Platelet Antithrombin Defect in Malignancy: Platelet Protein Alterations

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Sixty-eight patients with malignant disease were divided into two groups based on the results of the platelet antithrombin test (PAT). The normal group had a PAT clotting time ranging from 21.4 to 28.6 seconds, which was equivalent to 25% to 50% inactivation of the 2 U of thrombin added to the test system. The other group showed abnormal PAT clotting time, 21.4 seconds or <25% thrombin inactivation. The polypeptide composition of platelets from the two patient groups was analyzed by sodium dodecyl sulfate (SDS)-electrophoresis on 7.5% polyacrylamide gels. A polypeptide of 180,000 apparent mol wt was decreased or absent in both Coomassie blue- and Alcian blue-stained gels of the platelets from patients whose PAT was abnormal; this polypeptide comigrated with purified platelet thrombospondin. Tritium labeling of platelet surface glycoproteins by the periodate-borohydrate method followed by two-dimensional electrophoresis was performed on platelets of seven patients with abnormal PAT. When they were compared with ten patients with normal PAT, a glycoprotein of 140,000 apparent mol wt with a pi of 4.5 to 5.2 was decreased in platelets of all seven patients with abnormal PAT. Nitrocellulose replicas of one-dimensional gels of platelets from 13 of 14 patients with abnormal PAT showed decreased reaction with an anti-human platelet glyocalcin antiserum. Platelets of these same patients also showed a decreased or absent platelet agglutination induced by ristocetin. Patients with normal PAT had a mean agglutination slope of 1.25 ± 0.6 (n = 26) as compared with 0.37 ± 0.34 (n = 26) for the abnormal PAT group (P < .001). Results indicate that platelets from a subpopulation of tumor patients characterized by decreased platelet antithrombin activity have alterations in two platelet glycoproteins, identified as GPIb and thrombospondin.

The clotting activity of α-thrombin can be neutralized by intact human platelets. This activity is referred to as platelet antithrombin.1 Thrombin inactivation by normal platelets has been shown to be membrane dependent for two-thirds of the activity and secondary to the release of intracellular platelet fibrinogen for the other third.2 A simple two-stage assay has established the normal physiologic range3 and has been used to demonstrate a decrease in platelet antithrombin activity in certain pathologic states. This includes patients with primary (congenital) deficiencies4 as well as patients with apparent acquired deficiency secondary to malignant states.5 In a separate clinical study, one third of the patients with advanced disseminated adenocarcinomas, melanomas, and myeloproliferative disorders were found to have a consistent decrease in platelet antithrombin activity.6

The present study was designed to determine whether changes in platelet proteins could be correlated with abnormal platelet antithrombin activity. A group of patients with malignant disease and reduction in platelet antithrombin activity was compared with a group of patients with similar malignancies in whom thrombin inactivation by platelets was normal. The platelet proteins from these two groups of patients were analyzed by several electrophoretic and immunchemical techniques. The results demonstrate that two platelet proteins are altered in cancer patients whose platelets show defective inactivation of thrombin. One shows the characteristics of the membrane glycoprotein Ib (GPIb) and the other shows an α-granule component, thrombospondin.

Materials and Methods

Patient population. Sixty-eight patients from the medical oncology ward of the New England Deaconess Hospital were divided into two groups on the basis of platelet antithrombin test (PAT) results. All were part of a larger clinical study involving platelet antithrombin determinations on >200 patients with advanced malignant disease.6 Malignancies studied included ovary, lung, colon, melanoma, and lymphoma. Patients who were receiving aspirin, nonsteroidal anti-inflammatory agents, or vinca alkaloids were excluded. As established previously,4 results of PAT measurement follow a biomodal distribution. Patients with abnormal PAT had a clotting time <20.5 seconds (PAT measurement is also described in the Results section). Fourteen of the 37 patients with an abnormal PAT and 15 of the 31 patients with a normal PAT were not on current chemotherapy. The members of each group who were receiving chemotherapy had equal exposure to standard agents such as fluorouridine (FUDR), cytoxan, cisplatinum, 5-fluorouracil (5-FU), and Adriamycin.

