Defective collagen-induced platelet activation in two patients with malignant haemopathies is related to a defect in the GPVI-coupled signalling pathway

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Summary

The occurrence of a thrombocytopathy concomitantly to the development of a malignant haemopathy has been reported for some time, but little is known about the mechanism(s) involved in the platelet dysfunction. Platelet glycoprotein VI (GPVI) has now been identified as a principal platelet receptor for collagen. In this paper, we report the cases of two patients with a myelodysplasia and a B lymphopathy, respectively, who presented with thrombocytopathy in relation to a defective GPVI-mediated platelet reactivity to collagen. Thus, with regard to the different steps of adhesion, activation secretion or aggregation, patients' platelet responses to collagen and to the GPVI specific agonists, collagen related peptide (CRP) or convulxin were null or dramatically impaired. Platelet responses to other agonists ADP, TRAP, Arachidonic acid were normal or showed only a moderate decrease. GPVI content was repeatedly normal, and binding of specific ligands, such as convulxin, satisfactory. Nevertheless, specific activating monoclonal antibodies and convulxin failed to induce platelet secretion; collagen, CRP or convulxin were unable to provoke calcium mobilisation. Furthermore, using a perfusion chamber model, we showed that *ex vivo* collagen-induced thrombi formation was very impaired. Taken together, these data provide evidence, for the first time, of an acquired defect in GPVI-mediated platelet reactivity to collagen, which reflects data observed in constitutional GPVI deficiencies, in two patients with malignant haemopathies.

Keywords

Collagen, glycoprotein VI, platelet function, myelodysplasia, chronic lymphocytic leukaemia

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Introduction

Although a thrombocytopathy, characterized by defective platelet secretion and aggregation, has been reported for some time in myelodysplasias (1, 2), in acute myeloblastic leukaemias, or more seldom in acute lymphoblastic leukaemias and chronic lymphocytic leukaemias, little is known about the biochemical abnormalities leading to the platelet dysfunction. In contrast, collagen present in the vascular subendothelium, is a major determinant of the thrombogenicity of the blood vessel wall. Platelet-collagen interactions are multivalent and involve a first stage of contact, mediated at high shear rate by platelet glycoprotein (GP) Ib, via von Willebrand factor, followed by platelet firm adhesion and activation, mediated by $\alpha_2\beta_1$ integrin and GPVI. Recent data emphasize the importance of a concerted interplay of these receptors: GPIb in synergy with $\alpha_2\beta_1$ mediating primary adhesion reinforced by activation through GPVI, which further regulates the thrombus formation (4, 5). Secretion of platelet granules, recruitment of additional platelets by the released secondary agonists (ADP and thromboxane A2) and aggregation result in thrombus formation. Over the last few years, the role of GPVI has been shown to be crucial in platelet activation triggered by collagen (6). GPVI belongs to the superfamily of immunoglobulin cell receptors and is expressed as a non-covalent complex with the common signalling subunit of these receptors, the Fc receptor γ chain (FcRγ) (7, 8). Collagen-induced GPVI clustering results in the phosphorylation of the FcRγ chain on its ITAM (immunotyrosine based activation motif), allowing the re-

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cruitment of a large complex of signalling molecules, including kinases, such as P72syk and p85-PI3-kinase, and adapter proteins (i.e. LAT and SLP76) and leading to phospholipase $C\gamma_2$ ($PLC\gamma2$) activation. Finally, Ca^{++} mobilization and protein kinase C activation result in secretion, activation of $\alpha_{IIb}\beta_3$ and aggregation (9). These important advances were permitted by the description of rare patients presenting either auto-antibodies against GPVI (10–13) or deep constitutional deficiencies in GPVI (14, 15). Of note, after platelet adhesion, GPVI-mediated platelet activation is reinforced by an integrin $\alpha_2\beta_1$ mediated outside/in mechanism leading to the phosphorylation of many of the proteins in the GPVI-FcRγ-chain cascade, including Src, SLP-76 and $PLC\gamma_2$ as shown recently (16).

We report the cases of a 50-year-old patient with myelodysplasia and a 77-year-old patient with chronic lymphocytic leukaemia, both presenting with haemorrhagic manifestations in relation to a lack of platelet reactivity to collagen, although reactivity to the other agonists: ADP, TRAP, arachidonic acid was subnormal or only slightly decreased. This anomaly could be attributable, at least in part, to a defect in the GPVI-mediated signal transduction pathway. Despite a normal amount of platelet GPVI, platelet responses to the specific ligands of GPVI, that is collagen-related peptide (CRP) (17) or the snake venom protein convulxin (18, 19), were null or very impaired, thus suggesting a defect in GPVI-mediated signalling. This mechanism reported here for the first time, is in agreement with the functional platelet defect observed in exceptional constitutional deficiencies in GPVI, but could be more frequently involved in malignant haemopathies, where it might account for previously unexplained thrombocytopathies and haemorrhagic manifestations.

