The Value of Flow Cytometric Analysis of Platelet Glycoprotein Expression on CD34+ Cells Measured Under Conditions That Prevent P-Selectin–Mediated Binding of Platelets


In the present study, we show by adhesion assays and ultrastructural studies that platelets can bind to CD34+ cells from human blood and bone marrow and that this interaction interferes with the accurate detection of endogenously expressed platelet glycoproteins (GPs). The interaction between these cells was found to be reversible, dependent on divalent cations, and mediated by P-selectin. Enzymatic characterization showed the involvement of sialic acid residues, protein(s) containing O-linked glycans, and elastase-sensitive protein(s). The demonstration of mRNA for the P-selectin glycoprotein ligand 1 (PSGL-1) in the CD34+ cells by polymerase chain reaction (PCR) analysis suggests that this molecule is present in these cells. Under conditions that prevent platelet adhesion, a small but distinct subpopulation of CD34+ cells diffusely expressed the platelet GPIIb/IIIa complex. These cells were visualized by immunohistochemical studies. Furthermore, synthesis of mRNA for GPIIb and GPIIIa by CD34+ cells was shown using PCR analysis. The semiquantitative PCR results show relatively higher amounts of GPIIIa mRNA than of PF4 mRNA in CD34+CD41+ cells in comparison with this ratio in platelets. This finding is a strong indication that the PCR results are not caused by contaminating adhering platelets. MoAbs against GPIIa, GPIIb, GPIV, P-selectin, and the α-chain of the vitronectin receptor did not react with CD34+ cells. The number of CD34+ cells expressing GPIIb/IIIa present in peripheral blood stem cell (PBSC) transplants was determined and was correlated with platelet recovery after intensive chemotherapy in 27 patients. The number of CD34+CD41+ cells correlated significantly better with the time to platelet recovery after PBSC transplantation (r = −.83, P = .04) than did the total number of CD34+ cells (r = −.55). Statistical analysis produced a threshold value for rapid platelet recovery of 0.34 × 10^6 CD34+CD41+ cells/kg. This study suggests that if performed in the presence of EDTA the flow cytometric measurement of GPIIb/IIIa on CD34+ cells provides the most accurate indication of the platelet reconstitutive capacity of the PBSC transplant.

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MATERIALS AND METHODS

MoAbs. The following antibodies were used in this study. IgG1 and IgG2a isotype control antibodies were obtained from the CLB from the European Cancer Centre, Amsterdam, The Netherlands.

From the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; the Free University Hospital, Amsterdam, The Netherlands; the Netherlands Cancer Institute/Amsterdam and Leiden, The Netherlands; INSERM U.91 Hôpital Henri Mondor, Créteil, France; INSERM U.362 Institut Gustave Roussy, Villejuif, France; and the Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands.

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EVERAL STUDIES have shown that flow cytometric measurement of CD34+ cells can be used to establish the reconstitutive capacity of a stem cell transplant.1-4 In these studies, reinfusion of peripheral blood stem cells (PBSCs) resulted in rapid granulocyte reconstitution in almost all patients but not always in a swift independence of platelet transfusions. The observation that the time to platelet recovery varied strongly after reinfusion of small number of CD34+ cells suggests that an additional marker is required to more directly measure the megakaryocytic reconstitutive capacity of a PBSC transplant. The number of megakaryocyte progenitor cells can be determined by the use of the clonogenic assay for megakaryocytes (colony-forming unit-megakaryocyte [CFU-MK]). However, this assay is laborious, and results are not available until after 14 days. Therefore, the flow cytometric measurement of megakaryocytic precursor cells might be more practical and useful.5 This parameter could be used as an indicator for the platelet reconstitutive capacity of an autologous transplant after high-dose chemotherapy.

A number of studies have reported on the presence of platelet antigens on CD34+ cells.6-9 In these studies, a variable but, in most studies, predominant reactivity with CD41 and CD61 monoclonal antibodies (MoAbs) was found, ranging from 2% to 90% of the CD34+ cell population. These antibodies recognize the platelet glycoproteins (GP) IIb and GPIIIa, respectively. This high level of variation could be due to some pitfalls. It has been shown that platelets can adhere to monocytes, neutrophils, eosinophils, basophils, and subpopulations of T lymphocytes, causing spurious detection of platelet antigens on these cells.10-13 Moreover, Betz et al14 reported that CD41 reactivity on acute myeloid leukemic blasts was caused by adherent platelets or platelet fragments in 85% of the cases, whereas in only 15% of the cases the cells showed true diffuse membrane and cytoplasmic positivity on cytoxpin slides.

The aims of the present study were to evaluate the expression of the different platelet GPs on hematopoietic progenitor cells by flow cytometry and to investigate whether binding of platelets can interfere in this assay. Subsets of PB CD34+ cells were quantified, and the numbers of reinfused subsets of CD34+ cells were correlated with platelet recovery after intensive chemotherapy to obtain a more accurate indication of the reconstitutive capacity of a PBSC transplant.
Fluorescent isothiocyanate (FITC)-labeled HPCA-2 (CD34), phycoerythrin (PE)-labeled rat MoAb against the α light chain of mouse Ig, and MoAb Leu-8 (CD62L, L-selectin) were purchased from Becton Dickinson (San Jose, CA). MoAbs CLB-thromb/1 (CD17, CD61, GPIIIa), CLB-thromb/3 (CD49b, VLA-2), CLB-thromb/5 (CD62P, P-selectin), CLB-thromb/6 (CD62P, P-selectin), CLB-thromb/7 (6C9, CD41, GPIb), ES12F11 (CD31, GPIIIa*, PECA-1), ES4C7 (CD36, GPIV), SW16 (CD42d, GPV), CLB-3B9 (CD15, Le*, CD34-gran2 (CD15, Le*), and B13.9 (CD66b) were all produced in our laboratory (CLB) and clustered in the International Workshops on Leukocyte Differentiation Antigens.11,12 Biotin-labeled CD41 (CLB-thromb/7) and a biotin-labeled isotype control are MoAbs produced in our laboratory (CLB); MoAbs W6/32 (HLA class I), CSLEX-1 (CD15s), MB45 (CD42b, GPIbα), Y2.51 (CD61, GPIIb), and NKI-M7 (CD51) were obtained from the Fourth International Workshop studies.13,14 MoAbs Immu-133, Immu-409, 14G3, BNC5, CD34-9F2, HPCA2, 43A1, 581, 570, 553, 563, MD34.1, MD34.2, MD34.3, QBend10, 4A1, 9044, and 9049, directed against CD34, were obtained from the Fifth International Workshop studies.15,16