Platelet preparation. Venous blood was drawn from the 68 patients and from three normal individuals (healthy controls) and mixed with acid-citrate-dextrose (ACD) [National Institutes of Health (NIH), Formula A] and EDTA anticoagulant solution to yield final concentrations of 15% (vol) ACD and 5 mmol/L of EDTA. After centrifugation at room temperature at 130 g for 15 minutes, the platelet-rich plasma (PRP) was removed, a 20% vol of ACD-EDTA was added, and the platelets were pelleted by centrifugation at 1200 g for 15 minutes. Platelets were washed twice by resuspension in Tris-buffered saline (TBS) containing 10% ACD-EDTA was added, and the platelets were pelleted by centrifugation at 1200 g for 15 minutes. Platelets were washed twice by resuspension in Tris-buffered saline (TBS) containing 10% ACD-EDTA. Platelet aliquots used for the PAT were resuspended without anticoagulants and adjusted to 2 × 10^9/mL for the PAT and 15 of the 26 for the abnormal PAT group (P < .001). Results indicate that platelets from a subpopulation of tumor patients characterized by decreased platelet antithrombin activity have alterations in two platelet glycoproteins, identified as GPIb and thrombospondin.

Platelet preparations. Platelet antithrombin activity was measured by a two-stage assay described previously.4,6 Washed platelets (0.5 mL) were incubated for 10 minutes at room temperature.

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temperature with 0.5 mL of thrombin solution (Parke-Davis, Morris Plains, NJ) at 4 NIH U/mL. Commercial thrombin gave results comparable to those of purified human thrombin. After incubation, 0.2 mL of the reaction mixture was added to 0.2 mL of fibrinogen (2 mg/mL) prewarmed at 37°C for 5 minutes. The clotting time was then determined. The variation between duplicate samples averaged 0.7 seconds (range 0 to 1.5 seconds).

**SDS-Polyacrylamide gel electrophoresis (PAGE).** Polypeptides were fractionated by SDS-PAGE according to the procedure of Laemmli using a 7.5% polyacrylamide resolving gel and a 4% stacking gel. Washed platelets were solubilized with 3% SDS and boiled for 3 minutes and were then treated with 10 mmol/L of dithiothreitol for 1 minute at 100°C. Equivalent protein concentrations (20 to 80 μg) were applied in each analysis of platelets from patients with normal and abnormal PAT.

Gels were stained with Coomassie blue and Alcian blue. For Alcian blue staining, polyacrylamide gels were fixed in 12.5% trichloroacetic acid for 30 minutes and were then treated for 50 minutes at room temperature with 7% periodic acid in 3% acetic acid. The fixed gels were washed twice with 200 mL of distilled water and soaked in 0.5% metabisulfite for 30 minutes. The gels were washed and stained with 1% Alcian blue (in 3% acetic acid) overnight and destained in 7% acetic acid. Standard mol wt marker proteins were: spectrin heterodimer (240,000 and 220,000) — galactosidase (130,000), phosphorylase A (94,000), albumin (68,000), glutamate dehydrogenase (56,000), creatinine kinase (40,000), and carbonic anhydrase (29,000).

**Radiolabeling of surface carbohydrate moieties.** Surface sialic acid residues of glycoproteins were labeled with [3H]N-acetylglucosamine (GlcNAc) or 450 mmol/L of GlcNAc. The 450-mmol/L GlcNAc eluate was dialyzed against 6 mmol/L of potassium phosphate buffer (pH 6.8) with 0.2 mmol/L of EDTA for six changes over 72 hours and then applied to a column (2.5 × 8 cm) of freshly prepared hydroxyapatite. Stepwise elution was performed with 6, 40, and 200 mmol/L of potassium phosphate buffer containing 0.2 mmol/L of EDTA. The fraction eluted with 40 mmol/L of potassium phosphate showed a single band of 130,000 apparent mol wt when analyzed by SDS-PAGE followed by Alcian blue staining. Purified glycoprotein was stored at −20°C. Thrombospondin was isolated from the supernatants of washed platelets that had been treated with thrombin by the method described previously.14

**Antiserum preparation.** Antiplatelet glycoprotein antiserum (raised in rabbits) was obtained from PRP, Inc. (Newton, MA). Antithrombospondin antiserum was prepared in mice. Female BALB/c mice, aged 6 to 10 weeks, were immunized by intraperitoneal (IP) injections with 0.1 mg of pure human platelet thrombospondin and emulsified in complete Freund’s adjuvant followed by two booster IP injections (at 3-week intervals) of 50 μg of antigen in TBS/2 mmol/L of CaCl2 in incomplete Freund’s adjuvant. Serum containing antibody was prepared from blood collected 3 weeks after the second booster injection.

