Heterogeneity of Membrane Surface Proteins in Glanzmann’s Thrombasthenia

By Joseph R. Holahan and Gilbert C. White II

Studies in several laboratories have suggested that platelets from patients with Glanzmann’s thrombasthenia are deficient in two major membrane glycoproteins and that this membrane defect is uniform from patient to patient. We have used an improved electrophoretic technique to study further the surface composition of normal and thrombasthenic platelets. Platelets from three unrelated thrombasthenic patients were labeled by either lactoperoxidase-catalyzed iodination or the neuraminidase-galactose oxidase-[3H]NaBH4 technique and the labeled proteins were separated by two dimensional isoelectric focusing-SDS polyacrylamide gel electrophoresis. With both techniques, the major radiolabeled proteins were clearly separated from each other and were present as a horizontal collection of discrete spots that suggest charge heterogeneity. Most of the labeled proteins had an acidic isoelectric point. Compared to normal platelets, platelets from patients with Glanzmann’s disease contained no electrophoretically identifiable fibrinogen. In two patients with thrombasthenia, there was total absence of surface glycoproteins GPIIb and GPIIIa, while a third patient with thrombasthenia, who was clinically indistinguishable from the previous two patients, had decreased, but detectable, amounts of GPIIb and GPIIIa. These studies suggest that there are at least two phenotypic patterns of membrane abnormalities in Glanzmann’s thrombasthenia involving GPIIb and GPIIIa and may indicate genetic heterogeneity in this disease.

Materials

Neuraminidase (Vibrio cholerae) was purchased from Schwarz/Mann and was devoid of proteolytic activity. Galactose oxidase from United States Biochemical Corporation was further purified and assayed by the method of Hatton and Regoeczi. Tritiated sodium borohydride, 5–15 Ci/mmol was obtained from Research Products International and was dissolved in 0.001 M NaOH. Sodium dodecyl sulfate (SDS, 99.0% pure), 2-mercaptoethanol and glycerol were prepared by British Drug House and purchased from Seakem and pure human fibrinogen was a gift of Dr. J. McDonagh.

Platelet Samples

Normal platelets were obtained from healthy male or female volunteer donors with no evidence of liver or hematological disease. Thrombasthenic platelets were obtained from three unrelated patients with Glanzmann’s disease [(O.G., NCMH No. 39-39-00, J.W., NCMH No. 41-11-43, and S.H., NCMH No. 42-52-15)] All three patients had a life-long bleeding disorder characterized by prominent mucous membrane bleeding and bleeding secondary to surgery or trauma. In each, the diagnosis of Glanzmann’s disease...
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was established by a prolonged bleeding time, the failure of platelets to aggregate with adenosine diphosphate, epinephrine, collagen, and thrombin, and an abnormal clot retraction.

Whole blood was collected by the two syringe technique into plastic syringes containing acid-citrate-dextrose, and was centrifuged at 100 g for 20 min at room temperature. The platelet rich plasma was layered onto a plastic column of sepharose 2B-CL equilibrated with either iodination buffer or 125I for subsequent labeling or divalent cation deficient Tyrode's solution, pH 7.4, containing 0.001 M ethylenediaminetetraacetic acid (EDTA) for labeling with tritium.

Labeling of Platelets

Proteins on the surface of gel filtered platelets were labeled by either of two techniques.

Galactose oxidase technique. Gel filtered platelets (20 ml containing 2–4 × 1010 platelets per ml) were brought to 37°C. To the platelets was added 0.25 ml of a mixture containing 30 units of Vibrio cholerae neuraminidase, 20 U of galactose oxidase, 0.162 M CaCl2, and 0.04 M MgCl2. After 15 min, the platelets were diluted to 60 ml with Tyrode's solution, centrifuged at 500 g for 10 min, and resuspended in 1 ml of divalent cation deficient Tyrode's solution. The oxidized galactose and glucosamine residues were then reduced by incubation with one mCi (0.2 μmole) [3H]NaBH4 for 10 min at 37°C. The platelets were then washed three times with divalent cation deficient Tyrode's solution and resuspended in a final volume of 1 ml in preparation for electrophoresis.

