

Cell cycle activation of hematopoietic progenitor cells increases very late antigen-5-mediated adhesion to fibronectin

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Objective. Recent studies suggested that trafficking of hematopoietic progenitor cells is related to cell cycle status. We studied whether adhesion of progenitor cells to extracellular matrix proteins was modulated by cell cycle transit.

Materials and Methods. Mobilized peripheral blood CD34⁺ cells were stimulated ex vivo for 48 hours with stem cell factor, flt-3 ligand, and thrombopoietin and fractionated by adhesion to fibronectin or vascular cell adhesion molecule-1 (VCAM-1). Adherent and nonadherent cells were assayed for cell cycle status, long-term culture-initiating cell frequency, and integrin function.

Results. Binding to fibronectin, but not to VCAM-1, displayed a cell cycle selectivity as the adherent fraction to fibronectin was enriched in cycling CD34⁺ cells and in cycling long-term culture-initiating cells compared to the nonadherent fraction. Combined cell cycle and phenotypic analysis showed that the expression of VLA-5 was upregulated during S/G₂+M but that of VLA-4 remained constant. The selective binding of cycling CD34⁺ cells to fibronectin was reverted by anti-VLA-5 but not by anti-VLA-4 blocking antibodies. Also, cycling CD34⁺ cells preferentially adhered to the VLA-5 binding domain but not to the VLA-4 binding domain of fibronectin. Adhesion of cycling CD34⁺ cells to fibronectin was a reversible process modulated by cell cycle progression, because adherent cells could exit the cell cycle and return to a nonadhesive state within an additional 48-hour culture period.

Conclusion. The results indicate that the enhanced binding capacity of cycling progenitor cells to fibronectin is mediated by VLA-5. © 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Introduction

During cytokine or chemotherapy-induced mobilization, hematopoietic progenitor cell levels increase up to 50-fold in the peripheral blood (PB). It has been demonstrated that mobilized cells include stem cells with repopulating ability [1]. Intriguing differences in cell cycle status of CD34⁺ cells isolated from mobilized PB compared to bone marrow (BM) were reported by several investigators. In contrast to BM CD34⁺ cells, PB CD34⁺ cells are blocked in the G₀/G₁ phase of the cell cycle [2]. In particular, primitive long-term culture-initiating cells (LTC-IC) isolated from PB are quiescent, whereas significant numbers of BM LTC-IC are actively cycling [3]. Furthermore, using DNA/RNA fractionation, we observed that PB CD34⁺ cells are depleted of cells in late G₁ phase, compared to BM CD34⁺ cells [4].

The quiescence of PB progenitor cells is not due to the presence of proliferation inhibitors in the blood [5]. It still is not known whether mobilization occurs only in noncycling cells or whether cycling cells are effectively mobilized and rapidly cleared from the circulation. Most strikingly, engraftment of transplanted hematopoietic cells displays a similar selectivity for quiescent cells. It was first demonstrated by Ramshaw et al. [6] that murine stem cells acquired an engraftment defect when induced to proliferate by in vivo 5-fluorouracil treatment. Subsequently, Habibian and colleagues [7] demonstrated that this negative effect on engraftment was related to transit through S and G₂ phases of the cell cycle and was reverted after the completion of mitosis. As for human stem cells, we also observed that cell cycle progression was associated with a decrease of engraftment capacity in nonobese diabetic/severe combined immunodeficient recipient mice [8]. In light of these findings, it can be envisioned that both mobilization and engraftment are regulated by a cell cycle-based mechanism, the exact na-

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ture of which is not known at present. We thus postulated that the adhesive properties of hematopoietic cells to the BM stroma were modulated during cell cycle transit.

Within the BM, stem/progenitor cells are located in tight contact with stromal cells and extracellular matrix (ECM) proteins, most notably fibronectin (Fn). Very late antigen (VLA)-4 ($\alpha 4\beta 1$ integrin) was first recognized to mediate interaction between mouse repopulating cells [9] or human primitive progenitor cells [10] and Fn. More recently, it was shown that human and mouse stem cells bind to Fn through VLA-5 ($\alpha 5\beta 1$ integrin) as well [11]. VLA-4 also interacts with vascular cell adhesion molecule (VCAM)-1 expressed by stromal cells [12]. It has been reported that the expression of VLA-4 and VLA-5 by human hematopoietic progenitor cells is modulated during continuous cytokine exposure in ex vivo cultures [13]. However, the relationship between cell cycle transit and integrin expression and/or function was not directly established.

In the present study, we investigated whether adhesion of progenitor cells to defined ECM proteins was dependent on cell cycle status. Freshly isolated PB CD34⁺ cells were induced to cycle in suspension cultures in response to the combination of stem cell factor (SCF), flt-3 ligand (FL), and thrombopoietin (TPO), which has been shown to stimulate the proliferation of primitive progenitor cells [14,15]. We present evidence that adhesion of CD34⁺ cells and LTC-IC to Fn is enhanced during cell cycle transit, whereas adhesion to VCAM-1 is not modified. Furthermore, our data show that VLA-5-mediated but not VLA-4-mediated adhesion to Fn is increased during cell cycle transit. Finally, our results suggest that adhesion of replicating cells to Fn is reversible after completion of the cell cycle.

Materials and methods

Cells

After obtaining informed consent, mobilized PB samples were obtained from normal adult volunteers according to the guidelines established by the Ethical Committee of the University of Liège. Mobilization was achieved by daily granulocyte colony-stimulating factor (G-CSF) administration at 10 $\mu\text{g/kg}$ for 5 consecutive days. Cells were collected by apheresis on day 5. PB CD34⁺ cells were isolated by immunomagnetic selection using a large-scale Isolex 300i system (Baxter Healthcare, Newbury, United Kingdom) as per manufacturer's instructions. Cells were cryopreserved in small aliquots in 90% fetal calf serum (FCS; Life technologies, Merelbeke, Belgium) and 10% dimethylsulfoxide (Sigma Chemical Corp., St. Louis, MO, USA). In some instances, fresh PB samples were obtained from normal donors providing CD34-selected progenitor cells for related recipients. Thawed or fresh CD34⁺ cells were purified further using MACS CD34 isolation kits (Miltenyi Biotech, Auburn, CA, USA). In some experiments, cells recovered from the Isolex system were labeled with anti-CD34-fluorescein isothiocyanate (FITC; Becton Dickinson Immunocytometry Systems [BD], San Jose, CA, USA), anti-CD38-phycoerythrin (PE; BD), and propidium iodide (PI; Sigma). CD34⁺CD38^{low}PI^{neg}

cells were sorted on a FACStar Plus flow cytometer (BD). CD34⁺ cell purity in the final product always exceeded 95%.

