platelets

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ORIGINAL ARTICLE

Megakaryocytic emperipolesis and platelet function abnormalities in five patients with gray platelet syndrome

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Abstract

The gray platelet syndrome (GPS) is a rare congenital platelet disorder characterized by mild to moderate bleeding diathesis, macrothrombocytopenia and lack of azurophilic α-granules in platelets. Some platelet and megakaryocyte (MK) abnormalities have been described, but confirmative studies of the defects in larger patient cohorts have not been undertaken. We studied platelet function and bone marrow (BM) features in five GPS patients with *NBEAL2* autosomal recessive mutations from four unrelated families. In 3/3 patients, we observed a defect in platelet responses to protease-activated receptor (PAR)1-activating peptide as the most consistent finding, either isolated or combined to defective responses to other agonists. A reduction of PAR1 receptors with normal expression of major glycoproteins on the platelet surface was also found. Thrombin-induced fibrinogen binding to platelets was severely impaired in 2/2 patients. In 4/4 patients, the BM biopsy showed fibrosis (grade 2–3) and extensive emperipolesis, with many (36–65%) MKs containing 2–4 leukocytes engulfed within the cytoplasm. Reduced immunolabeling for platelet factor 4 together with normal immunolabeling for CD63 in MKs of two patients demonstrated that GPS MKs display an alpha granule-specific defect. Increased immunolabeling for P-selectin and decreased immunolabeling for PAR1, PAR4 and c-MPL were also observed in MKs of two patients. Marked emperipolesis, specific defect of MK alpha-granule content and defect of PAR1-mediated platelet responses are present in all GPS patients that we could study in detail. These results help to further characterize the disease.

Introduction

The gray platelet syndrome (GPS) is a rare autosomal recessive platelet disorder characterized by lack of α-granules in platelets [1]. Clinical characteristics of GPS are moderate thrombocytopenia, mild-to-moderate bleeding diathesis, splenomegaly and high levels of serum vitamin B12. Three independent next-generation sequencing approaches identified *NBEAL2* as the gene responsible for GPS in 23 families with autosomal recessive disease [2–4]. *NBEAL2* autosomal recessive mutations were then identified in four additional GPS families [5]. *NBEAL2* encodes for a neurobeachin-like-2 protein that is predicted to participate in vesicular trafficking and may be critical for the development of platelet α-granules. *Nbeal2* knockout (*Nbeal2*^{−/−}) mice, generated from three different groups, displayed a GPS-like phenotype [6–8]. More recently, one family with an autosomal-dominant

form of GPS associated with a dominant-negative GFI1B mutation was described [9].

Different abnormalities of platelet hemostatic function – defective response to thrombin [10–14] and collagen [10–12, 14–18], variable response to ADP and ristocetin [10–12, 14, 17, 18] and normal response to arachidonic acid [10, 11, 14] – have been described so far in GPS patients. In one case, a specific defect of GPVI associated with abnormal response to collagen and convulxin was described [17]. In another case, defective platelet responses to thrombin, protease-activated receptor (PAR)1-activating peptide (AP) and PAR4-AP were described [19]. In *Nbeal2*^{−/−} mice, it has been found impaired aggregation of platelets by CRP, collagen and PAR-4 AP [7] decreased P-selectin exposure upon stimulation by thrombin, CRP [7, 8] and convulxin [7], whereas the activation of α_{IIb}-β_3 [7] or the binding of the fibrinogen to platelets [8] induced by the same agonists were normal.

With regard to bone marrow (BM) features, myelofibrosis is the most frequently reported finding in GPS patients. In the largest GPS patient cohort reported so far, which included 25 patients, 8 patients underwent BM evaluation and 7 had variable degree of myelofibrosis [20]. Recently, myelofibrosis

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was also described in nine months old $Nbeal2^{-/-}$ mice [8], whereas in younger mice (four months old), it was not found [6]. Emperipolesis (the presence of intact leukocytes within the cytoplasm of megakaryocytes [MKs]) has been described for the first time in one GPS patient by Falik-Zaccai et al. [21]. More recently, emperipolesis was found in the BM of one patient from a GPS family with autosomal dominant inheritance and GFI1B mutation [9]. Emperipolesis has been described in all three different $Nbeal2^{-/-}$ mice [6–8].

In this study, we report the results of platelet function studies and BM morphology in five GPS patients with *NBEAL2* mutations, whose clinical and genotypic characteristics have been previously described [5]. We demonstrate that extensive BM emperipolesis and impaired platelet responses to PAR1-AP are present in all analysed patients. Our results and a review of the literature suggest that these abnormalities might represent common features of GPS.