**Patient characteristics.** The group of 27 patients studied (median age, 35 years; range, 19 to 52 years) included 9 patients with breast cancer, 8 patients with germ cell cancer, 4 patients with Hodgkin’s disease, 3 patients with non-Hodgkin’s lymphoma, 1 patient with t(1;14) carcinoma, 1 patient with medulloblastoma, and 1 patient with neuroblastoma. The patients were either in their first chemotherapy-induced (near) complete remission (patients with breast cancer, the mobilizing regimen consisted of ifosfamide (4 g/m2, day 1) and etoposide (100 mg/m2, days 1 through 4). In the remaining patients, PBSCs were mobilized with either high-dose chemotherapy regimens (CTC or BEAM) or until the WBC count in the PB exceeded 5 x 109/L. No significant differences in the rate of neutrophil or platelet recovery were found with either high-dose chemotherapy regimens (CTC or BEAM) or in patients with the different diagnoses (data not shown).

**Flow cytometry.** The percentage of cells expressing the CD34 antigen was determined in a sample of the leukocytapheresis product by direct immunofluorescence just before cryopreservation. After lysis of the erythrocytes with isomolar NH4Cl buffer for 10 minutes, 1 x 109 cells were incubated with MoAb CD34-FITC. All incubations were performed at 4°C. After each incubation, the cells were washed with phosphate-buffered saline (PBS) containing 0.2% (wt/vol) bovine serum albumin (BSA).

For double-color immunofluorescence analysis, the cells were incubated for 30 minutes with the primary MoAb, followed by incubation with PE-labeled rat-antimouse Ig. An isotype-matched mouse Ig served as control. Residual binding sites of rat-antimouse Ig were blocked with a mixture of irrelevant murine MoAbs of IgG1 and IgG2a subclasses. Subsequently, the cells were incubated with MoAb CD34-FITC. After each incubation, the cells were washed with PBS/EDTA/BSA.

Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). A minimum of 20,000 cells were acquired in list mode. Analysis of the five-dimensional data was performed with Consort 30 software (Becton Dickinson). The percentage of CD34+ cells present was assessed after correction for the percentage of cells reactive with an isotype control. For the determination of the phenotype of CD34+ cells, a minimum of 2,500 CD34+ cells were analyzed. A marker was set at the first log decade and the percentage of CD34+ cells coexpressing a specific antigen was assessed after correction for the percentage of cells reactive with an isotype control. Absolute numbers of CD34+ cells were calculated by multiplication of the total amount of nucleated blood cells in the leukocytapheresis product with the percentage of CD34+ cells in the total leukocytapheresis product.

**Isolation of cells.** Platelet-rich plasma (PRP) was removed from BM or leukocytapheresis samples by centrifugation at 200g for 20 minutes. The erythrocytes were lysed with isomolar NH4Cl buffer for 10 minutes at 4°C and subsequently washed twice with PBS containing 5 mmol/L EDTA and 0.2% (wt/vol) BSA (PBS/EDTA/BSA). BM MNCs were isolated by density centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden; density, 1.077 g/mL). To facilitate further analysis of GPs on PB CD34+ cells, we enriched...
Platelet glycoprotein expression on CD34+ cells

for immature cells by one or two rounds of immunomagnetic depletion of T cells and monocytes. MNCs (1 x 10^7/mL) containing 0.5% to 5% CD34+ cells were incubated for 40 minutes at 4°C with a mixture of CD2 and CD14 in the presence of DNase (20 U/μL) and 10 mmol/L MgCl2. The cells were washed twice and incubated with immunomagnetic beads coated with goat-anti-mouse Ig (ratio of beads:cells = 3:1; Dynal, Hamburg, Germany). This procedure did not affect the expression of GPs on CD34+ cells (data not shown).

Resting platelets were prepared from EDTA-anticoagulated whole blood by direct fixation in paraformaldehyde (PFA) at a final concentration of 1% (wt/vol) for 10 minutes at room temperature. Platelets were isolated from PRP and washed twice with PBS/EDTA/BSA. Activated platelets were prepared by treatment with 1 U/mL of human α-thrombin (Sigma Chemical, St. Louis, MO) or trypsin (GIBCO BRL, Grand Island, NY). After fixation with osmium tetroxide, dehydrated, and embedded in epon. Thin sections were examined with a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands) after lead citrate staining.