Short-term suspension cultures

CD34⁺ cells were plated in a serum-free medium consisting of Iscove's medium supplemented with 10 $\mu\text{g/mL}$ bovine serum albumin (BSA), 10 $\mu\text{g/mL}$ bovine insulin, 200 $\mu\text{g/mL}$ transferrin (all from Stem Cell Technologies, Vancouver, BC, Canada), 2 mM alanyl-glutamine, 1% (v/v) lipids cholesterol-rich, 1 mM sodium pyruvate (all from Sigma), 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 5×10^{-2} mM 2-mercaptoethanol (all from Biowhitaker, Petit-Rechain, Belgium). Cells were stimulated by a combination of 100 ng/mL SCF (Amgen, Thousand Oaks, CA, USA), 50 ng/mL TPO (Amgen), and 100 ng/mL FL (R&D Systems, Abingdon, United Kingdom) and maintained at 37°C in a 100% humidified atmosphere with 5% CO₂. The percentage of CD34⁺ cells up to 96 hours in culture was always greater than 90%.

Cell cycle fractionation with Hoechst 33342

Cultured CD34⁺ cells were resuspended at 1×10^6 cells/mL in a 1 $\mu\text{g/mL}$ solution of Hoechst 33342 (Hst; Molecular Probes, Eugene, OR, USA) in Hst Buffer. Hst buffer consisted of Hank's balanced salt solution (HBSS; Biowhitaker), 20 mM HEPES (Biowhitaker), 1 g/L glucose, and 10% FCS. After incubation at 37°C for 45 minutes, cells were washed once, resuspended in Hst buffer, and sorted on a FACS Vantage (BD) equipped with a multiline ultraviolet laser (351–364 nm) providing excitation for Hst. Hst signal was detected with a 424 ± 22 -nm bandpass filter.

Adhesion assays

Fn (Sigma) or 40-kDa and 120-kDa Fn fragments (Life Technologies) were adsorbed at 4°C to wells of nontissue culture-treated 24-well plates (BD) at 20 $\mu\text{g/cm}^2$ in phosphate-buffered saline (PBS; Biowhitaker). VCAM-1 (R&D) was coated at 0.15 $\mu\text{g/cm}^2$ in tissue culture-treated 24-well plates (BD). Coating was done at 4°C for 12 to 16 hours. Control plates were coated with 1% BSA (Life Technologies). The coating solution was removed by aspiration and plates were incubated with RPMI 1640 (Biowhitaker) containing 1% fraction V BSA (Life Technologies, Paisley, United Kingdom) at 37°C for 30 minutes to block nonspecific binding sites. After two washes in RPMI 1640, 1 to 2×10^5 unmanipulated or cultured CD34⁺ cells were plated in RPMI 1640 containing 0.1% BSA at 37°C. After 1-hour incubation, nonadherent cells were harvested by two standardized washes using warm PBS. Adherent cells were recovered after a 2-minute incubation in an enzyme-free cell dissociation buffer (Life Technologies) at 37°C followed by vigorous pipetting. Adherent and nonadherent cells were counted in Fuchs-Rosenthal hemocytometers (volume 3.2 μL). Percent adhesion was calculated as (number of adherent cells)/(number of adherent cells + number of nonadherent cells). Adhesion on BSA-coated control plates was less than 1%. In some experiments, CD34⁺ cells were preincubated with anti-human $\alpha 4$ integrin (clone P4C2; Life Technologies) at 1:400 dilution, anti-human integrin $\alpha 5$ (clone P1D6, Life Technologies) at 1:100 dilution or control mouse IgG₃ (Pharmingen, San Diego, CA, USA) for 30 minutes before the adhesion assay, as described by Prosper et al. [13].

LTC-IC and colony-forming cell assays

Absolute frequencies of LTC-IC were determined by limiting dilution analysis over M2-10B4 fibroblast feeder cells [16] (American Type Culture Collection Rockville, MD, USA). Briefly, M2-10B4

cells harvested from large-scale cultures in RPMI 1640 with 10% FCS were irradiated at 7,500 cGy and plated in 96-well plates at 15,000 cells/well in 100 μ L Myelocult (Stem Cell Technologies). Within 1 week, human test cells were plated in limiting dilution at 24 wells/cell dose in another 100 μ L Myelocult and maintained at 37°C in 100% humidified atmosphere containing 5% CO₂, with weekly half-medium change. After 5 to 6 weeks, medium was carefully aspirated from each well followed by the addition of 200 μ L of Methocult H4435 (Stem Cell Technologies). After an additional 2 weeks, wells were scored for the presence or absence of hematopoietic colonies and the frequency of LTC-IC was calculated using the maximum likelihood estimator [17]. Colony-forming cells (CFC) were assayed in Methocult H4435 as per manufacturer's instructions.

Cell cycle analysis of LTC-IC was performed by hydroxyurea (HU; Sigma) suicide assay as described by Cen and Levin [18], with minor modifications. In preliminary experiments with HU doses ranging from 0.1 to 10 mg/mL, we established the optimal dose allowing discrimination between quiescent and cycling progenitor cells, using PB and BM, respectively. At HU concentration of 2 mg/mL, killing of BM CFC was $25.4\% \pm 6.0\%$ ($n = 7$), whereas killing of PB CFC was $4.6\% \pm 4.3\%$ ($n = 4$). These data are consistent with previously published results [19]. In LTC-IC killing assays, 2 to 10×10^4 CD34⁺ cells were treated with 2 mg/mL HU in Iscove's medium and 1% BSA for 1 hour at 37°C. Control cells were incubated in medium only. After two washes in Iscove's medium with 1% BSA, cells were replated in limiting dilution in LTC-IC assays. The percentage of cycling LTC-IC (% kill) was estimated using the following calculation: % LTC-IC after HU treatment/% LTC-IC after incubation in control medium.

Flow cytometric analysis of integrin expression

FITC-conjugated anti-VLA-4 (CD49d) or anti-VLA-5 (CD49e) (both from Coulter Immunotech, Marseille, France) were used in combination with anti-CD34-PE (BD) to analyze integrin expression on CD34⁺ cells. As more than 90% of either fresh or cultured cells expressed CD34, anti-CD49e-FITC and anti-CD49d-PE (Pharmingen) also were analyzed, together without simultaneous CD34 staining. Cells were incubated with antibodies or isotype-matched control immunoglobulin G (IgG) for 20 minutes on ice in the dark. Cells then were washed in PBS 1% calf serum (Biowhitaker) and fixed in PBS 1% paraformaldehyde (Sigma). Data were acquired on a FACSsort (BD) flow cytometer and analyzed using Cellquest software (BD). Positive cells were identified as those displaying a fluorescence greater than 99% of the isotypic control. Integrin density was expressed as the mean channel fluorescence ratio (MCFR) defined as MCF of integrin expression divided by MCF of fluorescence-matched isotypic control.

