Role of β_1 and β_2 Integrins in the Adhesion of Human CD34^{hi} Stem Cells to Bone Marrow Stroma

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Abstract

Hematopoietic stem cell interaction with elements of the underlying stroma is essential for sustained normal hematopoiesis. Here we have determined that adhesion receptors in the integrin family play a role in promoting adhesion of human hematopoietic stem cells to cultured human marrow stromal cells. Enriched CD34^{hi} progenitor cells expressed VLA-4, VLA-5, and at least one or more β_2 integrins. Homogeneous marrow stromal cell monolayers capable of supporting proliferation of cocultivated CD34^{hi} cells expressed VCAM-1 and fibronectin (ligands for VLA-4 and VLA-5) as well as ICAM-1 (ligand for LFA-1 and Mac-1). Adhesion-blocking experiments indicated that VLA-4/VCAM-1, VLA-5/fibronectin, and β_2-integrin / ICAM-1 pathways all are important for CD34^{hi} cell attachment to stromal cells. Consistent with this suggestion, IL-1 stimulation of stromal cells caused both increased VCAM-1 and ICAM-1 expression and increased attachment by CD34^{hi} bone marrow cells. In addition, CD34^{hi} cells utilized VLA-4 to adhere to purified VCAM-1 and employed VLA-5 (and to a lesser extent VLA-4) to adhere to purified fibronectin. Together these results suggest that CD34^{hi} stem cells may utilize multiple integrin-mediated adhesion pathways to localize within specialized microenvironmental niches created by marrow stromal cells. (*J. Clin. Invest.* 1992. 90:358–367.) Key words: adhesion • CD34^{hi} • integrins • stroma

Introduction

Bone marrow consists of a complex mixture of stromal cells that create an inductive microenvironment necessary for maintenance, proliferation, and differentiation of hematopoietic stem cells (1–3). Long-term bone marrow cultures have been used to study stem cell regulation and reproduce the heterogeneous stromal population of endothelial fibroblasts, adipocytes, and macrophages in vitro (4, 5). In addition, marrow stromal cells enriched with a specific antibody (Stro-1) were found to support hematopoiesis in vitro (6). Preformed, fresh marrow-derived stromal monolayers have also been used to quantitate human stem cells in vitro (7, 8). Although stem cells are known to home to and adhere to stromal cells in vivo and in vitro, the mechanisms by which stem cells are restricted within specific areas in the marrow remain undefined (9, 10). Recent attention has begun to focus on the role of glycoproteins and integrins in this process (9–14).

Members of the integrin family of cell adhesion receptors mediate both cell–extracellular matrix and cell–cell adhesion and are involved in cell migration and localization during embryonic development, tissue organization, cell differentiation, inflammation, and metastasis (15–19). The integrin family consists of at least 17 distinct heterodimers formed by associations between 13 α subunits and 7 different β subunits, each with distinct ligand binding properties (15). Among integrins, the VLA-5 ($\alpha^5\beta_1$) complex is widely distributed and functions as a receptor for fibronectin (20, 21). In contrast, the VLA-4 ($\alpha^4\beta_1$) complex is expressed at substantial levels on normal peripheral blood B and T cells, thymocytes, monocytes, and some melanoma cells as well as on marrow blast cells and erythroblasts (11, 17, 22, 23). Ligands for VLA-4 are vascular cell adhesion molecule–1 (VCAM-1),[1] identified on the surface of activated endothelial cells (24–27) and CS-1, an alternately spliced domain within the Hep II region of fibronectin (28–30). Another group of integrins (CD11a/CD18, CD 11b/CD18, and CD11c/CD18) share the common β_2 chain and are variably expressed on peripheral T cells, monocytes, and mature granulocytes. Ligands for β_2-integrins include members of the Ig superfamily (ICAM-1 and ICAM-2) found on activated endothelial cells (19, 31, 32).

Recently, we have demonstrated the role of tissue-specific adhesion protein hemonectin in mediating adhesion of murine colony-forming cells with a high proliferative potential (33). In other studies, formation of day 12 colony-forming unit–spleen (CFU-s) by murine stem cells was inhibited by antibodies against integrin β_1 subunit (13). In addition, lymphopoiesis in murine long-term marrow cultures was inhibited and myelopoiesis retarded by addition of an anti-α^4 antibody (14). Adhesion of human erythroid progenitors to fibronectin is mediated by the integrins VLA-4 and VLA-5 (11) whereas adhesion of human B cell precursors to marrow stroma is mediated by VLA-4 to VCAM-1 (34). In addition, for human progenitors the adhesion molecules thrombospondin and heparan sulfate have also been implicated (35, 36). Although VLA-4 has been detected on immature blast cells and CD11a/CD18 found on a subpopulation of CD34$^+$CD33$^-$ cells, their role in mediating the interaction between human stem cells and underlying stroma remains to be defined (22, 37).

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Received for publication 28 October 1991 and in revised form 25 March 1992.

J. Clin. Invest.

0021-9738/92/08/0358/10 $2.00
Volume 90, August 1992, 358–367

1. *Abbreviations used in this paper:* EPO, erythropoietin; LTC-IC, long-term culture-initiating cells; PCR, polymerase chain reaction; PE, phycoerythrin; SBA, soybean agglutinin; SF, steel factor; VCAM-1, vascular cell adhesion molecule-1.

In the present study we used human $CD34^{hi}$ progenitors that have marrow repopulating ability together with cultured human marrow stromal cells capable of supporting hematopoiesis in an in vitro model to investigate the role of integrins in human stromal cell interaction with stroma.

Methods

Bone marrow cells. Normal human marrow cells were obtained from informed and consenting allogeneic bone marrow transplant patients and patients undergoing total hip replacement. Cells separated on Ficoll-Hypaque (1.077 g/dl) were added to primed polystyrene devices (Applied Immune Sciences, Menlo Park, CA) coated with soybean agglutinin (SBA). After 1 h of incubation the cells in the suspension (SBA^- fraction, which contained ~ 80% lineage committed progenitors) were harvested, washed, and added to flasks coated with anti-CD34 monoclonal antibodies (clone ICH3). The $CD34^+$ cells were recovered after 1 h of incubation by positive selection (38). Although enrichment of $CD34^+$ cells may not be as high with this protocol as that obtained in FACS-sorted cells, the $CD34^+$ cells recovered constituted ~ 1–3% of the total marrow cells. Based on FACS analysis 60–80% of the cells expressed high density of CD34 after positive selection compared with 14–20% of the cells in the SBA^- fraction.

Stromal cell cultures. Total human bone marrow cells were plated in 25-cm^2 flasks for 3 wk with media changes every week. The medium used was DME supplemented with 10% FCS and 10^{-5} M hydrocortisone sodium succinate. To generate homogeneous stromal monolayers the adherent cells from human bone marrow cultures were trypsinized, passaged for at least four passages, and either frozen in liquid nitrogen or used immediately for adhesion assays or cocultures with enriched $CD34^+$ cells. These cultures had a homogeneous population of large fibroblast-like cells and were devoid of nonspecific esterase-positive macrophages. Stromal cell culture expressed collagen type-I fibronectin, as detected by indirect immunostaining (P. Anklesaria, unpublished observations).