**Electroblotting and immune staining.** Washed platelets were solubilized with SDS and fractionated by SDS-PAGE on 7.5% slab gels. Fractionated polypeptides were electrophoretically transferred to nitrocellulose paper at 30 V for 20 to 24 hours at 10°C in pH 8.3 buffer containing 25 mmol/L of Tris, and 192 mmol/L of glycine as described, except that no methanol was used. Nitrocellulose replicates were washed in 0.15 mol/L of NaCl, incubated with phosphate-buffered saline (PBS; pH 7.5) containing 5% bovine serum albumin (PBS-albumin) for 1 hour at 22°C, then washed with five changes of 0.15 mol/L of NaCl solution over 30 minutes. The protein blots were incubated with either the monospecific antiserum against human platelet glycoprotein or thrombospondin, washed, then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (for glycoprotein), or goat anti-mouse IgG (for thrombospondin) (10 μl/10 mL TBS, Cooper Biomedical [Cappel Division], Malvern, PA) for 1 hour at 22°C. Bound anti-IgGs were detected with 4-chloro-1-naphthol (0.6 mg/mL) and 0.3% hydrogen peroxide in a solution of 20% methanol and 50 mmol/L of Tris-HCl (pH 7.6).

**Measurement of ristocetin-induced aggregation.** Formalin-fixed platelets were prepared for ristocetin aggregation by a method modified from Coller and co-workers using Gaintner’s buffer. A single batch of pooled normal citrate anticoagulated plasma (diluted 1:2) was used as the standard source of factor VIII von Willebrand factor (vWF). The platelet suspension (0.35 mL) was incubated with 0.1 mL of the plasma at 37°C, and aggregation...
was induced by the addition of ristocetin (50 μL of 1.5 mg/mL). The steepest slope of the agglutination curve was used to assess ristocetin agglutination as described by Weiss. 48

*Xylocaine treatment of platelets.* Washed platelets in TBS (1 x 10⁷/mL) were incubated with xylocaine (40 mmol/L) at 37°C for 3 hours as described by Coller. 49 Control samples were incubated in the presence of 5 mmol/L of EDTA without xylocaine. After incubation, platelets were washed twice in Tris-buffered saline and prepared for PAT measurement and surface leveling as described previously.

*Other methods.* Platelet sizing and WBC determinations were done electronically using a Coulter Counter (Model-S). All platelet preparations were 99.9% leukocyte-free. Protein determinations were performed by the method of Lowry and colleagues. 2 Individual polypeptides in electrophoresis gels and autoradiograms were estimated by densitometric scanning using a 580-nm filter on a Hoeffer gel densitometer.

**RESULTS**

The platelets from 68 tumor patients were studied. Of this group, 37 had abnormal platelet antithrombin. The PATs in the abnormal group ranged from 17.0 to 20.5 seconds with an average of 18.6 seconds (SD 0.96). The PATs of the normal group ranged from 21.4 to 29.8 seconds with an average of 25.4 seconds (SD 1.97). The coefficient of variation was 5.2% for the abnormal group and 7.8% for the normal group.

When the polypeptide composition of platelets from the 37 patients with abnormal PAT was analyzed by SDS-PAGE and compared with the protein composition of platelets from 31 patients with normal PAT and three healthy individuals, a marked reduction in a polypeptide of 180,000 apparent mol wt was demonstrated; this difference was evident when gels were stained with either Alcian blue or Coomassie blue (Fig 1A and B). Staining by Alcian blue was found to be more sensitive than that by Coomassie blue, however, in demonstrating alterations of 180,000 mol wt polypeptide. This polypeptide could be readily detected by Alcian blue stain when only 20 to 40 μg protein from normal platelets was applied to the gels, whereas 40 to 80 μg of normal platelet protein was required to detect this polypeptide by Coomassie blue staining. By means of Alcian blue staining, the 180,000-mol wt polypeptide was shown to be decreased or absent in 36 of 37 samples with abnormal PAT and present in the 34 control samples with normal PAT. Purified human platelet thrombospondin, analyzed in parallel with platelet samples, exhibited identical electrophoretic mobility and staining characteristics (under reducing conditions) to this 180,000-mol wt polypeptide (Fig 2). Furthermore, the nitrocellulose replicas of SDS gels of normal platelet proteins showed reaction of this 180,000-mol wt polypeptide with a monoclonal antisem against human platelet thrombospondin; this polypeptide also was decreased or absent in platelets from patients with abnormal PAT (Fig 2). On this basis, the decreased polypeptide in platelets from patients with abnormal PAT was identified as thrombospondin.

