

Cytokines Increase Human Hemopoietic Cell Adhesiveness by Activation of Very Late Antigen (VLA)-4 and VLA-5 Integrins

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Summary

Cytokines are known to be important regulators of normal hemopoiesis, acting in concert with components of the bone marrow microenvironment. Interactions with this microenvironment are known to regulate the proliferation, differentiation, and homing of hemopoietic progenitor (CD34⁺) cells. Adhesive interactions with the extracellular matrix retain CD34⁺ cells in close proximity to cytokines, but may also provide important costimulatory signals. Thus, the functional states of adhesion receptors are critical properties of CD34⁺ cells, but the physiological mechanisms responsible for regulating functional properties of cell adhesion receptors on primitive hemopoietic cells are still unknown. We confirm that the integrins very late antigen (VLA)-4 and VLA-5 are expressed on the CD34⁺ cell lines MO7e, TF1, and on normal bone marrow CD34⁺ progenitor cells, but in a low affinity state, conferring on them a weak adhesive phenotype on fibronectin (Fn). Herein, we show that the cytokines interleukin (IL)-3, granulocyte-macrophage CSF (GM-CSF), and KIT ligand (KL) are physiological activators of VLA-4 and VLA-5 expressed by MO7e, TF1, and normal bone marrow CD34⁺ progenitor cells. Cytokine-stimulated adhesion on Fn is dose dependent and transient, reaching a maximum between 15 and 30 min and returning to basal levels after 2 h. This cytokine-dependent activation is specific for VLA-4 and VLA-5, since activation of other β_1 integrins was not observed. The addition of second messenger antagonists staurosporine and W7 abolished all cytokine-stimulated adhesion to Fn. In contrast, genistein inhibited KL-stimulated adhesion, but failed to inhibit GM-CSF- and IL-3-stimulated adhesion. Our data suggest that cytokines GM-CSF and IL-3 specifically stimulate β_1 integrin function via an "inside-out" mechanism involving protein kinase activity, while KL stimulates integrin activity through a similar, but initially distinct, pathway via the KIT tyrosine-kinase. Thus, in addition to promoting the survival, proliferation, and development of hemopoietic progenitors, cytokines also regulate adhesive interactions between progenitor cells and the bone marrow microenvironment by modifying the functional states of specific integrins. These data are of importance in understanding the fundamental processes of β_1 integrin activation and cellular response to mitogenic cytokines, as well as on the clinical setting where cytokines induce therapeutic mobilization of hematopoietic progenitors.

In adults, normal hemopoiesis takes place in the bone marrow, where hemopoietic progenitors are retained in intimate contact with the stroma. The bone marrow stroma constitutes a microenvironmental niche where specific growth factors and inhibitors regulate quiescence, proliferation, and differentiation of hemopoietic stem cells (1-3). Stroma-free liquid and semisolid cultures have identified some of the various functions that growth factors (cytokines) and growth inhibitors induce in the progenitor population. Some of these cytokines, such as IL-1, IL-3, IL-6, and GM-CSF, are active

in soluble form, whereas others, such as CSF-1 and KIT ligand (KL)¹ (stem cell factor) can act in soluble or membrane-bound forms (for review, see reference 4).

Long-term culture of bone marrow progenitors on preestab-

¹ Abbreviations used in this paper: ECM, extracellular matrix proteins; Fn, fibronectin; HCCR, Hanson Centre for Cancer Research; HPCA-2-PE, PE-conjugated anti-CD34 mAb HPCA-2; KL, KIT ligand; VLA, very late antigen.

lished stromal monolayers has highlighted the importance of interactions between progenitor cells, stromal cells, and elements of the extracellular microenvironment. Such interactions are essential to sustain the long-term self-renewal of very primitive progenitors and support their differentiation into committed progenitors. Several studies have shown that the interaction of integrin receptors expressed by hemopoietic progenitors with their stromal ligands, or counterreceptors, is critical for adhesion and homing of myeloid and lymphoid progenitors to the bone marrow (for review, see reference 5). This is particularly the case for β_1 integrin receptors $\alpha_4\beta_1$ (very late antigen [VLA]-4, CD49d/CD29) and $\alpha_5\beta_1$ (VLA-5, CD49e/CD29), which interact with fibronectin (Fn) and vascular cell adhesion molecule-1, two major components of the bone marrow stroma. While VLA-5 is widely expressed, VLA-4 is more restricted to normal B and T cells, thymocytes, monocytes, and hemopoietic progenitors (6–9). Teixidó et al. (10) have shown that 90% of marrow CD34^{hi} progenitor cells express the β_1 integrin chain. These β_1 integrins are mainly comprised of VLA-4 and VLA-5 complexes, since 77% and 49% of CD34^{hi} cells coexpress α_4 and α_5 chains, respectively, with very little expression of α_1 , α_2 , α_3 , and α_6 chains (9, 10). The importance of VLA-4 in hemopoiesis was first demonstrated by Miyake et al. (11), who reported that the addition of anti-VLA-4 mAbs to long-term bone marrow cultures abrogated lymphopoiesis and retarded myelopoiesis. VLA-4-specific antibodies have also been shown to abrogate stroma-dependent erythropoiesis (12). Fn has been shown to increase the proliferation of burst-forming unit-E, CFU-E, and CFU-M, induced by IL-3 and erythropoietin in methyl cellulose cultures of enriched CD34⁺ cells, an effect reversed by Arg-Gly-Asp-Ser-containing peptides which antagonize the VLA-5/Fn interaction (13, 14).

Adhesion-stimulated signaling pathways thus appear to be an important component of the regulation of cell division and differentiation during normal hemopoiesis. Using hemopoietic progenitor cell (CD34⁺) models, we have investigated the interrelationship between cytokine-stimulated pathways and cellular events induced by the interaction of Fn with the β_1 integrins, VLA-4 and VLA-5. In this report, we show that specific cytokines can rapidly and transiently modify VLA-4 and VLA-5 function to promote adhesion on Fn in two growth factor-dependent myeloid cell lines, TF1 and MO7e, and in CD34⁺ progenitors purified from normal donor bone marrow aspirates. Thus, growth factors may regulate cellular events, such as cell adhesion, critical to normal hemopoiesis, in addition to proliferation and differentiation.