Patients

The first patient was 50 years old, and presented at St. Antoine's hospital with anaemia and leuconeutropenia in relation to a myelodysplasia without blast excess or cytogenetic abnormality. The occurrence some months later of a purpura of the inferior limbs in this patient, who had not previously experienced significant haemorrhages, led to haemostatic studies being carried out in our department, with informed patient consent, as required by French legislation. The platelet count was normal, but the bleeding time was repeatedly very prolonged (more than 15 min, 1440 microliters versus less than 4 min, 120 µl, in the normal range). VIIIc-von Willebrand complex was in the upper limit of the normal range (at about 140%). Platelet function studies repeatedly revealed a lack of reactivity to type I collagen although platelet aggregation to ADP, arachidonic acid, TRAP or ristocetin was satisfactory; the patient first received danazole, which was combined 2 years later with erythropoïetin. Three years after the onset of the disease, a pre-acutisation occurred (circulating blasts inferior to 5%); since platelet aggregations were unchanged, studies with CRP and convulxin were performed thrice during the preacutisation phase when the myelogram showed a slight excess of blasts (inferior to 15%) with or without circulating blasts (<5%) and with a platelet count superior to 100 G/L, and showed a defective GPVI-mediated signalling. Subsequent chemotherapy (Aracytine, Idarubicine and then Aracytine Topotecan) did not obtain medullar remission. Aplasia was very prolonged and severe; in particular thrombocytopenia was very deep, preventing further studies of platelet function. The transformation towards a rapid fatal myelomonoblastic leukaemia occurred 18 months after the onset of the preacutisation state.

The second patient was a 77-year-old woman with untreated chronic lymphocytic leukaemia, and no past history of bleeding, who presented at Bichat hospital with pulmonary embolism confirmed by angioscanner. At day 10 of treatment with LMW heparin, she developed extensive bleeding with ecchymoses and haematomas at the sites of injection. Heparin administration was stopped. Platelet count was 102 G/L but heparin-induced thrombocytopenia was ruled out. The PFA-100 closure times were prolonged (Col/Epi >300 sec, Col/ADP 191 sec), anti-platelet antibodies and anti-PF4/heparin antibodies were negative. Von Willebrand factor was normal as was ristocetin-induced platelet aggregation. However a complete lack of aggregation in response to 4 µg/ml collagen led to the analysis of platelet function. The patient passed away suddenly a few months after her hospitalization.

Reagents and methods

Reagents

Platelet agonists

ADP and ristocetin were purchased from Diagnostica Stago (Asnières, France); arachidonic acid from Sigma Laboratory (St Quentin Fallavier, France); thrombin receptor activating peptide (TRAP) from Neosystem (Strasbourg, France); human thrombin was prepared in the laboratory as described (20). Type I collagen was from General Diagnostics, Chronolog (France), Chemicon (Temecula, CA, USA), collagen Horm from Nycomed Pharma GmBH (Germany); type III collagen from Sigma and from Chemicon. Collagen-related peptide (CRP) was kindly provided by Dr Richard Farndale (Cambridge, UK). Convulxin was purified from the venom of Crotalus durissus terrificus (Latoxan, Valence, France).

Antibodies

To defect platelet antibodies, fluorescein conjugated rabbit polyclonal anti IgG or IgM purchased from Cappel (Tebu, France) was used.

Antiplatelet CD41, CD42a, CD42b, CD36 and CD62P respectively directed against platelet GPIIb/IIIa, GPIb/IX, GPIbα, GPIV and P-selectin, and anti–$\alpha_2\beta_1$ integrin (GPIa/IIa) (clone Gi9) were purchased from Beckman-Coulter-Immunotech (Marseille, France) and were directly coupled to FITC for flow cytometry studies . Anti-α_2 chain 6F1 was kindly provided by Prof B. S. Coller (Mount Sinai Medical Center, New York, NY) and AB1936 from Serotec (Oxford, UK); anti-β_1 subunit 31H4 and 16B4 were from Serotec and 4B4 from Beckman-Coulter-Immunotech.. Specific FITC-coupled anti-mouse antibody was from TEBU. Concerning platelet GPVI, the anti-GPVI FITC-coupled MoAb 3J24.2 already described (21) was used for flow cytometry, and MoAb 9E18.2 was used for activation. Polyclonal anti-GPVI IgGs, kindly provided by Prof M. Okuma were from the same batch as in previous studies (21) and anti FcR γ-chain was from Upstate Biotechnology (Lake Placid, NY).

Anti-phosphotyrosine MoAb (PY20) was from Transduction Laboratories (Lexington, FY),anti-Syk, PLCγ2 and SLP76 from Santa-Cruz Biotechnology (Santa Cruz, CA), anti-LAT from Upstate Biotechnology (Lake Placid, NY). Peroxydase-coupled secondary antibodies (anti-mouse and anti-rabbit IgGs) and protein A were from Amersham (Les Ulis, France).

Methods

Platelet aggregation and adhesion

Venous blood obtained from patients or from consenting healthy human donors (controls) was collected on 3.8% citrate anticoagulant or on ACD-A. Platelet rich plasma (PRP) was obtained from citrated blood after centrifugation at 80g during 10 min at 15°C-20°C. Washed platelets were prepared from blood collected on ACD-A as previously described (19, 22).

Platelet aggregation was performed in a Coultronics aggregometer, in PRP, with a platelet count between 150 and 400 x 10^9 /L in patients and controls. Aggregation was also measured using washed platelets (3 x 10^8/ml). Platelet adhesion to immobilized types I and III collagen or convulxin, was performed under static conditions (19, 22).

Antiplatelet antibodies

Platelet-associated Immunoglobulins (Ig) (IgG or IgM) were screened at 22°C using the platelet immunofluorescence test (23). Circulating platelet autoantibodies were screened by immunofluorescence for IgG or IgM and ELISA for IgG. In case of IgG, the characterization was done by a MAIPA test using specific anti GPIIb/IIIa, anti GPIb/IX, and anti GPIV monoclonal antibodies (24).