Patients and methods

Patients

We studied five patients from four unrelated families with GPS, confirmed by mutational screening of *NBEAL2*, whose main clinical and laboratory characteristics were previously detailed [5]. All the subjects or their legal guardians gave written informed consent according to the Declaration of Helsinki. Protocols were approved by the Institutional Review Boards of the centers in which patients were diagnosed and studied. Pedigrees of the families and the defective content of α-granules of probands are reported in Supplemental Figure 1.

Platelet studies

Platelet aggregation and ATP secretion

Platelet aggregation in platelet-rich plasma (PRP) was measured by light transmission aggregometry in probands of families 2 and 3, using different concentrations of ADP, collagen, epinephrine, ristocetin and PAR1-AP according to protocols routinely used at the institutions where the tests were performed. In the proband of family 2 platelet ATP secretion was measured by lumiaggregometry (Chrono-log, Havertown, PA), upon the addition of luciferin–luciferase reagent to PRP [22].

Flow cytometry

The expression of major platelet membrane glycoproteins (GPs) and of PAR1 was measured in probands of families 2 and 3. For studies in family 2, aliquots of PRP were incubated at room temperature (RT) for 30 minutes with: (i) APC-conjugated anti-GPIbα moAb HIP1 (Becton Dickinson, San Diego, CA); (ii) APC-conjugated anti-GPIIb HIP8 (Becton Dickinson); and (iii) unconjugated mouse anti-PAR1 ATAP2 (Invitrogen, Carlsbad, CA, USA), followed by 30-min incubation with FITC-conjugated goat anti-mouse IgG1 (BD Biosciences, San Jose, CA). Samples were run through a FACSCanto flow cytometer (Becton Dickinson).

In family 3, aliquots of PRP were incubated with PE-conjugated anti-PAR1 moAbs SPAN12 or WEDE15 (Beckman Coulter, CA) at RT for 30 minutes. Platelet population was identified by double-staining with FITC-GPIIIa. For platelet surface GP analysis, PRP was incubated with FITC- or PE-conjugated moAbs to GPIIIa, GPIbα and GPIX (BD Biosciences) and analysed by flow cytometry. Results are expressed as % of control platelet MFI.

Flow cytometric analysis of platelet activation

The exposure of P-selectin and the activation of integrin $α_{IIb}$-$β_3$ after stimulation by different agonists were analysed in probands of families 2, 3 and 4 using multiparameter flow cytometry.

For studies in probands of families 2 and 4, 10-fold Tyrode's buffer diluted PRP was incubated at RT for 15 minutes simultaneously with the agonist and a combination of: (i) PE-conjugated anti-P-selectin (clone CLB-Thromb/6, Beckman Coulter) plus FITC-conjugated anti-GPIb (Beckman Coulter) or (ii) FITC-conjugated PAC-1 (Becton Dickinson) plus PE-conjugated anti-GPIb (e-Bioscience, San Diego, CA, USA) moAbs. Samples were quench-diluted 1:10 with modified Tyrode's buffer and analysed by a FC500 Beckman-Coulter flow cytometer. Platelets were identified as GPIb-positive population.

In family 3, the same protocol as above was applied, except that FITC-conjugated PAC1 or FITC-conjugated anti-P-selectin (Becton Dickinson) were added together with ADP or PAR1-AP to platelet suspension for five minutes. PE-conjugated anti-GPIb (Becton Dickinson) was added at this step to identify the platelet population. In addition, platelets were fixed with 1% paraformaldehyde for 45 minutes before analysis by flow cytometry.

FITC-fibrinogen binding

This test was performed in patients of family 3. PRP was centrifuged in the presence of PGE1. Platelets were washed twice with modified Tyrode's buffer and adjusted to 10^8 platelets/mL. Washed platelets were stimulated by thrombin at RT for 15 minutes, and fibrinogen binding was studied using Alexa Fluor 488-conjugated human fibrinogen (Molecular Probes Inc., Eugene, OR). The platelet population was identified by PE-conjugated anti-GPIb (Becton Dickinson). Platelets were then fixed with 1% paraformaldehyde for 45 minutes and analysed by flow cytometry.

BM studies

BM biopsy samples were obtained from the probands of families 1, 3 and 4. Immunohistochemical analysis of paraffin-embedded specimens was performed by the avidin–biotin–peroxidase complex method. Expression of c-MPL, CD61, P-selectin, platelet factor 4 (PF4), CD63, PAR1, PAR4 on MKs was investigated using the following antibodies: mouse MoAb anti-human c-Mpl (R&D Systems, Minneapolis, MN), SZ21 anti-GPIIa (Immunotech, Marseille, France), mouse MoAb anti-P-Selectin 1E3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-PF4 (Chemicon International, Temecula, CA), ab1318 anti-CD63 antibody (NK1/C3) (Abcam, Cambridge, UK), ab32611 anti-human PAR1 and rabbit polyclonal anti-human PAR4 (GeneTex, Irvine, CA). The Abs were used according to the manufacturer's instructions.

