A simple, novel and robust test to diagnose type I Glanzmann thrombasthenia

Glanzmann thrombasthenia (GT) is an inherited bleeding disorder due to either absence or dysfunction of fibrinogen binding receptors, i.e either GPIIb (GPIIβ)[1] or GPIIIa (GPIIIα)[2] on platelet membrane. The complete fibrinogen receptor, i.e. GPIIβGPIII which binds fibrinogen on activated platelets involves association of these two glycoproteins. Absence of either or both of these receptors can, therefore, cause platelet dysfunction. In the endoplasmic reticulum, pro GPIIβ forms heterodimer with GPIIIα, a pre-requisite for surface expression. In the Golgi, pro- GPIIβ is cleaved into heavy and light chains, linked through a disulfide bridge (mature-GPIIβ). The membrane glycoprotein GPIIβ consists of four major domains, β-propeller, thigh, calf-1 and calf-2 domains.[3] The main contact site with GPIIβ is located in the β-propeller, while calf-1 and calf-2 domains contribute minor interfaces. In type I disease no GPIIIα; GPIIβ; receptors are reproduced or only a very low number, can be detected on the platelet surface, while in type II GT their number is measurable.

Due to the high rate of consanguineous marriages, GT is the commonest platelet function defect in places like India,[4] Arab countries and Israel.[5] In many parts of the world, the availability of a quick and robust test to detect Glanzmann thrombasthenia could be useful, and platelet aggregometry and flow cytometry are not only costly techniques, but also need experienced technicians who are in short supply.

We used a bifunctional bioengineered disintegrin/alkaline phosphatase hybrid protein ErAPv to evaluate 14 consecutive cases of Glanzmann thrombasthenia and 20 normal healthy controls. Disintegrins have high affinity and selectivity for integrins which selectively bind and inhibit integrin function. The eristostatin (Er) portion selectively binds to the GPIIβGPIIIα glycoprotein on the surface of the platelet membrane while the APv portions allow the development of a dot in a one-step method.[6] Platelet aggregation was performed by using agonists ristocetin (1.25 mg mL^{-1}), ADP (6 μM), collagen (4 μg mL^{-1}), epinephrine (4 μM) and arachidonic acid (0.75 mM). Flow cytometry analysis of platelet membrane surface receptors was carried out using FITC labeled antiGPIIβGPIIIα, antiβ3, anti-GPIβ, anti-GPIX (BD Biosciences, Pharmingen, San Diego, USA), antiGPIIβ and antifibrinogen antibodies (Dako, Glostrup, Denmark). For dot blot analysis, 3 microliters of washed platelets (2×10^8 mL^{-1}) were blotted onto a polyvinilydine difluoride [PVDF] membrane and left to dry. The PVDF strips were blocked with 5% non-fat milk in Tris-buffered saline, pH 8.2 (20mM Tris, 0.9% NaCl, 20mM NaN_3) or blocking solution for 2 hrs. at room temperature with mild agitation. ErAPv protein was added (40 μL/mL of blocking solution) and further incubated for 2 hrs. Membranes were washed 3×5 mins. with Tris-buffered saline with mild agitation to remove the unbound protein. NBT/BCIP alkaline phosphatase substrate (1:1.25) (Roche Diagnostics, Mannheim, Germany) was added until dots developed. A clinical severe type II GT homozygous for 1619 del C was taken as positive control (case 12). A substrate blank was also included as negative control.

Out of the 14 GT patients, 12 were type I and 2 were type II variants. Nine were born out of first degree consanguineous marriages. Five patients did not have any history of transfusion, while others had history of minimal transfusion. Fourteen patients showed classical features of GT, i.e. severely reduced or no aggregation with all the agonists except reduced or normal aggregation with ristocetin. The GPIIβGPIIIα content in 12 of the 14 patients was found to be <1% of normal. Two patients had a normal αIIββ3α content of 56% and 58% respectively, suggesting that these patients have a variant type of thrombasthenia with the GPIIbβGPIIIα receptors

Table 1. Flow cytometry and dot blot analysis data of Glanzmann thrombasthenia patients.