Flow cytometry

Washed platelets (3 x 10^8/ml) were incubated with saturating concentrations of monoclonal antibodies (25). Each antibody was detected either using FITC-coupled first monoclonal antibodies, or using a secondary FITC-conjugated anti-mouse antibody. Quantification of fluorescence was carried out using a FacScan Flow Cytometer (Becton Dickinson). Measurements were made at least three times during the clinical course. For GPIIb/IIIa, GPIb/IX, GPIbα and GPIV, results were recorded in arbitrary units of fluorescence, expressed as a percentage of mean normal values obtained in at least 30 normal subjects and GP quantification was performed using calibration beads (Byocytex, Marseille, France). GPVI expression was measured using FITC-coupled anti-GPVI MoAb 3J24.2 while GPVI functional expression was tested by measuring the binding of FITC-coupled convulxin (20nM) as already described (21).

Exocytosis of α-granules was investigated by measuring the expression of P-selectin (CD62P) after stimulation by different agonists. Washed platelets were activated by convulxin, human thrombin, or an activating anti-GPVI MoAb 9E18.2 (5 µg/ml) which induces 80% platelet aggregation in 10 min (C. Lecut, M. Jandrot-Perrus personal communication) before labeling with a FITC-coupled anti-CD62P antibody and flow cytometry.

Immunoblotting, ligand blotting and immunoprecipitation

Platelets (2.10^9/ml) were lysed in a buffer composed of 1% v/v NP40 in 12 mM Tris, 300 nM NaCl, 12 mM EDTA containing 0.2 mM PMSF, 2 µM leupeptin and 5 KIU aprotinin and 2% SDS for 5 min at 100°C. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (21). The membranes were incubated either with polyclonal anti-GPVI or anti-FcRγ chain IgGs, detected using peroxidase-coupled protein-A. Ligand blotting was performed as reported using ^{125}I-convulxin (19) and auto-radiography.

After stimulation by agonists, either in aggregation or adhesion tests, protein-tyrosine-phosphorylations were analyzed after platelet solubilisation in the presence of vanadate by immunoblotting with an anti-phosphotyrosine MoAb (22, 26)

For immunoprecipitation, platelet lysates were precleared with protein A/G sepharose. GPVI was immunoprecipitated using polyclonal anti-GPVI IgGs and protein A-sepharose. PLCγ2 was precipitated using corresponding specific antibodies (1 µg/ml) and protein A/G-sepharose. Proteins were analyzed by SDS-PAGE and immunoblotting as above.

Intracellular calcium concentration

Increases in intracellular calcium concentration ([Ca^{2+}] i) were measured using fura-2-loaded platelets as described (19). Fluorescence was measured at 37°C using two excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm on a Hitachi H-2000 spectrofluorometer (Sciencetec, Les Ulis, France). [Ca^{2+}]i was calculated using a Kd of 224 nM for the interaction between fura-2 and Ca^{2+} (27).

Platelet adhesion and thrombus formation in an *ex vivo* perfusion chamber

The model used has already been described (28). Briefly, native non-anticoagulated blood from a forearm vein was drawn straight from a 19-gauge butterfly infusion set (Venisystems, Abbot Lab, Ireland). This set was connected to the glass cylindrical chamber coated with human type I or type III collagen. Blood was perfused for 4.5 min at a shear rate of 1600 s^{-1}. At the end of the perfusion, chambers were rinsed, fixed, and double-embedded in epon. Semi-thin cross-sections were cut 5 mm downstream of the proximal end of the cylinder and stained (0.01% toluidine blue and 0.01% fuchsin) for light microscope computer-assisted morphometry. The microscope view of the thrombi was displayed on a color video monitor, (Microvitec, HL series) at a final magnification of X 1400 through a video camera (Sony, 3CCD) fitted onto the photographic lightpath of the microscope (Zeiss, Axioplan). Thrombi were automatically recorded and contrasted by a color effect generator (NS 15000) and data collected using the Lucie program (Microvision, Evry, France). The percentage of the inner chamber surface coverage with platelets and/or fibrin (i.e. % adhesion), and the average thrombus volume per unit area ($\mu m^3/\mu m^2$) were determined according to Sakariassen et al (29).

Results

Platelet aggregation

For patient 1, platelet aggregations (PRP) were repeatedly subnormal in response to ristocetin (1 mg/ml), to ADP (at 0.6 to 2.4 µM) or to TRAP (at 10 µM), decreased after arachidonic acid ($5x10^{-4}$ M) at 30% the normal values, but were constantly null

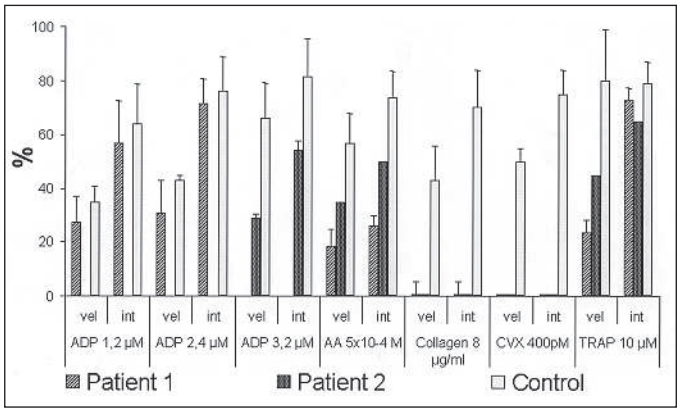

Figure 1: Main platelet aggregations in Platelet Rich Plasma (PRP). Aggregations were performed in PRP with a platelet count comprised between 150 and 400x109/L like in controls. ADP, Arachidonic acid (AA), TRAP, collagen and convulxin (CVX) were used. For collagen, type I collagen was considered from General Diagnostics, Chemicon or Horm, at concentrations ranging up to four times the usual ones. Here the data are shown for collagen from General Diagnostics (patient 1) or Horm (patient 2). Data are expressed in % (mean + SD). For patient 1 experiments were done five times with ADP, AA and collagen, twice with convulxin and TRAP. For patient 2 experiments were done twice. Data of controls correspond to n=20; except for convulxin n=10.

