Background and Objectives. Storage pool diseases (SPD) are heterogeneous disorders associated with an abnormal presence of intraplatelet granules, which cause mild to moderate bleeding diathesis. We investigated signaling through tyrosine phosphorylation of proteins occurring in platelets with total or partial absence of dense- and α-granules in response to activation.

Design and Methods. We included a patient with severe δ-SPD, a patient with severe α-SPD or gray platelet syndrome, and six patients with partial deficiency of dense or α-granules. SPD was confirmed by electron microscopy evaluation of platelet ultrastructure. Platelet function was evaluated by bleeding time determination and conventional aggregometry. Platelet suspensions were activated with collagen and thrombin to analyze changes in tyrosine phosphorylation of proteins by electrophoresis and Western-blotting.

Results. Bleeding times were prolonged in all the patients included. Aggregation responses were slightly decreased in δ-SPD and normal in the rest of patients. Tyrosine phosphorylation in platelets from patients with partial forms of SPD was equivalent to that observed in control platelets, absent in response to collagen and thrombin activation in δ-SPD, and deficient only to thrombin activation in α-SPD.

Interpretation and Conclusions. Tyrosine phosphorylation of proteins in activated platelets is highly dependent on the substances contained in the dense-granules and moderately dependent on those contained in the α-granules. A minimum amount of intraplatelet granules ensures signaling through tyrosine phosphorylation of proteins.

Key words: dense-granules, α-granules, gray platelet syndrome, collagen, thrombin.
them are receptors for extracellular ligands, and others are non-receptors such as Src, and Syk. In early stages of activation a marked increase in phosphorylation of tyrosine residues occurs in several cellular proteins including tyrosine kinases themselves and their substrates. Some tyrosine kinases are activated by agonists independently of the aggregation, such as Src and Syk, whereas others, like FAK, become active during aggregation. It is believed that tyrosine phosphorylation may regulate its target protein causing a conformational change, or may provide a binding site for specific domains present in other proteins (SH2 or PTB domains). The precise roles of tyrosine kinases and their specific substrates in platelet function are currently under investigation.

Protein phosphorylation in tyrosine residues has only been studied in limited cases of SPD including patients with Hermansky Pudlak syndrome,9 and gray platelet syndrome,10 and in platelets from mice and black cattle with Chediak-Higashi syndrome. In order to contribute to the knowledge of the relationship between protein phosphorylation and platelet release mechanisms, we analyzed tyrosine phosphorylation of proteins in platelets from a group of patients suffering from different types of SPD.

Design and Methods

Patients

Our study was carried out in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all the participants. Eight patients with SPD were enrolled in the study: 1 with severe platelet deficiency of dense-granules (δ-SPD), 1 with severe deficiency of α-granules (α-SPD) or gray platelet syndrome, and 6 with a partial deficiency of dense- or α-granules (δ/α-SPD). The diagnosis of the disease was established by clinical and laboratory findings including platelet granule ultrastructural evaluation. von Willebrand’s disease and other non-platelet primary hemostatic defects were excluded, as were kidney, liver, and myelodysplastic disorders.

All subjects included in our study were carefully interviewed to exclude any possible intake of drugs known to affect platelet function in the 10 days preceding our investigations.

Experimental design

Platelet function was evaluated by measuring the bleeding time, and by standard aggregometric techniques. Dense- and α-granule deficiencies were established by transmission electron microscopy. Signal transduction processes occurring through tyrosine phosphorylation were also investigated in platelets activated in suspension by two different agonists, collagen and thrombin. Blood samples from healthy volunteers were processed in parallel.

Platelet function

The bleeding time was measured by the Ivy method and was considered normal up to 9 minutes. Whole blood was obtained by venipuncture and anticoagulated with citrate-phosphate-dextrose (100 mM sodium citrate, 16 mM citric acid, 18 mM sodium hydrogen phosphate and 130 mM dextrose) (at a final citrate concentration of 19 mM). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) samples were obtained by centrifugation of citrated blood samples at 2000 × g for 10 minutes, or at 800 × g for 20 minutes, respectively, at 22°C. Aggregation studies were carried out under stirring conditions in a Hitachi-Aggrecorder aggregometer. Samples of PRP were placed in 6 mm-wide siliconized cuvettes. Platelet counts were normalized to the same value (2 × 10^5 platelets/µL). The minimum and maximum amplitudes of the recorder were adjusted with PRP (0% transmission) and PPP (100% transmission), respectively. Arachidononic acid (1.2 mM), ADP (4 µM), collagen (2.5 mg/mL), epinephrine (10 µM) and ristocetin (1 mg/mL) were used as inductors, under stirring. Results were expressed as percentages of maximum aggregation obtained after 5 min of stimulation.

