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Research Article

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Mobilization and Homing of Peripheral Blood Progenitors Is Related to Reversible Downregulation of $\alpha 4\beta 1$ Integrin Expression and Function

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Abstract

Despite the wide use of mobilized peripheral blood (PB) progenitor cells (PBPC) for clinical transplantation the mechanism(s) underlying their mobilization and subsequent engraftment are still unknown. We compared the adhesive phenotype of CD34⁺ colony-forming cells (CFC) in bone marrow (BM) and PB of normal donors before and after administration of granulocyte colony-stimulating factor (G-CSF) for 5 d. G-CSF-mobilized PB CFC cells adhered significantly less to BM stroma, fibronectin, and to the $\alpha 4\beta 1$ binding fibronectin peptide, CS1, because of decreased expression of the $\alpha 4$ integrin. Since incubation of BM CD34⁺ cells for 4 d with G-CSF at concentrations found in serum of G-CSF-treated individuals did not affect $\alpha 4$ -dependent adhesion, G-CSF may not be directly responsible for the decreased $\alpha 4$ -mediated adhesion of PB CFC. Culture of G-CSF-mobilized PB CD34⁺ cells with cytokines at concentrations found in BM stromal cultures upregulated $\alpha 4$ expression and restored adhesion of mobilized PB CFC to stroma, fibronectin, and CS1. Adhesion of cultured, mobilized PB CFC to stroma and CS1 could not be further upregulated by the $\beta 1$ activating antibody, 8A2. This indicates acquisition of a maximally activated $\alpha 4\beta 1$ integrin once PB CFC have been removed from the in vivo mobilizing milieu. Thus, decreased $\alpha 4$ expression on CD34⁺ CFC in PB may be responsible for the aberrant circulation of mobilized PB CD34⁺ cells. Reexpression of a maximally activated $\alpha 4\beta 1$ integrin on mobilized PB CFC removed from the mobilizing in vivo milieu may contribute to the early engraftment of mobilized PBPC. (*J. Clin. Invest.* 1998. 101:2456–2467.) Key words: mobilization • homing • bone marrow transplant • growth factors • adhesion receptors

Introduction

Studies demonstrating the presence of hematopoietic progenitors with long-term lymphomyeloid repopulating ability in the peripheral blood (PB)¹ have led to the use of PB progenitor cells (PBPC) in transplantation (1–6). Several stimuli including

stress, exercise, endotoxin, dextran-sulfate, and chemotherapy can increase the number of circulating progenitors (7–11). Likewise, administration of cytokines such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, or stem cell factor (SCF) greatly increases the frequency of CD34⁺ cells, committed colony-forming cells (CFC), and more primitive progenitors in the blood (9, 12, 13). Since a small number of progenitors can be found in the PB under steady state conditions, mobilization could be seen as an exaggeration of an otherwise physiological process. Alternatively, mobilization may be caused by specific changes in the interaction between progenitors and the microenvironment or changes in the microenvironment itself (14–17). Currently, it is unknown if cytokines act by expanding the pool of available bone marrow (BM) progenitors and their subsequent release in the circulation, by expanding a specific subpopulation of marrow progenitors which interact less strongly with the marrow microenvironment or by expanding and mobilizing progenitors present outside of the marrow (18).

Under steady state conditions, hematopoietic progenitors are mainly found in close contact with the marrow microenvironment (19). Steady state BM CD34⁺ cells express multiple adhesion receptors including the $\alpha 4$, $\alpha 5$, $\beta 1$, CD11a/CD18 and CD11b/CD18 integrins, L-selectin, PECAM-1, and CD44 (16, 20–23). We and others have demonstrated in vitro that adhesion of BM progenitors to BM stroma is at least in part due to the interaction of the $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins with stromal extracellular matrix (fibronectin) and cell surface expressed ligands (VCAM) (24–27). In vivo studies have demonstrated a dominant role for the $\alpha 4\beta 1$ integrin in the interaction between progenitors and the BM microenvironment. Repopulating stem cells fail to engraft when murine or sheep BM is incubated with anti- $\alpha 4$ antibodies before transplantation, and the number of progenitors found in the PB increases dramatically after infusion of anti- $\alpha 4$ antibodies into baboons (15, 28, 29). Several studies have examined cell adhesion receptor expression on CD34⁺ cells present in the PB after mobilization with cytokines or chemotherapy (14). Decreased levels of the $\alpha 4$ and CD11a/CD18 integrins and increased levels of the CD62L receptor have been described (16). In addition, several studies have demonstrated that incubation in vitro with GM-CSF, IL-3, and SCF may affect the adhesive behavior of progenitors by altering the affinity status of adhesion receptors (30–32). However, whether changes in receptor expression and/or function are functionally relevant and are responsible for mobilization of progenitors is unknown. Since cytokines may also up- or downregulate expression of adhesive ligands in the extracellu-

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1. *Abbreviations used in this paper:* BM, bone marrow; CFC, colony-forming cells; LTC-IC, long-term culture-initiating cells; MCFR, mean channel fluorescence ratio; MNC, mononuclear cells; PB, peripheral blood; PBPC, peripheral blood progenitor cells; PE, phycoerythrin; SCF, stem cell factor.

lar matrix or on stromal cells, alterations in the microenvironment may promote premature release of progenitors in the PB (25, 33, 34).

It is even less clear how progenitors that were mobilized in the PB after administration of cytokines can result in the clinically observed early engraftment after transplant. Engraftment requires that progenitors home to the BM microenvironment. This depends at least in part on adhesions through the $\alpha 4\beta 1$ integrin (28, 35–37). Since alterations in the expression and/or function of the same $\alpha 4\beta 1$ integrin may be involved in the mobilization of progenitors in the PB, one needs to assume that a fast reversal of the nonadhesive phenotype of mobilized hematopoietic progenitors that have been removed from the mobilizing milieu must underlie their potential for fast engraftment.

In this study, we examined the effect of mobilization with G-CSF on progenitors that remain in the BM as well as on progenitors that are induced to circulate in the PB. We demonstrate that decreased expression, but not change in function, of the $\alpha 4\beta 1$ integrin is responsible for decreased adhesion of PB progenitors to BM stroma, fibronectin, and CS1. This may explain the premature circulation of progenitors in the PB after in vivo administration of G-CSF. We also demonstrate, in an in vitro system, that $\alpha 4$ expression and function is upregulated on PB CD34⁺ cells, once removed from the in vivo milieu. This is associated with increased adhesion of PB CFC to BM stroma, fibronectin, and CS1 which in turn may be responsible for homing and engraftment of PBPC.

Methods

Samples

Donors. Normal healthy donors were selected using standard criteria of the American Association of Blood Banks for blood donors (38). Informed consent was obtained using guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota. All donors had a negative serologic test for HBsAg, anti-HCV, and anti-HIV and they had normal hemoglobin, white blood cell counts, white cell differentials, and platelet counts.

PBPC. Normal donors received a daily dose of 10 $\mu\text{g}/\text{kg}$ per day of human recombinant G-CSF (Neupogen; Amgen, Inc., Thousand Oaks, CA) subcutaneously for 5 d (days 1–5). G-CSF was given as a single morning dose. 60 ml of blood was obtained by venopuncture from each donor before the first injection of G-CSF (day 0) and on day +6.

BM. 50 ml of heparinized BM was obtained from each normal donor before the first injection of G-CSF (day 0) and on day +6.

Processing of samples

Steady state PB and BM as well as G-CSF–mobilized PB and BM hematocrit white blood cell and differential count were determined using an automated cell counter (S+IV; Coulter Electronics Inc., Hialeah, FL). Flow cytometry, clonogenic assays, and long-term cultures were performed on steady state and G-CSF–mobilized PBPC, as well as BM obtained before and after treatment with G-CSF.

Cell selection. Steady state and G-CSF–mobilized PB and BM mononuclear cells (MNC) were separated by Ficoll Hypaque centrifugation (specific gravity, 1077) (Sigma Chemical Co., St. Louis, MO). CD34 enrichment was performed using the MACS[®] CD34 isolation kit (Miltenyi Biotec Inc., Sunnyvale, CA) as described previously (12).

FACS[®] analysis of cell surface receptors. The following antibodies coupled to FITC or phycoerythrin (PE) were used: mouse antibodies directed to CD29 ($\beta 1$ integrin), CD34, CD44, CD49d ($\alpha 4$ integrin), CD49e ($\alpha 5$ integrin), and CD62L (L selectin). Antibodies

purchased from Becton Dickinson (San Jose, CA) were CD44, CD62L, and CD34; CD29, CD49d, and CD49e were from Immunotech (Marseilles, France). Enumeration of CD34⁺ cells in steady state or mobilized PB MNC and BM MNC was performed as described by Sutherland et al. (39). For dual-color analysis 100,000 column-selected CD34⁺ cells or cells recovered from ex vivo culture initiated 1–4 d earlier with CD34⁺ cells were resuspended in 100 μl of PBS plus 0.3% BSA labeled with 20 ng antiadhesion receptor antibodies in conjunction with 20 ng of anti-CD34 antibodies and incubated for 30 min at 4°C, washed, and then analyzed with a FACStarPlus[®] flow cytometer equipped with a CONSORT 32 computer. FITC- and PE-conjugated isotype-matched Igs were used as controls.