Flow cytometric analysis of cell cycle status

Conventional cell cycle analysis by DNA staining with PI was performed by incubating cells in PBS 0.6% IGEPAL CA-630 (Sigma) containing 50 μ g/mL PI and 1 mg/mL RNase (Boehringer, Mannheim, Germany). After a 30-minute incubation on ice in the dark, cells were analyzed on a FACSsort flow cytometer using FL-2 channel. The proportion of cells in the different phases of the cell cycle was determined using ModFit software (Verity Software, Topsham, ME, USA). Alternatively, cells were analyzed for Ki-67 expression and DNA content as described by Jordan et al. [20], with minor modifications. Cells were washed and resuspended in 1

mL PBS 0.4% paraformaldehyde. After 30 minutes at 4°C, 1 mL of PBS 0.2% Triton X-100 (Sigma) was added and cells were left overnight at 4°C. Cells then were washed twice in PBS 1% BSA and stained with FITC-conjugated anti-Ki-67 (clone MIB-1; Coulter Immunotech) for 60 minutes at 4°C. Isotype controls were stained in parallel. Finally, cells were washed and resuspended in PBS 1% BSA containing 5 μ g/mL 7-aminoactinomycin-D (7-AAD; Sigma). After a 3-hour incubation on ice, samples were run on a FACSsort flow cytometer using FL-1 and FL-3 channels for Ki-67 and 7-AAD, respectively.

Combined cell cycle analysis and cell surface staining was performed according to the following procedure. Cells were stained with FITC-conjugated anti-VLA-5 (CD49e, Coulter Immunotech), PE-anti-VLA-4 (CD49d, Pharmingen), or nonspecific isotype-matched IgG for 20 minutes at 4°C in the dark. After washing, cells were fixed and permeabilized with PBS containing 0.5% paraformaldehyde and 0.5% saponin (Sigma) at 4°C for 5 minutes. Cells were washed twice in PBS 1% BSA and stained with PBS 1% BSA containing 5 μ g/mL 7-AAD and 1mg/mL RNase for 3 hours on ice. Samples were run on a FACSsort flow cytometer. Isotypic limits were set to include 99% of the cells.

Statistical analysis

Results are reported as mean \pm SEM. Statistical analysis was performed using SigmaStat software (Jandel, San Rafael, CA, USA). All P values are two-sided.

Results

Adhesion of PB CD34⁺ cells

to Fn is increased after ex vivo culture

To analyze the consequences of mitotic activation on adhesion to ECM proteins, for these experiments we used PB CD34⁺ cells as a "baseline" population of nonadherent and mitotically inactive hematopoietic cells [13,21]. PB CD34⁺ cells were stimulated in suspension culture for 48 hours in a serum-free medium supplemented with SCF (100 ng/mL), TPO (50 ng/mL), and FL (100 ng/mL), a cytokine cocktail conducive for the onset of primitive progenitor cell replication [14,15]. In these conditions, both CD34 expression ($97.9\% \pm 2.1\%$ CD34⁺ cells at initiation vs $98.2\% \pm 1.4\%$ after 48 hours; $n = 12$) and CFC frequency ($40.5\% \pm 11.9\%$ at initiation vs $53.0\% \pm 7.9\%$ after 48 hours; $n = 12$) remained constant during culture.

Before and after stimulation, adhesion was determined by plating the cells onto ECM protein-coated dishes for 1 hour at 37°C and collecting adherent and nonadherent fractions. Adhesion of CD34⁺ cells to VCAM-1 ($44.7\% \pm 6.3\%$ of input for fresh cells) was not modified after ex vivo culture ($39.9\% \pm 9.6\%$ for cultured cells; $n = 4$). In contrast, there was a two-fold increase in adhesion of CD34⁺ cells to Fn after culture, from $7.0\% \pm 3.5\%$ of input for unmanipulated cells up to $14.4\% \pm 4.5\%$ of input for cultured cells ($n = 12$; $p < 0.05$). Similarly, adhesion of CFC to Fn rose from $5.2\% \pm 0.5\%$ at culture initiation to $9.5\% \pm 1.8\%$ after 48 hours ex vivo.

Actively cycling CD34⁺ cells

adhere preferentially to Fn but not to VCAM-1

The cell cycle status of PB CD34⁺ cells binding to Fn or VCAM-1 was assessed by PI staining and FACS analysis, before and after cytokine stimulation. More than 98% of freshly isolated PB CD34⁺ cells were blocked in the G₀/G₁ phase of the cell cycle. There was no difference in cell cycle status between adherent and nonadherent cells, whether adhesion was assessed on Fn or on VCAM-1 (data not shown). After 48 hours in culture, 34.3% ± 1.5% of the cells were engaged in S/G₂+M (n = 18). When adherent and nonadherent cells to Fn were analyzed separately, the adherent fraction was enriched in cycling cells: 45.1% ± 3.2% of adherent cells were in S/G₂+M vs 33.2% ± 2.1% of nonadherent cells (n = 18; *p* < 0.00001) (Fig. 1). Thus, transit into the replicative phase of the cell cycle was associated with increased adhesion to Fn. In contrast, when adhesion of cultured CD34⁺ cells was assessed on VCAM-1, no association between cycle activation and adhesion was observed. The proportion of cells in S/G₂+M was similar in adherent (32.1% ± 3.5%) and nonadherent (32.7% ± 3.3%) fractions (n = 6) (Fig. 1).

We next determined the proportion of CD34⁺ cells in G₀ in adherent and nonadherent fractions by using high-resolution cell cycle analysis by Ki-67/7-AAD staining. In this procedure, a DNA histogram is generated by 7-AAD and plotted against the expression of the nuclear antigen Ki-67, which is present in cycling cells but not in G₀ cells. To enrich the starting cell population in G₀ cells, PB CD34⁺CD38^{low/-} cells were isolated (instead of total CD34⁺ cells), cultured for 48 hours in the same conditions, and fractionated by adhesion to Fn. The proportion of cells remaining in G₀ was 15.0% in adherent cells compared to 24.6% in nonadherent cells, in a

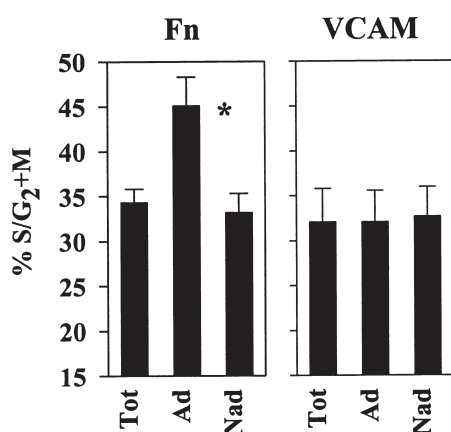


Figure 1. Binding to Fn, but not to VCAM-1, is increased in cycling cells. PB CD34⁺ cells were stimulated ex vivo during 48 hours and replated onto Fn (n = 18) or VCAM-1 (n = 6) coated plates and fractionated into adherent and nonadherent fractions. The percentage of cells in S/G₂+M was determined by PI staining in total (tot), adherent (Ad), and nonadherent (Nad) cells. **p* < 0.00001 compared to nonadherent cells.

representative experiment (of three) depicted in Figure 2. Therefore, not only the transit in S/G₂+M but also the G₀–G₁ transition was associated with increased cell binding to Fn.

