Rapid Communication

Cytokine Regulation of Proliferation and Cell Adhesion Are Correlated Events in Human CD34+ Hemopoietic Progenitors

By Jean-Pierre Lèvesque, David N. Haylock, and Paul J. Simmons

Adhesive interactions with the extracellular matrix of the bone marrow (BM) stroma are of critical importance in the regulation of hematopoiesis. In part, these interactions are presumed to play an important role in retaining CD34+ hematopoietic progenitor cells (HPCs) within the BM environment, in close proximity with BM stromal cells and the cytokines they produce. Evidence of a more direct role for cell adhesion in the regulation of hematopoiesis is provided by recent data showing that adhesive interactions can also provide important costimulatory signals. We have previously shown that normal CD34+ HPCs express high levels of fibronectin (Fn) receptors very late antigen-4 (VLA-4) and VLA-5 in a low-affinity state, which do not allow HPCs to strongly adhere to immobilized Fn, and that cytokines such as interleukin-3, granulocyte-monocyte colony-stimulating factor, and stem cell factor transiently activate these receptors, providing HPCs with an adhesive phenotype on Fn. Thus, knowledge of the functional states of adhesion receptors is critical to our understanding of the physiological mechanisms responsible for the regulation of normal hematopoiesis. Herein, we show that combinations of cytokines that synergize to stimulate the proliferation of CD34+ HPCs result in additive stimulation of the adhesion of these cells to Fn. Thus, the activation level of Fn receptors expressed by normal CD34+ HPCs is highly correlated with their proliferative state, suggesting a functional link between these two events. Therefore, we propose a 2-step model with an initial activation of VLA-4 and VLA-5 generated by cytokine receptors that is followed by a secondary signal resulting from Fn binding to VLA-4 and VLA-5, which may cooperate with those generated by cytokine receptors.

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In normal adults, hematopoietic progenitor cells (HPCs) reside in the bone marrow (BM), where their proliferation and differentiation are very finely regulated. They are in an intimate contact with the BM stroma. The various cellular elements of the stroma, together with their associated biosynthetic products including extracellular matrix (ECM) components and cytokines, constitute the hematopoietic microenvironment of the BM. Interactions mediated by at least two classes of molecules contribute to maintenance of steady-state hematopoiesis: (1) cytokines, an increasing number of which have been shown to exist as integral membrane glycoproteins on BM stromal cells or physically bound to stromal cell-derived ECM molecules such as proteoglycans, and (2) cell adhesion molecules (CAMs), which, through binding to their cognate ligands, support the physical association of HPCs with the BM stroma. CAMs include integrins, selectins, proteoglycans such as CD44 and Ig superfamilies molecules such as CD54.

Integrins belong to a superfamilly of receptors involved in cell-cell contacts and in cell adhesion on ECM proteins.

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They are heterodimers of one α chain noncovalently linked with a β chain. Among 15 identified α chains and 8 β chains, the specific pairing of a particular α chain with a β chain defines a unique integrin receptor with a unique repertoire of ligand specificity, cell distribution, and regulation. These receptors are involved in processes as diverse as cell attachment and homing, cell migration, and T- and B-cell activation.

In the human hematopoietic system, CD34+ HPCs mainly express the β1 integrins α2β1 (very late antigen-2 [VLA-2], CD49b/CD29), α6β1 (VLA-4, CD49d/CD29), αβ1 (VLA-5, CD49e/CD29), and αβ1 (VLA-6, CD49f/CD29). Although VLA-4 and VLA-5 are both receptors for fibronectin (Fn), VLA-4 has a second ligand, the counterreceptor vascular cell adhesion molecule-1, which is expressed by BM stromal cells. Several lines of evidence suggest their involvement in the control of hematopoietic progenitor cell proliferation. Indeed, Miyake et al have shown in long-term BM cultures that blocking anti-α5 antibodies completely abrogate lymphopoiesis and severely reduce myelopoiesis. In mice, blocking anti-β1-chain antibodies reduce in vivo colony formation in the spleen and medullar hematopoiesis from allogeneic HPCs. Moreover, in cultures of purified CD34+ progenitors, Fn has been shown to stimulate, in cooperation with interleukin-3 (IL-3), the formation of colonies derived from colony-forming unit-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), burst-forming unit-erythroid (BFU-E), CFU-erythroid (CFU-E), and CFU-macrophage (CFU-M), an effect reversed by Arg-Gly-Asp-Ser-containing peptides that block Fn/VLA-5 interaction.