Materials and Methods

Antibodies. mAbs 61-2C4 and 49-1B11, identifying the β_1 integrin chain (CD29) and L-selectin (CD62L), respectively, were made available by Dr. J. Gamble (Hanson Centre for Cancer Research [HCCR], Adelaide, Australia). Ig directed to IL-3 receptor α -chain (CD123), 9F5, and GM-CSF receptor α -chain (CDw116), 8G6, were provided by Dr. A. Lopez (HCCR). YB5.B8, an mAb specific for KIT receptor (CD117) (15), was kindly provided by Dr. L. Ashman (HCCR). P4C2, which binds to VLA-4 (CD49d/CD29), blocking adhesive function (16), P3G8, recognizing α_4 integrin

chain (CD51), P1B5, recognizing VLA-3 (CD49c/CD29), and P5D2, recognizing β_1 integrin chain, were generously provided by Dr. E. Wayner (University of Minnesota Medical School, St. Paul, MN). PHM2 (17), a function-blocking mAb directed to VLA-5 (CD49e/CD29), was a gift from Professor R. A. Aitkins (Monash Medical Centre, Melbourne, Australia). The anti- β_1 (CD29) integrin mAb, 8A2, previously shown to activate β_1 integrin function (18), was made available by Drs. J. Harlan and N. Kovach (University of Washington, Seattle, WA). PE-conjugated anti-CD34 mAb HPCA-2 (HPCA-2-PE) was purchased from Becton Dickinson & Co. (Mountain View, CA). TS2/7 recognizing VLA-1 (CD49a/CD29) and AC11, a blocking anti-VLA-2 (CD49b/CD29), was obtained from the American Type Culture Collection (Rockville, MD). Unless noted otherwise, all antibodies in this study were used as affinity-purified Ig. FITC-conjugated sheep F(ab)₂ fragments, directed toward mouse Ig, were purchased from Silenus Laboratories Pty., Ltd. (Hawthorn, Australia).

Chemicals and Cytokines. Recombinant human KL, IL-1, IL-3, IL-6, GM-CSF, and G-CSF were generously provided by Amgen Biologicals (Thousand Oaks, CA). Laminin and type IV collagens were purchased from Collaborative Biomedical Products/Becton Dickinson Labware (Bedford, MA). Human Fn was purchased from Boehringer Mannheim (Mannheim, Germany). Vitronectin was a generous gift from Dr. G. Burns (Royal Newcastle Hospital, Newcastle, Australia) and rat tail collagen I was from Dr. P. Kaur (Institute of Medical and Veterinary Science, Adelaide, Australia). Second messenger inhibitors staurosporine, W7, genistein, and calyculin A were purchased from Calbiochem-Novabiochem (San Diego, CA); okadaic acid and cholera toxin were from Sigma Chemical Co. (St. Louis, MO). All culture media were purchased from Life Technologies (Glen Waverley, Australia). BSA was from Commonwealth Serum Laboratories (Parkville, Australia).

Cell Lines. The erythroleukemia-derived cell line TF1 (19) was routinely grown in RPMI 1640 medium supplemented with 10% non-heat-denatured FCS, 10 mM Hepes, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin, gentamycin, and 2 ng/ml GM-CSF. The megakaryocytic leukemia-derived cell line MO7e (20) was routinely grown in DMEM, supplemented with 10% FCS, 10 mM Hepes, 2 mM L-glutamine, penicillin, gentamycin, and 10 ng/ml IL-3. Both cell lines were shown to be mycoplasma-free by using the Mycoplasma TC Rapid Detection System (Gen-Probe Inc., San Diego, CA). 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.

Normal Bone Marrow CD34⁺ Progenitor Cells. Bone marrow was collected from normal adult volunteers under a program approved by the Human Ethics Committee of the Royal Adelaide Hospital. Bone marrow samples were aspirated from donor sternum into heparinized (15 U/ml heparin; Wellcome, Beckenham, UK) tubes. Aspirates were diluted in HBSS-FCS, and light density mononuclear cells were recovered over a density gradient (Lymphoprep; Nycomed Pharmacia, Oslo, Norway). These cells were washed twice with HBSS-FCS, then incubated with HPCA-2-PE (anti-CD34, Becton Dickinson & Co., Mountain View, CA) at 4°C for 45 min. Unbound HPCA-2-PE was removed with two washes, using HBSS-FCS, and the cells were transferred to a flow cytometer (FACStar®; Becton Dickinson & Co.) equipped with an argon laser emitting 488 nm light at 200 mW. CD34⁺ cells within the lymphocyte/blast region (21) were sorted into IMDM, supplemented with 50 Kunitz U/ml DNase (No. D5025, Sigma Chemical Co.), and 20% FCS. A portion of the sorted cells was analyzed immediately to assess purity and viability. Cells were then

washed twice in IMDM, resuspended at 2×10^5 cells/ml, and starved overnight at 37°C in the following serum-free medium: IMDM supplemented with 10 mg/ml BSA (No. A2153; Sigma Chemical Co.), 200 µg/ml iron-saturated human transferrin (No. T2158; Sigma Chemical Co.), 10 µg/ml insulin, 10 µg/ml low density lipoproteins, 10^{-5} M 2-ME.

Adhesion to Extracellular Matrix Proteins. 96-well tissue culture-treated plates (Nunc, Roskilde, Denmark) were incubated overnight at 4°C with 40 µl/well of PBS containing 50 µg/ml of extracellular matrix proteins (ECM): Fn, collagen I, collagen IV, laminin, or vitronectin. The coating solution was removed by aspiration, replaced with 100 µl of RPMI 1640 containing 2% BSA, and incubated for a further 2 h at 37°C. After this blocking step, plates were washed three times using RPMI 1640 containing 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 min.

Cells starved overnight without growth factor were harvested, washed twice, and resuspended in 500 µl cell adhesion medium. 50–100 µCi of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear, Boston, MA) was added, and cells were incubated for 1 h at 37°C. After radiolabeling, cells were washed three times using adhesion assay medium, resuspended to $2\text{--}4 \times 10^5$ cells/ml, and chilled on ice for 10 min before assay. 100 µl of the labeled cell suspension was placed in triplicate into ECM-coated wells. Growth factors were added at the specified concentrations. The entire procedure was carried out on ice. Plates were centrifuged at 1,000 rpm for 5 min at 4°C to sediment cells into direct, uniform contact with treated surfaces. Plates were quickly warmed for 2 min to 37°C, using a heating block before transfer to a humidified (100%) incubator at 37°C for the indicated periods. Assay medium was removed by aspiration and wells were washed three times by addition of 150 µl of the adhesion assay medium and flicking off. After the last wash, cell adhesion and shape were examined using an inverted-contrast microscope before lysing in 150 µl 1% SDS, 1% NaOH solution. Lysates were counted after 10 min, 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 activity in the adherent fraction by the radioactivity contained in 100 µl of the initial labeled cell suspension.

