JCI The Journal of Clinical Investigation

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J Clin Invest. 1992;90(4):1232-1241. https://doi.org/10.1172/JCI115985.

Research Article

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Mechanisms Underlying Abnormal Trafficking of Malignant Progenitors in Chronic Myelogenous Leukemia

Decreased Adhesion to Stroma and Fibronectin but Increased Adhesion to the Basement Membrane Components Laminin and Collagen Type IV

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Abstract

We studied the adhesion of primitive and committed progenitors from chronic myelogenous leukemia (CML) and normal bone marrow to stroma and to several extracellular matrix components. In contrast to benign primitive progenitors from CML or normal bone marrow, Ph[1]-positive primitive progenitors from CML bone marrow fail to adhere to normal stromal layers and to fibronectin and its proteolytic fragments, but do adhere to collagen type IV, an extracellular matrix component of basement membranes. Similarly, multilineage colony-forming unit (CFU-MIX) progenitors from CML bone marrow do not adhere to fibronectin or its adhesion promoting fragments but adhere to collagen type IV. Unlike committed progenitors from normal bone marrow, CML single-lineage burst-forming units-erythroid and granulocyte/macrophage colony-forming units fail to adhere to fibronectin or its components but do adhere to both collagen type IV and laminin. Evaluation of adhesion receptor expression demonstrates that fibronectin receptors (α4, α5, and β1) are equally present on progenitors from normal and CML bone marrow. However, a fraction of CML progenitors express α2 and α6 receptors, associated with laminin and collagens, whereas these receptors are absent from normal progenitors. These observations indicate that the premature release of malignant Ph[1]-positive progenitors into the circulation may be caused by loss of adhesive interactions with stroma and/or fibronectin and acquisition of adhesive interactions with basement membrane components. Further study of the altered function of cell-surface adhesion receptors characteristic of the malignant clone in CML may lead to a better understanding of the mechanisms underlying both abnormal expansion and abnormal circulation of malignant progenitors in CML. (*J. Clin. Invest.* 1992. 90:1232–1241.) Key words: bone marrow stroma • extracellular matrix • integrins

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Received for publication 4 October 1991 and in revised form 16 April 1992.

J. Clin. Invest.

0021-9738/92/10/1232/10 $2.00
Volume 90, October 1992, 1232–1241

Introduction

Chronic myelogenous leukemia (CML)[1] is a malignant disorder of the human hematopoietic stem cell characterized by a reciprocal translocation between chromosome 9 and chromosome 22 (Ph[1]) (1). At the molecular level, juxtaposition of 5′ coding sequences of the *bcr* gene on chromosome 22 and 3′ coding sequences of the *c-abl* gene on chromosome 9 (*bcr/abl*) occurs (2). Clinically, CML is characterized by an abnormal expansion of the malignant Ph[1]-positive stem cell clone, although presumed normal, Ph[1]-negative stem cells may coexist in some patients with CML (3–5). Primitive and committed malignant Ph[1]-positive myeloid progenitors leave the bone marrow microenvironment prematurely and circulate in the peripheral blood of patients with CML (6). It has been demonstrated that primitive progenitors present in the peripheral blood of patients with CML have a diminished capacity to adhere to normal bone marrow derived stromal layers when compared with bone marrow–derived hematopoietic progenitors from normal individuals (7, 8). Studies evaluating the adhesion of bone marrow–derived CML progenitors to either intact irradiated stromal layers or to different components of such stroma have not been performed.

Bone marrow stromal cells and/or extracellular matrix molecules have an important role in the regulation of the human hematopoietic stem cell (9). Such stromal layers provide both proliferation and differentiation stimuli (9–11). Stroma provides also factors such as transforming growth factor-β (TGF-lgb), which have the capacity to inhibit the proliferation of primitive hematopoietic progenitors (10, 11). It has been suggested that mechanisms that either promote or restrict the generation of more mature hematopoietic elements are only operative when primitive hematopoietic progenitors are in close proximity to stromal elements (12). The dysregulated hematopoiesis in CML could, therefore, be secondary to the inability of malignant CML primitive progenitors to adhere to bone marrow stromal elements.

Bone marrow–derived stroma consists of multiple cell types (13) and a variety of extracellular matrix molecules (14–18). Several extracellular matrix components have been implicated in the adhesion of committed hematopoietic progenitors

1. *Abbreviations used in this paper:* BMMNC, bone marrow mononuclear cells; CML, chronic myelogenous leukemia; IMDM, Iscove's modified Dulbecco's medium; LTBMC long-term bone marrow culture(s).

(14–17). Recently, we have demonstrated that receptors which can interact with the 33/66-kD COOH-terminal heparin-binding cell-attachment fragment of fibronectin may play an important role in the adhesion of primitive hematopoietic cells with long-term in vitro repopulating capacity to irradiated stromal layers and hence, to the bone marrow microenvironment (18). Upon differentiation into more committed clonogenic cells, human hematopoietic progenitors alter their adhesive capacity and are capable of interacting with other cell-attachment sites of the fibronectin molecule (15, 16, 18) and other extracellular matrix components (17).

We have previously described (3) that selection of $CD34^+$ cells lacking antigens associated with commitment to myeloid, monocytoid, or lymphoid lineage and lacking expression of HLA-DR results in the selection of benign Ph^1-negative primitive progenitors from CML bone marrow. In contrast, $CD34^+$ cells expressing the HLA-DR antigen contain exclusively malignant Ph^1-positive progenitors (3). In this report we describe differences in adhesion of highly purified benign and malignant progenitors derived from bone marrow of CML patients to intact irradiated stroma and to several extracellular matrix components. We demonstrate that malignant Ph^1-positive primitive progenitors, but not their benign Ph^1-negative counterparts, fail to adhere to irradiated stromal layers. In contrast to normal hematopoietic progenitors, both primitive and committed Ph^1-positive CML bone marrow progenitors fail to adhere to fibronectin and its 33/66-kD heparin-binding or its 75-kD RGDS-containing cell-attachment fragments. CML progenitors do interact with collagen type IV and laminin which are extracellular matrix molecules associated with basement membranes of the vasculature. These observations indicate that abnormal adhesive characteristics of malignant CML primitive progenitors may underlie the abnormal expansion and trafficking of primitive and committed CML hematopoietic progenitors.

Methods

Bone marrow samples

Bone marrow was obtained from the posterior iliac crest of 22 CML patients and 28 healthy young volunteers after informed consent. Patient characteristics are summarized in Table I.