FACS[®] selection of CD34⁺ $\alpha 4^{+++}$ and CD34⁺ $\alpha 4^{+/-}$ cells. For FACS[®] selection, CD34⁺ cells were labeled with FITC-conjugated mouse anti- $\alpha 4$ and PE-conjugated mouse anti-CD34 (10 ng per 10⁶ cells), incubated for 30 min on ice, and then washed with cold PBS. Cells were selected on a FACStarPlus[®] laser flow cytometry system equipped with a CONSORT 32 computer into a CD34⁺ $\alpha 4^{+/-}$ and CD34⁺ $\alpha 4^{+++}$ population based on a mouse IgG1-PE and IgG1-FITC control fluorescence profile. For experiments in which cells were subjected to subsequent adhesion assays, the anti- $\alpha 4$ antibody used for selection was a nonblocking antibody, B5G10 (40), kindly provided by Dr. M. Hemler (Harvard University, Boston, MA). Cells were stained with B5G10, washed, and stained with a goat anti-mouse IgG-PE (Tago Inc., Burlingame, CA) secondary antibody washed and stained sequentially with anti-CD34–biotin (CellPro) and streptavidin-670 (SA-670; Life Technologies, Gaithersburg, MD). CD34⁺ $\alpha 4^{-}$ and CD34⁺ $\alpha 4^{+++}$ cells were selected.

Progenitor cultures

Short-term methylcellulose progenitor culture. CD34⁺ cells (2×10^3 cells/ml), MNC (2×10^5 cells/ml), or adherent and nonadherent fractions from adhesion assays were cultured in methylcellulose as described (12) in the presence of SCF (Amgen, Inc.), GM-CSF (Immune, Seattle, WA), and IL-3 (R&D Systems, Minneapolis, MN) each at a final concentration of 5 ng/ml and erythropoietin (Amgen, Inc.) 3 IU/ml.

Long-term culture. 2×10^6 PB or BM MNC (22 replicates: 61,224; 20,408; 6,802; and 2,267 cells/well) and 30,000 CD34⁺ cells (22 replicates: 900, 300, 100, and 33 cells/well) were plated in limiting dilutions onto previously irradiated M2-10B4 feeders in long-term BM culture (12, 24). After 8 wk, clonogenic culture medium was added and wells were scored for the presence or absence of secondary CFC 14 d later as described (12, 24). The absolute number of long-term culture-initiating cells (LTC-IC) present in the different cell populations was calculated as described (41, 42).

Purification and synthesis of fibronectin and peptides from fibronectin

Human plasma fibronectin was purified as a by-product of Factor VIII production by sequential ion exchange and gelatin chromatography (43). Peptides from fibronectin were synthesized at the Microchemical Facility of the University of Minnesota (44–46). Peptide CS1 which has the sequence DELPQLVTLPHPNLHPGEILDVPST (47) was chemically conjugated to ovalbumin (44, 46).

Cell adhesion assays

MNC and CD34⁺ cells from PB and BM were resuspended in serum-free IMDM and plated on preestablished, irradiated BM stromal layers for 2 h. BM stroma was established from normal human marrow donors, as previously described. Once confluent, feeders were irradiated at 1,250 rad, and subcultured in wells of 24-well plates (24). Nonadherent cells were harvested by three gentle washes with warm IMDM and adherent cells were recovered after short-term trypsinization, as described (24).

BSA, ovalbumin, fibronectin, and the CS1-ovalbumin peptide were diluted in Voller's carbonate buffer (pH 8.4) to the appropriate concentrations and adsorbed to wells of 48-well plates (43, 46).

CD34⁺ cells from PB and BM or CD34⁺α4⁻ and CD34⁺α4⁺⁺⁺ cells from Mob.PB were resuspended in serum-free IMDM and plated in contact with fibronectin or CS1 for 3 h in a humidified atmosphere at 37°C. Nonadherent cells were removed by four standardized washes using warm IMDM and adherent cells by trypsinization (46).

To determine the percentage of adherent CFC, adherent and nonadherent fractions were replated in methylcellulose assays (24, 46). Percent adhesion was calculated as: (number of CFC in the adherent fraction)/(number of CFC in adherent + nonadherent fractions) × 100.

Adhesion blocking experiments. In some experiments, PB and BM CD34⁺ cells were incubated with 8A2 (1:100,000 dilution), P4C2 (1:400 dilution), P4C10 (1:400 dilution), P1D6 (1:400) control mouse IgG, or with media alone for 30 min before adhesion assays (48, 49). Monoclonal antibody, 8A2 (a kind gift from Dr. Nicholas Kovach, University of Washington, Seattle, WA), was used as a dilution of mouse ascites. The integrin-blocking monoclonal antibodies P4C2 (murine anti-human α4), P4C10 (murine anti-human β1), and P1D6 (murine anti-human α5) were purchased from GIBCO-BRL (Gaithersburg, MD) and used as dilutions of mouse ascites (48). Control mouse IgG (Sigma Chemical Co.) was diluted in PBS and used at a concentration of 20 μg/ml.

Serum-free medium culture

Column-selected CD34⁺ cells were plated in IMDM containing BSA (20 mg/ml) (GIBCO BRL), insulin (10 μg/ml), transferrin (200 μg/ml) (Sigma Chemical Co.), 10⁻⁴ M 2-mercaptoethanol, penicillin 100 U/ml, and streptomycin 100 U/ml (GIBCO BRL) (50) (serum-free media). The following cytokines were added to the medium: GM-CSF (200 pg/ml), G-CSF (0–100 ng/ml), SCF (200 pg/ml) (Amgen, Inc.), LIF (50 pg/ml) (R&D Systems), MIP-1α (200 pg/ml) (R&D Systems), and IL-6 (1 ng/ml) (Genetics Institute, Boston, MA). The concentration of the different cytokines corresponds to the physiological levels at which they are present in Dexter-type culture (51, 52). After 24 h to 7 d, cells were harvested, immunophenotyped, and used in adhesion assays.

Statistics

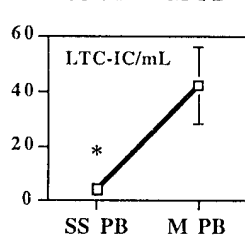
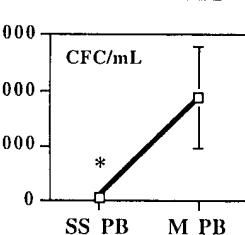
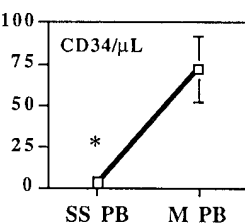
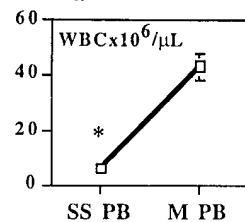
Results of experimental points obtained from multiple experiments were reported as the mean ± SEM. Significance levels were determined by two-sided Student's *t* test analysis.

Results

G-CSF increases the number of progenitors present in PB but not in BM. As we and others have demonstrated (9, 12, 14, 53–56), G-CSF induced a 22-fold increase in the number of CD34⁺ cells, a 26-fold increase in the number of CFC, and a 9-fold increase in week 8 LTC-IC per milliliter of PB in normal individuals (Fig. 1). Although a sevenfold increase in the number of white blood cells was seen per milliliter of BM of normal individuals after 5 d of G-CSF administration, the number of either CD34⁺ cells, CFC, or week 8 LTC-IC per milliliter of BM did not change (Fig. 1).