RNA isolation and synthesis of cDNA. RNA of CD34+CD41+ cells was isolated from 100,000 fluorescence-activated sorted cells essentially as previously described by Ziegler et al24 and Rappolee et al.25 Briefly, cells were transferred in 100 μL of lysis buffer (4 mmol/L guanidine thiocyanate, 25 mmol/L sodium citrate, pH 5.0, 0.5% sodium lauroyl sarcosinate, 0.1% nol/L β-mercaptoethanol) containing 20 μg of glycogen (Boehringer Mannheim, Mannheim, Germany) as carrier. The mixture was then thoroughly vortexed and layered on top of a 100 μL 5.7 mol/L CsCl cushion. Samples in 0.3 mL diethylpyrocarbonate (DEPC)-treated polycarbonate centrifuge tubes were centrifuged for 2.5 hours at 4°C at 55,000 rpm in a TLS-55 rotor on a Beckman TL-100 tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA). The RNA pellet was resuspended in DEPC-treated H2O, precipitated with 0.1 vol of potassium acetate and 2.5 vol 100% ethanol, washed in 70% ethanol, dried, and again resuspended in DEPC-treated H2O. First-strand cDNA was synthesized by incubating RNA at 37°C for 1 hour in a final volume of 10 μL in RT-buffer (50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl2) containing 100 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO/BRL, Gaithersburg, MD), 0.5 μg oligo(dT), 15 U RNAse inhibitor, 10 mmol/L diethylthioctetol (DTT), and 1.0 mmol/L of each deoxyribonucleoside triphosphate (dNTP).

cDNA-polymerase chain reaction (PCR) procedure. Oligonucleotide primers selected for the PCR were used to amplify those parts of the cDNA that contain the sequences corresponding to GPlib, GPlila, P-selectin glycoprotein ligand 1 (PSGL-1), and β3, respectively. The locations of the primers in the nucleotide sequence were as follows: GPlib (sense primer: positions 1025-1046, sequence 5′ATAGATCGCTAGCTATAGGAGAGCCG3′ in exon 12; antisense primer: positions 1580-1599, sequence 5′TAGAATTCTTAAGGATAGCTCTGAGG3′ in exon 16; product length: 575 bp); GPlila (sense primer: positions 379-401, sequence 5′TAGAATCCCAAGATGAGTCTACCTC3′ in exon 8; antisense primer: positions 675-679, 5′ATAAGCTCTTCTACCTCAACACATTCTTTC3′ in exon 10; product length: 279 bp); PSGL-1 (sense primer: positions 76-96, sequence 5′TCTGTTTGCTGTA-TCTACTGGG3′; antisense primer: positions 416-397, sequence 5′ATAAGCTGCTAAGCTGATCTG3′; product length: 341 bp); platelet factor 4 (F4; sense primer: positions 8-31, sequence 5′ATGGATCCCGGAATTCGCT3′; antisense primer: positions 316-339, sequence 5′AATGACACACAGTTAGGACGTAGT3′; product length: 332 bp); and β3-actin (sense primer: positions 144-163, 5′GGTGCGGCCCAGGCCCAAC3′ in exon 2; antisense primer: positions 683-660, 5′CTCCTTAATGTCACCCAGAT-TTC3′ in exon 4; product length: 540 bp).27 All PCR primers were for 30 minutes with a biotin-labeled CD41 (CLB-thromb57) MoAb. A biotin-labeled isotype-matched mouse Ig served as control. Subsequently, the cells were washed and spun on slides for 5 minutes at 350 rpm in a Shandon cytocentrifuge (Shandon Southern Products Ltd, Runcorn, Cheshire, UK). After fixation, the cells were incubated for 1 hour with peroxidase-conjugated avidin-biotin complex (Brunschwig Chemicals, Amsterdam, The Netherlands) and diluted in Tris-buffered saline (TBS; pH 7.8) to which 2.5% BSA and 0.5% Triton X-100 was added. Peroxidase was visualized with 3,3′-diaminobenzidine-tetrahydrochloride. All the rinsing procedures were performed with TBS. The sections were counterstained with haematoxilin.

Ultrastructural studies. Sixty thousand fluorescence-activated sorted cells were washed twice in Hank’s medium at 4°C, fixed in 1.25% glutaraldehyde in Gey’s buffer21 for 10 minutes, washed, and incubated in dinamobenzidine medium.22 Cells were then postfixed with osmium tetroxide, dehydrated, and embedded in epon. Thin sections were examined with a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands) after lead citrate staining.

Platelet Glycoprotein Expression on CD34+ Cells

Cell adhesion assay. The interaction between CD34+ cell and platelet adhesion assays was studied as described before.20 Briefly, fixed MNCs and platelets were immunostained with MoAb CD34-FTTC and MoAb W6/32 (anti-HLA class I), respectively. PE-labeled rat-antimouse Ig was used as conjugate for MoAb W6/32. Thereafter, the cells were resuspended in a buffer containing 25 mmol/L Tris-HCl, pH 7.0, 150 mmol/L NaCl, 0.2% (wt/vol) BSA, supplemented with either 1 mmol/L CaCl2 and 1 mmol/L MgCl2 (incubation buffer) or 5 mmol/L EDTA. Fifty microliters of the platelet suspension (1 x 10^7/mL) was mixed with 50 μL of the MNC suspension (1 x 10^6/mL), giving a platelet to MNC ratio of 1:10. After incubation for at least 30 minutes at room temperature, platelet MNC cell adhesion was quantitated by measuring doubly stained cells on a FACSscan cytometer (Becton Dickinson). Antibody inhibition studies were performed by preincubating of either the platelets or the MNCs with MoAb in optimal dilution. Then, without washing, platelets and MNCs were gently mixed, and the FACS analysis was performed as described above. After treatment with enzymess, the cells were washed twice with PBS and 0.2% BSA and fixed with PFA. Subsequently, platelets and enzyme-treated MNCs were gently mixed, and the FACS analysis was performed as described above. An isotype-matched control antibody was used to set a threshold (99% of events below threshold) for positive platelet fluorescence. The percentage of CD34+ cells that bound platelets was expressed as the percentage of CD34+ cells that reacted positively with antiplatelet antibodies. In binding inhibition studies, the percentage of CD34+ cells that bound platelets was expressed as the percentage of total CD34+ cells that reacted positively with antiplatelet antibodies. In binding inhibition studies, the percentage of bound activated platelets of CD34+ cells was calculated as a percentage of this value. The mean total platelet fluorescence (TPF) of positively reacting CD34+ cells was used as an estimate of the number of platelets bound per CD34+ cell.