To rule out the possibility that adhesion to Fn by itself was responsible for the higher cycling status of the adherent fraction, cultured CD34⁺ cells were first fractionated into cycling and noncycling cells after staining with Hoechst 33342 (Fig. 3) and then assayed for Fn binding capacity (Table 1). Adhesion was significantly increased in cycling cells (25.1% ± 2.6%) compared to noncycling cells (8.0% ± 1.1%, n = 3; *p* < 0.05).

LTC-IC are stimulated to

bind to Fn during cell cycle transit

Binding capacity and cell cycle status of primitive progenitor cells were assayed using LTC-IC assays by limiting dilution analysis. We first determined the absolute numbers of LTC-IC at days 0, 2, and 6 of ex vivo culture. We established that after 48 hours of suspension culture in SCF, FL, and TPO, there was an initial 51% ± 22% loss of LTC-IC; 4 days later, LTC-IC numbers had returned to 99% ± 32% of day 0 levels (n = 4). This suggests the existence of at least two subsets of LTC-IC: a first fraction that was not supported by the ex vivo conditions used (e.g., for lack of a particular cytokine and/or stromal accessory cells), and another

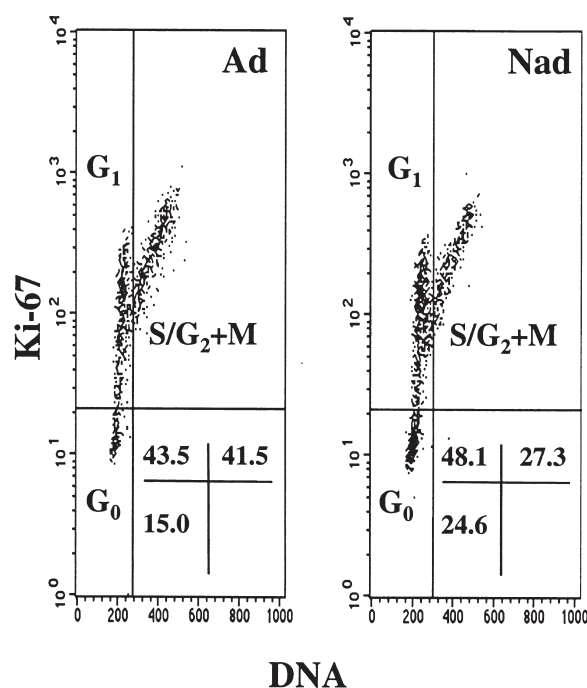


Figure 2. Dual-parameter cell cycle analysis of adherent and nonadherent CD34⁺ cells to Fn after 48 hours of ex vivo culture. PB CD34⁺CD38⁻ cells were stimulated ex vivo for 48 hours and then allowed to bind to Fn. The cell cycle status of cells recovered in the adherent and nonadherent fractions was assessed by simultaneous Ki-67 and 7-AAD staining. The percentages of cells in G₀, G₁, and S/G₂+M are shown in the lower right quadrant of both panels. A representative experiment is shown.

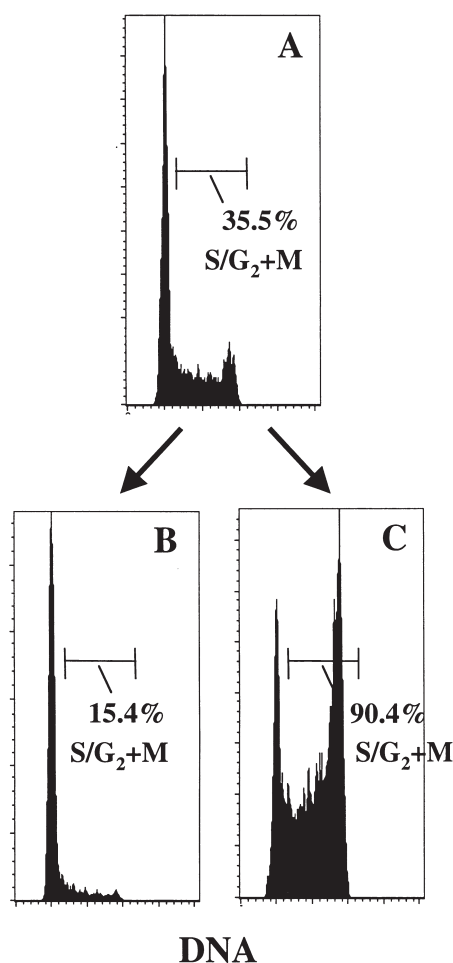


Figure 3. Cell cycle fractionation by Hoechst staining. Cultured CD34⁺ cells were stained with Hoechst 33342 and fractionated into noncycling (G₀/G₁) and cycling (S/G₂+M) fractions. The purity of sorted cells was assessed by PI staining of input (A) and sorted cells [(B) noncycling; (C) cycling]. A representative experiment is shown.

more responsive subset that eventually provided the recovery of initial LTC-IC numbers after several cell divisions. Thus, although no LTC-IC expansion could be observed in these experiments, LTC-IC replication occurred between days 2 and 6.

Adherent and nonadherent cells to Fn harvested either from freshly isolated CD34⁺ cells or from cells cultured for 48 hours were replated in LTC-IC assays. In unmanipulated cells ($n = 4$), the frequency of LTC-IC was similar in the adherent ($5.4\% \pm 1.4\%$) and the nonadherent fractions ($4.3\% \pm 1.2\%$). After 48 hours in culture ($n = 10$), the percentage of LTC-IC was higher in the adherent fraction ($4.8\% \pm 0.6\%$) compared to the nonadherent fraction ($2.7\% \pm 0.4\%$; $p = 0.0019$). LTC-IC assays also were performed after incubating the cells for 1 hour at 37°C on Fn-coated plates and pooling adherent and nonadherent cells. No difference in LTC-IC frequency could be demonstrated between cells exposed to Fn and control cells (data not

Table 1. Adhesion of FACS-sorted cycling and noncycling CD34⁺ cells

Experiment no.	Cell fraction		
	Total CD34 ⁺	G ₀ /G ₁ CD34 ⁺	S/G ₂ +M CD34 ⁺
1	12.1	6.0	21.1
2	17.3	8.3	24.3
3	14.5	9.7	29.9
Mean	14.6	8.0	25.1*
SEM	1.5	1.1	2.6

Cultured CD34⁺ cells were fractionated into noncycling (G₀/G₁) and cycling (S/G₂+M) fractions by Hoechst staining and cell sorting. Adhesion to fibronectin was measured as described in the Methods section. Data are from three independent experiments and are given as percent adherent cells.