Cell lines. U937, Ramos, Nalm 6, and Jurkat cell lines were grown in RPMI supplemented with 10% FCS (24). KMT2 cell line (39) was also grown in RPMI supplemented with 10% FCS and human recombinant (hr) IL-3 (5 ng/ml).

Cocultures and colony assays. For cocultures, marrow stromal cultures were plated in 96-well plates and enriched $CD34^{hi}$ cells were added at 1,000 or 500 cells per well (8). Cultures were maintained at 33°C for 5 wk in Fischer's medium supplemented with 25% FCS and 10^{-5} M hydrocortisone with weekly medium changes. At the end of 5 wk the number of wells with viable cells were counted. In some experiments cells from the SBA^- fraction were also cocultivated at similar densities. The plating efficiency for this SBA^- population was 10-fold lower.

Blast colony assays were conducted according to Leary and Ogawa (40). Enriched $CD34^{hi}$ cells in suspension were plated into 48-well plates at indicated concentrations without growth factors for 14 d after enrichment. Methylcellulose medium (Terry Fox Laboratories, Vancouver, BC) and growth factors were added on day 14 of culture and blast colonies counted after additional 14 d of incubation. Growth factors used were Steel factor (SF/mast cell growth factor) at 150 ng/ml (41), hrIL-3 at 10 ng/ml (both provided as a gift by Immunex Corp., Seattle, WA) and erythropoietin (EPO, 2.5 U/ml, Terry Fox Laboratories).

To determine whether adhered $CD34^{hi}$ cells could proliferate and differentiate on stromal cells, confluent marrow stromal cells were incubated with 1×10^6 enriched $CD34^{hi}$ cells for 30 min. Nonadherent cells were washed off and the dishes containing adherent (stroma and $CD34^{hi}$) cells were replenished with fresh Fisher's medium supplemented with 20% fetal bovine serum and 10^{-5} M hydrocortisone. Nonadherent cells were removed weekly, counted, and plated in colony assays to determine the number of secondary hematopoietic progenitors. Colony assays were done in methylcellulose medium supplemented with hrIL-3 and EPO (8). Results are expressed as cumulative number of cell counts and colony counts for up to 4 wk in culture.

Antibodies and matrix proteins. Monoclonal antibodies used were the following: TS2/7 (anti-VLA-1 [42]); P1H5 (anti-VLA-2 [43]); J-143 (anti-VLA-3 [44]); B-5G10 and HP2/1 (anti-VLA-4 [45, 46]); PID6 (anti-VLA-5 [21]), A-IA5 and MAB13 (anti-VLAβ_1 [47, 48]); 60.3 (anti-β_2 [49]); 4B9 (anti-VCAM-1 [27]); RR1/1 (anti-ICAM-1 [50]); P3 (isotype matched negative control [51]); anti-CD34 (Amac Inc., Westbrook, ME); anti-CD33PE; and anti-CD71FITC (Becton Dickinson Immunocytometry systems, San Jose, CA). Plasma fibronectin was purchased from Telios Pharmaceuticals, San Diego, CA. The FN-40 (heparin II-binding domain) fragment from fibronectin was prepared as previously described (30). Recombinant soluble VCAM-1 (sVCAM-1) was a gift from Dr. R. Lobb (Biogen Inc., Cambridge, MA). The latter was prepared by genetic alteration of the native molecule such that the transmembrane and the cytoplasmic regions are deleted (52).

Cell surface iodination and immunoprecipitation. Stromal cells and $CD34^{hi}$ were surface-labeled with ^{125}I using lactoperoxidase and lysed with 0.5% Nonidet P-40 (NP-40) in PBS containing 1 mM PMSF, 8 mM iodoacetamide, 10 μM leupeptin, 1 U/ml aprotinin. Lysates were precleared as previously described (53), specific antibodies were added, and the immune complexes harvested with Protein A-Sepharose and washed thrice with PBS containing 0.1% Triton and 0.02% SDS. Samples were analyzed on 8% SDS-PAGE as previously described (53).

Adhesion assays. For adhesion to stromal monolayers, suspended cells were labeled by incubation in complete media with 50–150 μCi/ml ^{51}Cr for 4 h and then washed once in PBS containing 0.5 mM EDTA and once in 0.1% BSA in RPMI, and finally were resuspended in the same solution. Labeled cells at a concentration of $3–5 \times 10^4$ were added to the stromal monolayer in 96-well microtiter plates, which had been previously incubated for 24 h in complete medium, alone, or with 50 pg/ml IL-1 (R&D Systems, Inc., Minneapolis, MN). After 25 min of incubation at 37°C, nonadherent cells were removed by washing three times with 0.1% BSA in RPMI. The bound cells were then lysed in NaOH/SDS solution and the radioactivity was analyzed in a gamma counter.

For assays of $CD34^{hi}$ cell adhesion to purified ligands, 96-well microtiter plates (Flow Laboratories, Inc., McLean, VA) were first incubated with sVCAM-1, FN-40, or fibronectin in 0.1 M $NaHCO_3$, pH 8.3, for 16 h at 4°C, and then for 2 h at 37°C. Next, 0.1% BSA in 0.1 M $NaHCO_3$, pH 8.3, was added for 2 h at 37°C to block nonspecific adhesion. Cells were labeled in complete media by incubation with 50 μCi/ml ^{51}Cr for 4 h and washed and resuspended as described for adhesion to stroma monolayers. After cells ($3–5 \times 10^4$) were incubated for 25 min at 37°C with the ligand-coated microtiter plates, wells were washed thrice with 0.1% BSA in RPMI to remove unbound cells. Remaining adhered cells were lysed and radioactivity was analyzed in a gamma counter. The number of bound cells per well was divided by 33 mm^2 per well to yield cells bound per mm^2. Cell adhesion assays were done in triplicate and results (cells bound per mm^2) reported as mean±SD.

Flow cytometry. To detect integrins, $CD34^+$ cells were double-stained with anti-CD34-FITC and with anti-VLA-4, anti-VLA-5, anti-VLA-β_1 or anti-β_2 mouse monoclonal antibodies. The latter were detected using biotin-conjugated goat anti-mouse antibodies and phycoerythrin (PE)-conjugated strepavidin. In control experiments cells were labeled with control antibody (P3) and either goat anti-mouse conjugated with FITC or goat anti-mouse biotin and strepavidin PE. To detect surface differentiation antigens, enriched $CD34^+$ cells were stained with each of the monoclonal antibodies against CD34, CD33 and CD71 conjugated with either FITC or PE. The staining procedure was done according to the manufacturer's recommendation. Briefly, cells were suspended in 100 μl of PBS supplemented with 5% FCS and incubated at appropriate concentrations of each antibody for 30 min at 4°C. After rinsing twice, the cells were incubated with FITC-labeled anti-mouse antibodies. Cells that were stained for two-color fluores-

cence were incubated with the second antibody which followed sequentially by biotin-conjugated anti-mouse antibodies and strepavidin-PE. Each incubation was at 4°C for 30 min and followed by two washes with PBS. Finally, immunofluorescently labeled cells were fixed in 1.5% paraformaldehyde for 10 min, washed, and resuspended in PBS. Single and two-dimensional flow cytometric analysis was performed on a FACscan (Becton-Dickinson & Co., Mountain View, CA).