For each subject, the percentage of MKs containing at least one leukocyte within the cytoplasm was calculated. Results obtained in patients were compared with those obtained in 10 consecutive subjects with newly diagnosed Hodgkin's lymphoma without BM involvement. Biopsies of patients and of controls were stained in parallel. Fibrosis was assessed according to the European consensus on grading BM fibrosis [23].

Results

Platelet studies

Immunofluorescence of α-granules content

The platelet α-granule content, as measured by identification of TSP-1-positive granules, was severely reduced in all the probands (Figure 1 supplementary data). The content of platelet PF4 was also measured in the probands of family 2 and of family 4; in all cases, the results were very similar to those obtained with TSP-1 [19].
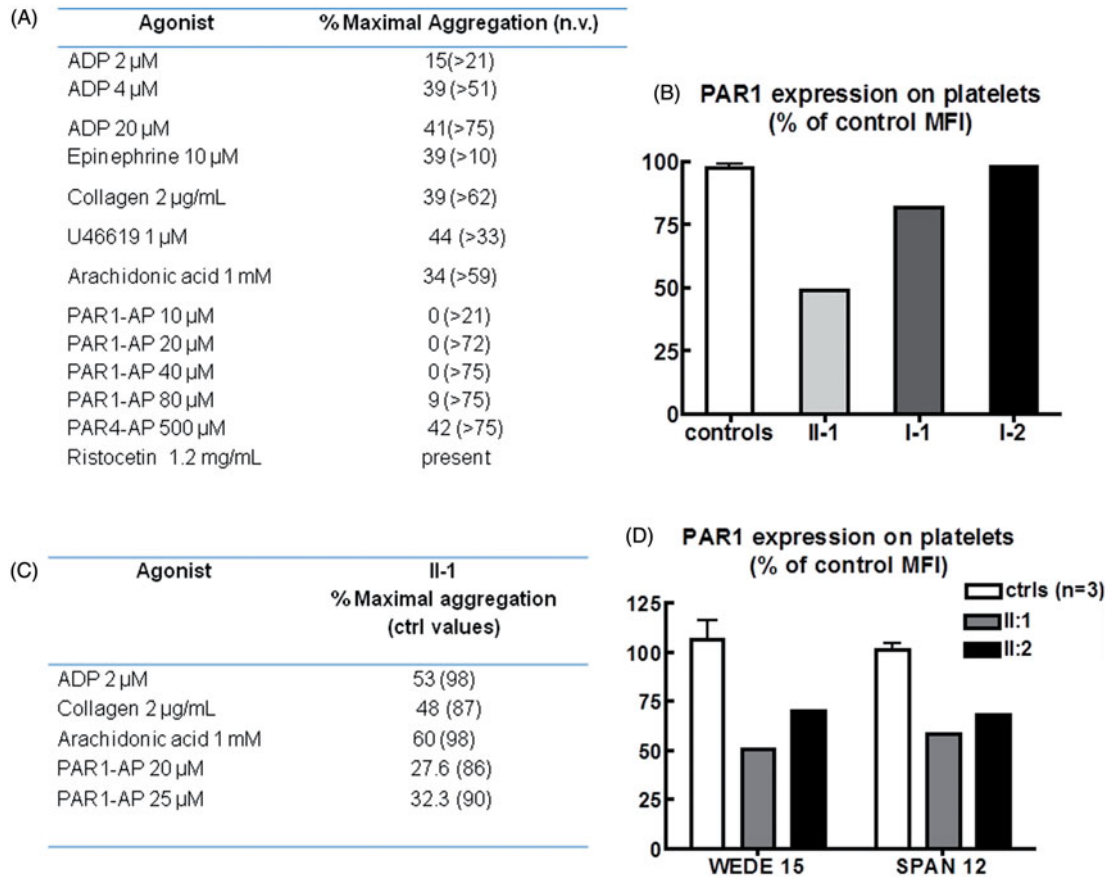
Figure 1. Results of platelet studies in families 2 and 3. (A) and (B) Family 2. (A) Platelet aggregation in the proband of family 2. Normal reference values were determined on 20 healthy subjects. (B) PAR1 receptors measurement on the platelet membrane of the proband and his parents by flow cytometry and FITC-conjugated anti-PAR1 MoAb. (C) and (D) Family 3. (C) Platelet aggregation in the proband II-1 of family 3 compared with a simultaneously tested control sample. (D) PAR1 receptors on the platelet membrane of the two probands II:1 and II:2 as measured by two anti PAR1 moAbs. These results are representative of two tests performed on different occasions.