Ultrastructural studies

Peripheral venous blood was collected directly in 1.25% glutaraldehyde in White’s buffer saline, without any anticoagulant, as previously described. Briefly, after the fixed PRP preparation, a platelet pellet was obtained and post-fixed in 1% osmium tetroxide in White’s saline. Specimens were then dehydrated in a series of graded alcohols and embedded in Epon 812 following standard methods. The ultrathin sections were stained with uranyl acetate and lead citrate before being examined in a transmission electron microscope at 80 Kv accelerating voltage. Using computer-assisted morphometry, the size and shape of the platelets were measured as were the size and number per platelet area (number/µm^2) of the dense- and α-granules. Other intraplatelet structures, such as the open canalicular system (OCS), mitochondria, lipid droplets, and glycogen aggregates were also measured. Ultrastructural morphologic features of the above and other structures, such as the dense tubular system (DTS), were included in the analysis. Morphologic and morphometric results were
Enhanced chemiluminescence (ECL) method18 and blots were developed by the USA. The excess of antibody was removed by extensive washing and blots were probed with horseradish peroxidase-conjugated anti-phosphotyrosine recombinant antibody (Transduction Laboratories, Lexington, KY, USA). After blocking non-specific binding, Western-blot analysis was carried out with purified type I collagen or thrombin was carried out under stirring conditions in the aggregometer. Aliquots of platelet suspensions were kept at 37°C for 20 minutes before activation.

Activation of platelet suspensions with purified type I collagen or thrombin was carried out under stirring conditions in the aggregometer. Aliquots were activated with either 20 µg/mL of purified type I collagen (Chrono-Log Co, Haverton, PA, USA) or 0.1 U/mL of thrombin (Sigma Chemical, St. Louis, USA) for 90 seconds. Platelet aggregation was recorded and results expressed as a percentage of maximal aggregation.14 Activation was stopped by addition of Laemmli's buffer (125 mM Tris-HCl, 2% SDS, 5% glycerol and 0.003% bromophenol blue) containing 2 mM orthovanadate and 5 mM N-ethylmaleimide, and heated for 5 minutes at 90°C. Samples were kept at -20°C until electrophoretic analysis.

Evaluation of tyrosine-phosphorylated proteins

Equal amounts of proteins in non-activated and activated platelets were resolved by 8% SDS-polyacrylamide gel electrophoresis16 and transferred to nitrocellulose membranes7 (BioRad, Hercules, CA, USA). After blocking non-specific binding, Western-blotts were probed with horseradish peroxidase-conjugated anti-phosphotyrosine recombinant antibody (RC20) (Transduction Laboratories, Lexington, KY, USA). The excess of antibody was removed by extensive washing and blots were developed by the enhanced chemiluminescence (ECL) method18 (Amersham Pharmacia Biotek, Essex, UK).

**Results**

Clinical evaluation, platelet function and ultrastructural studies

Clinical characteristics, platelet counts, and ultrastructural platelet granule analysis are described in Table 1. Patient #1 was a female with a severe decrease of dense granules (δ-SPD) and absent release of ATP after stimulation of platelets with 2 mg/mL of collagen. Patient #2 was a man with a severe decrease of α-granules (α-SPD), previously described as a case of gray platelet syndrome,15 with an associated hereditary deficiency of plasma factor V. Patients #3 to 8 presented partial forms of SPD (δ/α-SPD), including dense- (5 cases) and α- (2 cases) granule deficiencies. Some of the patients belonged to families with other members affected.

Most patients had a moderate bleeding diathesis, combined in some cases with thrombocytopenia. Bleeding time was prolonged in four cases, including those patients with the most severe defects. Aggregometry was normal or slightly decreased in most cases, with the exception of patient #1 who showed marked aggregometric anomalies to the different agonists used.

Morphometric measurements on ultrastructural images (Figure 1, Table 1) of patients' platelets showed different numbers of dense- or α-granule per µm² of platelet area, with respect to normal individuals. Platelets from patient #1 contained normal α-granules but the dense-granules were decreased to 6% and revealed low electron density. Platelets from patient #2 had normal numbers of dense-granules without morphologic anomalies. However, the platelets contained only scarce α-granules with a marked reduction in size. A partial deficiency of dense-granules was observed in patients #3 to 6 and was sometimes associated with morphologic anomalies in these granules, such as fragmented dense cores or decreased electron density. A partial deficiency of α-granules was observed in patients #7 and 8 which was associated, in the former, with very irregularly sized and shaped α-granules and also many elongated forms.