Detection of growth factor mRNA by polymerase chain reaction (PCR). Total cellular RNA was prepared according to Huang and High (54). 1 μg of total RNA was reverse-transcribed to cDNA using MMLV-RT and subsequently amplified using specific primers for human IL-3, IL-6, and IL-1 purchased from Clonetech Laboratories, Palo Alto, CA. Both reverse transcription and amplification were done using the GeneAmp RNA PCR kit (Perkin Elmer Cetas Corp., Norwalk, CT) according to the manufacturer's recommendation.

Immunohistochemical detection of IL-1α. Confluent cultures of stromal cells grown on coverslips were fixed with 3% paraformaldehyde in PBS for 5 min, washed three or four times and incubated with 1:10 dilution of anti-IL-1α (Oncogene Science, Inc., Manhasset, NY). This was followed by biotin-labeled rabbit anti-mouse secondary antibody, strepavidin-linked alkaline phosphatase, and developed with fast red substrate according to the manufacturers directions (BioGenix Labs, San Ramon, CA) and counterstained with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, MO).

Results

Expression of β_1 and β_2 integrins on CD34$^+$ cells. To determine co-expression of integrins on enriched CD34$^+$ human hematopoietic cells, dual-color immunofluorescence staining was done (Fig. 1). Cells were stained with anti-CD34-FITC and antibodies to α and β subunits of the integrin family. Of the cells expressing CD34, 77% co-expressed VLA-4, < 3% of total cells expressed VLA-4 but not CD34, and ~ 9% of the popula-

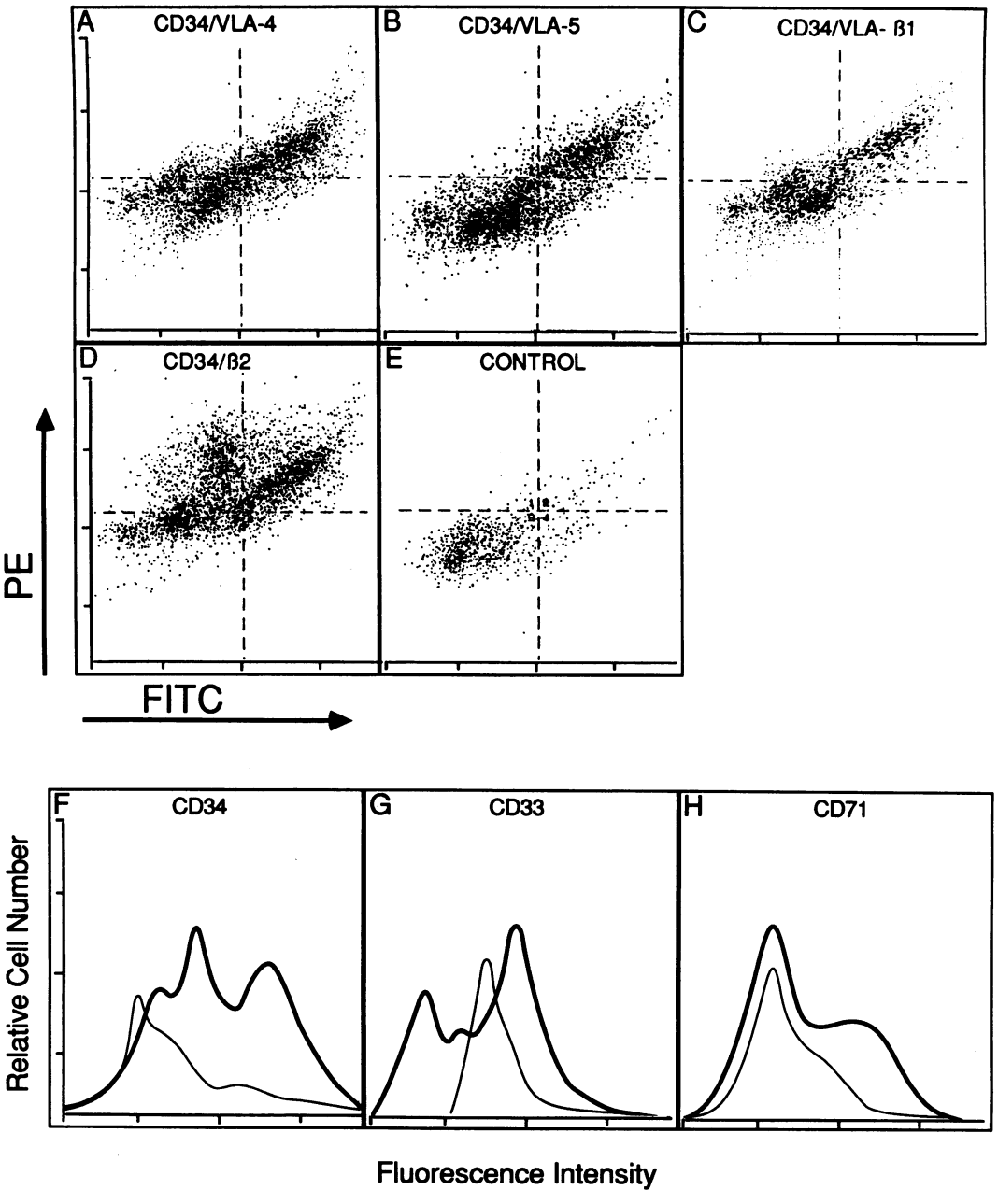

Figure 1. Flow cytometric analysis of enriched CD34$^+$ cells. Coexpressed integrins on CD34$^+$ cells were determined by two-dimensional immunofluorescence using anti-CD34 FITC and anti-α^4-biotin strepavidin-PE (*A*, $n = 4$); anti-CD34-FITC and anti-α^5-biotin-strepavidin-PE (*B*, $n = 4$); anti-CD34-FITC and anti-β_1-biotin-strepavidin-PE (*C*, $n = 6$) anti-CD34-FITC and anti-β_2-biotin-strepavidin-PE (*D*, $n = 3$). CD34 cells were stained with control antibody P3, anti-mouse-FITC, and P3-biotin-strepavidin-PE (*E*, $n = 10$). Enriched CD34$^+$ stained with anti-CD34-FITC (F, n = 10), anti-CD33-PE (*G*, $n = 6$) anti-CD71-FITC (*H*, $n = 3$) and analyzed by one-dimensional immunofluorescence. Thick lines represent staining with specific antibodies and thin lines represent staining with isotype-matched control antibodies (*F–H*). Results are from a representative experiment.

