Bone Marrow Transplantation (2002) 30, 381–387

www.nature.com/bmt

Platelet measurements

Flow cytometric findings in platelets of patients following allogeneic hematopoietic stem cell transplantation

R Pihusch[1], H Wegner[1], C Salat[1], M Pihusch[1], E Holler[2], HJ Kolb[1,3] and E Hiller[1]

[1]*Medizinische Klinik III – Groβhadern, Klinikum der Ludwig Maximilians-Universität München, Germany;* [2]*Abteilung für Hämatologie und Internistische Onkologie, Klinikum der Universität Regensburg, Germany; and* [3]*Klinische Kooperationsgruppe Hämatopoietische Zelltransplantation, GSF, Forschungszentrum für Umwelt und Gesundheit, Neuherberg, Germany*

Summary:

Following allogeneic hematopoietic stem cell transplantation (HSCT) patients may have an increased bleeding tendency in spite of a normal platelet count. Moreover, an association between chronic graft-versus-host disease (cGVHD) and a thrombophilic state has been observed. Platelet receptors and granules from 27 patients following HSCT (13 without cGVHD, 14 with cGVHD) were evaluated by flow cytometric analysis and compared to 62 healthy controls. Platelets from HSCT patients stained weakly with mepacrine indicating a reduced content of dense bodies, whereas no significant degranulation reaction of alpha granules and lysosomes was detectable. In addition, a lower surface expression of GP Ia/IIa was observed, indicating an acquired thrombocytopathy. The surface receptors are activated in HSCT patients, which could be seen by the lower surface expression of GP Ib internalized during the activation process and elevated levels of LIBS-1 and PAC-1 antibody binding. Patients with cGVHD had a seven-fold increased ratio of microparticles. This study demonstrates platelet receptor and granule defects in patients following HSCT. The key role of platelets in HSCT-associated hemostatic disorders is underscored by the high levels of circulating microparticles in cGvHD patients which might explain the thrombophilic state in these patients.

Bone Marrow Transplantation (2002) **30,** 381–387. doi:10.1038/sj.bmt.1703663

Keywords: platelets; graft-versus-host reaction; storage pool disease; microparticles

Platelet function is strongly dependent on glycoproteins (GPs) exposed on the surface of activated platelets.[1–3] The GP IIb/IIIa complex is the inducible fibrinogen receptor on platelets and plays a central role in platelet aggregation. This receptor has the capacity to shift through conformational changes from a low-affinity state to a ligand-competent state on activated platelets, which can be detected by PAC-1-antibody. Furthermore, fibrinogen binding to GP IIb/IIIa itself modifies the conformation of this integrin, exposing neoepitopes known as ligand-induced binding sites (LIBS). The GP Ib/V/IX complex is the von Willebrand factor (vWF) receptor responsible for adhesion of platelets to a damaged vessel wall, whereas the glycoprotein complex Ia/IIa (GP Ia/IIa) is a major collagen receptor on platelets. The latter plays an important role in platelet adhesion and activation following initial interaction of the platelet with the vessel wall which is mediated by vWF.[4] During aggregation platelets degranulate and express multiple granule-stored GPs on their surface that mediate platelet interactions with other vascular cells including leukocytes and the endothelium. Following intensive activation, platelets liberate minute membrane vesicles. These microparticles can be detected in the circulation and induce a significant thrombosis risk by providing a large phospholipid surface which serves as substrate for plasmatic coagulation.[5]

A common clinical observation following hematopoietic stem cell transplantation (HSCT) is an impaired bleeding time in spite of a normal platelet count. On the other hand, patients with chronic graft-versus-host disease (cGVHD) have a 13-fold thrombosis risk in spite of thrombocytopenia.[6]

In this study, we evaluated changes in membrane GPs on circulating platelets and the liberation of microparticles in long-term survivors of allogeneic hematopoietic stem cell transplantation and found profound changes which could well explain the clinical observations.