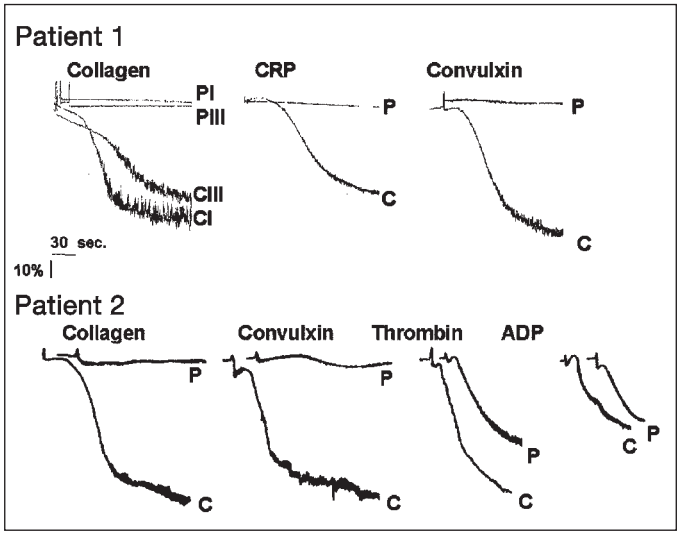

Figure 2: Platelet aggregation on washed platelets. Aggregation of washed platelets (3 x 10^8/ ml) was initiated by the addition of: Upper panel: 40 µg/ml of type I collagen (Chemicon) or 10 µg/ml of type III collagen (Chemicon); CRP (250 µg/ml) or convulxin (1 nM). Lower panel: 2 µg/ml of Horm collagen, convulxin (2.5 nM), human thrombin (0.5nM) or ADP (2µM). C: platelets from a control subject, P: patient's platelets. For patient 1, platelet aggregations to types I and III collagens are shown (respectively PI and PIII curves) in comparison with the control's platelet aggregations to type I and III collagen (respectively CI and CIII curves).

after stimulation by type I collagen for concentrations reaching 4-times the usual higher concentrations, suggesting a platelet defect specifically affecting collagen-induced responses (Fig. 1). In the case of patient 2, platelet aggregation in PRP in response to 3.2 µM ADP, to TRAP and to arachidonic acid was decreased by about 30% when compared to controls. Collagen, at concentrations reaching four times the usual, also failed to induce platelet aggregation (Fig. 1). Nevertheless, a slight response was obtained at very high concentration: 10 µg/ml, 5-fold the usual ones (velocity 15%; intensity 25% versus respectively 70% and 75% in controls).

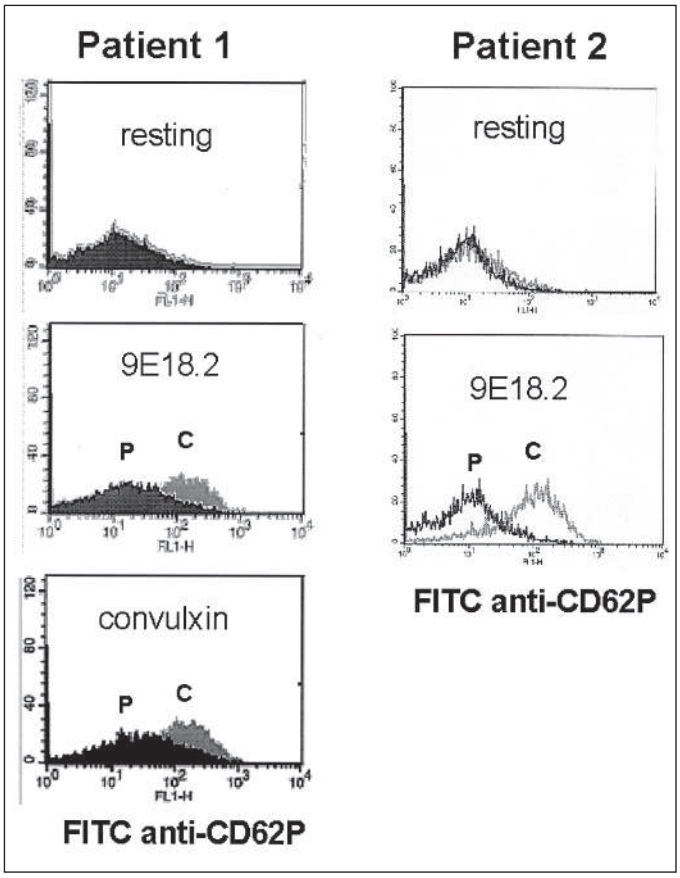

Figure 3: Platelet Alpha granule secretion. Washed platelets (5 x 10^7 /ml) from the patient (P) or from a control subject (C) were incubated with an activating anti-GPVI MoAb 9E18.2 (10 µg/ml) or convulxin (1 nM) for 10 min. FITC labeled anti-Pselectin was then added and platelets analyzed by flow cytometry.

In both cases, the patient's serum neither activated platelets nor inhibited collagen-induced platelet activation of platelets from control subjects and no specific platelet autoantibodies were detected. A defect in platelet granules was ruled out: the number of dense granules and their content in serotonin were normal; platelet α-granules were present and their content in β-thromboglobulin was also within the normal range.