Purification of progenitor populations

Bone marrow mononuclear cells (BMMNC) were obtained after Ficoll-Hypaque separation (s.g. 1.077). BMMNC from patients 5–15 were purified further in an initial counterflow elutriation step as previously described (3). Negative and positive selections for primitive progenitors were performed using previously described methods (3, 18, 19). In short, BMMNC or cells from fractions 9–12 recovered after counterflow elutriation were labeled with 25 ng/10^6 cells anti-CD2, anti-CD3, anti-CD11b, anti-CD15, anti-CD16, anti-CD19, anti-CD56, and anti-CD71 monoclonal antibodies (Becton Dickinson & Co., Mountain View, CA) and lineage-specific cells removed using goat anti-mouse IgG and IgM coated immunomagnetic beads (30–40 beads per cell) (Advanced Magnetics Inc, Cambridge, MA). This lineage-negative population contained < 2% residual cells labeled with low levels of lineage-specific mouse antibodies. The resultant lineage-negative cells were labeled with anti-CD34 antibody (250 ng/10^6 cells) (Becton-Dickinson & Co.), followed by FITC-conjugated goat $F(ab)'_2$ anti-mouse IgG (250 ng/10^6 cells) (Tago Inc., Burlingame, CA) and anti-HLA-DR-PE antibody (250 ng/10^6 cells) (Becton-Dickinson & Co.). Cells were sorted on a FACS-Star laser flow cytometry system (Becton-Dickinson & Co.) equipped with a consort 40 computer. Cells were initially selected for low vertical and very low/low horizontal light scatter properties. Cells in this window were then sorted into fractions expressing high-density CD34 antigen and either lacking (DR^- population) or bearing (DR^+ population) HLA-DR (9). These windows were chosen on the basis of the fluorescence pattern of control samples labeled with mouse IgG (Sigma Chemical Co., St. Louis, MO), FITC-conjugated goat $F(ab)'_2$ anti-mouse IgG and finally mouse IgG2a-PE antibodies. Fewer than 0.1% residual cells labeled with low levels of lineage-specific mouse IgG used in the lineage negative immunomagnetic depletion step were included in the $CD34^+$ fraction.

Table I. Characteristics of CML Patients

Characteristic	Number
Disease stage	
Chronic phase	20
<1 yr	12
>1 yr	8
Accelerated phase*	2
Treatment‡	
None	4
Hydroxyurea‡	16
Interferon-α§	2
Others	2

* Accelerated phase CML was diagnosed according to established criteria (1). ‡ All treatment stopped > 5 d before study. § Treatment was stopped 3 and 6 mo before study. ‖ Patients were treated with busulphan 1 and 4 mo before study.

Short-term methylcellulose assay

Cells recovered in the adherent fraction of adhesion assays performed with normal DR^- cells and CML or normal DR^+ cells were plated in clonogenic methylcellulose assay supplemented with 3 IU recombinant erythropoietin (Epoetin) (Amgen, Inc., Thousand Oaks, CA) and 10% conditioned media from the bladder carcinoma cell line 5637 as previously described (3, 18, 19). Cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 18–21 d. The cultures were assessed at days 18–21 of culture for the presence of colony-forming units CFU-MIX and CFU-GM, and burst-forming units-erythroid (BFU-E) as previously described. Likewise, cells harvested at week 5 from long-term bone marrow cultures (LTBMC) initiated with CML or normal DR^+ or DR^- cells were plated in short-term methylcellulose culture and secondary CFU-MIX, CFU-GM, and BFU-E were enumerated at days 14–16 of culture.

LTBMC

Plating. CML and normal DR^+ and DR^- cell populations and cells recovered in the adherent fraction of adhesion assays performed with CML DR^+ and normal DR^- cells were cultured in LTBMC as described (3, 18, 19). Cells were plated on allogeneic irradiated stroma in 1 ml of LTBMC media Iscove's modified Dulbecco's medium [IMDM] with 12.5% fetal calf serum, 12.5% horse serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, penicillin 1,000 U/ml, streptomycin 100 U/ml (Gibco Laboratories, Grand Island, NY), and 10^{-6} M hydrocortisone (A-Hydrocort) (Abbott Laboratories, North Chicago, IL).

Panning. Alternatively, CML and normal DR^+ and DR^- cell populations were panned on allogeneic irradiated stroma in LTBMC media for 2 h, the stroma was washed extensively with warm IMDM and the nonadherent cells were recovered in the panning effluent (7, 8, 19). The cells recovered in the panning effluent were then plated onto sec-

ondary stromal layers in order to determine the number of LTBMC-initiator cells (IC) which failed to adhere to normal allogeneic stroma during the initial panning experiment.

Maintenance of cultures. All LTBMC cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. At weekly intervals the cultures were fed by removing half of the supernatant and replacing it with fresh media. Nonadherent cells recovered in the supernatant as well as adherent cells recovered from selected stromal layers after treatment with 0.1% collagenase were assayed weekly in short-term methylcellulose assay for the presence of colony-forming cells.

It is believed that committed progenitor cells present in the initial bone marrow population fail to produce clonogenic cell growth in LTBMC 4–5 wk after initiation of such cultures. The number of clonogenic cells recovered from both adherent and nonadherent layers of LTBMC at 5 wk of culture may therefore represent progeny of more primitive progenitors which can sustain long-term in vitro hematopoiesis and are termed LTBMC initiator cells or LTBMC-IC (19–21).

Purification of collagen type I, collagen type IV, laminin, fibronectin, and fibronectin fragments.

Human plasma fibronectin was purified as a by-product of factor VIII production by sequential ion exchange and gelatin chromatography (22). A 75-kD tryptic fragment containing the cell binding fragment RGDS was purified from long-term (90′) tryptic digestion as previously described (22). The 33- and 66-kD COOH-terminal heparin binding fragments were purified from short-term (15′) tryptic/catheptic digests of intact fibronectin by sequential heparin and antibody column chromatography as described (22). The purity of intact fibronectin, the 33/66-kD fragment and the 75-kD fragment of fibronectin was verified by SDS-PAGE and Coomassie Brilliant Blue staining.

Collagen type IV and laminin were isolated from the Engelbreth-Holm-Swarm tumor grown subcutaneously in Swiss Webster mice (Simonson Laboratories, CA). The mice were made lathyritic by adding 0.1% β-amino proprionitrile to their water, using previously described techniques (23).

Collagen type I (Vitrogen) was obtained from the Collogen Corp., Palo Alto, CA.

Cell adhesion assays

Substrata were prepared as follows: collagen type I, collagen type IV, laminin, fibronectin, and proteolytic fragments from fibronectin were diluted to the appropriate concentrations in Voller's carbonate buffer (22). Proteins were adsorbed to wells of 48-well plates (Costar, Cambridge, MA) overnight in a humidified atmosphere at 37°C. Nonspecific sites were blocked the following day with 5 mg/ml BSA (BSA, fatty acid free, Miles, Inc., Naperville IL) in PBS, pH 7.4, for 2–3 h. Control wells were adsorbed with BSA 5 mg/ml. DR^- cells and DR^+ cells were suspended at 4×10^4 cells/ml in IMDM + 1% BSA (Sigma Diagnostics, St. Louis, MO). 250 μl of cell suspension was plated in ligand coated or control wells for 3 h at 37°C and 5% CO_2. The nonadherent and loosely attached cells were removed after 3 h by four or five consecutive washings with warm IMDM after standardized horizontal shaking of the plates (30 s, 100 excursions per minute). Cells present in the adherent fractions of adhesion assays initiated with CML DR^+ cells or normal DR^- cells were evaluated for the presence of LTBMC-IC by culturing in long-term cultures for 5 wk and evaluating these cultures for the presence of secondary clonogenic cells. Cells recovered in the adherent fraction of adhesion assays initiated with normal and CML DR^+ cells were evaluated for the presence of single lineage BFU-E and CFU-GM progenitors and multilineage CFU-MIX progenitors by culturing in short-term methyl cellulose culture. Percent adhesion was calculated as: (the number of progenitor cells adherent to ligand coated wells divided by the total input of progenitor cell) × 100.