Patients and methods

Patients

A random series of 27 patients which had undergone allogeneic hematopoietic stem cell (HSCT) transplantation months ago were evaluated. Patients were regularly monitored on an outpatient basis. Fourteen patients showed signs of chronic GVHD (skin, liver, gut). Of the remaining 13 patients having no evidence of cGVHD, six patients were thrombocytopenic due to a delayed platelet take. All

Correspondence: Dr med R Pihusch, Medizinische Klinik III – Groβhadern, Klinikum der Ludwig Maximilians-Universität München, Marchioninistr. 15, 81377 München, Germany
Received 18 February 2002; accepted 29 May 2002

patients were in complete remission from their underlying disease and had a complete chimerism. None of the patients had any bacterial, fungal or viral infections within 2 months prior to the beginning of the study, nor did any have implanted stents or a history of antirheumatic medication. Platelet transfusions had not been given within the last 10 days. In addition, a control group of 62 healthy individuals recruited from the medical staff was evaluated. The patient and transplant characteristics are given in Table 1.

Laboratory parameters

Laboratory investigations included a routine platelet count (H 6000 analyser, Coulter, Miami, FL, USA) and platelet flow cytometry. Platelet flow cytometry is an *in vitro* technique which allows analysis of surface epitopes on platelets with specific fluorochrome tagged antibodies.[7] The method used was based on that of the European Working Group on Clinical Cell Analysis[8] with additional *in vitro* stimulation of platelets to gain information on the platelet function.

Blood sampling and processing

Citrated blood was collected from the patients in 4.3 ml Primavette S coagulation tubes (Kabe Labortechnik, Nuembrecht-Elsenroth, Germany). To avoid platelet activation by shear stress during blood sampling, the blood was taken from the cubital vein using a cuff pressure of 40 mmHg with a 21 Gauge canule thoroughly avoiding negative pressure in the tube. Immediately after sampling the blood was processed in the cytometry laboratory. By addition of warm phosphate-buffered saline (pH 7.4, 37ºC) the sample was diluted to a platelet density of 10 g/l. After standardized incubation (5 min at 37ºC) with the specific, fluorescein-tagged primary antibody and a phycoerythrin-tagged CD41-antibody (P2, 5 μg/ml, Immunotech, Hamburg, Germany) for platelet identification, the reaction was stopped by adding 2 ml paraformaldehyde (2% solution, pH 7.4) and the probe was scanned immediately in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with a 15 mW argon ion laser. By electronic gating (see Figure 1), 30 000 CD41-positive single platelets were acquired from each sample and analyzed using CellQuest software (Becton Dickinson).

Specific antibodies and in vitro stimulation

Fluorescein-tagged antibodies against the following antigens were used (all Immunotech, Hamburg, Germany):

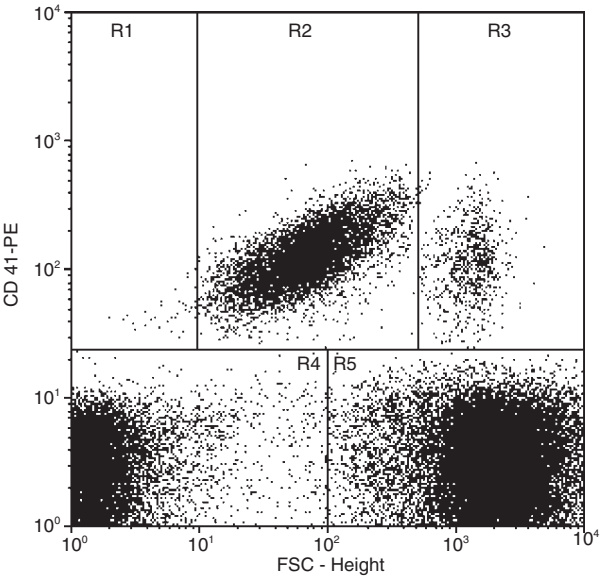

Figure 1 Flow cytometric dot plot demonstrating the gating technique. CD41-PE negative events (R4 = debris, R5 = erythrocytes) and platelet aggregates (R3) were electronically excluded. R2 represents the platelet population, R1 the platelet microparticles. The border between microparticles and platelets was set at a FSC height of 10. To exclude a drift of the FSC height, the cytometer was gauged daily by sized beads (see methods).