Differences in adhesion receptor expression between steady state or mobilized PB or BM CD34⁺ cells. To determine which adhesion molecules may be involved in mobilization of hematopoietic progenitors we examined the expression of adhesion receptors on CD34⁺ cells obtained from PB or BM of normal individuals before and after in vivo administration of G-CSF. No significant differences were seen in expression of α2, α5, β1, CD11b, CD11c, or P-selectin. In vivo treatment with G-CSF increased L-selectin expression on CD34⁺ cells present in PB or BM (percent CD34⁺ cells positive: steady state PB: 26 ± 12% and mobilized PB: 64 ± 9%; steady state

Peripheral Blood



Bone Marrow

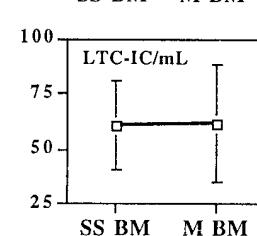
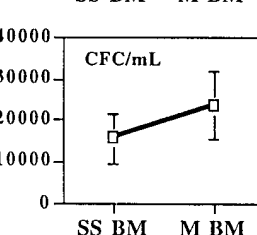
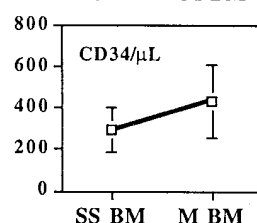
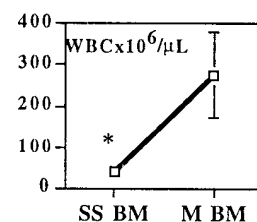


Figure 1. Treatment of normal donors with G-CSF increases the number of progenitors in the PB but does not increase the number of progenitors in the BM. Normal donors (*n* = 6) received 10 μg/kg of G-CSF daily for 5 d. BM and PB samples were obtained before treatment with G-CSF and 12–20 h after the last dose of G-CSF. The number of CD34⁺ cells, CFC, and LTC-IC per volume was calculated as: (number of CD34⁺ cells, CFC, or LTC-IC per 10⁵ MNC × number of MNC per ml)/10⁵. The number of LTC-IC was evaluated after 8 wk in long-term culture (week 8 LTC-IC). Results represent the mean ± SEM of six experiments. SS, Steady state; M, mobilized. **P* < 0.01 comparison between the number of progenitors per volume before and after treatment with G-CSF.

BM: 25 ± 14% and mobilized BM: 61 ± 8%; *P* < 0.001) and decreased expression of the β2 integrin (CD11a/CD18) (percent CD34⁺ cells positive: steady state PB: 54 ± 7% and mobilized PB: 42 ± 4%; steady state BM: 62 ± 5% and mobilized BM: 44 ± 4%; *P* < 0.05). In contrast, irrespective of G-CSF treatment, α4 integrin expression was lower on CD34⁺ cells present in PB than in BM (percent CD34⁺ cells positive: steady state PB: 35 ± 6% and mobilized PB: 28 ± 5%; steady state BM: 47 ± 4% and mobilized BM: 45 ± 4%). Aside from the fraction of CD34⁺ cells that expressed α4 integrin, the density of α4 integrin on CD34⁺ cells (mean channel fluorescence of α4) was also lower on mobilized and steady state PB CD34⁺ cells than on steady state or mobilized BM CD34⁺ cells (Fig. 2).

Because CFC represent only 10–20% of all CD34⁺ cells, we FACS[®] selected CD34⁺ cells from steady state BM and

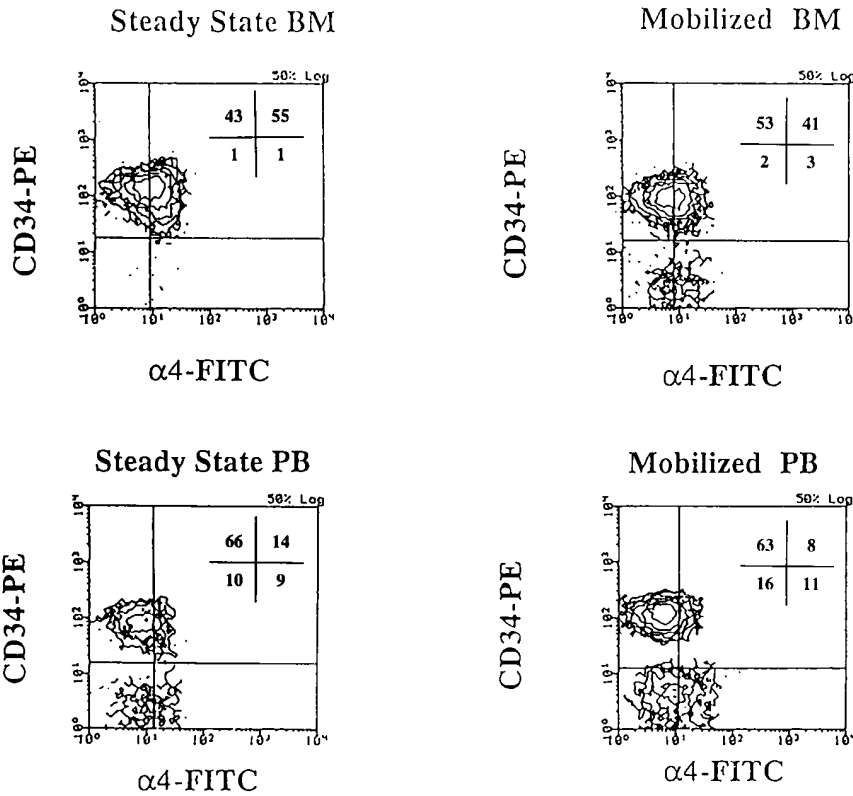


Figure 2. Expression of $\alpha 4$ integrin in $CD34^+$ cells from BM and PB before and after mobilization with G-CSF. Column-selected $CD34^+$ cells from steady state and mobilized BM and PB were stained with FITC-conjugated anti-human $\alpha 4$ integrin antibody and PE-conjugated anti-human $CD34$ antibody as described in Methods. One representative experiment is depicted.

G-CSF-mobilized PBPC based on their expression of $\alpha 4$ integrin to determine if $\alpha 4$ integrin expression was different between PB and BM CFC. In accordance with previous reports (27), > 80% of steady state BM CFC were recovered in the $CD34^+/\alpha 4^{+++}$ subpopulation. In contrast, > 80% of CFC were recovered in the $CD34^+/\alpha 4^{+/-}$ fraction of G-CSF-mobilized

PB (Fig. 3). These studies indicate that BM $CD34^+$ cells and CFC express significantly more $\alpha 4$ integrin than mobilized PB $CD34^+$ cells and CFC irrespective of G-CSF treatment. This led to the hypothesis that the aberrant localization of steady

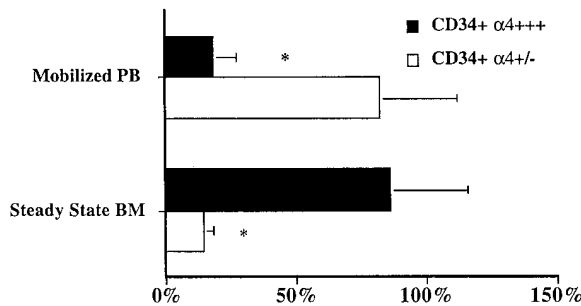


Figure 3. Significantly less mobilized PB than steady state BM CFC are $CD34^+/\alpha 4^{+++}$. Steady state BM and mobilized PB $CD34^+$ cells were selected by FACS[®] based on $\alpha 4$ integrin expression into $CD34^+/\alpha 4^{+++}$ and $CD34^+/\alpha 4^{+/-}$ cells. Cells were then plated in methylcellulose progenitor assay. The total number of CFC that express $\alpha 4$ integrin was calculated as: $(CFC \text{ in } CD34^+/\alpha 4^{+++} \text{ cells}) \times (\text{percentage of } CD34^+/\alpha 4^{+++} \text{ cells}) / (\text{total number of CFC in } CD34^+ \text{ cells}) \times 100$. The number of CFC/2,000 plated cells was: BM 276 ± 100 for $CD34^+/\alpha 4^{+++}$ cells and 164 ± 60 for $CD34^+/\alpha 4^{+/-}$ cells; mobilized PB 142 ± 44 for $CD34^+/\alpha 4^{+++}$ cells and 270 ± 112 for $CD34^+/\alpha 4^{+/-}$ cells. The percentage of $CD34^+$ cells present in the $CD34^+/\alpha 4^{+++}$ window was $71 \pm 8\%$ for BM and $37 \pm 6\%$ for PB. * $P < 0.01$ comparison between total CFC present in $CD34^+/\alpha 4^{+++}$ and $CD34^+/\alpha 4^{+/-}$ cells in steady state BM and mobilized PB ($n = 4$).