Cytometric Analysis of Antigen Expression. The cell surface expression of adhesion and cytokine receptors was measured on CD34^+ cells starved overnight at 37°C after treatment in suspension with or without 10 ng/ml IL-3, GM-CSF, or KL, as described above. All the following staining procedures were performed on ice with ice-cold solutions. Immediately after the indicated incubation times with cytokines, cells were chilled on ice, pelleted, and resuspended in PBS containing 5% sheep serum and 0.1% NaN_3 . After 1 h incubation, cells were pelleted and resuspended in RPMI 1640, 10% FCS, 0.1% NaN_3 containing equal volumes of hybridoma supernatants or a 1:100 dilution of ascites fluid. After a 1-h incubation, cells were washed twice in PBS, 0.1% NaN_3 and incubated for 1 h in a 1:100 dilution of fluorescein-conjugated sheep F(ab)₂ fragments directed toward mouse Ig in RPMI 1640, 10% FCS, 0.1% NaN_3 . Cells were then washed three times in PBS, 0.1% NaN_3 and fixed in 1% formaldehyde in PBS before analysis on an Epics-Profile II (Coulter Corp., Hialeah, FL). Antibody binding was measured relative to isotype-matched, irrelevant control antibodies.

Results

β₁ Integrins Responsible for Attachment of CD34⁺ Hemopoietic Cells to Fn are in a Low Affinity State. Previous studies

have demonstrated that, despite the high level of expression of β_1 integrins by human CD34^+ progenitor cells, relatively few of these cells spontaneously attach to Fn (9, 22). In the current study, we sought to examine the mechanisms responsible for this apparent discrepancy and further to determine by what means the β_1 integrin-mediated adhesion of CD34^+ cells to Fn could be increased. As a prelude to these investigations, we examined by flow cytometry the expression of β_1 integrins on normal bone marrow CD34^+ progenitor cells and to CD34^+ cytokine-dependent cell lines TF1 and MO7e. In parallel, we measured the adhesion of these cells to immobilized intact human plasma Fn. In accord with previous observations, although VLA-4 and VLA-5 were expressed at high levels by all three populations (Table 1), only 6.1%, 9.9%, and 10.0% of bone marrow CD34^+ progenitor, TF1, and MO7e cells, respectively, demonstrated spontaneous adhesion to Fn (Table 2). However, after treatment with mAb 8A2, an anti-CD29 antibody shown by others to activate the ligand-binding function of β_1 integrins through an "outside-in" mechanism (18, 23), all three populations of CD34^+ cells exhibited markedly increased attachment to Fn (Table 2). Collectively, these data suggest that the majority of β_1 integrins expressed by these cells are in a low affinity state incapable of binding immobilized Fn.

Cytokine-induced Adhesion to Fn is Dose Dependent and Transient. Several studies have shown that integrin-function in CD34^+ progenitor cells can be activated by various stimuli, such as PMA (9) or anti-CD31 polyclonal antibodies (24). Fig. 1 shows that a number of cytokines, including KL, IL-3, and GM-CSF, stimulate adhesion of MO7e and TF1 cells to Fn in a dose-dependent manner. IL-1, IL-6, and G-CSF were without effect over an equivalent range (data not shown). In the case of MO7e cells, half-maximal cytokine-stimulated adhesion to Fn was observed with 0.05 ng/ml GM-CSF, 0.3 mg/ml KL, and 2 ng/ml IL-3, whereas, with TF1 cells, concentrations of 0.03 ng/ml GM-CSF, 1 ng/ml KL, or 3 ng/ml IL-3 were required to stimulate half-maximal adhesion of TF1 cells to Fn. Attachment to the control ligand, BSA (nonspecific binding), remained below 1% under all conditions (data not shown).

In the absence of exogenous cytokine, the basal attachment of MO7e and TF1 cell lines in direct contact with plastic-immobilized Fn was maximal within 15 min incubation at 37°C and remained stable with extended incubation (Fig. 2). 8A2-treated cells bound Fn with similar saturation-binding kinetics and demonstrated stable adhesion over the 2-h time course of the assay. IL-3-, GM-CSF-, and KL-stimulated attachment to Fn was also maximal (three to four times basal levels) within 15–30 min incubation at 37°C, but, in contrast to 8A2-stimulated adhesion, was transient, returning to unstimulated levels after 2 h.

In an attempt to understand the transient nature of cytokine-stimulated attachment to Fn, we measured the expression of β_1 integrin and cytokine receptor expressions on TF1 and MO7e cell lines after a 2-h incubation in the presence of IL-3, GM-CSF, or KL when cell adhesiveness returned to close to basal level (Table 3). The transient nature of this adhesive-

Table 1. Expression of β_1 and α_v Integrins on Starved TF1, MO7e, and Normal Bone Marrow CD34⁺ Progenitor Cells

mAb	Antigens	Cell lines		
		TF1	MO7e	CD34 ⁺ progenitors
49.1B11	E-selectin	0.291	0.304	0.10
TS2/7	VLA-1	0.280	0.270	0.11
AC11	VLA-2	0.299	1.848	3.93
P1B5	VLA-3	0.296	0.309	0.22
P4C2	VLA-4	5.037	4.824	15.6
PHM2	VLA-5	3.238	13.03	11.2
GoH3	VLA-6	0.359	4.954	0.32
61.2C4	CD29	8.666	20.13	29.6
P3G8	CD51	0.800	2.904	0.19

Cells were starved and labeled as described in Materials and Methods. Results are expressed as mean fluorescence intensities.

ness cannot be explained by a drop in the level of VLA-4 or VLA-5 expression, since they remained unaltered after a 2-h incubation in the presence of 10 ng/ml IL-3, GM-CSF, or KL. IL-3 receptor α chains and the common β_c chains of IL-3 and GM-CSF receptors were not downregulated in both cell lines, while GM-CSF receptor α chain was partially downregulated by GM-CSF. Finally, the KIT receptor was strongly downregulated on TF1 cells by KL, but only partially on MO7e cells.

Cytokine-stimulated Adhesion Is Refractory to Autologous, but not Heterologous, Secondary Stimulation. To determine if cytokine-stimulated integrin activity was refractory to a secondary cytokine stimulation, TF1 cells were incubated for 2 h at 37°C in Fn-coated wells in the presence of 10 ng/ml GM-CSF, KL, or without cytokine, and then restimulated by a second addition of cytokine when adhesion had returned to near-basal levels. Cells left for 2 h in the adhesion assay medium without

exogenously added cytokine remained responsive to all the stimuli to the same extent as fresh cells. Cells treated with GM-CSF for 2 h could not be restimulated with either GM-CSF or IL-3 (Fig. 3). These cells remained sensitive, however, to stimulation with KL. Similarly, cells stimulated with KL were refractory to restimulation with KL, but remained sensitive to GM-CSF or IL-3. mAb 8A2 consistently induced maximal Fn attachment, irrespective of any cytokine pre-treatment.