* $p < 0.05$ compared to G₀/G₁ CD34⁺ and total CD34⁺ cell populations (one-way ANOVA followed by Student-Newman-Keul's tests for multiple pairwise comparisons).

shown), indicating that, in our conditions, the increase in LTC-IC frequency in adherent cells was not the result of a growth-supporting effect mediated by Fn, as reported elsewhere [22,23].

We next examined the cell cycle status of LTC-IC collected in the adherent and nonadherent fractions by suicide assay. Cells were treated with HU for 1 hour at 37°C in order to kill cells during S-phase transit. Control cells were incubated for 1 hour in medium only. Cells then were replated in limiting dilution in LTC-IC assays and the proportion of cycling LTC-IC (% kill) determined by calculating the ratio between the LTC-IC frequency of HU-treated cells and the LTC-IC frequency of control cells. In freshly isolated PB CD34⁺ cells, no loss of LTC-IC was observed after HU treatment, confirming these to be a quiescent population [3] (Fig 4). After 48 hours in culture, the adherent fraction was significantly enriched in cycling LTC-IC (% kill = $58.7\% \pm 6.7\%$) compared to the nonadherent fraction (% kill = $19.7\% \pm 6.5\%$, $n = 4$; $p < 0.05$) (Fig. 4). These results indicate that, after a 2-day ex vivo culture, not only was the adherent cell population enriched in LTC-IC, but, more importantly, the Fn binding capacity of cycling LTC-IC was higher than that of quiescent LTC-IC.

Enhanced adhesion of replicating progenitor cells to Fn is mediated by VLA-5

The expression of VLA-4 and VLA-5 integrins at the surface of PB CD34⁺ cells was analyzed before and after 48 hours of cytokine stimulation. The vast majority ($99.3\% \pm 0.5\%$, $n = 5$) of freshly isolated CD34⁺ cells were VLA-4⁺ and after ex vivo culture there was a slight, but not statistically significant, reduction in VLA-4 expression ($93.6\% \pm 3.6\%$ VLA-4⁺ cells). VLA-4 density, expressed as MCFR, increased from 14.8 ± 2.2 in fresh cells to 30.5 ± 10.9 in cultured cells, but the difference did not reach statistical significance ($n = 5$; $p > 0.05$). On the contrary, VLA-5 expression, which was detectable in only $1.8\% \pm 2.4\%$ of un-

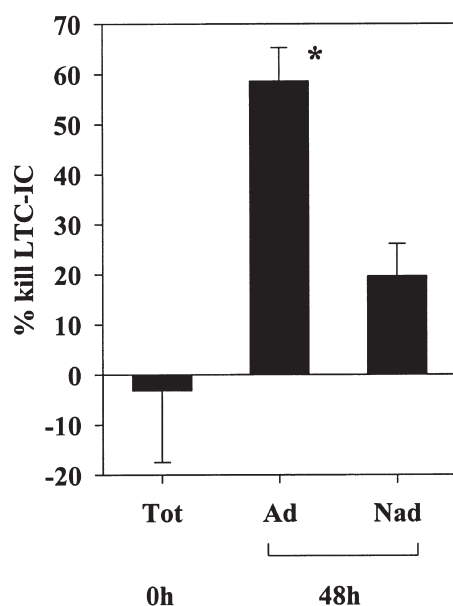


Figure 4. Enhanced binding of cycling LTC-IC to Fn. PB CD34⁺ cells were cultured ex vivo for 48 hours and then replated on Fn-coated dishes. The cell cycle status of LTC-IC was determined by HU suicide assay in total CD34⁺ cells before culture (Tot) and in cultured CD34⁺ cells recovered in the adherent (Ad) and in the nonadherent (Nad) fractions to Fn (n = 4). The percentage of cycling LTC-IC was estimated by the proportion of LTC-IC killed by HU treatment. **p* < 0.05 compared to nonadherent cells.

manipulated CD34⁺ cells, increased after 48 hours of cytokine stimulation ($32.9\% \pm 2.2\%$ VLA-5⁺ cells, n = 9; *p* < 0.001) (Fig. 5).

We next analyzed in more details whether adhesion of replicating CD34⁺ cells to Fn was mediated by VLA-4 or by VLA-5. VLA-4 and VLA-5 expression in cultured CD34⁺ cells was measured separately in G₀/G₁ and in S/G₂+M. VLA-4 was expressed by more than 90% of the cells in either phase of the cell cycle and with similar density (MCFR 22.9 ± 2.7 in noncycling cells vs 23.4 ± 4.4 in cycling cells, n = 3; *p* > 0.05). VLA-5 was expressed at higher levels in actively

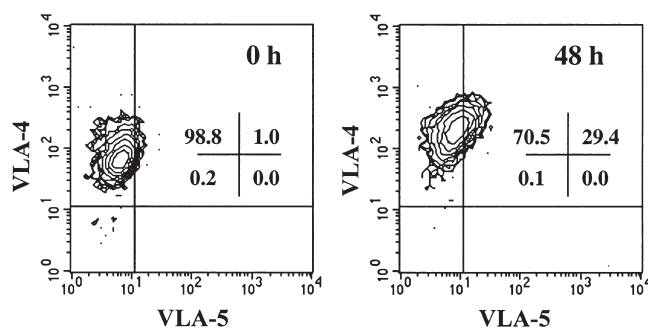


Figure 5. Changes of VLA-4 and VLA-5 expression in CD34⁺ cells during ex vivo culture. VLA-4 and VLA-5 expression was determined in freshly isolated (0 h) and in cultured (48 h) CD34⁺ cells. The percentage of cells in each quadrant is indicated. A representative experiment is shown.

cycling cells: $59.8\% \pm 9.6\%$ of CD34⁺ cells in S/G₂+M were VLA-5⁺ vs only $23.0 \pm 5.2\%$ of cells in G₀/G₁ phase (n = 3; *p* = 0.02) (Fig. 6). These data document a modulation of VLA-5 expression, but not of VLA-4, by cell cycle progression.

