

Defective expression of GPIb/IX/V complex in platelets from patients with May-Hegglin anomaly and Sebastian syndrome

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Background and Objectives. May-Hegglin anomaly (MHA) and Sebastian syndrome (SBS) are inherited macrothrombocytopenias with Döhle-like bodies in leukocytes. MHA-SBS are due to mutations of the gene (*MYH9*) for the heavy chain of non-muscle myosin IIA (NMMHC-IIA), the only myosin II expressed in platelets. The bleeding tendency is often more severe than expected on the basis of platelet count, but no abnormality of platelet function has been identified. To characterize platelet abnormalities deriving from *MYH9* mutations better, we studied surface glycoproteins (GPs) in platelets from MHA-SBS patients.

Design and Methods. Eight patients from 4 unrelated families were studied. Platelet surface GPs were studied by flow cytometry in both the whole platelet population and subpopulations of platelets identified according to their size.

Results. Flow cytometry identified a defect of the GPIb/IX/V complex in the whole platelet population in 7 of 8 patients. Moreover, in all patients the subpopulation of large platelets had defective expression of this complex.

Interpretation and Conclusions. These findings indicate that *MYH9* mutations may be responsible for reduced surface expression of GPIb/IX/V. This defect could contribute to the bleeding tendency of these patients. The identification of a GPIb/IX/V defect in MHA-SBS platelets raises the question of the differential diagnosis from heterozygous Bernard-Soulier syndrome.

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Key words: platelets, May-Hegglin anomaly, Sebastian syndrome, GPIb/IX/V complex, inherited thrombocytopenias.

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May-Hegglin anomaly (MHA) and Sebastian syndrome (SBS) are autosomal dominant inherited disorders defined by platelet macrocytosis, Döhle-like leukocyte inclusions and possible thrombocytopenia. The differentiation of MHA from SBS is based on subtle differences in the ultrastructural features of the Döhle-like bodies.^{1,2}

The MHA-SBS genetic defect has been recently mapped on the long arm of chromosome 22³ and then identified in the gene (*MYH9*) encoding the heavy chain of non-muscle myosin IIA (NMMHC-IIA).⁴⁻⁶ *MYH9* mutations are associated with two other macrothrombocytopenic syndromes: Fechtner syndrome,⁷ characterized by nephritis, sensorineural hearing loss, cataract and polymorphonuclear inclusion bodies, and Epstein syndrome,⁸⁻¹⁰ differing from Fechtner syndrome by the absence of cataract and Döhle-like bodies.

Vertebrates possess two isoforms of NMMHC-II, which are widely and differentially expressed in tissues and have overlapping but distinct intracellular localizations.¹¹ Only NMMHC-IIA is expressed in human platelets and leukocytes.¹² In normal subjects it has a diffuse and homogeneous distribution in leukocyte cytoplasm and platelets, while in patients with *MYH9* mutations it is clustered in a finite number of spots.¹³ On this basis, it has been suggested that Döhle-like bodies observed in leukocytes from patients with MHA-SBS and Fechtner syndrome are due to the aggregation of NMMHC-IIA in the cytoplasm.

Most patients with MHA-SBS have mild to severe thrombocytopenia, but this feature is not constant, since the platelet count may also be within the normal range.² The clinical spectrum of MHA-SBS ranges from a severe bleeding tendency to no bleeding diathesis, with the majority of patients suffering from easy bruising, prolonged menstrual periods and mild epistaxis. A bleeding tendency and prolonged bleeding time have also been observed in patients with a normal or slightly reduced platelet count, and on this basis it has

been suggested that these conditions derive from a functional defect of platelets.^{1,2} However, the mechanism of platelet dysfunction remains unknown. Glycoproteins (GPs) on the platelet surface play a key role in platelet function: the GPIb-IX/V complex is essential for initial adhesion of platelets to subendothelial von Willebrand factor, while the GPIIb/IIIa complex is required for both platelet spreading onto subendothelium and platelet aggregation induced by fibrinogen binding. Moreover, GPIa and GPIV participate in platelet adhesion and aggregation, respectively.¹⁴

In order to obtain further information on platelet dysfunction in MHA-SBS, we studied surface expression of these GPs in 8 patients from 4 families with defined *MYH9* mutations.