Lactoperoxidase iodination technique. Gel filtered platelets (20 ml containing 2–4 × 1010 platelets per ml) were incubated with 1 mCi of [125I] and 50 μl of 0.0002 M lactoperoxidase at room temperature. The iodination reaction was initiated and maintained by the addition of 20 μl of freshly prepared 0.03% hydrogen peroxide at 30–sec intervals for 120 sec. The platelets were washed twice in iodination buffer and resuspended in a final volume of 1.0 ml of iodination buffer in preparation for electrophoresis.

Platelet Electrophoresis

High resolution two-dimensional electrophoresis by isoelectric focusing in one dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension (2D-IEF/SDS PAGE) was performed as described by O'Farrell. Isoelectric focusing gels were prepared in 120 x 2.5 (ID) mm glass tubing and contained 5% (w/v) urea, 3.8% (w/v) acrylamide, 0.2% (w/v) bis, 2% (v/v) NP-40, 4% (v/v) ampholines (pH 5–7), and 1% (v/v) ampholines (pH 3.5–10). The second dimension gel was a discontinuous slab gel measuring 150 x 150 x 0.7 mm. The running gel was 7.5% T and 2.6% C while the stacking gel was 3.5% T and 2.6% C*

Samples were prepared for electrophoresis within 4 hr of venipuncture. Labeled platelets (100 μl containing 109 platelets per ml) were added to 100 mg urea. After the urea went into solution, 100 μl of lysis buffer containing 5% 2-mercaptoethanol was added and a portion of the sample applied to the isoelectric focusing gel. Electrophoresis in the IEF dimension was performed overnight for 5400 V-hr and then for 1 hr at 800 V. The gels were extruded, equilibrated with reducing sample buffer for 1 hr and then applied to the second dimension SDS-PAGE gel with 1% agarose. Electrophoresis in the second dimension slab gel was carried out at 20 mA.

One dimensional SDS-PAGE was performed according to the technique of Laemmli. Two dimensional unreduced-reduced SDS-PAGE was performed according to the technique of Phillips and Agin.

Gels were stained with 0.1% Coomassie Brilliant Blue in 50% TCA and destained with 7% acetic acid. Gels with [3H] labeled proteins were equilibrated with PPO, dried and exposed to x-ray film at ~70°C. Gels with [125I] labeled proteins were dried and exposed to x-ray film at ~70°C.

The pH gradient in IEF gels were determined by cutting the IEF gels into 5 mm lengths that were placed in tubes containing 0.5 ml degassed deionized water, capped and agitated gently for 30 min. The pH of the solution was measured with a pH meter.

Miscellaneous

With the ampholyte mixture and methods employed, a stable and reproducible pH gradient was formed between pH 5.0 and pH 7.0. The major radio-labeled proteins all had isoelectric points in this pH range and were clearly delineated. When the IEF gels were stained for protein, actin was identified as the most prominent spot and had a pI of 5.7. Myosin was present on the basic end of the gel with a pI of approximately 6.8.

Normal Platelet Proteins

Two dimensional electrophoresis of NP-40 solubilized proteins from intact, normal platelets resulted in the formation of a reproducible pattern of protein spots (Fig. 1A). When a portion of the solubilized platelets containing 150–200 μg of protein was electrophoresed and stained for protein, more than 75 spots were identified ranging in molecular weight from 30,000 to greater than 250,000 daltons. The major protein in Coomassie Blue-stained gels was actin that appeared as two closely migrating spots consisting of predominantly beta-actin (estimated pI 5.7) with smaller amounts of the more basic gamma-actin (estimated pI 5.8). Two high molecular weight, acidic proteins in Coomassie-stained gels were identified as GPIIb and GPIII* by their molecular weights and radioactivity. The gamma chain of fibrinogen was identified by electrophoresis of purified fibrinogen.