MO7e- and TF1-augmented Adhesion Is Mediated by VLA-4 and VLA-5. The specificity of cytokine-activated adhesion of β_1 integrins was tested for comparison with mAb 8A2, which activates all β_1 integrins (18). We analyzed β_1 integrin-mediated cell adhesion to other ligands, including the ECM proteins, collagen types I and IV (VLA-1, VLA-2, and VLA-3), Fn (VLA-4 and VLA-5), laminin (VLA-6), and vitronectin (α_v integrins) (25). mAb 8A2 promotes MO7e cell adhesion to collagen I, Fn, laminin, and vitronectin (Fig. 4 A). These cells were found to express their corresponding receptors, VLA-2, -4, -5, -6, and α_v integrins (Table 1). In contrast to mAb 8A2, cytokines selectively stimulated VLA-4 and VLA-5 only (Fig. 4 A). The same selectivity was observed with TF1 cells (Fig. 4 B), which only express VLA-4, VLA-5, and α_v integrins (Table 1).

To characterize the receptors mediating cytokine-induced attachment to Fn, integrin-specific, function-blocking antibodies were included in attachment assays. Attachment of MO7e cells to Fn induced by IL3, GM-CSF, and KL was completely inhibited by anti-VLA-5 mAb PHM2, indicating that VLA-5 is the primary Fn-receptor in stimulated cells. mAb P4C2, a function-blocking anti-VLA-4 mAb, only partially blocked MO7e adhesion to Fn (Fig. 5 A). The control mAb AC11, a function blocking anti-VLA-2 antibody, had no effect. The same experiment performed using TF1 cells showed attachment to Fn was only partially inhibited in the presence of PHM2 or P4C2 when used individually. Complete inhibition of cell attachment was observed only when

Table 2. mAb 8A2 Stimulates MO7e, TF1, and Normal Bone Marrow CD34⁺ Hemopoietic Progenitor Cell Attachment to Fn

Cell lines	Stimuli	
	Nil	8A2
MO7e	10.0 ± 1.1	83.6 ± 1.7
	<i>0.6 ± 0.2</i>	<i>1.0 ± 0.1</i>
TF1	9.9 ± 1.2	70.7 ± 1.5
	<i>1.9 ± 0.9</i>	<i>0.5 ± 0.3</i>
Normal CD34 ⁺ progenitors	6.1 ± 1.0	83.1 ± 4.3
	<i>0.6 ± 0.2</i>	<i>0.4 ± 0.1</i>

Cell attachment to immobilized Fn and BSA (*italics*) was measured as described in Materials and Methods after a 20-min incubation at 37°C with or without 1 μ g/ml 8A2 mAb. Data represent the mean ± SEM.

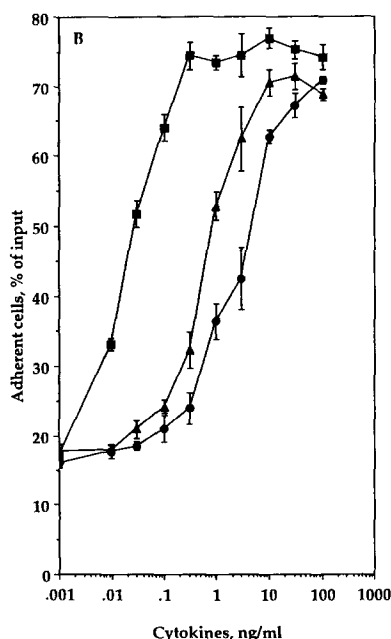
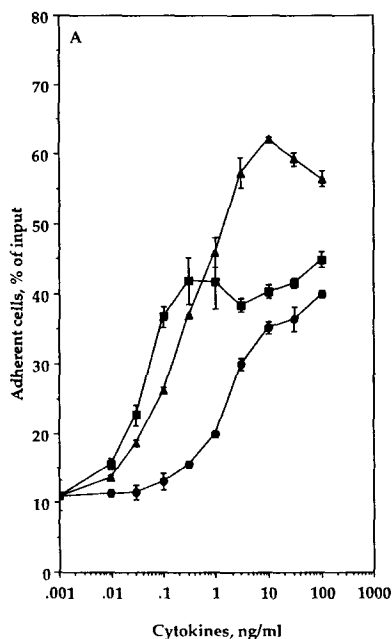


Figure 1. Cytokine-dependent activation of MO7e attachment to Fn is dose dependent. MO7e (A) and TF1 (B) cells were incubated for 20 min at 37°C in the presence of the indicated concentrations of IL3 (●), GM-CSF (■), or KL (▲). Data represent the mean \pm SD of triplicate samples from a representative experiment of four.

both PHM2 and P4C2 were present (Fig. 5 B). The presence of both antibodies together was required to inhibit cell adhesion to Fn induced by mAb 8A2 in all CD34⁺ cells. In another experiment (not shown), we observed that 61.2C4, a function blocking anti-CD29 (β_1 chain) mAb, was also able to block cytokine- and 8A2-induced MO7e and TF1 cell adhesion to Fn. Collectively, these data show that VLA-4 and VLA-5 mediate both “inside-out” and “outside-in” signal-enhanced attachment to Fn.

VLA-4- and VLA-5-mediated Enhanced Adhesion Is Not Due to Changes in Cell Surface Expression. Cytokine-augmented

cell attachment to Fn is rapid, suggesting that VLA-4 and VLA-5 might be mobilized to the cell surface from pre-existing intracellular pools. To address this possibility, we analyzed cell surface expression on MO7e and TF1 cells after a 20-min treatment with 10 ng/ml IL-3, GM-CSF, or KL at 37°C. Table 4 shows that the expression of CD29, VLA-4, VLA-5, and α_n integrins was not dramatically altered by the 20-min cytokine treatments.