Treatment of cells with enzymes. MNCs were treated with elastase (Elastin products, Pacific, MO) or trypsin (GIBCO BRL, Grand Island, NY) as described.21 Neuraminidase (Vibrio cholerae; Behringwerke, Marburg, Germany) treatment of cells was performed by incubating 1 x 10^7 cells in 100 μL of PBS and 0.2% BSA with 0.2 U/mL neuraminidase for 60 minutes at 37°C. Treatment with glycoprotease Pasteurella haemolytica (generous gift from Dr A. Mellors, Guelph, Ontario, Canada) was performed by incubating 1 x 10^7 cells in 100 μL of PBS and 0.2% BSA with 0.05 mg glycoprotease for 60 minutes at 37°C.

Immunocytochemical staining. For immunocytochemical analysis of CD34+ cells, the fluorescence-activated sorted cells were incubated for 30 minutes with a biotin-labeled CD41 (CLB-thromb57) MoAb. A biotin-labeled isotype-matched mouse Ig served as control.
synthesized on a DNA synthesizer (Applied Biosystems model 392, Palo Alto, CA) and purified with oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA). A small portion of the RT-products (1 µL) was mixed with 1.25 U of Taq DNA polymerase (GIBCO/BRL), 1 µmol/L of each appropriate primer, 200 µmol/L of each dNTP in a buffer containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 5 mmol/L MgCl₂, and 0.01% (wt/vol) BSA in a 50 µL volume. For the PCR reaction of the P-selectin ligand, 2 U of Taq DNA polymerase from Promega (Madison, WI) and purified with oligonucleotide purification cartridges were analyzed on 6% polyacrylamide gels. Fragments were visualized by UV illumination after ethidium bromide staining. One-hundred basepair ladders were used as markers.

For the semiquantitative PCR, a sample of 10 µL was taken after 21, 24, 27, and 30 cycles of the GPIIb, PF4, and β-actin PCRs. The samples were denatured in 0.5 mol/L NaOH and 0.15 mol/L NaCl for at least 10 minutes and neutralized in 0.5 mol/L Tris, pH 8.0, and 0.15 mol/L NaCl. Immediately thereafter the samples were dot-blotted using a broad dot-blot apparatus to Hybond N⁺ positively charged nylon membranes (Amersham, Buckinghamshire, UK). After drying, the DNA was cross-linked to the membranes with UV light for 5 minutes. The filters were then prehybridized in 6× SSC buffer containing 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt’s, and 100 µg/mL denatured salmon sperm DNA for at least 1 hour at 60°C. After 1 hour, the mixture was replaced by the hybridization mix that contains all of the ingredients of the hybridization mix except for the Denhardt’s. 32P-radioactively labeled probes were added to the hybridization mixture. The following sequences of probes were used: for GPIIb, TAGGATCCGTGAGGAAACAACTACACCGC; for PF4, TCCATGAAAGGATGATCTGTGG; and for β-actin, GATGACCCAGATCATGTTGT. The labeled probe that gets through the sephadex column. The labeled probe that gets through the sephadex column by UV illumination after ethidium bromide staining. One-hundred basepair ladders were used as markers.

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Statistical analysis. For nonnormal distributed values, data were summarized by means of median and ranges; otherwise, the arithmetic mean and standard deviation were used. Differences were calculated by means of the Mann-Whitney U test. The correlations are Spearman Rank correlations. Differences were calculated by means of a z-test. A P value less than α = .05 was considered significant. To determine the relative influence of parameters (multivariate analysis), a Cox’s proportional hazards model was used. The models were built in a stepwise procedure.

Thresholds for rapid platelet recovery (arbitrarily set as at the time to platelet transfusion independence within 14 days after PBSC transplantation) were defined by the optimum of both sensitivity and specificity of a tested parameter in the receiver operating characteristic curve.

RESULTS

Flow cytometric assay for adhesion of thrombin-activated platelets to CD34⁺ cells. A double-color immunofluorescence assay was used to investigate whether platelets can adhere to CD34⁺ cells. Platelets were thrombin-activated and PE-labeled and then added to FITC-labeled CD34⁺ cells in a buffer containing Ca²⁺ and Mg²⁺. Without additional washing, platelet-cell adhesion was quantified using flow cytometry.

It appeared that nearly half of the BM CD34⁺ cells bound thrombin-activated platelets, whereas directly fixed nonactivated platelets bound to only low numbers of CD34⁺ cells (Table 1). Similar results were obtained with PB CD34⁺ cells.

The involvement of divalent cations in the interaction of platelets and CD34⁺ cells was studied by incubation of MNCs and platelets in the presence of EDTA. Binding of activated platelets to BM and PB CD34⁺ cells was almost completely abolished by 5 mmol/L EDTA. A slight reduction of binding of nonactivated platelets to CD34⁺ cells was also observed (Table 1).

As an estimate for the number of platelets bound per CD34⁺ cell, the TPF per CD34⁺ cell was also determined. The mean TPF for activated platelets bound to BM CD34⁺ cells was 5 times as high as for nonactivated platelets. Similar results were obtained with PB CD34⁺ cells (Table 1).

To investigate whether platelet-CD34⁺ cell interaction is reversible, activated platelets were bound to CD34⁺ cells and, after incubation for 30 minutes, the cells were washed twice in the presence of 5 mmol/L EDTA. These wash steps abolished platelet-CD34⁺ cell interaction and reduced the percentage of cells with bound platelets and the TPF to values comparable with those found when MNCs and platelets were incubated in the presence of EDTA (Table 1).