Table 1 Demographic and transplantation characteristics of the patients under study and controls

	HSCT patients		*Controls*
	cGVHD	*No cGVHD*	
Total	14	13	62
Gender (male/female)	9/5	8/5	34/28
Age (median years (range))	38 (26–48)	42 (27–60)	39 (24–62)
Diagnosis			
Chronic myelogenous leukemia	6	6	
Acute myelogenous leukemia	1	4	
Acute lymphatic leukemia	2	1	
Non-Hodgkin's lymphoma	2	2	
Myelodysplastic syndrome	2	0	
Osteomyelofibrosis	1	0	
Type of allogeneic transplantation			
Related	5	7	
Unrelated HLA identical	8	5	
Unrelated HLA different	1	1	
Time after HSCT (median weeks (range))	123 (103–193)	84 (63–106)	

If not stated otherwise, the numbers give cases.
HSCT = hematopoietic stem cell transplantation; cGVHD = chronic graft-versus-host disease.

CD42b (SZ2, 7.5 μg/ml), CD49 b (GI9, 4 μg/ml), CD62P (CLB-Thromb/6, 5 μg/ml), CD63 (CLB-gran12, 5 μg/ml), CD29 (Gi9, 10μg/ml). In addition, an antibody against a neoepitope appearing after activation of the GP IIb/IIIa receptor (PAC-1, clone SP-2; Becton Dickinson, 10 μg/ml) and one against the ligand-induced binding site 1 (LIBS-1), a neopepitope appearing after fibrinogen binding to the GP IIb/IIIa receptor (anti LIBS-1 antibody; M Ginsberg, Scripps Research Institute, La Jolla, CA, USA, dilution 1:10) were used. For measurement of platelet reactivity, some samples were additionally stimulated with ADP (CD62P: 8 μM, CD 63: 18 μM, anti-LIBS-1: 0.8 μM) for 10 min at 37ºC immediately before incubation with the antibodies. The reaction was stopped immediately after antibody staining by adding paraformaldehyde.

Mepacrine staining

Dense bodies were quantified by mepacrine staining (5 μM, Sigma, Deisenhofen, Germany) for 30 min at 37ºC in the dark. For identification of platelets, the tube additionally contained the phycoerythrin-tagged CD41-antibody (P2, 5 μg/ml, Immunotech). At the end of the incubation time, platelets were fixed with 2 ml paraformaldehyde (2% solution, pH 7.4) and immediately scanned in the flow cytometer. For measurement of the degranulation reaction of dense bodies, the sample was additionally incubated with the hexapeptide thrombin receptor-activating peptide (TRAP, 20 μM) for 10 min at 37ºC immediately before mepacrine staining.

Microparticles

The CD41-positive microparticles were distinguished from intact normal platelets on the basis of their size as decribed by Abrams *et al*.[9] To quantify and discriminate between platelets and microparticles, the limit was set at the lower border of the forward scatter profile of a normal platelet population (see Figure 1), which is between the first and second quartile of a four decade logarithmic forward scatter scale. The number of the microparticles was expressed as the percentage of particles below this limit to the total number of CD41-positive fluorescent particles counted (ie, platelets plus microparticles).

Quality controls

The instrument settings were gauged daily by use of standard beads (Quantum 24, FCSC, Fishers, IN, USA). This enables standardized results independent of a possible long-term instrument drift and, except for the LIBS-1 antibody and the mepacrine staining (arbitrary units (AU)), makes it possible to express the fluorescence intensities in absolute values (standard units: molecules of equivalent soluble fluorochrome (MESF)). All data were collected using four-decade logarithmic amplification. To calibrate size measurement of microparticles, a daily calibration was made by running fluorescent-labeled beads of known size (Flow-Check; Coulter) and adjusting the gain such that the 1.0 μm beads fall at the beginning of the second decade of a four-decade log forward-angle light scatter scale.

Medication

The two HSCT groups were comparable with regard to their medication: In the cGVHD group, seven (50%) patients were on aciclovir medication (4×500 mg/day orally), two (14.3%) on ketoconalzole (1×200 mg/day orally), seven (50%) on cyclosporine A (oral dose adjusted to plasma levels of 150 to 200 ng/ml), three (21.4%) on mycophenolic acid (4×500 mg/day orally) and seven (50%) on steroids (methylprednisolone 20–100 mg/day orally). Of those patients without signs of GVHD, nine (69.2%) were on aciclovir, four (30.8%) on ketoconazole, six (46.2%) on cyclosporine A, five (38.5%) on mycophenolic acid and eight (61.5%) on steroids.