Platelet aggregation and ATP secretion

Platelet aggregation and ATP secretion (not shown) induced by PAR1-AP were severely decreased in the proband of family 2, while those induced by other agonists were moderately reduced or normal (Figure 1A).

In family 3, platelet aggregation was performed only in subject II:1, as II:2 had a very low platelet count. Patient II:1 showed a severe defect of PAR1-AP-induced platelet aggregation together with mild-moderate reduction of platelet aggregation induced by other agonists (Figure 1C).

Expression of platelet membrane PAR1 receptors and major GPs

The expression of PAR1 on the platelet membrane was about 50% of normal in proband of family 2 and in subject II:1 of family 3 and 70 % of normal in subject II:2 of family 3 (Figure 1B and D). In these patients, the amounts of platelet GpIb, GpIIb, GPIIIa and GPIX were normal (not shown).

Flow cytometric analysis of platelet activation

P-selectin exposure induced by PAR1-AP or ADP was severely impaired in all patients (Figure 2A–C), while activation of α_{IIb}-β_3 was severely reduced when induced by PAR1-AP and normal (proband families 3 and 4) or only slightly reduced (proband of family 2) when induced by ADP (Figure 2A–C).

FITC–fibrinogen binding

Thrombin-induced FITC–fibrinogen binding to washed platelets of GPS patients from family 3 was severely reduced (Figure 2A).

BM histology and immunohistochemistry

Marrow fibrosis

BM biopsies of probands of family 1 and 4 had normal to increased cellularity and normal number of MKs. Staining for reticulin showed grade 2 fibrosis in both cases. The BM samples from both probands of family 3 displayed grade 3 fibrosis. The older age of these two patients (67 and 72 years) could account for the more severe degree of BM fibrosis (not shown).

Emperipolesis

Extensive emperipolesis was the most peculiar finding in all the four BM samples analysed. In particular, BM biopsy of the proband of family 1 had about 40% of MKs containing 1–3 leukocytes engulfed within the cytoplasm (Figure 3D), and the BM biopsy of the proband of family 4 had about 65% of MKs containing 2–4 leukocytes (Figure 3A–C). Staining for MK lineage-specific markers – CD61 and c-MPL – clearly showed that other intact cells were within the MK cytoplasm (Figure 3B and C). In the two probands of family 3, the marked fibrosis allowed the analysis of only a few BM areas. Extensive emperipolesis was also evident in the few MKs that could be identified in these samples (36–37% of MKs; Figure 3E).