Cytokine-augmented Cell Adhesion Is Differentially Inhibited by Inhibitors of Second Messengers. To clarify the nature of the putative “inside-out” transduction pathways with a role

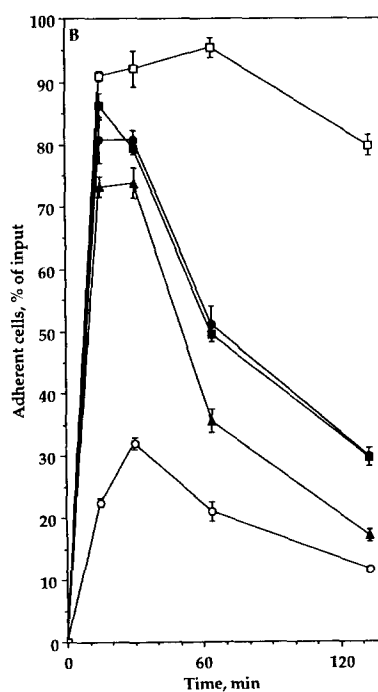
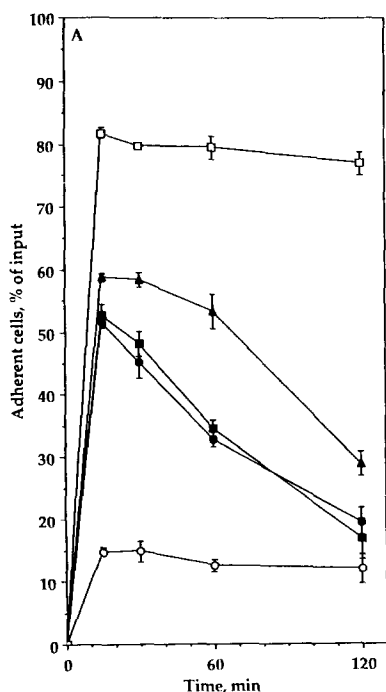


Figure 2. Kinetics of adhesion on Fn induced by cytokines and mAb 8A2. MO7e (A) and TF1 (B) cells were incubated at 37°C in the presence of 10 ng/ml of IL3 (●), GM-CSF (■), KL (▲), 1 μ g/ml 8A2 mAb (□), or without stimulus (○). At the indicated times, cell adhesion was measured in triplicate, as described in Materials and Methods. This experiment is representative of two experiments.

Table 3. TF1 and MO7e Cell Antigen Expression after a 2-h Incubation at 37°C with IL-3, GM-CSF, or KL

mAb	Antigens	TF1				MO7e			
		Nil	IL-3	GM-CSF	KL	Nil	IL-3	GM-CSF	KL
49.1B11	E-selectin	0.333	0.338	0.357	0.403	0.677	0.507	0.559	0.488
YB5.B8	KIT/CD117	3.136	2.714	3.164	0.857	16.48	18.16	18.76	9.673
3D7	β_c	5.194	5.444	5.569	4.962	0.949	0.712	0.717	0.775
9F5	IL-3R α	1.336	1.195	1.429	1.092	1.089	0.975	1.062	1.057
8G6	GM-CSFR- α	3.674	3.029	1.208	2.248	1.291	1.103	0.814	1.151
P4C2	VLA-4	6.365	6.476	6.526	6.261	5.533	5.658	5.886	5.445
PHM2	VLA-5	4.707	5.302	6.116	3.978	11.77	11.53	11.16	11.07
P5D2	CD29	12.26	13.26	13.62	12.61	21.10	19.36	21.09	22.85

Starved cells were treated as described in Materials and Methods and incubated at 37°C for 2 h without cytokine or in the presence of 10 ng/ml of IL-3, GM-CSF, or KL. Cells were then labeled and analyzed as described in Materials and Methods. Results are expressed as mean fluorescence intensities.

in the β_1 integrin activation process, we treated cells with inhibitors of putative second messenger pathways. As shown in Fig. 6, cholera toxin, an activator of adenylate cyclase (26), calyculin A, and okadaic acid, inhibitors of protein phosphatases

PP1 and PP2A (27, 28), failed to alter cytokine-augmented cell attachment to Fn. Conversely, staurosporine, a broad inhibitor of protein kinases (29), and W7, a calmodulin antagonist (30), strongly inhibited GM-CSF- and KL-induced attachment to Fn. Interestingly, genistein, a specific inhibitor of tyrosine kinases (31), strongly inhibited KL-augmented adhesion of MO7e and TF1 cells to Fn, whereas GM-CSF-stimulated adhesion was only marginally affected, suggesting a divergence in cytokine-stimulated transduction pathways. With the exception of W7, all inhibitors failed to alter 8A2-induced cell adhesion, confirming its "outside-in" mode of action.

Adhesiveness of Steady-state CD34⁺ Progenitors Is Increased by Cytokines. Finally, we investigated whether cytokines also promoted the adhesion of normal bone marrow CD34⁺ progenitor cells to Fn. In agreement with the results obtained with the CD34⁺, MO7e and TF1 cell lines, IL-3, GM-CSF, and KL significantly increased the binding of bone marrow CD34⁺ cells to Fn (Fig. 7). As observed with leukemic cell lines, the activation was transient, since adhesion returned to the basal level after 150 min. As with MO7e cells, cytokine-activated adhesiveness of normal progenitors was significantly but partially inhibited by the anti-VLA-4 P4C2 mAb with a 45–57% reduction of cell attachment, and was almost completely blocked by the anti-VLA-5 mAb PHM2 with an 80–85% reduction. The combination of both of these mAbs completely abolished adhesion on Fn. Contrary to transformed cell lines, normal cells retained their round shape (data not shown).

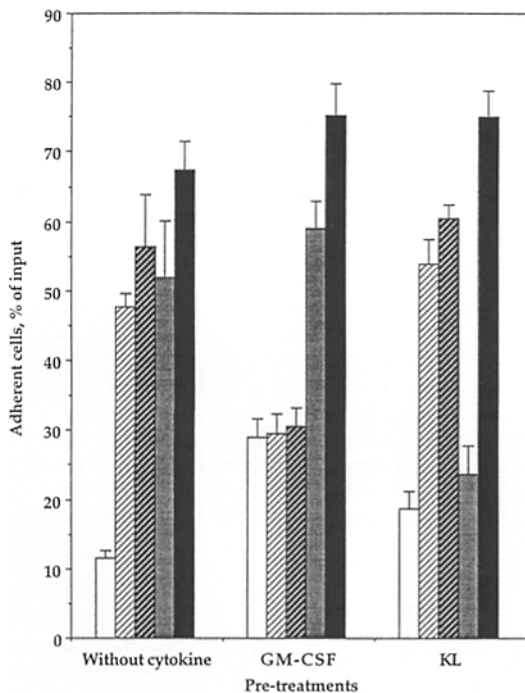


Figure 3. Cytokine-stimulated adhesion is refractory to autologous, but not heterologous, restimulation. TF1 cells were pretreated for 2 h at 37°C in Fn-coated wells with either 10 ng/ml GM-CSF or 10 ng/ml KL, or in the absence of cytokine. Then, cells were incubated for another 20-min period after the addition of 10 ng/ml IL-3 (▨), GM-CSF (■), KL (■), 1 μ g/ml 8A2 mAb (■), or without second stimulus (□). Cell attachment was measured as described in Materials and Methods. Data represent the mean \pm SD of triplicate samples from a representative experiment of two.