Table I. Functional Characterization of Enriched CD34^{hi} Cells and Cultured Stromal Cells

	Number of cells plated per well					
	1,000	500	100	50	10	1
Clonogenic cells*	NT	NT	3±1.7	1.7±1	1±0.5	0.33 (16/48)§
LTC-IC‡	1.0	1.0	0.66	NT	0.12	NT

* The number of clonogenic cells was determined by plating cells in methycellulose cultures 14 d after enrichment. The growth factors used were hrIL-3, SF, and EPO. Blast cell colonies were counted at 28 d after initial plating. Results are mean±SD of at least 16 wells plated for each cell concentration. ‡ LTC-IC was determined by plating enriched CD34^{hi} cells onto performed irradiated (1,000 cGy) stromal monolayers. After 5 wk the cultures were scored for number of wells with viable cells. Results are the ratio of the number of wells with viable cells per total wells plated. There were no detectable viable cells after 5 wk in cultures plated without stromal underlayer. § Number of wells containing at least one blast cell colony was considered positive. The values in parentheses denote number of positive wells per number of wells plated. NT, not tested.

tion expressed CD34 but not VLA-4 (Fig. 1 *A*). In cell surface iodination and immunoprecipitation experiments, anti-α^4 antibodies co-immunoprecipitated the α^4 and β_1 subunits indicating that CD34^{hi} cells express the VLA-4 complex (data not shown). Similarly, 49% of total cells co-expressed CD34 and VLA-5, < 2% of the population expressed VLA-5 but not CD34, whereas 32% of cells expressed CD34 but not VLA-5. (Fig. 1 *B*). The VLA-1, VLA-2, and VLA-3 complexes were not co-expressed on CD34^{hi} population (data not shown). The common β_1 and β_2 chains were expressed on 92.6±5% and 80±6.5%, respectively, of enriched CD34^{hi} cells (Fig. 1, *C* and *D*).

Primitive hematopoietic progenitors (1–3% of total marrow cells) have been described as those that do not bind to SBA, express high levels of CD34, form blast colonies with high plating efficiency, and are enriched in long-term culture-initiating cells (LTC-IC [8]). To determine whether our CD34$^+$ population was enriched for primitive stem cells, their functional properties were evaluated. The CD34$^+$ cells were incubated for 14 d in culture medium at indicated cell densities and then plated in methylcellulose with a cocktail of growth factors (hrIL-3, SF, and EPO). The enriched CD34$^+$ cells formed blast colonies with a 20–30% plating efficiency (Table I). In addition as seen in Fig. 2 adherent CD34^{hi} cells could proliferate and give rise to secondary nonadherent colony-forming progenitors in culture for up to 4 wk.

From previous studies (55), it is clear that only 1% of the CD34$^+$ cells (or 0.01% of total marrow population) do not co-express differentiation antigens such as CD33 (myeloid lineage), CD71 (erythroid lineage), and CD10 and CD5 (lymphoid B and T lineage). Furthermore, a decrease in CD34 expression during maturation is associated with increased expression of one or more of the differentiation antigens (55). For the CD34$^+$ cells used in our studies, > 75% expressed high CD34 density, 20–30% expressed low densities of CD33, and < 20% expressed low densities of CD71 (Fig. 1, *F–H*). There were no cells detected that expressed CD5 and < 5% of cells expressed CD10 (data not shown). These data indicate that a population of enriched CD34^{hi} cells with a high proliferative capacity was obtained.

Adhesion molecules expressed by human stromal cells. The primary human bone marrow stromal monolayer in culture is a complex mixture of several different cell types including fibroblasts, endothelial cells, and macrophages. Distinct stromal cell types are associated with erythroid and granulocytic cells in vivo (56, 57). To determine the contribution of a specific stromal cell subtype toward stem cell adhesion and maintenance, homogeneous fibroblast-like, stromal cell cultures devoid of macrophages were established from human marrow. As shown in Table I, these cultured stromal cultures could support proliferation of cocultivated CD34^{hi} cells for up to 5 wk. In separate experiments these stromal cells also supported production of progenitors and mature hematopoietic cells from cocultivated CD34^{hi} population (Fig. 2).

Expression of VCAM-1, a ligand for VLA-4 was analyzed by iodination and immunoprecipitation of cell surface proteins. VCAM-1 was expressed on resting stromal monolayers, as detected by the anti-VCAM-1 MAb 4B9 (Fig. 3 *A*, lane *b*). Preincubation of the stromal cultures with IL-1 resulted in a two- to threefold increase in expression of VCAM-1 (Fig. 3 *A*, lane *c*). In comparison, ICAM-1, a ligand for LFA-1 and Mac-1 was expressed at very low levels on resting cells but its expression was dramatically induced by incubation of stromal monolayers with IL-1, as detected with anti-ICAM-1 MAb RR1/1 (Fig. 3 *A*, lanes *d* and *e*). In contrast to VCAM-1 and ICAM-1, which were upregulated upon activation of stroma with IL-1, extracellular matrix proteins such as fibronectin and hemonectin were constitutively expressed and were not up-regulated by IL-1 (data not shown).

To determine whether endogenous growth factors in resting stromal cells might cause basal VCAM-1 and ICAM-1 expres-

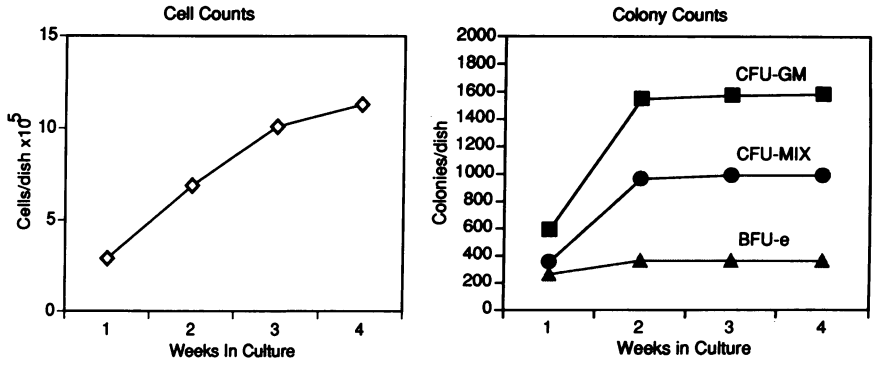

Figure 2. Adhesion and subsequent differentiation of CD34$^+$ cells on human marrow stromal cells. Confluent marrow stromal cells were incubated with enriched CD34^{hi} cells (1×10^6) for 30 min. Nonadherent cells were washed off and the cultures were replenished with fresh medium. Weekly nonadherent cells were removed, counted, and plated in secondary colony assays in methycellulose medium supplemented with human rhIL-3 and EPO. Results are expressed as cumulative number of cell counts and colony counts for 4 wk.