Design and Methods

Patients

Eight patients from 4 unrelated families were studied. The *MYH9* mutations in families A, C and D have been previously reported,⁵ while the mutation in family B has been recently identified (*Savoia, personal communication*) (Table 1). The bleeding diathesis was mild or absent in all but one patient (A/1), who suffered from severe post-partum bleeding requiring blood transfusion. In symptomatic patients, easy bruising and prolonged menstrual periods were the most common symptoms. All patients were thrombocytopenic, and the whole blood counter (DASIT CC800, Kobe, Japan) greatly underestimated platelet counts compared to the values obtained with a hemocytometer using phase microscopy (Table 1). This difference occurred because the electronic counter failed to recognize very large platelets. For the same reason, the electronic counter was not reliable for the assessment of platelet volume. Therefore, the degree of platelet macrocytosis was calculated by counting the percentages of platelets with a diameter below 4 μm , between 4 and 8 μm , and greater than 8 μm . Normal values of this parameter were determined in 30 healthy donors. Two hundred platelets were examined for each subject. This method revealed that all patients had severe macrocytosis, with 7 to 44% of platelets larger than red cells (Table 1). *In vitro* platelet aggregation after collagen, ADP or ristocetin stimulation was normal in all subjects.²

Flow cytometry

Surface expression of platelet GPs was investigated by flow cytometry in platelet-rich plasma with an Epics XL flow cytometer (Coulter Corporation, Miami, FL, USA) as previously reported.¹⁵ The

Table 1. Patients' characteristics.

Family/ Patient	Diagnosis	<i>MYH9</i> mutation	Platelet count ($\times 10^9/L$)		Platelet size* (% of platelets)
			Counter	Microscopy	
A/1	SBS	N93K	15	26	A: 20.9, B: 35.1, C: 43.9
B/1	MHA/SBS ^o	E1945X	28	68	A: 69.7, B: 21.7, C: 8.6
B/2	MHA/SBS ^o	E1945X	8	63	A: 55.0, B: 32.0, C: 13.0
C/1	MHA	R1165C	14	88	A: 63.1, B: 27.9, C: 9.0
C/2	MHA	R1165C	10	110	A: 43.0, B: 38.0, C: 19.0
D/1	MHA	E1841K	21	59	A: 69.4, B: 21.8, C: 8.8
D/2	MHA	E1841K	6	48	A: 68.5, B: 24.1, C: 7.4
D/3	MHA	E1841K	21	59	A: 17.0, B: 47.0, C: 36.0

*A: platelet diameter less than 4 μm ; B: diameter 4 - 8 μm ; C: diameter > 8 μm . Normal values (range) in 30 healthy subjects: A: 87-100%, B: 0-13%, C: 0-1.5%.
^oIn family B, ultrastructural studies for differentiation of MHA from SBS have not been possible.

following monoclonal antibodies (mAbs) were used: SZ21 (Immunotech SA, Marseille, France) that recognizes GPIIIa (CD61); AP2 (Immunotech) recognizing GPIIb/IIIa (CD41/61); MB45 (CLB, Amsterdam, the Netherlands) and SZ2 (Immunotech) against GPIb α (CD42b); FMC25 (kindly provided by Zola H, Adelaide, Australia) and SZ1 (Immunotech) that recognizes GPIX (CD42a); SW16 (CLB) against GPV (CD42d); FA6-152 and Gi9 (both from Immunotech) against GPIV (CD36) and GPIa (CD49b), respectively. MO₂ (Coulter) was used as a negative control. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (GAM-FITC) was purchased from the Coulter Corporation.