Discussion

The physiological importance of interactions between hemopoietic progenitors and the bone marrow stroma is well documented, although little is known of the molecular events and the regulation of these events. "Inside-out" signals have been shown to modify integrin function, specificity, and ligand

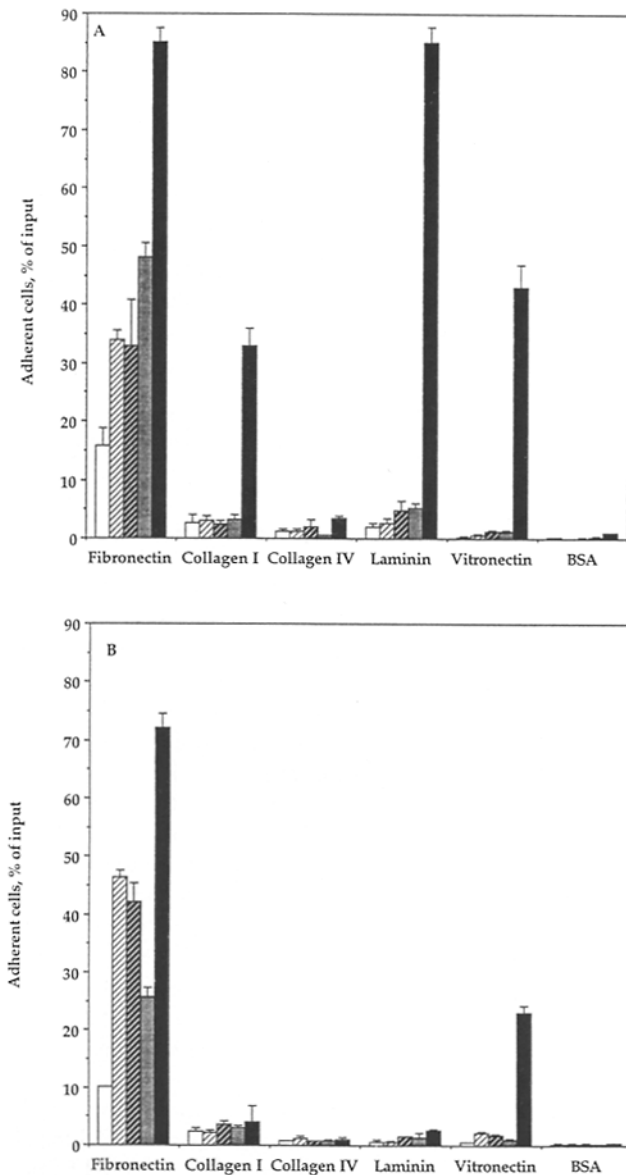


Figure 4. Cytokines selectively increase cell adhesion to Fn. MO7e (A) and TF1 (B) cells were incubated in the presence of 10 ng/ml IL-3 (▨), GM-CSF (■), KL (▩), 1 μ g/ml 8A2 mAb (■), or without stimulus (□) in wells coated overnight with indicated ECM. Cell attachment was measured as described in Materials and Methods at 37°C after 20 min contact with immobilized proteins. Data represent the mean \pm SD of triplicate samples from a representative experiment of two.

affinity in other cellular systems (25, 32, 33). This is particularly well documented for the platelet integrin $\alpha_{IIb}\beta_3$ (34–36), β_1 integrins, such as VLA-6 (37, 38), and β_2 integrins, such as LFA-1 (39). Agonists which stimulate β_1 integrin-mediated adhesiveness on hemopoietic and immune cells include activators of protein kinase C, such as PMA (9, 40), cross-linking of antibodies specific for CD31 (24, 41), the T cell receptor, CD7, CD28 (42), and certain activating anti-CD29 mAb (18, 43–45).

In this study, we show that hemopoietic cytokines, which

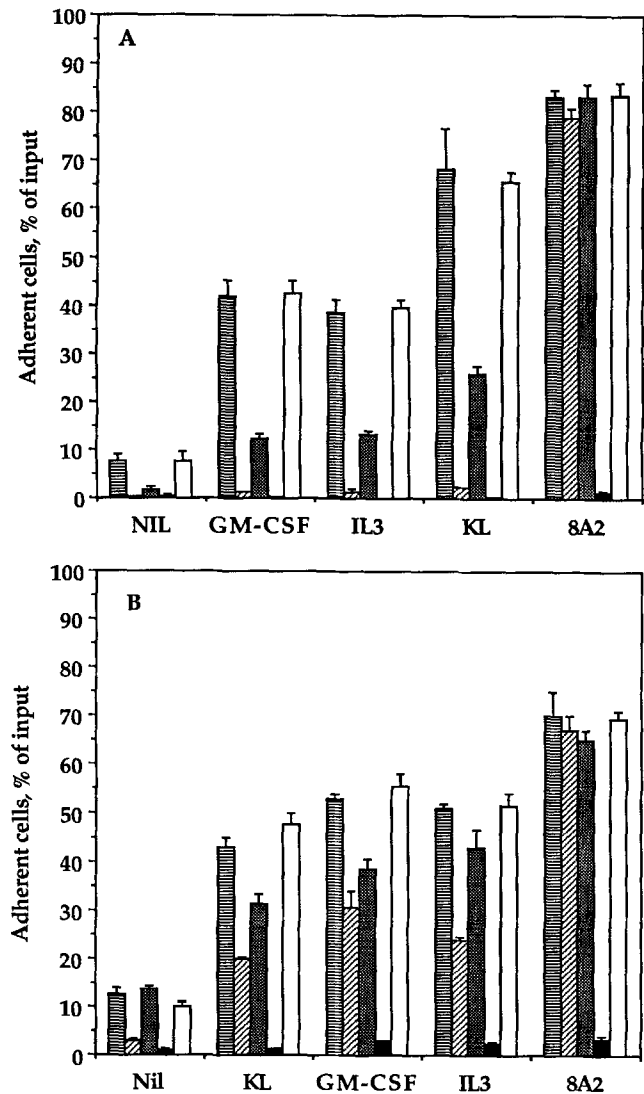


Figure 5. Adhesion of MO7e (A) and TF1 (B) cells on Fn is inhibited by P4C2 (anti-VLA-4) and PHM2 (anti-VLA-5) mAbs. Cells were labeled with ^{51}Cr , as described in Materials and Methods, and preincubated in suspension for 30 min at room temperature without addition (□) or in the presence of PHM2 (▨), P4C2 (▩), PHM2 and P4C2 (■), or AC11 (□), as described in Materials and Methods. Antibody-treated cells were transferred to Fn-coated wells containing 10 ng/ml of the indicated cytokines or 1 μ g/ml 8A2. Cell attachment was measured as described in Materials and Methods at 37°C after 20 min contact with immobilized Fn. Data represent the mean \pm SD of triplicate samples from a representative of two experiments.