Platelet activation was further investigated using isolated washed platelets (Fig. 2). Type I and type III collagen (for concentrations ranging up to 10-fold the usual concentrations) failed to induce patient 1's platelet aggregation. Similarly, platelets from patient 2 failed to aggregate in response to type I collagen at a concentration up to 4-fold the normal concentration. In contrast, ADP and thrombin-induced platelet aggregation were subnormal. In PRP and washed platelets, GPVI-specific agonists were then tested. In the case of patient 1, neither CRP (250 ng/ml) nor convulxin (up to 5-fold the normal concentration) induced platelet aggregation, and platelets from patient 2 were unable to aggregate in response to convulxin.

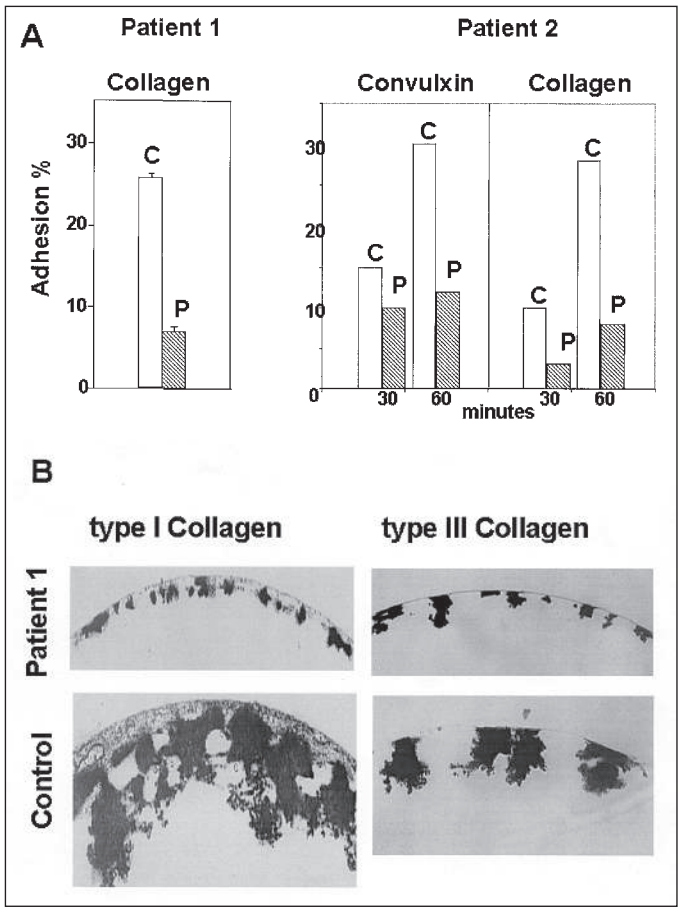

Figure 4: Platelet adhesion. A: Wells in a microtiter plate were coated either with fibrillar type I collagen (10µg/ml) or convulxin (20 µg/ml). Platelet suspensions ($3x10^8$ /ml) were added to the wells and incubated for 30 min to 1 hour at room temperature. After washing the number of adherent platelets was determined . C: platelets from a normal subject, P: patient's platelets.
B: Platelet adhesion and thrombus formation in a perfusion chamber. Blood from a control subject or from the patient was allowed to flow at 1600 $^{s-1}$ through a capillary tube coated with type I (A) or type III collagen (B) (see methods). At the end of the experiment semi-thin sections stained with toluidine/fuschin (x 1400) are shown.

Table I: *In vivo* thrombi formation. Thrombi formation in the patient was tested at the onset of the preacutisation phase of the disease. The % of adhesion represents the % of the inner chamber surface covered with platelets and/or fibrin, and the thrombi volume per unit area (µm3/µm2) was determined according to Sakariassen et al. as described in the § Methods. Data observed in the control are in the range of that observed in the cohort of normal controls.

	Type I Collagen		Type III Collagen	
	Adhesion %	Thrombi volume ($\mu m^3/\mu m^2$)	Adhesion %	Thrombi volume ($\mu m^3/\mu m^2$)
Patient 1	70	5.8	58	3.9
Control	100	16.6	53	12.1

GPVI-triggered platelet activation / P-selectin exposure and activation of GPIIbIIIa

These results suggested a defect affecting GPVI. GPVI-triggered platelet activation was further investigated by measuring P-selectin exposure after stimulation by an activating anti-GPVI MoAb (Fig. 3). MoAb 9E18.2 induced a right shift in the fluorescence associated to the anti-P-selectin on control platelets indicating α-granule exocytosis. The exposure of P-selectin at the surface of platelets from both patients was almost null, when compared to control platelets, while it was normal in response to human thrombin (data not shown). The P-selectin exposure in response to convulxin was similarly reduced at the surface of platelets from patient 1.

Platelet adhesion

GPVI is required for platelet activation by collagen and also contributes to platelet adhesion (30, 31). We have thus investigated platelet adhesion in static conditions for both patients (Fig. 4A), and in flow conditions for patient 1 (Fig. 4B). Adhesion of platelets from both patients to immobilized fibrillar type I collagen was considerably reduced. In addition, a decreased binding of platelets from patient 2 to immobilized convulxin was observed. *Ex-vivo* thrombi formation was studied in a perfusion chamber model (shear rate: 1600 s^{-1}) where capillaries were coated either with type I or type III collagen: the percentage of covering adhesion was slightly reduced in the case of patient 1 compared to the controls on type I collagen coated capillaries, and was normal on type III coated capillaries (Table I). However, thrombi volume was largely decreased in response to type I and type III collagen (being about one third of the normal values) (Table I and Fig. 4B).

