Elevated Levels of Circulating Procoagulant Microparticles in Patients With Paroxysmal Nocturnal Hemoglobinuria and Aplastic Anemia

By Bénédicte Hugel, Gérard Socié, Thi Vu, Florence Toti, Eliane Gluckman, Jean-Marie Freyssinet, and Marie-Lorraine Scrobohaci

Paroxysmal nocturnal hemoglobinuria (PNH), frequently occurring during suppressed hematopoiesis including aplastic anemia (AA), is a clonal disorder associated with an increased incidence of thrombotic events. Complement-mediated hemolysis, impairment of the fibrinolytic system, or platelet activation are thought to be responsible for the associated thrombotic risk. We investigated here the elevation of membrane-derived procoagulant microparticles in the blood flow of such patients. Elevated levels of circulating microparticles were in fact detected in both de novo PNH patients and AA subjects with a PNH clone, but not in those with AA without a PNH clone. The cellular origin of the microparticles was determined in PNH samples; most stemmed from platelets. Glycoprotein A* particles were rarely detected. Therefore, platelet activation, resulting in the dissemination of procoagulant phospholipids in the blood flow, could be one of the main causes for the elevated thrombotic risk associated with PNH. These observations suggest that shed membrane particles can be considered a valuable biological parameter for the assessment of possible thrombotic complications in patients with PNH.

PATIENTS AND METHODS

Patients. Details of the patient clinical courses, treatment, and evolution have been previously reported in detail. In brief, patients were considered to have PNH if the Ham-Dacie’s test was positive at diagnosis. Patients with a previous history of AA who later developed a positive Ham-Dacie’s test and/or had evidence of defective expression of GPI-linked proteins by flow cytometry were considered to have an AA/PNH syndrome. Of the 29 patients with either PNH (n = 12) or an AA/PNH syndrome, 2 patients (P1 and P4) had a history of thrombotic complications. Patient P1 had PNH and developed a Budd-Chiari syndrome. Patient P4, with an AA/PNH syndrome, had a positive Ham-Dacie’s test 4 years after diagnosis and evidence of a deficiency in GPI-anchored proteins. Almost 10 years after being treated by immunosuppressive therapy, she had thrombosis of the lower limb.

Materials. The monoclonal antibody (MoAb) against glycoporphin A was from Immunotech S.A. (Marseille, France). The MoAbs to human platelet glycoprotein Ib (GPIb) and glycoprotein IIIa (GPIIIa) were from Biogenesis S.A. (High Wycombe, United Kingdom). Endogenous GPIbα was detected with the MoAb 27-420, a protein showing a strong affinity for phosphatidylserine, through which capture was feasible. Such circulating particles carry membrane antigens specific for the cells they stem from and through which capture was also achieved to determine their cellular origin.
were kind gifts from Dr F. Lanza (Unité 311 INSERM, Strasbourg, France). The irrelevant biotinylated Ig (IgG1B) was from Leinco Technologies (Ballwin, MO). Purified human blood coagulation factors were the same as those used in a recent study reported by our group.18 Factor V was a product from Diagnostica Stago (Asnières, France). Recombinant human annexin V was purchased from Euromedx (Souffelwyersheim, France) and conjugated with fluorescein isothiocyanate (FITC; annexin VFITC) following the procedure described by Dachary-Prigent et al.19 High binding capacity streptavidin-coated microtiter plates, 1-O-n-octyl-β-D-glucopyranoside, biotin-X-OSu, and Chromozym TH were from Boehringer Mannheim (Mannheim, Germany). Human serum albumin (HSA), the streptavidin-R-phycocerythrin conjugate, phosphatidylcholine, and phosphatidylserine from bovine brain were products from Sigma Chemical Co (St Louis, MO). Calcium ionophore A23187, D-phenylalanyl-prolyl-arginyl chloromethyl ketone (FPR.CK), and 1,5-dansyl-glutamyl-glycyl-arginyl chloromethyl ketone (Dns-EGR.CK) were obtained from Calbiochem (San Diego, CA). All other reagents were of the highest available purity grade.

