (data not shown). The protein concentration was determined with the Bio-Rad Protein Assay Kit according to Bradford (21). The concentration of glycocalcin was calculated from its protein concentration and the fact that glycocalcin contains 60% carbohydrate by weight (6).

**Biotin labeling of monoclonal antibody.** HPL-7 antibody was biotinylated as previously described (11, 12). We mixed 1 mL of a 1 g/L solution of HPL-7 antibody in 0.1 mol/L NaHCO_{3} with 120 μL of a 1 g/L solution of N-hydroxysuccinimidobiotin in dimethylsulfoxide. The reaction mixture was incubated at room temperature for 4 h and then dialyzed overnight at 4 °C against phosphate-buffered saline (PBS; per liter, 8 mmol of Na_{2}HPO_{4}, 2 mmol of KH_{2}PO_{4}, 3 mmol of KCl, and 0.14 mol of NaCl, pH 7.4). The labeled antibody was kept at −20 °C until used.

**Coating of solid phase.** Flat-bottomed 96-well microtiter plates (Nunc Immuno Plate; Nunc AS, Kamstrup, Denmark) were coated with 0.5 μg of HPL-16 antibody in 100 μL of coating buffer (50 mmol/L carbonate–bicarbonate buffer, pH 9.6) per well and incubated overnight at 4 °C. We then emptied the plates and removed unbound antibodies by washing with wash solution (Tween 20, 100 μL/L, in PBS). The washing procedure was performed with an automated washer (ELISA washer; International Reagents Co., Kobe, Japan). The remaining binding sites on the plates were blocked by incubation with 200 μL of BSA solution (20 g/L in PBS) overnight at 4 °C. After again washing the plates with the wash solution, we stored them at −20 °C. The plates could be stored for as long as three months without measurable loss of activity.

**ELISA of glycocalcin.** The procedure was as follows: pipette 100 μL of the standard or plasma sample (diluted twofold with PBS) into the plate wells. Cover the plate with plastic film and mix the solutions in the wells gently, then incubate for 45 min at 37 °C in an incubator. Wash the plate thoroughly with the wash solution and tap to remove excess moisture. Next add 100 μL of the biotin-labeled HPL-7 antibody (1 mg/L in diluent buffer: 5 g of BSA per liter of PBS) and incubate for 45 min at 37 °C. Again wash the plate, add to each well 100 μL of the avidin–horseradish peroxidase conjugate (diluted 1000-fold in diluent buffer), and re-incubate for 15 min at 37 °C. Wash the plate, and add to each well 100 μL of the buffered enzyme substrate solution: 0.4 g of p-phenylenediamine and 0.4 mL of H_{2}O_{2} (300 mL/L solution) per liter of citrate/phosphate buffer (0.1 mol of citric acid and 0.2 mol of Na_{2}HPO_{4} per liter, pH 5.0). Incubate this in the dark at room temperature for 10 min, then stop the reaction by adding 50 μL of 2 mol/L H_{2}SO_{4} reagent to each well. Mix the content of the wells and measure the absorbance at 490 nm with a microtiter plate reader (we used a Vmax kinetic microplate reader; Molecular Devices Co., Menlo Park, CA).

**Results**

**Preparation of plasma samples.** To determine the centrifugal force required to obtain platelet-free or platelet microparticle-free plasma, we measured glycocalcin concentration in plasma obtained by centrifugation of blood at various conditions (all performed at 4 °C). Although plasma obtained by centrifugation at 2000 × g for 10 min contained slightly more glycocalcin (0.35 mg/L) than that centrifuged at greater speeds, plasma obtained at 3000 × g for 30 min contained the same amount as samples treated with greater centrifugal force (10 000 × g for 30 min or 100 000 × g for 1 h): 0.33, 0.32, and 0.33 mg/L, respectively.

**Standard curve.** Figure 1 illustrates a typical standard curve obtained with ELISA for glycocalcin. The limit of detection, calculated from 16 consecutive assays, was 7 μg/L (3 SD from the zero standard), as generated by a nonlinear polynomial regression method (22). The range of determinations was 0.01 to 1 mg/L.