Most of the integrins expressed on mature blood cells such as β1 integrins and the platelet fibrinogen receptor αIIbβ3 are expressed in a nonactive, nonligand-binding form. We have recently shown that VLA-4 and VLA-5 expressed by normal CD34+ HPCs and cytokine-dependent myeloid cell lines MO7e and TF1 are in a low-affinity state unable to strongly adhere to Fn, and that cytokines such as IL-3, granulocyte-macrophage CSF (GM-CSF), and stem
cell factor (SCF; KIT ligand/Steel factor) are transient and selective activators of both VLA-4 and VLA-5. Short exposure to these cytokines dramatically increases VLA-4 and VLA-5 avidity to Fn, thus conferring on these cells a transient adherent phenotype to Fn. To further investigate the possible interrelationship between the mitogenic response of HPCs to cytokines and the activation of $\beta_2$ integrins VLA-4 and VLA-5, we have studied adhensive and proliferative responses of cytokine-independent and cytokine-dependent cell lines and of normal CD34+ HPCs to various combinations of mitogenic cytokines. Herein, we show that the activation state of Fn receptors expressed by these cells is strongly correlated with their proliferative state. By using normal CD34+ HPCs, combinations of cytokines with the greatest capacity to promote proliferation were the most efficient in stimulating their adhesion to Fn. Therefore, we propose a 2-step model with an initial activation of VLA-4 and VLA-5 via an “inside-out” mechanism generated by cytokine receptors that is followed by the production of a secondary “outside-in” signal resulting from VLA/Fn binding that may synergize with those generated by cytokine receptors.

MATERIALS AND METHODS

Chemicals and antibodies. Recombinant human erythropoietin (Epo), IL-1$\beta$, IL-3, IL-6, GM-CSF, granulocyte-CSF (G-CSF), and SCF were kindly provided by Amgen Biologicals (Thousands Oaks, CA). Human plasma Fn was purchased from Boehringer Mannheim (Mannheim, Germany). Mononclonal antibody (MoAb) 49-1B11 was made available by Dr J. Gamble (Hansson Centre for Cancer Research [HCCR], Adelaide, Australia). MoAbs directed to IL-3 receptor $\alpha$ chain (CD123), 9F5, to GM-CSF receptor $\alpha$ chain (CD116), 8G6, and to their common $\beta_2$ chain, 8B8, were kindly provided by Dr A. Lopez (HCCR). YB5.B8, an MoAb specific for KIT receptor (CD117) was kindly provided by Dr L. Ashman (HCCR). P3G8, recognizing the $\alpha$ integrin chain (CD51), P5D2, recognizing the $\beta_1$ chain (CD29), and P4C2, a blocking anti-VLA-4 MoAbs, were generously provided by Dr E. Wayner (University of Minnesota Medical School, St Paul, MN). The activating anti-$\beta_2$ integrin chain MoAb 8A2 was a generous gift of Drs N. Kovach and J. Harlan (University of Washington, Seattle, WA). PHM2, a blocking anti-VLA-5 MoAb, was a gift from Prof R.A. Aitkins (Monash Medical Centre, Melbourne, Australia). Fluorescein isothiocyanate-conjugated sheep F(ab); fragments directed towards mouse Ig were purchased from Silenus Laboratories Pty, Ltd (Hawthorn, Australia).

Normal BM CD34+ progenitor cells. BM was collected from normal adult volunteers under a program approved by the Human Ethics Committee of the Royal Adelaide Hospital. The purification procedure has been previously described. Before adhesion assays, purified CD34+ HPCs were resuspended at 2 × 10$^5$ cells/mL and starved overnight at 37°C in a serum-free medium consisting of Iscove’s modified Dulbecc’s medium (IMDM) supplemented with 10 mg/mL bovine serum albumin (BSA) (no. A2153; Sigma Chemical Co, St Louis, MO), 200 $\mu$g/mL human transferrin (no. T2158; Sigma Chemical Co), 10 $\mu$g/mL insulin, 10 $\mu$g/mL low-density lipoproteins, and 10$^{-2}$ mol/L 2-mercaptoethanol without the addition of cytokine.