P-selectin–mediated adhesion of thrombin-activated platelets to CD34⁺ cells. Platelets were preincubated with MoAbs against P-selectin before incubation with MNCs to study the involvement of P-selectin in the interaction of activated platelets and CD34⁺ cells. Antibody CLB-thromb/6 almost completely blocked adhesion of activated platelets to CD34⁺ cells from BM or PB, reducing the mean TPF to 6.1 ± 3.6 and 5.7 ± 4.5 (on a linear arbitrary scale), respectively (Table 1). Antibody CLB-thromb/5, which recognizes a nonfunctional epitope of P-selectin, did not inhibit the platelet-CD34⁺ cell interaction. The number of bound activated platelets as measured by the TPF was then the same as in the presence of control MoAbs. Thus, P-selectin is involved in the platelet-CD34⁺ cell interaction.

Putative CD34⁺ cell receptors for binding platelets. MNCs were preincubated with MoAbs against P-selectin to characterize the P-selectin ligand present on CD34⁺ cells. When MoAbs against CD15 antigen (CLB-3B9 and CLB-gran/20) were preincubated with the MNCs, binding to activated platelets was not inhibited (Table 1). Similar results were obtained with anti-sialyl Lewisα MoAb CSLEX-1 in different concentrations (range, 1.5 to 300 µg/
PLATELET GLYCOPROTEIN EXPRESSION ON CD34+ CELLS

Table 1. Effect of EDTA and MoAbs Against P-Selectin on Platelet Adhesion to CD34+ Cells

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<tr>
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<th>BM (n = 5)</th>
<th>Leukocytapheresis Samples (n = 10)</th>
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<tr>
<td></td>
<td>% Adhesion (SD)</td>
<td>TPF</td>
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<tr>
<td>Resting platelets</td>
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<tr>
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<td>8.5 (4.3)</td>
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<td>EDTA</td>
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<td>Activated platelets</td>
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<td>EDTA</td>
<td>6.4 (3.4)</td>
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<tr>
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<td>Activated platelets</td>
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<td>Two EDTA wash steps</td>
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Abbreviation: SD, standard deviation.
* In binding inhibition studies, the percentage of CD34+ cells with adherent activated platelets was set as 100%, and inhibition of platelet adhesion was expressed as a percentage of this value.

with CD34+ cells and loss of binding of activated platelets to neutrophils present in the same cell sample as the CD34+ cells (data not shown).

Vascular CD34 is a ligand for L-selectin and could be a ligand for P-selectin as well. To study the involvement of CD34 in the interaction of activated platelets and CD34+ cells, MNCs were preincubated with a panel of MoAbs against all three classes of CD34 epitopes before incubation with platelets. No blocking effects of these MoAbs was observed on platelet-CD34+ cell interaction (data not shown).

The involvement of carbohydrates was studied further by enzymatic treatment of the MNCs with neuraminidase from Vibrio cholerae, with glycoprotease derived from Pasteurella haemolytica, or by treatment with the proteolytic enzymes trypsin or elastase (Table 2). Treatment with neuraminidase, which hydrolyses terminal N-acetylneuraminic acids, partially inhibited binding of activated platelets to BM CD34+ cells and reduced the number of bound platelets to CD34+ cells as the TPF decreased by 54% ± 8%. This finding indicates that sialic acid residues are present on the P-selectin ligand of CD34+ cells. The efficacy of the treatment with neuraminidase was established by the complete loss of reactivity of anti-sialyl LewisX antibody CSLEX-1 with CD34+ cells and loss of binding of activated platelets to neutrophils present in the same cell sample as the CD34+ cells (data not shown).

Table 2. Effect of Enzymatic Treatment on Platelet Adhesion to CD34+ Cells

<table>
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<th></th>
<th>BM (n = 5)</th>
<th>Leukocytapheresis Samples (n = 10)</th>
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<tr>
<td>—</td>
<td>47.9 (9.6)</td>
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<td>Trypsin</td>
<td>26.8 (8.3)</td>
<td>18.3</td>
</tr>
<tr>
<td>Elastase</td>
<td>5.8 (3.8)</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Abbreviation: SD, standard deviation.
* In binding inhibition studies, the percentage of CD34+ cells with adherent activated platelets was set as 100%, and inhibition of platelet adhesion was expressed as a percentage of this value.

To obtain evidence for the synthesis of PSGL-1 by CD34+ progenitor cells, messenger ribonucleic acid (mRNA) was isolated from fluorescence-activated cell sorted CD34+ cells and PCR analysis was performed. The analysis of RT-PCR products showed the expression of mRNA for PSGL-1 (Fig 1).

Platelet GP expression on CD34+ cells from BM and PB.
Expression of GPs on CD34+ cells from BM and PB was examined in a double-color fluorescence assay with a panel of MoAbs recognizing platelet membrane GPs Ia, Ibα, IIaβ', IIIa, IIb/IIIa, IV, and V (Table 3). GP expression was measured under two conditions, i.e., either in the absence of EDTA or after removal of platelets by density centrifugation and washing in EDTA-containing buffer. In the absence of EDTA, there was significant reactivity of BM- and PB-CD34+ cells with the platelet-reactive MoAbs against GPIIa' (CD31), GPIIb (CD41), GPIIIa (CD61), and GPIV (CD36), whereas reactivity with MoAbs against GPIa, GPIbα, VNRα, and P-selectin was weak. Binding of all these MoAbs was reduced when antigen expression was measured after the removal of platelets by density centrifugation and washing in EDTA-containing buffer. Under these conditions, a small and distinct subset of CD34+ cells (2% to 12%) still reacted with MoAbs against the GPIIb (CD41) and GPIIIa (CD61) (Fig 2), whereas virtually no staining was seen with MoAbs recognizing GPIa, GPIbα, GPV, VNRα, and P-selectin either in BM or in PB. The expression of platelet GPs was not significantly different on CD34+ cells obtained from BM or PB.