Statistical analyses

All analyses were performed with SPSS 10.0 for Windows software (SPSS, Chicago, IL, USA). All results are expressed as median values (range). Comparisons between groups were done using the Mann–Whitney test and the Kruskal–Wallis test. A $P < 0.05$ was considered statistically significant. All significant differences are given as two-sided values.

Results

Platelet count

The platelet count was within the normal range in the control group, whereas transplanted patients with and without cGVHD had a significantly lower platelet count (Table 2).

Platelet surface receptors

The surface expressions of the integrins CD29 (GP IIa) and CD49b (GP Ia) were significantly lower in HSCT patients with and without cGVHD as compared to controls, whereas the integrin CD41 (GP IIb) was comparable in all groups (Table 2, Figure 2). The surface expression of CD42b, which is part of the Gp Ib/V/IX complex, was significantly lowered in HSCT patients with and without cGVHD. The LIBS-1 antigen and the antigen detected by the PAC-1 antibody, which are both neoepitopes appearing after activation of the GP IIb/IIIa receptor, were elevated in all HSCT patients when compared to the normal control group. After platelet activation by ADP, LIBS-1 was expressed at a significantly higher level in all HSCT patients in general when compared to the control group. All platelet flow cytometric results were independent of the platelet count and intake of medication by the patient.

Platelet granules

CD62P (P-selectin), a marker of alpha granule degranulation, was similar in all three groups, as was CD63 (GP 53) surface density, a marker of degranulation of the lysosomes (Table 2, Figure 3). Mepacrine staining intensity, which is proportional to the number of dense bodies, is significantly lowered in all HSCT patients when compared to the control

Table 2 Density of platelet surface receptors and markers of platelet degranulation as determined by flow cytometry

	HSCT patients		*Controls*
	cGVHD	*no cGVHD*	
n	14	13	62
Platelet count (g/l)	161.5*	145.4*	266.0
	43.0–241.0	25.0–251.0	150.0–324.0
Platelet receptors			
CD 49b (GP Ia)	32.5*	33.0*	44.5
	19.8–43.3	22.5–47.0	21.3–86.0
LIBS-1 [AU]	447.2*	441.8*	131.7
	111.9–1176.7	42.9–1168.1	84.4–240.1
ADP	960.1*	859.1*	341.8
	124.3–1910.7	312.4–1678.1	223.8–477.1
PAC-1	19.4^{+}	26.4*	16.6
	11.2–47.6	20.0–41.3	7.6–30.5
Platelet granules			
CD62P (P-Selectin)	11.9	13.4	12.0
	8.6–14.3	9.6–15.3	7.4–16.0
ADP	37.1	57.3	46.0
	6.0–66.7	24.8–114.3	16.3–100.7
CD63 (GP 53)	20.6	21.4	21.6
	13.2–25.0	16.2–35.4	15.8–37.1
ADP	37.5	40.1	34.2
	16.1–54.5	21.1–164.4	24.0–88.3
Mepacrine (AU)	47.2*	38.5*	67.0
	22.6–64.5	25.9–48.1	33.8–230.1
TRAP	40.0*	35.6*	45.2
	19.7–48.5	23.2–48.0	20.3–186.8

All values are medians (range) of MESF (molecules of equivalent soluble fluorochrome), if not stated otherwise. AU = arbitrary fluorescence units; ADP = result after stimulation with ADP; TRAP = result after stimulation with thrombin receptor activating peptide; GP = glycoprotein; *significantly different ($P < 0.05$) with respect to the controls; $^{+}$significantly different ($P < 0.05$) with respect to HSCT patients without cGVHD.

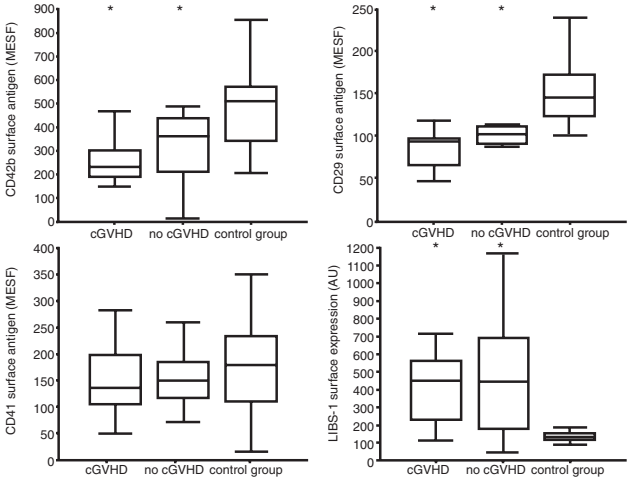

Figure 2 Platelet surface receptor expression as determined by flow cytometry in HSCT patients with and without chronic GVHD and controls. For experimental details see Materials and methods. The data are given as boxplots, which show the 1st and 3rd quartile, the median and the minimum and maximum values of the fluorescence intensities. *Significantly different ($P < 0.05$) with respect to the controls. AU = arbitrary fluorescence units; MESF = molecules of equivalent soluble fluorochrome.