As far as concerns the other ultrastructural features analyzed, the main anomalies observed were those affecting platelet size, and the following intraplatelet structures: OCS, DTS, and lipid droplets (Figure 1). All the patients except the one with δ-SPD (patient #1) had variably larger platelets with an increased amount of OCS. Some individual elements of OCS were dilated, giving swiss-cheese platelet images in the α-SPD patient, and in another with δ/α-SPD (patient #4). DTS was also more developed than normal in α-SPD, and in δ-SPD platelets (patients #1, and 2), as well as in 2 patients with partial SPD (patients #3, and 5). A lot of DTS complexes were observed in 3 of these patients (patients #1, 2, and 5). Finally, lipid droplets increased, to a variable extent, in all the cases studied.
Tyrosine phosphorylation after activation of platelets with collagen and thrombin

Activation of platelet suspensions by purified type I collagen and thrombin resulted in phosphorylation at tyrosine residues of multiple proteins, including p160, p150, p125, p100, p97, p85, p72, p66, p64, pp62, pp60, p58, p56 and p39 (Figure 2, lanes 2 and 3).

Only a total deficiency in δ-granules resulted in almost a total absence of signaling through tyrosine phosphorylation of proteins. Most of these proteins observed in control platelets after activation were not detected in profiles corresponding to platelet suspensions from patient #1, which were activated with collagen or thrombin (Figure 2, lanes 5, and 6). Interestingly, platelets deficient in α-granules, from patient #2, with gray platelet syndrome, responded significantly to collagen but not to thrombin in terms of tyrosine phosphorylation of proteins (Figure 2, lanes 8 and 9, respectively).

Activation of platelets from patients #3 to 8, with partial SPD, did not result in significant differences with respect to control samples. Profiles were qualitatively identical to those in control platelets, although intensity of phosphorylation at tyrosine residues was only slightly decreased (Figure 2, lanes 11 and 12 are representative of the 6 different patients with partial SPD).

Discussion

Storage pool diseases are heterogeneous disorders that cause a mild to moderate bleeding diathesis. SPD are difficult to identify by the methods commonly used in the laboratory to assess platelet function, due to the variability in response that in many cases may be even normal. We report studies on signaling through tyrosine phosphorylation of proteins. The studies were performed on platelets with a total or partial defect of intraplatelet granules, as demonstrated by ultrastructural analysis and morphometric measurements. Our present results indicate that signaling through tyrosine phosphorylation of proteins is absent only in those platelets with a total defect of dense granules.

Absence of the second wave of aggregation with ADP and epinephrine, and impaired response to collagen are aggregation patterns that have been commonly associated with SPD. However, it is currently accepted in the literature that SPD cannot be excluded by simple aggregation studies, since aggregation profiles are within the normal range in a considerably high number of patients with SPD.20,21 Our results using turbidimetric techniques...
are in agreement with those from previous reports, since aggregation responses were normal in most of the patients and diminished only in one patient, whose platelets exhibited a total absence of dense-granules.

Careful ultrastructural and morphometric studies combined with morphologic observation provide useful diagnostic tools, especially in those cases in which aggregation patterns are not confirmatory of the disease. In partial forms of SPD this methodology confirms the dense- or α-granule deficiency and can also quantify the degree of the defect as well as detect structural anomalies in the granules or in other platelet organelles. In the present study, two patients were diagnosed as having a severe SPD – one of dense-granules (δ-SPD) and another of α-granules (α-SPD) –, and six could be grouped as having partial forms of dense- or α-granule deficiency (δ/α-SPD). Morphologic anomalies were found in the granules (Figure 1, Table 1), but also in the development of OCS and DTS. Increased lipid droplets were detected in platelets from some of the patients included in the study. The ultrastructural anomalies found in OCS and DTS may represent structural defects that could play a role in the platelet dysfunction present in these patients. The presence of large platelets with highly developed OCS and DTS, found in some of the patients, could provide an extended membrane reservoir facilitating the inside-out mediated events. Whether these abnormal features are causative, reactive, or could just partially compensate the platelet dysfunction observed in SPD platelets, is still unknown.

Although platelets with SPD are deficient in granules, aggregating responses are within the normal range in a high proportion of cases. In the present study, we analyzed the signaling processes occurring through tyrosine phosphorylation of proteins after activation of platelets from patients with SPD. Although the group of patients included in the study is rather heterogeneous, it is representative of the nature of the disease, and the results obtained are quite conclusive. Our results indicate that a minimal amount of granules ensures signaling through phosphotyrosine proteins in response to collagen and thrombin. Dense granules are storage organelles for adenine and guanine nucleotides, amines such as serotonin and histamine, and bivalent cations.