Platelet surface glycoproteins from seven of the cancer patients whose platelets showed an abnormal PAT and those from ten individuals with a normal PAT were radiolabeled with 3H and analyzed by two-dimensional isoelectric focusing/SDS-electrophoresis and autoradiography (Fig 3). Under the precise experimental conditions described previously in the Materials and Methods section, platelets from patients with normal PAT showed three major radiolabeled polypeptides. These polypeptides had apparent reduced mol wts of 140,000, 120,000, and 100,000, and relative pls of 4.5 to 5.2, 5.3 to 5.5, and 5.3 to 5.5, respectively. The polypeptide of ~140,000 mol wt and pl 4.5 to 5.2 was diminished or absent in all seven patients with low PAT. Quantitation of labeled polypeptides by densitometric scanning of autoradiograms showed that the ratio of the densities of the 140,000- to the 120,000-mol wt polypeptide was significantly lower in the abnormal group than in the normal patient group, whereas the densities of the 120,000-mol wt polypeptide were similar in both groups.

Studies were undertaken to examine platelets of the two patient groups with a specific immunochemical probe for

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**Fig 1.** Polypeptide composition of platelets from individuals with normal and abnormal platelet antithrombin test (PAT). Washed platelets were solubilized in 3% sodium dodecyl sulfate (SDS) and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gels under reducing conditions. Gels were stained with Coomassie blue (A) and Alcian blue (B): lane 1, healthy control; lane 2, patient with normal PAT; lanes 3 and 4, patients with abnormal PAT. A polypeptide at 170,000 apparent mol wt is diminished in platelets from patients with abnormal PAT (arrows). For the Alcian blue-stained gels, the tracking dye was run off the leading edge for 30 minutes to improve resolution of high mol wt components. Positions of standard mol wt proteins are as indicated.
GPIb—a polyclonal antiserum against purified human platelet glycocalcin. The antiserum (raised in rabbits) reacted with highly purified glycocalcin (mol wt 130,000) and a single, $^{3}$H-labeled polypeptide of apparent reduced mol wt 140,000 when evaluated by staining of nitrocellulose replicas of SDS gels containing isolated glycocalcin and the fractionated polypeptides of $^{3}$H-labeled, normal platelets (Fig 4).

Platelet GPIb from 14 tumor patients with abnormal PAT and seven normal control subjects were analyzed by the immune replica technique, using the monospecific antiglycocalcin antiserum. There was a reduction in the intensity of staining of the polypeptide corresponding to GPIb in 13 of the 14 patients with abnormal PAT when compared with...
platelets from seven normal control subjects (Fig 5, lanes 3 through 6). Degradation products of GPIb were visible (indicated by low mol wt polypeptide that reacted with the antiglycocalcic antiserum) in four patients with abnormal PAT (Fig 5, lanes 3 and 4). The apparent mol wt for two degradation polypeptides shown by the two diffuse bands in Fig 5, lane 4 were 120,000 and 105,000.

Ristocetin-induced platelet agglutination was significantly lower in patients with abnormal PAT. For the group with normal PAT, the average steepest slope of agglutination was $1.25 \pm 0.6$ (n = 28). For the group with abnormal PAT, the average slope was $0.37 \pm 0.34$ (n = 26) (Fig 6). The difference between those two groups was statistically significant ($P < .001$).

Attempts to reproduce the PAT defect in normal platelets in vitro were made by exposing platelets to xylocaine, which is known to stimulate proteolysis of GPIb.$^{19}$ In two experiments, xylocaine treatment produced a decrease of antithrombin activity in platelets. The PAT was reduced from 31 seconds before treatment to 21 seconds after treatment in one experiment and from 23 seconds to 18 seconds in another. The effect of the xylocaine treatment could be seen on the two-dimensional PAGE and autoradiogram of $^3$H-labeled platelets as an absence of a protein of 140,000 mol wt at pI of 4.5 to 5.2 (Fig 7).

**DISCUSSION**

Platelet antithrombin activity denotes the ability of platelets to neutralize the clotting activity of thrombin.$^1$ Previous reports$^{21,22}$ have implicated GPIb as the protein responsible for antithrombin activity. Glycocalcin, a proteolytic product of GPIb, has been shown by other investigators to prolong the thrombin clotting time and inhibit competitively ristocetin-induced platelet aggregation$^{22,23}$: it has been implicated as the site of thrombin and vWF binding within the GPIb molecule.$^{13}$ Recently, Takamatsu and colleagues,$^{24}$ by chemical cross-linking studies, demonstrated one major thrombin receptor on the platelet surface and showed that the receptor binding is blocked by antiglycocalcic antibody.