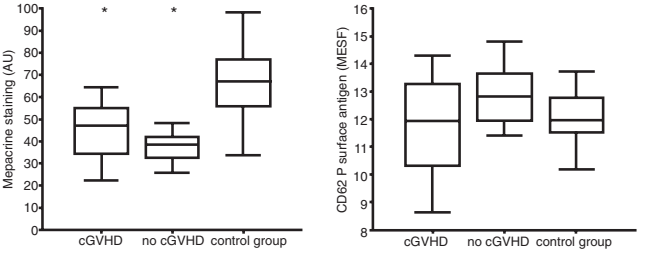

Figure 3 Dense bodies (mepacrine staining) and alpha granule secretion (surface expression of CD62 P) in patients under study and controls. For experimental details see Materials and methods. The data are given as boxplots, which show the 1st and 3rd quartile, the median and the minimum and maximum values of the fluorescence intensities. *Significantly different ($P < 0.05$) with respect to the controls. AU = arbitrary fluorescence units, MESF = molecules of equivalent soluble fluorochrome.

group. Accordingly, after stimulation with TRAP, the platelets of all patients had a significantly lower content of dense bodies than those of normal individuals.

Microparticles

Patients in the cGVHD group had a seven-fold increased ratio of circulating microparticles over the normal baseline value (Figure 4), whereas HSCT patients without cGVHD had a normal ratio.

Discussion

In this study, flow cytometric parameters of circulating platelets were evaluated in patients following hematopoietic stem cell transplantation. Using whole blood flow cytometry for the minimal manipulation of platelets, the major findings of this study can be summarized as follows: (1) following HSCT loss of dense granules caused an acquired storage pool disease. In addition, a reduced surface expression of the major collagen receptor GP Ia/IIa was

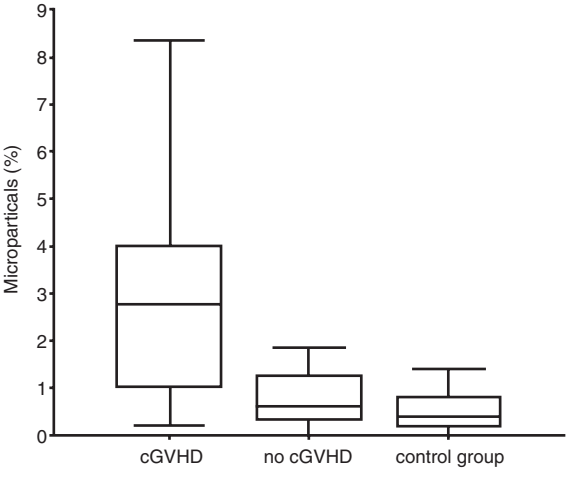

Figure 4 Percentage of the highly thrombogenic microparticles circulating in HSCT patients and controls. The boxplots show the 1st and 3rd quartile, the median and the minimum and maximum values of the percentage of microparticles when compared to all CD41-positive events. *Significantly different ($P < 0.05$) with respect to the controls.

observed. (2) Platelets were activated in patients following HSCT, but significant loss of alpha granules and lysosomes was not detected. (3) In chronic GVHD an elevated level of circulating microparticles was observed.