are more relevant physiological stimuli, transiently increase the adhesive function of integrins VLA-4 and VLA-5 for their common ligand, Fn. VLA-4 and VLA-5 are normally expressed by the CD34⁺ cell lines MO7e and TF1 in a low affinity configuration which bind Fn poorly. The magnitude of cell attachment to immobilized Fn stimulated by the anti-CD29 mAb 8A2 (18, 23) indicates that the majority of VLA-4 and VLA-5 on these cells is functionally inactive. Similarly, VLA-4 and VLA-5 are inactive on steady-state normal bone marrow CD34⁺ progenitor cells (9). Therefore, these adhe-

Table 4. TF1 and MO7e Integrin Expression after a 20-min Activation by GM-CSF or KL

mAb	Antigens	TF1				MO7e			
		Nil	IL-3	GM-CSF	KL	Nil	IL-3	GM-CSF	KL
49.1B11	E-selectin	0.362	0.322	0.296	0.312	0.590	0.640	0.612	0.565
P4C2	VLA-4	4.888	5.552	5.267	5.130	4.119	5.262	5.629	5.110
PHM2	VLA-5	3.294	3.298	3.877	3.804	10.07	9.711	10.85	10.95
P5D2	CD29	10.07	10.85	10.33	10.38	18.47	18.10	21.72	20.57
P3G8	CD51	1.366	1.371	1.456	1.433	3.333	2.845	3.348	4.019

Starved cells were treated as described in Materials and Methods and incubated at 37°C for 20 min without cytokine or in the presence of 10 ng/ml of IL-3, GM-CSF, or KL. Cells were then labeled and analyzed as described in Materials and Methods. Results are expressed as mean fluorescence intensities.

sive receptors require activation through a physiological mechanism to function *in vivo*. Indeed, blocking anti-VLA-4 mAbs (11, 12, 46) and RGDS-containing peptides (13, 14) impair hemopoiesis both *in vivo* and *in vitro*, suggesting that VLA-4 and VLA-5 must, even transiently, exist in a high affinity

state to be able to bind ligands and transduce specific signals that regulate hemopoiesis.

We have used two CD34⁺ cell lines, MO7e and TF1, which serve as models for hemopoietic progenitors, to investigate the role of relevant cytokines in adhesive interactions between hemopoietic progenitor cells and their marrow micro-environment. Indeed, like normal bone marrow CD34⁺ progenitor cells, MO7e and TF1 are cytokine dependent (IL-3, GM-CSF, or KL) and express the VLA-4 and VLA-5 receptors in a low affinity state. We have shown that these cytokines transiently activate (Fig. 2), in a dose-dependent manner (Fig. 1), both VLA-4 and VLA-5 enabling treated cells to interact with immobilized Fn. mAbs to both VLA-4 and VLA-5 are required to completely inhibit TF1 cell attachment to Fn, indicating that both receptors contribute to the adhesive phenotype in these cells. However, in MO7e and normal bone marrow CD34⁺ progenitor cells, attachment is mediated primarily by VLA-5. This difference in integrin usage could be due to the relative levels of activated VLA-5 versus VLA-4 in these different cell types. It is noteworthy that even with cytokine stimulation, not all available receptors are switched to the activated conformation. This is only achieved in the presence of mAb 8A2.

Since the cell surface expression of VLA-4 and VLA-5 is unchanged by cytokine treatment, alterations in cell adhesiveness likely result from modification of the affinity of VLA-4 and VLA-5 for their ligands. Modification of integrin affinity could be regulated by “inside-out” signaling triggered by occupancy of IL-3, GM-CSF, and KIT receptors. The transient nature of cytokine-stimulated activation is evidence for such a mechanism. Moreover, the transient nature of this cytokine-dependent activation cannot be explained by a correlated loss of cytokine receptor expression (Table 3), but rather by a mechanism similar to the transient activation of protein kinases acting downstream of cytokine receptors, such as JAK2 (47), p42^{mapk} and p44^{mapk} (48), p53/p56^{lyn}, or the phosphoinositide 3OH kinase (49), the activities of which peak between 5 and 15 min after cytokine stimulation. The nonphysiological activation conferred by the mAb 8A2 induces an “outside-in” stable change in the β_1 chain conformation, leading to a stable increase of both VLA-4 and VLA-5 affinities for Fn

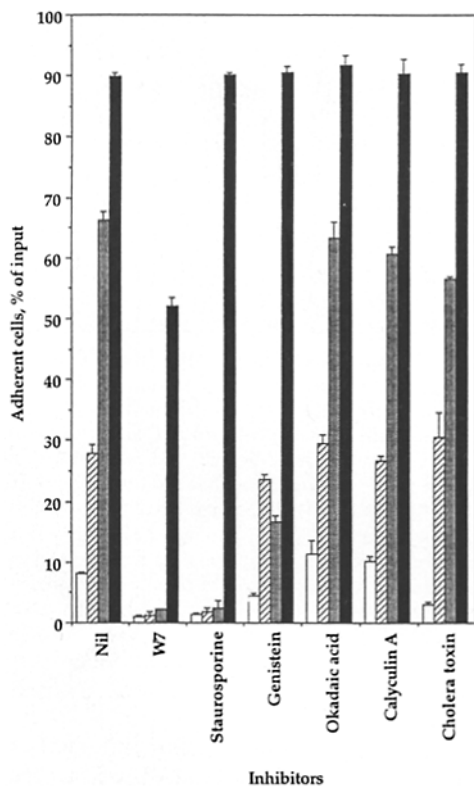


Figure 6. Effect of inhibitors on MO7e cell adhesiveness on Fn. After labeling with ⁵¹Cr, cells were preincubated for 30 min at 37°C in the presence of 50 μ M W7, 0.5 μ M staurosporine, 100 μ M genistein, 0.2 μ M okadaic acid, 0.2 μ g/ml calyculin A, or 0.2 μ g/ml cholera toxin. Then, cells were plated in Fn-coated plates, as described in Materials and Methods, and incubated for 20 min at 37°C in the absence (□), or presence, of 10 ng/ml GM-CSF (▨), 10 ng/ml KL (■), or 1 μ g/ml 8A2 (■). Data represent the mean \pm SD of triplicate samples from a representative of four experiments.