Patient N.	*Flow cytometry data*						*Type*	*Dot blot data*
	GPIIb	*GPIIIa.*	*GPIIb.GPIIIa*	*Fibrinogen*	*GP Ib*	*GP IX*		
1	1.7	38.82	0.58	39.47	82.89	81.01	1	Negative
2	0.23	9.55	0.09	54.9	78.08	75.68	1	Negative
3	0.81	26.15	0.33	60.84	89.05	88.02	1	Negative
4	2.18	25.06	0.83	57.43	86.36	89.78	1	Negative
5	1.82	22.8	0.35	39.06	79.6	94.86	1	Negative
6	0.47	3.72	0.11	44.27	77.44	90.54	1	Negative
7	2.03	15.31	0.24	60.85	82.61	95.53	1	Negative
8	2.55	32.87	0.51	44.92	78.55	92.15	1	Negative
9	1	4.35	0.29	22.06	90.5	85.25	1	Negative
10	0.17	0.43	0.1	9.1	85.06	90.21	1	Negative
11	0.17	0.43	0.09	12.92	87.05	85.36	1	Negative
12	71.01	94.46	56.17	55.92	80.72	95.76	Variant	Positive
13	68.91	93.64	58	58	77.64	95.48	Variant	Positive
14	4.05	34.52	0.79	62.88	88.1	89.21	1	Negative
Mean	1.43	17.83	0.36	44.47	83.11	89.2	-	-
SD	1.18	13.98	0.27	18.12	4.56	5.87	-	-
Normals (N=20)								
Mean	85.56	83.98	90.85	70.56	77.35	91.58	-	-
SD	4.3	3.8	5.2	4.5	6.2	2.3	-	-

Data from variant GT (Patients 12 and 13) were excluded while calculating mean and standard deviation for αIIβ, β3α and αIIββ3α. In both controls and patients the background binding percentage of fibrinogen is not deducted.

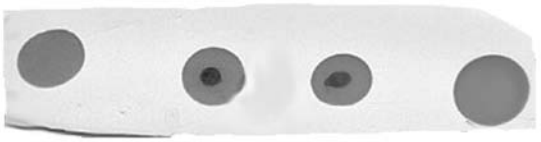

Figure 1. Dot Blot ELISA for diagnosis of type I GT. Index case is type I GT shows absence of central dot compared with to the positive control (Glanzmann thrombasthenia type II homozygous for 1619 del C) and mother of the index case. Negative control is the substrate without platelet lysate.

being quantitatively within the normal range but functionally defective. The other 12 patients were shown to be type I GT patients as per the 1990 classification.[7] Four of the 14 patients had almost undetectable levels of αIIβ and β3α surface receptors, and the rest of the patients showed almost undetectable GPIIβ and reduced levels of GPIIIα. The latter group is suggestive of the fact that there may be a defect in the GPIIβ receptor because absence of GPIIβ does not lead to complete absence of GPIIIα on the membrane surface (Table 1). But a defect in the GPIIIα leads to the presence of uncomplexed GPIIβ due to the lack of a suitable partner with which to form heterodimer, complete its process of maturation and be transported to the cell surface.

The dot blot procedure showed absence of dots in all type I GT patients. The variant type of GT showed marked dots compared with those of the controls. All the normal controls gave normal results, i.e., a marked dot indicating presence of a normal amount of the membrane glycoprotein GPIIbGPIIIα on their surface (Figure 1).

Platelet function tests using platelet aggregometry are required to detect platelet function disorders and platelet flow cytometry can reliably detect the presence or absence of specific glycoprotein antigens or inadequate generation of ligand binding sites on platelet activation.[8] Unfortunately both these tests are expensive, require considerable technical expertise and are generally not available in small coagulation laboratories in many countries. The present test was found to be highly specific (100%) and robustly sensitive (100%) to detect type I GT, where GPIIβGPIIIα receptors are almost absent. One drawback of the test is that it doesn't detect the variant type of GT where the receptors are quantitatively normal but functionally defective. It may be possible to detect variant type GT with this simple dot blot test using a similar disinterring excitation isolated from the venom of *Echis carinatus* coupled with alkaline phosphatase.[9] Eristostatin isolated from *Eristocophis macmahoni* venom binds with the same affinity to resting and to activated platelets, whereas the binding affinity of echistatin for platelets increases several fold after activation of GPIIbGPIIIα.[10]

In conclusion, this simple one-step dot blot test could be an alternative, less expensive test for Glanzmann thrombasthenia diagnosis.

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