**immunochemical and ultrastructural studies.** Immunochemical and ultrastructural studies were performed to confirm that, when EDTA is omitted from the buffers, platelets attached to CD34+ cells could attribute to the detection of platelet GPs on CD34+ cells from PB. When cytocentrifuge preparations of fluorescence-activated sorted CD34+ cells, stained in the absence of EDTA, were labeled with CD41, some diffusely stained CD34+ cells were visualized (Fig 3A), but, in addition to these cells, several CD34+ cells

**Table 3. Expression of Platelet Antigens on PB and BM CD34+ Cells in the Absence or Presence of EDTA**

<table>
<thead>
<tr>
<th>GP</th>
<th>CD</th>
<th>MoAb</th>
<th>EDTA -</th>
<th>EDTA +</th>
<th>EDTA -</th>
<th>EDTA +</th>
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<tr>
<td>Ia</td>
<td>49b</td>
<td>CLB-thromb/4</td>
<td>2.1</td>
<td>0.6</td>
<td>4.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Ibα</td>
<td>42b</td>
<td>MB45</td>
<td>2.5</td>
<td>1.3</td>
<td>6.2</td>
<td>1.9</td>
</tr>
<tr>
<td>IIaβ</td>
<td>31</td>
<td>ES12F11</td>
<td>39.6</td>
<td>86.1</td>
<td>94.5</td>
<td>91.3</td>
</tr>
<tr>
<td>IIb/IIIa</td>
<td>CLB-thromb/7</td>
<td>14.3</td>
<td>6.8</td>
<td>19.5</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>IIIa</td>
<td>41</td>
<td>CLB-thromb/1</td>
<td>7.2</td>
<td>4.9</td>
<td>11.8</td>
<td>4.9</td>
</tr>
<tr>
<td>IV</td>
<td>36</td>
<td>ES4C7</td>
<td>6.8</td>
<td>3.9</td>
<td>11.8</td>
<td>4.9</td>
</tr>
<tr>
<td>V</td>
<td>42d</td>
<td>SW16</td>
<td>12.2</td>
<td>11.5</td>
<td>16.7</td>
<td>15.0</td>
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<tr>
<td>VNRα</td>
<td>51</td>
<td>NKI-M7</td>
<td>1.2</td>
<td>0.3</td>
<td>4.5</td>
<td>1.1</td>
</tr>
<tr>
<td>P-selectin</td>
<td>62P</td>
<td>CLB-thromb/6</td>
<td>5.7</td>
<td>0.9</td>
<td>2.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**Values are the median percentage of cells expressing platelet antigen as determined in five experiments, with the range of platelet antigen expression in parentheses.**
with a patchy localized staining were identified, strongly suggesting the appearance of platelets and platelet particles adhering to the cell membrane. Similar cells were found when thrombin-activated platelets were added to the sorted CD34⁺ cells (Fig 3C). In the CD34⁺ cell preparation, labeled in the presence of EDTA after removal of platelets by density centrifugation, only diffusely stained cells and no cells with a patchy appearance were detected (Fig 3B). In addition, the interaction between thrombin-activated platelets and sorted CD34⁺ cells was blocked by EDTA (Fig 3D).

Electron microscopy on CD34⁺CD41⁺ sorted cells, stained in the absence of EDTA, showed numerous platelet membrane fragments adhering to cell membrane. Large nucleoli were observed in the nuclei of these cells and all granules showed strong peroxidase staining. Therefore, these cells were identified as promonocytes (Fig 4A). In contrast, when CD41-labeling of CD34⁺ cells was performed under conditions that prevent platelet adherence, no membrane fragments attached to CD34⁺CD41⁺ sorted cells were detected. Megakaryocytic cells were identified in this fraction by platelet-peroxidase staining of the endoplasmatic reticulum and of the area around the nuclear envelope (Fig 4B). Platelet organelles were not observed in these cells. In addition to these promegakaryoblasts, pro-
monocytes with small peroxidase-positive granules were still found to be present in this fraction. However, all of these cells were free of attached membranes or membrane fragments of platelets.

**Detection of GPIIb and GPIIIa by PCR.** To obtain evidence for the synthesis of platelet GPs by progenitor cells, PCR analysis was performed on mRNA isolated from CD34+CD41− fluorescence-activated cell sorted cells that were stained under conditions that prevent platelet adherence to CD34+ cells. The analysis of RT-PCR products showed the expression of mRNA for GPIIb and GPIIIa (Fig 1). The same products were detected in CD34+CD41− fluorescence-activated cell sorted cells (data not shown). To exclude the possibility that the expression of GPIIb and GPIIIa was mainly due to platelets adhering to the CD34+CD41− sorted cells, the mRNA expression of GPIIb and the platelet-specific protein PF4 were compared in a semiquantitative PCR assay by measuring the obtained PCR products after 21, 24, 27, and 30 PCR cycles in platelets and sorted CD34+CD41+ cells (Fig 5). In CD34+CD41+ cells, relatively more GPIIb mRNA was present than PF4 mRNA in comparison with the expression in platelets, eg, at 24 cycles the ratio of GPIIb/PF4 PCR products in CD34+CD41+ cells as measured by fosafluorimaging analysis was 2.6, whereas in platelets this ratio was 0.1. In all cell types, similar amounts of β-actin mRNA were present (data not shown). These results indicate that mRNA for GPIIb is mainly synthesized by CD34+ progenitor cells. Also, in CD34+CD41− cells, some GPIIb mRNA expression was detected, although at a lower level, whereas PF4 mRNA could not be detected.