Cell lines. The erythroleukemia-derived cell line TF1 was routinely grown in RPMI 1640 medium supplemented with 10% non-heat-denatured fetal calf serum (FCS), 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 12 $\mu$g/mL penicillin, 16 $\mu$g/mL gentamicin, and 2 ng/mL GM-CSF. The UT7 cell line$^9$ was routinely grown in IMDM supplemented with 10% FCS, 2 mmol/L L-glutamine, penicillin, gentamicin, and 2 ng/mL GM-CSF. The erythroblastic leukemia cell lines HEL$^{23}$ and K562,$^{24}$ the myeloid cell line KG1a,$^{25}$ and the granulomonocytic cell line HL60$^{26}$ were cultured without added cytokine in RPMI 1640 medium supplemented with 10% heat-denatured FCS, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, penicillin, and gentamicin. To minimize the effects of endogenous cytokines, cells were washed once in their respective culture medium without growth factor, resuspended to 4 × 10$^5$ cells/mL, and incubated overnight at 37°C before experimentation.

Adhesion assays. The 96-well tissue-culture–treated plates (Nunc, Roskilde, Denmark) were incubated overnight at 4°C with 40 $\mu$L/well of phosphate-buffered saline containing 50 $\mu$g/mL of Fn. The coating solution was removed by aspiration, replaced with 100 $\mu$L of RPMI 1640 containing 10 mmol/L HEPES and 2% BSA (Commonwealth Serum Laboratories, Parkville, Victoria, Australia), and incubated for a further 2 hours at 37°C. After this blocking step, plates were washed 3 times using RPMI 1640 containing 10 mmol/L HEPES and 0.2% BSA, referred to as cell adhesion medium. At this stage, plates were chilled on ice, and the adhesion assays were performed within 20 minutes.

Cells starved overnight without growth factor were harvested, washed twice, and resuspended in 500 $\mu$L cell adhesion medium. A total of 50 to 100 $\mu$Ci of Na$^{14}$CO$_3$ (New England Nuclear, Boston, MA) was added, and cells were incubated for 1 hour at 37°C. After radiolabeling, cells were washed 3 times using adhesion assay medium, resuspended to 1 to 3 × 10$^5$ cells/mL, and chilled on ice for 10 minutes before assay. The labeled cell suspension (100 $\mu$L) was placed in triplicate into Fn-coated wells. Growth factors were added at the specified concentrations. The entire procedure was performed on ice. Plates were centrifuged at 1,000 rpm for 5 minutes at 4°C to sediment cells into direct, uniform contact with treated surfaces. Plates were quickly warmed for 2 minutes to 37°C using a heating block before transfer to a humidified incubator at 37°C for 30 minutes. Assay medium was removed by aspiration, and the cells were washed 3 times by addition of 150 $\mu$L of the adhesion assay medium and vigorous flicking-off. After the last wash, cell adhesion and shape were examined using an inverted-contrast microscope before lysing in 150 $\mu$L 1% sodium dodecyl sulfate and 0.1 mol/L NaOH solution. Lysates were counted after 10 minutes using a $\gamma$ counter. Nonspecific cell adhesion was determined in wells treated with BSA only. The percentage of adherent cells was determined by dividing the radioactivity in the adherent fraction by the radioactivity contained in 100 $\mu$L of the initial labeled cell suspension.