To test directly VLA-4 and VLA-5 function in stimulated CD34⁺ cells, cells were preincubated with blocking antibodies against $\alpha 4$ (clone P4C2) or $\alpha 5$ (clone P1D6) integrins and then assayed for adhesion to Fn. The selectivity of both blocking antibodies was confirmed with two non-overlapping Fn fragments. The 40-kDa chymotryptic Fn fragment contains the CS-1 binding site for VLA-4 but lacks the RGDS binding sequence for VLA-5 [24]. The 120-kDa Fn fragment contains the RGDS binding site but

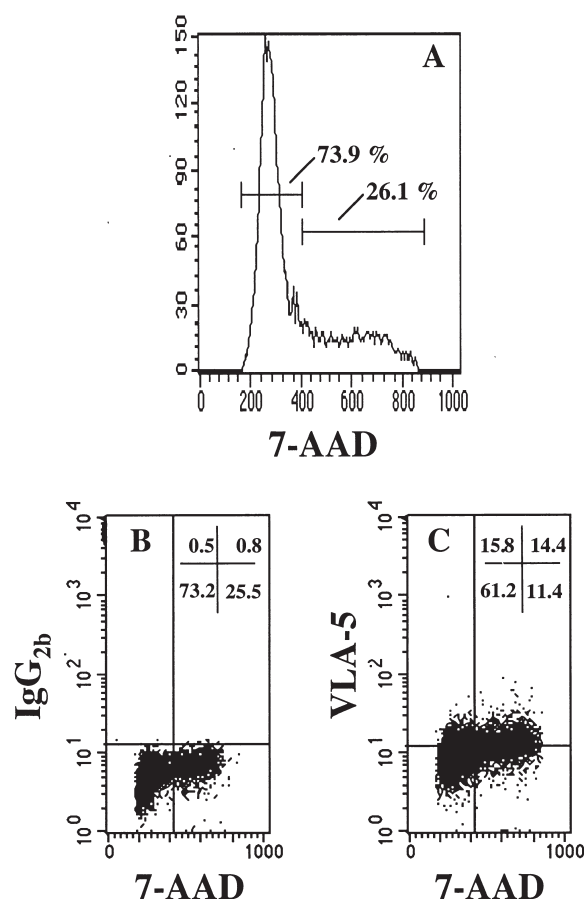


Figure 6. VLA-5 expression in cultured CD34⁺ cells is upregulated during S/G₂+M. VLA-5 expression was analyzed in combination with DNA staining by 7-AAD in cultured CD34⁺ cells. A representative experiment is shown. (A) DNA histogram showing the distribution of cells in G₀/G₁ (73.9%) and in S/G₂+M (26.1%) phases of the cell cycle. (B) Same sample showing simultaneous staining with control IgG. The horizontal line indicates the limit for nonspecific staining. The vertical line separates cells in G₀/G₁ and those in S/G₂+M. (C) Combined VLA-5 and 7-AAD staining. The proportion of cells expressing VLA-5 in G₀/G₁ is 20.5% [$15.8/(15.8 + 61.2) \times 100$] compared to 55.8% [$14.4/(14.4 + 11.4) \times 100$] in S/G₂+M.

not the CS1 sequence [25]. Anti-VLA-4 blocked CD34⁺ cell binding to the 40-kDa Fn fragment but not to the 120-kDa Fn fragment, whereas the reverse was observed for anti-VLA-5 antibody (data not shown). Compared to non-specific IgG, adhesion of cultured CD34⁺ cells to intact Fn was predominantly inhibited by anti-VLA-5 and to a lesser extent by anti-VLA-4 (Fig. 7A), indicating that VLA-4, although present, was not functionally active on a high proportion (>50%) of the cells. When cells were treated simultaneously with both anti- α 4 and anti- α 5, adhesion to Fn was completely abolished, which excludes the possibility that another receptor played a significant role in our experimental conditions.

To analyze separately the contribution of VLA-4 and VLA-5 in mediating adhesion of replicating cells to Fn, cultured CD34⁺ cells were incubated with a blocking antibody against either α 4 or α 5. Cells then were allowed to bind to Fn and the cell cycle status of adherent and nonadherent cells was assessed by PI staining ($n = 4$). When α 4 was blocked, i.e., cells were bound via VLA-5 only, the frequency of cycling S/G₂+M cells in the adherent fraction ($47.0\% \pm 3.8\%$) was similar to that observed after incubation with mouse IgG ($45.9\% \pm 5.4\%$; $p > 0.05$) (Fig. 7B). In contrast, when α 5 was blocked and cells were bound to Fn solely by VLA-4, there were only $28.7\% \pm 5.7\%$ cycling cells in the adherent fraction ($p < 0.05$ compared to adherent cells after IgG or anti- α 4 treatment) and cycling cells then were predominantly collected in the nonadherent fraction (Fig. 7B).

Similar results were obtained when the contribution of VLA-4 and VLA-5 was assessed using Fn fragments. CD34⁺ cells, either fresh or stimulated for 48 hours, were allowed to adhere onto plates coated with 40-kDa or 120-kDa Fn fragments containing the CS-1 and RGDS binding sites, respectively. Binding of CD34⁺ cells to the 40-kDa fragment was similar before ($6.7\% \pm 1.1\%$ adherent cells) and after cytokine stimulation ($7.4\% \pm 1.9\%$ adherent cells; $p > 0.05$), whereas adhesion to the 120-kDa Fn fragment was significantly increased after ex vivo culture, from $3.7\% \pm 0.8\%$ to $10.4\% \pm 2.6\%$ adherent cells ($p < 0.05$) (Fig. 7C). In addition, cell cycle analysis of adherent and nonadherent CD34⁺ cells to each fragment revealed that there were more cycling cells in the adherent fraction to the 120-kDa fragment ($50.1\% \pm 1.7\%$ S/G₂+M cells) than in the adherent fraction to the 40-kDa fragment ($33.3\% \pm 0.5\%$ S/G₂+M cells; $p < 0.05$) (Fig. 7D). Taken together with the blocking antibodies experiments, these data demonstrate that the increased adhesion capacity of cycling CD34⁺ cells to Fn was primarily mediated by VLA-5.

Fn binding of replicating

CD34⁺ cells is a reversible process

Finally, we wanted to study whether the increase in VLA-5-mediated adhesion to Fn in cultured CD34⁺ cells could be reverted after completion of cell division. To this end, in a final series of experiments ($n = 5$), adherent cells were collected after a 2-day ex vivo culture and transferred to fresh