Platelet membrane glycoproteins expression

The expression of the main platelet membrane glycoproteins was investigated several times by flow cytometry. On platelets from both patients, $\alpha_{IIb}\beta_3$ and GPIb/IX amounts were normal; $\alpha_2\beta_1$ level on patient 1 was 60% of the mean value found for a normal population (that is 976 sites vs 1620±765) and was similarly low on patient 2's platelets. GPIV was repeatedly null at the surface of platelets from patient 1 (patient 2 not tested). Surprisingly in both cases, a normal expression of GPVI was observed using a FITC-labeled monoclonal anti-GPVI antibody (Fig. 5) and the binding of FITC-coupled convulxin was subnormal (Fig. 5).

Platelet GPVI content analyzed by immunoblot was similar to that in controls when tested by immunoblot using either polyclonal anti-GPVI IgGs (Fig. 6) or a monoclonal antibody. Binding of ^{125}I-labeled convulxin in ligand-blotting experiments was normal too (Fig. 6). The FcRγ chain was present in amounts within the normal range in platelets from both patients. The association of both proteins was investigated by immunoprecipitation and FcRγ chain was found to be coprecipitated with GPVI in both cases (shown for patient 2 on Fig. 6).

GPVI-coupled signalling pathway

The preceding observations indicated that despite the normal GPVI expression and binding of specific ligands, platelet activation did not occur and suggested a qualitative defect in the coupling of GPVI to its signalling pathway. We measured cal-

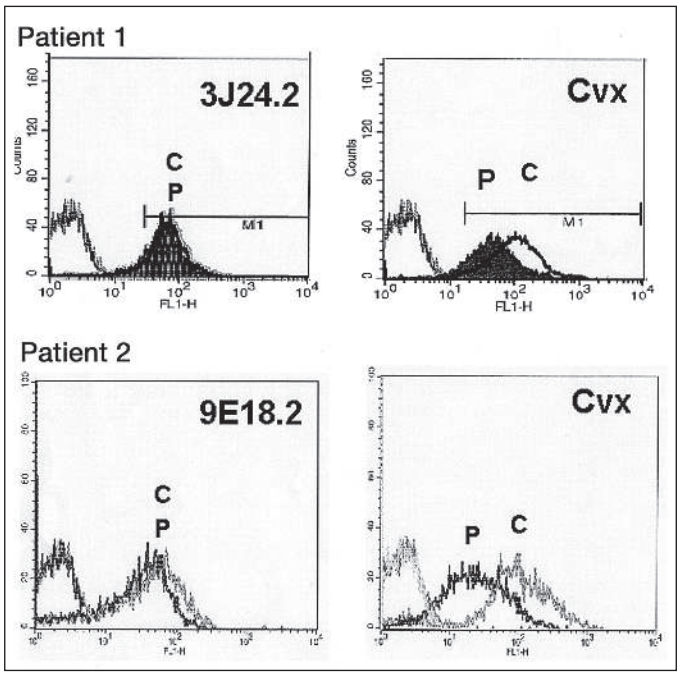

Figure 5: Surface expression of GPVI. Left panels: Washed platelets from a normal subject (C) or from patient 1 (P) were incubated with a FITC-coupled anti-GPVI MoAb (3J24.2) or an FITC-coupled isotypic IgG. Platelets from patient 2 and the corresponding control were incubated with a non-labeled anti-GPVI MoAb (9E18.2) or an isotypic IgG followed by a FITC labelled anti mouse antibody. Samples were analysed by flow cytometry. An overlay of the traces is presented, the peaks on the left corresponding to the isotypic control. Right panels: Washed platelets from normal subjects (C) or from patients (P) were incubated with FITC-coupled convulxin or an FITC-coupled irrelevant protein and analyzed by flow cytometry. An overlay of the traces is presented, the peaks on the left corresponding to the labeling by the irrelevant protein.

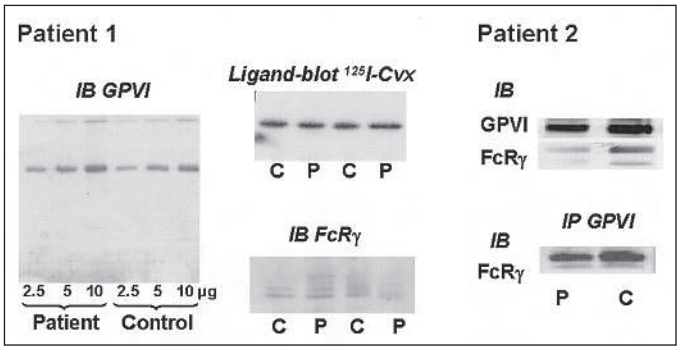

Figure 6: Platelet content in GPVI and FcRγ chain. Left panel: proteins from lysates of platelets were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were then incubated with a polyclonal anti-GPVI antibody (IB GPVI) , or a polyclonal anti FcRγ-chain (IB FcRγ) followed by peroxydase-coupled protein A revealed by chemiluminescence. Alternatively, membranes were incubated with ^{125}I-labeled convulxin (ligand-blot) detected by autoradiography. C: platelets from a normal subject; P: platelets from the patient.
Right panel: IB- platelet lysates were treated as above except that the anti-GPVI antibody was a monoclonal IgG (3J24.2). IP- GPVI was immunoprecipitated from platelet lysates using polyclonal anti-GPVI IgGs. After separation and transfer of precipitated proteins to nitrocellulose, the FcRγ chain was revealed by immunoblotting using the polyclonal anti-FcRγ antibody.

cium mobilization as a reflect of PLCγ_2 activation. In the patients' platelets, neither collagen (5 µg/ml) nor convulxin (1 nM) induced the increase in [Ca^{2+}]i observed in control platelets while thrombin (0.5 nM) induced a normal response, thus excluding an anomaly in calcium storage or mobilization (Fig. 7). In order to specify the level of the defect in the activation cascade, protein tyrosine phosphorylations were studied in adhesion or aggregation conditions (Fig. 8). A decreased intensity of the bands phosphorylated upon stimulation by collagen was observed, when compared to controls, which was more marked for patient 1, while a defective phosphorylation of phospholipase Cγ_2 was evidenced in patient 2 (Fig. 8).