When adhesion assays were performed using CD34+ HPCs before the culture of adherent cells, the procedure was modified from that previously described by omitting the labeling step with Na$^{14}$CO$_3$ and performing all manipulations under sterile conditions, thereby facilitating subsequent colony assays of HPCs. Before performing adhesion assays, CD34+ HPCs were starved overnight at 37°C in the previously serum-free medium described above. This step is necessary to eliminate the “noise” caused by transduction signals generated by interactions between Fn and cytokines contained in FCS with their respective receptors. This starvation step does not alter the viability and the proliferative response of colony-forming cells (CFCs) and very primitive progenitors such as pre-CFU (unpublished data). Then, HPCs were washed twice in adhesion assay medium and were resuspended in this medium at 10$^5$ cells/mL. Cells were chilled on ice for 10 minutes, and 100 $\mu$L of the cell suspension was placed in each Fn-coated well before the addition of the specified cytokines to give a final concentration of 10 ng/mL each. The entire procedure was performed on ice. Plates were spun down for 5 min-

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utes at 1,000 rpm at 4°C and were then incubated for 30 minutes at 37°C to permit adhesion of cells. Plates were then washed 3 times in adhesion assay medium. The efficiency of the washes was verified by observing the presence of residual cells in BSA-coated wells on an inverted microscope. Adherent cells were detached by gentle pipetting after a 2-hour incubation at 37°C in 50 μL of IMDM supplemented with 10% FCS and 50 Kunitz U/mL DNAse (no. DS025: Sigma Chemical Co). Again, the efficacy of detachment was visually verified on an inverted microscope. Resuspended cells were then dispersed in 1 mL of colony assay medium in the presence of IL-1β, IL-3, IL-6, GM-CSF, G-CSF, and SCF (10 ng/mL each) and 4 U/mL Epo. In parallel, 100 μL of the original cell suspension was directly placed into 1 mL colony assay medium to measure the number of CFUs and the different committed progenitors in the input. After 14 days at 37°C in humidified atmosphere containing 5% CO₂, colonies were scored according to the standard criteria. All assays were performed in triplicate.

**CD34⁺ HPC proliferation assays.** After an overnight starvation in serum-free medium, CD34⁺ HPCs were washed twice in serum-free medium and resuspended at 10⁴ cells/mL. Cultures (100 μL) were established in duplicate in 96-well plates (Nunc) precoated with Fn and in the presence of the indicated cytokines, each at 10 ng/mL. After an overnight incubation at 37°C without cytokine, the number of CFUs was determined by scoring the three plates from each triplicate well from the original liquid culture.

**Antigen analysis by flow cytometry.** Cytokine-dependent cell lines were labeled after an overnight incubation at 37°C without cytokine as described above, whereas cytokine-independent cell lines were labeled without this preliminary step because they were routinely cultured in absence of cytokine. The entire procedure for antigen labeling and analysis has already been described. All the primary antibodies used in this study were mouse IgG1. Antibody 49.1B11, a specific anti-E-selectin (CD62E), was used as an isotypic control since this antigen is not expressed on hematopoietic cells.

**Statistical analysis.** Linear and exponential regressions, determinations of correlation coefficients, and significances were calculated with a Statview Student software (Abacus Concept Inc, Berkeley, CA) running on an Apple PowerBook Computer (Macintosh, Cupertino, CA).

**RESULTS**

Cytokine-independent cell lines are spontaneously adherent to Fn, whereas cytokine-dependent ones are induced by cytokines to adhere. As a prelude to this study, we used myeloid cell lines as a model system, to determine whether there is a correlation between the capacity of a given cytokine to stimulate cell proliferation and its ability to activate containing 5% CO₂, cell proliferation was quantified by using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, 15 μL of MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) reagent was added in each well, and the plates were then incubated at 37°C for 4 hours. Solubilization solution (100 μL) was added to each well, and the plates were left overnight at room temperature to facilitate solubilization of newly formed formazan salts. The absorbance at 570 nm was measured on an enzyme-linked immunosorbent assay plate reader with a reference at 650 nm as specified by the manufacturer.

**Pre-CFU assays.** This procedure has been already described. Briefly, sorted CD34⁺ cells were resuspended at 10⁵ cells/mL in pre-CFU medium (IMDM supplemented with 30% heat-denatured FCS, 1% BSA, 3 mmol/L l-glutamine, and 5 × 10⁻³ mol/L 2-mercaptoethanol). One-milliliter cultures were established in triplicate in 24-well plates (Nunc) in the presence of 10 ng/mL of the indicated cytokines alone or in combination. After 7 days at 37°C in 5% CO₂, the content of each well was resuspended and washed to remove residual cytokines. Ten percent of the washed cells were then plated in triplicate in 35-mm Petri dishes in 1 mL IMDM supplemented with 0.9% methyl cellulose, 30% FCS, 3 mmol/L l-glutamine, 50 μL 5637-conditioned medium, 1 ng/mL IL-3, and 4 U/mL Epo. After 14 days of culture at 37°C in 5% CO₂, the number of CFU-GM generated was determined by scoring the three plates from each triplicate well from the original liquid culture.