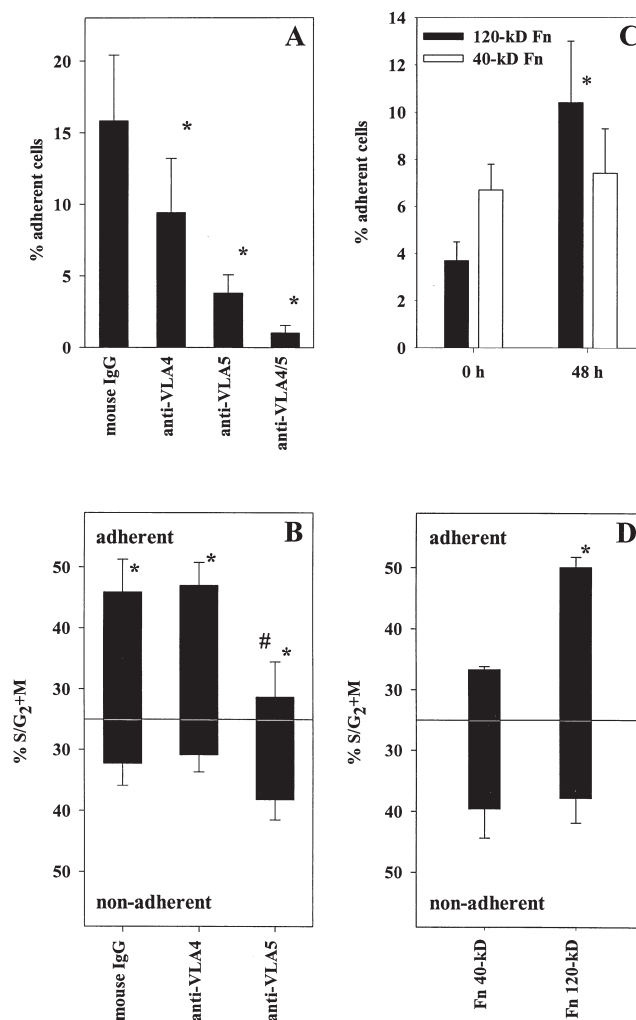


Figure 7. Enhanced binding of cycling CD34⁺ cells to Fn is mediated by VLA-5. (A) Cultured CD34⁺ cells were treated with isotype-matched mouse IgG or with blocking antibodies against VLA-4, VLA-5, or both, and replated on Fn-coated dishes. The percentage of cells recovered in the adherent fraction is indicated in each condition ($n = 8$). * $p < 0.05$ compared to mouse IgG. (B) Cultured CD34⁺ cells were treated with isotype-matched mouse IgG or with blocking antibodies against VLA-4 or VLA-5 and replated onto Fn-coated dishes for 1 hour, after which adherent and nonadherent cells were harvested. The percentage of cells in S/G₂+M was determined in adherent and in nonadherent fractions by PI staining ($n = 4$). # $p < 0.05$ compared to adherent cells recovered after mouse IgG or anti-VLA-4 treatment; * $p < 0.05$ compared to nonadherent cells in the corresponding treatment group. (C) Fresh (0 h) and cultured (48 h) CD34⁺ cells were plated on the 40-kD or the 120-kD fragment of Fn, containing CS-1 and RGDS sites, respectively. The percentage of adherent cells was measured in each condition. *Binding to 120-kD Fn increased after culture ($p < 0.05$ compared to adhesion at 0 h). (D) Cultured CD34⁺ cells were plated on the 40-kD or the 120-kD Fn fragment for 1 hour, after which adherent and nonadherent cells were collected. The percentage of cycling cells was determined in each fraction. *Percentage of S/G₂+M cells was higher in cells binding to the 120-kD fragment compared to cells binding to the 40-kD Fn fragment ($p < 0.05$).

cultures for an additional 48 hours. The percentage of CD34⁺ cells up to 96 hours in culture was always greater than 90%. Progeny cells resulting from these secondary cultures again produced an adherent and a nonadherent population. Both cell fractions were assayed for cell cycle status. Most progeny cells (80.6% \pm 3.6%) had lost their binding capacity to Fn. These nonadherent cells were depleted in cycling cells (25.7% \pm 3.0% S/G₂+M cells) compared to the starting adherent population (39.6% \pm 4.1% in S/G₂+M; p < 0.05) and compared to cells that were still able to bind to Fn (34.6% \pm 2.6% in S/G₂+M; p < 0.05). A representative experiment is depicted in Figure 8. These results suggest that adhesion of CD34⁺ cells to Fn during ex vivo culture is transient and reversible after completion of the cell cycle.

Discussion

We examined whether the adhesive properties of progenitor cells to ECM proteins are modulated by cell cycle transit. PB CD34⁺ cells were used for these experiments, as they are blocked in G₀/G₁ and have very low adhesiveness to Fn when freshly isolated. After a 2-day ex vivo culture, we observed the appearance of a population of adherent cells to Fn. In this adherent population, there was a significant enrichment in cycling (S/G₂+M) CD34⁺ cells and, more importantly, in cycling primitive progenitor cells assayed as LTC-IC. Moreover, quiescent CD34⁺ cells remaining in G₀ after 48 hours of cytokine stimulation were more frequent in the nonadherent population. Thus, transition from a resting state to an activated state in the cell cycle is associated with a potentiation of adhesion to Fn. Both growth-supporting and growth-inhibiting effects of Fn have been reported [22,23,26]; thus, it may be argued that Fn binding by itself may alter the proportion of progenitors in the different phases of cell cycle. To rule out this possibility, we sorted cycling and noncycling CD34⁺ cells after ex vivo culture and showed that cycling cells had a three-fold higher binding capacity as compared to noncycling cells. This shows unambiguously selective adhesion of cycling cells. However, cell cycle activation does not directly cause adhesion to Fn, because there also were many cycling cells in the nonadherent fraction. Still, both cycling CD34⁺ cells and cycling LTC-IC have a higher tendency to bind to Fn than their noncycling counterparts. These observations are in line with a recent report of Yamaguchi et al. [27], who described preferential binding of proliferating CD34⁺ cells to MS-5 stromal layers. Our data further identify Fn, but not VCAM-1, as a preferential ligand for cycling cells. We did not observe any quantitative change in adhesion to VCAM-1 after cytokine stimulation, and binding to VCAM-1 was not enhanced in cycling CD34⁺ cells. Thus, enhanced adhesiveness of cycling progenitor cells is restricted to specific ligands. It remains to be determined whether other ECM proteins or stromal cell adhesion molecules also are involved in this cell cycle selectivity.