Immunohistochemistry

This evaluation was possible on the BM samples of probands of families 1 and 4. Distribution of P-selectin was irregular and accumulated in clusters in most MKs (Figure 4). MKs from the
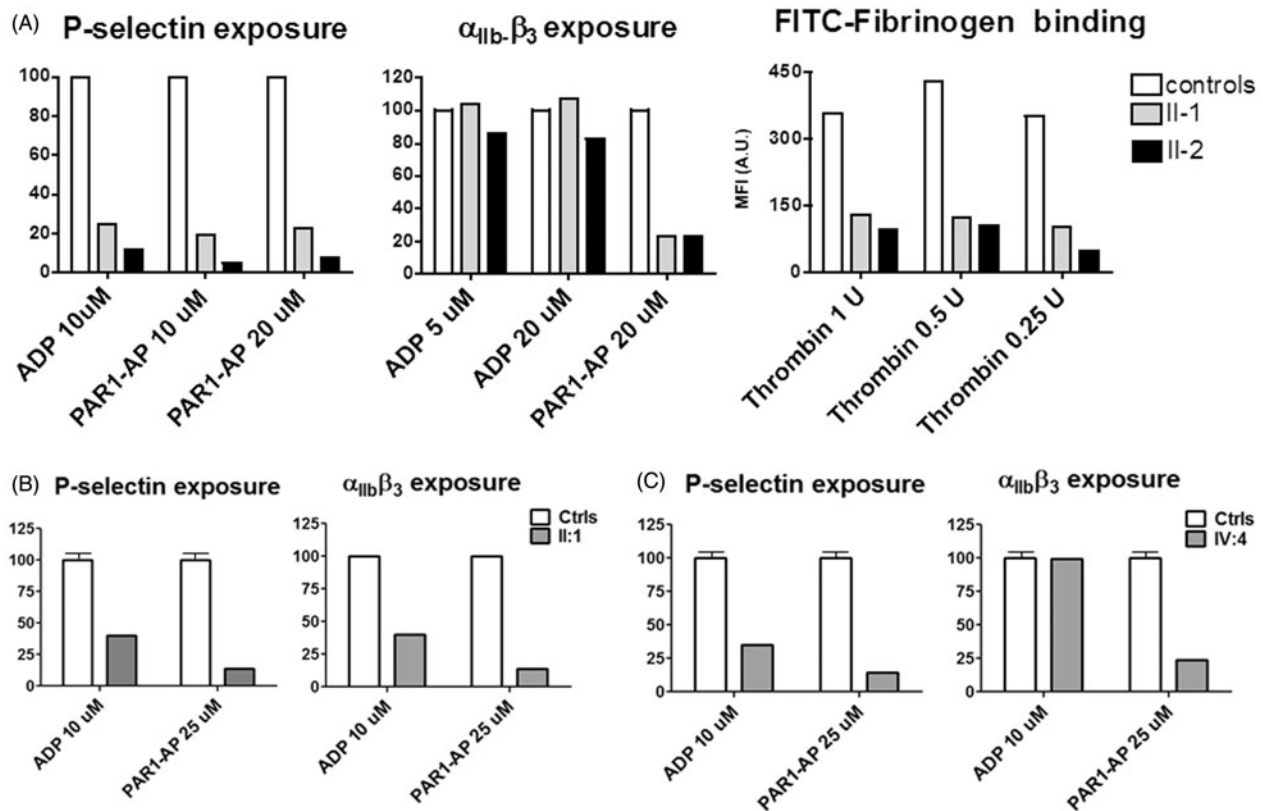
Figure 2. Platelet activation studies in probands of families 2, 3 and 4. (A) Activation studies in probands of family 3. Platelet P-selectin exposure and $\alpha_{IIb}\beta_3$ activation induced by different agonists are shown in the graphs on the left and in the middle, respectively. Results are expressed as % of MFI values of controls. Binding of FITC-conjugated fibrinogen to platelets induced by different thrombin concentrations is shown in the graph on the right. Results are expressed in arbitrary units. (B) and (C) Activation studies in the proband of families 2 (B) and 4 (C). Platelet P-selectin exposure and $\alpha_{IIb}\beta_3$ activation induced by different agonists are shown. Results are expressed as % of MFI values of controls.

proband of family 4 had increased immunoreactivity for P-selectin, while peripheral platelets of this patient had 30% of normal P-selectin content [19].

The secretory α-granule protein PF4 was significantly reduced compared with controls and appeared irregularly distributed within the cytoplasm (Figure 4). On the contrary, the staining for the lysosomal CD63 antigen was similar in GPS and control MKs (Figure 4).

Immunolabeling for PAR1 and PAR4 showed marked reduction of both receptors on GPS MKs, with some unstained MKs (*) (Figure 2 supplementary data). Several MKs were PAR1-negative in proband of family 4. In this patient, reduced amount of PAR1 in peripheral platelets was previously demonstrated [19]. Finally, c-MPL staining on MKs was reduced compared with controls (Figure 3C, controls not shown).

Discussion

The study of the platelet function in three GPS patients from two unrelated families showed that a defect of PAR-1-mediated platelet responses was the most consistent finding that can be paralleled by milder defective responses to other agonists. In one patient reported in this series (proband family 4), a severe isolated defect of PAR1- and PAR4-mediated platelet responses was previously described [19]. Noteworthy, although in $Nbeal2^{-/-}$ platelets the activation by CRP, collagen and PAR4-AP was decreased, the one by PAR4-AP was the most severely impaired [8]. Activation of α_{IIb}-β_3 by PAR1-AP was defective in all patients and was paralleled by normal or moderately reduced activation by ADP. Reduced binding of fibrinogen to

thrombin-activated platelets was also found in two patients. P-selectin exposure was defective upon stimulation by both ADP and PAR1-AP. This result in GPS patients mirrored the decreased P-selectin exposure upon platelet stimulation by several agonists in $Nbeal2^{-/-}$ mice [7, 8] and it is in agreement with reduced total content of P-selectin pool in GPS platelets.