Table 1. Clinical characteristics, blood platelet counts, and ultrastructural platelet granule analysis.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Sex</th>
<th>FP</th>
<th>Clinical bleeding</th>
<th>Platelets (x 10^9/L)</th>
<th>Bleeding time (min)</th>
<th>Platelet granules (%)</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>EB</td>
<td>MB</td>
<td>ST</td>
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<td>δ-SPD</td>
<td>1</td>
<td>57</td>
<td>F</td>
<td>−</td>
<td>++</td>
<td>+++</td>
<td>±</td>
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<tr>
<td>α-SPD</td>
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<td>29</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>δ/α-SPD</td>
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<td>F</td>
<td>+</td>
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<td>F</td>
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</table>

δ-SPD: δ-storage-pool disease; α-SPD: α-storage-pool disease; δ/α-SPD: partial dense- or α-storage-pool disease; FP: familial presentation; EB: easy bruising; MB: mucous bleeding; epistaxis, menorrhagia, Bleeding gums; ST: surgical or traumatic bleeding; *: granules with dysmorphic features.
The quantitative defect in signaling through tyrosine phosphorylation of proteins could be attributed to the absence of such substances. In this regard, it should be considered that adenine nucleotides are the main source of phosphate for protein kinases. Therefore, the enzymatic processes of protein phosphorylation could be expected to be abnormal. In addition, the tyrosine kinase Src has been described to be present in the dense-granules, apart from other locations, suggesting its involvement in the secretion process.

We also evaluated signaling through tyrosine phosphorylation in platelets from an α-SPD patient, with gray platelet syndrome (GPS). This rare disease is characterized by an almost complete deficiency of platelet α-granules. The link between the α-granule and functional defects has not yet been explained. Some patients with GPS have normal platelet aggregation, however, delayed aggregation with epinephrine and reduced aggregation with collagen and thrombin have been described in other patients. Interestingly, in our studies of a GPS patient, tyrosine phosphorylation patterns in response to collagen were both qualitatively and quantitatively normal, although quantitatively inhibited in response to thrombin. This finding could be in agreement with previous reports on GPS, in which delayed platelet aggregation with thrombin was described. In those cases, the abnormal response to thrombin was associated with a delay in calcium mobilization and secretion, and abnormal calcium transport into microsomes. The authors of those reports postulated that, apart from the absence of α-granules,
platelets with GPS could have a specific defect of thrombin receptor-mediated activation of phospholipase C. In normal platelets, this event leads to generation of second messengers such as IP3 and increases of intraplatelet calcium, which play an essential role in promoting both cytoskeletal organization and signaling through tyrosine phosphorylation of proteins. Defects at this level may compromise intraplatelet signaling.

Our present results are consistent with the concept that even a minimal number of intraplatelet granules guarantee signaling through phosphotyrosine proteins. In addition, our studies indicate that phosphorylation of proteins at tyrosine residues is highly dependent on substances contained in the dense-granules and only moderately dependent on those contained in \( \alpha \)-granules. Defects in signaling through tyrosine phosphorylation imply abnormal aggregating responses. Further studies on tyrosine phosphorylation occurring in platelets with SPD exposed to adhesive substrate under flow will be useful to understand the functional impact of these processes on platelet function.

Contributions and Acknowledgments

GA was the principal investigator. She carried out the experimental part of the study, and participated in the design, analysis and interpretation of the results. MDR and GE were responsible for the conception of the study and for its critical revision. We thank AO for his contribution to the conception of the discussion and critical revision of the study. NPM was responsible for the handling and interpretation of data and direct supervision. GA, MDR and NPM wrote the manuscript. All the authors approved the version sent to be published.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.


**Peer Review Outcomes**

**Manuscript processing**

This manuscript was peer-reviewed by two external referees and by Professor Carlo Balduini, Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Balduini and the Editors. Manuscript received March 11, 2002; accepted April 26, 2002.

**What is already known on this topic**

Mechanisms of platelet activation in storage pool diseases are poorly known since few patients have been studied in this respect.

**What this study adds**

The present study of eight patients showed that a minimum amount of intraplatelet granules is required to ensure signaling through tyrosine phosphorylation in activated platelets.

**Potential implications for clinical practice**

Evaluation of signaling through tyrosine phosphorylation after activation of platelets with collagen or thrombin will help to determine the severity of the disease.

Carlo Balduini, Associate Editor