FACS analysis of cell surface adhesion receptors

Cells obtained after Ficoll-Hypaque separation, counterflow elutriation (fractions 0–12), and T cell depletion using sheep erythrocyte rosetting from seven CML patients and seven normal individuals were labeled with antibodies against the α2, α4, α5, α6, and β1-integrin receptors in conjunction with antibodies against the CD34 and HLA-DR antigens as follows: 150×10^3 cells were sequentially labeled with 75 ng of unconjugated antibodies against integrins (1:20 dilution for anti-β1), 75 ng of goat anti-mouse-FITC, 10 μg of mouse IgG (Sigma Chemical Co.), 75 ng of anti-HLA-DR-biotin, 75 ng allophycocyanin, and 75 ng anti-CD34-PE. Control stains consisted of IgG1-PE, IgG2a-biotin followed by APC and mouse-IgG followed by FITC. Antibodies against the receptor β1 (P4C10) were a generous gift from Dr. Elizabeth Wayner (University of Minnesota); antibodies against the α2, α4, α5, and α6 receptors were obtained from AMAC Inc., Westbrook, MA; anti-CD34-PE, anti-HLA-DR-biotin and APC were obtained from Becton Dickinson & Co., goat anti-mouse-IgG-FITC was obtained from Tago Inc. Control samples were labeled with IgG1-PE, IgG2a-biotin followed by APC or mouse IgG followed by goat anti-mouse-FITC.

Statistical analysis

Results of experimental points obtained from multiple experiments were reported as the mean±1 SEM. Significance levels were determined by two-sided Student's *t* test analysis.

Results

Adhesion of LTBMC-IC to normal allogeneic irradiated stroma. In a first set of experiments we evaluated the adhesion of primitive progenitors capable of long-term in vitro hematopoiesis (LTBMC-IC) present in the CML DR^-, CML DR^+, and normal DR^- bone marrow populations to normal irradiated allogeneic stroma. FACS-sorted bone marrow fractions were panned for 2 h on irradiated allogeneic stroma (7, 8, 19). Stromal layers were then washed extensively, and adherent cells cultured further for 5 wk. At 5 wk the cultures were evaluated for the presence of secondary committed progenitors.

LTBMC-IC present in both the normal and the CML DR^- cell fraction adhered to irradiated stroma (Table II). The number of secondary clonogenic cells recovered from long-term cultures initiated with panned CML DR^- cells was similar to that recovered from long-term cultures initiated with plated CML DR^- cells at any time during the culture. Cells recovered in the panning effluent after panning with CML DR^- cells were significantly depleted of LTBMC-IC. These results are similar to those previously presented for LTBMC-IC derived from the DR^- fraction of normal bone marrow. This suggests that LTBMC-IC present in the DR^- cell fraction of CML bone marrow have similar adhesive characteristics as LTBMC-IC present in bone marrow of normal individuals (Table II).

In contrast, LTBMC-IC present in the DR^+ fraction of CML bone marrow did not adhere to irradiated stroma. Panning of CML DR^+ cells followed by culture of the adherent cells for 5 wk in long-term cultures resulted in the recovery of significantly fewer committed cells compared with experiments in which CML DR^+ cells were plated on irradiated stroma (Table II). Moreover, virtually all LTBMC-IC present in the CML DR^+ cell fraction were recovered in long-term cultures initiated with cells recovered in the panning effluent after panning stromal layers with CML DR^+ cells (Table II). These findings indicate that in contrast to benign DR^- LTBMC-IC present in bone marrow from CML patients or from normal individuals, malignant DR^+ primitive progenitors from CML bone marrow fail to adhere to stromal layers. A possible explanation for these differences in adhesion between LTBMC-IC in the DR^- fraction of CML and normal bone

Table II. Adhesion of CML and Normal LTBMC-IC to Normal Allogeneic Irradiated Stromal Layers

Cell population	Number of LTBMC-IC/10^6 cells		
	Plated	Panned	Panning effluent
CML DR^- cells	11,946±3,272 (14)*	12,651±4,668 (9)*	1,506±550 (9)
CML DR^+ cells	24,077±4,145 (15)‡	1,510±914 (10)	31,186±1,577 (10)‡
Normal DR^- cells	40,203±2,874 (19)§	40,453±3,771 (11)§	4,658±1,489 (6)

Values in parentheses are number of experiments. * $P = 0.01$: comparison of cultures initiated from plated or panned CML DR^- cells with cultures initiated from DR^- cells recovered in panning effluent. ‡ $P \leq 0.001$: comparison of cultures initiated from plated CML DR^+ cells or cultures initiated from CML DR^+ cells recovered in panning effluent with panned CML DR^+ cells. § $P \leq 0.001$: comparison of cultures initiated from plated or panned normal DR^- cells with cultures initiated from normal DR^- cells in panning effluent.

marrow and in the DR^+ fraction of CML bone marrow could be that CML DR^+ LTBMC-IC are cells at a different stage of differentiation. However, LTBMC-IC in the DR^+ fraction of CML bone marrow have the same in vitro growth and proliferation characteristics as LTBMC-IC in the DR^- fraction of either CML or normal bone marrow. We believe therefore that the observed decreased adhesion of DR^+ CML LTBMC-IC is not related to differences in differentiation status between DR^+ and DR^- progenitors but to an abnormal adhesive behavior of primitive malignant DR^+ cells.

Adhesion of primitive LTBMC-IC to extracellular matrix components. We then evaluated the adhesion of benign and malignant LTBMC-IC to different extracellular matrix components. Because the DR^- content in CML bone marrow is 10–100-fold smaller than that of normal bone marrow (3) secondary to the expansion of the more mature myeloid cell compartment, recovery of sufficient numbers of CML DR^- cells to assess their adhesion to ligand-coated wells is technically impossible. The adhesion of LTBMC-IC present in the DR^- fraction of normal and CML bone marrow to irradiated stromal layers is similar. Consequently, we compared the adhesion of malignant LTBMC-IC present in the DR^+ bone marrow fraction of CML patients with that of benign LTBMC-IC present in the DR^- fraction from normal individuals.

Cells were plated in wells coated with increasing concentrations of fibronectin and its proteolytic fragments, collagen type IV, laminin, and collagen type I. Adherent fractions were then cultured in LTBMC to enumerate the number of LTBMC-IC present in the adherent cell populations. Results shown represent the degree of adhesion of different progenitor populations to wells coated with the highest concentrations of ligands.

In contrast to LTBMC-IC in normal bone marrow (Fig. 1 *B*) (18), malignant CML LTBMC-IC failed to adhere to either intact fibronectin ($P = 0.11$), its 33/66-kD COOH-terminal heparin-binding fragment ($P = 0.07$) or its 75-kD RGDS-dependent cell-binding fragment ($P = 0.1$) (Fig. 1 *A*). However, a small but significant fraction of CML LTBMC-IC adhered to the extracellular matrix component collagen type IV ($P = 0.004$) (Fig. 1 *C*) whereas normal LTBMC-IC failed to do so ($P = 0.3$) (Fig. 1 *D*). Adhesion of either malignant CML LTBMC-IC or LTBMC-IC derived from normal bone marrow to wells coated with collagen type I and laminin (Fig. 1, *C* and *D*) was not significantly greater than that to control BSA-coated wells.