**Correlation with platelet recovery.** After high-dose chemotherapy, a median number of 6.0 × 10^6 CD34+ cells/kg (range, 1.6 to 39.4 × 10^6/kg) was reinfused in 27 patients. This resulted in a rapid platelet recovery (≤14 days) to platelet transfusion independence (defined as the platelet count remaining ≥20 × 10^9/L without platelet transfusions) in 15 of 27 patients (n = 27; median, 14 days; range, 7 to 37 days).

The expression of CD41 and CD61 antigens on CD34+ cells was measured in the presence of EDTA on all 92 leukocyteapheresis samples from these patients. Under these conditions, a median of 6.3% of the CD34+ cells expressed the CD41 antigen (range, 0% to 18.1%), whereas the CD61 antigen was detected on a median of 3.5% of CD34+ cells (range, 0% to 10.3%). These percentages were used to calculate the absolute number of CD34+ cell subsets per kilogram of body weight that were reinfused into a patient.

The correlation between the total number of CD34+ cells per kilogram and the time to platelet transfusion independence was r = −0.55 (95% confidence interval [CI], −0.68 to −0.34). When subsets of CD34+ cells, defined by the expression of the megakaryocytic lineage-associated antigens CD41 or CD61, were correlated with the time to platelet recovery, both subsets correlated better (CD34+CD41− cells, r = −0.83 [95% CI, −0.89 to −0.73]; CD34+CD61+ cells, r = −0.78 [95% CI, −0.85 to −0.65]) than did the total number of CD34+ cells. This difference was significant only for the subset of CD34+ cells defined by CD41 (P = .04), but failed to reach significance for the subset defined by the expression of CD61 (P = .09). Using a stepwise procedure in a Cox-regression analysis with the numbers of CD34+ cells, CD34+CD41− cells, and CD34+CD61+ cells, respectively, as covariates, only the number of CD34+CD41− cells was a significant covariable (β = .0012, SE = .003, P = .005).

To assess a threshold for rapid platelet recovery the optimal sensitivity and specificity of the number of reinfused CD34+ cells per kilogram of body weight was calculated. The threshold of CD41-expressing CD34+ cells for rapid platelet recovery was calculated to be 0.34 × 10^6 CD34+CD41− cells/kg. In the 17 patients who received more than the threshold of 0.34 × 10^6 CD34+CD41− cells/kg, the time to platelet recovery was significantly shorter (n = 17; median, 11 days; range, 7 to 16 days) as compared with the time to platelet recovery of the patients who received fewer CD34+CD41− cells (n = 10; median, 20 days; range, 13 to
Fig 5. Dot-blot analysis of PCR products from GPllb and PF4 hybridized to \(^{32}\)P-labeled probes as detected after 21, 24, 27, and 30 cycles. In all three cell types similar amounts of \(\beta\)-actin PCR products were detected after 24, 27, and 30 cycles, whereas no expression of \(\beta\)-actin was detected after 21 cycles (data not shown). Lane 1, CD34\(^+\)CD41\(^-\) cells; lane 2, CD34\(^+\)CD41\(^+\) cells; lane 3, platelets; lane 4, H\(_2\)O.

37 days; \(P < 0.0001\); Fig 6). None of these 10 patients had recovered within 2 weeks.

DISCUSSION

The expression of platelet GPs on CD34\(^+\) cells was analyzed to develop a simple flow cytometric assay for the number of platelet precursor cells. This study shows that the detection of endogenously expressed platelet GPs can be hampered by the adherence of activated platelets to CD34\(^+\) cells. Firstly, we observed that binding of the platelet-reactive MoAbs was reduced when antigen expression was measured after the removal of platelets by density centrifugation and washing in EDTA-containing medium. Secondly, both immunocytochemical and ultrastructural studies showed the presence of platelet membranes and membrane fragments attached to CD34\(^+\) cells when EDTA is omitted from the buffers. Most of these cells were identified as promonocytes. In contrast, when CD41-labeling of CD34\(^+\) cells was performed after removal of platelets by density centrifugation and washing in EDTA-containing buffer, membrane fragments attached to the sorted cells were not detected. We have quantified the platelet-cell interaction and showed that, when thrombin-activated platelets were added to the MNC fraction of BM or PB, about 50% of the CD34\(^+\) cells bound activated platelets. This binding of platelets to CD34\(^+\) cells is dependent on divalent cations and is reversible, because thrombin-activated platelets adherent to CD34\(^+\) cells could be removed by subsequent washing with EDTA in high concentration. Therefore, these studies indicate that platelet adherence could be attributed to the detection of platelet GPs on CD34\(^+\) cells and could explain why, when measured in the absence of EDTA, strong expression of platelet antigens on CD34\(^+\) cells has been found by others.\(^5,6\)

Furthermore, the platelet interaction with CD34\(^+\) cells derived from BM or PB was characterized. This interaction was shown to be mediated by P-selectin, because MoAbs against P-selectin almost completely blocked this interaction. Platelets adherence was similar to CD34\(^+\) from BM or PB. P-selectin is known to interact with carbohydrate ligands such as sialyl Lewis\(^x\) and related carbohydrates presented on a polypeptide backbone.\(^5,35\) Recently, a specific GP (PSGL-1) on myeloid cells has been recognized as a ligand for P-selectin.\(^36\) This P-selectin ligand carries large numbers of sialylated O-linked oligosaccharides and is functionally active only when it is appropriately glycosylated. The sialic acid(s) on this ligand is required for binding to P-selectin but appears to be somewhat resistant to sialidase treatment.\(^35\) Our results are in accordance with these findings. We showed that treatment of CD34\(^+\) cells with glycoprotease derived from Pasteurella haemolytica, which cleaves cell surface proteins containing O-linked glycans, completely inhibited the binding of activated platelets to CD34\(^+\) cells. In addition, this binding was only partially inhibited by neuraminidase, as was previously found for monocytes, lymphocytes, eosinophils, and basophils.\(^6\) Treatment with the proteolytic en-
zyme trypsin partially inhibited and with elastase completely inhibited the binding of activated platelets to CD34⁺ cells, although the amount of sialyl Lewis x was unaffected. In addition to the enzymatic characterization of the P-selectin ligand on CD34⁺ cells, our PCR results suggest the expression of mRNA for PSGL-1 by the CD34⁺ progenitor cells.