The defective response to PAR1-AP was paralleled by partially decreased (50–30% reduction) PAR1 expression on GPS platelets. A similar pattern is observed when platelets are activated by thrombin, as a rapid reduction of intact PAR1 receptors on the platelet surface, with at least 40% of the cleaved receptors remaining on the surface, together with refractoriness to both thrombin and PAR1-AP occur under physiological conditions [24]. These findings, together with reduced PAR1 and PAR4 immunolabeling on MKs shown in the BM of two patients, suggest that PARs on MKs might be activated and undergo internalization or shedding in the BM, so that only a fraction of PAR receptors remain on nascent platelets. Since thrombin is not present in the BM, we might speculate that other enzymes or metalloproteases able to interact with PARs, like elastase or cathepsin G in neutrophils [25, 26] or MMP1 [27] or disintegrin and metalloprotease 17 (ADAM17)/TACE [28] in MKs, can be responsible for PARs activation/cleavage/shedding when released close to the MK membrane, as a consequence of emperipolesis (see below in the discussion). Noteworthy, a ''pro-inflammatory'' phenotype of $Nbeal2^{-/-}$ MKs, characterized by increased expression of inflammatory cytokines, has been recently reported by Guerrero et al. [8]. This change of GPS MKs could be responsible for the observed decrease of PARs, through mechanisms to be further investigated.
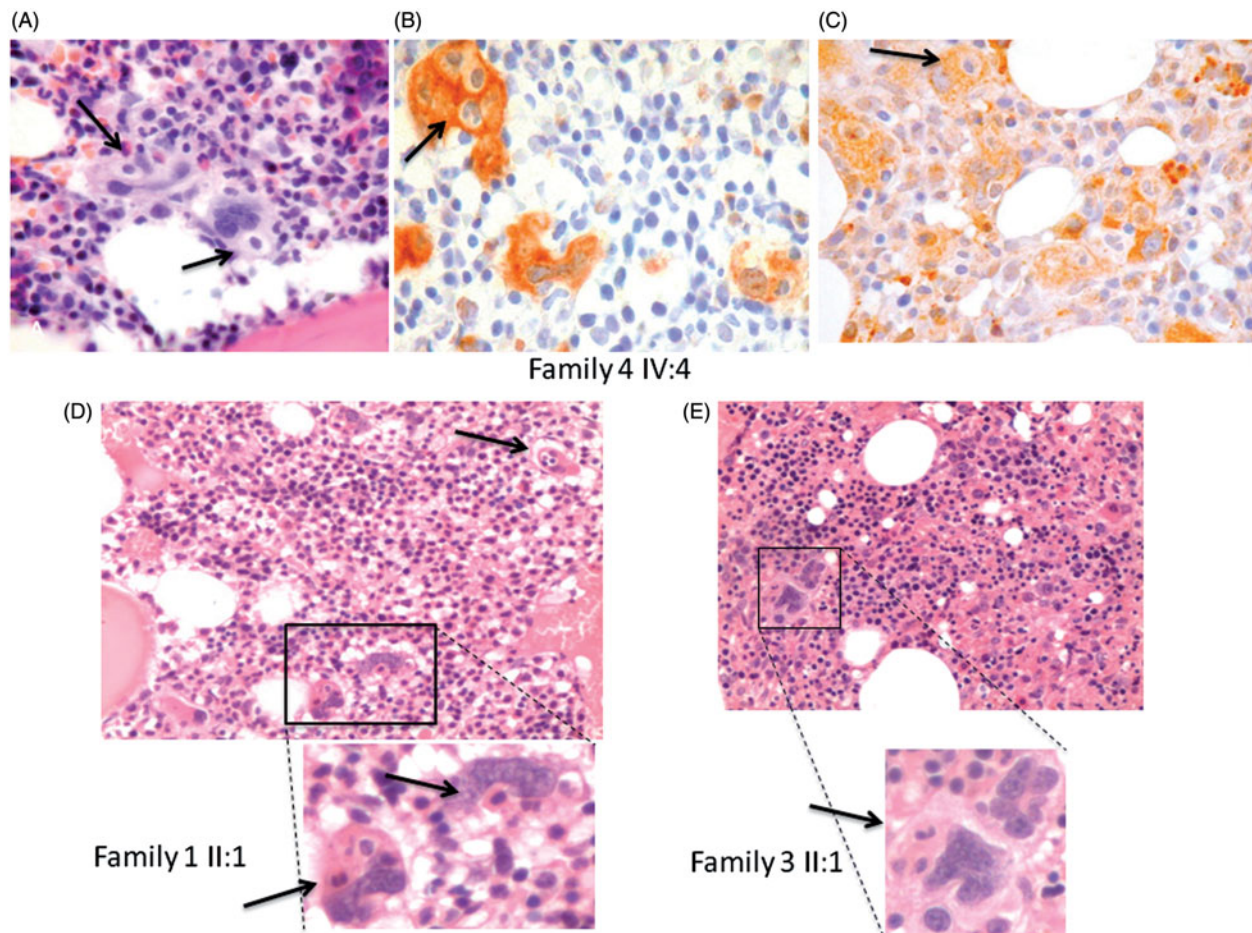
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Figure 3. Emperipolesis images on BM biopsies. (A–C) Proband of family 4. (A) Hematoxylin–eosin staining, (B) CD61 staining (C) cMPL staining. (D) Proband of family 1 and (E) Patient II-1 of family 3, hematoxylin–eosin staining. Arrows indicate leukocytes within MK cytoplasm. The most significant emperipolesis images are enlarged (insets).