In this study, a platelet glycoprotein interacting with antiglycocalcic antibody and having a mol wt of 140,000 and a pI of 4.5 to 5.2 was decreased in patients with an abnormal PAT. The electrophoretic pattern (as described previously), which showed characteristics similar to those of
GPIb as reported by others, along with positive staining of the immunoblot by antiglycocalicin antibody, suggests that this protein is GPIb.

The mechanism that produces the GPIb defect as demonstrated in this study is not known. It may be due to quantitative reduction of GPIb on the platelet surface or to a structural alteration of the molecule so that it no longer reacts to monospecific antibody against glycocalicin. GPIb is a prominent surface membrane glycoprotein, rich in sialic acid content and, as such, can be readily labeled with tritiated borohydride after generation of reactive carbonyl groups with sodium periodate. When tritiated platelets were analyzed by two-dimensional PAGE, a deficiency of GPIb also was demonstrated in platelets from patients with low PAT. The latter results may reflect less glycosylation of GPIb. Disialylation by neuraminidase treatment, however, caused a retardation of electrophoretic migration of GPIb in SDS-PAGE. Results from the present surface labeling and immunoblotting studies failed to show such effects.

Ristocetin-mediated binding of vWF to normal platelets is dependent on the integrity of GPIb. Antibodies prepared against GPIb have prevented platelet agglutination in response to ristocetin. Platelets of patients with Bernard-Soulier syndrome who are deficient in GPIb also fail to respond to ristocetin and bind less thrombin than do normal patients. Decreased concentrations of GPIb have been described in thrombocytopenia by other investigators. This is consistent with the previously reported decreases in PAT in that disease. Additional supporting evidence that GPIb is the platelet protein accounting for most platelet antithrombin activity comes from experiments dealing with exposure of normal platelets to xylocaine. This procedure causes cleavage of glycocalicin from GPIb (Fig 7) as well as diminished platelet antithrombin activity.

Both thrombin and plasmin are known to degrade GPIb. Therefore, the depletion of GPIb from platelets may be the result of the generation of circulating proteolytic enzymes in cancer patients. No direct evidence confirms proteolysis of GPIb of platelets with reduced PAT; however, the demonstration of low mol wt fragments of GPIb is consistent with proteolytic degradation of this surface glycoprotein. Release of calcium-dependent protease (CDP) from platelets can also cause depletion of GPIb. Because all platelet samples were prepared and lysed in the presence of EDTA and iodoacetamide, which are inhibitors of CDP, it is doubtful that depletion of GPIb from platelets is an in vitro phenomenon caused by the release of CDP during washing and solubilization of platelets.

Diminished platelet sialic acid content has previously been associated with malignancy, as has increased plasma sialic acid. The latter has been correlated with the extent of tumor mass. Because glycocalicin is found to circulate in normal plasma, it would be of interest to determine if any portion of the increased plasma sialic acid is accounted for by glycocalicin.

Decreased amounts of a second protein of apparent mol wt of 180,000 were also found in platelets of patients with low PAT. This protein was identified as thrombospondin on the basis of a comparison of its electrophoretic, immunologic, and staining properties with those of purified thrombospondin. Thrombospondin is an α-granular protein, composed of three apparently identical chains. It is released and expressed on the platelet surface in response to thrombin stimulation. Thrombospondin is deficient in platelets from patients with gray platelet syndrome as well as from patients with thrombocytopenia. Thrombospondin has been postulated to have multiple functions. The presence of thrombospondin on the platelet membrane was found to increase fibrinogen binding. Because surface-bound fibrinogen accounts for 25% of the platelet antithrombin activity, the absence of thrombospondin could indirectly account for the decrease in PAT.

It is unknown at this time whether the similar protein changes account for platelet antithrombin defect in nonmalignant disease. In addition, the unequivocal proof of a cause-effect relationship between platelet antithrombin deficiency and the depletion of GPIb and thrombospondin is still lacking. Nevertheless, this study provides the first evidence to link the antithrombin defect and alterations of glycoproteins in platelets from patients with malignant disease.

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REFERENCES

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