**Intra- and interassay precision.** The intra-assay CV, determined by analyzing three plasma samples having glycocalcin concentrations ranging from 0.15 to 0.90
mg/L, in 10 replicates in the same run, ranged from 3.6% to 5.2% (Table 1). The interassay CV ranged from 5.4% to 8.0%, estimated by determining glycocalicin in three plasma samples on 10 different occasions.

Analytical recovery. To determine the analytical recovery of the assay, we assayed a plasma pool to which had been added concentrations of glycocalicin ranging from 0 to 1.0 mg/L. Recovery ranged from 88% to 100% (mean 96%) (Table 2).

Clinical applications. We measured the concentration of glycocalicin in plasma from healthy volunteers. In these subjects the mean value for glycocalicin was 0.36 (SD 0.07) mg/L, with a median value of 0.36 mg/L and a range of 0.25 to 0.54 mg/L (Table 3). We also assayed plasma samples from patients with aplastic anemia (n = 2) and those with idiopathic thrombocytopenic purpura (n = 3). Plasma from the patients with aplastic anemia contained 0.02 and 0.01 mg/L of glycocalicin, whereas that from the patients with idiopathic thrombocytopenic purpura had a mean glycocalicin concentration of 0.34 mg/L (Table 3). Despite the low platelet count in the peripheral blood of both groups, the patients with aplastic anemia had the lower glycocalicin concentration. The normal concentration of glycocalicin in the patients with idiopathic thrombocytopenic purpura indicates that this concentration does not simply reflect the peripheral platelet count, a finding that agrees with that of Steinberg et al. (9).

Discussion

This is the first report on a noncompetitive ELISA for glycocalicin, a proteolytic fragment of platelet membrane glycoprotein Ib, in human plasma. The murine monoclonal antibodies, HPL-7 and HPL-16, used in the present ELISA recognize two different sites on glycoprotein Ib of the human platelet and show no cross-reactivity (16–18). We used HPL-16 as the capture antibody and HPL-7 as the labeled antibody, antibodies having the highest titration for glycoprotein Ib of the 12 clones of monoclonal anti-glycoprotein Ib antibodies investigated (16–18). Thus we were able to construct a non-competitive sandwich-type ELISA of high specificity and sensitivity.

Avidin has an extraordinarily high affinity for biotin (affinity constant = 10^{-18} mol/L), and each avidin mol-

Table 1. Intra- and Interassay Precision of the ELISA

<table>
<thead>
<tr>
<th>Glycocalicin, mg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.90</td>
<td>0.047</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td>0.37</td>
<td>0.015</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.005</td>
<td>3.6</td>
</tr>
<tr>
<td>Interassay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.88</td>
<td>0.048</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>0.42</td>
<td>0.032</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>0.16</td>
<td>0.013</td>
<td>8.0</td>
</tr>
</tbody>
</table>

n = 10 each.

cule can bind four biotin molecules (23). Biotin can be covalently coupled to antibody to a high specific activity without affecting the antigen-binding capacity of the antibody (11). Thus, the avidin–biotin technique allows for heightened assay sensitivity. The use of this system in the present assay may contribute to both the high sensitivity (7 μg/L) and the relatively short performance time (2 h) of this quantitative method.

The detection limit of the present ELISA (7 μg/L) was much lower (6.3% of the normal value) than that of previously published RIA for glycocalicin (8, 9). The determination range (0.01–1 mg/L) was also much wider than that of the RIA (6.3% to 100% of normal values). The intra- and interassay CVs (mean 4.3% and 7.0%, respectively) and analytical recovery (mean 96%) indicate that the present ELISA is precise and accurate.