In contrast to reduction of PARs, all our patients had normal amounts of major GPs (GpIb, GpIIb, GpIIA and GpIX) on platelet surface. Accordingly, normal amounts of αIIbβ3 and GpV (which is associated with GpIb in a multimeric complex) were recently reported on platelets form *Nbeal2*$^{-/-}$ mice [8]. It has to be outlined that the autosomal dominant GPS case associated with GFI1B mutation had reduced expression of GpIb on platelets [9]. Hence, *NBEAL2* mutation-associated GPS seems to differ from *GFI1B* mutation-associated GPS for the amount of platelet GpIb.

A review of the literature revealed that impaired platelet aggregation by thrombin and PAR1-AP was found in all GPS patients in whom this test was performed [10–14, 17, 21, 29, 30]. Therefore, we hypothesize that the defective PAR1-mediated platelet response, either isolated or combined to defective responses to other agonists, is a common defect in GPS patients and might be paralleled by reduced expression of PAR1 on the platelet surface. It is still to be investigated whether this feature is present also in the *GFI1B* mutation-associated GPS and in the other inherited macrothrombocytopenias.

We analysed BM samples from four GPS patients. All samples displayed severe BM fibrosis (grade 2–3), in agreement with previous reports [20]. BM fibrosis was traditionally considered non progressive in GPS [30], but it has been recently reported that development and progression of fibrosis in GPS could have a clinical and prognostic relevance because of the worsening of thrombocytopenia [20]. In our series, the two oldest GPS patients (probands of family 3) had the most severe degree of fibrosis and of thrombocytopenia.

The most peculiar feature in the BM biopsies of all our patients was the presence of extensive emperipolesis. In this study, for the first time, we report emperipolesis in a series of four patients with *NBEL2* autosomal recessive GPS. In our patients, emperipolesis could be observed in up to 65% of MKs. Increased and pathologic emperipolesis is frequently observed also in the BM of patients with primary myelofibrosis (PMF), although it has been found in only 11–18% of PMF MKs [31], and in the BM of animal models of myelofibrosis [32, 33].

The presence of marked emperipolesis in GPS patients, irrespectively of their molecular defect, and in all three differently generated *Nbeal2*$^{-/-}$ mice suggest that it may have a role in the pathogenesis and/or the phenotype of the disease.