Results

Table 2 shows that binding of mAbs against GPIIb/IIIa to platelet surface was higher in all patients with MHA-SBS than in controls. Similar results were obtained with mAbs recognizing GPIa/IIa and GPIV (*data not shown*). This is consistent with platelets from patients with MHA-SBS having a larger volume and surface area than platelets from healthy subjects. In this case, the number of surface receptors per cell is expected to be increased if their density (number of molecules per surface area unit) is normal. Conversely, the binding of mAbs to the components of the GPIb/IX/V complex was found to be lower in 5 of 8 patients than in controls. However, when we normalized the data of GPIb/IX/V to those of GPIIb/IIIa, a defect of GPIb/IX/V was observed in all patients but one. In order to identify a possible correlation between platelet volume and GPIb/IX/V deficiency, in some patients (B/1, C/1, D/1, D/2 and D/3) we studied surface GPs in platelet subpopulations identified on the basis of their forward scatter, which reflects platelet volume. This analysis revealed that the

Table 2. Platelet surface glycoproteins in patients with MHA or SBS.

Family/patient	Mean fluorescence intensity - % of control (values normalized to AP2 - % of control)			
	GPIIb-IIIa (AP2)	GPIb α (SZ2)	GPIX (FMC25)	GPV (SW16)
A/1	181	33 (18)	28 (15)	25 (14)
B/1	120	66 (55)	86 (72)	75 (62)
B/2	221	70 (32)	98 (44)	74 (33)
C/1	123	139 (113)	198 (161)	99 (80)
C/2	179	74 (41)	93 (52)	79 (44)
D/1	139	101 (73)	n.d.	n.d.
D/2	127	87 (68)	n.d.	n.d.
D/3	141	112 (79)	n.d.	n.d.
Heterozygous BSS	97-207	23-67 (17-64)	24-96 (24-76)	28-98 (20-77)

Mean fluorescence intensity (arbitrary units) obtained with mAbs against GPIIb/IIIa, GPIb α , GPIX and GPV are expressed as % of control platelets. Values in brackets are the mean fluorescence values for single components of the GPIIb/IX/V complex normalized to those of GPIIb/IIIa and expressed as percentage of control platelets. Results obtained with AP2, SZ2 and FC25 are representative of those obtained with the other mAbs against GPIIb/IIIa or GPIIb/IX/V. For comparison, we report the values previously observed in a case series of patients with heterozygous BSS.¹⁵

GPIIb/IX/V deficiency was more severe in large platelets than in medium-sized ones and minimal or null in small platelets (Figure 1). Moreover, a defect of GPIIb/IX/V was shown in fractionated large thrombocytes also in the patient with normal values in unfractionated samples.

Discussion

Although the gene responsible for MHA-SBS has been identified,⁴⁻⁶ we still have little information on the pathogenetic mechanisms of this condition, in that we do not know why patients have a low platelet count and giant platelets in peripheral blood. Moreover, we do not know why patients present a bleeding tendency that is often more severe than expected from the platelet count,² in that *in vitro* platelet function has been investigated by several authors, and no significant abnormality has been identified except for the failure of stimulated platelets to undergo a shape change.² Apart from macrocytosis, platelet morphology is roughly normal in MHA-SBS,¹² but immunofluorescence

with antibodies against tubulin and ultrastructural studies revealed that platelet microtubules are distributed unevenly instead of being organized in a circumferential band at the cell periphery.^{2, 17} Moreover, immunocytochemical studies recently showed that also the platelet distribution of NMMHC-IIA is abnormal, in that in some platelets this molecule was severely reduced or absent, while in some others it was clustered in a few spots located at the cell periphery instead of being uniformly distributed within the platelet.¹³ This observation supported the hypothesis that NMMHC-IIA mutations affect the correct assembly or stability of the quaternary myosin complex and prevent its normal distribution both in platelets and leukocytes.⁵ All together, these findings indicate that a complex defect of cytoskeleton organization is the basic anomaly of MHA-SBS platelets.