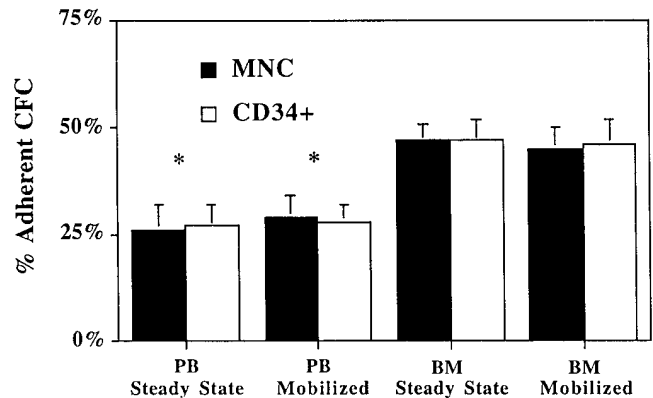


Figure 4. Steady state or G-CSF-mobilized PB CFC adhere significantly less to stroma than CFC from BM obtained before and after G-CSF. Steady state or mobilized BM or PB $CD34^+$ or MNC were plated for 2 h on irradiated BM stromal feeders. Adherent and non-adherent fractions were collected separately and replated in methylcellulose culture. The percentage of adherent CFC was calculated as described in Methods. * $P < 0.01$ comparison between adhesion of PB CFC and adhesion of BM CFC ($n = 6$). No differences were found between experiments performed with $CD34^+$ cells and MNC. The number of CFC per 100,000 MNC plated was: steady state BM 192 ± 37 ; mobilized BM 150 ± 45 ; steady state PB 31 ± 8 ; mobilized PB 181 ± 64 . The number of CFC per 1,000 $CD34^+$ cells plated was: steady state BM 131 ± 28 ; mobilized BM 109 ± 35 ; steady state PB 57 ± 18 ; mobilized PB 148 ± 29 .

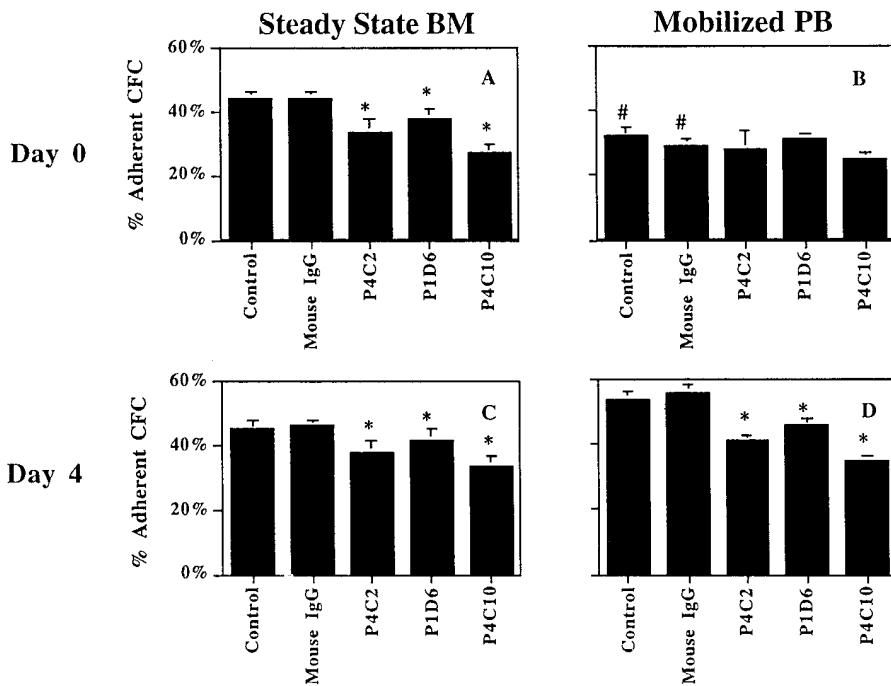


Figure 5. Role of the $\alpha 4\beta 1$ integrin in steady state BM and mobilized PB CFC adhesion to BM stroma. Steady state BM ($n = 6$) and mobilized PB ($n = 6$) CD34⁺ cells were tested either immediately after column selection (day 0 shown in A and B) or after culture ex vivo for 4 d in serum-free medium with cytokines at concentrations found in Dexter cultures (G-CSF at 250 pg/ml) (day 4 shown in C and D). 6,000 column-selected CD34⁺ cells were incubated for 30 min with blocking anti $\alpha 4$ (P4C2), $\alpha 5$ (PID6), or $\beta 1$ (P4C10) antibodies, mouse IgG, or no antibody. Cells were then plated in the continued presence of the antibodies on irradiated stroma for 2 h. Adherent and nonadherent fractions were collected, washed, and replated in methylcellulose assay. The percentage of adherent CFC was calculated as described in Methods. The number of CFC per 1,000 CD34⁺ cells on day 0 was: steady state BM 172 ± 54 ; mobilized PB 158 ± 32 . The number of CFC per 1,000 CD34⁺ cells on day 4 was: steady state BM 214 ± 62 ; mobilized PB 195 ± 57 . * $P < 0.01$ comparison between CFC adhesion in the presence of antibodies and no antibody control; # $P < 0.01$ comparison between adhesion of BM and PB CFC.

state and G-CSF-mobilized PB CD34⁺ CFC may be related to the decrease in $\alpha 4$ integrin expression.

Decreased $\alpha 4$ integrin expression is responsible for decreased PB CFC adhesion to stroma and fibronectin. We next examined the capacity of CFC in PB and BM before and after treatment with G-CSF to adhere to BM stromal feeders (Fig. 4). $47 \pm 4\%$ of steady state BM CFC adhered to BM stroma, which is in accordance with our previous studies (24, 49). Likewise, $45 \pm 5\%$ of CFC present in BM obtained after 5 d of in vivo treatment with G-CSF adhered to stromal feeders. In contrast, only 26 ± 6 and $29 \pm 5\%$ of CFC present in PB before and after treatment with G-CSF adhered to stroma. No differences were seen in adhesion of CFC (Fig. 4) when assays were performed with MNC or CD34⁺ enriched cells, suggesting that the selection with the QBEND-10 antibody did not alter the functional status of adhesion receptors.

Adhesion of steady state BM CD34⁺ cells to BM stroma could be blocked in part by antibodies against the $\beta 1$ integrin or $\alpha 4$ integrin but to a lesser extent by antibodies against the $\alpha 5$ integrin (Fig. 5). The total number of CFC in the adherent plus nonadherent fraction was similar for cells exposed to medium only, mouse IgG, anti- $\alpha 4$, - $\alpha 5$, or - $\beta 1$ antibodies indicating that the short-term exposure to these antibodies does not affect progenitor growth in subsequent methylcellulose cultures. These studies confirm that adhesion of steady state BM CFC to BM stroma is mediated by $\alpha 4$ -fibronectin or $\alpha 4$ -VCAM interactions (25, 26). The low level adhesion of mobilized PB CFC to stroma was not affected by blocking antibodies against the $\alpha 4$, $\alpha 5$, or $\beta 1$ integrin (Fig. 5), supporting the hypothesis that defects in $\alpha 4\beta 1$ -mediated adhesion may underlie the decreased adhesion to BM stroma and premature circulation of PB CFC.

To further characterize the decreased adhesion of PB progenitors, we next compared the adhesion of steady state BM and mobilized PB CFC to purified fibronectin (adhesion through $\alpha 4\beta 1$ and $\alpha 5\beta 1$) and the fibronectin peptide CS1 coupled to ovalbumin (CS1-OVA) (adhesion through $\alpha 4\beta 1$). Mobilized PB CFC adhered significantly less to fibronectin and to CS1-OVA than steady state BM CFC ($P < 0.01$) (Figs. 6 and

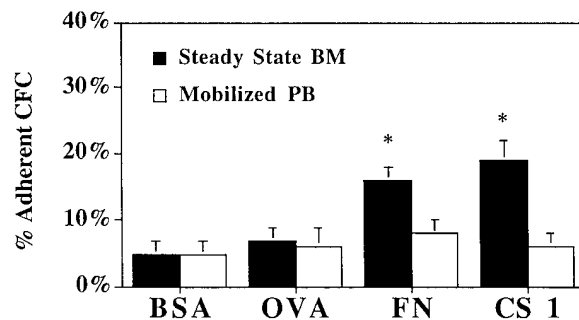


Figure 6. Mobilized PB CFC adhere significantly less to fibronectin and CS1 than steady state BM CFC. Steady state BM ($n = 10$) or G-CSF mobilized PB ($n = 9$) CD34⁺ cells were plated in wells of 48-well plates previously adsorbed with BSA, ovalbumin, fibronectin (50 $\mu\text{g/ml}$), or CS1 (20 $\mu\text{g/ml}$). 6,000 CD34⁺ cells were plated per well and incubated for 3 h in a humidified atmosphere. Adherent and nonadherent fractions were harvested as described in Methods and replated in methylcellulose assays. The percentage of adherent cells was calculated as described in Methods. The number of CFC per 1,000 CD34⁺ cells was: steady state BM 189 ± 15 , mobilized PB 205 ± 35 . OVA, Ovalbumin; FN, fibronectin. * $P < 0.01$, comparison between adhesion of steady state BM and mobilized PB CFC.