Microparticles derived from platelet membranes are present even in normal human plasma (24, 25) and might interfere with measurements of glycocalicin. Thus, it is very important to exclude platelet microparticles from plasma during sample preparation. We found plasma obtained by centrifugation of blood at 3000 × g for 30 min at 4 °C contained the same amount of glycocalicin as observed in plasma obtained at greater force. Although this centrifugal force to prepare plasma for glycocalicin might be insufficient to sediment all residual microparticles, there was no decrease in the concentration of glycocalicin even after ultracentrifugation (100 000 × g for 60 min). A previous study by Coller et al. (8) also showed that their standard centrifugation procedure (2000 × g for 10 min) was enough to remove both platelets and platelet fragments. We thus conclude that centrifugation at 3000 × g for 30 min is sufficient to obtain plasma for glycocalicin determination by the present ELISA.

In previous assays, glycocalicin was measured by RIA

Table 2. Analytical Recovery of Glycocalicin by the ELISA

<table>
<thead>
<tr>
<th>Glycocalicin, mg/L</th>
<th>Added*</th>
<th>Recovered</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.94</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.10</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.04</td>
<td>88</td>
<td></td>
</tr>
</tbody>
</table>

* Added to a plasma pool.

Table 3. Glycocalicin Concentrations in Normal Subjects and Patients with Thrombocytopenia, as Measured by the ELISA

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Glycocalicin, mg/L</th>
<th>Platelet count, × 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>36</td>
<td>0.36 (0.07)</td>
<td>225 (54)</td>
</tr>
<tr>
<td>AA</td>
<td>2</td>
<td>0.02, 0.01</td>
<td>6, 5</td>
</tr>
<tr>
<td>ITP</td>
<td>3</td>
<td>0.34 (0.07)</td>
<td>33 (9)</td>
</tr>
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</table>

AA, aplastic anemia; ITP, idiopathic thrombocytopenic purpura.

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or enzyme immunoassay (8–10), both based on the competitive inhibitory action of glycocalcin in plasma against binding of anti-glycoprotein Ib antibody to the glycoprotein Ib molecule on platelets. Adelman et al. (10) used an enzyme immunoassay to determine the concentration of glycoprotein-related antigen in supernates of plasm-in-treated washed platelets in vitro, but the method was not validated for clinical applications. The RIA study described by Coller et al. was the first to measure glycocalcin in plasma. In contrast to enzyme immunoassay, however, RIA requires special facilities for handling or disposing of radioactive isotopes. Thus, we developed the present ELISA.

Glycocalcin reportedly is susceptible to degradation by proteases such as plasmin or trypsin (20). In comparison with the study done by Coller et al. (8, 9) or with another study involving competitive binding assay (10), our estimate of glycocalcin in plasma was lower. We partially attribute this difference to the method we used. In our ELISA study, the method was based on the direct reaction between two different kinds of monoclonal antibodies and the antigen to be detected in the plasma. Other assays based on competitive binding could not possibly eliminate the contaminating degraded fragments of glycocalcin in plasma. In the present ELISA, however, glycocalcin might be detectable more precisely as an intact form by the two monoclonal antibodies. This also may be one of the reasons that our estimated glycocalcin value was lower than the results reported by Coller et al., who used only one monoclonal antibody.

The glycocalcin concentration in plasma is reportedly useful in classifying the mechanism of thrombocytopenia (9). We confirmed that finding with the present ELISA: patients with aplastic anemia, with underproduction of platelets, had a decreased concentration of plasma glycocalcin, whereas patients with idiopathic thrombocytopenic purpura, with an increase in the rate of platelet destruction, had a normal concentration of glycocalcin in plasma. Glycocalcin has not been studied systematically in various patient populations, but the present ELISA should make it easy to analyze in a large number of plasma samples (80 samples per plate), if it can be standardized. Furthermore, additional extensive quantitative studies of glycocalcin should help to clarify the metabolism of glycocalcin released from platelet membranes, not only in normal conditions but also in various diseases.

We thank Dr. Makoto Handa of the Blood Center, Keio University School of Medicine, Tokyo, for his generous gift of purified glycocalcin.

References