An additional defect identified by our study in MHA-SBS platelets is the reduced surface expression of the GPIIb/IX/V complex, which was more evident in large platelets than in small ones. Our results are at variance with those obtained 20 years ago by Coller and Zarrabi,¹⁸ who observed no GP abnormalities in two patients with MHA. However, they studied platelet GPs by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and this method is less sensitive than flow cytometry at detecting small defects. Moreover, they studied unfractionated samples, while flow cytometry offered us the possibility to analyze large and small platelet subpopulations separately.

The GPIIb deficiency we observed was not sufficient to affect *in vitro* platelet aggregation after ristocetin stimulation. We cannot exclude that this defect hampers *in vivo* platelet adhesion to subendothelium because of a defective GPIIb-von Willebrand factor interaction.

The correlation between a defect of the *MYH9* gene and reduced surface expression of GPIIb/IX/V is not clear, although it is well known that the cytoplasmic tail of GPIIb α interacts with the cytoskeleton of resting platelets.¹⁹ Several examples of primary alterations of the cytoskeletal components that result in a decreased expression of membrane proteins have been reported.²⁰⁻²² The most pertinent comparison can be made between MHA-SBS platelets and a melanoma cell line lacking actin-binding protein (ABP). Transfection studies in this cell line showed that ABP was essential for the surface expression of GPIIb/IX and that this effect did not involve a direct interaction of ABP with the complex, because GPIIb/IX membrane expression was restored also when the ABP-deficient cells