Hematopoietic stem cell transplantation (HSCT) is an important treatment modality for hematologic disorders, which may later eventually lead to an increased bleeding tendency even under situations with normal platelet counts. In our study, the platelets of patients following HSCT showed a significantly reduced fluorescence after staining with mepacrine, a probe which is specifically incorporated into dense bodies. This implies a reduced content of dense bodies present in the platelets of these patients. The granule defect was restricted to dense bodies only, since upon *in vitro* activation alpha granules and lysosomes degranulized with a normal amount of P-selectin and gp 53. Dense bodies are essential for primary hemostasis, as they contain ADP, ATP, serotonin and divalent cations. They are formed in the precursor megakaryocyte cell[10] and the release of the organelle contents upon platelet activation acts to amplify the original stimulus. A defect of dense bodies, termed storage pool deficiency, is usually inborn, but cases of an acquired storage pool deficiency have been described in end-stage renal failure,[11] myeloproliferative disorders[12,13] and chronic lymphatic leukemia.[14] Our data indicate that following allogeneic HSCT patients also have an acquired storage pool deficiency.

Not only a defect in the content of dense bodies but also a reduced surface expression of the GP Ia/IIa was found in HSCT patients. The integrin receptor GP Ia/IIa is the major collagen receptor. Especially at high shear rates it is involved in the adhesion of platelets to various collagen types,[15] thus eventually leading to platelet fixation and activation. A reduced surface expression of this essential receptor has been described in inborn platelet abnormalities[16] and in myelodysplastic syndromes[17] and leads to an increased bleeding risk. Therefore, the observed GP Ia/IIa deficiency aggravates the bleeding tendency in HSCT patients.

The HSCT-associated storage pool disease and the GP Ia/IIa deficiency were independent of the presence of chronic GVHD, the platelet count and the intake of antiviral, antifungal and immunosuppressive medication. This might be speculated to be the consequence of a pertubation of platelet synthesis following allogeneic HSCT. In HSCT patients, disturbances of the marrow microenvironment and stromal cells have been described.[18,19] However, their influence on platelet production has not been studied so far.

Besides these findings indicating an increased bleeding risk in HSCT patients, experimental evidence was found suggesting an *in vivo* activation of platelets. Activation of blood platelets by physiological stimuli induces inside-out signalling resulting in a conformational change of the integrin receptor GP IIb/IIIa on the platelet surface from an inactive to an active state capable of binding soluble fibrinogen. These neoepitopes are recognized by the PAC-1 antibody. Furthermore, ligand binding to the receptor initiates outside-in signalling and additional conformational changes, thereby unraveling extracellular neoepitopes termed ligand-induced binding sites (LIBS). One of these LIBS is recognized by the anti-LIBS-1 monoclonal antibody used in this study. PAC-1 and LIBS-1 epitopes were detectable far more frequently on platelets from patients following HSCT than in controls. Thus, circulating platelets appeared preactivated following HSCT. In line with these findings, circulating platelets of HSCT patients expressed a reduced number of GP Ib. The GP Ib receptor, part of the GP Ib/V/IX complex, is the major receptor for von-Willebrand factor, which mediates platelet adhesion to the damaged vessel wall.[20] Following platelet activation, the surface expression of the GP Ib/V/IX complex is reduced,[21,22] possibly due to internalization of the complex.

In addition, platelets from HSCT patients mobilized significantly higher levels of the LIBS-1 epitope on their surface after *in vitro* stimulation with ADP. As the total amount of GP IIb/IIIa receptors was comparable between controls and patients following HSCT, these patients have an enhanced platelet reactivity to activating stimuli within the circulation and may, therefore, express far more activated fibrinogen receptors than normal platelets. Fibrinogen receptors are essential for platelet aggregation. However, the mechanism by which platelet surface receptors are activated in HSCT patients remains unclear.

Interestingly, we found no evidence of increased lysosomal or alpha granule secretion *in vivo*. Recently published results from an animal model[23] demonstrated that reinfused thrombin-activated platelets expressing P-selectin continued to circulate and rapidly lost surface P-selectin. However, this effect, which may lead to an underestimation of the degree of *in vivo* degranulation, does not explain our findings. In addition to normal P-selectin surface expression, our data also indicate a normal GP53 surface expression and a normal degranulation reaction upon *in vitro* stimulation with ADP. We conclude, that in contrast to surface membrane receptors, the alpha granule and lysosomal axis was not preactivated in HSCT patients. This may be explained by the fact that a higher intensity of platelet stimulation is needed for degranulation than for the activation of surface receptors. In line with this hypothesis, a recent study found surface P-selectin to be an insensitive marker of platelet activation.[24]

Chronic graft-versus-host disease remains the most common late complication of allogeneic stem cell transplantation (SCT). Depending on the donor type,[25] it occurs in approximately 40–70% of patients following unmanipulated transplants, and means substantial morbidity and mortality for affected patients. A recent study from our group[6] found a 13.1-fold elevated thrombosis risk in patients with chronic GVHD, the reason for this distinctive thrombophilia remaining unclear.