**Table 1. Expression of β1 Integrins and Cytokine Receptor on Myeloid Cell Lines**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Antigens</th>
<th>HEL</th>
<th>K562</th>
<th>KG1a</th>
<th>HL60</th>
<th>TF1</th>
<th>UT7</th>
<th>M07e</th>
</tr>
</thead>
<tbody>
<tr>
<td>49.1B11</td>
<td>E-selectin</td>
<td>0.317</td>
<td>0.407</td>
<td>0.385</td>
<td>0.481</td>
<td>0.303</td>
<td>0.203</td>
<td>0.677</td>
</tr>
<tr>
<td>YB5.88</td>
<td>KIT/CD117</td>
<td>0.872</td>
<td>0.396</td>
<td>0.386</td>
<td>0.482</td>
<td>3.136</td>
<td>2.162</td>
<td>16.48</td>
</tr>
<tr>
<td>3D7</td>
<td>β1</td>
<td>0.426</td>
<td>0.404</td>
<td>0.337</td>
<td>1.079</td>
<td>5.194</td>
<td>0.395</td>
<td>0.949</td>
</tr>
<tr>
<td>9F5</td>
<td>IL-3Rα</td>
<td>0.353</td>
<td>0.375</td>
<td>2.582</td>
<td>1.114</td>
<td>1.336</td>
<td>0.331</td>
<td>1.089</td>
</tr>
<tr>
<td>8G6</td>
<td>GM-CSFRα</td>
<td>0.317</td>
<td>0.380</td>
<td>1.008</td>
<td>2.022</td>
<td>3.674</td>
<td>2.162</td>
<td>1.291</td>
</tr>
<tr>
<td>P4C2</td>
<td>VLA-4</td>
<td>12.70</td>
<td>0.585</td>
<td>20.47</td>
<td>37.42</td>
<td>6.365</td>
<td>11.55</td>
<td>5.533</td>
</tr>
<tr>
<td>PHM2</td>
<td>VLA-5</td>
<td>25.48</td>
<td>13.63</td>
<td>36.43</td>
<td>53.89</td>
<td>4.707</td>
<td>6.426</td>
<td>11.77</td>
</tr>
<tr>
<td>P5D2</td>
<td>CD29</td>
<td>44.87</td>
<td>13.99</td>
<td>81.96</td>
<td>53.77</td>
<td>12.26</td>
<td>15.50</td>
<td>21.10</td>
</tr>
</tbody>
</table>

Results are expressed as mean fluorescence intensities. 49.1B11 was used as an isotype-matched negative control because E-selectin is not expressed in the hematopoietic system.

Abbreviations: IL-3Rα, IL-3 receptor α chain; GM-CSFRα, GM-CSF receptor α chain.
the adhesive properties of Fn receptors VLA-4 and VLA-5. TF1, UT7, and M07e cells require a cytokine such as IL-3, GM-CSF, or SCF to proliferate, whereas HEL, KG1a, K562, and HL60 cell lines spontaneously divide without exoge-