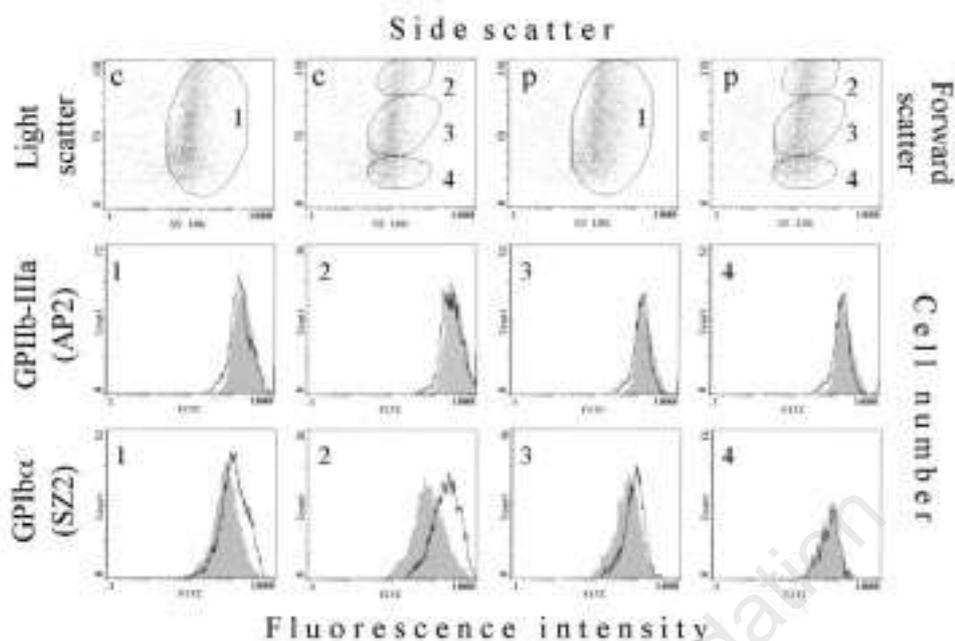


Figure 1. Flow cytometry analysis of platelet glycoproteins. Tracings obtained in patient B1 are reported. Upper line: linear forward scatter (vertical axis) versus log side scatter (horizontal axis). For both the control (c) and the patient (p) an electronic gate was drawn around the whole platelet cloud (1) and around platelet subpopulations with large (2), medium (3) or small volume (4). Intermediate and lower lines: linear cell count (vertical axis) versus log fluorescence (horizontal axis). Tracings refer to platelet fluorescence obtained in the patient (grey curve) and in a control (white curve) when the whole platelet population (1) or subpopulation 2, 3 or 4 was examined. In the patient, GPIIb/IIIa surface expression (intermediate line) was slightly increased compared to that of a control both in the whole platelet population (1) and in the three platelet subpopulations (2, 3 and 4), as shown by the shifting of the curves to the right. In contrast, surface expression of GPIb α (lower line) in whole platelet population (1) was lower in the patient than in the control. The GPIb α defect was much more severe in large platelets (2) than in medium-sized platelets (3). GPIb α expression of small platelets was similar in the patient and the control (4).

were transfected with a fragment of ABP lacking the GPIb/IX binding sites.²² On this basis, the authors concluded that defects of single components can alter the entire structural organization of the cytoskeleton with consequent effects on the expression of membrane GPs. Our observation that cells with a severe defect of both NMMHC-IIA and tubulin had a reduced surface expression of GPIb/IX/V further supports this conclusion.

The degree of GPIb/IX/V deficiency in MHA-SBS platelets is similar to that observed in a case series of patients with heterozygous Bernard-Soulier syndrome (BSS),¹⁵ a macrothrombocytopenia due to mutations of GPIb α , GPIb β or GPIX genes. Patients with the classical form of homozygous BSS have a severe bleeding tendency, little or no platelet GPIb/IX/V and defective platelet aggregation after ristocetin stimulation, while heterozygous subjects have a mild bleeding diathesis, a reduction of GPIb/IX/V to 20-70% of control values and a normal platelet response to ristocetin. Thus, although the molecular defects of MHA-SBS and BSS are different, both illnesses are characterized by a reduction of platelet GPIb/IX/V associated with platelet macro-

cytosis. Moreover, the platelet volume is greater in homozygous (severe deficiency of GPIb/IX/V) than in heterozygous (partial defect of the complex) BSS patients, and in MHA the defect of GPIb/IX/V is more evident in large platelets than in small ones. These results could suggest that a link exists between the production of macrothrombocytes and the defect of GPIb/IX/V, but further investigation is required to strengthen this hypothesis.

From a practical point of view, the identification of a GPIb/IX/V defect in MHA-SBS platelets raises the question of the differential diagnosis from heterozygous BSS. The two autosomal dominant macrothrombocytopenias undiscernibly share a partial defect of the GPIb/IX/V complex, normal *in vitro* platelet aggregation, and a mild bleeding tendency. A careful search for Döhle-like bodies in polymorphonuclear leukocytes and/or molecular biology techniques are, therefore, required to differentiate patients with heterozygous BSS from those with MHA-SBS.

Contributions and Acknowledgments

MDP and IFC performed the flow cytometry stud-

ies, AP was in charge of the immunocytochemistry. AS and MS performed the molecular biology investigations and contributed to the final writing of the paper. Other clinical and laboratory investigations were performed by PN. CLB was responsible for interpreting the results and writing the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no overlapping with previous papers.

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What is already known on this topic

May-Hegglin anomaly and Sebastian syndrome are inherited macrothrombocytopenias caused by mutations of the gene (*MYH9*) for the heavy chain of non-muscle myosin IIA (NMMHCIIA), the only myosin II expressed in platelets.

What this study adds

MYH9 mutations may be responsible for reduced surface expression of glycoprotein Ib/IX/V.

Potential implications for clinical practice

Since diagnosis of the heterozygous state for Bernard-Soulier syndrome is based on demonstration of reduced surface expression of glycoprotein Ib/IX/V, the present study indicates that patients with May-Hegglin anomaly and Sebastian syndrome could be misdiagnosed using this approach. A careful search for Döhle-like bodies in polymorphonuclear leukocytes and/or molecular biology techniques are, therefore, required to differentiate patients with heterozygous BSS from those with MHA-SBS.

Mario Cazzola, Editor-in-Chief