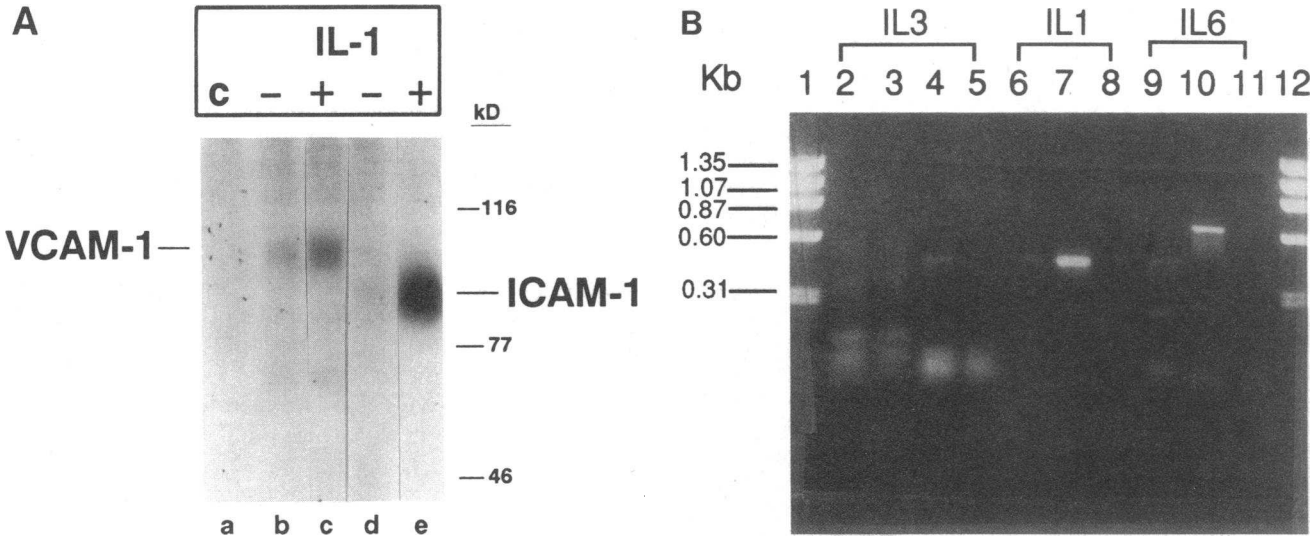

Figure 3 (A). Cell surface expression of VCAM-1 and ICAM-1 on stromal monolayers. Cells were preincubated for 24 h in the presence (+) or in the absence (−) of IL-1. After detaching with PBS/2 mM EDTA, cells were washed with PBS and surface proteins were labeled with ^{125}I as described in methods. Cell lysates were immunoprecipitated with control antibody P3 (lane *a*), anti-VCAM-1 MAb 4B9 (lanes *b* and *c*), or anti-ICAM-1 MAb RR1/1 (lanes *d* and *e*). Immunoprecipitates were analyzed by SDS-PAGE using nonreducing conditions. The positions of VCAM-1 and ICAM-1 are indicated. (*B*) Analysis of normal human stromal cell cultures for cytokine RNA by PCR. Total RNA was isolated from the same stromal cell cultures that were used for adhesion (*A* and Fig. 5) and functional assays (Table I). The cDNA generated was tested for the presence of sequences corresponding to IL-3 (450 bp, lanes *2* and *3*), IL-1α (408 bp, lane *6*) and IL-6 (636 bp, lane *9*) by using PCR and specific primers. Lanes *5, 8,* and *11* represent PCR reactions run with primers specific for IL-3, IL-1α and IL-6 in the absence of stromal cell cDNA. Lanes *4, 7,* and *10* represent PCR reactions run with specific primers for IL-3, IL-1α, and IL-6 in the presence of positive control cDNA supplied by the manufacturer. Products of the reactions were analyzed on a 1.5% agarose gels. Lanes *1* and *12* are the molecular size markers.

sion, cDNA prepared from stromal cell mRNA was screened for IL-3, IL-1, and IL-6 by PCR analysis. As seen in Fig. 3 *B*, there were no detectable sequences specific for IL-3 (lanes *2* and *3*) or IL-6 (lane *9*). However, mRNA specific for IL-1 (Fig. 2 *B*, lane *6*) was detected. These results were further confirmed by indirect immunostaining using specific MAbs to human IL-1α (data not shown). Thus a small amount of IL-1α produced by resting stromal cells might be responsible for basal expression of VCAM-1 as seen in Fig. 3 *A*.

Adhesion of human CD34^{hi} cells to stroma is mediated by VLA-4, VLA-5, and β_2 integrins. To address whether integrins expressed on CD34^{hi} cells can promote their attachment to marrow stromal cells, adhesion assays were carried out using CD34^{hi} cells and stromal cells. In some experiments only a moderate amount of CD34^{hi} cells bound to stromal cells. To rule out the possibility that the bound cells were not representative of the whole population, labeled CD34^{hi} cells were incubated for 30 min on stromal cells in the presence of nonspecific mouse IgG. The number of cells bound per mm^2 at the end of the first incubation was enumerated as 157±29. The nonadherent cells were subsequently removed from each of the wells and added to separate wells containing stromal cells for another 30 min. In two successive rounds the number of cells bound per mm^2 was 125±25 and 135±24, respectively. These results indicate that approximately the same number of nonadherent CD34^{hi} cells adhered to marrow stroma in presence of non specific mouse IgG. Thus, relatively low numbers of adherent cells observed in some of our assays (Figs. 4–6) are due to the limitation in the assay and not because subpopulations of cells are inherently nonadhesive.

The CD34^{hi} cells exhibited significant adhesion to stromal monolayers which was blocked by anti-α^4 antibody HP2/1 (~ 40%), and to a lesser extent by anti-VCAM-1 MAb 4B9 (Fig. 4 *A*). Induction of VCAM-1 expression by pretreatment of stromal cells with IL-1 resulted in increased CD34^{hi} cell adhesion, which was also significantly inhibited by anti-α^4 (−40%, $P < 0.05$) and anti-VCAM-1 MAbs (Fig. 4 *A*). Maximal adhesion as seen in Fig. 4 *A*, represented 28% of total cells added, or 35% of VLA-4$^+$ cells. In a separate experiment, adhesion to stroma was somewhat lower, as 6% of the total cells, or 12% of the VLA-5$^+$ cells were adherent (Fig. 4 *B*). As shown, preincubation with an anti-α^5 antibody caused significant ($P < 0.05$) inhibition (50%) of the binding of CD34^{hi} cells (Fig. 4 *B*). Additional experiments showed that an anti-β_1 MAb inhibited adhesion of CD34^{hi} cells to stroma by 63±10% (Table II). These results suggest that VCAM-1 and fibronectin on stromal cells and VLA-4 and VLA-5 on CD34^{hi} cells may play a role in the adhesion of CD34^{hi} marrow progenitors to stroma. In addition to β_1 integrins, β_2 integrins expressed on CD34^{hi} cells are also involved, based on partial blocking of CD34^{hi} adhesion to stroma an anti-β_2 antibody (Fig. 4 *C*). Inhibition by anti-β_2 was more obvious (35%) when stromal cells were pretreated with IL-1. Together these results suggest that at least three different integrins, and multiple adhesion pathways are important for CD34^{hi} attachment to certain stromal cells in the marrow microenvironment.