Fig 2. Effect of cytokines alone or in combinations on the short-term proliferation of CD34+ HPCs and their adhesion to Fn. (A) Adhesion assay. CD34+ HPCs, after an overnight starvation in serum-free medium, were labeled with 51Cr and then incubated for 30 minutes at 37°C in contact with immobilized Fn in the presence of indicated cytokines at 10 ng/mL each. Adhesion was measured as described in Materials and Methods. (B) Proliferation assay. After an overnight starvation in serum-free medium, 10⁵ CD34+ HPCs per milliliter were cultured for 3 days in Fn-coated microwells in the presence of the indicated cytokines at 10 ng/mL each. Cell proliferation was then measured by absorbance at 570 nm after reduction of MTT reagent by proliferating cells (see Materials and Methods). (C) Correlation between cytokine induction of CD34+ HPC proliferation and adhesion to Fn. Results of (A) in ordinate and of (B) in abscissa were plotted for each of the 31 cytokine combinations. The optimal linear regression was determined by the least-square method.
were adherent to Fn (Fig 1), reflecting the high level of VLA-4 and VLA-5 receptors expressed on these cell lines (Table 1). However, whereas cytokine-dependent cell lines TF1, UT7, and MO7e could be significantly stimulated by IL-3, GM-CSF, or SCF to adhere to Fn, high proportions (50% to 90%) of the factor-independent cell lines HEL, KG1a, K562 and HL60 were spontaneously adherent to Fn. Moreover, the proportion of cells adhering to Fn was not altered by the addition of these cytokines (Fig 1). To determine whether the absence of stimulation of these cytokine-independent cells could be because of the absence of cytokine receptors, receptor expression was analyzed by flow cytometry. HEL, KG1a, and HL60 cells expressed at their surface the receptor for at least one cytokine, whereas K562 did not express IL-3, GM-CSF, or KIT receptors (Table 1).

To control for the specificity of cell adhesion in these experiments, cytokine-independent cells were preincubated in the presence of P4C2, a blocking anti-VLA-4 MoAb, and PHM2, a blocking anti-VLA-5 MoAb, either alone or in combination. Inhibition of VLA-5 by PHM2 was sufficient to abolish the spontaneous adhesion of K562 and KG1a cells to Fn; however, inhibition of both VLA-4 and VLA-5 was required to obtain the same effect on basal or augmented adhesion of HEL, HL60, TF1, UT7, and MO7e cells (data not shown).

Cytokines that stimulate adhesion of CD34+ HPCs to Fn also stimulate their proliferation to the same extent. Because it appeared from the previous experiments that the level of the avidity for Fn of VLA-4 and VLA-5 expressed by transformed myeloid cell lines was dependent on the proliferative state of these cells, we tested the effect of 31 different combinations of six cytokines on both the proliferation of normal CD34+ HPCs and the activation of their adhesion to Fn. Individual cytokines did not maximally stimulate either HPC adhesion to Fn (Fig 2A) or proliferation (Fig 2B). On the other hand, the combinations of cytokines that synergized to maximally increase HPC adhesion (up to 50.7% ± 1.8% of the input) were those that were most efficient at stimulating HPC proliferation after 3 days in culture. The similar patterns of these two previous histograms (Fig 2A and 2B) prompted us to plot the proliferation of CD34+ progenitor cells versus the percentage of adherent...
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HPCs in response to a 30-minute stimulation by these same 31 combinations of cytokines (Fig 2C). The resulting correlation coefficient between these two series of data was equal to 0.926, with a high level of significance ($F = 180.23; P < .0001; df = 31$).

In another set of experiments, we had analyzed the effect of these 31 combinations of cytokines on the formation of CFU-GM in a pre-CFU assay (Fig 3A). Again, we found that the induction of CFU-GM production in a 21-day pre-CFU assay was significantly correlated with the activation of HPC adhesion to Fn ($r = .901; P < .0001; df = 31$; see Fig 3B).

Cytokines stimulate adhesion of CFCs to Fn. Because CD34+ HPCs were stimulated by mitogenic cytokines to adhere to Fn, we examined whether the adherent subsets contained CFCs. In this experiment, purified CD34+ HPCs were incubated with the indicated cytokines to adhere to Fn as described in Materials and Methods. Then, adherent cells were harvested and cultured for 14 days in a colony assay in the presence of Epo, IL-1β, IL-3, IL-6, GM-CSF, G-CSF, and SCF, which supported an optimal development of CFCs. As shown in Fig 4A, CFCs were present within the population of CD34+ HPCs stimulated to adhere to Fn by exposure to cytokines. In accord with the previous observations, single cytokines were relatively poor in their ability to stimulate adhesion of CFCs as compared with that for the combinations of IL-1/IL-3/SCF or the six cytokines together.