Methods. The preparation of platelet-free plasma samples, the biotinylation of annexin V and MoAbs, the capture of microparticles by immobilized annexin V or MoAbs, and the prothrombinase assay for the estimation of the amount of captured microparticles are detailed in Aupeix et al.11 It has to be mentioned that different incubation times were used for microparticle capture by annexin V (30 minutes) and MoAbs (2 hours). However, to exclude that complement attack of cells might occur during the blood drawing procedure and the separation of plasma from cells by centrifugation, some PNH and control blood samples were drawn into both EDTA and citrated anticoagulants. The particle capture procedure involving insolubilized AV as well as the assay of their phosphatidylserine content based on the possible biological link between this disorder and PNH. High to very high levels of circulating particles were indeed detected in some PNH samples when compared with the control group (P < .00007). The mean ± SD for the control group was 5.3 ± 2.2 nmol/L phosphatidylserine equivalent, that of the aplastic anemia group without a PNH clone (AA) was 1.9 ± 1.6 nmol/L, that of the aplastic anemia with a PNH clone group (AA/PNH) was 11.1 ± 9.1 nmol/L, and that of the PNH group 14.4 ± 10.5 nmol/L phosphatidylserine equivalent. Interestingly, a clear difference was established between the two AA groups (P < .02). Some AA/PNH samples contained high microparticle levels, comparable with those of the PNH group, whereas AA samples were measured at lower values than the control group (P < .0004).

Therefore, we searched for a possible link between the level of the PNH clone expression and the proportion of circulating particles. This was performed taking into account the proportions of GPI-deficient polynuclear cells, monocytes, platelets, or erythrocytes. Correlation coefficients never exceeded 0.7. No clear correlation could therefore be evidenced.

The cellular origin of these circulating particles was deter-

![Fig 1. Amount of circulating microparticles in peripheral blood samples from AA and PNH patients.](image-url)
CIRCULATING MICROPARTICLES IN PNH 3453

The present study clearly demonstrates the presence of elevated levels of phosphatidylserine-bearing microparticles in

Fig 3. Procoagulant phospholipid externalization and membrane vesiculation in calcium ionophore-stimulated RBC from 4 PNH patients (P) and 2 healthy subjects (T) measured by prothrombinase assay. Cells were stimulated by 5 μmol/L calcium ionophore A23187 in the presence of 2 mmol/L external CaCl2 for 90 minutes at 37°C. Stimulated cells were centrifuged for 30 seconds at 12,000g. Microparticle release was measured in the supernatant, whereas phosphatidylserine externalization was measured on the pelleted cells. Data (n = 3) represent the increase of prothrombinase activity after stimulation and are expressed as the ratio between the activity before and after ionophore stimulation. The basal activity of the RBC before stimulation was 2.2, 4.5, 0.6, 0.2, 1.0, and 0.3 mmol/L phosphatidylserine equivalent for P1, P2, P3, P4, T1, and T2, respectively. In the supernatant, corresponding basal activities were measured at 1.2, 1.3, 0.4, 0.2, 0.4, and 0.4 mmol/L phosphatidylserine equivalent.

DISCUSSION

The presence of glycophorin A+ particles was captured in most PNH samples (individuals having received recent blood transfusions were, of course, excluded from the study), although PNH RBC are believed to be one of the targets of the complement membrane attack complex leading to hemolysis (Fig 2). However, the presence of glycophorin A at the surface of PNH RBC was verified by flow cytometry analysis. The expression of glycophorin A in PNH RBC was not impaired when compared with that of normal erythrocytes, without or after stimulation by calcium ionophore (data not shown). In contrast, very high levels of platelet-derived particles bearing the GPIIb-specific marker were captured in several PNH samples. The antibody directed to GPIIIa yielded basically identical results (data not shown). The means of the amount of GPIIb+ particles were not significantly different between PNH and control group at the .05 level, but the variances comparison (using the ratio method) showed a clear difference in the distribution of the values at the .002 level. This is consistent with a situation in which some patients are in the range of the control group, whereas others present very high levels of circulating GPIIb+ particles. It has to be emphasized that no direct comparison between capture by annexin V and antibodies could be performed because preincubation times and affinities for the respective ligands are different.11