*The nomenclature used in this paper is based on that recommended by Phillips and Agin. The glycoproteins are glycoprotein Ia (GPIa), glycoprotein Ib (GPIb), glycoprotein Ic (GPIc), glycoprotein IIa (GPIIa), glycoprotein IIb (GPIIb), glycoprotein III (GPIII), glycoprotein IV (GPIV), and glycoprotein V (GPV). In the nomenclature of Clementson et al., GPIII is GPIIIa and GPIV is GPIIIb. Mosher and coworkers have described an alternate system of nomenclature based on Mr by polyacrylamide gel electrophoresis. Thus, GPIIb is termed GPI40 or SP132.
Fig. 1. Two dimensional separation of platelet proteins. The second dimension slab gel was stained for protein with Coomassie Brilliant Blue. The pattern of radiolabeled surface proteins determined by radioautography was superimposed on the photograph of the second dimension gel by tracing the radioautogram on the photograph of the Coomassie Blue stained gel. Spots drawn with solid lines signify radiolabeled proteins that were heavily exposed on the radioautogram while the broken lines signify proteins that were lightly exposed. (A) Normal platelets labeled with lactoperoxidase and $^{[35]}S$. The sample was 40 $\mu$l of platelets containing 200 $\mu$g of protein and 200,000 cpm. The gel was exposed to x-ray film for 14 days. (B) Thrombasthenic platelets (J.W.) labeled with lactoperoxidase and $^{[35]}S$. The sample was 40 $\mu$l of platelets containing 200 $\mu$g of protein and 75,000 cpm. The gel was exposed to x-ray film for 14 days.
Fig. 2. Two dimensional separation of platelet proteins. (A) Normal platelets labeled with neuraminidase-galactose oxidase-[\(^{3}H\)]borohydride. Approximately 40 \(\mu\)l of platelets containing 200 \(\mu\)g of proteins were applied to the gel. (B) Thrombasthenic platelets (O.G.) labeled with neuraminidase-galactose oxidase-[\(^{3}H\)]borohydride. The sample was 40 \(\mu\)l of platelets containing 150 \(\mu\)g of protein. The gel was subjected to fluorography for 7 days.
Also shown in Fig. 1A is the radioautographic pattern obtained when intact platelets labeled with lactoperoxidase and $[^{125}I]$ were electrophoresed by 2D-IEF/SDS PAGE. The major radiolabeled proteins were located on the acidic side of the gel, an observation consistent with the high sialic acid content of the proteins on the platelet surface. The most acidic protein of the radioautogram corresponded to the protein that stained most heavily with periodic acid Schiff reagent on the one dimensional Laemmli gel and was therefore identified as GPIb. The other labeled proteins were identified by their estimated molecular weights, relative radioactivity, and by comparison to one dimensional and two-dimensional unreduced-reduced SDS-PAGE gels (not shown). GPIV was identified by its resistance to trypsin digestion$^{19}$ (not shown).

When intact normal platelets were labeled with neuraminidase-galactose oxidase-$[^{3}H]$borohydride and electrophoresed as described above, the protein pattern that was observed was similar to that seen with iodinated platelets, but the pattern of radiolabeled, and therefore surface-exposed, proteins was different (Fig. 2A). Several of the radiolabeled proteins were present as discrete spots in the horizontal direction indicating charge heterogeneity. The most striking difference was a shift of GPIV toward the basic side of the gel in the neuraminidase-galactose oxidase treated platelets. Glycoprotein IIb was also changed to a slightly more basic position in the gel by treatment with neuraminidase and galactose oxidase (pI 5.5 to pH 5.6).

**Platelet Proteins of Thrombasthenic Platelets**

Platelets from three patients with Glanzmann's disease were studied by two dimensional electrophoresis. In all three patients, there was a complete absence of platelet fibrinogen in gels stained for protein (Fig. 1B, 2B, and 3).

Figure 1B shows the pattern of radiolabeled proteins that was obtained when iodinated platelets from a patient (J.W.) with Glanzmann's disease were analyzed by two-dimensional electrophoresis. The thrombasthenic platelets were deficient in two membrane glycoproteins, GPIIb and GPIII. Even in gels exposed for up to five times as long as gels containing equivalent amounts of normal platelet proteins, there was no detectable radioactivity in the area normally occupied by these two proteins. GPIIb and GPIII were also absent in Coomassie-blue stained gels. The remainder of the radiolabeled proteins from the thrombasthenic platelets were present in normal amounts and positions in the gel and demonstrated charge heterogeneity that was similar to that observed with normal platelets. No new radiolabeled spots were observed in the gel.