As observed in the previous experiments in which the percentage of adherent CD34+ HPCs was evaluated (Fig 2A), the most active single cytokines to stimulate the adhesion of the total pool of CFCs to Fn were IL-3, GM-CSF, G-CSF, or SCF alone, whereas IL-1β or IL-6 alone showed little or no activity (Fig 4A). A maximal adhesion of CFCs was obtained with the combinations of IL-1 plus IL-3 and SCF or with the six cytokines together (Fig 4A), two combinations of cytokines that had proven in the previous experiments (Fig 2A) to strongly enhance CD34+ HPC adhesion to Fn. Again, these similarities of trends prompted us to plot the percentage of adherent CD34+ HPCs versus the percentage of adherent CFCs in response to these nine combinations of cytokines. The correlation coefficient was equal to 0.979 and was highly significant ($F = 160.32; P < .0001; df = 8$). Moreover, a slope coefficient close to 1 suggested that the majority of CD34+ HPCs induced to adhere by cytokines were CFCs.

DISCUSSION

A large body of data supports the idea of considerable interdependence, functional overlap, and similarity between hematopoietic growth factors and CAMs. For instance, hematopoietic growth factors can influence ECM biosynthesis and degradation, whereas cell adhesion has been shown to induce the production of cytokines in many cell types. In addition, many cytokines are bound in a biologically active state by ECM proteins that allow them to mediate HPC adhesion. Moreover, some cytokines, notably macrophage-CSF and SCF/KIT ligand/Steel factor (SCF) can exist in transmembrane isoforms that promote cell attachment to cells expressing their respective receptors FMS and KIT. Finally, we have recently shown that cytokines such as IL-3, GM-CSF, and SCF can modulate the activation state of the adhesion receptors VLA-4 and VLA-5 expressed by normal CD34+ HPCs and by cytokine-dependent cell lines TFI and MO7e, allowing the physiological transition of these two receptors from an inactive, nonbinding state to a high-affinity, Fn-binding state.

During the course of this previous study, it appeared that cytokines which regulated avidity of integrins VLA-4 and VLA-5 were those that most efficiently promoted the proliferation of cytokine-dependent cell lines and, at least, of some subsets of normal CD34+ HPCs. In this report, we show that there is a high and significant correlation between the mitogenic state of normal and transformed hematopoietic cells and the avidity for Fn of VLA-4 and VLA-5 expressed by these cells. Indeed, cell lines that proliferated spontaneously in the absence of cytokine, such as HEL, KG1a, K562.
and HL60, showed high constitutive adhesion to Fn via activated VLA-4 and/or VLA-5. Moreover, the addition of IL-3, GM-CSF, or SCF, which did not increase the proliferation of these cells, also had no significant effect on their adhesion. Conversely, in the absence of these cytokines, TF1, M07e, and UT7 cell lines did not proliferate and adhered poorly to Fn, whereas the addition of these cytokines promoted both cell division and a transient and strong cell adhesion to Fn mediated by both VLA-4 and VLA-5.

In studies using normal CD34+ HPCs, we confirm and extend our previous observations regarding the capacity of cytokines to stimulate VLA-4/VLA-5-dependent adhesion of these cells to Fn. Single cytokines such as IL-3, GM-CSF, G-CSF, and in particular SCF were active in stimulating adhesion to Fn, whereas others such as IL-1β and IL-6 showed no such capacity. The latter two cytokines are regarded as synergistic factors whose effects on HPC proliferation are observed only in combination with other cytokines, an observation confirmed by the colorimetric 3-day proliferation assay used in the current study. Although IL-3, GM-CSF, G-CSF, or SCF used alone was able to promote HPC adhesion to Fn, SCF presented the unique property of activating cell adhesion to much a greater degree than proliferation. A similar observation was previously reported by Kinashi and Springer who showed that this cytokine is able to activate mast cell adhesion to Fn at 100-fold lower concentrations than those required for growth stimulation.

We have further found that combinations of cytokines that synergized to promote HPC entry into cell cycle also synergized to promote VLA-4/VLA-5-dependent CD34+ HPC adhesion to Fn to the same extent (Fig 2C). The high and significant correlation between these two assays suggests a functional link between activation of CD34+ HPC proliferation and adhesion to Fn. This correlation remains very high between integrin activation and the de novo generation of CFU-GM precursors from very primitive HPCs as determined by the pre-CFU assay (Fig 3B). This concept of a functional link between activation of proliferation and adhesion to Fn in CD34+ HPCs is reinforced by the fact that the HPCs induced to adhere to Fn by cytokines were CFCs (Fig 4B).