The present study demonstrates a pronounced number of circulating microparticles in the blood of patients with chronic GVHD, but not in patients without chronic GVHD. Microparticles are membrane vesicles shed from the outer platelet membrane.[26] They are highly thrombogenic[27] as they provide a large catalytic surface for the assembly of procoagulant enzymes[28,29] and activate other platelets.[30] They have recently been associated with prothrombotic conditions such as heparin-induced thrombocytopenia,[5] thrombotic thrombocytopenic purpura,[31] paroxysmal nocturnal haemoglobinuria[32] and myocardial infarction.[33] In conclusion, the seven-fold increased percentage of microparticles in the circulation of chronic GVHD patients

causes a considerable thrombophilic risk which could explain the thrombophilia of these patients.

Moreover, besides activating and amplifying the coagulation cascade, microparticles produce proinflammatory changes in the endothelium, lead to an increased expression of adhesion molecules[30] and have strong mitogenic effects on smooth muscle cells independent of PDGF.[34] As all these effects are also characteristic findings in chronic GVHD, the presence of microparticles might contribute significantly to the severe structural damage in tissues affected by chronic GVHD.

In patients with chronic GVHD, the observed thrombophilic state due to circulating microparticles and, at the same time, the HSCT-associated platelet defect which is attributed to an increased bleeding tendency, may seem paradoxical. However, a disturbed platelet function mainly impairs the formation of a primary hemostatic plug, which plays a key role in immediate hemostasis, whereas the enormous phospholipid surface provided by microparticles mainly activates the plasmatic coagulation, which is known to be of specific importance in the pathogenesis of thrombosis. Therefore, patients with cGVHD can very well have a bleeding tendency and a thrombophilic state at the same time, which is in accordance with clinical observations and explained by the findings of our study.

In summary, the present study suggests that platelet disturbances may explain the bleeding tendency found in patients following HSCT and the thrombophilic state observed in patients with chronic GVHD. Further studies are needed to clarify the molecular mechanisms by which platelet receptor expression, granule contents and generation of microparticles are altered in HSCT.

Acknowledgements

This work was supported by the 1998 research award of the Deutsche Gesellschaft für Thrombose- und Hämostaseforschung (GTH) and is part of the doctoral thesis of H Wegner. We thank Dr Mark Ginsberg (VB-2, The Scripps Research Institute, 10550 N, Torrey Pines, La Jolla, Ca 92037, USA) for the LIBS-1 antibody. The costs of producing the antibody have been covered by a grant from the NIH (No. HL-48728).