Assessment of phosphatidylserine exposure in individual PNH samples. The ability of PNH RBC to externalize phosphatidylserine to and vesiculate was examined in a functional prothrombinase assay with 4 PNH samples: P1, P2, P3 (already used for the control of glycophorin A expression), and P4. PNH RBC showed impaired procoagulant phospholipid externalization in 2 cases (P2 and P4) among the 4 samples tested (Fig 3). Vesiculation, assessed in the supernatant of stimulated RBC, was almost undetectable in 3 (P2, P3, and P4) among the 4 PNH RBC samples. Finally, in 1 PNH RBC sample (P1), procoagulant phospholipid externalization and membrane vesiculation appeared normal. It is of interest to notice that the absence of activability of P2 RBC is probably related to its high basal stimulation state (see legend of Fig 3). This was not the case for P4 and P3 samples.

To confirm the prothrombinase results, procoagulant phospholipids were probed with AVFITC on stimulated PNH and control RBC and on derived microparticles from the same individuals (Fig 4). The flow cytometry analysis showed lower to moderate levels of procoagulant microparticles in the supernatant of ionophore-stimulated PNH RBC compared with control RBC.

Finally, 3 PNH RBC samples of 4 (P2, P3, and P4) showed an impaired vesiculation in a functional prothrombinase assay (Fig 3) and 3 PNH RBC samples of 3 (P1, P2, and P3) were unable to shed normal levels of particles as deduced from flow cytometry analysis (Fig 4). P1 sample led to apparent contradictory results. The proportion of shed membrane microparticles after stimulation was lower than in controls when measured by flow cytometry, but the functional prothrombinase assay did not show any vesiculation impairment.
the peripheral circulation of several PNH patients or subjects with AA with a PNH clone. The procoagulant potential disseminated by these particles in the blood flow could be responsible, at least in part, for the high incidence of associated thrombotic complications. The basal level of circulating particles detected in control subjects probably reflects a balance between cell proliferation, stimulation, and death and concerns microparticles that transiently escape destruction by phagocytosis, phospholipases, or confinement by specific adhesion. In pathological situations in which apoptosis or cell stimulation is known to occur at a high degree, the elimination systems could be saturated, giving rise to increased levels of circulating shed microparticles. Interestingly, samples from AA patients who did not develop a PNH clone were measured under the control level. Therefore, the susceptibility of RBC and platelets lacking GPI-linked complement inhibitors of the membrane attack complex may account for the high levels of shed microparticles. But again, the proportion of circulating microparticles is probably dependent on the relative efficiency of clearance systems. Hence, individual variability of the elimination response may account for the lack of clear correlation between the PNH clone level and the particle proportion.

Very low amounts of particles bearing the RBC marker glycophorin A were captured in PNH plasmas, whereas hemolysis often occurs during the course of PNH. The normal expression of glycophorin A on PNH RBC and the specificity of the corresponding antibody were controlled because of the qualitative abnormality of glycophorin A reported by Parker et al. The circulating microparticles did actually not originate from lysed RBC to a significant extent. On the other hand, very high levels of particles of platelet origin were detected in several PNH samples. Hence, platelet activation, already reported in PNH, could be one of the main causes of the high incidence of thrombosis associated with PNH. It has to be emphasized that platelet-derived microparticles were easily detectable in control samples and probably account for an important part of the basal particle level.