In this report, we have shown a strong correlation between the capacity of a particular combination of cytokines to induce immature HPC proliferation and the capability to activate their Fn receptors VLA-4 and VLA-5. These data leave unanswered the crucial question: Why are βintegrins VLA-4 and VLA-5 activated by cytokine combinations in a manner that so closely reflects their ability to activate proliferation? Future studies will be necessary to determine the molecular basis and the biological function underlying these correlations.

We have previously shown that this activation step, or “inside-out” signaling, requires functional protein kinases activated by cytokine receptors. Stauroporin, a broad protein kinase inhibitor, was able to completely abolish cytokine activation of Fn receptors, whereas genistein, a tyrosine kinase inhibitor, prevented βintegrin activation by SCF but not by IL-3 or GM-CSF. Reports by other groups have shown that, on mast cells, the activation of Fn receptors by SCF was partially dependent on phosphatidyl inositol 3-(OH) kinase binding and activation by the KIT receptor. Conversely, by using nonhematopoietic systems, the results of a growing number of studies have shown that integrins are signaling molecules, triggering transducers that are also targets of mitogenic growth factor receptors (“outside-in” signaling). For instance, the Na+/H+ antiporter and mitogen-activated protein kinases have been shown to be activated by βintegrins. Furthermore focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase activated by integrins, directly associates with and activates phosphatidylinositol 3-(OH) kinase and the adaptor Grb2, which in turn leads to the activation of Ras and Raf-1/mitogen-activated protein kinase pathways. Moreover, adhesion to Fn leads to the accumulation of phosphatidylinositol 4.5 biphosphate that plays a role in cytoskeleton regulation and is the substrate for phospholipase Cγ, which in turn generates the second messengers diacylglycerol and inositol triphosphate. Finally, Symington has shown in a subclone of K562 cells expressing very high levels of VLA-5 that Gly-Arg-Gly-Asp-Ser, a peptide ligand for VLA-5, specifically stimulates p34cdc2-associated and cyclin A-associated kinase activities suggesting that integrins link the extracellular

**Fig 5.** A 2-step model with initial activation of βintegrins by an “inside-out” signal generated by cytokine receptors that is followed by a secondary “outside-in” signal resulting from the integrin-ligand binding that cooperates with those generated by cytokine receptors is shown.
environment and intracellular growth regulators. Alternatively, β integrins could trigger survival signaling necessary for the completion of the cell cycle previously initiated by cytokine binding. Indeed, it has been recently shown that VLA-5 can support cell survival by upregulating Bcl-2 expression. Therefore, we propose a 2-step model involving the initial activation of β integrins by an “inside-out” signal generated by cytokine receptors that is followed by a secondary “outside-in” signal resulting from the integrin-ligand binding and integrin aggregation that may synergize with and/or complete those generated by cytokine receptors, as modeled in Fig 5.

Finally, we would like to emphasize the clinical importance of this work, particularly with reference to the design of clinical protocols for stem cell mobilization from the BM. A growing number of cytokines, including SCF, IL-3, GM-CSF, and G-CSF, are used in clinical protocols to promote stem cell peripheralization. Previous studies have emphasized the importance of β integrin-mediated adhesion in HPC mobilization. Indeed, it has recently been shown that in vivo administration of anti-VLA-4 antibodies in baboons promotes a rapid and dramatic release of HPCs from the BM into the peripheral blood. More recently, Kovach et al have shown, in agreement with our previously published data, that in MO7e cells, high concentrations of SCF induce a biphasic response of VLA-4 and VLA-5 consisting of their short and transient activation followed by their inactivation. In this study, we have found that the cytokines shown to be good mobilizers when used alone, such as G-CSF and SCF, are also those that modulate most efficiently CD34+ HPC adhesion to Fn. In addition, we have found that combinations of cytokines are much more potent modulators of VLA-4 and VLA-5. These data predict that these combinations might be extremely effective in promoting HPC mobilization into peripheral blood.

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