Adhesion of committed progenitors to extracellular matrix components. We then evaluated the adhesion of committed multilineage CFU-MIX and single-lineage BFU-E and CFU-GM progenitors in the DR^+ fraction of either CML or normal bone marrow to fibronectin, collagen type IV, laminin, and collagen type I. DR^+ cells were allowed to adhere to ligand coated wells for 3 h. The adherent cell fraction was then plated in methylcellulose culture to evaluate the number of adherent CFU-MIX, BFU-E, and CFU-GM progenitors.

As previously reported (18), adhesion of normal CFU-MIX progenitors occurred only to the 33/66-kD heparin-binding adhesion fragment of fibronectin (Fig. 2 *B*). No significant adhesion was observed to fibronectin nor its 75-kD proteolytic fragment, collagen type IV ($P = 0.15$), laminin ($P = 0.35$), or collagen type I ($P = 0.15$) (Fig. 2, *B* and *D*). In contrast, CML CFU-MIX progenitors failed to adhere to the 33/66-kD fragment of fibronectin (Fig. 2 *A*) but 22% adhered to collagen type IV ($P \leq 0.001$) (Fig. 2 *C*). No significant adhesion was observed to laminin (Fig. 2 *C*) and collagen type I (Fig. 2 *C*).

Normal BFU-E and CFU-GM adhered to a significant extent to fibronectin and both its proteolytic fragments (Figs. 3 *B* and 4 *B*) (18). Adhesion of these normal progenitors to the other tested extra-cellular matrix molecules was insignificant (Figs. 3 *D* and 4 *D*). In contrast, adhesion of CML single-lineage BFU-E (Fig. 3 *A*) and CFU-GM (Fig. 4 *A*) to fibronectin and its proteolytic fragments did not reach significance. 20–30% of CML BFU-E ($P \leq 0.001$) and CFU-GM ($P = 0.003$) adhered, however, to collagen type IV (Figs. 3 *C* and 4 *C*) and in addition to the extracellular matrix molecule laminin ($P \leq 0.001$ for BFU-E and $P = 0.017$ for CFU-GM), another component of basement membranes (Figs. 3 *C* and 4 *C*). Adhesion of such progenitors to collagen type I (Figs. 3 *C* and 4 *C*) was not significant.

Cell-surface adhesion receptor expression. We finally evaluated the presence of adhesion receptors from the β1-integrin family on CML DR^+ and normal DR^+ and DR^- progenitors. Partially purified bone marrow populations from seven chronic phase CML patients and seven normal individuals were sequentially labeled with antibodies against integrin receptors, the CD34 antigen, and the HLA-DR antigen and then analyzed using three-color FACS. Results were analyzed by sequential gating on cells with low–very low horizontal light scatter properties and low vertical light scatter properties. Using antibody control stains, a second selection was performed for cells expressing high levels of the CD34 antigen and either presence or absence of the HLA-DR antigen. This final population was then evaluated for the expression of integrin receptors by frequency histogram. Fig. 5 demonstrates the histograms

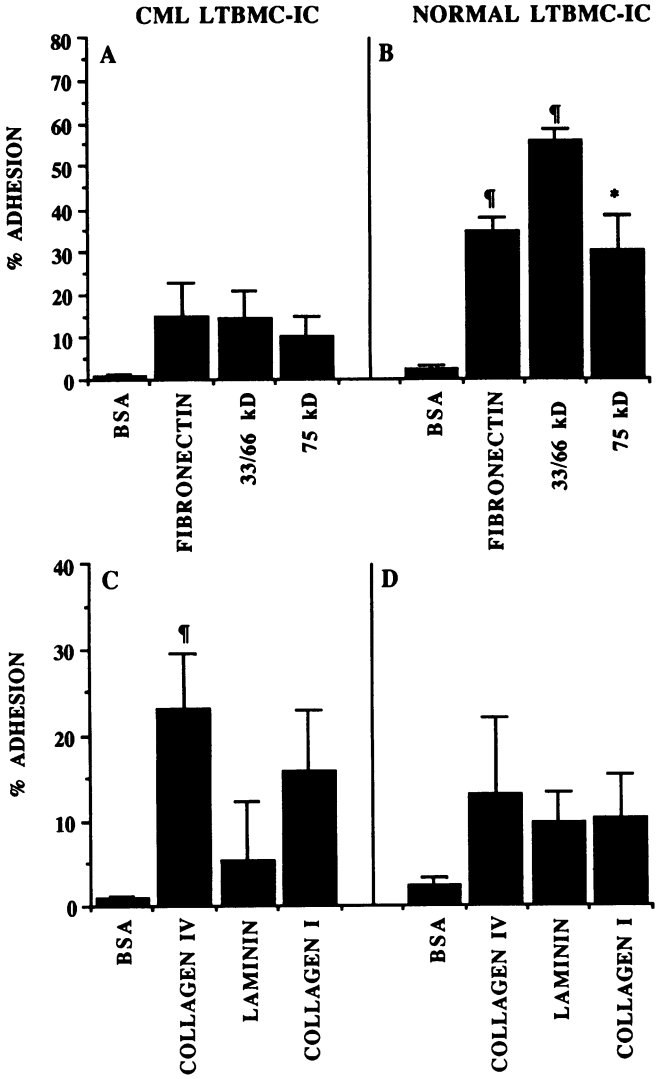

Figure 1. 10^4 cells from the CML DR$^+$ bone marrow fraction (*A* and *C*) or from the normal DR$^-$ bone marrow fraction (*B* and *D*) were plated in wells coated with collagen type I (100 μg/ml), collagen type IV (80 μg/ml), laminin (100 μg/ml), fibronectin (100 μg/ml), its 33/66-kD proteolytic fragment (60 μg/ml), its 75-kD proteolytic fragment (40 μg/ml), or BSA control wells for 3 h at 37°C and 5% CO_2. Adherent cells were cultured in LTBMC culture and the number of LTBMC-IC present enumerated at week 5 of culture. Percent adhesion was calculated as: (the number of LTBMC-IC adherent to ligand-coated wells/the total number of input LTBMC-IC) × 100. Mean number LTBMC-IC present in the starting population was $273/10^4$ cells for CML DR$^+$ cells and $374/10^4$ cells (fibronectin + fragments) or $266/10^4$ cells (other extracellular matrix proteins) for normal DR$^-$ cells. Results are the mean±SEM for five experiments in CML and four experiments for the adhesion of normal LTBMC-IC to collagen type I, collagen type IV, or laminin, and six experiments for the adhesion of normal LTBMC-IC to fibronectin and its fragments. Significance levels: comparison of adhesion to specific ligand and BSA control well ¶$P < 0.001$; *$P < 0.01$; **$P < 0.05$.