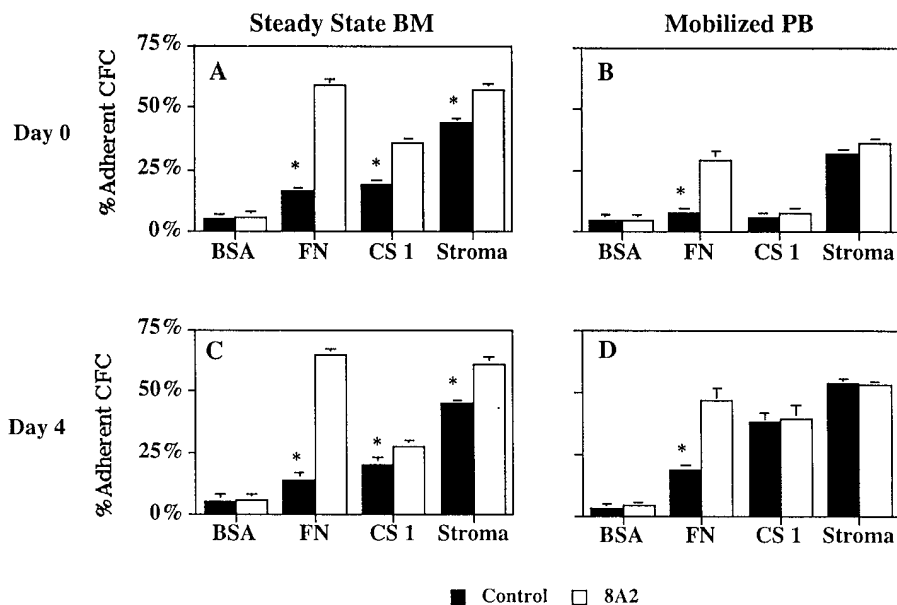


Figure 7. Restored adhesion of mobilized PB progenitors removed from the in vivo milieu as a result of reexpression and maximal activation of the $\alpha 4\beta 1$ integrin. Steady state BM ($n = 10$) and mobilized PB ($n = 9$) cells were tested either immediately after column selection (day 0 shown in *A* and *B*) or after culture ex vivo for 4 d in serum-free medium with cytokines at concentrations found in Dexter cultures (G-CSF at 250 $\mu\text{g}/\text{ml}$) (day 4 shown in *C* and *D*). 6,000 column-selected $\text{CD}34^+$ cells were incubated for 30 min with the $\beta 1$ activating antibody, 8A2, or mouse IgG before adhesion assays. Cells were then plated in the continued presence of the antibodies onto stroma or on wells of plates previously adsorbed with fibronectin (50 $\mu\text{g}/\text{ml}$) or CS1 (20 $\mu\text{g}/\text{ml}$) and incubated for 3 h in a humidified atmosphere. Adherent and nonadherent fractions were harvested as described in Methods and replated in methylcellulose assays. The percentage of adherent cells was calculated as described in Methods. FN, Fibronectin. * $P < 0.01$ comparison between adhesion of CFC incubated with and without 8A2.

7, *A* and *B*). In fact, adhesion of mobilized PB CFC to CS1-OVA was not significantly different than adhesion to BSA or ovalbumin (Fig. 6). In two experiments, we also showed that adhesion of mobilized BM CFC to fibronectin and OVA-CS1 is similar to that of steady state BM, but significantly higher than that of mobilized PB CFC (Table I). This confirms the notion that $\alpha 4$ integrin-mediated adhesion is defective in mobilized PB CFC but not in mobilized BM CFC.

Over the last 5 yr, it has become clear that the presence of an adhesion receptor on cells does not necessarily mean that

Table I. G-CSF Mobilized PB CFC, but Not G-CSF Mobilized BM CFC, Do Not Adhere to the CS1 Binding Site of Fibronectin

Ligand		Mobilized BM $\text{CD}34^+$	Mobilized PB $\text{CD}34^+$	Steady state BM $\text{CD}34^+$
Stroma	IgG	46 \pm 5%	28 \pm 5%	47 \pm 5%
	8A2	57 \pm 11%	36 \pm 2%	57 \pm 3%
BSA	IgG	3%, 7%	5 \pm 2%	5 \pm 2%
	8A2	7%, 7%	5 \pm 2%	6 \pm 2%
FN	IgG	30%, 34%	8 \pm 2%	16 \pm 2%
	8A2	65%, 62%	29 \pm 4%	59 \pm 3%
CS1	IgG	28%, 23%	6 \pm 2%	19 \pm 2%
	8A2	41%, 32%	8 \pm 2%	32 \pm 2%

G-CSF-mobilized BM $\text{CD}34^+$ cells ($n = 6$ for stroma and $n = 2$ for fibronectin and CS1), G-CSF-mobilized PB $\text{CD}34^+$ cells ($n = 6$ for stroma and $n = 10$ for fibronectin and CS1), or steady state BM $\text{CD}34^+$ cells ($n = 6$ for stroma and $n = 10$ for fibronectin and CS1) were allowed to adhere to BM stroma, BSA, FN, or CS1 after preincubation with either mouse IgG or 8A2. Percent adhesion was determined. FN, Fibronectin.

these receptors have functional relevance, i.e., they do not allow binding and/or adhesion to ligands (31, 47, 49, 51, 56, 57). The function of integrins can be upregulated by the ligand itself, activating antibodies, or through inside-out signals originating from other adhesion receptors or cytokine signals (30, 31, 58–60). Thus, decreased integrin-mediated adhesion of mobilized PB CFC could be due not only to decreased $\alpha 4$ expression but also decreased $\beta 1$ integrin function. We used the $\beta 1$ activating antibody 8A2 to determine if abnormal function rather than expression is responsible for the defect in PB CFC adhesion. Incubation of either steady state BM $\text{CD}34^+$ cells or mobilized BM $\text{CD}34^+$ cells with 8A2 increased adhesion of CFC to stroma, fibronectin, and CS1-OVA (Fig. 7 *A* and Table I). As we have reported previously, 8A2 did not increase or decrease colony growth in subsequent methylcellulose culture (not shown) (48). 8A2-induced adhesion of BM CFC to fibronectin could be significantly inhibited by anti- $\alpha 4$ or anti- $\beta 1$ antibodies but not by anti- $\alpha 5$ antibodies. Combined addition of anti- $\alpha 4$ and anti- $\alpha 5$ antibodies resulted in almost complete abrogation of adhesion which was now similar to that seen with the anti- $\beta 1$ antibody (Fig. 8). This indicates that, as for adhesion of BM CFC to stroma, the $\alpha 4\beta 1$ integrin is the most important receptor for adhesion of steady state BM CFC to fibronectin.

Although incubation with 8A2 significantly increased adhesion of mobilized PB CFC to fibronectin, the percentage of mobilized PB CFC incubated with 8A2 adherent to fibronectin was still significantly lower than that of 8A2-treated BM CFC. 8A2 did not affect adhesion of PB CFC to BM stroma or CS1 (Fig. 7 *B*). Furthermore, unlike BM CFC, 8A2-induced adhesion of mobilized PB CFC to fibronectin could not be inhibited by anti- $\alpha 4$ blocking antibodies but was inhibited by anti- $\alpha 5$ or $\beta 1$ blocking antibodies (Fig. 8). These results strongly suggest that the decreased adhesion of mobilized PB CFC to stroma and fi-

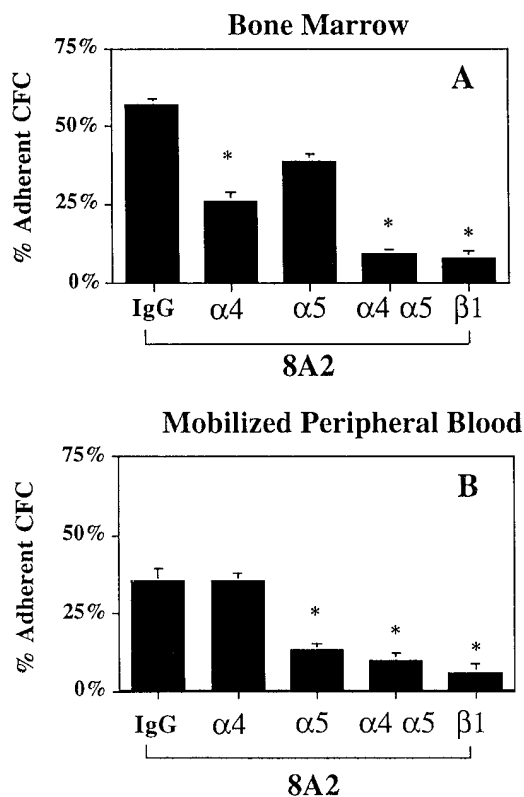


Figure 8. Role of the $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin in steady state BM and mobilized PB CFC adhesion to fibronectin. 6,000 column-selected $CD34^+$ cells from steady state BM ($n = 3$) or mobilized PB ($n = 3$) were incubated for 30 min with the $\beta 1$ activating antibody, 8A2, with either blocking anti $\alpha 4$ (P4C2), $\alpha 5$ (P1D6), and $\beta 1$ (P4C10) or mouse IgG before adhesion assays. Cells were then plated in the continued presence of the antibodies on wells of 48-well plates previously adsorbed with fibronectin (50 $\mu\text{g}/\text{ml}$) and incubated for 3 h in a humidified atmosphere. Adherent and nonadherent fractions were harvested as described in Methods and replated in methylcellulose assays. The percentage of adherent cells was calculated as described in Methods. * $P < 0.01$ comparison between adhesion to fibronectin in the presence of blocking antibodies against $\alpha 4$ (P4C2), $\alpha 5$ (P1D6), or $\beta 1$ (P4C10) and control (mouse IgG).