The absence of RBC-derived particles in PNH samples led us to investigate the ability of RBC to externalize phosphatidylserine and to vesiculate. An impaired ability of RBC to vesiculate has already been reported by Whitlow et al for 2 PNH patients lacking CD59 and CD55. We also observed a very weak ability of some PNH RBC to vesiculate using a functional prothrombinase assay, and we assume that it could explain the absence of circulating RBC-derived microparticles. The heterogeneity of the responses of the RBC samples to ionophore is probably linked to the random selection of the patients. The different stages of evolution of the disease and current treatments might, at least in part, explain such an heterogeneity. The impaired ability to vesiculate might also be related with the resistance to apoptosis observed in PNH granulocytes.

The use of the prothrombinase assay concomitantly with flow cytometry showed apparent contradictory results with the RBC sample P1. Vesiculation was lower than in control samples when measured by flow cytometry, but normal in a functional prothrombinase assay. This observation points to the fact that
the two analyses do not measure the same parameters. Flow cytometry enables us to estimate the proportion of particles, whereas the prothrombinase assay detects their procoagulant potential. Therefore, the P1 RBC actually show an impaired ability to vesiculate, but the microparticles shed in the supernatant might bear an increased procoagulant potential. Interestingly, patient P1 recently experienced several thrombotic events. On the other hand, P4 RBC showed impaired ability to externalize phosphatidylserine and to vesiculate by prothrombinase assay, but patient P4 also developed thrombosis.

Whitlow et al. also evidenced an impaired ex vivo vesiculation of platelets completely lacking GPI-linked proteins, whereas Wiedmer et al. were able to induce complement-mediated platelet membrane vesiculation in platelet samples lacking CD59 antigen. Here, we have detected very high levels of in vivo circulating platelet-derived microparticles in several PNH blood samples. In our case, platelet vesiculation was not directly investigated using isolated platelets. It can be reasonably assumed that our assay measures a balance between the shedding of particles in the blood flow and elimination by the various clearance systems. Therefore, it is conceivable that significant levels of particles of platelet origin are consistently released in the blood flow, although higher or equivalent amounts of particles of RBC origin are shed, but, for unknown reasons, the latter might be more efficiently eliminated from the circulation. The description by Simons and Ikonen of functional sphingolipid-cholesterol rafts in cell membranes might explain such differences, especially in a context of absence of GPI-anchored proteins. CD55 and CD59 have been precisely shown to be sorted and shed in exosomes during reticulocyte maturation.

Circulating particles, giving rise to disseminated potential prothrombotic seats, are certainly not neutral with respect to the response of the coagulation system of an individual. In several PNH patients investigated, these particles seem to originate massively from platelets and their amount could be responsible in part for thrombotic events. Moreover, platelet microparticles released after complement activation were shown to be enriched in the membrane receptor for coagulation factor Va, the latter involving phosphatidylserine. On a therapeutic point of view, the measurement of the proportion of circulating microparticles can be of interest with regard to the choice of the anticoagulant treatment. The balance between hemorrhage and thrombosis is believed to be subtle in the PNH pathology. It is probably related to the frequent association of pancytopenia with severe cell activation. Therefore, if high levels of circulating microparticles increase the thrombotic tendency, anti-vitamin K treatment should be the more appropriate preventive approach. Moreover, PNH patients with pancytopenia and high levels of circulating microparticles should not be at risk of bleeding episodes, whereas PNH patients with pancytopenia but low levels of circulating microparticles might probably be. Elevated levels of circulating platelet microparticles were precisely found protective against bleeding in patients with autoimmune thrombocytopenia but were associated with the occurrence of small cerebral vessel infarcts when very high. Helpful indications can be deduced from the new biological parameter consisting of the level of circulating procoagulant microparticles. This system may enable us to assess an instant in vivo thrombotic risk associated with PNH owing to the possibility of massive release of procoagulant microparticles shed from activated platelets in the blood flow. However, it would be interesting to investigate both circulating microparticles and other coagulation parameters in more homogeneous groups of patients who previously developed thrombosis to evaluate the multifactorial character of the mechanisms of thrombosis in the PNH pathology. But, the relative rare occurrence of this disorder is certainly a limit for such studies.

REFERENCES

15. Sims PJ, Faiioni EM, Wiedmer T, Shattil SJ: Complement...