Adhesion of various leukocyte cell lines to stromal cells. To confirm and extend the finding that stromal cell monolayers support VLA-4/VCAM-1–dependent cell adhesion, leukocyte cell lines in the myeloid and lymphoid lineages were analyzed. A human IL-3–dependent cell line KMT-2, which expresses CD34, was inhibited by anti-VLA-4 and anti-VCAM-1 antibodies, only when the stromal monolayers were preincubated with IL-1. Thus, these cells must utilize other adhesion path-

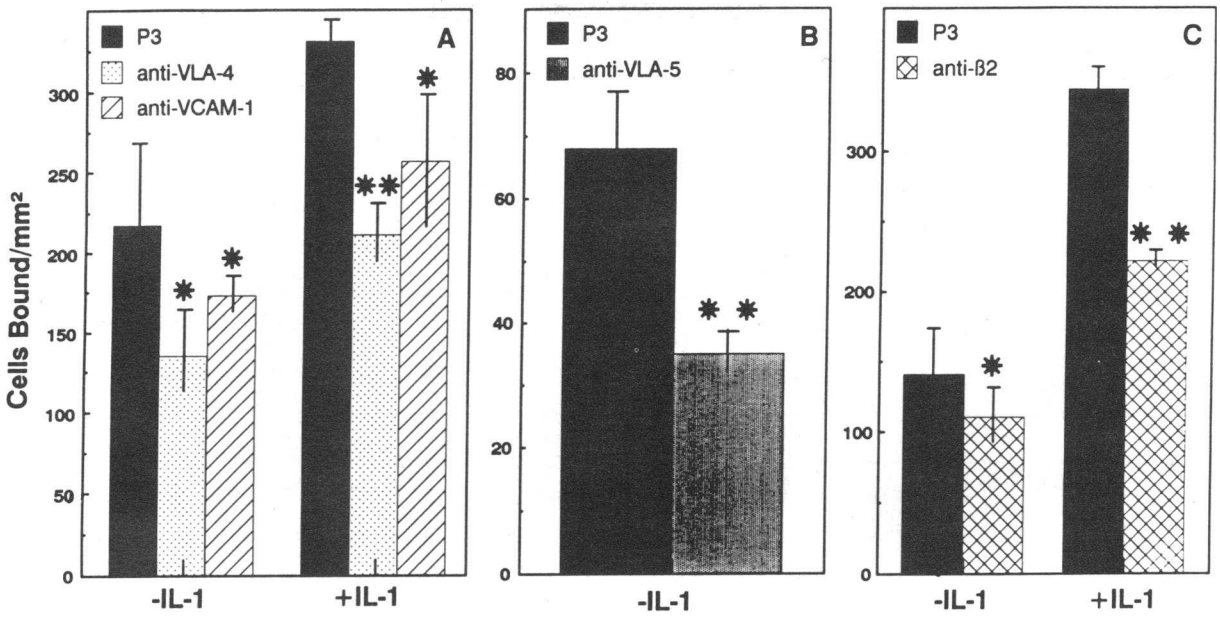

Figure 4. Involvement of VLA-4, VLA-5, and β_2 integrins in adhesion of CD34^{hi} to stromal cells. CD34^{hi} cells were labelled with ^{51}Cr and incubated with stromal monolayers that had been preincubated for 24 h with or without IL-1 in 96-well plates in the presence of optimal concentrations of (*A*, $n = 4$) control antibody P3, anti–VLA-4 (HP2/1), or anti–VCAM-1 MAb (4B9); (*B*, $n = 1$) control antibody (P3) or anti–VLA-5 MAb (PID6) and (*C*, $n = 4$) control antibody (P3) or anti-β_2 MAb (60.3). After adhesion for 25 min, bound cells were solubilized and their incorporated radioactivity measured in a γ-counter. **Adhesion was significantly inhibited, $P < 0.05$ according to the Student's two-tailed t test. *Inhibition was observed, but was not quite low enough to meet $P < 0.05$ confidence level.

ways to interact with resting stroma (Fig. 5 *A*). Other myeloid (U937) and lymphoid cell lines (preB Ramos, Nalm 6; T cell Jurkat) showed different degrees of adhesion to resting and activated stromal monolayers, and each was partly or almost completely inhibited by anti–VLA-4 and anti–VCAM-1 antibodies (Fig. 5, *B–E*). In addition, as seen in the case of CD34^{hi} progenitors, monoclonal antibodies to the β_2 integrins also inhibited binding (by 5–10%) of some of the leukocyte cell lines to IL-1–activated stromal cells (data not shown).

Adhesion of CD34^{hi} cells to purified proteins. Indirect evidence in Fig. 4 strongly suggested that VLA-4/VCAM-1 and VLA-5/fibronectin pathways are important for CD34^{hi} cell attachment to stromal cells. To provide a more direct evaluation of the function of VLA-4 and VLA-5 on CD34^{hi} cells, their adhesion to optimal concentrations of purified ligands sVCAM, FN-40, and intact fibronectin was tested. The anti-α^4 MAb HP2/1 completely inhibited adhesion of CD34^{hi} cells to sVCAM (Fig. 6 *A*). The anti-α^4 MAb also substantially inhibited adhesion of CD34^{hi} cells to the FN-40 fragment of fibronectin coated onto microtiter wells (Fig. 6 *B*). Adhesion of CD34^{hi} cells to intact fibronectin was markedly inhibited by an anti-α^5 MAb and also partially by anti-α^4 MAb (Fig. 6 *C*). In Fig. 6, *A*, *B*, and *C*, adherent cells represented 13%, 14%, and 44% of the total population, respectively. Considering only VLA-4$^+$ cells, adherent cells represented 17%, 18%, and 55%, respectively (Fig. 6, *A*, *B*, and *C*). Of the VLA-5$^+$ cells, 87% bound to fibronectin (Fig. 6 *C*).

Discussion

This study establishes that (*a*) enriched CD34^{hi} cells from human marrow express VLA-4, VLA-5, and β_2 integrins and (*b*) these integrins provide at least three distinct adhesion pathways for binding to specific ligands on human marrow stromal cell cultures. First, CD34^{hi} cells used VLA-4 to adhere to VCAM-1 expressed on the surface of stromal cells. Second, CD34^{hi} progenitors may adhere to stroma by interaction with fibronectin deposited by stromal cells in the extracellular matrix. For this purpose they use mainly VLA-5 and to a lesser extent VLA-4. Third, they can also use one or more β_2 integrins to interact with ICAM-1 expressed on IL-1–activated stromal cells.

Several lines of evidence in our studies indicate that VLA-4/VCAM-1, VLA-5/FN and β_2/ICAM-1 mediate adhesion between the CD34^{hi} cells as opposed to more differentiated progenitors (CD34^{lo}CD33^{hi}), and stroma: (*a*) Differentiation antigens such as CD33 and CD71 were expressed at very low densities on < 20% of the enriched CD34^{hi} population. (*b*) Close to 80% of the enriched CD34$^+$ population co-expressed

Table II. Inhibition of CD34^{hi} Cells to Marrow Stroma by Anti-β_1

	Percent inhibition		
Antibody	1	2	3
Anti-β_1	51	62	77
Anti-α^4	40	49	52
Anti-β_2	20	42	30

Enriched CD34$^+$ cells were labeled with ^{51}Cr and incubated with stromal cells as described in legend to Fig. 4. Results of three experiments are shown and triplicate or quadruplicate wells were used for each condition. In experiments 2 and 3 the stromal cells were pretreated with IL-1 for 24 h. Results are expressed as percent inhibition relative to adhesion of CD34^{hi} cells to stroma in the presence of nonspecific mouse IgG.