bronectin is due to decreased expression but not function of the $\alpha 4$ integrin on mobilized PB progenitors. To demonstrate this further, we selected $CD34^+\alpha 4^-$ and $CD34^+\alpha 4^{+++}$ cells from mobilized PB collections using a nonblocking anti- $\alpha 4$ antibody, B-5G10 (40) (Fig. 9). Cells were then subjected to adhesion assays in the presence of mouse control IgG or the activating anti- $\beta 1$ antibody, 8A2. Mobilized PB $CD34^+\alpha 4^{+++}$ cells, like BM $CD34^+$ cells, adhered to both intact fibronectin and CS1, and this adhesive interaction could be increased with the activating antibody 8A2 (Table II). In contrast, mobilized PB $CD34^+\alpha 4^-$ cells failed to adhere to CS1 and adhesion could not be increased with 8A2, whereas their baseline adhesion to fibronectin was increased after treatment with 8A2. The observation that $\alpha 4$ integrin expressing mobilized PB CFC adhere to CS1 in a similar fashion as steady state BM CFC indicates further that decreased adhesion of the largely $\alpha 4$ -negative mobilized PB CFC is caused by lack of expression rather than decreased function of the $\alpha 4$ integrin.

Ex vivo exposure of BM $CD34^+$ cells to G-CSF does not decrease expression of $\alpha 4$ integrin nor decrease CFC adhesion to BM stroma. To determine if G-CSF per se induces the decrease in $\alpha 4$ receptor expression and lack of adhesion of PB CFC to BM stroma, we cultured steady state BM $CD34^+$ cells in serum-free medium and cytokines at concentrations found in supernatants of stromal feeders which may reflect cytokine concentrations found in the BM microenvironment. These cultures were performed with increasing concentrations of G-CSF (up to 100 ng/ml) for 1–4 d. Expression of $\alpha 4$ integrin on $CD34^+$ cultured cells was compared with $\alpha 4$ expression on fresh $CD34^+$ cells. We defined the mean channel fluorescence ratio (MCFR) as the ratio between MCF for $\alpha 4$, $\alpha 5$, or $\beta 1$ expression divided by MCF for their respective negative control. *Ex vivo* culture of steady state BM $CD34^+$ cells in defined medium with cytokines and 250 $\mu\text{g}/\text{ml}$ G-CSF did not change the expression of $\alpha 5$ or $\beta 1$ integrins but increased the $\alpha 4$ receptor density ($\alpha 4$ integrin MCF ratio: steady state BM 6.2 ± 0.5 and after 4 d in culture 10.5 ± 0.7) ($P < 0.05$). When the concentration of G-CSF added to the *ex vivo* culture was increased from 250 pg/ml to 100 ng/ml, a similar increase in $\alpha 4$ expression was seen (MCFR after 4 d in culture with G-CSF at 100 ng/ml: 9.8 ± 1.5). Thus, steady state BM $CD34^+$ cells did not acquire the $\alpha 4^{+/-}$ phenotype seen on mobilized PB $CD34^+$ cells.

Adhesion of steady state BM $CD34^+$ cells cultured *ex vivo* with 250 pg/ml G-CSF remained unchanged when analyzed on days 1, 2, 3, 4, or 7 after culture (Fig. 10). As we had seen for uncultured BM CFC, adhesion of cultured BM CFC to stroma could be inhibited by anti- $\alpha 4$ and anti- $\beta 1$ antibodies (Fig. 5) and could be enhanced by the activating antibody 8A2 (Fig. 7). These results indicate that the concentrations of cytokines used in the *ex vivo* culture system may reflect concentrations of cytokines present in the *in vivo* BM microenvironment milieu. When G-CSF was added to this cytokine mixture in concentrations up to 100 ng/ml, adhesion of BM CFC remained unchanged (percentage of CFC adherent to BM stroma after a 4-d culture with 100 ng/ml of G-CSF $44 \pm 3\%$), and continued to be dependent on $\alpha 4\beta 1$ integrins (adhesion in the presence of anti- $\beta 1$ integrin antibody $28 \pm 4\%$; adhesion in the presence

Table II. $CD34^+\alpha 4^-$ -mobilized PB CFC, but Not $CD34^+\alpha 4^{+++}$ -mobilized PB CFC, Do Not Adhere to the CS1 Binding Site of Fibronectin

Ligand		Mob. PB $CD34^+\alpha 4^-$	Mob. PB $CD34^+\alpha 4^{+++}$	Mob. PB $CD34^+$	SS BM $CD34^+$
BSA	IgG	2%, 2%	5%, 4%	$5\% \pm 2$	$5\% \pm 2$
	8A2	2%, 7%	8%, 2%	$5\% \pm 2$	$6\% \pm 2$
FN	IgG	2%, 11%	15%, 18%	$8\% \pm 2$	$16\% \pm 2$
	8A2	40%, 46%	59%, 52%	$29\% \pm 4$	$59\% \pm 3$
CS1	IgG	1%, 6%	13%, 18%	$6\% \pm 2$	$19\% \pm 2$
	8A2	3%, 4%	24%, 22%	$8\% \pm 2$	$32\% \pm 2$

In two experiments, mobilized PBPC $CD34^+$ cells were sorted in an $\alpha 4^-$ and $\alpha 4^{+++}$ population using a nonblocking antibody, B-5G10 (40). Alternatively, in 10 experiments steady state BM $CD34^+$ cells and mobilized PB $CD34^+$ cells were tested irrespective of $\alpha 4$ antigen expression. Cells were allowed to adhere to BSA, FN, or CS1 after preincubation with either mouse IgG or 8A2. Percent adhesion was determined. *Mob.*, Mobilized; *SS*, steady state; *FN*, fibronectin.

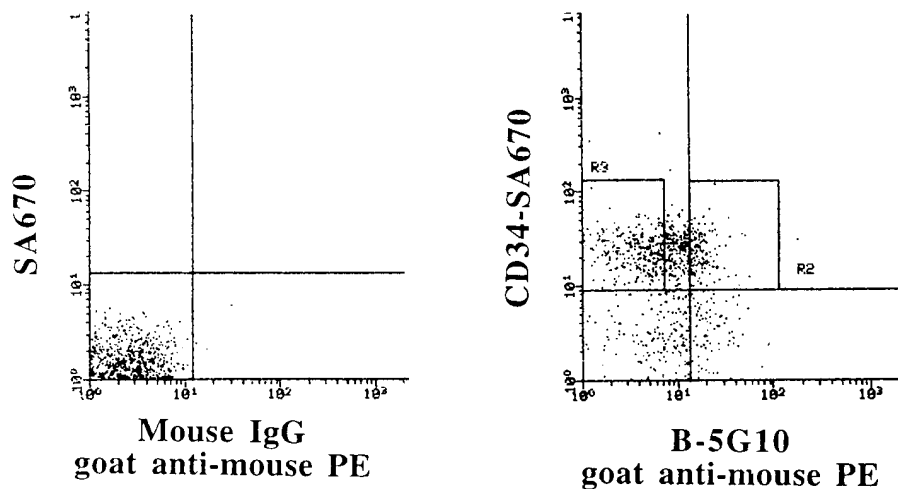


Figure 9. Selection of CD34⁺α4⁻ and CD34⁺α4⁺⁺⁺ cells. Mobilized PB cells enriched for CD34⁺ cells were stained with a nonblocking anti-α4 antibody, B5G10 (40), and goat anti-mouse IgG PE (*horizontal axis*) and a murine biotinylated IgM anti-CD34 antibody and SA670 (*vertical axis*). CD34⁺α4⁻ and CD34⁺α4⁺⁺⁺ cells were selected based on isotype control staining.

of anti-α4 integrin antibody 34±5%). Thus, consistent with the observation that the adhesion of the majority of CFC present in the BM after treatment with G-CSF remains unchanged, these studies indicate that *in vitro* exposure to G-CSF per se does not induce the nonadherent phenotype, characteristic for PB CD34⁺ cells and CFC, on the majority of BM CD34⁺ cells.