Our data do not exclude the presence of P-selectin ligands other than PSGL-1 on CD34⁺ cells. L-selectin, which has multiple ligands, including other selectins, may function as a ligand for P-selectin. In addition, it was recently reported that vascular CD34 functions as a ligand for L-selectin. Analogous with this report, hematopoietic CD34 could function as a ligand for P-selectin. In our study, no evidence was found for a role of L-selectin or hematopoietic CD34 as ligand for P-selectin. The reactivity of the L-selectin reactive MoAb Leu-8 with CD34⁺ cells was not decreased by elastase treatment, whereas the binding of activated platelets to CD34⁺ cells was completely inhibited. In addition, no blocking effect of platelet CD34⁺ cell interaction by a panel of anti-CD34 MoAbs was observed, suggesting that hematopoietic CD34 is not a major ligand in the P-selectin-mediated interaction of activated platelets and CD34⁺ cells.

After showing that the binding of platelets to CD34⁺ cells is reversible and dependent on divalent cations, we were able to measure platelet GP expression on CD34⁺ cells using double-color flow cytometry after the removal of platelets by density centrifugation and washing in EDTA-containing buffer. Under these conditions, a small but distinct population of CD34⁺ cells, ranging from 2% to 12%, reacted with CD41 and CD61 MoAbs, which recognize GPs IIb and IIIa. The percentage of cells reactive with CD41 MoAb was higher than that reactive with CD61 MoAb. This discrepancy is probably caused by a higher affinity of the CD41 MoAb. We cannot rule out the possibility that, in the presence of EDTA, a small percentage of CD34⁺CD41⁺ or CD34⁺CD61⁺ cells are cells to which platelet fragments are still bound, because some contaminating promonocytes were still present in the CD34⁺CD41⁺ sorted cell fraction as observed by electron microscopy. However, several lines of evidence indicate that the number of CD34⁺ cells with attached platelets in this population is negligible and that the reactivity with GPIb/IIIa antibodies is caused by protein synthesis by CD34⁺ cells. Firstly, no CD34⁺ cells reacted with MoAbs recognizing GPIbα or with MoAbs against GPIa and GPV, GPs known to be present on platelets. Using purified CD34⁺ cells, Debili et al. have shown that the more mature megakaryocyte progenitors are present in the CD34⁺CD41⁺ cell population, whereas immature megakaryocyte progenitors are present in the CD34⁺CD41⁻ cell population.

No staining of CD34⁺ cells was seen with MoAbs recognizing GPIa, GPIbα, or GPV. GPIIIa can also be associated with the α-chain of the vitronectin receptor (CD51). 38,39 CD34⁺ cells did not react with CD51 antibodies, indicating that most if not all GPIIIa on CD34⁺ cells is associated with the platelet-specific GP GPIIb. CD34⁺ cells also reacted with MoAbs against GPIIb (CD31) and GPIV (CD36). However, these MoAbs cannot be used for determining the number of platelet progenitor cells, because GPIIb was found to be present on myeloid progenitors, and GPIV on erythroid precursors.

Current evidence indicates that GPIIIb and GPIIIa are detected first during megakaryocytic differentiation, whereas the platelet GP Iba appears to be a slightly later marker of differentiation. 42-44 Using purified CD34⁺ cells, Debili et al. showed that, in culture, a very small population of CD34⁺ express GPIbα. These cells are extremely rare and might not be detectable using the present technique. Moreover, among acute megakaryoblastic leukemic cells, cells showing reactivity with both GPIIb/IIIa and GPIbα antibodies as well as cells expressing only GPIb/IIIa have been found.45,46,47 The results of our experiments are concordant with those of these earlier reports, because we show that only GPIb/IIIa is present on CD34⁺ cells, indicating that these cells represent an early stage in the megakaryocyte lineage.

We assumed that, if performed in the presence of EDTA, the flow cytometric measurement of CD34⁺ cells expressing platelet antigens could be used as an indicator for the number of megakaryocytic progenitors. Indeed, we found that CD34⁺CD41⁺ cells correlated significantly better with the time to platelet recovery after autologous PBSC transplantation than the total number of CD34⁺ cells. This observation suggests that the CD34⁺CD41⁺ cells represent megakaryocytic precursors that are responsible for platelet recovery after PBSC transplantation. An important clinical issue is to establish a practical minimum number of cells required for rapid platelet recovery. In this study, the threshold for rapid platelet recovery was calculated to be 0.34 × 10⁹ CD34⁺CD41⁺ cells/kg and reinfusion of more than the threshold of CD34⁺CD41⁺ cells per kilogram resulted in a significantly faster recovery. Together with the significantly better correlation of the number of reinfused CD34⁺CD41⁺ cells per kilogram with platelet recovery than of the total number of CD34⁺ cells per kilogram, this study indicates that determining the number of megakaryocytic precursor cells by flow cytometry most accurately represents the platelet reconstitutive capacity of the PBSC transplant. The value of determining the number of CD34⁺CD41⁺ cells by flow cytometry has to be confirmed in studies with a larger number of patients receiving different types of high-dose chemotherapy.

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The value of flow cytometric analysis of platelet glycoprotein expression of CD34+ cells measured under conditions that prevent P-selectin-mediated binding of platelets

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