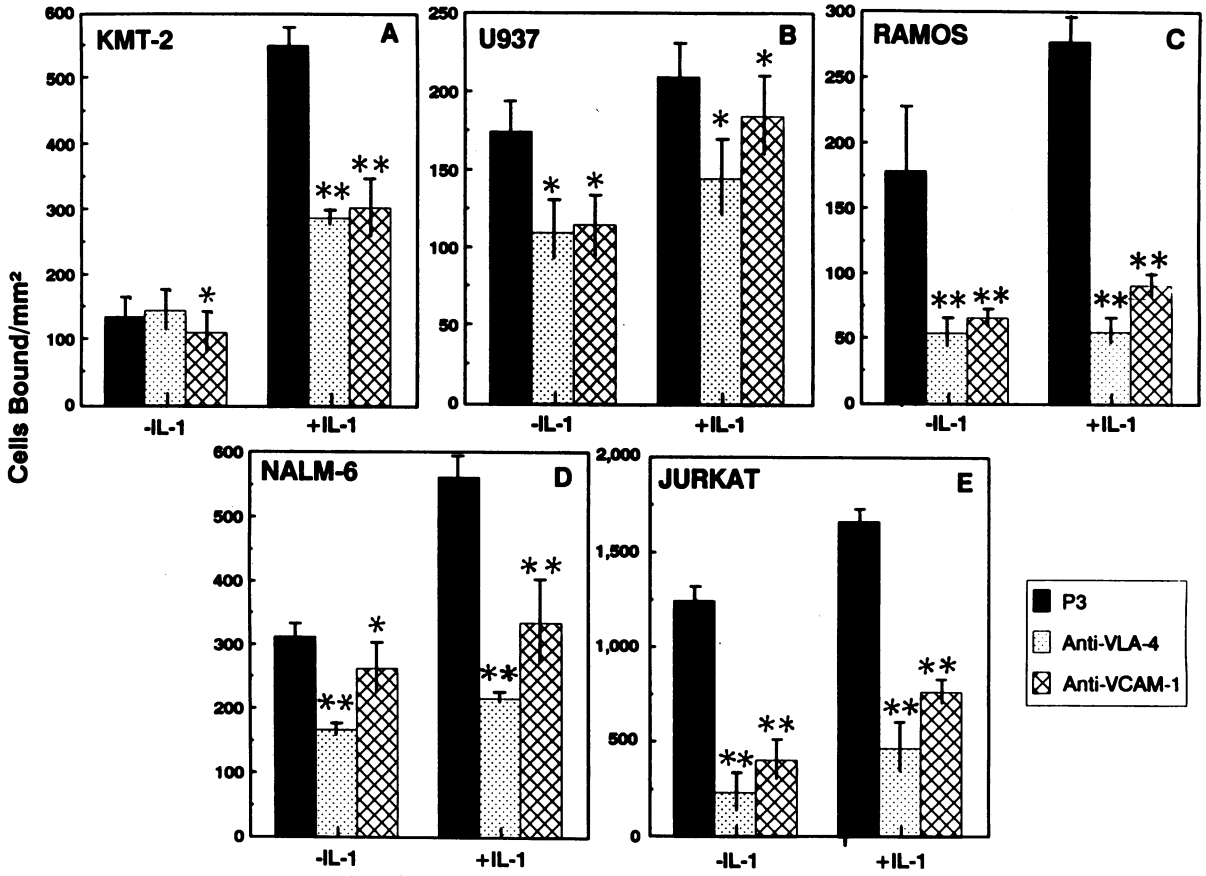

Figure 5. Adhesion of myeloid and lymphoid cells to stromal monolayers. Stromal monolayers were preincubated for 24 h with or without IL-1. Adhesion of indicated cell lines to stromal monolayers was carried out in 96-well microtiter plates in the presence of control antibody P3, anti-α^4 MAb HP2/1 or anti-VCAM-1 MAb 4B9 was measured as described in the legend to Fig. 3. Percentage of cells bound ranged from 15% (for U937 cells) to nearly 100% (for Jurkat cells). **Adhesion was significantly inhibited, $P < 0.05$ according to the Student's two-tailed t test. *Inhibition was observed, but was not quite low enough to meet $P < 0.05$ confidence level.

high densities of CD34 and the VLA-4 complex ($\alpha^4\beta_1$), whereas > 50% of $CD34^{hi}$ cells co-expressed VLA-5, β_1, and β_2. In contrast, VLA-4 was expressed in only a small percentage of cells in the SBA^- population ($CD34^{lo}CD33^{hi}$). (*c*) Up to 30–35% of $CD34^{hi}$ cells bound to stroma during a 25-min assay, thus indicating specific high avidity binding. Again the SBA^- population bound with less avidity to stroma and their adhesion was not significantly inhibited by anti-VLA4 MAbs (data not shown). The $CD34^+$ human hematopoietic stem cell population is heterogeneous. The majority of this $CD34^+$ population expresses CD34 and CD38 and may contain a small percentage of cells committed toward a specific lineage (55). The percentage of very primitive stem cells $CD34^+CD38^-$ found in the marrow (0.01%) was too small to allow us to study their adhesive properties in the present experiments. The enriched $CD34^+$ population used in these studies were capable of forming blast colonies at a high plating efficiency as well as initiating long-term cultures and giving rise to secondary clonogenic cells. Both these properties are associated with primitive stem cells (8).

Recent studies on stem cell adhesion have focused on single receptor–ligand interactions using purified fragments of adhesion molecules (12, 13), which perhaps do not reflect a true physiologic condition. Verfaillie et al. (12) have demonstrated that primitive LTBMC-IC adhere to intact fibronectin, although they do not indicate the receptors that might be involved. In contrast, Ryan et al. (34) have shown that VLA-4/VCAM-1 but not VLA-4/FN plays a role in adhesion of human B cell precursors to stroma. Experimental data from the murine system indicate that cells capable of forming day 12 CFU-s bind to the CS-1 fragment of fibronectin but not to intact fibronectin (13). The role of VCAM-1 in stem cell adhesion was not evaluated in these studies (13). The studies of Mikaye et al. (14) do not clarify whether retardation of myelopoiesis by anti-VLA-4 antibodies in murine long-term marrow cultures was a result of inhibition of stem cell binding to VCAM-1 or fibronectin. Reappearance of progenitor cells and eventual normalization of myelopoiesis in those studies suggests the role of other integrins and perhaps nonintegrin receptors mediated adhesion of stem cell to stromal cells. The differences between murine progenitors and human $CD34^{hi}$ cells are not clear at the present time. It is plausible that since the stem cell population is heterogeneous (58–60), different cell populations are being studied using different in vivo and in vitro assays.