Removal from the in vivo milieu reverses the nonadherent phenotype of PB CFC. Studies in animals have demonstrated that the α4 integrin is involved in homing of hematopoietic progenitors to the BM (28, 29). Since mobilized PB progenitors can home and engraft, we hypothesized that removal from the *in vivo* milieu may reverse the nonadhesive phenotype of mobilized PB CFC. To evaluate this hypothesis we cultured G-CSF mobilized PB CD34⁺ cells in defined medium and cytokines as described above including 250 pg/ml of G-CSF. Expression of the α4 integrin on mobilized PB CD34⁺ increased significantly after 48 h of culture and was maximal after 4 d in culture (α4 MCFR on day 0: 4.7±0.8; on day 4: 7.5±1.1; *P* < 0.001) (Fig. 11). Expression of the β1 integrin was also upregulated (β1 MCFR on day 0: 6.9±1.2; on day 4: 8.1±1.8) although to a lesser extent than α4 and there was no significant change in α5 integrin expression (α5 MCFR on day 0: 2.9±1.6; on day 4: 3.9±1.7) (Fig. 10).

We next examined adhesion of cultured PB CFC to stroma, fibronectin, and CS1. In contrast to steady state BM CFC, *ex vivo* culture of mobilized PB CD34⁺ (Fig. 10) resulted in an increase in the fraction of CFC that adhered to BM stroma, noticeable after 24 h of *ex vivo* culture and maximal between days 3 and 4 after removal from the *in vivo* milieu. Adhesion of mobilized PB CFC that had been cultured for 4 d *ex vivo* to stroma could be blocked with antibodies against either the α4 or β1 integrin (Fig. 5), suggesting that increased adhesion of cultured mobilized PB depends, at least in part, on increased expression of α4 integrin. This was confirmed in experiments using purified ligands. Adhesion of mobilized PB CFC cultured in defined medium for 4 d to fibronectin (*P* < 0.01) and CS1 (*P* < 0.001) was significantly higher than that of freshly obtained mobilized PB CFC (day 0) (Fig. 7).

Interestingly, adhesion of *ex vivo* cultured mobilized PB CFC to BM stroma and CS1 surpassed that of steady state BM CFC before and after *ex vivo* culture (Figs. 7 and 10). Unlike what we observed for BM CD34⁺ cells where 8A2 increased

integrin-mediated adhesion, adhesion of cultured PB CFC to stroma or CS1 could not be upregulated by 8A2 (Fig. 7). Even though adhesion of cultured mobilized PB CD34⁺ cells to fibronectin could be increased by 8A2, this occurred to a lesser extent than observed for cultured BM CFC (Fig. 7). This suggests that aside from an increase in α4 expression, removal of mobilized PB CFC from the *in vivo* milieu also results in upregulation of the function of the α4β1 but not the α5β1 integrin.

Discussion

That cytokines result in the temporary mobilization of committed and primitive progenitors in the peripheral circulation has been well established (9, 12, 13). Although these cells are likely released from the BM, the origin and mechanism(s) through which these cells are released in the PB are not well understood. It is also unclear how these cells re-adhere to the BM microenvironment, a phenomenon known as homing. In this study we demonstrate that *in vivo* administration of G-CSF to normal individuals minimally affects the majority of BM progenitors since: (a) the absolute number of CD34⁺, CFC, and LTC-IC present per milliliter of BM is unchanged after G-CSF; (b) *in vivo* exposure to G-CSF has no effect on adhesion of BM progenitors, as adhesion of BM CFC to stroma, fibronectin, and the α4β1 integrin binding domain of fibronectin, CS1, is the same before and after G-CSF treatment; and (c) *ex vivo* exposure of BM progenitors to high concentrations of G-CSF does not induce the nonadherent phenotype characteristic of PB progenitors. In contrast, progenitors found in the PB, both before and after *in vivo* treatment with G-CSF, fail to adhere to BM stroma, fibronectin, and the α4β1 binding peptide CS1 due to a decreased α4 integrin expression. However, removal of these progenitors from the *in vivo* milieu results in increased adhesion of PB progenitors to stroma, fibronectin, and CS1. The increase in adhesion of PB progenitors removed from the *in vivo* milieu is associated with a significant increase in α4 integrin expression as well as a maximal activation of the α4β1 integrin.

The finding that the absolute number of CD34⁺ cells, CFC, and LTC-IC in BM obtained after 5 d of *in vivo* G-CSF administration is unaltered is consistent with recent studies in nonhu-

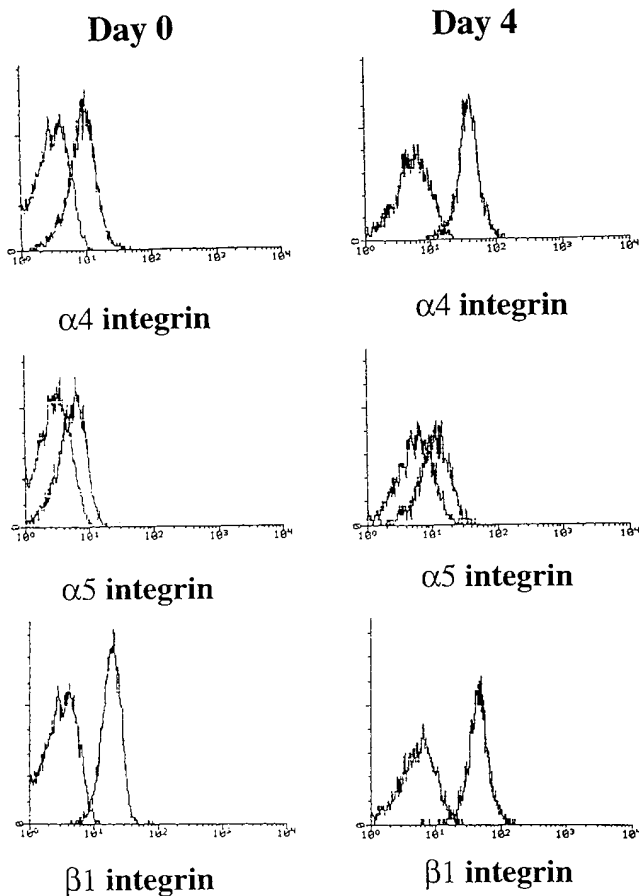


Figure 10. Mobilized PB CD34⁺ cells cultured ex vivo in defined medium upregulate expression of $\alpha 4$ and $\beta 1$ integrin. Mobilized PB CD34⁺ cells were labeled with PE-conjugated anti-CD34 antibody and FITC-conjugated antibodies against $\alpha 4$, $\alpha 5$, and $\beta 1$ integrins or controls before and after culture for 4 d in serum-free media. One representative experiment is depicted.

man primates (61). These studies demonstrated that treatment of baboons with G-CSF, SCF alone, or in combination did not change the number of CD34⁺ cells present per milliliter of BM. This suggests that mobilization of progenitors in the PB is not likely the result of an expansion of progenitors in the BM and subsequent overflowing of progenitors in the PB. Since no depletion of progenitors in the BM is noted, G-CSF-induced mobilization may also not be the result of the displacement of a large fraction of BM progenitors into the PB. Using an in vitro assay in which BM CD34⁺ cells are exposed to G-CSF at concentrations found in the circulation of individuals treated with G-CSF (62), we also provide evidence suggesting that G-CSF itself does not induce the nonadhesive phenotype of mobilized PB CFC. Culture of steady state BM CD34⁺ cells in the presence of defined medium and 1–100 ng/ml of G-CSF did not decrease $\alpha 4$ expression nor adhesion to stroma of BM CD34⁺ cells and CFC. These studies support the concept that mobilization of progenitors in the blood may be due to expansion of a small subpopulation of CD34⁺ $\alpha 4^{+/-}$ cells present in the BM which, even under steady state conditions, is released in the blood due to lack of $\alpha 4$ -mediated interactions. Studies are currently underway to determine if such a progenitor pool

is present in steady state BM which, unlike the majority of CD34⁺ $\alpha 4^{+++}$ BM progenitors, is expanded in BM of individuals treated with G-CSF. Our studies do not exclude the possibility that the adhesive phenotype ($\alpha 4$ expression and $\alpha 4$ -mediated adhesion) of BM CD34⁺ CFC is changed 1–2 d after G-CSF treatment has started and leads to the release of these cells into the blood on days 4–6 of G-CSF treatment. However, our in vitro culture studies failed to induce a decrease in $\alpha 4$ antigen expression and a decreased adhesion of steady state BM progenitors cultured for 1 or 2 d in the presence of up to 100 ng/ml of G-CSF.