obtained from one CML patient and one normal individual. As can be seen in this figure, normal DR$^+$ and DR$^-$ cells express similar amounts of α4, α5, and β1 fibronectin receptors but do not express α2 and α6 receptors, associated with adhesion to laminin and collagens. Similarly, DR$^+$ cells from the CML patient express α4, α5, and β1 receptors to a similar degree as the normal individual. However, a fraction of CML DR$^+$ cells does express α2 and α6 receptors. Results for CML DR$^-$ cells are hard to interpret in that the percent of DR$^-$ cells is small, even after partial purification of the bone marrow progenitor fraction. Data for all individuals studied were then

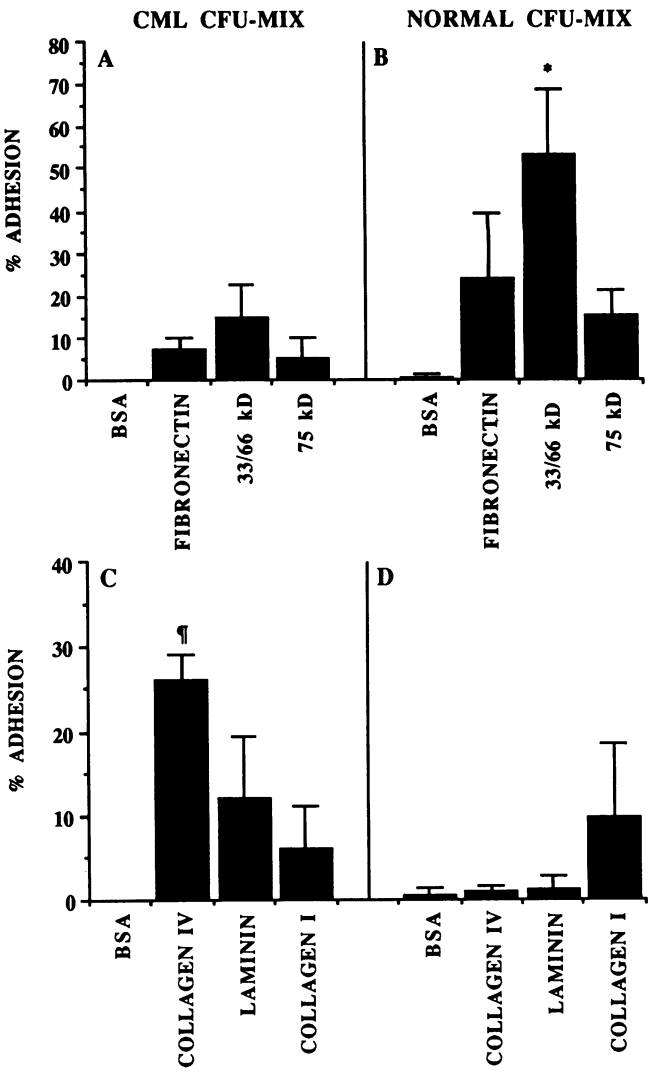

Figure 2. 10^4 CML DR$^+$ cells (*A* or *C*) or normal DR$^+$ cells (*B* and *D*) were plated in wells coated with collagen type I (100 μg/ml), collagen type IV (80 μg/ml), laminin (100 μg/ml), fironectin (100 μg/ml), its 33/66 kD proteolytic fragment (60 μg/ml), its 75-kD proteolytic fragment (40 μg/ml), or BSA control wells for 3 h at 37°C and 5% CO_2. Adherent cells were cultured in short-term methylcellulose culture and the number of CFU-MIX colonies present enumerated at days 16–21 of culture. Percent adhesion was calculated as: (the number of CFU-MIX adherent to ligand-coated wells/the total number of input CFU-MIX) × 100. Mean number of CFU-MIX present in the starting population was 2.2±9.2/10^3 cells for CML DR$^+$ cells and 4.6±1.7/10^3 cells (fibronectin + fragments) or 1.6±0.7/10^3 cells (other extracellular matrix proteins) for normal DR$^+$ cells. Results are the mean±SEM for six experiments in CML or five experiments for the adhesion of normal CFU-MIX to collagen type I, collagen type IV, or laminin, and six experiments for the adhesion of normal CFU-MIX to fibronectin and its fragments. Significance levels: comparison of adhesion to specific ligand and BSA control well ¶$P < 0.001$; *$P < 0.01$; **$P < 0.05$.

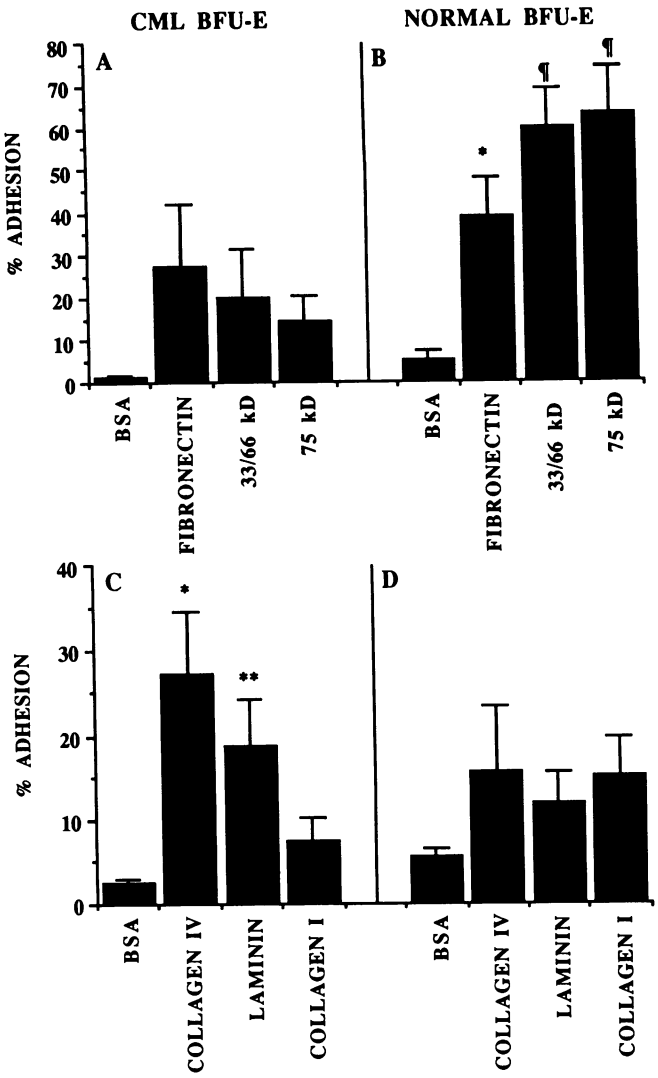

Figure 3. 10^4 CML DR^+ cells (*A* or *C*) or normal DR^+ cells (*B* or *D*) were plated in wells coated with collagen type I (100 μg/ml), collagen type IV (80 μg/ml), laminin (100 μg/ml), fibronectin (100 μg/ml), its 33/66-kD proteolytic fragment (60 μg/ml), its 75-kD proteolytic fragment (40 μg/ml) or BSA control wells for 3 h at 37°C and 5% CO_2. Adherent cells were cultured in short-term methylcellulose culture and the number of BFU-E colonies present enumerated at days 16–21 of culture. Percent adhesion was calculated as: (the number of BFU-E adherent to ligand coated wells/the total input BFU-E) × 100. Mean number of BFU-E present in the starting population was $24.7\pm4.3/10^3$ for CML DR^+ cells and $17.4\pm6.7/10^3$ cells (fibronectin + fragments) or $17.5\pm80.7/10^3$ cells (other extracellular matrix proteins) for normal DR^+ cells. Results are the mean±SEM for six experiments in CML or five experiments for the adhesion of normal BFU-E to collagen type I, collagen type IV, or laminin, and six experiments for the adhesion of normal BFU-E to fibronectin and its fragments. Significance levels: comparison of adhesion to specific ligand and BSA control well $^{\P}P < 0.001$; $^{*}P < 0.01$; $^{**}P < 0.05$.