Fluorograms of thrombasthenic platelets (patient O.G.) labeled with neuraminidase-galactose oxidase-$[^{3}H]$borohydride also showed that GPIIb and GPIII were absent (Fig. 2B). Again, GPIIb and GPIII were not detected in Coomassie-blue stained gels. In addition, thrombasthenic platelets labeled with neuraminidase-galactose oxidase-$[^{3}H]$borohydride were deficient in a surface glycoprotein with Mr $= 93,000$ that had a pi of 5.7 to 5.8. The other radiolabeled proteins from the thrombasthenic platelets maintained their normal positions, charge heterogeneity, and relative radioactivity.

When platelets from a third patient with Glanzmann's disease (S.H.), who was clinically indistinguishable from the previous patients, were labeled with either $[^{3}H]$ or $[^{125}I]$ and analyzed by two dimensional electrophoresis, a different pattern of radioactivity was observed (Fig. 3). Glycoproteins GPIIb and GPIII were decreased in amount but were present. The amount of radioactivity in these two proteins was decreased by approximately 50%. GPIIb and GPIII were decreased but present in Coomassie-blue stained gels. As in the platelets from the previous thrombasthenic patients, the $[^{3}H]$ labeled protein of Mr $= 93,000$ and pl 5.7–5.8 was found to be absent. The other radiolabeled proteins in this patient did not change their isoelectric point, charge heterogeneity or relative labeling. One dimensional SDS-polyacrylamide gels also showed that the two proteins, GPIIb and GPIII, were present in decreased amounts (not shown).

**DISCUSSION**

The results of the present study define further the molecular defect in platelets from patients with Glanzmann's disease. It is apparent from these studies that the deficiency of membrane glycoproteins GPIIb and GPIII, which was initially described by Phillips and Agin$^{23}$ and Nurden and Caen,$^{28}$ is heterogeneous. The possibility of additional membrane defects in thrombasthenic platelets is raised by the demonstration that platelets labeled with neuraminidase-galactose oxidase-$[^{3}H]$borohydride showed deficient labeling of an as yet unidentified surface protein with Mr $= 93,000$. The three patients described in this report were similar by clinical and laboratory criteria yet had different membrane surface patterns. Caen has stated that there appear to be two types of thrombasthenia.$^{29}$ In patients with type I disease, platelet fibrinogen and clot retraction were virtually absent. In patients with type II disease, platelet fibrinogen and clot retraction were intermediate between normal values and the very low values seen in type I. Kunicki and Aster have also
Fig. 3. Two dimensional separation of platelet proteins. (A) Thrombasthenic platelets (S.H.) labeled with lactoperoxidase and $^{125I}$. The sample was 40 μl of platelets containing 200 μg of protein and 125,000 cpm. The gel was exposed to x-ray film for 14 days. (B) Thrombasthenic platelets (S.H.) labeled with neuraminidase-galactose oxidase-$^{3}H$-borohydride. Approximately 40 μl of platelets containing 200 μg of protein was applied to the gel. The gel was subjected to fluorography for 7 days.
described two groups of abnormalities in thrombasthenia. One group was completely deficient in membrane proteins, GPIIb and GPIII, and the platelet antigen P1A, while the other group of patients had intermediate amounts of these membrane proteins and the P1A antigen. In addition, Hagen has recently reported the use of an immunoelectrophoretic technique to study platelet proteins. With this technique, patients with Glanzmann’s disease were divided into two types. Type I had no clot retraction, platelet fibrinogen, or immunoprecipitate 16 [GPIIb + GPIII]; while type II subjects had intermediate values of platelet fibrinogen, clot retraction and immunoprecipitate 16. Peterson et al., however, have found more detectable, but decreased, amounts of GPIIb and GPIII but had detectable, although decreased, amounts of platelet fibrinogen; and one patient had detectable, but decreased, amounts of GPIIb and GPIII but did not have any platelet fibrinogen.