CD34⁺ cells present in PB both before and after treatment with G-CSF expressed significantly less $\alpha 4$ integrin than CD34⁺ cells found in steady state or G-CSF-treated BM. Further, CFC circulating in the PB adhered significantly less to BM stroma and the $\alpha 4\beta 1$ binding fibronectin peptide CS1. Thus, decreased expression of the $\alpha 4$ integrin and lack of $\alpha 4$ -dependent adhesion correlate with the abnormal circulation of CFC in the PB, and may therefore underlie mobilization. This is consistent with studies in baboons demonstrating that anti- $\alpha 4$ antibodies mobilize progenitors in the blood (15). Furthermore, this is consistent with observations in CML where the abnormal trafficking of progenitors is associated with decreased $\alpha 4\beta 1$ - and $\alpha 5\beta 1$ -mediated adhesion to BM stroma (49, 56). We demonstrate that similar to CML, CFC in mobilized PB adhere poorly to BM stroma, fibronectin, and CS1. In contrast to CML, where lack of adhesion is caused by a functional defect in the $\alpha 4\beta 1$ integrin (48, 49, 56), our data indicate that lack of adhesion of PB progenitors is due mainly to decreased expression but not decreased function of $\alpha 4$ integrins since: (a) the majority of mobilized PB CFC, unlike steady state BM CFC, are present in the CD34⁺ $\alpha 4^{+/-}$ population; (b) in comparison with BM CFC, mobilized PB CFC fail to adhere to the $\alpha 4\beta 1$ binding fibronectin peptide CS1; (c) unlike adhesion of BM CFC, adhesion of mobilized PB CFC to BM stroma could

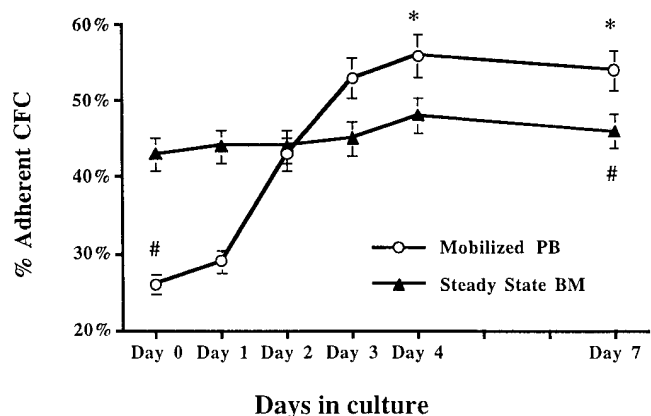


Figure 11. Ex vivo culture increases adhesion of G-CSF-mobilized PB CFC but not steady state BM CFC to BM stromal feeders. Steady state BM and G-CSF-mobilized PB CD34⁺ cells were cultured in serum-free medium in the presence of cytokines and adhesion experiments were performed after 1, 2, 3, 4, and 7 d in culture. For these experiments, the G-CSF concentration in ex vivo cultures was 250 pg/ml ($n = 6$). * $P < 0.01$ comparison between adhesion of freshly selected mobilized PB CFC and ex vivo cultured mobilized PB CFC. # $P < 0.05$ comparison between adhesion of mobilized PB and steady state BM CFC.

not be inhibited by anti- $\alpha 4$ antibodies; and (d) the $\beta 1$ integrin activating antibody 8A2 did not increase adhesion of mobilized PB CFC to stroma or CS1 but could increase $\alpha 5\beta 1$ -mediated adhesion to fibronectin. However, the small subpopulation of $\alpha 4$ expressing mobilized PB CFC adheres to CS1 and the activating antibody 8A2 enhances this adhesive interaction, similar to what is seen for steady state marrow-derived CFC.

We also present evidence that the nonadherent phenotype of progenitors found in PB can be reverted once progenitors are removed from the in vivo milieu, which may then allow homing and engraftment. The mechanism(s) involved in homing of hematopoietic stem and progenitor cells is unclear. It is thought that homing of HSC is a two-step process where initial attachment to the marrow microvasculature may be mediated by lectins, while $\beta 1$ integrins are responsible for securing hematopoietic cells in the BM (28, 35–37). The importance of $\alpha 4$ -VCAM or $\alpha 4$ -fibronectin interactions has been demonstrated in studies in which anti- $\alpha 4$ antibodies prevent HSC engraftment in murine and ovine transplant models (28, 29). We demonstrate here that culture of mobilized PB progenitors with physiological concentrations of growth factors upregulates expression and function of the $\alpha 4\beta 1$ integrin. Increased expression of the $\alpha 4\beta 1$ integrin results in increased $\alpha 4$ -dependent adhesion of mobilized PB CFC. In this in vitro model, maximal upregulation of $\alpha 4\beta 1$ -mediated adhesion required 2 to 4 d. Adhesion through $\alpha 4\beta 1$ integrin is thought to be responsible for securing hematopoietic progenitors in the BM, while the initial steps of the homing process may depend on other receptors such as L-selectin, P-selectin, and PECAM-1 (22, 63, 64). L-selectin and PECAM-1 are more highly expressed on PB or BM progenitors obtained after in vivo treatment with G-CSF and may allow the initial phase of the homing process. We hypothesize that upregulation of the $\alpha 4\beta 1$ integrin expression and function in vivo may occur within the BM microenvironment once the cells have migrated into the BM space (28). This hypothesis is currently being tested in a xenogeneic in vivo transplant model.

In vivo studies suggest that adhesion of progenitors to the BM microenvironment depends for a large part on the $\alpha 4\beta 1$ integrin but not the $\alpha 5\beta 1$ integrin, even though $\alpha 5\beta 1$ integrins are present on CD34⁺ cells (15, 28, 29). Likewise, our in vitro assays demonstrate that the $\alpha 4\beta 1$ integrin, rather than the $\alpha 5\beta 1$ integrin, is responsible for CFC–stroma and CFC–fibronectin interactions. First, blocking antibodies against the $\alpha 4$ integrin decreased adhesion of BM CFC to BM stroma to a greater extent than antibodies against the $\alpha 5$ integrin. Second, adhesion of BM CFC activated with 8A2 to fibronectin could be inhibited significantly with antibodies against the $\alpha 4$ integrin but not with antibodies against the $\alpha 5$ integrin. This suggests a dominant role for the $\alpha 4\beta 1$ integrin in the adhesion of progenitors to stroma and fibronectin. However, $\alpha 4$ and $\alpha 5$ integrins cooperate, since CFC adhesion to fibronectin was blocked to a much larger extent after addition of both anti- $\alpha 4$ and - $\alpha 5$ antibodies. Mobilized PB CFC, containing a majority of $\alpha 4^{+/-}$ cells, adhere to fibronectin solely through the $\alpha 5$ integrin and likely explain the lower percent adhesion of mobilized PB CFC to fibronectin and the lesser increase in adhesion induced by the activating antibody 8A2. Of interest is the observation that the adhesion of ex vivo cultured mobilized PB CFC to stroma and CS1 was not increased in the presence of 8A2, but that 8A2 increased their adhesion to fibronectin, although to a lesser extent than that of uncultured or ex vivo

cultured BM CFC. This indicates that only the $\alpha 5\beta 1$ -mediated adhesion, but not the $\alpha 4\beta 1$ -mediated adhesion, could be increased by 8A2. As has been shown in other biological systems (65), our studies suggest that the ability to adhere through an $\alpha\beta$ heterodimer is, at least in part, dictated by the α chain associated with the $\beta 1$ chain, a hypothesis that is currently being examined.

In summary, the data presented in this study support the concept that PB and BM progenitors may represent two discrete populations which differ in expression of $\alpha 4$ and in their ability to adhere to BM stromal elements, fibronectin, and peptides derived from fibronectin. Circulation of CD34⁺ progenitors in the blood is associated with an $\alpha 4^{+/-}$ phenotype and inability of progenitors to interact through the $\alpha 4\beta 1$ integrin with stromal feeders and purified ligands. After removal from the in vivo milieu, expression and function of the $\alpha 4\beta 1$ integrin is upregulated on mobilized PB progenitors. This results in the reestablishment of an adhesive phenotype that may allow engraftment. Future studies will address the origin of mobilized PB CD34⁺ cells as well as the mechanisms underlying the decreased expression of the $\alpha 4\beta 1$ integrin on circulating CD34⁺ cells and the upregulation of the $\alpha 4\beta 1$ integrin expression and function once PB CD34⁺ cells have been removed from the in vivo milieu.

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