compiled in Table III. Results are given both as the percent of cells expressing the integrin receptors and as the mean channel of fluorescence for the specific stain divided by the mean channel of fluorescence of the mouse IgG control stain (numbers between brackets). Normal primitive DR^- cells express the fibronectin receptors α4, α5, and β1-integrin receptors but express neither the α2 nor α6-integrin receptors. Similarly, normal DR^+ progenitors express the α4, α5, and β1 receptors but not the α2 nor α6 receptors. In contrast, > 20% of CML DR^+ progenitors express the α2 and α6 receptors. Although CML DR^+ progenitors do not adhere to fibronectin or its fragments, DR^+ cells derived from CML bone marrow express the α4, α5, and β1 receptors. The expression of α4, α5, and β1 receptors is,

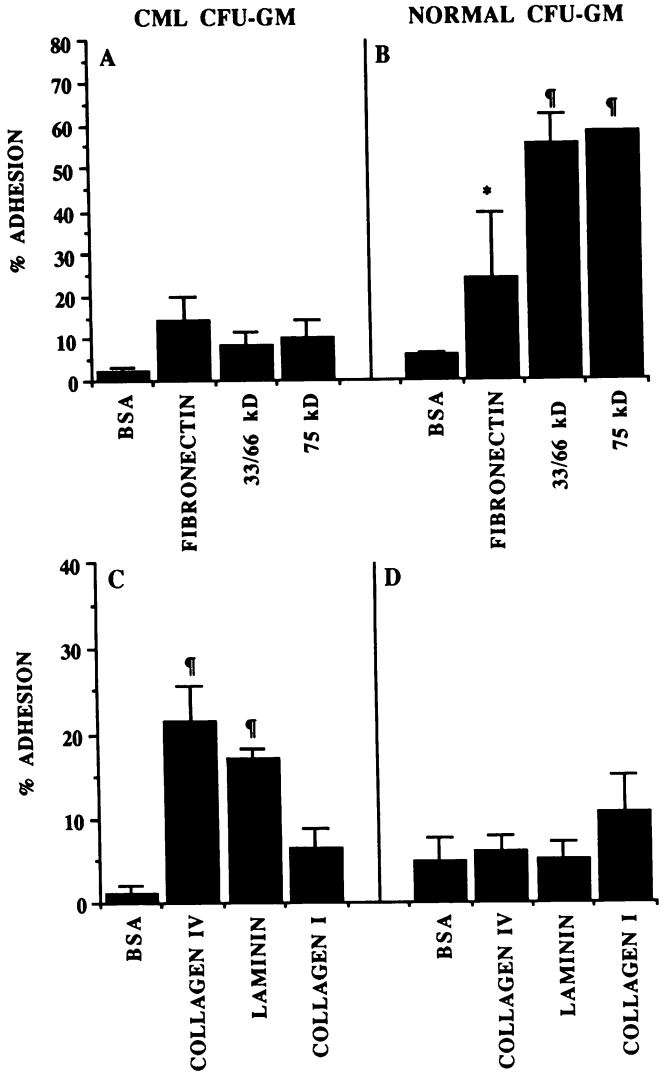

Figure 4. 10^4 CML DR^+ cells (*A* or *C*) or normal DR^+ cells (*B* and *D*) were plated in wells coated with collagen type I (100 μg/ml), collagen type IV (80 μg/ml), laminin (100 μg/ml), fibronectin (100 μg/ml), its 33/66-kD proteolytic fragment (60 μg/ml), its 75-kD proteolytic fragment (40 μg/ml), or BSA control wells for 3 h at 37°C and 5% CO_2. Adherent cells were cultured in short term methylcellulose culture and the number of CFU-GM colonies present enumerated at days 16–21 of culture. Percent adhesion was calculated as: (the number of CFU-GM adherent to ligand coated wells/the total input CFU-GM) × 100. Mean number of CFU-GM present in the starting population was $155\pm65/10^3$ cells for CML DR^+ cells and $106\pm49/10^3$ cells (fibronectin + fragments) or $162.3\pm42.2/10^3$ cells (other extracellular matrix proteins) for normal DR^+ cells. Results are the mean±SEM for six experiments in CML or five experiments for the adhesion of normal CFU-GM to collagen type I, collagen type IV, or laminin, and six experiments for the adhesion of normal CFU-GM to fibronectin and its fragments. Significance levels: comparison of adhesion to specific ligand and BSA control well $^{\P}P < 0.001$; $^{*}P < 0.01$; $^{**}P < 0.05$.