Immunohistochemical staining performed on BM samples of our patients showed that the α-granule secretory protein PF4 was reduced in GPS MKs. The GP CD63, a marker of δ-granules and lysosomes, was normal, thus confirming the presence of a specific α-granule defect in these MKs. This is in agreement with previous report of lack of α-granule secretory proteins in GPS MKs [34, 35]. On the contrary, the α-granule membrane protein P-selectin was increased and irregularly distributed in GPS MKs, as already reported in another GPS patient with emperipolesis [21]. Noteworthy, P-selectin was reported to be abnormally distributed in platelets of *Nbeal 2*$^{-/-}$ mice [6]. Increased or mislocalized P-selectin on the GPS MKs membrane might, in principle, cause emperipolesis and play a key role in the development of marrow fibrosis, according to mechanisms hypothesized for patients with PMF and for mouse models of myelofibrosis-like syndromes. Mislocalization of P-selectin could result in the trapping of
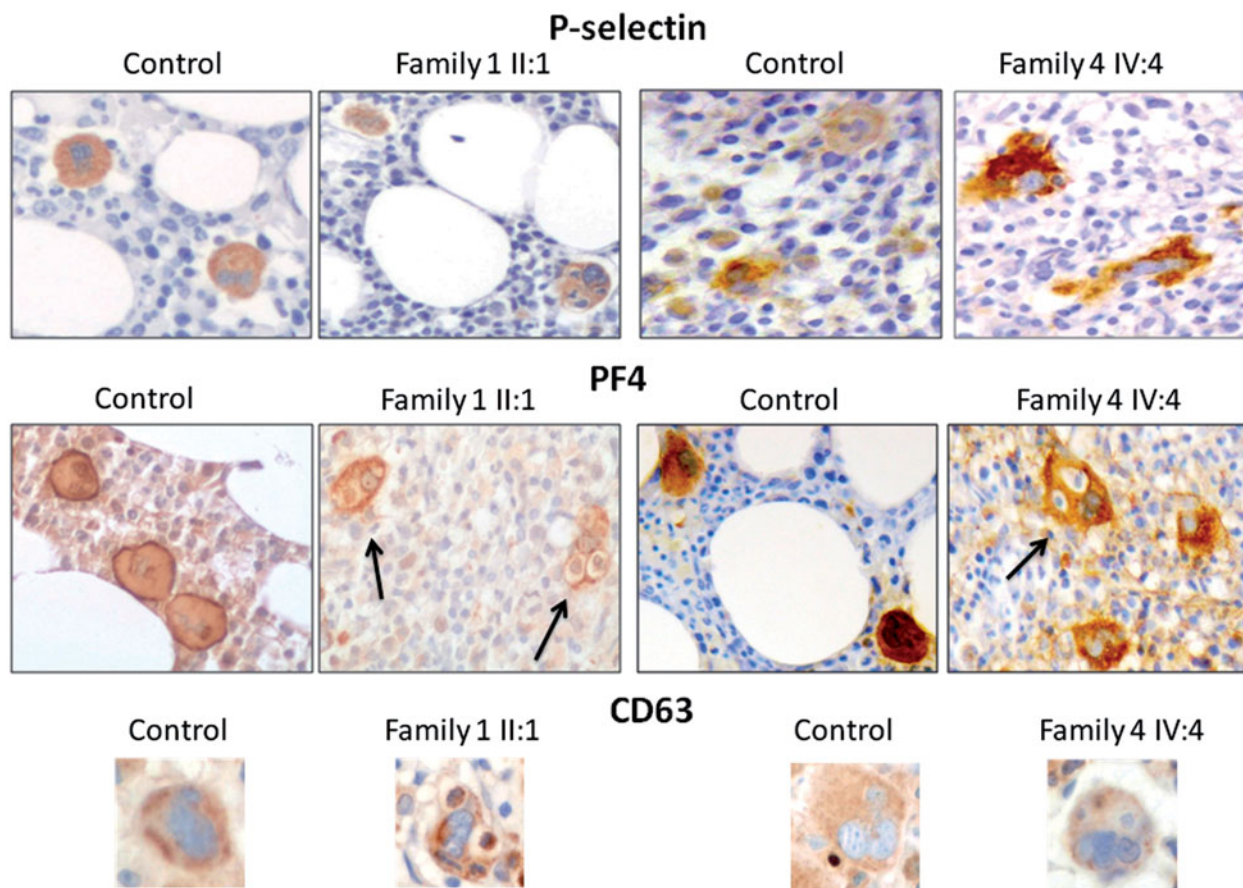
P-selectin

PF4

CD63

Figure 4. P-selectin and PF4 content in MKs of probands of families 1 and 4. Patients samples and controls were stained in parallel. P-selectin staining was increased in proband of family 4 and irregularly distributed within the cytoplasm. PF4 staining was reduced in both patients compared with controls, whereas CD63 was similar in the two GPS patients compared with controls. Arrows indicate emperipolesis. These images are representative of three different slides.

neutrophils – through the P-selectin GP ligand 1 – during their physiological BM exit through the MK cytoplasm, resulting in increased emperipolesis. Leukocytes trapped within the MKs can favor the release of fibrogenic MK cytokines, such as PDGF and TGF-β, and of neutrophil proteases within the BM environment, thus driving the development of fibrosis [31, 32, 36]. Indeed, it has been recently demonstrated in *Nbeal2*$^{-/-}$ mice that the lack of α-granules is due to their loss from mature MKs and that the leak of proteins from α-granule causes the development of BM fibrosis [8].

Interestingly, there are other features of GPS that are similar to PMF, in addition to fibrosis and emperipolesis: (1) splenomegaly is frequent both in PMF and GPS and extramedullary hematopoiesis was also reported in one GPS patient [18]; (2) c-Mpl expression may be reduced both in GPS [37] and PMF; (3) serum Vit B12 levels are increased in both conditions; and (4) the presence of a pro-inflammatory phenotype. Further studies are required to investigate whether these symptoms/signs might have common mechanisms in the two conditions.

Conclusions

We show that extensive emperipolesis is the most interesting feature in the BM of four GPS patients with *NBEAL2* biallelic mutations, and we hypothesized that it may have a role in the pathogenesis of the disorder.

In addition, we show in three more patients that a defect of PAR1-mediated platelet responses, isolated or combined to defective responses to other agonists, is a consistent finding in

GPS patients and can be paralleled by PAR1 decreased amount on platelet membrane.

Declaration of interest

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Supplementary material available online
Supplemental Figures 1 and 2.