As for integrin receptors binding to Fn, VLA-4 and VLA-5

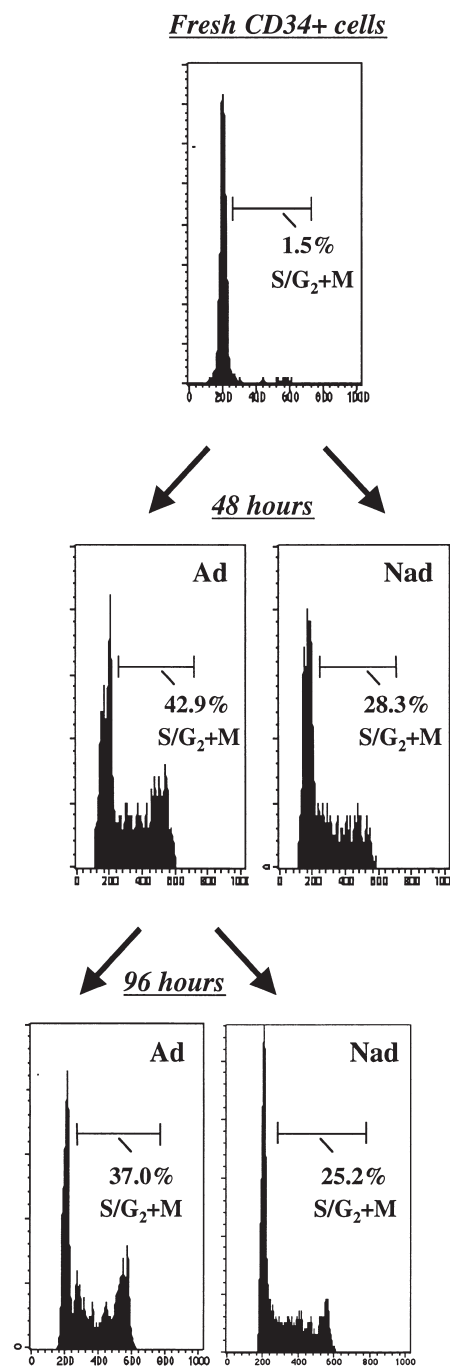


Figure 8. Binding of CD34⁺ cells to Fn during cell cycle transit is reversible. Freshly isolated PB CD34⁺ cells were stimulated ex vivo for 48 hours and plated over Fn. Adherent (Ad) and nonadherent (Nad) cells were harvested separately. Adherent cells were replated in suspension culture for an additional 48 hours. Cells from these secondary cultures were fractionated further into adherent and nonadherent cells to Fn. The percentage of S/G₂+M cells was determined in each fraction by PI staining. A representative experiment is shown.

were affected differently by cell cycle activation. The proportion of cells expressing VLA-5 was increased during a 2-day ex vivo culture, specifically on the surface of cells traversing S/G₂+M. VLA-4 was expressed on the vast ma-

jority of both unmanipulated and cultured CD34⁺ cells and with a higher density on cultured cells (although this was not statistically significant). However, VLA-4 density and the proportion of VLA-4 expressing cells were not different in cycling and noncycling cells. Prosper et al. [13] reported upregulation of VLA-4, but not of VLA-5, at the surface of PB CD34⁺ cells after a 4-day ex vivo culture. This discrepancy with our results may arise from differences in the culture conditions used. The cocktail of SCF, FL, and TPO at high doses used in the present study was selected to induce maximal proliferation of primitive progenitor cells [14,15]. In contrast, a combination of granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, SCF, leukemia inhibitory factor, macrophage inhibitory protein-1a, and interleukin-6 at low doses was used in the study by Prosper et al. to promote the survival of progenitor cells with minimal proliferation. Therefore, it should not be surprising that the increase of VLA-5-mediated adhesion to Fn was not observed by Prosper et al., because it occurs mainly in cycling progenitor cells. A formal comparison of integrin changes after progenitor cell stimulation with physiologic or pharmacologic cytokine doses should be undertaken to provide further support for this interpretation.

There has been controversies in the literature as to the extent of VLA-4 expression on PB CD34⁺ cells. In several reports, VLA-4 was found on a smaller proportion of PB CD34⁺ cells as compared to BM [13,28], whereas in other studies [27,29], including our own, it was reported that VLA-4 was expressed on the vast majority of PB CD34⁺ cells. These discrepancies are likely due to the fact that VLA-4 expression is quite homogeneous, with no clear separation between negative and positive cells. Depending on the sensitivity of the method and/or the equipment used, PB CD34⁺ cells labeled with anti-VLA-4 may or may not overlap with isotypic controls, whereas BM CD34⁺ cells are brightly positive. Lichterfeld et al. [29] reported that VLA-4 is expressed by the majority of PB CD34⁺ cells but at a lower density (or MCFR) than in BM CD34⁺ cells.

VLA-4 and VLA-5-mediated adhesion to Fn was functionally assessed by blocking antibodies. When $\alpha 4$ integrin was blocked, there was a 50% reduction in adhesion of cultured CD34⁺ cells compared to nonspecific IgG, indicating that although expressed on more than 90% of the cells, VLA-4 was not fully active. In that case, binding to Fn occurred via VLA-5 only and was still increased in cycling cells. When $\alpha 5$ integrin was blocked, adhesion to Fn was reduced by approximately 80%, demonstrating a predominant role of VLA-5 in Fn binding of cultured CD34⁺ cells. Most importantly, cycling cells were less able to attach to Fn and then were predominantly collected in the nonadherent fraction. These data demonstrate that the preferential binding of cycling CD34⁺ cells to Fn is more specifically mediated by VLA-5. This was further shown by using Fn fragments. Following cytokine stimulation, adhesion of CD34⁺ cells was increased on the 120-kDa Fn fragment recognized by

VLA-5 but not on the 40-kDa fragment recognized by VLA-4. In addition, binding to the 120-kDa Fn fragment was increased in cycling cells compared to noncycling cells, whereas adhesion to the 40-kDa Fn fragment was not.

VLA-4 and VLA-5 are expressed by resting progenitor cells in a low-affinity state providing weak interaction with Fn. Cytokines such as interleukin-3, GM-CSF, SCF, and TPO [30–33] increase transiently the affinity of VLA-4 and VLA-5 for Fn without concomitant changes in the expression of these receptors. In this study, we show that, in addition to the regulation of integrin affinity, cytokine stimulation allows overexpression of activated VLA-5 once the cells are engaged in cell replication. Therefore, we propose that fast changes in the affinity of integrin receptors occur independently of cell cycle status, whereas slower modulation of integrin expression (particularly of VLA-5) takes place during cell proliferation.

Many reports suggest that trafficking of hematopoietic stem cells in and out of the BM is modulated by cell cycle status. First, only resting progenitor cells are found in PB [2,5]. Our results support the hypothesis that the absence of cycling hematopoietic cells in PB may result from their selective retention in the BM stroma or from their selective uptake in extramedullary tissues onto Fn-coated sites. Second, engraftment of transplanted cells is impaired during cell replication [7,8,34]. When viewed in the context of ex vivo expansion and gene therapy, our results show that after 48 hours of culture in conditions promoting stem cell proliferation, adhesion of replicating cells to Fn is significantly augmented. Whether enhanced VLA-5-mediated adhesion to Fn could possibly modify migration and/or homing and engraftment of cultured cells remains to be tested in vivo. Interestingly, several reports now have established a reciprocal relationship between adhesion and migration. When adhesion is maximal, the cell is immobilized and cannot migrate, whereas at intermediate adhesiveness, dynamic disruption of cell–substratum interactions may allow cell locomotion [35,36]. If the same applies to primitive hematopoietic cells, higher expression of VLA-5 during cell replication in ex vivo conditions subsequently may limit the intramedullary migrating ability and hence the capacity to lodge to specific niches in the BM microenvironment.

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