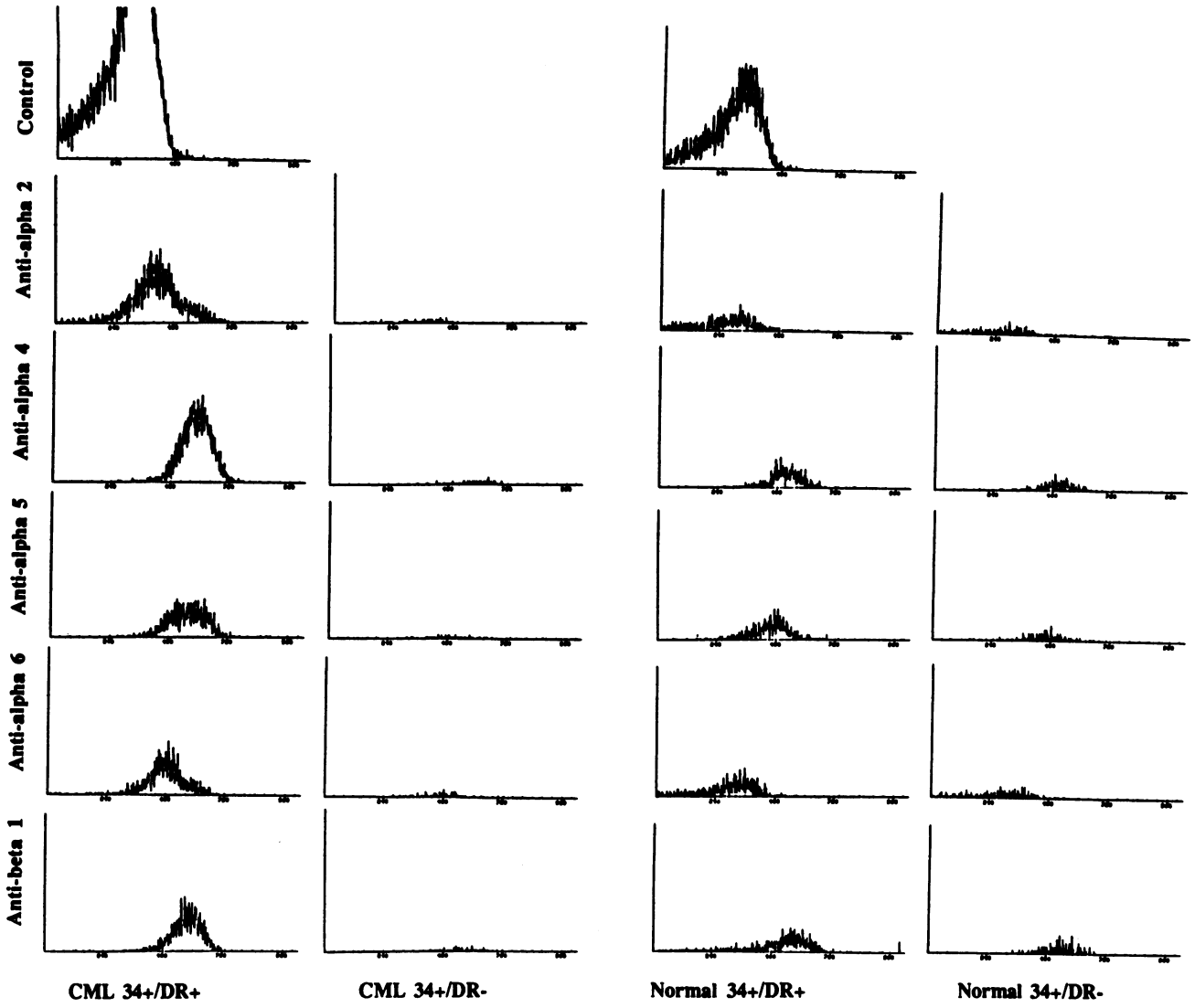

Figure 5. Representative example of frequency histograms demonstrating the expression of integrin receptors on $CD34^+/DR^-$ and $CD34^+/DR^+$ cells from one CML patient and one normal individual. Cells obtained after Ficoll-Hypaque separation, counterflow elutriation (fractions 0–12) and T cell depletion using sheep erythrocyte rosetting were labeled with antibodies against the $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\beta 1$-integrin receptors in conjunction with antibodies against the CD34 and HLA-DR antigens. Results were analyzed by sequential gating on cells with low–very low horizontal light scatter properties and low vertical light scatter properties. Using antibody control stains, a second selection was performed for cells expressing high levels of the CD34 antigen and either presence or absence of the HLA-DR antigen. This final population was then evaluated for the expression of integrin receptors by frequency histogram.

moreover, quantitatively similar in CML and normal progenitors in that the shift in mean channel fluorescence between control stain and specific stain is equivalent for the $\alpha 4$, $\alpha 5$, and $\beta 1$ receptors in DR^+ and DR^- cells from normals and the DR^+ fraction from CML marrow.

Discussion

The adhesion of cells to adjacent cells or to the surrounding extracellular matrix is fundamentally important for the maintenance of normal adult tissue structure and function. Ordered hematopoietic development is believed to depend not only on the interaction of the most primitive hematopoietic progenitors with specific cellular and extracellular components of the bone marrow microenvironment, but also on the trafficking of less primitive hematopoietic progenitors to specific sites of differentiation within the bone marrow. Ultimately mature blood elements are released from the bone marrow into the peripheral blood. Such processes require multiple, discrete recognition events which occur in a cell type– and developmental stage–specific fashion. The most primitive hematopoietic progenitors which can reconstitute long-term in vitro hematopoiesis found in the DR^- fraction of human bone marrow are capable of adhering within 2 h to bone marrow–derived stroma (7, 8, 19). This adhesion to stroma is in part mediated through cell surface adhesion receptors that are responsible for the interaction of such progenitors with the 33/66-kD COOH-terminal heparin-binding cell-attachment fragment of fibronectin (18). Differentiation and maturation of the normal hematopoietic stem cell into clonogenic cells and ultimately into mature blood elements is associated with changes in the capacity of these more mature cells to interact with irradiated stroma (8, 9, 19) or its extracellular matrix components (15–18, 24–28).

Table III. Expression of Integrins on CML and Normal CD34⁺ Progenitors

	CML	Normal	
	DR⁺	DR⁺	DR⁻
	% positive (% increase in mean fluorescence channel)		
Control	0.6±0.1 (0±0)	0.3±0.1 (0±0)	
Anti-α2	22.8±3.8‡ (36±9)*	0.3±0.1 (8±4)	0.6±0.4 (3±4)
Anti-α4	78.2±3.8 (76±8.7)	58.5±2.6 (88±3)	44.3±5.5 (69±16)
Anti-α5	74.3±3.5 (69±7)	45.7±4.7 (65±9.9)	33.4±3.8 (63±5.9)
Anti-α6	23.4±8.5‡ (37±7.5)‡	1.3±0.7 (6±3.6)	0.99±0.4 (1±3.1)
Anti-β1	87.6±2 (77±8)	55.9±10.3 (74±16)	50.7±8.9 (69±4)

Cells obtained after Ficoll-Hypaque separation, counterflow elutriation (fractions 0–12) and T cell depletion using sheep erythrocyte rosetting from seven CML patients and seven normal individuals were labeled with antibodies against the α2, α4, α5, α6, and β1-integrin receptors in conjunction with antibodies against the CD34 and HLA-DR antigens. Results were analyzed by sequential gating on cells with low–very low horizontal light scatter properties and low vertical light scatter properties. Using antibody control stains, a second selection was performed for cells expressing high levels of the CD34 antigen and either presence or absence of the HLA-DR antigen. This final population was then evaluated for the expression of integrin receptors by frequency histogram. Results are given both as the percent of cells expressing the integrin receptors and as the mean channel of fluorescence for the specific stain divided by the mean channel of fluorescence of the mouse IgG control stain (results inside parentheses). Significance levels: comparison of CML $34^+/DR^+$ and normal $34^+/DR^+$ or $34^+/DR^-$ cells: * $P < 0.001$; ‡ $P < 0.01$.

CML is characterized by the premature release and circulation of vast numbers of malignant, primitive, and more differentiated hematopoietic progenitors from the bone marrow microenvironment into the peripheral circulation (5). This aberrant behavior indicates that recognition mechanisms found in normal hematopoietic development are not operative in CML hematopoiesis. Moreover, immature CML hematopoietic progenitors must possess the capacity to interact with endothelial cells lining the bone marrow sinusoids in order to leave the bone marrow microenvironment. Hence, the malignant phenotype of hematopoietic progenitors in CML is likely to be accompanied by altered adhesion mechanisms and by alterations in the function or expression of cell surface adhesion receptors.

Ph^1-negative, *bcr/abl* nonrearranged primitive progenitors can be separated from their malignant counterparts on the basis of HLA-DR expression (3). Such benign progenitors in the DR^- fraction of CML bone marrow demonstrate similar adhesion to irradiated stromal layers as their counterparts found in the DR^- fraction of bone marrow obtained from normal individuals. In contrast, the majority of malignant, primitive progenitors in the DR^+ population of CML bone marrow fail to adhere to preformed, stromal layers. These observations are consistent with the reports from Gordon and co-workers, who demonstrated that primitive progenitors with self-renewal capacity derived from CML peripheral blood adhere poorly to preformed stromal layers (7, 8). Because we used preestablished, irradiated stromal layers generated from the bone marrow of normal individuals, we believe that an intrinsic adhesive defect in malignant CML hematopoietic progenitors underlies their reduced adhesion. The adhesive properties of malignant primitive progenitors from CML bone marrow are reminiscent of those of committed progenitors found in the DR^+ fraction of normal bone marrow (19). However, the majority of clonogenic cells in normal individuals are usually retained in the bone marrow microenvironment, whereas uncommitted and committed malignant CML progenitors are circulating. This suggests additional differences in adhesive properties between both uncommitted and committed progenitors in CML and hematopoietic progenitors from normal individuals.