The VLA-4/VCAM-1 adhesion pathway has been implicated in important physiological processes involving migration and localization of immune cells (24, 26, 27, 61–66) and some tumor cells (23, 67). In the present study we have established that this adhesion pathway also plays a role in mediating adhe-

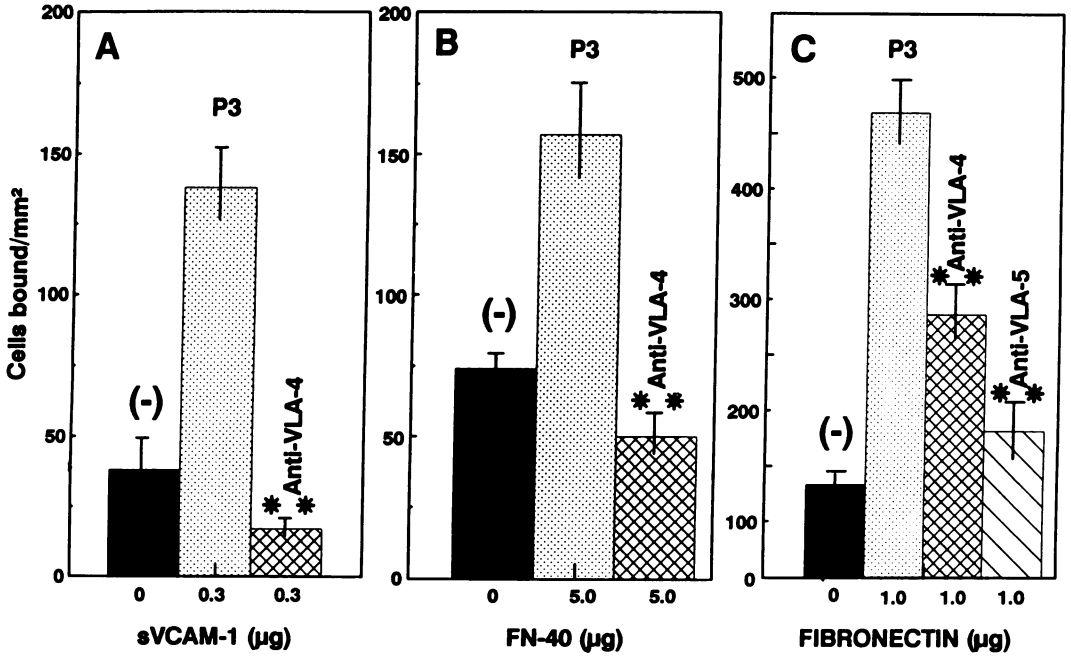

Figure 6. Adhesion of $CD34^{hi}$ cells to soluble VCAM-1, FN-40, and fibronectin. Cells were labeled with ^{51}Cr and incubated in a 96-well microtiter plates coated with BSA (*black bars*) or with indicated amounts of s-VCAM-1 (*A*), FN-40 (*B*) and fibronectin (*C*), in the presence of control antibody P3, anti-α^4 (MAb HP2/1) or anti-VLA-5 (P1D6) as shown. In each case, concentration of ligand was chosen which supports near maximal adhesion (see reference 84). After adhesion for 25 min, bound cells were solubilized and incorporated radioactivity measured in a gamma counter. Results are expressed as mean±SD. **Adhesion was significantly inhibited, $P < 0.05$ according to the Student's two-tailed *t* test.

sion of stem cells to bone marrow stroma, which could be critical for initiation and maintenance of hematopoiesis in the bone marrow. Although our data do not identify the specific β_2 integrins that may be involved in stem cell adhesion, it is most likely that the CD11a/CD18 is involved in this interaction in that it has been the only β_2 integrin detected on early stem cells (37). At this time we have not ruled out the possibility that other β_2 ligands, such as ICAM-2 (32), may play a role in stem cell adhesion to stroma. Further, while participation of VLA-4, VLA-5, and β_2 accounted for almost 100% of the adhesion between $CD34^{hi}$ and stromal cells, it is still possible that other integrins may also make a minor contribution to this process. Further experiments using multiple antibody combinations may help resolve this question. In addition, we have shown that expression of VCAM-1 and ICAM-1 adhesion molecules was possibly regulated by endogenous IL-1 produced by marrow stromal cells in culture. Other studies have demonstrated that some human stromal cell lines established by SV-40 transformation express small amounts of IL-6 and IL-1β message (68). It remains to be determined whether human stromal cells express IL-1 under steady-state conditions in vivo.

Although the role of diffusible and stroma-derived growth factors in stem cell regulation has been extensively studied (69–74), the functions of adhesion molecules remain to be defined. The influence of fibronectin on proliferation and differentiation of erythroid cells has been documented (75, 76). Adhesion of $CD4^-CD8^-$ thymocytes to thymic stromal cells through FN-FN receptor interaction is known to be critical for their differentiation (77). Integrin receptors on primitive stem cells could be involved in (*a*) homing and sequestration of stem cells within specific inductive areas within the marrow (78), (*b*) reinforcing stem–stromal cell interactions that have been induced by membrane-anchored growth factors (79, 80), (*c*) transducing signals to induce either proliferation and/or differentiation of progenitors, and (*d*) maintaining stem cells in a quiescent state under limited growth factor concentrations. The in vitro model developed in this study using enriched $CD34^{hi}$ progenitors and homogeneous stromal cells provides a system to study the relative biological and functional contributions of each of the adhesion molecules and their receptors.

Disruption of normal adhesion pathways between stem and stromal cells has been implicated as a critical factor in pathogenesis of chronic myeloid leukemia (81, 82). Furthermore, after chemotherapy and/or total body irradiation, the observed extravascation of stem cells into peripheral blood (83) might result from their interaction with VCAM-1 and/or ICAM-1 on activated venule endothelium. The diverse nature of adhesion pathways used by stem cells to interact with stroma underscores their regulatory role in normal stem cell differentiation.

Acknowledgments

We are grateful to Dr. Kirk Johnson (Memorial Hospital, Worcester, MA) for providing bone marrow samples, Dr. D. Williams (Immunex Corp., Seattle, WA) for providing recombinant MGF(SF), Dr. T. Suda (Jichi Medical School, Tokyo) for the KMT2 cell line, and Marcia McFadden and Tammy Krupoch for FACS analysis. We thank Drs. S. Neben and M. E. Rybak for helpful discussions. We acknowledge Francisco Sanchez-Madrid (Hospital de la Princesa, Madrid) for providing valuable monoclonal antibodies; Roy Lobb (Biogen Inc., Cambridge, MA) for providing soluble VCAM-1, S. Yamada (National Institutes of Health) for MAb13, and the American Red Cross Blood Services-Northeast Region, for providing human plasma for the use in preparation of fibronectin fragments.

This work was supported by grants from the National Institutes of Health to Dr. Greenberger (CA-39851 and DE-08798), to Dr. Hemler (CA42368), and a BRSG grant to Dr. Anklesaria.

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