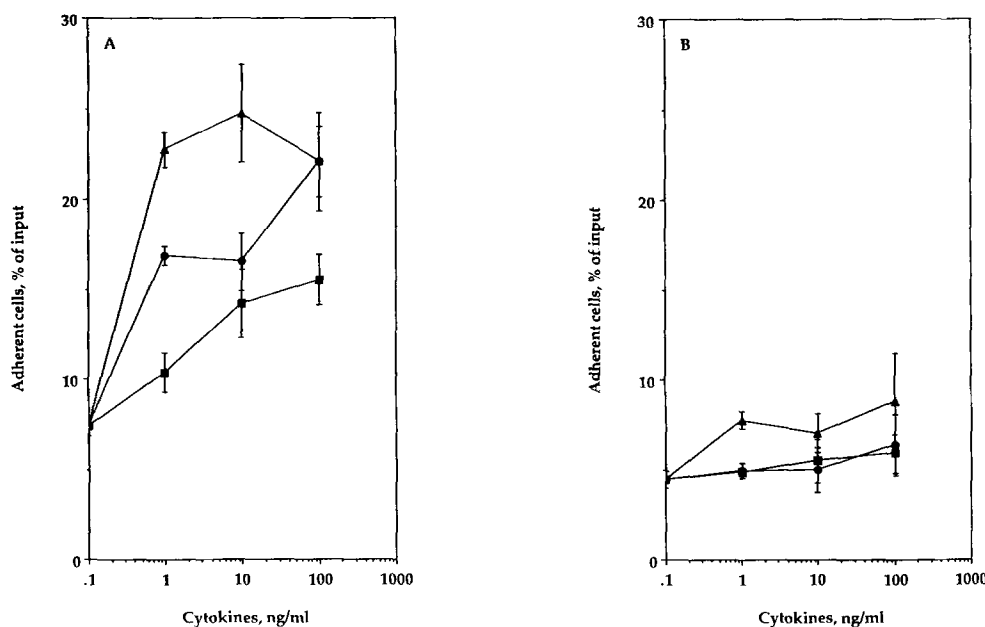


Figure 7. Cytokines increase adhesion of normal bone marrow CD34⁺ hemopoietic progenitors to Fn. CD34⁺ donor bone marrow cells, enriched using FACS[®] and starved overnight in serum-free medium, as described in Materials and Methods, were incubated for 30 min (A) or 2.5 h (B) at 37°C in contact with Fn in the presence of the indicated concentrations of IL-3 (■), GM-CSF (●), or KL (▲). Nonadherent cells were removed as described and adherent cells were counted. These data represent the mean \pm SD of triplicate samples from a representative of two experiments. Adhesion on BSA remained below 1% in all the tested conditions.

(23, 50). This is indicated by the action of the blocking anti-VLA-4 and anti-VLA-5 mAbs, P4C2 and PHM2. Used individually, neither mAb is able to alter 8A2-activated cell adhesion to Fn; the combination of both being required to inhibit adhesiveness. MO7e cells treated with 8A2 also adhere to type I collagen via VLA-2, laminin via VLA-6, and vitronectin via $\alpha_v\beta_1$, yet fail to recognize these ECM ligands after treatment with IL-3, GM-CSF, or KL. Thus, mAb 8A2 is a panactivator of β_1 integrins, whereas "inside-out" signals induced by cytokines selectively activate α_4 - and α_5 -containing integrins.

Our results demonstrate that the activation pathway induced by cytokines requires some functional protein kinases other than cAMP-dependent protein kinase, while protein phosphatases PP1A and PP2B are unnecessary. Indeed, staurosporine, a broad inhibitor of protein kinases, completely abolished cytokine-mediated β_1 integrin activation. It is noteworthy that genistein, a tyrosine kinase inhibitor, strongly inhibited activation mediated by KIT, which is a tyrosine kinase receptor, while marginally inhibiting activation through the GM-CSF receptor, which does not possess any intrinsic tyrosine kinase activity. The protein kinases involved in this "inside-out" signaling pathway act downstream of the cytokine receptors and belong to an as yet undefined cascade leading to VLA-4 and VLA-5 activation. The molecular mechanism of β_1 integrin activation remains obscure, since β_1 integrins are constitutively phosphorylated (Lévesque, J.-P., unpublished data). Furthermore, there is no evidence that phosphorylation is induced by, or as a consequence of, integrin activation or ligand binding (38, 51). cAMP-dependent protein kinase does not seem to belong to this "inside-out" pathway, since cholera toxin has no effect. On the other hand, it is not clear whether calmodulin-dependent protein kinases are directly involved in β_1 integrin activation or in postreceptor cytoskeletal rearrangements leading to the strengthening of cell adhesion (51). Indeed, we noticed that W7, a calmodulin

antagonist, inhibited the cytokine-dependent induction of cell adhesion, but also partially inhibited 8A2-activated adhesiveness. Moreover, after activation with mAb 8A2 in the presence of W7, we noticed that the adherent cells retained a rounded shape, rather than the flattened morphology observed with or without the other inhibitors used in this study (not shown), suggesting an effect on the cytoskeleton.

It is tempting to speculate that this cytokine-regulated activation of VLA-4 and VLA-5 occurs with normal CD34⁺ bone marrow progenitors. Several groups have shown that anti-VLA-4 antibodies strongly inhibit hemopoiesis both in vitro (11, 12) and in vivo (46), while RGDS-containing peptides reduce colony formation in methyl cellulose cultures (13, 14). Moreover, in a previous study, Verfaillie et al. (22) have shown that long-term bone marrow culture-initiating cells, CFU-mix, burst-forming unit-E, and CFU-GM, adhere to intact Fn and that specific changes in adhesion to VLA-4- and VLA-5-binding domains of Fn are associated with differentiation of primitive multipotent progenitors into single lineage committed progenitors. In their study (22), the adhesion assays were performed in a serum-free medium without cytokine stimulation. Significantly, we have observed that, as with MO7e and TF1 cells, the adhesion of normal bone marrow CD34⁺ progenitors to Fn can be significantly augmented by IL-3, GM-CSF, and KL. This stimulation is transient and dose dependent.

In conclusion, this work points to the intricate relationships between cytokine receptors and cytoadhesive receptors, and cell proliferation and cell adhesion. The specificity of VLA-4 and VLA-5 integrin activation suggests an exquisite control of specific transduction pathways resulting in a subtle regulation of hemopoiesis at a molecular level. The activation of β_1 integrins and augmentation of cell adhesion to specific ligands could be an important regulator of the proliferation and differentiation of hemopoietic cells in response to cytokines. Conversely, the modulation of adhesive properties

by cytokines could be involved in the regulation of hemopoietic progenitor cell homing, or release from the bone marrow,

as observed in vivo with therapeutic protocols using perfusion of high doses of cytokines (5, 52, 53).

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