In addition to their inability to interact with intact stromal layers, malignant DR^+ CML progenitors demonstrate significantly decreased adhesion to fibronectin. In contrast to normal multipotential LTBMC-IC and CFU-MIX, which adhere significantly to the 33/66-kD COOH-terminal heparin-binding cell-attachment fragment of fibronectin (18), malignant LTBMC-IC and CFU-MIX from CML bone marrow fail to do so. We demonstrate also that malignant BFU-E and CFU-GM from CML bone marrow fail to adhere to either intact fibronectin, its 75-kD RGDS-containing or 33/66-kD heparin-binding cell-attachment fragments. However, similar bone marrow fractions from normal individuals have the ability to interact with fibronectin and with both proteolytic fragments (18). Receptors involved in the adhesion of normal hematopoietic progenitors to fibronectin are in part responsible for their adhesion to intact stromal layers (18). Failure of malignant progenitors from CML bone marrow to interact with fibronectin may therefore in part explain their premature release from the bone marrow into the peripheral circulation.

The second major difference observed between hematopoietic progenitors from normal bone marrow and malignant progenitors in CML bone marrow is the ability of a small fraction of the latter to adhere to collagen type IV and laminin. Both laminin and collagen type IV are almost exclusively found in basement membranes where they comprise the majority of proteins (29, 30). Malignant transformation of cells is generally accompanied by molecular alterations in the adhesive behavior of the tumor cells compared with their non-malignant counterparts (31, 32). These alterations generally enable tumor cells to escape from their usual environment and to disseminate. During the process of intra- or extravasation, malignant cells must penetrate subendothelial basement membranes. Malignant tumor cells are therefore likely to acquire the ability to adhere to basement membrane proteins and to express adequate numbers of adhesion receptors which interact with collagen type IV or laminin (33). The capacity of 20–30% of malig-

nant CML hematopoietic progenitors to adhere to laminin and collagen type IV may facilitate their egress from the bone marrow into the circulation. However, not all CML malignant progenitors are capable of adhering to these extracellular matrix components under the above described test conditions. This may indicate that other as yet not identified mechanisms may have a separate or accessory role in the abnormal trafficking of CML progenitors.

The adhesive phenotype of malignant CML progenitors resembles that of mature blood elements. This is consistent with the clinical observation that numerous immature progenitors circulate in the blood of patients with CML. The mechanism(s) by which normal mature blood elements are released from the bone marrow microenvironment into the circulation are not well known. A developmentally regulated decrease in function and/or expression of adhesion receptors on hematopoietic progenitors may result in their release in the circulation. Upon maturation into reticulocytes and mature red blood cells, erythroid precursors progressively loose their capacity to adhere to fibronectin (15, 16). Similarly, it has been demonstrated that granulocytic progenitors adhere to hemonectin whereas mature circulating granulocytes have lost this capacity (17). However, before entering the circulation, mature blood elements need to cross the basement membrane of the capillary endothelium lining the venous sinusoids of the bone marrow. Mature leukocytes (27), monocytes (25), and lymphocytes (34) in the circulation have the capacity to adhere to laminin. This enables these cell populations to interact transiently with basement membranes and diapedese out of the blood vessels into tissue compartments. Similarly, platelets adhere to the subendothelial matrix of injured vessel walls as an initial step in the formation of a hemostatic plug (35). This adhesion involves interaction with laminin and collagen type IV through integrin receptors (25–27, 34). The same adhesive mechanisms may in part be responsible for the release of normal mature blood elements from the normal bone marrow microenvironment and malignant immature hematopoietic progenitors from the CML bone marrow microenvironment.

We are currently defining the cell-surface receptors responsible for the adhesion of normal LTBMC-IC and their more committed progeny to intact stromal layers or to fibronectin. To elucidate the molecular events underlying the altered adhesion of CML progenitors, it will be necessary to evaluate both quantitative and qualitative differences in cell-surface adhesion receptors between CML progenitors and their benign counterparts. Decreased expression of receptors involved in cellular adhesion such as the LFA-3 molecule, has been demonstrated in CML (35). Here we describe an additional aberration in the adhesive phenotype of malignant CML progenitors. In contrast to normal $34^+/DR^-$ and $34^+/DR^+$ progenitors, 22% of CML $34^+/DR^+$ progenitors express high levels of $\alpha 2$ and $\alpha 6$-integrin receptors, associated with adhesion to collagens and laminin. Surprisingly, adhesion receptors associated with fibronectin ($\alpha 5\beta 1$ = RGD dependent and $\alpha 4\beta 1$ = CS1 dependent) are expressed to the same extent on CML progenitors and immature hematopoietic progenitors from normal bone marrow. The malignant phenotype of CML progenitors may therefore be associated not only with aberrant expression of adhesion receptors but also with changes in the functional status of such receptors. Anchorage-independent growth of fibroblasts has been demonstrated after transfection of NIH-3T3 fibroblasts with Rous sarcoma virus which results in the generation of a neo-protein, $pp60^{SRC}$. This neo-protein has increased tyrosine kinase activity similar to that of the $p^{210bcr/abl}$ kinase. This enzyme can be found in close proximity with the cell membrane, can induce phosphorylation of integral membrane proteins and can alter their function (36). Similarly, transformation of the hematopoietic progenitor in CML results in the abnormal distribution of the $p^{210bcr/abl}$ tyrosine kinase in the cell cytoplasm rather than the normal distribution of the p^{156} tyrosine kinase product from *c-abl* in the nucleus (37, 38). This altered distribution may change the functional status of cell surface adhesion receptors resulting in impaired adhesion of the malignant population to bone marrow–derived stromal elements. Further studies evaluating the molecular alterations resulting in the abnormal adhesive properties of malignant CML hematopoietic progenitors will help to elucidate the abnormal differentiation and circulation of hematopoietic progenitors in CML.

Acknowledgments

The authors thank L. Burch, K. Boland, M. Hupke, D. Mickelson, and K. Myhre for their excellent technical help.

Catherine Verfaillie is a Special Fellow of the Leukemia Society of America and a Special Fellow of the "Fundacion Internacional Jose Carreras Para La Lucha Contra La Leucemia." This work was supported in part by National Institutes of Health grants R01-CA-4581401, PO1-CA-21737, and RO1-CA-43924. We also acknowledge the support of the Gambel-Skogmo Foundation, the Paul Christiansen Foundation, the University of Minnesota Bone Marrow Transplant Research Fund, the Minnesota Medical Foundation, The Leukemia Task Force, the Children's Cancer Research Fund, The Graduate School of the University of Minnesota, and the American Cancer Society.

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