References

1 George JN, Nurden AT, Phillips DR. Molecular defects in interactions of platelets with the vessel wall. *New Engl J Med* 1984; **311**: 1084–1098.
2 Ginsberg MH, Loftus JC, Plow E. Cytoadhesins, integrins and platelets. *Thromb Haemost* 1988; **59**: 1–6.
3 McEver RP. The clinical significance of platelet membrane glycoproteins. *Hematol Oncol Clin North Am* 1990; **4**: 87–103.
4 Nurden P, Bihour C, Combrie R, Gralnick et al. Glycoprotein Ia-IIa (VLA-2) and glycoprotein Ib–IX complexes are processed independently during thrombin-induced platelet activation. *J Lab Clin Med* 1994; **124**: 579–588.
5 Warkentin TE, Hayward CPM, Boshkov LK *et al*. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood* 1994; **84**: 3691–3699.
6 Pihusch R, Salat C, Schmitt E, Göhring P *et al*. Hemostatic complications in bone marrow transplantation: a retrospective analysis of 447 patients. *Transplantation* (in press).
7 Michelson AD. Flow cytometry: a clinical test of platelet function. *Blood* 1996; **87**: 4925–4936.
8 Schmitz G, Rothe G, Ruf A *et al*. European Working Group on Clinical Cell Analysis: Consensus protocol for the flow cytometric characterisation of platelet function. *Thromb Haemostasis* 1998; **79**: 885–896.
9 Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 1990; **75**; 128–138.
10 Fukami MH. Dense granule factors. In: *Platelets and their Factors*. Springer Verlag: Berlin, 1997, pp 419–429.
11 Gawaz MP, Bogner C, Gurland HJ. Flow-cytometric analysis of mepacrine-labelled platelets in patients with end-stage renal failure. *Haemostasis* 1993; **23**: 284–292.
12 Mohri H. Acquired von Willebrand disease and storage pool disease in chronic myelocytic leukemia. *Am J Hematol* 1986; **22**: 391–401.
13 Schafer AI. Bleeding and thrombosis in the myeloproliferative disorders. *Blood* 1984; **64**: 1–12.
14 Carr ME, Hines S, Carr SL *et al*. Storage pool disease in chronic lymphocytic leukemia: abnormal aggregation and secretion without bleeding. *Am J Med Sci* 1997; **313**: 176–181.
15 Saelman EUM, Nieuwenhuis HK, Hese KM *et al*. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa ($\alpha 2\beta 1$-integrin). *Blood* 1994; **83**: 1244–1250.
16 Nieuwenhuis HK, Sakariassen KS, Houdijk WP *et al*. Deficiency of platelet membrane glycoprotein Ia associated with a decreased platelet adhesion to subendothelium: a defect in platelet spreading. *Blood* 1986; **68**: 692–695.
17 Handa M, Watanabe K, Kawai Y *et al*. Platelet unresponsiveness to collagen: involvement of glycoprotein Ia-IIa (alpha 2 beta 1 integrin) deficiency associated with a myeloproliferative disorder. *Thromb Haemostasis* 1995; **73**: 521–528.
18 Carlo-Stella C, Tabilio A, Regazzi E *et al*. Effect of chemotherapy for acute myelogenous leukemia on hematopoietic and fibroblast marrow progenitors. *Bone Marrow Transplant* 1997; **20**: 465–471.
19 Marsh JC, Harhalakis N, Dowding C *et al*. Recurrent graft failure following syngeneic bone marrow transplantation for aplastic anaemia. *Bone Marrow Transplant* 1989; **4**: 581–585.
20 Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* 1996; **84**: 289–297.
21 Hourdille P, Heilmann E, Combrie R *et al*. Thrombin induces a rapid redistribution of glycoprotein Ib–IX complexes within the membrane systems of activated human platelets. *Blood* 1990; **76**: 1503–1513.
22 Michelson AD, Barnard MR. Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb–IIIa complex. *Blood* 1987; **70**: 1673–1678.
23 Michelson AD, Barnard MR, Hechtman HB *et al. In vivo* tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci* USA 1996; **93**: 11877–11882.
24 Michelson AD, Barnard M, Krueger LA *et al*. Circulating monocyte-platelet aggregates are a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin. *Circulation* 2001; **104**: 1533–1537.
25 Sullivan K, Agura E, Anasetti C. Chronic graft-versus-host

disease in other late complications of bone marrow transplantation. *Semin Hematol* 1991; **28**: 250–259.
26 Hughes M, Hayward CP, Warkentin TE *et al*. Morphological analysis of microparticle generation in heparin-induced thrombocytopenia. *Blood* 2000; **96**: 188–194.
27 Warner MN, Pavord S, Moore JC *et al*. Serum-induced platelet procoagulant activity: an assay for the characterization of prothrombotic disorders. *J Lab Clin Med* 1998; **133**: 129–133.
28 Gilbert GE, Sims PJ, Widemer T *et al*. Platelet-derived microparticles express high affinity receptors for Factor VIII. *J Biol Chem* 1991; **266**: 17261–17268.
29 Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem* 1988; **263**: 18205–18212.
30 Barry OP, Practico D, Lawson JA, Fitzgerald GA. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest* 1997; **99**: 2118–2127.
31 Galli M, Grassi A, Barbui T. Platelet-derived microvesicles in thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Thromb Haemostasis* 1996; **75**: 427–431.
32 Hugel B, Socie G, Toti F *et al*. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. *Blood* 1999; **93**: 3451–3456.
33 Mallat Z, Benamer H, Hugel B *et al*. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* 2000; **101**: 841–848.
34 Weber A, Koppen HO, Schror K. Platelet-derived microparticles stimulate coronary artery smooth muscle cell mitogenesis by a PDGF-independent mechanism. *Thromb Res* 2000; **98**: 461–466.