Discussion

We describe here two patients from two different institutions presenting platelet dysfunction, a major characteristic of which is a lack of reactivity to collagen. The defect was probably acquired, since the patients did not present any previous significant haemorrhagic manifestations.

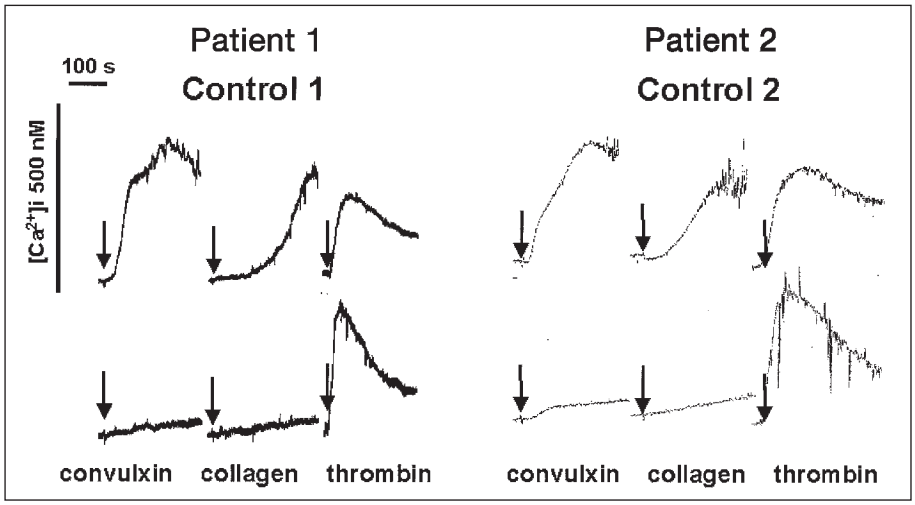

Figure 7: Intracellular calcium concentration. Platelets from a normal subject or from the patients were loaded with Fura 2AM. After washing, platelets (3x 10^8/ml) were activated by convulxin (0.1 nM), collagen (1 µg/ml) or human thrombin (0.5 nM) in the presence of 2 mM external $CaCl_2$. Variations in fluorescence was measured using two excitations wavelengths and intracellular calcium concentration calculated as described in methods.

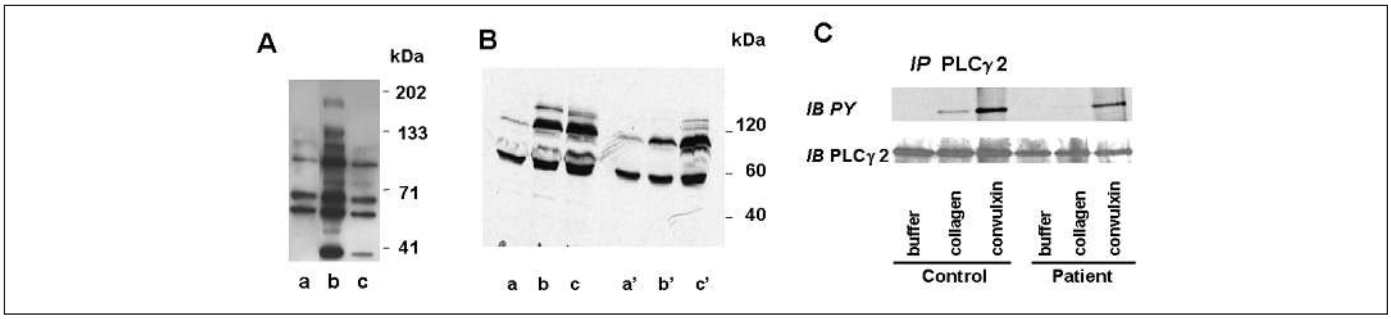

Figure 8: Protein tyrosine-phosphorylation. Platelets proteins were separated by SDS-PAGE, transferred to PVDF and membranes were blotted with an anti-phosphotyrosine MoAb followed by a peroxydase-coupled anti-mouse antibody revealed by chemiluminescence. A: Resting platelets (a) or platelets from a normal subject (b) or patient 1 (c) adherent to immobilized type I collagen. B : Platelets from a normal subject (a-c) and from patient 2 (a'-c') were lysed after incubation with buffer (a,a'), collagen type I 1µg/ml (b,b') or 10 µg/ml (c,c') for 2 min. in the aggregometer. After protein separation by SDS-PAGE, Tyr-phosphorylated proteins were detected as above. In C, platelets were incubated with buffer, collagen 1 µg/ml or convulxin 0.3 nM. phospholipase Cγ2 was immunoprecipitated from the lysates and immunoblotted using an anti-phophotyrosine antibody as above. Membrane were reprobed with the anti-PLCγ2 to control protein loading.