The present study confirms the existence of phenotypic heterogeneity in Glanzmann’s disease. Our patients displayed two types of membrane protein abnormalities. Two patients had no detectable GPIIb or GPIII. These two patients also had decreased clot retraction and one patient had a P1A antigen of less than 1%. The third patient, who also had decreased clot retraction and a severe bleeding tendency had decreased but detectable amounts of GPIIb and GPIII and a P1A antigen of 25%. Platelet fibrinogen could not be detected on the 2D-IEF/SDS PAGE of any patient. Thus, two of our patients with thrombasthenia are probably the type I patients described by Caen and by Hagen and coworkers. In addition, they probably belong to the first group of patients described by Kunicki and Aster who were deficient in membrane proteins GPIIb and GPIII and the P1A antigen. The third patient (S.H.) was different from the type II patients of Caen and of Hagen and coworkers. She lacked platelet fibrinogen but had detectable, although decreased, amounts of GPIIb and GPIII.

Table 1. Phenotypic Patterns in Patients With Glanzmann’s Disease

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<tr>
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<th>GPIIb/GPIII</th>
<th>Platelet Fibrinogen</th>
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<td>S.H.</td>
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*Membrane glycoproteins GPIIb and GPIII were assessed qualitatively by SDS-PAGE (this study, see also references 29 and 31) or by crossed immunoelectrophoresis and were graded as absent (-), reduced (+ +), or present (+ + + +).†Platelet fibrinogen was determined by crossed immunoelectrophoresis or 2D-IEF/SDS PAGE (this study and reference 31) and was graded as present (P) or absent (A).§Platelet fibrinogen was determined as previously described. In this study clot retraction was determined with a whole blood modification of Benthaus. Normal was greater than 65%.

Phenotypic heterogeneity in Glanzmann’s disease is uncertain in view of the patient described by Peterson and coworkers who had absent GPIIb and GPIII with detectable fibrinogen and our patient (S.H.) who had detectable GPIIb and GPIII with no fibrinogen. In addition, patient S.H. is an example of the heterogeneity in the P1A antigen first described by Kunicki and Aster and provides additional support for their contention that the P1A antigen is structurally related to GPIII. Further studies will be required to determine the relationship between GPIIb/GPIII and platelet fibrinogen, and between the membrane defects and clot retraction. Although biochemically complex, the observed discrepancies among the amounts of GPIIb/GPIII, platelet fibrinogen, and the P1A antigen have important genetic implications and suggest that there is genetic heterogeneity in Glanzmann’s disease.

ACKNOWLEDGMENT

The authors wish to thank Renee Williams for excellent secretarial assistance. The determination of platelet P1A antigen in the patients with Glanzmann’s disease was kindly performed by Drs. T. Kunicki and R. Aster, Milwaukee Blood Center, Milwaukee, Wisc. Purified human fibrinogen was provided by Dr. J. McDonagh.
Fig. 23.—Sagittal MR image (1500/40) shows numerous mural myomas (m). Other images confirmed displaced but intact endometrial echo. (CT scan of this patient is shown in Fig. 20.)

Fig. 24.—Large submucous fibroid with cystic degeneration. Midsagittal MR image (1500/40) of uterus shows large submucous fibroid (arrows) bulging into high-signal-intensity endometrial cavity (e). High signal intensity (asterisk) in center of fibroid indicates cystic degeneration. Additional myomas of low signal intensity are seen in cervix (f) and posterior corpus (F).

Fig. 25.—A, Hysterosalpingogram shows large polypoid filling defect (arrows) in cervical region. Cervical fibroids may be difficult to visualize unless they protrude into cervical canal.

B, Sagittal MR image (1500/40) clearly depicts myoma (asterisk) in anterior cervix of retroverted uterus (solid arrows). High-intensity rim (open arrow) most likely is due to vascular congestion.

Fig. 26.—A, Sagittal MR image (1500/40) shows multiple low-signal-intensity masses in submucous (asterisk), mural (m), and subserous (s) locations. Note that submucous myoma impinges on endometrial signal. Another submucous myoma found at surgery was seen on other MR images.

B, Hysterosalpingogram shows only two submucous myomas (m).
Heterogeneity of membrane surface proteins in Glanzmann's thrombasthenia

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