In both cases, platelet granule content was normal. The presence of platelet antibodies was carefully ruled out since it is well demonstrated in mice, that specific antibodies against GPVI lead to the *in vivo* depletion of the receptor at the platelet membrane (32). The identification of an anomaly affecting collagen receptors was thus attempted. GPIb and vWF were not involved in the patients' defect. The two other main collagen receptors are $\alpha_2\beta_1$ integrin and GPVI; $\alpha_2\beta_1$ integrin is involved in the stable platelet adhesion to collagen (31, 33), while the importance of GPVI coupled signals for collagen-induced activation is now established (34, 35). The amount of $\alpha_2\beta_1$ on platelets from both patients corresponded to the lower limit of the normal range, possibly due to a genetic polymorphism of $\alpha_2\beta_1$; this fact was well demonstrated for patient 1, who was heterozygous for the 807C polymorphism associated with a low expression of $\alpha_2\beta_1$ (36). This amount of $\alpha_2\beta_1$ cannot however explain the total lack of platelet responses to type I collagen observed in our patients while, in contrast, a complete lack of $\alpha_2\beta_1$ was reported to account for platelet unresponsiveness to collagen in one patient with a myeloproliferative disorder (37). Concerning the GPVI-FcRγ chain complex, it also appeared to be normally expressed on the surface of platelets from both patients as indicated by flow-cytometry using different anti-GPVI antibodies, by immunoblotting and immunoprecipitation using anti-GPVI and anti-FcRγ chain antibodies. Thus, our cases differ from those where GPVI is lacking either constitutionally (14, 15) or in relation to the presence of specific autoantibodies (10–13), or possibly in relation to a protease degradation like in the Gray Platelet syndrome (38). By contrast, in our patients, although a normal amount of GVPI functional exploration indicated that the GPVI-triggered activation was deficient, the GPVI-specific agonists CRP, convulxin and anti-GPVI antibodies, even used at high concentrations did not induce platelet activation as shown by the lack of aggregation, P-selectin expression and increase in $[Ca^{2+}]i$. Such a defect could be the result of a defective binding of specific ligands to GPVI. However, binding of convulxin to GPVI was preserved in both patients. Alternatively, a functional defect might have affected a step in the signalling pathway coupled to GPVI. Calcium mobilization in response to GPVI agonists was absent, while the thrombin-induced increase in $[Ca^{2+}]i$ was normal, indicating that PLCγ2 was not activated in response to GPVI stimulation. PLCγ2 activation occurs downstream of the phosphorylation on Tyr residues of several proteins. The intensity of the bands corresponding to phosphorylated proteins was decreased in patients' platelets stimulated by collagen, confirming a low efficiency of the signalling pathway in favor of a defect in the GPVI-coupled signalling pathway. Of note, in patient 2 it was possible to obtain a normal protein phosphorylation when collagen concentrations were considerably increased (up to 5-fold the usual values).

The rapid fatal outcome of these patients prevented us from defining the precise enzymatic abnormality(ies), involved in the pathway signalling. In both patients, collagen-induced responses were predominantly affected but responses to other agonists (in particular arachidonic acid) were also more or less decreased. This may be due to the fact that the signalling proteins involved in the GPVI and FcRγII common pathway may also contribute to the amplification of pathways coupled to other receptors (39). For example, PI3 kinase plays an important role in the GPVI-coupled signalling pathway. It has also been shown to contribute to the pro-aggregatory properties of P2Y12 by potentiating the P2Y1-induced response (40). Furthermore, an ADP-dependent-activation PtdIns(3–4)P2 accumulation is important for PAR-1-induced irreversible aggregation (41).

The respective role of α2β1 and GPVI in platelet adhesion to collagen has recently been revisited. It appears now that $\alpha_2\beta_1$ as other integrins needs to be activated to bind collagen with a good affinity (42), and that GPVI activation may contribute to α2β1 activation (30, 43). In agreement with this model, we observed that, for both patients, platelet adhesion to immobilized collagen was reduced. In addition, *ex vivo* thrombi formation using whole blood under flow conditions either to type I or type III collagen (28, 44) was largely decreased in the case of patient 1. These data concur with other data reporting abolished or very impaired *ex vivo* thrombus formation under flow conditions respectively in GPVI null or FcRγ null mice (45, 46) and in a patient presenting a deep deficiency in GPVI (47). For both patients, the discovery of the deficiency in collagen induced platelet activation was made during a bleeding event. Patients with an isolated GPVI

deficiency do not present a significantly increased bleeding time, and have been reported to suffer of mild bleeding symptoms. However, in the present cases the simultaneous low level of integrin $\alpha_2\beta_1$ which was shown recently to participate not only in platelet adhesion but also in platelet activation (16) and impaired function of GPVI may have resulted in deeper haemorrhagic manifestations in the same way that a low level of $\alpha_2\beta_1$ integrin associated with a decrease in von Willebrand factor was shown to correlate with an impaired platelet function and haemorragic complications (48). In addition, in the first case, GPIV, whose role in collagen interaction is controversial (5), was repeatedly undetectable on the patient's platelets and this could have emphasized the consequences of the simultaneous low level of integrin $\alpha_2\beta_1$ and impaired GPVI signalling.

Finally, to our knowledge, this is the first time that an acquired defect mainly due to an abnormal GPVI-mediated response to collagen has been reported in malignant haemopathies. The difference in signalling defect in our two patients may be explained, for example, by the fact that it was possible to obtain phosphorylation of the main proteins involved after stimulation by high doses of collagen in patient 2, but not in patient 1, but our observations point to the possibility that platelet functional defects often associated to malignant haemopathies could result from an alteration in the tight regulation of the signalling pathways. The relationship with different malignant haemopathies should constitute an area of interest for future studies.

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